Bluetongue

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N. James MacLachlan & James E. Pearson, Editors

Part II

Veterinaria Italiana, Volume 40 (4), October-December 2004
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<td>Å</td>
<td>Angstrom (a unit of length equal to 1 hundred-millionth of a centimetre, or 1 hundredth of a nanometre)</td>
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<tr>
<td>AA</td>
<td><em>Aedes albopictus</em> (cells)</td>
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<tr>
<td>AAVLD</td>
<td>American Association of Veterinary Laboratory Diagnosticians</td>
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<tr>
<td>ABADRI</td>
<td>Arthropod-Borne Animal Diseases Research Laboratory (USDA/ARS, USA)</td>
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<tr>
<td>ac-ELISA</td>
<td>antigen capture ELISA</td>
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<tr>
<td>ACIAR</td>
<td>Australian Centre for International Agricultural Research</td>
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<tr>
<td>AcNPV</td>
<td>nuclear polyhedrosis virus of <em>Autographa californica</em></td>
</tr>
<tr>
<td>AFSSA</td>
<td>French Agency for Food Safety (<em>Agence française de Sécurité sanitaire des Aliments</em>)</td>
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<tr>
<td>AGE</td>
<td>agarose gel electrophoresis</td>
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<td>AGID</td>
<td>agar gel immunodiffusion</td>
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<td>AHS</td>
<td>African horse sickness</td>
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<td>AHSV</td>
<td>African horse sickness virus</td>
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<td>AKAV</td>
<td>Akabane virus</td>
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<td>AMV</td>
<td>avian myeloblastosis virus</td>
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<td>ANDV</td>
<td>Andasibe virus</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APC</td>
<td>antigen presenting cells</td>
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<tr>
<td>APHIS</td>
<td>Animal and Plant Health Inspection Service (USDA)</td>
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<tr>
<td>AQIS</td>
<td>Australian Quarantine and Inspection Service</td>
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<tr>
<td>ARC</td>
<td>Agricultural Research Council, Onderstepoort, South Africa</td>
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<tr>
<td>ARS</td>
<td>Agricultural Research Service (USDA)</td>
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<tr>
<td>asl</td>
<td>above sea level</td>
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<tr>
<td>ASL</td>
<td>local veterinary office (<em>Azienda sanitaria locale</em>), Italy</td>
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<tr>
<td>AVHRR</td>
<td>advanced very high resolution radiometer</td>
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<tr>
<td>AWS</td>
<td>automatic weather station</td>
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<tr>
<td>BAV</td>
<td>Banna virus</td>
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<tr>
<td>BCS</td>
<td>blind calf syndrome</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney</td>
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<tr>
<td>Bm-1 CPV</td>
<td><em>Bombyx mori</em> cytoplasmic polyhedrosis virus-1</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSR</td>
<td>a clone of baby hamster kidney cell</td>
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<tr>
<td>BT</td>
<td>bluetongue</td>
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<tr>
<td>BTV</td>
<td>bluetongue virus</td>
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<tr>
<td>CCF</td>
<td>cross-correlation function</td>
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<tr>
<td>CCID</td>
<td>cell culture infective dose</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CEID</td>
<td>chick embryo infective dose</td>
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<tr>
<td>c-ELISA</td>
<td>competitive ELISA</td>
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<td>CESME</td>
<td>National Reference Centre for Exotic Diseases, Teramo, Italy (<em>Centro Studi Malattie Esotiche</em>)</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CFIA</td>
<td>Canadian Food Inspection Agency</td>
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<tr>
<td>CFT</td>
<td>complement fixation test</td>
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<tr>
<td>cfu</td>
<td>colony-forming unit</td>
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<td>CGLV</td>
<td>Changuinola virus</td>
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<tr>
<td>CGV</td>
<td>Chobar Gorge virus</td>
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<tr>
<td>CIRAD</td>
<td>Centre for international co-operation in agronomic research for development, France (Centre de coopération internationale en recherche agronomique pour le développement)</td>
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<tr>
<td>CLP</td>
<td>core-like particle</td>
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<td>CNUV</td>
<td>Chenuda virus</td>
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<td>COI</td>
<td>cytochrome oxidase subunit I</td>
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<td>CORV</td>
<td>Corriparta virus</td>
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<td>COV</td>
<td>Codajas virus</td>
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<tr>
<td>CPAE</td>
<td>calf pulmonary artery endothelium</td>
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<td>CPE</td>
<td>cytopathic effect</td>
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<td>CPV</td>
<td>cytoplasmic polyhedrosis virus</td>
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<td>CRI</td>
<td>clinical reaction index</td>
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<tr>
<td>Cryo-EM</td>
<td>cryoelectron micrographs</td>
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<td>CT</td>
<td>cycle threshold</td>
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<td>CTFV</td>
<td>Colorado tick fever virus</td>
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<td>CTL</td>
<td>cytotoxic T lymphocyte</td>
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<td>CVO</td>
<td>Chief Veterinary Officer</td>
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<tr>
<td>DB</td>
<td>dissemination barrier</td>
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<tr>
<td>DC</td>
<td>dendritic cells</td>
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<tr>
<td>DDSV</td>
<td>Departmental Directorate of Veterinary Services, France (Direction départementale des services vétérinaires)</td>
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<tr>
<td>DEM</td>
<td>digital elevation model</td>
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<tr>
<td>DGAL</td>
<td>National Food Directorate, France (Direction générale de l’Alimentation)</td>
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<tr>
<td>DIA</td>
<td>dot immunobinding assay</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dpi</td>
<td>days post infection</td>
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<tr>
<td>ds</td>
<td>double-stranded</td>
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<tr>
<td>dsRNA</td>
<td>double-stranded RNA</td>
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<td>EC</td>
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<td>ECE</td>
<td>embryonated chicken egg</td>
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<td>EDTA</td>
<td>ethylene-diaminetetra-acetic acid</td>
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<td>EEV</td>
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<td>EGF</td>
<td>epidermal growth factor</td>
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<td>EHDV</td>
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<td>EI</td>
<td>extrinsic incubation period</td>
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<td>ELD</td>
<td>egg lethal dose</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EM</td>
<td>electron microscopy</td>
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<td>Description</td>
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<tr>
<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
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<tr>
<td>EMEM</td>
<td>Eagle’s minimum essential medium</td>
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<td>ENSO</td>
<td>El Niño/Southern Oscillation</td>
</tr>
<tr>
<td>epg</td>
<td>eggs per gram</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>EST</td>
<td>expressed sequence tag</td>
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<td>EU</td>
<td>European Union</td>
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<td>Eubenangee virus</td>
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<td>EXANDIS</td>
<td>Exotic Animal Disease Preparedness Program, Australia</td>
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<td>FAO</td>
<td>Food and Agriculture Organization</td>
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<tr>
<td>FBS</td>
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<td>fg</td>
<td>femtogram</td>
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<td>FP</td>
<td>forward primer</td>
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<td>fluorescence resonance energy transfer</td>
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<td>GEL</td>
<td>gelsolin</td>
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<td>GI</td>
<td>GenInfo identifier</td>
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<td>GIS</td>
<td>geographic information system</td>
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<td>GIV</td>
<td>Great Island virus</td>
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<td>global positioning system</td>
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<td>GuSCN</td>
<td>guanidine thiocyanates</td>
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<td>haematocrit</td>
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<td>HGB</td>
<td>haemoglobin</td>
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<td>HI</td>
<td>haemagglutination inhibition</td>
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<td>HIT</td>
<td>herd immunity threshold</td>
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<td>Hyg B</td>
<td>hygromycin B</td>
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<td>IAH</td>
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<td>International Committee for the Taxonomy of Viruses</td>
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<td>IDW</td>
<td>inverse distance weighted</td>
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<td>i-ELISA</td>
<td>indirect ELISA</td>
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<td>IERIV</td>
<td>Ieri virus</td>
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<td>IETS</td>
<td>International Embryo Transfer Society</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<td>IFEV</td>
<td>Ife virus</td>
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<tr>
<td>IF/R-1</td>
<td>internal primers</td>
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<td>IGM</td>
<td>Military Geographical Institute, Santiago, Chile (Instituto Geográfico Militar)</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>INTA</td>
<td>National Institute of Agricultural Technology, Argentina (Instituto Nacional de Tecnología Agropecuaria)</td>
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<tr>
<td>IP</td>
<td>immunoperoxidase</td>
</tr>
<tr>
<td>ISVP</td>
<td>infectious subviral particles</td>
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<tr>
<td>ITS</td>
<td>internal transcribed spacer</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>ITUV</td>
<td>Itupiranga virus</td>
</tr>
<tr>
<td>IU</td>
<td>international unit</td>
</tr>
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<td>IZS</td>
<td>Istituto Zooprofilattico Sperimentale, Italy</td>
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<td>IZSA&amp;M</td>
<td>Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’, Teramo, Italy</td>
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<td>JAPV</td>
<td>Japanaut virus</td>
</tr>
<tr>
<td>JE</td>
<td>Japanese encephalitis</td>
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<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
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<td>KDV</td>
<td>Kadirovo virus</td>
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<td>KMPV</td>
<td>Kammavanpettai virus</td>
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<td>KNMI</td>
<td>Royal Netherlands Meteorological Institute, De Bilt, The Netherlands (Het Koninklijk Nederlands Meteorologisch Instituut)</td>
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<tr>
<td>KOH</td>
<td>potassium hydroxide</td>
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<tr>
<td>L1</td>
<td>large segment</td>
</tr>
<tr>
<td>LCV</td>
<td>Lake Clarendon virus</td>
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<tr>
<td>LD</td>
<td>lethal dose</td>
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<tr>
<td>LDCC</td>
<td>Local Disease Control Centre, Italy</td>
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<tr>
<td>LDR</td>
<td>light-dependent resistor</td>
</tr>
<tr>
<td>LEBV</td>
<td>Lebombo virus</td>
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<tr>
<td>LED</td>
<td>light-emitting diode</td>
</tr>
<tr>
<td>LOD</td>
<td>limit of detection</td>
</tr>
<tr>
<td>LSD</td>
<td>least significant difference</td>
</tr>
<tr>
<td>LST</td>
<td>land surface temperature</td>
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<tr>
<td>M4</td>
<td>medium segment</td>
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<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
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<tr>
<td>MARU</td>
<td>Middle America Research Unit</td>
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<tr>
<td>MATV</td>
<td>Matucare virus</td>
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<tr>
<td>MCH</td>
<td>mean cellular haemoglobin content by mass</td>
</tr>
<tr>
<td>MCHC</td>
<td>mean cellular haemoglobin concentration</td>
</tr>
<tr>
<td>MCV</td>
<td>mean red blood cell volume</td>
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<td>MEB</td>
<td>mid-gut escape barrier</td>
</tr>
<tr>
<td>MeHgOH</td>
<td>methyl mercury hydroxide</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MEVP</td>
<td>membrane enveloped virus particles</td>
</tr>
<tr>
<td>MIB</td>
<td>mid-gut infection barrier</td>
</tr>
<tr>
<td>MIR</td>
<td>middle infra-red reflectance</td>
</tr>
<tr>
<td>MLV</td>
<td>modified-live virus</td>
</tr>
<tr>
<td>MMLV</td>
<td>Moloney murine leukaemia virus</td>
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<td>MMOH</td>
<td>methyl mercury hydroxide</td>
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<td>MODIS</td>
<td>moderate resolution imaging spectroradiometer</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MRV</td>
<td>mammalian orthoreovirus</td>
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<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
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<tr>
<td>MT SN</td>
<td>microtitre serum neutralisation</td>
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<td>MTT</td>
<td>3-(4,5, dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide</td>
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<td>NAMP</td>
<td>National Arbovirus Monitoring Program, Australia</td>
</tr>
<tr>
<td>NAMRU</td>
<td>Naval Medical Research Unit, USA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>NAQS</td>
<td>Northern Australia Quarantine Strategy</td>
</tr>
<tr>
<td>NASA</td>
<td>National Aeronautics and Space Administration, USA</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information, USA</td>
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<tr>
<td>NDCC</td>
<td>National Disease Control Centre, Italy</td>
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<tr>
<td>NDEV</td>
<td>Ndelle virus</td>
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<tr>
<td>NDVI</td>
<td>normalised difference vegetation index</td>
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<tr>
<td>NERC</td>
<td>Natural Environmental Research Council, Oxford, UK</td>
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<tr>
<td>NIS</td>
<td>National Information System, Italy</td>
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<tr>
<td>NLRV</td>
<td>Nilaparvata lugens reovirus</td>
</tr>
<tr>
<td>nPCR</td>
<td>nested PCR</td>
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<tr>
<td>NS</td>
<td>non-structural</td>
</tr>
<tr>
<td>NS1</td>
<td>non-structural (virus protein) 1</td>
</tr>
<tr>
<td>NSP</td>
<td>non-structural protein</td>
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<td>NSW</td>
<td>New South Wales, Australia</td>
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<tr>
<td>NT</td>
<td>nucleotide</td>
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<tr>
<td>NTC</td>
<td>no template control</td>
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<td>NVSL</td>
<td>National Veterinary Services Laboratory, Ames, USA</td>
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<td>OBP</td>
<td>Onderstepoort Biological Products, Onderstepoort, South Africa</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>OIE</td>
<td>Office International des Epizooties (World Organisation for Animal Health)</td>
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<tr>
<td>opg</td>
<td>oocysts per gram</td>
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<td>ORUV</td>
<td>Orungo virus</td>
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<td>OVI</td>
<td>Onderstepoort Veterinary Institute, Onderstepoort, South Africa</td>
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<tr>
<td>PALV</td>
<td>Palyam virus</td>
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<tr>
<td>PAUP</td>
<td>phylogenetic analysis using parsimony</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>pc</td>
<td>post challenge</td>
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<tr>
<td>PCB</td>
<td>printed circuit board</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>pfu</td>
<td>plaque-forming units</td>
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<tr>
<td>pg</td>
<td>picogram</td>
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<tr>
<td>PHSV</td>
<td>Peruvian horse sickness virus</td>
</tr>
<tr>
<td>pi</td>
<td>post inoculation</td>
</tr>
<tr>
<td>$P_i$</td>
<td>monophosphate</td>
</tr>
<tr>
<td>pI</td>
<td>post infection</td>
</tr>
<tr>
<td>PPi</td>
<td>pyrophosphate</td>
</tr>
<tr>
<td>PSEK</td>
<td>porcine stable equine kidney</td>
</tr>
<tr>
<td>pv</td>
<td>post vaccination</td>
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<tr>
<td>R&amp;D</td>
<td>research and development</td>
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<td>RAPD</td>
<td>random amplification of polymorphic DNA</td>
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<tr>
<td>RAPID</td>
<td>ruggedised advanced pathogen identification device</td>
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<td>RBC</td>
<td>red blood cell</td>
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<td>RDCC</td>
<td>Regional Disease Control Centre, Italy</td>
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<td>RDV</td>
<td>rice dwarf virus</td>
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<td>RE</td>
<td>restriction endonuclease digest</td>
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Abbreviations

RELSU Reggio Emilia Local Sanitary Unit, Italy
REML restricted maximum likelihood
RNA ribonucleic acid
RPL ribosomal protein L
RRSV rice ragged stunt virus
RSA Republic of South Africa
RT reverse transcriptase
RV rotavirus
S7 small segment
SC seroconversion
SCC somatic cell count
SCRV St Croix River virus
SeaWiFS Sea-viewing Wide Field-of-view Sensor (NASA)
SECYT Science and Technology Secretary, Argentina (Secretaría de Ciencia, Tecnología e Innovación Productiva)
sem standard error means
SENASA National Animal Health Service, Argentina (Servicio Nacional de Sanidad y Calidad Agroalimentaria)
SGEB salivary gland escape barrier
SGIB salivary gland infection barrier
SN serum neutralisation
SNT serum neutralisation test
SPF specific pathogen-free
SPM spatial process model
SPS serologically positive sentinel
SR seroconversion rate
ssRNA single-stranded RNA
STD standard deviation
TAIR air temperature
TC tissue culture
transcriptase complex
TCID tissue cell infective dose
TF/R-1 terminal primers
Tm temperature
TMEV Tembe virus
TNF tumour necrosis factor
TOT transovarial transmission
TOTB transovarial transmission barrier
TPF total photon flux
TRV Tracambe virus
UK United Kingdom
UMAV Umatilla virus
UNESP University of São Paulo State, Brazil (Universidade Estadual Paulista 'Júlio Mesquita Filho')
UNMIK United Nations interim administration mission in Kosovo
USA United States of America
USAHA United States Animal Health Association
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>USDA</td>
<td>United States Department of Agriculture</td>
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<tr>
<td>VAERS</td>
<td>Vaccine Adverse Event Reporting System (Centers for Disease Control and Prevention and the Food and Drug Administration, USA)</td>
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<td>Vero</td>
<td>African green monkey cell lines</td>
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<td>VI</td>
<td>virus isolation</td>
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<td>VIB</td>
<td>viral inclusion bodies</td>
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<td>VLP</td>
<td>virus-like particle</td>
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<td>VMRD</td>
<td>Veterinary Medical Research and Development, Pullman, United States of America</td>
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<td>VN</td>
<td>virus neutralisation</td>
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<td>VNT</td>
<td>virus neutralisation test</td>
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<td>VP</td>
<td>viral protein</td>
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<td>VNT</td>
<td>virion protein</td>
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<td>VS</td>
<td>viral suspension</td>
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<td>v/v</td>
<td>volume/volume</td>
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<td>WALV</td>
<td>Wallal virus</td>
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<td>WARV</td>
<td>Warrego virus</td>
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<td>WC-1</td>
<td>white collar-1</td>
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<td>WGRV</td>
<td>Wongorr virus</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<td>WMV</td>
<td>Wad Medani virus</td>
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<td>WTO</td>
<td>World Trade Organization</td>
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<td>YOV</td>
<td>Yunnan orbivirus</td>
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</table>
A comparison of different orbivirus proteins that could affect virulence and pathogenesis

H. Huismans, V. van Staden, W.C. Fick, M. van Niekerk & T.L. Meiring

Department of Genetics, University of Pretoria, Pretoria 0002, South Africa

Summary

The factors that determine the virulence and pathogenesis characteristics of bluetongue virus (BTV), African horse sickness virus (AHSV) and other orbiviruses are not well known. With respect to the viral proteins that are expected to play a role it may be assumed that proteins, such as the outer capsid proteins VP2 and VP5, that are involved in the attachment of virus particles to target cells and influence replication efficiency are particularly important. Equally important are viral proteins such as non-structural protein NS3 that influence the release of virus particles from a target host or vector cell. The authors compare the amino acid sequence variation, structural motifs and some phenotypic characteristics of proteins VP2, VP5 and NS3 of different orbiviruses, such as AHSV, BTV and equine encephalosis virus (EEV). The most variable protein is VP2 and a pair-wise alignment of VP2 sequences of different serotypes of both BTV and AHSV indicated variation of between 48% to 64% and 46% to 52% for most isolates, respectively. Several regions of high variability can be identified. VP5 of BTV is much less variable than VP2 but still more so than the cognate AHSV VP5. In contrast, the NS3 protein of AHSV is much more variable than its BTV or EEV counterpart with maximum levels of NS3 variation up to 36% as compared to 10% for BTV. The AHSV NS3 variation is clustered into three discreet phylogenetic groups. All orbivirus NS3/NS3A proteins share a number of highly conserved structural features that include two hydrophobic domains (HD1 and HD2) that are involved in the interaction with the membrane. Most of the NS3 variation is located in HD1 and the adjacent variable region between HD1 and HD2. In the case of AHSV this region only has 13% identity compared to 64% in the case of BTV. NS3 of AHSV is also a highly toxic protein and mutation analysis has indicated that the toxicity is associated with the two hydrophobic domains. Expression of NS3 deletion mutants in bacterial cells has shown that both HD1 and HD2 are necessary for cytotoxicity and that removal of the adjacent N-terminal domains increases cytotoxicity. Preliminary results with different AHSV strains and the corresponding NS3 equivalent have indicated that the membrane permeabilisation effect of the individual NS3 proteins correlate with the permeabilisation effect of the corresponding viruses. These results would suggest that characterisation of the NS3 protein by itself might predict some phenotypic characteristics and potential membrane destabilisation effect of the corresponding virus.

Keywords


Introduction

Although the pathogenesis and the clinical aspects related to the disease caused by orbiviruses such as bluetongue virus (BTV), African horse sickness virus (AHSV) and equine encephalosis virus (EEV) have been well characterised (7, 10, 24, 38), very little is known about the molecular basis of virulence and pathogenesis of these viruses (22). Most molecular studies have been focused on BTV, the orbivirus prototype, but in recent years an increasing number of the other orbivirus proteins have been studied in greater detail. The increase in sequence data within a serogroup has also enabled more comparative and functional analysis of the cognate proteins in different serogroups (43). The authors report on the
sequence variation in a few of the orbivirus proteins that are presumed to be associated with virulence or pathogenesis. The possible involvement of an orbivirus protein in the disruption of the cellular membrane is also addressed.

The factors involved in the pathogenesis and virulence of orbiviruses are complex and multifactorial (22, 29, 37, 50). All the different steps in the viral replication cycle are potentially involved. The first of these is the interaction of the outer capsid proteins with cellular receptors resulting in viral entry and penetration into the cell (17). Also important in pathogenesis are the steps associated with removal of the outer capsid layer, the release of core particles in the cytoplasm (16) and the general velocity of viral replication. Equally important are possible differences in the cytopathogenicity of different virus strains and the budding and release of viruses from a cell (37). The latter may have a major impact on pathogenesis as it determines the spread of infection within and between organs. The different steps of viral replication are in turn influenced by the intracellular milieu, induced cellular functions and the capacity of the host to develop a proper immune response (37).

Pathogenesis may therefore be determined by any or a selected combination of the orbivirus viral proteins and host factors. Some viral proteins are nevertheless more likely than others to be important in determining pathogenicity. These are the proteins involved in the interaction with cellular receptors as well as those involved in uncoating and the spread of virus particles in and between cells. Proteins that affect membrane destabilisation are also of particular importance since these proteins often induce cytopathogenicity that could play a role in disease symptoms. In orbiviruses, this focuses the attention on the two outer capsid proteins involved in virus entry, as well as on the non-structural protein involved in the release of virus particles from a cell.

Orbivirus particles are icosahedral structures composed of a core particle or inner capsid surrounded by an outer capsid layer (20, 34, 35, 48, 49). The core is composed of two major structural proteins (VP7 and VP3) and three minor structural proteins (VP1, VP4 and VP6) that enclose a genome of ten dsRNA segments. The minor proteins have different enzymatic activities such as RNA polymerase (VP1), RNA capping (VP4) and helicase activity (VP6) that all support the role of the core proteins in viral replication and transcription (30, 31, 39). Outer capsid protein VP2 is primarily involved in cell attachment and virus penetration (17). VP2 is also the major determinant of serotype specificity (9, 17, 19) and involved in the induction of a neutralisation-specific antibody immune response. After entry of the virion into the cell, the virus is enclosed in endocytotic vesicles in which the outer capsid is removed, resulting in the release of transcriptionally active core particles into the cytoplasm. The other outer capsid protein, VP5, appears to play a major role in the destabilisation of the membrane of endocytosed vesicles (16).

In addition to the structural proteins, the viral genome encodes four non-structural proteins, NS1, NS2, NS3 and NS3A (2, 34, 35). NS3 and NS3A are encoded from two in-phase overlapping reading frames from the smallest of the ten genome segments (44). BTV NS3 is proposed to play a role in the final stages of BTV morphogenesis and release of virions from the cell (21). Baculovirus expressed AHHSV NS3 is membrane-associated and cytotoxic in insect cells (41, 46). It has been suggested that it plays a role in virulence and influences the timing of virus release from infected cells (25, 29).

The orbivirus proteins that are assumed to play an important role in virulence and pathogenesis are therefore the major cellular attachment protein VP2 and the membrane destabilising proteins VP5 and NS3. In this paper, the sequence variation in these proteins is compared, identifying some of the conserved and variable amino acid sequence patterns and motifs on these cognate proteins. A unique feature of AHHSV NS3 proteins is that it is highly variable amongst the different AHHSV serotypes (42, 43). We have also compared different AHHSV NS3's and NS3 deletion mutants with respect to their cytotoxic effect on bacterial and eukaryotic cells.

**Percentage variation in the VP2, VP5 and NS3 proteins of different African horse sickness virus, bluetongue virus and equine encephalitis virus isolates**

The VP2, VP5 and NS3 amino acid sequences of BTV, AHHSV and EEV used in the analysis were obtained from GenBank. The origins of the various sequences are summarised in Table I and the proteins compared by means of pairwise alignment (PAUP version 4.0b8). The results are displayed in Figure 1. VP2 is the most variable of the proteins, with more than 80% of all BTV isolates showing a variation of between 48% to 64% between different serotypes. The minimum level of variation between serotypes is about 28%. The AHHSV VP2 proteins are less variable, with almost 90% of all isolates varying between 46% to 52% with a minimum of 28% variation between different serotypes.
Variable and conserved domains of VP2, VP5 and NS3

The observed variation in the different sequences is not evenly distributed over the total length of the different proteins. As illustrated in Figure 2 in the case of protein VP2, regions of high sequence diversity are flanked by more conserved sequences. The result shows the percentage of identical conserved amino acids in 150 amino sections of the different VP2 proteins of BTV and AHSV for which sequence data is available. The C-terminal region is the most conserved region of VP2, both for AHSV and BTV. In the case of AHSV, the 150 amino acids at the N-terminus are also more conserved. The most variable regions in AHSV are in the region of amino acid 600 to 690, as well as in the region of amino acid 220 to 410. The currently identified neutralisation specific epitopes fall within these variable regions (4, 9, 26, 27, 47).

The most variable BTV VP2 region is also from amino acids 590 to 690 with another highly variable region from amino acid 150 to 290. These regions also include the epitopes that determine serotype specificity (5, 12, 18, 28, 33). The AHSV and BTV proteins differ in length, complicating any comparisons. Furthermore, the very high variability of VP2 makes it very difficult to assign or identify specific VP2 virulence markers.

The results obtained with VP5 (not shown) are very similar, although the proteins are much less variable. A uniquely conserved feature is the presence of the amphipathic helices at the N-terminal ends of both BTV and AHSV. This region is involved in
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membrane destabilisation (16) and therefore potentially important in determining virulence characteristics. It is a highly conserved region and the limited number of virulent and avirulent AHSV isolates that were compared did not reveal any mutations in the amino acids that affect the amphipathic helices.

The variable and conserved domains of BTV, AHSV and BTV NS3 have been very well characterised (2, 3, 44, 45) and are shown in Figure 3. These regions include the amphipathic helix at the N terminus, the in-phase NS3/NS3A overlapping reading frames in the NS3 encoding genes, the proline rich region, the conserved domain, the two hydrophobic domains predicted to be transmembrane regions, and the variable region between the two domains. The percentage identity of the domains shown in Figure 3 reflects the result in Figure 1, illustrating that AHSV NS3 is more variable than the cognate EEV and BTV proteins. By far the most variable domains in AHSV NS3 are hydrophobic domain HD1 and the variable region between HD1 and HD2. In the case of BTV and EEV NS3 the sequences of HD1 are 73% and 64% conserved, respectively, whereas the AHSV HD1 domain shows no more than 35% identity. Even more variable is the region situated between the two hydrophobic domains, with only 13% identity in the case of AHSV compared to 64% and 61% in the corresponding BTV and EEV regions. It is not yet known if and how proteins such as NS3 contribute to the overall pathogenesis of the disease. If it does play a role, the sequence variation in hydrophobic

![Figure 2](image-url)

**Figure 2**

Variation in the VP2 amino acid sequence of bluetongue virus and African horse sickness virus

The numbers shown in the blocks are sequence identity based on the percentage of aligned identical amino acids in 150 amino acid sections of the 9 AHSV and the 10 BTV VP2 sequences indicated in footnotes (b) and (c) in Table I. The dark bars indicate the broad regions where neutralisation specific epitopes have been located in both viruses. Numbers below indicate amino acid residues.

![Figure 3](image-url)

**Figure 3**

Comparison of the conserved amino acid sequence elements of the NS3 proteins of different orbiviruses

Numbers within the sections shown the percentage sequence identity for that region, based on the percentage of aligned identical amino acid sequences of the NS3 proteins indicated in footnotes (f), (g) and (h) in Table I.
domains and the intermediate region could reveal strain differences that could be important in determining phenotypic characteristics of the virus associated with virulence and disease.

**Possible relevance of NS3 and NS3 variation in disease**

NS3 has a cytotoxic effect on its host cells when expressed in a baculovirus system (45), resulting in the death of approximately 90% of an infected cell population at 48h post infection. Site-specific mutations in either of the two hydrophobic domains, predicted to be transmembrane regions, abrogated this cytotoxicity. However, modifications to other regions did not affect the detrimental effect of NS3 on its host cells. As illustrated in Figure 4, this includes deletion of the N-terminal 13 amino acids of NS3 that are predicted to form an amphipathic α-helix and mediate interaction with a cellular membrane trafficking protein in BTV (3). Therefore the cytotoxicity of AHSV NS3 is dependent on its membrane topography, and this involves both hydrophobic domains HD1 and HD2 (41).

Figure 4
Effect of recombinant baculoviruses expressing African horse sickness virus NS3 or NS3 mutants on viability of Sf9 insect cells
Cells were infected with recombinant baculoviruses expressing AHSV-3 NS3 (Bac-NS3), a truncated NS3 lacking the 11 N-terminal amino acids (Bac-NS3A), NS3 with 4 non-polar amino acids substituted with charged amino acids in the first hydrophobic domain (Bac-HD1) or in the second hydrophobic domain (Bac-HD2)
Mock refers to uninfected Sf9 cells
Cell viability was determined by staining aliquots of cells at three-hourly intervals over a 48-h period with 0.2% trypan blue and counting stained (non-viable) cells

The mechanism whereby NS3 causes cell death is not known, however it shares many structural properties with a class of molecules termed viroporins. Viroporins are small viral proteins that interact with membranes, thereby modifying cellular membrane permeability (1, 6, 11, 13). This leads to changes in the metabolism and morphology of the cell, and promotes the release of viral particles (6, 13). This raises the questions of whether NS3 causes cell death by modification of the membrane permeability of the host cell and whether the high level of sequence variation reflects on any phenotypic, virulence or disease related properties of either the NS3 protein or the parental virus. One NS3 protein representing each of the three discreet phylogenetic clusters α, β and γ were selected, namely AHSV-4 (α), AHSV-3 (β) and AHSV-2 (γ) and investigated for its effect on mammalian cell membrane permeability.

The NS3 genes encoding the relevant proteins were cloned and expressed in Sf9 insect cells using the baculovirus expression system. Crude cell extracts from Sf9 cells expressing the three different NS3 proteins were prepared, and approximately equimolar amounts added externally to Vero cells. The permeabilisation of the Vero cell membranes was investigated using the hygromycin B (Hyg B) translation inhibition assay (23, 32). Only permeabilised cells allow the entry of the translation inhibitor Hyg B to inhibit the [35S]-methionine uptake of cellular proteins. Using this method the percentage of permeabilised Vero cells could be calculated from the [35S] incorporation in the presence of Hyg B divided by the control [35S] incorporation in the absence of Hyg B for each sample. The results are shown in Figure 5. There were distinct differences in the degree of membrane permeabilisation caused by the different NS3 proteins. The addition of lysates containing AHSV-2 NS3 resulted in 72% permeabilisation of cells, AHSV-3 caused 62% permeabilisation and

Figure 5
Membrane permeabilisation of Vero cells 180 min after addition of Sf9 cell lysates without NS3 or with NS3 from three different African horse sickness strains, measured using the protein synthesis inhibitor hygromycin B
The percentage [35S]-methionine incorporated into the permeabilised cells was calculated as follows:

\[
\% \text{ permeabilisation} = \frac{[35S] \text{ incorporation in the presence of Hyg B}}{[35S] \text{ incorporation in the absence of Hyg B}} \times 100
\]
Bluetongue virus and disease

AHSV-4NS3 caused 47%. NS3-free lysates from wildtype baculoviruses caused only a slight 15% increase in membrane permeabilisation after 3 h.

It was subsequently investigated whether the effect of the different NS3 proteins correlated with the effect of AHSV infection. The corresponding AHSV-2, AHSV-3 and AHSV-4 virus strains were used to infect Vero cells, and the Hyg B assay described above used to monitor the membrane permeabilisation at different times post infection (Fig. 6). Infection with AHSV-2, AHSV-3 and AHSV-4 resulted at 24 h post infection in 49%, 29% and 9% membrane permeabilisation, respectively, compared to the 2% in the case of mock-infected cells. The two results are diagrammatically compared in Figure 7. In both cases, AHSV-2 (whole virus or AHSV-2 NS3) has the most drastic effect on cell membrane permeability, followed by AHSV-3 with an intermediate effect and AHSV-4 with the least severe effect. Although permeability as the result of virus infection could be the result of other viral proteins and the interaction of multiple factors, and does not necessarily relate directly to pathogenesis, it is tempting to speculate that NS3 acts as a viroporin, and is a key determinant of the cellular cytopathogenicity of AHSV.

![Figure 6](image6.png)

**Figure 6**
Membrane permeabilisation of Vero cells following infection with AHSV-2, AHSV-3 or AHSV-4 compared to mock infected control cells

Membrane permeabilisation assayed as in Figure 5

The effect of NS3 on bacterial cells

The cytotoxic nature of NS3 was further investigated in bacterial cells, using the pET vector expression system (40). This tightly regulated inducible system is particularly amenable for the synthesis of toxic proteins and has been used for analysing the cytotoxic proteins of a number of viruses. This includes the p10 protein of avian reovirus (6), NSP4 of rotavirus (8), Vpu and gp41 of HIV-1 (14) and the M2 protein of influenza virus (15).

![Figure 7](image7.png)

**Figure 7**
Comparison of the effect of externally added NS3 proteins (A) versus infection with the African horse sickness virus strains harbouring the corresponding NS3 genes (B) to Vero cell membrane permeability

The full-length NS3 gene and a series of NS3 truncated mutants (M1, M2, M3, M4, M5 and M6) were prepared and cloned in pET41c vectors (Fig. 8). The mutants represent different N-terminal and C-terminal truncations, including deletions of either one or both hydrophobic domains HD1 and HD2 (see diagrammatic representation in Fig. 8). The wild-type and mutant proteins were expressed in *Escherichia coli* cells and the effect on cell growth monitored at various times after induction by measuring the optical density at 600 nm. A growth curve indicating the percentage increase compared to a non-cytotoxic control was constructed for each of the mutants (results not shown). The results clearly indicated that the full-length NS3 protein was detrimental to *E. coli* growth. The results at 4 h post induction are summarised in Figure 8. The growth of cells expressing full-length NS3 was inhibited by 35% compared to the control. The expression of the C-terminal truncated M1 and M2 mutants that did not contain either the HD1 or HD2 hydrophobic domains did not inhibit bacterial growth in any way. The expression of the M3 and M4 mutants that each contained only one of the two hydrophobic domains had very little effect on cell growth, inhibiting growth by 10% and 5%, respectively. However, a dramatic inhibitory effect was seen in the case of the M5 and M6 mutants that each contained both hydrophobic domains. As early as 1 h after induction, cell density was already noticeably affected and by 4 h post induction, growth in the M5 and M6 mutants were inhibited by as much as 92% and 70%, respectively. Truncation of the regions adjacent to the hydrophobic domains therefore appears to
The cytotoxic effect of expressing different NS3 truncation mutants in *Escherichia coli* cells

A diagrammatic representation of the different mutants M1 to M6 is shown.

The mutants were prepared and cloned in pET41c vectors.

The percentage inhibition in cell growth after expressing the wild-type NS3 or the respective mutants M1 to M6 is given on the right.

Cell growth was measured by optical density readings at 600 nm, 4 h post induction.

Figure 8

The cytotoxic effect of expressing different NS3 truncation mutants in *Escherichia coli* cells.

The results also suggest that NS3 cytotoxicity requires both the hydrophobic domains.

**Conclusion**

The comparison between the variability of different orbivirus proteins that could be involved in virulence or pathogenicity indicated that VP2 and VP5 of BTV are more variable proteins than the cognate AHSV proteins. Distinct regions of high variability were identified in the internal sequences of VP2 of both viruses. In contrast, the NS3 protein of AHSV is much more variable than its BTV counterpart. AHSV NS3 is also a highly toxic protein and the expression of NS3 in bacterial cells appears to mimic the cytotoxic effect observed in mammalian cells.

Mutation analysis indicated that cytotoxicity is associated with the presence of both the hydrophobic domains. Preliminary results have also suggested that the membrane permeabilisation effect of an individual NS3 protein correlates with the permeabilisation effect of the corresponding virus. These results would suggest that characterisation of an NS3 protein by itself might predict some phenotypic characteristics and potential membrane destabilisation effect of the corresponding virus. If the NS3 cytotoxicity can be linked to membrane permeabilisation and disease in future studies, the NS3 sequence might be able to predict some of the disease characteristics of the corresponding virus.

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**References**


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Bluetongue virus replication, molecular and structural biology

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Summary

The icosahedral bluetongue virus (BTV) particle (~80 nm diameter) is composed of three distinct protein layers. These include the subcore shell (VP3), core-surface layer (VP7) and outer capsid layer (VP2 and VP5). The core also contains ten dsRNA genome segments and three minor proteins (VP1[Pol], VP4[CaP]and VP6[Hel]), which form transcriptase complexes. The atomic structure of the BTV core has been determined by X-ray crystallography, demonstrating how the major core proteins are assembled and interact. The VP3 subcore shell assembles at an early stage of virus morphogenesis and not only determines the internal organisation of the genome and transcriptase complexes, but also forms a scaffold for assembly of the outer protein layers. The BTV polymerase (VP1) and VP3 have many functional constraints and equivalent proteins have been identified throughout the Reoviridae, and even in some other families of dsRNA viruses. Variations in these highly conserved proteins can be used to identify members of different genera (e.g. by comparing the polymerase) and different virus species (serogroups) within the genus Orbivirus (e.g. by comparison of VP3). This has helped to identify three new genera within the Reoviridae and two new Orbivirus species. In contrast, sequences of the BTV outer capsid proteins (involved in interactions with neutralising antibodies) are much more variable (particularly VP2) and comprehensive sequence analyses for the 24 types demonstrate that they can be used to identify BTV serotype. The 21 species (158 serotypes) currently recognised within the genus Orbivirus are listed, along with 11 unassigned viruses.

Keywords


Introduction: bluetongue virus classification and virion structure

The family Reoviridae currently contains twelve genera of multi-segmented dsRNA viruses, including pathogens of a wide range of insects, reptiles, fish, crustaceans, mammals (including humans), plants and fungi (31), many of which are of economic, veterinary or medical importance. These viruses can be distinguished and identified by a number of different characteristic features, including capsid structure, number and size distribution of genome segments, host range, serological properties, protein composition, disease symptoms and most recently by sequence analyses and comparisons of individual genome segments. Indeed, these sequencing studies and phylogenetic comparisons have helped to identify three new genera within the family Reoviridae and two new species of Orbivirus (Tables I and II) that will be included in the Eighth Report of the International Committee for the Taxonomy of Viruses (ICTV), due to be published in 2004.

The orbiviruses (which are classified as members of the genus Orbivirus, within the family Reoviridae) characteristically have a ten-segmented dsRNA genome that is packaged as one copy of each segment within an icosahedral protein capsid (~85 nm diameter). Bluetongue virus (BTV) is the prototype species of twenty-one different Orbivirus
### Table I

**Virus genera of the family Reoviridae**

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. of genome segments</th>
<th>No. of species</th>
<th>No. of types (serotypes)</th>
<th>Tentative or unassigned isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Orthoreovirus</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>2. Orbivirus</td>
<td>10</td>
<td><strong>21</strong></td>
<td><strong>160</strong></td>
<td><strong>11</strong></td>
</tr>
<tr>
<td>3. Cypovirus</td>
<td>10</td>
<td>16</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>4. Aquareovirus</td>
<td>11</td>
<td>6</td>
<td>unknown</td>
<td>5</td>
</tr>
<tr>
<td>5. Rotavirus</td>
<td>11</td>
<td>5</td>
<td>&gt;23</td>
<td>2</td>
</tr>
<tr>
<td>6. Calicivirus</td>
<td>12</td>
<td>2</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>7. Seadornavirus</td>
<td>12</td>
<td>3</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>8. Fijivirus</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>9. Phytoreovirus</td>
<td>12</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>10. Oryzavirus</td>
<td>10</td>
<td>2</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>11. Mycoreovirus</td>
<td>11 or 12</td>
<td>3</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>12. Idnoreovirus*</td>
<td>10</td>
<td>5</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

* the creation of the three new genera *Seadornavirus*, *Mycoreovirus* and *Idnoreovirus* has been approved by the International Committee for the Taxonomy of Viruses. The name *Idnoreovirus* is derived from ‘Insect derived non-occluded reovirus’

### Table II

**The Orbivirus species**

<table>
<thead>
<tr>
<th>Orbivirus species currently recognised</th>
<th>No. of serotypes/strains</th>
<th>Tentative species/ unassigned viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. African horse sickness virus (AHSV)</td>
<td>9 serotypes</td>
<td>Andasibe virus (ANDV)</td>
</tr>
<tr>
<td>2. Bluetongue virus (BTV)</td>
<td>24 serotypes</td>
<td>Ife virus (IFEV)</td>
</tr>
<tr>
<td>3. Changuinola virus (CGLV)</td>
<td>12 serotypes</td>
<td>Itupiranga virus (ITUV)</td>
</tr>
<tr>
<td>4. Chenuda virus (CNUV)</td>
<td>7 serotypes</td>
<td>Japanaut virus (JAPV)</td>
</tr>
<tr>
<td>5. Chobar Gorge virus (CGV)</td>
<td>2 serotypes</td>
<td>-</td>
</tr>
<tr>
<td>6. Corriparta virus (CORV)</td>
<td>6 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Kammavanpattai virus (KMPV)</td>
</tr>
<tr>
<td>7. Epizootic haemorrhagic disease virus (EHDV)</td>
<td>10 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Lake Clarendon virus (LCV)</td>
</tr>
<tr>
<td>8. Equine encephalosis virus (EEV)</td>
<td>7 serotypes</td>
<td>Matucare virus (MATV)</td>
</tr>
<tr>
<td>9. Eubenangee virus (EUBV)</td>
<td>4 serotypes</td>
<td>Tembe virus (TMEV)</td>
</tr>
<tr>
<td>10. Ieri virus (IERIV)</td>
<td>3 serotypes</td>
<td>Codajas virus (COV)</td>
</tr>
<tr>
<td>11. Great Island virus (GIV)</td>
<td>36 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>Tracame virus (TRV)</td>
</tr>
<tr>
<td>12. Lebombo virus (LEBV)</td>
<td>1 serotype</td>
<td>Yunnan orbivirus (YOV)</td>
</tr>
<tr>
<td>13. Orungo virus (ORUV)</td>
<td>4 serotypes</td>
<td>-</td>
</tr>
<tr>
<td>14. Palyam virus (PALV)</td>
<td>13 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>15. Peruvian horse sickness virus (PHSV)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 serotype</td>
<td>-</td>
</tr>
<tr>
<td>16. St Croix River virus (SCRV)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 serotype</td>
<td>-</td>
</tr>
<tr>
<td>17. Umatilla virus (UMAV)</td>
<td>4 serotypes</td>
<td>-</td>
</tr>
<tr>
<td>18. Wad Medani virus (WMV)</td>
<td>2 serotypes</td>
<td>-</td>
</tr>
<tr>
<td>19. Wallal virus (WALV)</td>
<td>3 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>20. Warrengo virus (WARV)</td>
<td>3 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>21. Wongorr virus (WGRV)</td>
<td>8 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>21 virus species</td>
<td>160 serotypes/strains&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> in some species the serological relationships between strains has not been fully determined

<sup>b</sup> two new species of *Orbivirus* (SCRV and PHSV) have recently been recognised by the International Committee for the Taxonomy of Viruses, based primarily on genome segment sequence analyses and comparison (3, 33); Ndelle virus (previously classified as an *Orbivirus*) was also reclassified as an *Orthoreovirus* on this basis (2)
species now recognised by the ICTV. It has a capsid composed of three distinct protein layers (Figs 1 and 2): the subcore, composed of VP3 (T2); the core-surface layer, composed of VP7 (T13); and the outer capsid layer, composed of VP2 and VP5.

The earlier development of effective purification methods for BTV virus particles and cores (Figs 1 and 3) (28) not only provided material for electrophoretic analyses and biochemical studies of viral proteins/RNAs. It also provided homogeneous particles in sufficient quantity and purity for crystallisation (Fig. 3) and X-ray diffraction studies of the core particle (5) leading to the resolution of its atomic structure (6, 10, 13). It has not been possible to crystallise intact BTV virus particles and consequently it has not yet been possible to derive a high-resolution atomic structure for components of the outer capsid shell. However, the BTV outer capsid structure has previously been determined by cryo-electron microscopy (EM) (15, 16). These studies demonstrated that it is composed of 180 copies of the Mr $111 \times 10^3$, ‘sail-shaped’ VP2 protein, arranged as trimeric ‘triskellion’ structures, together with 360 copies of an inter-dispersed and underlying VP5 protein (Mr $59 \times 10^3$), which also appears to be arranged as 120 trimers. Figure 2 shows a schematic representation of the BTV particle structure derived from these X-ray crystallography and EM structural studies. Additional biochemical data concerning the individual BTV proteins are available on the dsRNA virus page on the Institute for Animal Health website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/Orbivirus.htm).

Figure 1
Electron micrographs of negatively stained virus and core particles of BTV-1, purified Mertens et al. (28)

Figure 2
Schematic diagram illustrating the structure of the bluetongue virus particle derived from biochemical, X-ray crystallography and cryo-electron microscopy

VP2 trimer
VP5 trimer
VP7 (T13) trimer
P3 (T2) decamer
Transcriptase complex
VP6 hexamer?
VP1(Pol) monomer
VP4(Cap) dimer
10 segments of dsRNA, as 4 stacked spirals of five-fold axes

Figure 3
A self forming CsCl gradient used to purify core particles of BTV-1 and a typical crystal of native BTV-10 core particles

The presence of only a single band that contains ~20 mg of core particles demonstrates the homogeneity and purity of the core preparation

The image on the right shows a typical crystal of native BTV-10 core particles, approximately 0.5 mm in diameter

>1 000 of these crystals were used in studies to determine the atomic structure of the BTV core (13)

The bluetongue virus outer-capsid proteins and determination of serotype

The structure of the outer capsid layers of the different species and genera of viruses within the
family Reoviridae are highly variable (31), reflecting their responsibility for virus transmission between individuals of widely different host species, and their role in mediating cell attachment and penetration of different cell types. In many cases, it is impossible to identify exactly comparable outer capsid proteins from the different genera. In mammalian hosts, these outer surface proteins also interact with components of the immune system of the hosts, inducing neutralising antibodies. Consequently they are subject to antibody selective pressure, leading to higher levels of sequence variation even within a single virus genus or species.

Indeed, the components of the BTV outer capsid, proteins VP2 and VP5, are the most variable of the viral proteins. VP2, in particular, contains neutralising epitopes and by controlling the specificity of virus particle interactions with neutralising antibodies, determines the identity of the 24 BTV serotypes that are currently recognised using serum neutralisation (SN) assays. Sequence analyses of genome segment 2 (23, 24, 25) and segment 6 (37) from representative isolates of all 24 BTV serotypes have recently been completed. Phylogenetic comparisons have demonstrated that variations in the nucleotide sequences of segment 2 and 6 and in the amino acid sequences of VP2 and VP5 show a high correlation with virus type as determined by SN assays (23, 24, 25, 37). This is particularly true for BTV genome segment 2, where oligonucleotide primers designed for reverse transcriptase-polymerase chain reaction (RT-PCR) assays and sequencing studies can be used to rapidly and reliably identify virus serotype (23, 24, 25).

**Bluetongue virus cell entry and initiation of virus replication**

A schematic representation of the BTV replication cycle is shown in Figure 4 (27). Infecting BTV particles are taken up via an endosomal route (19). The reduction of pH within the early endosome is thought to release outer capsid components from the virus core, which is then released into the host cell cytoplasm. Individually expressed VP5 is toxic, causing cell fusion (14) and is consequently thought to play a role in penetration of the endosomal membrane and the release mechanism. However, the BTV core particle contains no VP5, but it is also infectious in its own right for both Culicoides and some mammalian cell systems (30). Antibodies to the outer core protein VP7 will bind to and neutralise core particles but not fully intact virus (18). This suggests that although VP7 (which is not exposed on the surface of fully intact virions) (18) can mediate both cell attachment and penetration of the BTV core, it is not involved in cell attachment of the intact virion. The BTV core can bind to the cell surface via the interaction of VP7 with glycosaminoglycans, although other receptors may also be required for cell entry (18). Core particle infectivity, unlike that of the intact virus, is also independent of the pH reduction in the endosome (18) and is unaffected by treatment of cells with either ammonium sulphate or Concanamycin A (a drug

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**Figure 4**

Schematic diagram representing the lytic replication cycle of bluetongue virus (27)
that specifically blocks the vacuolar ATPase, thereby raising the pH of the endosome). It appears likely that this reflects the absence of an outer capsid layer that must be removed in order to activate the core-associated RNA-dependent RNA polymerase. It also suggests that release of the outer capsid proteins or exposure of VP7 may be essential for transport of the core into the cell cytoplasm from the endosome. However, it is also possible that the BTV core enters cells via a different route.

**Virus replication and transcription**

In order to infect and successfully replicate in their target host species, the dsRNA viruses have to overcome a number of specific biochemical problems. dsRNA molecules are ineffective as mRNA for translation and cannot themselves function as templates for host cell transcriptases. BTV must therefore provide its own transcription and capping enzymes and carry them into the host cell at the initiation of infection, in order to synthesise the mRNAs required for synthesis of viral proteins. However, many host cells also contain antiviral defence mechanisms (20) including induction of apoptosis, interferon production, modification of host-cell translation mechanisms and even RNA silencing (8, 9). Many of these mechanisms recognise and would be activated by naked dsRNA within the host cell cytoplasm (20). Indeed, the ubiquitous nature of these defences in many different animals and plants suggests that the dsRNA viruses are themselves an ancient lineage that has diversified, along with their host species, from common ancestors.

In order to avoid exposure to the host cell cytoplasm and activation of host defences, many of the dsRNA viruses (including the orbiviruses) retain their genomes, and mRNA synthesising enzymes, within stable closed-protein capsids. These ‘nano’ transcription machines form the basic infectious unit of the virus and must be delivered intact into the host cell cytoplasm in order to initiate replication. The removal of viral proteins VP2 and VP5 during cell entry activates the transcriptase functions of the core particle, allowing it to synthesise and cap full length mRNA copies of the ten genome segments, while they are still packaged within the core itself. Some further disassembly of cores may occur (17), although this is not thought to be a functional part of the replication process and the majority of core particles appear to remain intact throughout the replication cycle (7).

**The major protein components of the bluetongue virus core structure**

The structures of the core of BTV serotypes 1 (BTV-1) and 10 (BTV-10) (containing ~1 000 protein molecules) have been determined to a resolution of 3.5 Å and 6.5 Å respectively, the latter using crystals with unit cell parameters in excess of 1 000 Å (10, 13). These structures have revealed the organisation of the protein bilayer that makes up the icosahedral capsid of the BTV core.

The innermost complete capsid shell of the BTV core (the subcore) is composed of 120 copies of the viral protein VP3 (901 amino acids, 103 kD), arranged with T=2 icosahedral pseudo-symmetry (Fig. 5). This requires VP3 to occupy two different conformations, identified as A and B, in order to form a complete capsid shell. The VP3 A and B molecules are chemically identical but undergo a conformational shift between their internal ‘apical’, ‘carapace’ and ‘dimerisation’ domains, allowing them to interact and close the surface of the icosahedron.

![Figure 5](image-url)

a) Diagram showing the domain structure of VP3 (T2)

The radial view shows the molecule as if it were in the subcore when viewed from outside the particle looking towards the centre

The tangential view shows how thin the molecule, and consequently the subcore, shell is compared to the volume it encloses

b) The bluetongue virus subcore, made up of 120 copies of VP3 (T2)

The ten conformationally distinct A and B molecules making up a decamer at the uppermost five-fold axis are outlined

The icosahedral five-fold, three-fold and two-fold symmetry axes have been marked

Grimes et al. (13)
The subcore can be considered as an assembly of twelve VP3 decamers, each containing five copies of the triangular VP3-A molecules around the five-fold apex of the icosahedron, leaving a small central pore. These are interspersed with five VP3-B molecules that are slightly more distant from the five-fold axis, creating a dish-shaped decamer. Each of the 12 decamers has a zigzag outer edge that ‘zips’ together with its neighbours to form the intact icosahedral shell.

The BTV subcore layer can self-assemble when VP3 is synthesised separately from the other viral proteins (for example as expressed by recombinant baculovirus in insect cells) (P. Roy, personal communication). Subcore particles of some orbiviruses are also stable in the absence of the outer capsid layers (Fig. 6). Structure-based modification of the VP3 molecule to remove the dimerisation domain responsible for inter-decamer contacts still allows decamer formation but prevents assembly of the intact subcore shell (P. Roy, personal communication) supporting a model for subcore assembly from 12 interacting decamers.

The architecture of the BTV subcore shell and the overall shape of the subcore shell protein (VP3 of BTV) show remarkable similarities to the innermost capsid shell and inner capsid protein of many other dsRNA viruses. This suggests that these dsRNA viruses evolved from a common ancestor that originally developed a simple and elegant mechanism for the assembly of an inner capsid shell. This now represents an important and characteristic step in the virion assembly pathway. The amino acid sequence of VP3 must determine not only the fold and overall structure of the protein, but also it represents the ‘information’ that allows the subcore shell to self-assemble. Once completed, the BTV subcore can act as a scaffold for the attachment of VP7 and subsequently the outer capsid proteins VP2 and VP5, thereby influencing the structure of whole capsid. The VP3 (T2) layer also dictates the internal organisation of the viral genome and the components of the transcriptase complexes (VP1, VP4 and VP6) that are attached, or interact with it internally. This protein therefore carries an enormous functional load and as a result it is highly conserved within the different genera of the Reoviridae. Indeed functionally and structurally similar subcore shell proteins can be identified in many other dsRNA viruses. This allows them to be used in phylogenetic analyses of the ancestral relationships of these viruses, as has previously been described for BTV (11, 12). By comparing the sequences of genome segment 3 and the VP3 protein, it is possible to distinguish different species within the genus Orbivirus and such comparisons provided important evidence to support the classification of St Croix River virus as a new species of Orbivirus (3).

The outer surface of the BTV core contains 780 copies of the VP7, arranged (as 260 trimers) with \( T = 13 \) icosahedral symmetry. Individual trimers can occupy five different positions in the core surface layer, which are identified with increasing distance from the five-fold axis, starting with P through Q, R and S, to T at the three-fold axis (Fig. 7).

The VP7 molecules are layered onto the scaffolding of the intact VP3 (T2) subcore shell. As a result of mismatched symmetry between these layers of protein, the base of the VP7 monomers interact...
with the surface of the underlying subcore VP3 (T2) molecules in thirteen different orientations. These interactions curve the layer of VP7 trimers around the subcore particle surface, as a series of six component, ring-shaped capsomers, finishing with rings of five VP7 trimers around the five-fold axes. The significance of VP3-VP7 interactions in forming the core surface layer is suggested by VP7 of African horse sickness viruses (AHSV) (members of another closely related Orbivirus species), which form crystals of VP7 within the cell cytoplasm. In the absence of any interactions with VP3 (T2), the VP7 trimers form into large flat hexagonal arrays of exclusively six-membered rings (4). Only at the three-fold icosahedral axes of the BTV core do the symmetries of the two protein layers of the core particle coincide. At this point, the interactions between the VP7 (T13) layer and VP3 (T2) layer are also the most extensive, suggesting that the T trimer is added first and the other trimers are added sequentially towards the five-fold axes.

**RNA packaging**

Electron density maps of the core of both BTV-1 and BTV-10 (10, 13) have revealed layers of density within the central space of the subcore that cannot be modelled as viral proteins (Fig. 8). These layers are made up of multiple strands, which in many places have a helical structure that is not only very similar for the two virus serotypes but are also consistent with layers of the packaged genomic dsRNA (Fig. 9).

From a detailed knowledge of the volume and internal contents of the BTV core (13), it was possible to calculate the concentration of the dsRNA within the central cavity of the BTV particle at ~410 mg/ml.

The properties of concentrated solutions of dsDNA are relatively well characterised. It is established that it forms liquid crystalline arrays at high concentration, with the phase and helix-helix packing distance being a simple function of concentration (22) (although less is known about the properties of dsRNA). At concentrations of ~400 mg/ml, the liquid crystalline packing arrangement for DNA has been shown to be columnar hexagonal, with an inter-helix packing distance of approximately 30 Å and it appears likely that the behaviour of dsRNA would be similar at high concentrations. This ordering of the RNA may be essential if it is to function effectively as a template for the virion-associated transcriptase activities, without becoming tangled and jamming the mechanism. One potentially relevant property of this packing structure is that in the presence of suitable counter ions the nucleic acid chains would glide over each other with very little friction (22). The phosphate backbone of the dsRNA carries a negative charge that would presumably be neutralised by counter ions within the particle. However, a scanning proton microprobe, used to detect the presence of metal ions within the crystals of BTV core particles (10), failed to detect magnesium. Although both calcium and zinc were present, they are only detected at approximately one one-hundredth of the level of phosphorous. This would be insufficient to neutralise the charge on the phosphate backbone of the RNA and suggests that
an organic cation such as spermidine may be present, although this has not yet been confirmed.

The model proposed for the packing of the BTV genome (derived from X-ray crystallography studies) (10) represents ~80% of the 19219 bp (a total length ~6 μm). This model implies that there is a particular organisation of the dsRNA strands within the core. Such ordering appears to be at least partially imposed by chemically featureless grooves that form tracks for the RNA on the inside of the VP3 (T2) layer. Specific RNA/protein interactions are evident at only two points in the icosahedral asymmetric unit (13) and there are very few basic residues on the inner surface of VP3 (T2). This paucity of specific interactions may also facilitate the movement of RNA within the core (for example during transcription).

There are striking similarities in the interactions that the structurally distinct A and B copies of VP3 (T2) make with the RNA (although there are also some differences reflecting the specific conformational changes between the two protein subunits). Since the two molecules lie at different radial distances from the five-fold axes, the protein-RNA interactions generate a spiral structure that is observed in the RNA. The model proposed by Gouet et al. (10) shows each dsRNA strand in the outer shell of packaged RNA, leaving the transcriptase complex (TC) situated on the inner surface at the five-fold axis of the subcore shell, then spiralling around it (10). At a certain diameter away from the five-fold axis, the RNA effectively fills the outer layer and interacts with a neighbouring genome segment. This is thought likely to redirect the RNA strands inward to lay down a second discrete layer, spiralling back toward the TC at the five-fold axis. Further switching would lay down a second discrete layer, spiralling back toward the TC at the five-fold axis. Additional switching would lay down a second discrete layer, spiralling back toward the TC at the five-fold axis. Finally, the newly synthesised mRNAs are capped by VP4(Cap), which has nucleotide phosphohydrolase, guanylyltransferase and two transmethylase activities that are required for synthesis of ‘Cap 1’ structures (21, 26, 29, 32, 34).

The BTV genome segments have very different lengths (from 822 to 3954 bp) and there is little free space within the particle. Some of the longer dsRNA molecules must therefore trespass into the volume of neighbouring segments (as well as into the volume around the two five-fold axes that are empty of genome). This suggests that simple steric clashes with neighbouring segments might be one of the most important factors limiting the lateral expansion of the spiral (particularly in the inner layer of the RNA), although it is possible that layer switching in the outer RNA layer might also be facilitated by interaction with a flexible loop of VP3-B, close to the icosahedral three-fold axis.

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**The minor protein components of the bluetongue virus core**

The core particle represents a biochemically active compartment. It contains the ten dsRNA genome segments, as well as enzymes needed to synthesise cap and methylate ssRNA copies of each segment. These mRNAs are released into the cell cytoplasm, where they are either translated into viral proteins, initiating the molecular events of virus replication and morphogenesis, or are processed by the enzyme components of nascent core particles and packaged as part of new viral genome segments within progeny virus particles (Fig. 4).

The BTV core contains a small number (10 to 12 copies) of transcriptase complexes (TC) (39) which are activated by removal of the outer capsid proteins. The TCs are composed of three minor structural proteins: the polymerase VP1(Pol) (36, 40) the capping enzyme VP4(Cap) (29, 32) and the helicase VP6(Hel) (38). The polymerase, which requires Mg2+, transcribes positive (+ve) sense ssRNA copies from the dsRNA genome segments (Fig. 4). At a later stage of virus replication it also uses these mRNAs as a template for the production of new dsRNA genomic material, which are packaged within the progeny virus subcore. During RNA synthesis the polymerase uses NTPs as substrates and produces pyrophosphate (PP) as a by-product. The newly synthesised mRNA strands are capped by VP4(Cap), which has nucleotide phosphohydrolase, guanylyltransferase and two transmethylase activities that are required for synthesis of ‘Cap 1’ structures (21, 26, 29, 32, 34).

The nucleotide phosphohydrolase activity removes the gamma phosphate from the 5′G residue at the end of the +ve sense RNA strand, releasing monophosphate (P1) as a by-product. The guanylyltransferase activity then takes GDP or GTP substrates and adds GMP to the 5′end-forming GpppG as a 5′ to 5′ linked structure, releasing Pi or PP, as by-products. Finally the methyltransferase activities use S-adenosyl-L-methionine (AdoMet) as substrate, adding two methyl groups to the Cap structure, releasing S-adenosyl-L-homocysteine (AdoHcy) as a further by-product. VP6(Hel) has helicase and ATPase activities, using the energy released by the hydrolysis of ATP to ADP and P, to separate dsRNA into its component strands.

In addition to the RNA of the viral genome, a distinct but unlayered region of electron density was detected within the central space, below the internal five-fold axes of the icosahedral BTV subcore, (Fig. 8). This is thought to represent the protein components of the TCs (10). These complexes
appear to be attached to the inner VP3 (T2) surface immediately below a pore at the five-fold axis. By analogy with the cypoviruses (41) and complying with the model for packing of BTV RNA described above (10), each TC is thought to be closely associated with a single genome segment (29, 40). These complexes composed of the minor core proteins, transcribe the ten genome segments, producing exactly full length mRNA copies, which are extruded from the core surface.

During transcription, each dsRNA genome segment must move through the active site of the polymerase enzyme. The fully conservative nature of BTV transcriptase imposes certain topological requirements on the process. The two strands of the parental dsRNA segments must initially be unwound prior to transcription to allow the -ve sense template strands to enter the polymerase active site. However, the resulting parental-daughter strand duplexes must also be separated, so that the nascent RNA chains can be exported from the core particle through the pores in the VP3 (T2) subcore shell at the five-fold axes (10, 13) and the parental strands of the dsRNA duplex re-annealed. It appears likely that the helicase activity of VP6(Hel) (38), is involved in one or both of these processes.

The BTV core faces several logistical problems during transcription. It must provide entry routes and mechanisms to continuously feed the viral NTP and AdoMet substrates to the internal enzyme complexes, maintain appropriate levels of certain metal ions and allow the reaction by-products to escape. It must also simultaneously propel the ten nascent mRNA molecules into the infected cell cytoplasm so that they can be translated or packaged within the next generation of virus particles. Further X-ray crystallography studies of the biochemically active core particles within intact crystals have identified sites of binding as well as entry and exit routes for the substrates and products of the core-associated enzymes. These are thought to be situated as twelve transcriptase complexes inside the subcore shell just below the pores that allow exit of the viral mRNAs (6).

The polymerase (VP1 of BTV) serves a central and vital role in the replication of dsRNA viruses. It is responsible both for the synthesis of the viral mRNAs as templates for translation of viral proteins, as well as being responsible for minus strand synthesis, usually within the nascent progeny virus particles. Consequently, the polymerase protein and the RNA segment from which it is translated are amongst the most highly conserved of the viral proteins/RNAs and can be identified across the whole of the Reoviridae and even in other families of dsRNA viruses. Comparisons of nucleotide and amino acid sequences of the polymerase gene/protein can therefore be used to distinguish and identify the members of different genera within the family Reoviridae (Fig. 10). Indeed, such a comparison formed a central component of the evidence that the seadornaviruses are distinct from members of the genus Coltivirus (1), leading to their classification within a distinct genus by the ICTV (Fig. 10).

**Virus assembly and particle release**

BTV subcore and core particles are assembled within large granular matrices, or viral inclusion bodies (VIB), within the cytoplasm of infected cells. The manner in which the genome segments and the three viral enzymes are selected for packaging within the subcore is still unresolved and represents one of the challenges that faces molecular virology. The outer capsid proteins appear to be added to the progeny core as it is released from the VIB surface.

BTV particles are released from the infected mammalian cell either by budding, or by direct cell membrane penetration, which appears to damage the cell, culminating in cell lysis. Previous expression studies have demonstrated that BTV protein NS3 (encoded by genome segment 10) can mediate virus release from insect cells. Since BTV infection does not cause cell lysis in Coltivirus cells, NS3 may be necessary, both for cell exit and for virus spread from the initial site of infection in the insect mid-gut to the salivary glands of the insect. It may therefore control the ability of the insect to transmit the virus. Indeed studies of reassortant virus strains of AHSV, containing variants of NS3, showed considerable differences in their abilities to cause a systemic infection in adult *Culicoides* when administered via an oral route (35). This suggests that variations in NS3 sequence may influence the efficiency of virus transmission in different insect vector populations.

**Conclusion**

The structure and structural proteins of the multi-segmented dsRNA viruses within the family Reoviridae, appear to show a gradient of variability. The most conserved of these proteins are situated within the central compartment of the virion (the subcore), reflecting similar biochemical replication strategies and processes (e.g. the polymerase) or similar particle assembly mechanisms (e.g. the ‘T2’ subcore shell protein). The conservation evident in these proteins/segments allows them to be used to identify distantly related viruses, from different genera within the Reoviridae (by comparison of the
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polymerase), or from different species for example within the genus Orbivirus (by comparison of VP3).

In contrast, the structure of the outer capsid layers and the sequence of outer capsid proteins/genes are more variable, reflecting the different host species targeted by each virus and differences in their transmission or infection strategies. The outer capsid proteins may also interact with the immune system of the hosts, becoming subject to antibody selective pressure leading to greater variability even within a single virus species. As a result, variability in these outer capsid proteins can often be used to identify even closely related viruses. The completion of representative sequences of genome segment 2 and 6 (encoding outer capsid proteins VP2 and VP5) for each the 24 BTV serotypes, provides a database for the rapid identification of BTV serotypes and even the identification of vaccine and field strains from recent outbreaks in Europe (23, 24, 25, 37).

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References


Information concerning the individual proteins and RNAs of double stranded RNA viruses from several different genera is available on the dsRNA virus page on the Institute for Animal Health website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/).
Genetic diversity of bluetongue viruses in Australasia

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Summary

The authors have characterised the genetic diversity of the bluetongue virus (BTV) RNA segments 3 and 10 from Indonesia, Malaysia and Australia. Analysis of RNA segment 3, which codes for the core protein VP3, showed conserved sequences in the previously defined Australasian topotype, but which further divided into four distinct clades or genotypes. Certain genotypes appeared to be geographically restricted while others were distributed widely throughout South-East Asia. Ongoing surveillance programmes in Australia have identified the movement of Indonesian genotypes into northern Australia and possible reassortment among them. Similarly, analysis of RNA segment 10, which codes for the non-structural protein NS3/3A, showed they were also conserved and grouped into five clades or genotypes, three Asian and two North American/South African.

Keywords


Introduction

Bluetongue (BT) is an arthropod-transmitted disease of wild and domestic ruminants caused by BT virus (BTV). BTV is a member of the Orbivirus genus, one of nine genera in the family Reoviridae (22). They have a segmented, double-stranded RNA (dsRNA) genome (32) and 24 serotypes have been described (8). BTV is transmitted between vertebrate hosts by Culicoides biting midges (18). The virus particle contains three protein layers, the outer capsid layer comprised of two proteins, VP2 and VP5 (11, 20), where VP2 is the major neutralising protein and determinant of serotype specificity (12). The bi-layered core particle is made up of two proteins, VP7 and VP3, three minor proteins, VP1, VP4, VP6, and ten species of dsRNA (20). There are three non-structural proteins, NS1, NS2 and NS3/3A, which are expressed in virus-infected cells (19). The RNA segment 10 codes for NS3/3A protein which mediates the release of virus particles from infected cells (14).

The BTVs have a wide distribution throughout both tropical and sub-tropical regions. In Australia, BTV was first isolated in 1975 (29) and currently eight serotypes have been reported. Six of these (3, 9, 15, 16, 20, 23) have only been found in the north of the Northern Territory, while two serotypes (1, 21) are widely distributed across the northern and eastern coastal regions of Australia (33).

In Indonesia, an outbreak of BT occurred in 1981 in Suffolk sheep imported from South Australia. Subsequently BTV was shown to be widespread throughout Indonesia with seasonal patterns of infection (21, 26). Serotypes 1, 7, 9, 12, 21 and 23 have been isolated from sentinel cattle or Culicoides species (26) with serological evidence in cattle, buffalo, goats and sheep (26). The presence of BTV in Malaysia was first indicated serologically in 1977 and clinical BT was reported in imported Australian sheep (4, 27). Subsequently, serotypes 1, 2, 3, 9, 16 and 23 were isolated from sentinel cattle (27). Eleven of the 24 known serotypes of BTV have now been confirmed in Australia, Indonesia and Malaysia.

Molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) (5, 16, 34, 35) have been used to show that geographic separation has resulted in significant divergence in RNA segment 3 sequences (9, 10, 25). Significantly, the
RNA segment 3 sequences could be used to determine the topotype of the BTV isolates (9). The three topotypes identified were the Australasian topotype, North American/South African topotype and another topotype characterised by BTV serotype 15 isolates in Australia (9). The nucleotide sequence variation between topotypes was defined as greater than 15%. Similarly analysis of the RNA segment 10 of field isolates of BTV from the United States of America (USA) demonstrated that virus strains isolated in a restricted geographic region had evolved independently (24) and could be segregated into three distinct monophyletic groups, including two USA groups and one Asian group (3). More recently, the South African BTV isolates have grouped with the North American clusters (I and II), while the Indian BTV isolates grouped with the Asian cluster (III) (31).

We characterised the pattern of distribution and the genetic relationships among BTV isolates from the Australasian region using the nucleotide sequences of RNA segments 3 and 10.

Methods

Virus isolation and identification

Viruses were isolated from sentinel cattle in embryonated chicken eggs, *Aedes albopictus* C6/36 and baby hamster kidney 21 (BHK-21) cells. The serotypes were determined by plaque reduction neutralisation tests (7). Sera from sentinel cattle were tested for BTV-specific antibodies by competitive enzyme-linked immunosorbent assay (c-ELISA) (15).

Molecular analysis

Nucleic acids were extracted from BTV-infected cells using the RNeasy kit (Qiagen) in accordance with the instructions of the manufacturer. The RT-PCR was performed with the One-Step RT-PCR kit (Qiagen) and BTV-specific primers used were A196 (5’ accgcacagcttaatgatgttag3’) and A203 (5’taatgcctgctccgagtccttacc3’) for RNA segment 3 and B49 (5’gttaaaaagtgtcgctgccatgct3’)/B50 (5’gtaagtgtatagcgccgcaca3’) for RNA segment 10. PCR products from RNA segments 3 and 10 were purified from agarose gels with Qiaquick PCR kits (Qiagen), and sequenced on an AB377 automated sequencer. BTV sequences were aligned using Clustalw1.6 (30), and the phylogenetic relationships were determined using programs in the Phylip package (6) and the TreeView program (23).

Results

Sequence analysis of RNA gene segment 3

BTVs from Australia, Indonesia and Malaysia were isolated over a number of years in an ongoing surveillance programme (Table I). The majority of nucleotide changes for RNA segments 3 and 10 were synonymous as was shown previously (9, 17, 25). The history of isolation, genotype and GenBank accession numbers are listed in Table I. Genetic analysis showed that the RNA segment 3 sequences in this study could be grouped into the Australasian group or topotype with further groupings into genotypes (Table II; Fig. 1). The genotypes were given names based on the region of first isolation and within a genotype we observed nucleotide sequence variation of less than 6%, while between genotypes the variation was greater than 6% (Table II). Nucleotide sequence analysis of their RNA segment 3 showed the Malaysian BTV isolates, all from 1991, were almost identical and comprised a single genotype, named Malaysia A, while the Indonesian BTV isolates, from 1988-1992, showed much greater genetic variation, and grouped into several genotypes, Java A, C and Australia A (Fig. 1).

Movement of Indonesian genotypes into Australia

Since BTV was first isolated in Australia, the predominant genotype has been Australia A (Table III). This was from 1975 when BTV serotype 20 was first isolated until 1992, 17 years later, when BTV-20 was again isolated (Table III). This BTV serotype 20 had RNA 3 sequences closely related to a Java A genotype from Indonesia. Then further isolations of BTV serotype 20 were obtained in 1995 with the Malaysia A genotype. Neither of these genotypes were isolated again.

Similarly, BTV-21 was not isolated for 10 years, between 1984 and 1994. In 1993/1994, early virus isolations of serotypes 1 and 21 had the Australia A genotype. However, towards the end of the wet season, in June 1994, a novel serotype 21 was isolated with a sequence closely related to another Indonesian genotype Java C (Table III).

During the next wet season, serotype 21 viruses with genotype Java C were the most abundant, until March 1995, when the serotype 20 viruses were again isolated with the Malaysia A genotype (Table III). At the end of this wet season we isolated a BTV serotype 20 with the Java C genotype and RNA gene segments 2, 9 and 10 with altered electrophoretic mobilities in polyacrylamide gels (data not shown).
# Table I

**Genotype, GenBank accession numbers and origin of bluetongue virus isolates**

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ND not done
Table II
Per cent nucleotide sequence variation for RNA segment 3 among South-East Asian and Australian bluetongue virus genotypes
Figures in brackets are the percent nucleotide variation within each genotype

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<td>–</td>
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<td>9-12</td>
<td>24</td>
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<tr>
<td>Australia B (0)</td>
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Figure 1
Phylogenetic analysis of bluetongue virus isolates using RNA segment 3
Bootstrap values were not included for clarity
Additional sequences were obtained from McColl and Gould (16)
Table III
Genotypes of bluetongue virus from surveillance in Australia

<table>
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Sequence data showed that the RNA gene segments 9 and 10 of this virus were also closely related to Indonesian viruses (data not shown). A reassortment appears to have occurred which possibly conferred a selective advantage on this virus in this region.

Subsequently, in 1995/1996, another reassortment occurred between this virus (serotype 20/Java C genotype) and a serotype 1/Australian A genotype, which resulted in a reassortant BTV serotype 1 with a Java C genotype. Again the sequences of gene segments 3, 9 and 10 were closely related to Indonesian genotypes (Table III).

There has been a long history of certain serotypes being restricted in their movement within Australia. It is known that serotypes 1 and 21 have been established across the top and along the east coast of Australia, whereas serotypes 20, 3, 9, 16, 23 and 15 have been confined to the northern area of the Northern Territory.

Sequence analysis of RNA gene segment 10

The Indonesian BTV RNA segment 10 sequences (NS3/3A) also segregated into the Asian and North American/South African groups, which could be further clustered into genotypes. Significantly, the key structural components of the NS3/3A protein were conserved. These include the N-linked glycosylation site (150) and two hydrophobic domains H1 (118-141) and H2 (162-182), which are potential membrane spanning sequences (1, 13, 24). In addition, there are conserved tryptophan (159), cysteine (137 and 181) and proline residues near the amino terminus. There is also a hypervariable region (153-158) between the two hydrophobic domains, H1 and H2. Greater heterogeneity occurred among the Asian isolates than the North American isolates (Fig. 2) and the Chinese, Indian and Indonesian BTV appear to share a common ancestor and have evolved independently from the North American isolates (Fig. 2).

Discussion

The studies reported in this paper were undertaken to improve the understanding of the regional distribution and molecular epidemiology of BTV in South-East Asia and Australia. Between 1988 and 1992, seven serotypes were isolated from Malaysia and Indonesia. While in Malaysia and Irian Jaya (4 000 km to the east of Java), similar serotypes were isolated during the same period indicating that these viruses are endemic throughout the region.

Previously, partial segment 3 sequences were used to group BTV isolates into three distinct groups or topotypes; Australasian, North American/South African and BTV serotype 15 (9, 10). BTV isolates within the Australasian topotype had nucleotide sequences greater than 15% different from isolates from the Caribbean, North America and South Africa, and from Australian BTV serotype 15 (25). While the RNA segment 3 sequences in this study could be grouped into the Australasian topotype, there were further groupings or clades with nucleotide sequence variation greater than 6%, but less than 15% (Table II). These grouping were defined as genotypes, and have been given names based on the region of first isolation (Table II).

Work conducted under the National Arbovirus Monitoring Program (NAMP) has detected possible incursions of Indonesian genotypes into northern Australia on several occasions, in 1992, 1994, 1995 and 2000. Further studies over a longer time frame...
are needed to determine if these genotypes circulate continuously or periodically and would assist in the study of the complex interactions which give rise to the evolution of these viruses, their regionalisation and the role of host/vectors. Phylogenetic analysis of RNA segment 3 has shown the evolutionary relationships among the BTV isolates from South-East Asia and Australia. There appears to have been significant movement of genotypes throughout the region. The progenitor of the Australia A genotype shares a common ancestor with the Java A genotype and Indonesian isolates RIVS46 and 1163 (Fig. 1), but the evolutionary history of the Australia B genotype (BTV-15 Australia) is not yet known (25). Similarly, some Japanese encephalitis (JE) virus genotypes in this region have spread across Asia while others are localised and it has been suggested that JE virus originated from its ancestral virus in the Indonesia-Malaysia region (28).

Figure 2
Phylogenetic analysis of bluetongue virus isolates using gene segment 10
Bootstrap values were not included for clarity
Additional sequences were obtained from Bonneau et al. (3) and Van Niekerk et al. (31)
The BTV RNA segment 10 sequences, which code for the non-structural proteins NS3/3A, were also analysed and appeared to cluster according to their geographic origin (3) and may be related to distinct vector species (31). The North American prototype viruses were more closely related to the Asian viruses, while BTV serotype 12 and 15 from China formed independent branches of the Asian group (3). Greater heterogeneity was observed among the Australasian segment 10 sequences than that shown for the North American isolates. Clearly the Chinese, Indian and Australasian viruses share a common ancestor and have evolved independently of the North American viruses. Significantly, the key structural components of the NS3/3A protein were conserved. The NS3/3A protein mediates the release of virus from infected cells and the correct folding at residues 142-161 is thought to be required for transport from the endoplasmic reticulum (ER) to the golgi and then to the cell surface (1, 2). It has been proposed that the RNA segment 10 may have co-evolved with insect vectors (3, 31).

As shown previously, Australasian BTV isolates appear to have evolved from a gene pool distinct from those in other parts of the world. In this region, they appear to have evolved into closely related genotypes primarily based on their geographic isolation. The distribution of BTV within Australasia is also dependent on the movement of infected vectors and/or hosts within naturally constrained ecosystems. Some genotypes were found to be widespread throughout Australasia, while others were confined to discrete niches. Furthermore, the genetic variation of RNA segment 10 sequences may be related to their geographic region and/or vector species responsible for propagation since different vector species predominate in different regions. There appears to be a complex relationship between these viruses and their environment.

Acknowledgements

The authors acknowledge the support of the Research Institute for Veterinary Science, Bogor, Indonesia, the International Atomic Energy Agency (Project No. INS/78/074), the Australian Quarantine and Inspection Service (AQIS) under the Northern Australia Quarantine Strategy (NAQS), the United States Naval Medical Research Unit, Detachment II, Jakarta and the Australian Centre for International Agricultural Research (ACIAR) for its support of the research. The Intensive Research and Priority Area Project Funds of Malaysia provided additional funding. We gratefully acknowledge the expert technical assistance of Kim Newberry and Tony Pye.

References

Genetic diversification of field strains of bluetongue virus

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Summary

The considerable genetic heterogeneity of field strains of bluetongue virus (BTV) occurs as a consequence of both genetic drift and shift. Comparison of strains of BTV from the People’s Republic of China and North America showed that viruses from the two regions were readily distinguished by sequence analysis of their S10 (which encodes the NS3/3A proteins) but not their L2 (which encodes the serotype-specific VP2 protein) genes. Subsequent laboratory studies showed that individual BTV genes evolve through a combination of genetic drift coupled with founder effect in vector insects. This model explains the diversification of BTV gene segments within each region, and can be extrapolated to explain diversification of BTV into distinct topotypes worldwide.

Keywords


Genetic heterogeneity of field strains of bluetongue virus (BTV) occurs as a consequence of both genetic drift and shift. The consequence of these two phenomena is a remarkable heterogeneity amongst strains of BTV that circulate in endemic regions such as California, even amongst virus strains that co-circulate (1, 5, 6, 7, 9). Reassortment of BTV genes is responsible for genetic shifts amongst strains of BTV, and has been demonstrated after infection of either the ruminant host or insect vector with different strains or serotypes of BTV (10, 11). Accumulation of nucleotide substitutions within individual BTV genes leads to genetic drift of each.

Australian workers first proposed the term of virus ‘topotypes’ for the region-specific grouping of BTV strains that they observed after sequence analysis of the L3 gene of each virus (8). To further evaluate the region-specific grouping (topotype clustering) of field strains of BTV, we first compared the S10 and portions of the L2 genes of Chinese and North American strains of BTV (2). Phylogenetic analysis of the S10 gene segregated the Chinese viruses into a monophyletic group distinct from the American viruses, whereas analysis of the L2 gene segregated strains of BTV according only to serotype, regardless of geographic origin. These studies showed not only that BTV genes evolve independently of one another, but also confirmed that BTV strains from distinct geographic locations can be classified as topotypes based on the sequence of a conserved gene that assigns a virus isolate to a specific geographic region, regardless of serotype.

In subsequent studies to further characterise the genetic diversity of field strains of BTV that co-circulate at a single site, we directly amplified and sequenced the S10 gene of field strains of BTV contained within Culicoides sonorensis (C. sonorensis) collected from a dairy in southern California (4). Phylogenetic analysis established that the S10 gene of BTV in C. sonorensis collected from the site existed as a heterogeneous but related population, probably arising from genetic drift. Thus, we hypothesised that viral genes undergo genetic drift during alternating passage of BTV in its ruminant and insect hosts. To test this hypothesis, variation in the consensus sequence and quasispecies heterogeneity of the L2 and S10 genes of BTV was determined during alternating infection of a sheep and calf with BTV that was transmitted by C. sonorensis (3). This study demonstrated that individual BTV gene segments evolve independently of one another by genetic drift in a host-specific fashion, generating quasispecies populations in the ruminant and insect hosts. A unique viral variant randomly ingested by C. sonorensis that fed on the viraemic sheep resulted in fixation of a novel genotype, thereby demonstrating founder effect. Therefore, genetic drift coupled with founder effect offers a model for the diversification
of BTV gene segments at a single site, and can be extrapolated to explain diversification of BTV into distinct topotypes worldwide (1).

Acknowledgements

The authors gratefully acknowledge all of the collaborators who made the studies described in this review possible, especially Udeni Balasuriya, Alec Gerry, Brad Mullens and NianZu Zhang.

References


A comparison of laboratory and ‘wild’ strains of bluetongue virus – is there any difference and does it matter?

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Summary

Bluetongue (BT) viruses (BTV) have been propagated in laboratory culture systems for more than 50 years, especially for the production of vaccines. An important outcome of the adaptation of these viruses to laboratory culture is attenuation of their virulence. As a consequence of this modification, it has been possible to produce vaccines that have reduced the clinical impact of BT in a number of countries. Unfortunately, the adaptation of these viruses has also introduced undesirable properties. In particular, modified live BT vaccine viruses have a high capacity to cross the ovine placenta and cause congenital abnormalities in the foetus. Modified strains of BTV have also been found in the semen of bulls and rams. It is possible that there are also other undesirable properties, including the potential to infect non-ruminant hosts. Because these characteristics are not properties of naturally occurring BTVs, the use of laboratory-adapted strains is not recommended when the biological properties of BTV are being studied.

Keywords

Attenuation – Bluetongue – Cell culture – Laboratory modified – Foetal defect – Semen contamination – Virus.

Introduction

The development of laboratory-based systems for the investigation of animal diseases such as bluetongue (BT) has been crucial to our understanding of the infectious agent and the disease process. Without the use of a wide range of laboratory tools, our knowledge would be very limited and research would progress very slowly. As a result of the adaptation of BT viruses (BTV) to grow in laboratory systems such as cell culture and embryonated chicken eggs (ECE), it has been possible to develop a range of diagnostic tests and vaccines. Studies of the structure of the virus, virus replication, interactions between virus and cells and other aspects of the basic biology of BTV have all been possible. The information gained from these studies has allowed the development of sophisticated molecular diagnostic procedures and the production of virus-like particles using recombinant DNA technology. Such advances would not have been possible if it was necessary to rely on virus that was amplified in its natural ruminant hosts.

An important consideration during the amplification of BTV in laboratory systems such as cell culture or ECE is that the progeny virus should retain the main characteristics that are representative of the parent virus. For both diagnostic tests and vaccines, the antigenic characteristics of the virus should be altered as little as possible. On the other hand, for vaccine production, there is a deliberate attempt to remove undesirable traits, especially the ability of BTV to cause disease in sheep. During the adaptation of the virus to a laboratory system and any modification to reduce the virulence of the virus (attenuation), it is desirable that no other change should occur to the biological or antigenic characteristics of the virus. For BTVs, it is probable that there have in fact been undesirable changes to the biological characteristics of these viruses. The purpose of this presentation is to review the adaptation of BTVs to laboratory systems, to present evidence of undesirable characteristics and to discuss the implications of these features.
Diagnostic reagents

During the development of diagnostic tests (other than virus detection by animal inoculation), there is no practical alternative to the use of BTV that has been amplified in a laboratory system such as cell culture or ECE. The main consideration of virus that is used for the production of diagnostic antigens is that the virus is faithfully representative of its serotype. During the passaging of viruses used for the production of diagnostic antigens, any significant deviation in antigenicity may be limited by the use of a seed lot system and by restricting the number of times that a seed is amplified. Variations in antigenic properties of the virus may be monitored by testing of the progeny virus against one or more type-specific reference antisera. Generally there are no changes of significance to virus used in this way for the production of diagnostic antigens.

Vaccines

Bluetongue was first described as a disease more than a century ago (24). It was feared as a major epidemic disease and consequently placed on the OIE List ‘A’. Despite this ranking, and many reports of disease incidents to the OIE, there have been remarkably few outbreaks of BT described in the scientific literature. The situation with the development of vaccines presents a marked contrast. There are many reports of attempts to develop vaccines for BT. The earliest vaccines were developed in South Africa and were delivered as blood from infected animals. For about 40 years from 1907, strains of low initial virulence were utilised, maintained solely by passage in sheep (57). Deficiencies in these vaccines (occasional severe disease, and vaccine failure due to infections with other serotypes) led to their replacement. Laboratory culture systems have since been used to produce a large volume of virus of known quality. Both live and inactivated vaccines have been evaluated. Although inactivated vaccines are very safe, they have generally been less effective and more expensive than live vaccines and have not been used on a commercial scale. In many respects, antigens for inactivated BTV vaccines are similar to antigen used for diagnostic reagents. Limits on the extent of repeated passaging and the use of seed stocks presumably restricts the occurrence of major antigenic changes. Provided steps are taken to maintain appropriate antigenicity, there can be no deleterious effects arising from the field use of inactivated vaccine because there is no real potential for the introduction of undesirable genetic material into the environment. While not proven, it is also unlikely that other non-infectious vaccines (e.g. subunit, virus-like particles or possibly DNA vaccines) will transfer unwanted genetic material to mammalian hosts. The situation with viruses used to produce live vaccines may, however, be quite different.

Modified live vaccines and attenuation

The biological characteristics of BTVs are extremely complex. The BTV serogroup is relatively large and, although all of the viruses, by definition, share certain features, there is also considerable diversity. Shared antigenic characteristics unite these viruses, but the assignment of a virus to a serotype also denotes that there are differences between members of the group. Interestingly, the elements that define serotype are not directly linked to those that influence perhaps the most important elements – the determinants of pathogenicity and virulence. Within a serotype, there can be virus strains that are highly pathogenic and others that, at best, cause very mild disease. For example, strains of BTV-1 in South Africa or the People’s Republic of China have caused large disease outbreaks while there are Australian serotype 1 viruses that are non-pathogenic.

There have been two main considerations during the development of live BT vaccines. These have been safety and efficacy. Safety considerations have predominantly focused on ensuring that viruses that are amplified for vaccine production do not cause disease, or, at worst, that the clinical signs that occur are very mild. A secondary consideration has been the reversion to virulence, based on concerns that a modified virus may resume virulence characteristics after repeated passage in mammalian hosts. During attempts to reduce the virulence of a ‘wild-type’ virus by manipulation in laboratory systems, there has often been a delicate balance between achieving an acceptable degree of attenuation and maintaining an appropriate degree of immunogenicity. Even today, at a molecular level, the basis for attenuation is still poorly understood and the outcome of attempts to modify a virus cannot be precisely controlled.

There have been attempts to attenuate BTVs for more than 50 years (3). Two main systems have been utilised, namely: propagation in ECE and in cell cultures of various types. Adaptation of a virus to growth in ECE or cell culture induces desirable changes that result in attenuation. BTV adapted to growth in ECE and repeatedly passaged to achieve an appropriate reduction in virulence was used for vaccine production for several decades in South Africa (3, 21). Once cell cultures became available, these were also used to serially propagate viruses. Both ECE and cell culture propagated vaccines were
used concurrently in South Africa (23), although little data has been published on their safety and efficacy. In 1952, BTV was officially recognised in the United States of America (USA), and vaccines were developed. Importation of vaccines from South Africa was not permitted. However, vaccines based on ECE propagation of USA strains (32) were manufactured locally. Later, high passage BTV that had been propagated in ECE was used as the starting material for further passage in cell culture (27). More recently, candidate USA vaccines of solely cell culture derivation have been produced and evaluated (31). These vaccines have been beneficial and have significantly reduced the impact of BTV infection in countries where they have been used. Other countries have also developed modified live vaccines after propagation in ECE or cell culture (63).

Mechanism of virus attenuation

The molecular basis by which attenuation of BTV strains is achieved is not clear. Furthermore, there are no precise criteria that have been followed to uniformly achieve the required end result of a vaccine virus with minimal virulence but optimal immunogenicity. Nevertheless, there are some trends in practices that have been adopted to develop modified live vaccines. Most vaccines have been based on seeds that are the product of repeated passage of virus in laboratory culture systems, usually involving cells of species different from the target mammalian host. As there is considerable variation in the virulence of field strains, it is not unexpected that there have been different levels of passage required to achieve suitable attenuation. However, the passage level for ECE-adapted viruses has usually ranged from 30 to 68, with earlier passage levels producing unacceptable reactions in vaccinated animals (3, 32, 37, 66). There is also some evidence, that excessive passage in ECE may lead to over-attenuation of the virus (66). The temperature of incubation of the eggs also affects the passage level at which optimal attenuation occurs (3). In one study (15) a single passage in ECE, followed by another single passage in cell culture, was sufficient to achieve attenuation. In other situations, more extensive passaging in combinations of host systems has been followed (13, 27). Adequate attenuation of Australian BTVs has been achieved after about 20 passages in BHK-21 cells (30, 63) but ‘wild’ strains of these viruses are generally of lower virulence than the same serotypes in South Africa (18).

There have been few studies to identify the determinants of virulence and mechanisms of attenuation of BTV at the molecular level. There are some indications that genome segments 2 (51) or 2 and 6 (22) might be involved, but sequencing studies did not support this (15). The most convincing work to date has involved mouse-adapted variants of USA serotype 11 (UC-2 and UC-8) in a model system involving new-born mice and in subsequent studies in cattle. These studies indicated that segment 5 of the genome was associated with virulence (58, 59, 60, 61, 62).

Foetal infections and teratogenicity

While it has been possible to achieve desirable modifications to a range of different BTVs, a key issue is whether there have been any adverse outcomes arising from the amplification of BTVs in laboratory systems. Unfortunately, the passage of BTVs in ECE or cell culture can induce undesirable properties. Some of these changes appear to occur after relatively limited manipulation in laboratory systems. One of the most prominent features of laboratory-adapted virus (for example, some attenuated or modified live vaccine viruses) is the ability of the virus to cross the placenta, causing foetal abnormalities, abortion and perhaps other reproductive losses. Concerns about the teratogenicity of attenuated BTV vaccines first arose following the use of an ECE-adapted vaccine (55). The teratogenic effects of modified live vaccines for BTV are now well recognised (39) and vaccination of pregnant ewes is contraindicated.

Natural infection of sheep and cattle with ‘wild’ strains of bluetongue virus

Foetal infection following natural exposure of sheep, cattle or goats to ‘wild-type’ strains of BTV seems to be a very rare occurrence. Sometimes abortion has occurred in sheep after infection with pathogenic strains of BTV, but this has been considered to be secondary to the febrile illness affecting the ewe. In countries where live vaccines have not been used, there is no evidence of virus crossing the placenta. For example, in Australia, in some years up to 0.5 million cattle may be infected with a strain of BTV, without adverse sequelae.

Experimental infection of sheep with ‘wild’ strains of bluetongue virus

When sheep have been infected experimentally with virus that has been derived directly from the field, and has not been knowingly passaged in a laboratory culture system, there are some apparently conflicting results. The inoculum used for such studies has been blood that contains virus that has been maintained by repeated passage in sheep or cattle. It has always been assumed that repeated passage between mammalian hosts, without a cycle through the arthropod vector, does not alter the virulence of the virus and presumptively does not alter the
characteristics of the virus. In studies in the USA (4, 8), clinical signs were observed in a high proportion of ewes that were inoculated with unadapted virus. The virus did cross the placenta and caused foetal death in up to 40% of lambs (4). Another study in Cyprus (49) compared both laboratory-adapted and field strains. Both strains crossed the placenta, infected the foetus and caused lesions in a range of organs including the brain. These results are in marked contrast to those of three similar studies, two of which were conducted in Australia (25, 26) and one in the USA (45). In each instance, ewes of similar stages of pregnancy (ranging from 25 to 45 days) were infected and many of the ewes showed signs of BT. However, there was either no evidence of foetal infection (25) or a very low (<5%) incidence of abortion and occasional isolation of BTV from foetal or placental specimens.

Infection of sheep with laboratory-adapted viruses
Since the early reports (9, 16, 54, 55) that described a significant incidence of foetal infections and congenital defects in lambs following vaccination of ewes with modified live vaccines, a number of experimental studies have been conducted (12, 26, 49, 65). These investigations were performed in several countries and used either ECE or cell culture adapted virus and studied a number of different serotypes of BTV. There were similar results and agreement that laboratory-adapted virus could readily cross the placenta and infect the foetus, with devastating results. Congenital defects, especially hydranencephaly, were consistently observed. The peak period of susceptibility was around 35-42 days of gestation.

Infection of cattle with laboratory-adapted viruses
Unlike the situation with sheep, there have only been a few studies of experimental infections of pregnant cattle with laboratory-adapted BTVs. Cattle were infected at several different stages of gestation, at times when a teratogenic agent would be likely to infect a foetus. There were no reports of adverse effects, in particular any evidence of transplacental infection, in any of these studies (44, 46, 50). There have been other studies that involved direct inoculation of the foetus in utero but these have not been considered in this review as they artificially bypass the placental barrier.

Bluetongue virus in semen
Another of the well-known properties of BTV that has had a profound impact on trade between countries is the excretion of virus in semen. Concern arose because of studies that suggested that some bulls may undergo a persistent infection and intermittently excrete virus in their semen (29). The results of these studies are now of doubtful significance (33, 41). Nevertheless, there are many reports of studies of the testing for BTV in the semen of both naturally and experimentally infected bulls.

Natural infections of bulls
In both Australia and the USA, a large number of semen samples have been collected commercially from known seropositive bulls (28, 35, 47, 53). These represent mature bulls that have been infected with a range of different serotypes. BTV has not been isolated from any of these samples. Prospective studies (14, 34, 36) of the monitoring of sentinel bulls over periods of up to 15 years have also been described. Both blood and semen samples were collected regularly to monitor the occurrence of BTV viraemia and to detect BTV in semen. Over the time span of those studies, bulls were naturally infected with five different serotypes of BTV. There was only one possible infection of semen in one bull during the period of viraemia, but it was thought that this was probably an artefactual finding (36).

Experimental infection of bulls
With intense interest in the possible excretion of BTV in the semen of bulls and its potential ramifications for international trade, there have been many investigations of experimentally infected bulls (5, 6, 17, 19, 20, 28, 35, 42, 43). Unfortunately, parameters such as the age of the bulls or the passage history of the viruses have not always been clearly documented (6, 19, 43). Many of these studies appear to have utilised laboratory-adapted strains of BTV. It is now recognised that these factors may contribute to the possibility of BTV being detected in the semen (5). Consequently, a more recent study was specifically designed with some of these issues in mind (28, 35). Collectively these studies of experimentally infected bulls have shown that virus may be found in semen only when it is also present in the blood or during the period of viraemia. Virus was detected in semen intermittently and only in the semen of a proportion of bulls, even though all animals became viraemic.

Apart from the investigation of natural infections, there is little data available with which to systematically compare the potential for laboratory-adapted and field strains of BTV to traverse the reproductive tract of bulls and contaminate the semen. There is only one extensive study in which both ‘wild’ and laboratory-adapted viruses (each from two serotypes) were compared concurrently and in both young and old bulls (28, 35). Virus was never detected in the semen of young bulls, whether infected with ‘wild’ strains of BTV or with cell
culture adapted viruses. In comparison, virus was found in the semen of many of the old bulls infected with cell culture adapted virus. Virus was also detected in the semen of some of the bulls infected with one of the ‘wild’ serotypes. However, these bulls were quite old (10 to 12 years) and blood was generally detected in the semen at the same time. It is believed that virus may be present in the semen of old bulls as a result of inflammatory changes that occur in older animals (5, 6) or if there is detectable blood in the semen (28, 35) perhaps also as a result of damage to the reproductive tract. Collectively, these studies suggest that laboratory-adapted strains of BTV are often found in semen, whereas ‘wild’ strains are infrequently found in semen, and only during the period of viraemia in old bulls.

**Infection of rams**

There is little published data on the infection of rams with BTV. However, in a limited study, laboratory-modified vaccine virus was detected intermittently in the semen of six-year-old rams during the period of viraemia, but not in the semen of young rams (S. Johnson, personal communication).

**Bluetongue virus in non-ruminant species**

BTV is traditionally considered to be a virus that infects ruminants but it can be adapted by laboratory modification to replicate in cells of other hosts, and in vivo in non-ruminant species (60, 61, 62). This was exemplified unexpectedly in 1993, when several cases of severe and fatal disease, characterised by pulmonary oedema, occurred in bitches that were in the late stages of pregnancy (1, 7, 11, 64). There were a large number of abortions but there was minimal evidence of viral replication observed in the placentae, and none in the aborted foetuses (7). BTV-11 was conclusively found to be the cause of the problem. All of the dogs had been vaccinated with a modified live multivalent canine vaccine and circumstantial evidence pointed strongly to the vaccine as the source of the BTV-11. Contamination of the cell culture used to produce the vaccine and/or the foetal calf serum used in the medium was suggested as a possible cause of the contamination. Following this incident, extensive serosurveillance of carnivores in Africa was performed to determine whether carnivore species in their natural environment (2) had been infected with BTV. Neutralisation tests indicated that many species had been infected naturally, and the route was conjectured as being oral, as a result of eating naturally infected ruminants. While there is no direct proof, there is speculation that the disease in the dogs was also a result of infection with a laboratory-adapted BTV.

**Discussion and conclusions**

It is clear from the numerous studies that have been undertaken that strains of BTV that have undergone adaptation to laboratory culture systems (either ECE or cell culture) have different biological characteristics to true ‘wild-type’ viruses. Attenuation of the virulence of wild strains has been the basis of the development of many effective vaccines for the control of BT. However, there is a similar body of evidence that shows that laboratory-adapted strains of BTV acquire undesirable properties, in particular the ability to cause foetal abnormalities, or excretion (or at least presence) of the virus in semen and perhaps a capacity to cause disease in non-ruminant species. Whether these undesirable traits are exclusively confined to laboratory-adapted viruses is not definite but it is very apparent that these features are markedly more pronounced in laboratory-modified strains of BTV compared to ‘wild’ strains.

Are there any other implications arising from these undesirable properties of laboratory-adapted virus? For simplicity and convenience, it has been usual practice to undertake studies of BTV with viruses that have been passaged in laboratory systems, mainly cell cultures. As there appear to be profound differences between cell culture passaged virus and true ‘wild’ strains, any *in vivo* studies of the basic biology of BTVs must include a parallel study of animals infected with virus that has not been adapted to laboratory systems. However, in some countries, there may be concerns that a BTV acquired from the field could contain genes from a laboratory-adapted virus. There are a number of documented examples of reassortment occurring between field viruses (10, 38, 52, 56). It is also probable that vaccine viruses have been spread by insect vectors. While it has been suggested that the very low titre viraemias of some vaccine viruses prevent transmission by vectors, this is somewhat contentious. There is evidence that some Australian field viruses (e.g. BTV-1) produce very low titre viraemias (less than 2.5 log10/ml of blood) in cattle (C.F. Williams and P.D. Kirkland, unpublished observations) but are transmitted widely by *Culicoides brevitarsis*, a relatively inefficient vector. During infection of either the arthropod vector or vertebrate hosts, vaccine viruses may have reassorted with true field strains (40, 48) and may have persisted in the field for many years (40). These reassortant viruses have effectively become modified field strains and it may now be difficult to acquire true ‘wild’ strains under these circumstances. If laboratory-adapted viruses, or reassortant viruses containing genes from laboratory-modified viruses are used in studies of the basic biology of BTVs, it is quite possible that the results may not be indicative
of natural BTV infection and incorrect conclusions may be drawn.

References


A potential overwintering mechanism for bluetongue virus – recent findings

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Summary

Bluetongue virus (BTV) is transmitted between its mammalian hosts almost exclusively via bites from the adults of certain species of Culicoides biting midges. Theoretically, the spread of BTV into the more northerly areas of Europe should therefore be terminated by the harsh winters experienced in these regions, when adult midges disappear for extended periods of time. However, it has been shown that BTV can survive for periods as long as 9 to 12 months in such locations in the absence of adult insect vectors, with no detectable cases of viraemia, overt disease or seroconversion in the host species. Virus survival in this manner throughout the winter is called ‘overwintering’ but the mechanism involved has not been satisfactorily explained. With knowledge currently available and results from a series of preliminary experiments, the authors discuss a possible overwintering mechanism.

Keywords


Overwintering of bluetongue virus

It has been established that the members of several virus species that belong to the genus Orbivirus, including bluetongue virus (BTV) and African horse sickness (AHS) virus (AHSV), are transmitted in the field almost entirely by certain species of Culicoides biting midges. Adult midges can only become infected by ingestion of a blood-meal from an infected mammalian host and are only capable of transmitting the virus to a new host when they take a subsequent blood-meal. If adult vectors are absent for a period of time that is longer than the maximum duration of viraemia in the mammalian host, newly emerging insects should not become infected. The life-cycle of these viruses should therefore be broken and they would be unable to survive. Indeed, epidemiological studies have shown that in many areas of the world where adult vector insects are effectively absent during winter periods, viruses (such as BTV and AHSV) and the diseases they cause also ‘disappear’. However, these viruses and diseases frequently also reoccurdece annually, after quiescent periods that can last as long as 8 to 12 months (30, 31). These periods are significantly longer than the maximum published duration of viraemia, namely: <50 days for BTV in sheep (12, 25), <100 days for BTV cattle (16, 25), 18 days for AHSV in horses (18), and 40 days in zebra (20), confirming that a mechanism must exist that allows the virus to ‘overwinter’ in these locations.

The re-introduction of either infected adult midges or viraemic vertebrate hosts from other enzootic areas could give the appearance of overwintering. Although this possibility is often difficult to exclude with certainty, in at least some cases it cannot be reconciled with the available epidemiological data. For example, during the outbreak of AHS in Spain from 1987 to 1990, annual disease episodes were caused by serotype 4, which had never previously been recorded outside southern Africa. At the time
Survival of bluetongue virus in the winter

One possible overwintering mechanism could involve the long-term survival of infected adult vector insects. However, the life-span of an adult midge is usually less than 10 days. In exceptional conditions, some midges can survive for several weeks but there is no evidence to suggest that adult midges can survive for an entire intra-epizootic period of 9 to 12 months (17). The survival of BTV in this way is therefore considered to be exceedingly unlikely. Culicoides midges usually survive winter periods as 4th instar larvae but there is no evidence of transovarial or vertical transmission of BTV, AHSV or indeed any other arbovirus in these insects. Persistence of virus in the immature insect stages is therefore also considered to be highly improbable (17). A further possibility is that an unknown vector or vertebrate host species could be involved, providing a natural reservoir in which the virus could persist. This is also most unlikely, as the ‘winter’ conditions that result in the absence of the adults of known BTV and AHSV vector species of Culicoides, will have precisely the same effect on other less abundant and less efficient vectors (24). In respect of unknown vertebrate reservoirs, attempts to infect a wide range of animals (mice, rats, hamsters, guinea-pigs, rabbits, ferrets, dogs, other carnivores, camel and elephant) have been made at one time or another with either AHSV or BTV. In all cases these species are not considered to play a significant role in the epidemiology of either disease (1, 2, 6). In the absence of an identifiable overwintering reservoir, an alternative vector or mammalian host species, we have considered the possibility that orbiviruses might persist via some unidentified mechanism in their usual vertebrate hosts.

The overwintering mechanism – a hypothesis

Cattle and other ruminants were indeed incriminated as possible hosts for overwintering as early as towards the end of the 1960s (4). However, after the publication and retraction of controversial work indicating both latent BTV infection and a ‘showering’ phenomenon in cattle (13), the overwintering mechanism is a subject that has been largely avoided. For successful BTV transmission and recrudescence of the disease to occur, the BTV overwintering mechanism must make infectious virus particles available to feeding midges at a time when sufficient adult insects are available for successful transmission of the disease. A possible overwintering mechanism was recently suggested (29) based on the following observations:

a) ovine and bovine lymphocyte cultures (including \(\gamma\delta\) T-cells) can be persistently infected with BTV in vitro (26, 27, 29) without apparent cytopathogenic effect (CPE), or host cell protein shut-off, as has also been observed in persistently BTV-infected Culicoides cells (15)
b) BTV-infected \(\gamma\delta\) T-cells can be isolated from experimentally infected sheep, at least during viraemic periods (29)
c) persistently BTV-infected \(\gamma\delta\) T-cells can be converted to a more productive, lytic infection in vitro by co-culturing with anti-white collar-1 (WC-1) antibody, or with certain skin fibroblasts (29) (note: orthoreovirus structural protein \(\sigma-1\) is required for cell lysis and CPE. Orthoreovirus \(\sigma-1\) blocks the host cell cycle at the G1 phase (23). Anti-WC-1 antibody induces G1 cell growth arrest of proliferating \(\gamma\delta\) T-cells) (9, 10, 11, 28)
d) feeding of Culicoides midges induces skin inflammation in both ruminants and horses (19, 29; P.S. Mellor & M.H. Jeggo, unpublished observation)
e) skin inflammation recruits activated \(\gamma\delta\) T-cells into the inflamed areas (3, 7, 29)
f) BTV can be isolated for at least 9 weeks after the termination of detectable viraemia from cultures of activated \(\gamma\delta\) T-cells that were derived from skin biopsy sites on previously infected sheep, then cultured with interleukin-2 (IL-2) (29)
Bluetongue virus and disease

Proteases released by inflammation of the skin (8) may cleave the outer capsid protein of BTV to form infectious subviral particles (ISVP) (5, 14, 21, 22), which are ~100 times more infectious to midge cells and adult midges than intact BTV particles (22).

Thus we hypothesise that the inflammation induced at the site of midge feeding recruits γδ T-cells that are persistently infected with BTV, where interaction with skin fibroblasts converts the persistent infection to a lytic form. This results in an increase in BTV replication and virus production. The released BTV is then modified by inflammatory proteases, generating ISVP, the more infectious particle type (for insects), thereby increasing the likelihood that an infection will be established in the vector midges. The transmission of BTV from a fully infected adult midge to a susceptible mammalian host is very efficient, requiring only a single bite. The infection of even a single midge from a host in which the virus has successfully survived the winter may therefore be sufficient to reinitiate an outbreak of disease (Fig. 1).

Questions to be answered

We believe this hypothesis is the most logical and reasonable explanation of BTV overwintering mechanism based on currently available knowledge. A number of the component steps in the proposed mechanism remain to be explored and confirmed, so this still remains as only a hypothesis. These include:

1. Where are persistently infected γδ T-cells localised in the host during vector free periods?
2. How do persistently infected γδ T-cells (and possibly other infected cell types) remain undetected by immune surveillance?
3. Is there any role in this mechanism for other lymphocyte subsets, such as CD4+ and CD8+ T-cells, which can be persistently infected with BTV in vitro?
4. Are some species/breeds of ruminant more likely to support overwintering of BTV than others?
5. Are some BTV types/strains better adapted to overwintering than others?
6. What is the molecular mechanism by which BTV fails to shut-down persistently infected γδ T-cells, and how does WC-1 signalling enhance BTV replication?
7. Are sufficient numbers of infectious BTV particles released at the biting site to initiate infection in an adult vector insect?

It appears likely that in the absence of cell lysis BTV would exit persistently infected lymphocytes (e.g. γδ T-cells) by budding through the cell

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**Figure 1**

A possible overwintering mechanism for bluetongue virus

BTV can persistently infect ruminant γδ T-cells. Skin inflammation induced by biting midges has been shown to induce a severe inflammatory response in the ruminant host, resulting in recruitment of activated γδ T-cells (some of which may be infected with BTV) into the inflamed areas. Interaction between skin fibroblasts and γδ T-cells appears likely to occur via binding of WC-1 on the γδ T-cell surface, to a WC1 ligand on skin fibroblasts (inflammation may also up-regulate expression of the WC1 ligand). This interaction causes host cell shut-off in the activated γδ T-cells, accompanied by conversion to a lytic and more productive form of BTV-infection. This will result in the release of BTV particles at skin locations where midges are feeding. Inflammation associated proteases may also cleave the BTV outer capsid protein ‘VP2’, generating infectious subviral particle (ISVP) that are ~100 times more infectious for Culicoides midges than intact virus, further increasing the infection rate of the feeding midges.
membrane and would become coated by the host lymphocyte membrane in the process – ‘membrane enveloped virus particles’ (MEVP) (15). This suggests some possible answers to the questions listed above (Fig. 2). For example:

1. Since the BTV outer capsid would be coated with a lymphocyte membrane, neutralising antibodies may be unable to recognise the concealed outer capsid proteins (VP2 and VP5).

2. Lymphocyte surface membranes contain a number of important molecules associated with lymphocyte-lymphocyte interactions. Thus, the lymphocyte-derived MEVP may specifically bind to and infect other lymphocytes generating further persistently infected cells.

3. Interaction between persistently infected and uninfected lymphocytes may also result in transmission of BTV infection via fusion between the cellular and budding membranes (interaction between γδ T-cells and CD4+ T-cells has been reported).

4. MEVP may be less effectively detected by conventional BTV isolation methods (but could be detected by reverse transcriptase-polymerase chain reaction [RT-PCR]).

5. MEVP that may bind to or be taken-up by dendritic cells (DC), may induce peripheral tolerance or anergy, due to ‘self-antigen’ on the virus surface. Consequently DC could suppress anti-BTV immune responses.

6. It appears likely that host lymphocyte membranes would be removed by proteases associated with inflammation and may not therefore affect the ability of the resulting modified virus particles (ISVP) to infect midges.

A vital component of the suggested mechanism for BTV overwintering is the requirement for persistent or latent BTV-infection of γδ T-cells, which has been demonstrated in vitro. However, for this mechanism to be effective, persistently infected γδ T-cells (or possibly other lymphocytes) must exist in vivo for periods of up to 9 to 12 months after the termination of viraemia. A demonstration that these cells can exist for long periods in the post-viraemic mammalian host is still required and would strongly support the overwintering hypothesis.

**Figure 2**

A possible mechanism to maintain persistent infection of bluetongue virus in mammalian host species.

Lymphocytes from cattle and sheep can be persistently infected with BTV. Although this persistent infection does not result in host cell shut-off or lysis (so there is little or no sign of CPE) the virus can still escape by budding through the cell membrane, a mechanism that has previously been observed in both mammalian and insect cells. In doing so, the virus particles initially become coated with host cell membranes and have been identified as membrane enveloped virus particles (MEVPs). The BTV outer capsid components (VP2 and VP5) are likely to be masked by this envelope, making MEVPs less likely to be neutralised by antibodies. On the other hand, when taken-up by dendritic cells (DC) or other antigen presenting cells (APC), this could result in peripheral tolerance, or anergy due to ‘self-antigen’ on the virus surface. Lymphocyte membranes contain a number of important molecules associated with lymphocyte-lymphocyte interactions. As a consequence, MEVP may be able to bind to other lymphocytes, enhancing their infectivity for these cells. Direct interactions between persistently infected and uninfected lymphocytes could also result in BTV transmission.
It is recognised that BTV infection in cattle is clinically milder than sheep and longer viraemic periods have been reported. It therefore appears likely that the virus may be better adapted to bovines than ovines. Thus cattle may be a better mammalian host species for further study of BTV persistence and overwintering. Epidemiological studies of specific breeds of sheep, cattle or goats from locations where BTV overwintering has been observed may also identify other species/breeds of ruminant host that are more susceptible to persistent infection and overwintering. Molecular biological studies (sequencing and/or generation of reassortant virus strains) with BTV isolates from locations where overwintering has been observed, may also contribute to our understanding of the mechanism involved. Field isolates from the current outbreaks in Eastern Europe may therefore provide valuable reagents allowing us to study and elucidate the overwintering mechanism in the laboratory. The design of fully effective anti-BTV strategies may only be possible with a full understanding of the mechanisms involved in virus persistence in the field. We therefore hope, by proposing this hypothesis, to trigger an international effort to solve the mystery of BTV overwintering.

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References


Bluetongue: pathogenesis and duration of viraemia

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Summary

Bluetongue (BT) is a non-contagious, insect-transmitted disease of domestic and wild ruminants caused by bluetongue virus (BTV). Whereas BTV infection of the haematophagous *Culicoides* insect vector is persistent (life-long), BTV infection of ruminants is transient. The prolonged viraemia that occurs in many BTV-infected ruminants occurs through a novel interaction of the virus with erythrocytes and, initially, other blood cell types. The presence of BTV in ruminant blood can readily be detected by polymerase chain reaction which provides a very conservative assay for the screening of ruminants prior to movement to BTV-free regions as animals remain positive by PCR assay long after all infectious virus is cleared from their blood. BT disease occurs in sheep and some wild ruminant species and is characterised by vascular injury with haemorrhage, oedema and tissue necrosis. Inherent, species-specific differences in the susceptibility and responses of endothelial cells may be responsible for the occurrence of BT disease in BTV-infected sheep but not cattle. Although BT was once considered to be a global emerging disease that was spread by animal movement and trade, it now is clear that BTV exists throughout tropical, subtropical and some temperate regions of the world in distinct, relatively stable, ecosystems where different strains of the virus have co-evolved over long periods of time with different species of insect vector.

Keywords


Introduction

Bluetongue (BT) virus (BTV) is the aetiological agent of BT, a non-contagious, insect-transmitted disease of sheep and some species of wild ruminants (18, 19, 24, 30, 33, 41). BT was first recognised and comprehensively described in southern Africa, and BTV has subsequently been isolated from ruminants and/or vector insects from all continents except Antarctica (reviewed in 21). As BTV infection of ruminants is not contagious, the global distribution of BTV coincides with the distribution of competent *Culicoides* insect vectors and hot or warm climatic conditions. Although BTV infection of domestic and wild ruminants occurs throughout much of the world with minimal occurrence of disease, BT is just one of 16 diseases classified in List A by the Office International des Épizooties (OIE). As a direct consequence of its inclusion in OIE List A, BT continues to have an impact on the global trade of ruminants and their germplasm (2). Concerns over the potential impact of BT on animal production have been heightened by the recent re-emergence of BT in Mediterranean and south-eastern Europe (6).

History

The disease of BT was first described as ‘malarial catarrhal fever’ and ‘epizootic catarrh of sheep’ in the original written descriptions by investigators in South Africa. The name of ‘bluetongue’ was later used to describe the distinctive cyanotic tongue of some severely affected sheep. The first descriptions of BT were published in the late 19th and early 20th centuries, although farmers in South Africa recognised the disease soon after the introduction of fine-wooled European breeds of sheep to that region of the world (18, 41). Prior to the 1940s, BT was thought to be confined to southern Africa. The first well-documented epizootic of BT outside Africa occurred amongst sheep on Cyprus in 1943. The disease was recognised in Texas soon thereafter, and an extensive epizootic occurred on the Iberian Peninsula in 1956-1957. Subsequently, the disease was recognised in the Middle East, Asia and southern Europe. These epizootics were interpreted in the middle of the 20th century to reflect the emergence of BT disease from its presumed ancestral origin in Africa, leading to ‘doomsday’
scenarios regarding putative global spread of BT that justified its inclusion in OIE List A. It is now clearly evident that BTV infection occurs throughout tropical and subtropical regions of the world, extending also into many temperate regions as well. BT disease, however, is either rare or non-existent in many regions with endemic BTV infection. Furthermore, it is clearly apparent that the global spread of BTV was not a recent event, and that different serotypes and strains of BTV have evolved in different regions of the world, coincident with the presence of distinct species of Culicoides insect vectors (reviewed in 4, 21, 43).

The pathogenesis of bluetongue virus infection of ruminants

The pathogenesis of BTV infection is similar in sheep and cattle, and most probably, all species of ruminants (5, 24, 28, 35). There are marked differences in the severity of disease that occurs in different ruminant species after BTV infection, however, with cattle being especially resistant to expression of BT disease. After cutaneous instillation of virus through the bite of a BTV-infected Culicoides vector, the virus travels to the regional lymph node where initial replication occurs. The virus then is disseminated to a variety of tissues throughout the body where replication occurs principally in mononuclear phagocytes and endothelial cells. Viraemia in BTV-infected ruminants is highly cell associated, and viraemia is prolonged but not persistent (reviewed in 5, 9, 24, 40). The virus promiscuously associates with all blood cells, thus titres of virus in each cell fraction are proportionate to the numbers of each cell type; specifically, BTV is quantitatively associated most with platelets and erythrocytes and, because of the short lifespan of platelets, virus is most associated with erythrocytes late in the course of BTV infection of ruminants. BTV infection of erythrocytes facilitates both prolonged infection of ruminants and infection of haematophagous insect vectors that feed on viraemic ruminants (10, 11). Interestingly, BTV nucleic acid may be detected by polymerase chain reaction (PCR) in the blood of infected cattle and sheep for many months after it no longer can be detected by virus isolation in cell culture or inoculation of susceptible sheep. Furthermore, ruminant blood that contains BTV nucleic acid as determined by PCR assay, but not infectious BTV as determined by virus isolation, is not infectious to vector insects by either intrathoracic inoculation or oral feeding (9, 24, 26, 44). Ruminant blood that is positive by PCR assay but not by virus isolation is also not infectious to other ruminants (25, 26).

Ruminants infected with BTV develop a prompt and high titered antibody response to a variety of viral proteins. Serotype-specific neutralising antibodies are directed against VP2, and these can be detected by the serum neutralisation test (12). Antibodies directed against core protein VP7, as well as other structural and non-structural proteins, may be detected with serogroup-reactive assays such as the agar gel immunodiffusion and competitive enzyme-linked immunosorbent assay (c-ELISA). A positive serological result confirms only that an animal was previously infected with BTV. Thus, although BTV infection of cattle and sheep often is prolonged, there is no credible evidence of truly persistent BTV infection of ruminants (5, 9, 21, 26, 40, 44) and the vast majority of seropositive cattle and sheep from BTV-endemic regions are not infected with the virus.

Clinical signs and lesions in BTV-infected sheep possibly reflect virus-mediated endothelial injury, as BTV replicates in endothelial cells causing cell injury and necrosis (28, 35). Similarly, white-tailed deer, which are highly susceptible to BT, develop consumptive coagulopathy as a consequence of BTV-induced damage to endothelial cells (22). Consumptive coagulopathy (disseminated intravascular coagulation) in BTV-infected sheep and deer predisposes to the bleeding tendency that characterises fulminant BT. Endothelial injury is also probably responsible for increased vascular permeability leading to oedema in tissues, such as the lung (pulmonary oedema), and vascular thrombosis leads to tissue infarction.

Foetal infection was once proposed to lead to persistent, immunotolerant infection of cattle. However, this theory has been thoroughly and repeatedly discredited (reviewed in 25). It now is increasingly clear that only strains of BTV that have been modified by growth in cell culture, such as modified live virus (MLV) vaccine strains, have the capacity to cross the ruminant placenta. Once MLV BTV strains cross the placenta they cause embryonic or foetal death, and cerebral malformations after infection of older foetuses that survive congenital infection (reviewed in 27).

Bluetongue disease of ruminants

Although BTV infection of both wild and domestic ruminants occurs throughout tropical, subtropical and temperate regions of the world, BT disease is uncommon or not recognised in many areas where BTV is endemic. For example, BTV infection occurs throughout extensive regions of northern and eastern Australia yet outbreaks of BT disease have
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not yet been described. Similarly, there are very few descriptions of BT amongst ruminants in the Caribbean islands or in Central and South America, despite endemic BTV infection in much of this region.

BT occurs principally in sheep and some species of wild ruminants, whereas BTV infection of cattle, goats and most wild ruminant species is typically asymptomatic or subclinical. The signs of BT in sheep reflect congestion, oedema and haemorrhage as a consequence of virus-mediated vascular injury. Thus, sheep with BT have any combination of fever, serous to bloody nasal discharge, oral erosions and ulcers, lameness with hyperaemia of the coronary band, and weakness secondary to muscle necrosis. Lesions present at post-mortem of affected sheep can include hyperaemia, haemorrhages, erosion and ulceration of the mucosa of the upper gastrointestinal tract (oral cavity, oesophagus, forestomachs); subintimal haemorrhages in the pulmonary artery; pulmonary oedema; pleural and/or pericardial effusion; oedema within the fascial planes of the muscles of the abdominal wall; necrosis of skeletal and cardiac muscle, with the papillary muscle of the left ventricle being an especially characteristic site (reviewed in 18, 19, 24, 28, 30, 33, 35, 41).

It is to be emphasised that most BTV-infected sheep develop mild or no obvious disease, especially in BTV-endemic areas. Outbreaks of BT typically occur either when susceptible sheep are introduced into BTV-endemic regions, or when the virus spreads into immunologically naive sheep populations at the interface of BTV-endemic and non-endemic regions. Expression of BT disease reflects a variety of virus, host and vector factors.

**Virus factors**

Field strains of BTV in endemic areas such as California exhibit remarkable genetic heterogeneity, even amongst strains that co-circulate (4, 8, 16, 17, 34). This genetic variation amongst field strains of BTV has arisen as a consequence of both genetic drift and reassortment of individual viral genes. Reassortment of BTV genes has been demonstrated after infection of either the ruminant host or insect vector with different strains or serotypes of BTV (37, 38). It is clear in endemic areas that gene segments, other than the L2 gene, evolve and reassort independently of serotype amongst field strains of BTV, and that individual genes also evolve and reassort independently of one another. Accumulation of nucleotide substitutions within each BTV gene leads to genetic drift of each. We recently have established that genetic drift of BTV genes occurs by selective acquisition and amplification in vector insects of specific viral variants from the quasispecies virus population that arises in the blood of infected ruminants through the process of founder effect (7, 9). It is logical that this considerable genetic variability of BTV is reflected by differences in phenotypic properties of each virus strain, including their virulence to susceptible ruminants.

**Ruminant factors**

Selected breeds of sheep and wild ruminant species (e.g. white-tailed deer) are most susceptible to BT disease. Sheep that are native to tropical and subtropical regions of the world where BTV is endemic are usually resistant to BT, whereas fine-wooled European breeds such as the Merino are highly susceptible. Nutritional status, immune status and age also influence the severity of BT in individual sheep, as can environmental stress such as high temperature and ultraviolet radiation.

A fundamental question that has vexed scientists for many years is why virulent strains of BTV produce disease in sheep but not in cattle (5, 36). The fact that the pathogenesis of BTV infection of sheep and cattle is similar further emphasises this obvious paradox. Fundamental differences have recently been identified in the inherent susceptibility of endothelial cells from cattle and sheep to BTV infection (13, 14, 15). To facilitate these studies, we isolated and propagated pure cultures of endothelial cells from the microvasculature of sheep and cattle, and then evaluated their responses to infection with BTV. Lung microvascular endothelial cells were selected because pulmonary oedema and microvascular injury are both highly characteristic of BT disease. Interestingly, whereas BTV infection of the bovine endothelial cells resulted in endothelial activation, with the increased transcription of genes encoding a variety of vasoactive and inflammatory mediators and increased expression of cell surface adhesion molecules, similar infection of sheep endothelial cells resulted in minimal activation of endothelial cells. Furthermore, the ratio of thromboxane to prostacyclin, which is indicative of enhanced coagulation and possible consumptive coagulopathy, was significantly greater in sheep than in cattle that were experimentally infected with BTV.

**Vector factors**

_Culicoides_ vectors are critical to the survival and transmission of BTV as infection is not contagious and there is no credible evidence of long-term maintenance of BTV in ruminants. Thus, BTV infection occurs only where competent vectors are present. Furthermore, both BTV infection and BT
disease usually occur during late summer and early autumn when numbers of insect vectors are highest in BTV-endemic areas such as California (20, 42). In an extensive field study in California, virtually all (approximately 98%) isolations of BTV from ruminants were made between August and the end of November (32, 42).

Climatic conditions also have a significant impact on the transmission of BTV. For instance, insect survival is inversely related to temperature so that Culicoides insects survive for longer periods in cool temperatures. In contrast, higher ambient temperatures stimulate insect feeding and promote virogenesis of BTV in insects, both of which enhance virus transmission (31). Lastly, it is to be stressed that the environmental conditions that produce the highest numbers of vector insects are likely to optimise the transmission of BTV amongst ruminants. The species of Culicoides that transmit BTV in different regions of the world are clearly very different, as may be the environmental factors that promote population expansions of each.

The role of ruminants in the epidemiology of BTV infection

Both insects and ruminants are essential to the life-cycle of BTV. Vector Culicoides insects become persistently infected with BTV for their entire lifespan after acquiring infection through feeding on a BTV-infected ruminant. Although venereal and vertical transmission of BTV can occur in ruminants, these routes are unimportant to the maintenance of BTV and the distribution of BTV in the world coincides only with that of competent vector insects (24, 43). Climatic conditions also dictate the global distribution of BTV, thus the virus exists in an extensive band that includes tropical, subtropical and temperate regions of the world between latitudes of approximately 40°N and 35°S. Exceptions are areas of North America and Eurasia, where BTV infection of ruminants can occur as far as 50°N. The species of vector insects that transmit BTV differ between regions, and are especially poorly characterised in much of both Europe and Asia (23, 39).

Culicoides insects are biological vectors of BTV, thus the virus replicates within the tissues of each insect after infection from feeding on the blood of a BTV-infected ruminant (reviewed in 29). Vector insects can only transmit BTV to another susceptible ruminant after an extrinsic incubation period of approximately 10 to 14 days, during which time the virus is disseminated from the gut of the insect to its salivary glands. The external incubation period is shorter when insects are held at high ambient temperatures. Ambient temperature has a profound effect on the survival of vector insects, their feeding activity and the replication of BTV in the insect vector (31). Thus, temperature-dependent control of virogenesis and other similar insect-dependent factors possibly limit the expansion of BTV into regions outside its current range, even into areas where apparently competent vector insects occur. Global warming, however, would be predicted to expand the northern and southern extremes of global BTV distribution (21), as has recently been the case in southern Europe (6).

It is increasingly evident that BTV has not recently been spread globally through international trade and movement of ruminants. Rather, the virus exists in distinct, relatively stable ecosystems in different regions of the world where specific strains of the virus have probably co-evolved with different species of insect vector (4, 21, 43). Thus, in the Americas, the serotypes of BTV that circulate in the United States are different from those in adjacent regions of the Caribbean and Central America, despite the lack of any substantial geographic barrier between the regions and the movement of enormous numbers of cattle between the two regions without any testing for BTV. The essential difference lies in the different species of vector insects in the two regions: Culicoides sonorensis is the vector of BTV serotypes 10, 11, 13 and 17 in the United States, whereas Culicoides insignis is the vector of BTV serotypes 1, 3, 4, 6, 8, 12, 14 and 17 in the Caribbean and Central/South America.

A variety of other hosts have been implicated in the life-cycle of BTV infection. Serological evidence indicates that large African carnivores are infected with BTV, whereas smaller predators that co-habit with them are not, suggesting that large carnivores are infected through feeding on BTV-infected ruminants (3). Inadvertent contamination of a canine vaccine with BTV confirmed that dogs are susceptible to BTV infection, indeed pregnant bitches that received this contaminated vaccine typically aborted and died (1). There is no evidence, however, that dogs or other carnivores are important to the natural cycle of BTV infection.

References

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S10 segment sequence analysis of some Greek bluetongue virus strains

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Summary

Sequence analyses of the non-structural protein gene NS3/NS3A of eight Greek bluetongue (BTV) virus (BTV) field isolates from the 1979 and 1999-2001 epizootics provide preliminary molecular data on the epidemiology of BT in Greece. These isolates from infected sheep belonged to serotypes BTV-1, BTV-4, BTV-9 and BTV-16. Phylogenetic analysis of the NS3/NS3A gene segregated these Greek isolates of BTV into two monophyletic groups. The first group was formed by all isolates of BTV-4; all were identical in their sequences, regardless of the area and year of isolation in Greece, and clustered with strains from Tunisia and Corsica. The isolates of BTV-1, BTV-9 and BTV-16 segregated into a second monophyletic group and clustered with Asian strains, showing a high homology (97-99%). From an epidemiological point of view, these preliminary results infer that one group of isolates is Mediterranean, whilst the second appears to be of Asian origin.

Keywords


Introduction

Bluetongue (BT) virus (BTV) is the causative agent of BT, an arthropod-transmitted disease of wild and domestic ruminants. BTV is the prototype virus of the genus Orbivirus in the family Reoviridae. There are at least 24 serotypes known worldwide (5). Isolates belonging to four serotypes (BTV-1, BTV-4, BTV-9 and BTV-16) have been identified to occur in Greece thus far (authors, unpublished data).

The BTV double-stranded RNA genome consists of 10 unequal segments that are encapsulated by a double-layered icosahedral shell, and encodes 7 structural (VP1-VP7) and 4 non-structural (NS1, NS2, NS3/NS3A) proteins (11). The smallest genome segment, S10, encodes the proteins NS3/NS3A of 229/216 amino acid lengths. These two proteins mediate the release of BTV particles from infected cells (7). Therefore, they may play an important role in the transmission of the virus and its subsequent dissemination, thus playing a major role in the epidemiology of BT.

Different workers have published phylogenetic analyses of NS3/NS3A sequences of BTV. Comparison of strains from the United States of America (USA) and China showed that these segregated into different monophyletic clusters based on geographical location (1). Moreover, it was found that factors such as serotype, host species or year of isolation did not influence cluster formation (14).

BT occurs throughout the temperate and tropical regions of the world, in an area that parallels the distribution of the competent vector, Culicoides spp. (3). In Greece, BT occurred for the first time on the east Aegean island of Lesbos in the autumn of 1979 (8) and, following the application of a BT control programme, the island was declared officially free of the disease in 1991. New BT epizootics reappeared in the late 1990s (1998-1999) and during 2000-2001 not only on the East Aegean islands, but also in the north central, north-west, west and mainland Greece (authors, unpublished data). Culicoides imicola is considered to be the main vector species linked with BTV transmission in Greece, as is the case in most other European countries (10). The purpose of this...
study was to investigate the epidemiology of BT in Greece, based on the phylogenetic analysis of the NS3/NS3A sequences of BTV isolates originating in different epizootics. Moreover, it was also considered of interest to study the extent of sequence variation in these Greek isolates and to compare them with those of other published strains (1, 14, 17) and on GenBank.

Materials and methods

Virus isolates

A total of eight BTV isolates belonging to four serotypes (BTV-1, BTV-4, BTV-9, BTV-16) were obtained from blood or spleen of naturally infected sheep, using the embryonated chicken egg (ECE) method and tissue culture techniques, and serotyped as described in the Office International des Épizooties (OIE) protocol (12). The isolates were stored at –70°C until further use. Details of all Greek isolates used in this study are shown in Table I. GenBank accession numbers of strains not otherwise published in the literature are provided in Fig. 1.

RNA extraction

Total RNA was extracted from BTV-infected cell cultures using the Trizol™ LS reagent (Invitrogen GmbH) in accordance with the instructions of the manufacturers. Each isolate was processed independently to minimise any sample-to-sample contamination. For each isolate, the amount of total RNA was measured in sterile RNAse free water, using the BioPhotometer (Eppendorf) and stored at –70°C.

Table I
Details of the Greek field isolates used in this study

<table>
<thead>
<tr>
<th>Virus</th>
<th>Serotype</th>
<th>Area</th>
<th>Year</th>
<th>Tissue</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR79LS</td>
<td>BTV-4</td>
<td>Lesbos</td>
<td>1979</td>
<td>Spleen</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR308/99RS</td>
<td>BTV-16</td>
<td>Rhodes</td>
<td>1999</td>
<td>Blood</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR408/99ChS</td>
<td>BTV-9</td>
<td>Chalkidiki</td>
<td>1999</td>
<td>Blood</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR395/99LS</td>
<td>BTV-4</td>
<td>Lesbos</td>
<td>1999</td>
<td>Spleen</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR692/99EvS</td>
<td>BTV-4</td>
<td>Evia</td>
<td>1999</td>
<td>Spleen</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR457/99PS</td>
<td>BTV-4</td>
<td>Pieria</td>
<td>1999</td>
<td>Spleen</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR631/99MaS</td>
<td>BTV-4</td>
<td>Magnesia</td>
<td>1999</td>
<td>Blood</td>
<td>Sheep</td>
</tr>
<tr>
<td>GR15/01Gre</td>
<td>BTV-1</td>
<td>Grevena</td>
<td>2001</td>
<td>Spleen</td>
<td>Sheep</td>
</tr>
</tbody>
</table>

Figure 1
Phylogeny of the NS3/NS3A sequences of the Greek field isolates and other strains used for reference purposes in this study

The NS3 gene of EHDV-1 was included as the outgroup in the analysis
Branch lengths are indicative of the genetic distances between the sequences
Accession numbers not available from the bibliography (1, 14, 17) are provided in the Figure
Reverse transcription and amplification of the S10 segment

The primers were designed based on the sequences of the S10 segment published by Hwang et al. (6) and synthesised at MWG, Germany. The forward primer (F10P) identified nt 1-22 and the reverse primer (F10M) identified nt 798-822 on the S10 gene sequence. Both the reverse transcription and the PCR reactions were performed using the One Step RT-PCR kit (Qiagen GmbH, Germany) following the instructions of the manufacturer. Approximately 5 µg of total RNA was used for each reaction. The PCR product was run on a 1.7% agarose gel where the presence of a band (approximately above the 800 bp band on the 100 bp DNA ladder) indicated the amplified product.

Sequencing of the PCR product

The PCR amplification products were cleaned using the MinElute PCR Purification Kit (Qiagen GmbH) following which both strands were sequenced using the forward and reverse primers. The nucleic acids were analysed, aligned and a dendrogram created using the Clustal W Software program (16). The TreeView program of Page (13) was used to view and edit the dendrogram.

Results

Overall, among the Greek isolates, the NS3/NS3A sequence homologies ranged between 84-100%. The five isolates that belonged to serotype BTV-4 (GR79LS, GR395/99LS, GR692/99EvS, GR457/99PS and GR631/99MaS) presented 100% homology. The isolates that belonged to serotypes BTV-1, BTV-9 and BTV-16 (GR15/01Gre, GR408/99ChS and GR308/99RS, respectively), presented homologies of 97-99% in the NS3/NS3A sequences. The variation in the nucleotide sequences between the former and the latter group of isolates ranged between 15% and 16%.

Phylogenetic analysis separated these isolates into two main clusters (Fig. 1): one that consisted of isolates belonging to serotype BTV-4 and the other that included isolates belonging to serotypes BTV-1, BTV-9 and BTV-16 (referred to as Group I and Group II, respectively, for the purpose of this study). Group I isolates clustered closely with the Corsican and the Tunisian strains and, at some distance, with the Group A United States field isolates of Pierce et al. (14) and the United States prototype strain of serotype BTV-2. On the other hand, Group II isolates clustered with the Asian strains (Indian, Chinese and Australian). The South African strain of BTV-1 used in this analysis also fell into this cluster.

Discussion

The objective of this study was to examine the sequence variation of the NS3/NS3A gene of Greek isolates and to investigate the epidemiology of BT in Greece during the epizootics of 1979 and 1999-2001 by molecular and phylogenetic analyses of this gene.

The NS3/NS3A gene is considered to be one of the conserved genome segments of BTV as the proteins encoded by this gene tolerate little variation (14). However, variation of up to 20% in nucleic acid sequence and up to 10% in amino acid sequence has been reported in certain cases (6, 14, 17). In this study, variation of 15% to 16% was observed among the Greek isolates and therefore is in agreement with the above reports.

The results of the present work divided the eight Greek BTV field isolates into two distinct groups on the basis of their NS3/NS3A gene sequences. All isolates of serotype BTV-4, regardless of the year and the area of isolation, were included in Group I and were identical in their sequences. Group II consisted of isolates of three different serotypes (BTV-1, BTV-9 and BTV-16) with homologies in the nucleic acid sequences of 97-99%. Our finding regarding 100% identity in the NS3/NS3A sequences among the Greek BTV-4 isolates from the 1979 and 1999 epizootics indicate that this serotype of BTV must have existed in the neighbouring areas during the past 20 years, without significant mutations so confirming the resistance of this gene to selective environmental pressure. It is noteworthy that two of these isolates, GR79LS and GR395/99LS, came from the same island (Lesbos), with a gap of 20 years, even though an eradication programme was implemented following upon the 1979 epizootic and, after extensive serological surveillance, Greece was declared free of the disease in 1991.

Phylogenetically, the isolates of Group I clustered closely with the serotype BTV-2 strains from Tunisia and Corsica and more distantly to the United States Group A strains of Pierce et al. (14) that included serotypes BTV-2, BTV-10 and BTV-13. This suggests that in spite of different origins and serotypes, all viruses in this cluster might have a common ancestor as has also been reported by others (17). On the other hand, the isolates of Group II were closely related to the Asian group, thereby indicating their origin from the countries of the East. Interestingly, the Greek BTV-4 isolates did not fall into the same cluster as the Chinese BTV-4 strain. This would mean that, despite being the same serotype, these two strains have different origins with regard to the NS3/NS3A gene.
Sequence analysis of a conserved gene, such as NS3/NS3A, may be used to assign a BTV isolate as a ‘topotype’ in a given geographic region, regardless of its serotype (4). Accordingly, it may be said that the isolates of Group I and Group II form two different BTV ‘topotypes’.

Previous studies have shown that the BTV populations that circulated in a particular geographic region form one or two monophyletic groups (1, 14, 17). This is likely to be the consequence of the co-evolution of the virus populations and the specific insect vector that occurs in each particular region (3). In this study, the two distinct monophyletic groups that were identified could be related to the occurrence of different Culicoides vector species in Greece. As in other Mediterranean countries, C. imicola is the common BTV vector in Greece and it has been identified on the islands of Lesbos, Rhodes and Chios and on mainland Greece (2, 18). Culicoides obsoletus (C. obsoletus) is also suspected to be a potential vector of BTV transmission in different European countries (9, 18). Moreover, it has recently been reported that a member of the C. obsoletus species complex is co-involved with C. imicola in the transmission of BTV in Italy but that in Bulgaria and the neighbouring territories of the Former Yugoslav Republic of Macedonia, Serbia, Montenegro and Croatia, only C. obsoletus is involved (15). Therefore, it is possible that the presence of these two species of Culicoides has contributed to the formation of the two different monophyletic groups of BTV in Greece.

In conclusion, based on the NS3/NS3A sequence analysis, the various populations of the four serotypes of BTV identified in Greece during the epizootics of 1979, 1999 and 2001 are separable into two monophyletic groups. From an epidemiological aspect, it may be inferred that one group is related to other Mediterranean strains and that the other is of Asian origin.

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References


VP2 gene sequence analysis of some isolates of bluetongue virus
recovered in the Mediterranean Basin during the 1998-2002 outbreak

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Summary
Since 1998, five serotypes of bluetongue virus (BTV), BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16, have been reported in countries surrounding the Mediterranean Basin. Preliminary data on the sequencing analysis of the VP2-genes of BTV isolates recovered during the 1998-2002 epizootic of BT in Italy, Greece and Israel were studied. The VP2-genes of the Italian BTV-2 and BTV-9, Greek BTV-4 and BTV-9, Israeli BTV-4 and BTV-16 and South African BTV-2, BTV-4, BTV-9 and BTV-16, together with those of their corresponding South African serotype reference and vaccine strains, were cloned and the sequences of their terminal ends determined. These sequences, as well as those of all BTV VP2-gene sequences currently available on GenBank, were used to compile a phylogenetic tree to determine the probable geographic origins of the BTV incursions into Europe. The Italian isolates included in this study were from different regions, animal hosts and years (2000-2002). The results demonstrated that sequencing of the terminal end of the VP2-gene of BTV can be used for topotyping. According to the phylogenetic analysis, the Italian BTV-2 and BTV-9 isolates were stable across all species, irrespective of geographic origin and year of isolation. The sequencing data of the Italian isolates were identical to those of a BTV-2 isolate from Corsica. There was 97% homology between the Italian and Corsican BTV-2 isolates and the BTV-2 vaccine and reference isolates from South Africa. Italian BTV-9 isolates were also identical to the Greek BTV-9 isolates (99% homology). Surprisingly these BTV-9 isolates had only 67% homology with the reference BTV-9 isolate from South Africa. Conversely, BTV-9 field isolates from Australia and elsewhere in Europe had 89% homology with the Italian isolate at the nucleic acid level. Greek and Israeli BTV-4 isolates were almost identical (98% homology) and shared a 90% homology with the BTV-4 South African reference and vaccine strains. Israeli BTV-16 and South African BTV-16 reference strains were also similar. From these results, it may be concluded that Italian and Corsican BTV-2, Israeli and Greek BTV-4, and South African and Israeli BTV-16 had a common origin. The Greek BTV-9 isolate had more than 99% homology with the isolates from Italy, indicating these isolates to have had a common origin. The European BTV-9 isolates, grouped as ‘eastern isolates’, were more similar to the Australian isolates than to the South African reference strains.

Keywords

Introduction
The Mediterranean Basin is currently experiencing a severe and long-lasting outbreak of bluetongue (BT) disease in sheep. Since 1998, BT has been reported in many European countries. In Italy, the first evidence of BT infection was reported in Sardinia in August 2000 and since then numerous outbreaks
have been described, including the regions of Sicily, Calabria, Basilicata, Puglia, Campania, Lazio, Tuscany, Abruzzo and Molise (2, 3). Five serotypes of the bluetongue virus (BTV): (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16) have been reported from many of the countries surrounding the Mediterranean Basin (2, 3, 4). Several hypotheses have been formulated regarding the possible origin of these incursions into Europe, but little data are available. It is crucial to know more about the possible origin of the various BTV serotypes recovered in these countries in order to better understand the epidemiology of the infection for the implementation of more effective control measures in the future. This study provides preliminary data on the sequencing analysis of the VP2 genes of BTV isolates recovered during the 1998-2002 epizootic of BT in Italy, Greece and Israel (1, 6, 7).

Materials and methods

Virus isolates

A phylogenetic tree was compiled using the BTV VP2 gene sequences of the Italian, Greek, Israeli, South African BTV-2, BTV-4, BTV-9 and BTV-16 reference and field isolates; also included were all BTV VP2 gene sequences currently available on GenBank. Italian isolates were from different regions, animal species (cattle, goats, sheep and deer) and years (2000-2002). Table I lists the various isolates sequenced.

dsRNA extraction

The isolates of BTV were propagated in a 75 cm² flask containing a monolayer of BHK-21 cells. From each isolate, the total RNA was extracted using Tri-Reagent (MRC), following the protocol of the manufacturer. The ssRNA was then removed from the total RNA by precipitation with 2M LiCl. The dsRNA was purified from the supernatant using a Gel extraction Kit (QIAGEN). The purified full-length VP2 gene PCR products were cloned into pGEM-T easy (Promega), according to the specifications of the manufacturer. Positive clones were selected based on insert size and restriction analysis.

Table I

<table>
<thead>
<tr>
<th>No.</th>
<th>NRV</th>
<th>Serotype</th>
<th>Year</th>
<th>Region</th>
<th>Province</th>
<th>Animal host</th>
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<tbody>
<tr>
<td>1</td>
<td>1155</td>
<td>2</td>
<td>2002</td>
<td>Sicily</td>
<td>ME</td>
<td>Goat</td>
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<tr>
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</tr>
<tr>
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<td>ME</td>
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<td>9</td>
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<td>CZ</td>
<td>Sheep</td>
</tr>
<tr>
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<td>1617</td>
<td>2</td>
<td>2000</td>
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<td>OR</td>
<td>Sheep</td>
</tr>
<tr>
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<td>1853</td>
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<td>Basilicata</td>
<td>PZ</td>
<td>Sheep</td>
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<tr>
<td>8</td>
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<td>9</td>
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<td>Vaccine</td>
<td>RC</td>
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<tr>
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<tr>
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<td>ME</td>
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<tr>
<td>15</td>
<td>3820</td>
<td>2</td>
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<tr>
<td>20</td>
<td>8340</td>
<td>2</td>
<td>2000</td>
<td>Sardinia</td>
<td>CA</td>
<td>Sheep</td>
</tr>
</tbody>
</table>

Israeli isolates

3C  16  1999  Beit Dagan  Sheep
35C  4  2001  Nahalal  Sheep
38C  4  2001  Hatzav  Sheep

Greek isolates

BTV-4  4  2001  Sheep
BTV-9  9  1999  Sheep

South African isolates

BTV-2, BTV-4, BTV-9, BTV-16 reference and vaccine strains
5  1373  4  2002  Vaccine  Cartile
8  1931  9  2001  Vaccine  Sheep

NRV identification number for the IZSA&M Virology Department
ME  Messina  CZ  Catanzaro
OR  Oristano  PZ  Potenza
RC  Reggio Calabria  PA  Palermo
LT  Latina  NU  Nuoro
GR  Grosseto  RM  Roma
VT  Viterbo  SA  Salerno
CA  Cagliari  BTV  bluetongue virus
Table II
Primers used for the reverse transcriptase-polymerase chain reaction of full-length bluetongue virus VP2

<table>
<thead>
<tr>
<th>BTV serotype</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<td>BTV-2 (South Africa)</td>
<td>5′-GTTAAAACAGGATCGGATGGATGACGC-3′</td>
<td>5′-GTAAGTTCAACAGATCGGACCTGC-G-3′</td>
</tr>
<tr>
<td>BTV-2 (Italy)</td>
<td>5′-GTTAAAACAGGATCGGATGGATGACGC-3′</td>
<td>5′-GTAAGTTCAACAGATCGGACCTGC-G-3′</td>
</tr>
<tr>
<td>BTV-4 (South Africa)</td>
<td>5′-GTTAAAACAGGATCGGATGGATGACGC-3′</td>
<td>5′-GTAAGTTCAACAGATCGGACCTGC-G-3′</td>
</tr>
<tr>
<td>BTV-9 (South Africa)</td>
<td>5′-GTTAAAACAGGATCGGATGGATGACGC-3′</td>
<td>5′-GTAAGTTCAACAGATCGGACCTGC-G-3′</td>
</tr>
<tr>
<td>BTV-9 (Italy)</td>
<td>5′-GTTAAAACAGGATCGGATGGATGACGC-3′</td>
<td>5′-GTAAGTTCAACAGATCGGACCTGC-G-3′</td>
</tr>
</tbody>
</table>

Sequencing and sequence analysis
Purified PCR-products were either sequenced directly using the forward type-specific primer for the corresponding serotype or each of the VP2 gene-clones were sequenced using M13 forward and reverse primers (Promega). Sequencing reactions were analysed with an Abi Prism® 377 DNA sequencer (Perkin-Elmer Applied Biosystems).

Resulting sequences were subjected to BLAST analysis (NCBI). Phylogenetic analysis was performed using Dnman (Lynnon Biosoft).

Results
Amplification and cloning of BTV VP2 cDNA
The full-length VP2 gene amplicons of all of the BTV-2, BTV-4 and BTV-9 isolates could be amplified in a one-step RT-PCR using primers based on sequences from previously cloned and sequenced BTV-2 and nine Italian isolates and the sequence from the reference strain of BTV-4 (Fig. 1). Only isolate 8 (BTV-9) could not be amplified using the primers against the Italian BTV-9. The whole
genome of this isolate and other isolates from Israel (3C, 35C and 38C) could be amplified using the method of Potgieter et al. (5) (Fig. 1). All the full-length BTV VP2 genes were cloned in the plasmid pGEM-T easy (Promega).

**Sequencing and phylogenetic analysis**

Approximately 500 bp of the terminal ends of each of the VP2-genes of all the BTV isolates were sequenced. An analysis of each of these nucleotide sequences shows that all the cloned VP2 cDNAs were full-length copies of the VP2-genes of each isolate. Each gene contained the 5′GTT and TAC-3′ sequences. A BLAST (NCBI) analysis also confirmed the serotype of each of the VP2-genes. Phylogenetic analysis demonstrated that all the Italian BTV-2 isolates grouped together and no significant sequence changes were apparent. They were also identical to those of a BTV-2 isolate from Corsica. There was 96% homology between the European BTV-2 isolates and the BTV-2 South African reference and vaccine strains. The analysis of the Italian BTV-9 isolates showed clearly that these were almost identical to the BTV-9 isolates from 2001. More significant however was the fact that a BTV-9 isolate from Greece showed a very high homology (99%) with all the Italian BTV-9 isolates. Australian and European BTV-9 field isolates have 89% homology at the nucleic acid level but the two groups of isolates have only a 67% homology with the reference BTV-9 isolate from South Africa. Greek and Israeli BTV-4 were almost identical and grouped together with the South African BTV-4 isolates (90% homology). The BTV-16 isolate from Israel also grouped with the South African reference strain showing identical homology for the first 500 bp of the terminal end (Figs 2 and 3).

**Discussion and conclusions**

Results of this study demonstrated that the sequencing data analysis of terminal ends of VP2 genes of BTV can be used for topotyping (8). During the two years of the Italian outbreak, the isolates were stable despite originating from different animal hosts and from different regions; this would indicate that the outbreaks of BTV-2 in Italy, together with the Corsican outbreak, may have had a common source (Fig. 4).

The fact that the BTV-9 isolate from Greece has an homology of more than 99% with the isolates from Italy indicates that the outbreaks of this serotype in Italy may have originated in Greece. In addition, the recent isolates from Italy were almost identical to the isolates from the previous year, indicating their subsequent spread from a common source. All the
BTV-9 isolates are together referred to as ‘eastern isolates’ since they were more similar to the Australian isolates than to the South African reference strain.

The BTV-4 isolates from Greece and Israel were very similar with a 98% homology, demonstrating a close relationship. These isolates, as a group, had only a 90% homology with the South African reference and vaccine strains, indicating that outbreaks of these ‘western isolates’ cannot be linked to the South African vaccine strain.

However, the Israeli BTV-16 isolate had a 100% homology with the South African reference and vaccine strains showing it to be definitely a ‘western isolate’. Whether this isolate can be linked to vaccination cannot be excluded but will only be confirmed if studies on other BTV gene segments are performed, such as on S8 and S10, which encode for NS2 and NS3, respectively.

It is recommended that more comprehensive sequencing studies of field isolates of BTV circulating in countries bordering the Mediterranean Basin be conducted to determine virus movement(s) and source(s) of BT epizootics in Italy. Not only should the VP2 genes be fully sequenced but studies on other BTV gene segments encoding for VP3, VP5, NS2 and NS3 should be conducted to obtain a more complete epidemiological ‘picture’.

**References**


Phylogenetic analysis of bluetongue virus genome segment 6

(encoding VP5) from different serotypes

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Summary
Full-length cDNAs were sequenced for genome segment 6 from representative isolates of all 24 bluetongue virus (BTV) serotypes. Segment 6 is 1635 to 1645 nucleotides in length with a single open reading frame in all isolates. The deduced VP5 protein sequence is 526 amino acids long for most BTVs, except BTV-15 (isolate number RSArrrrr/15), which is 527 amino acids long, and BTV-12 (isolate number RSArrrrr/12) and BTV-22 (isolate number Nig1982/11), which were 529 amino acids long. Sequence comparisons have revealed the level of genetic diversity in segment 6, between different BTV serotypes and between isolates within a single serotype. The resulting sequences can be used to design RT-PCR primers for amplification and identification of segment 6 from new BTV isolates, providing potentially valuable diagnostic and research tools. These data are available on the international databases and accession numbers are listed on the website of the Institute for Animal Health (iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv_sequences.htm).

Keywords

Introduction
Bluetongue virus (BTV) is an arbovirus (transmitted by certain species of Culicoides biting midges), which infects both domesticated and wild ruminants. The virus causes clinical disease in sheep, particularly in the improved European breeds used for wool or mutton, but it is usually mild or inapparent in cattle and goats. BTV is the prototype species of the genus Orbivirus, within the family Reoviridae. Twenty-four distinct serotypes of BTV have been recognised, which can be distinguished by serum neutralisation (SN) assays. BTV is currently prevalent in the Mediterranean region, causing outbreaks of disease involving serotypes 1, 2, 4, 9 and 16, with strong evidence that the disease is gradually penetrating further north (12). The virus is also endemic in the Indian sub-continent involving as many as 21 different serotypes.

The BTV particle is icosahedral, non-enveloped and has three concentric capsid layers (4, 10). The BTV core contains the 10 dsRNA segments of the virus genome and the three minor protein components of the transcriptase complex, VP1(Pol), VP4(Cap) and VP6 (Hel) (6, 9). Cores also have two major protein components, VP3 (T2) (which forms the innermost subcore shell) and VP7 (T13) (forming the outer core-surface layer) (4). In the intact virus particles the core is surrounded by an outer capsid layer, composed of two additional major proteins (VP2 and VP5). These proteins, which are more variable than the core proteins, or the three non-structural virus proteins (NS1, NS2 and NS3) (9), are involved in cell attachment and penetration during initiation of infection and contain epitopes that bind neutralising antibodies (10). The specificity of interactions between the outer capsid proteins (particularly VP2) and neutralising antibodies can be evaluated in SN assays, thereby distinguishing the different virus serotypes (7, 14). Although VP2 contains the majority of the neutralising epitopes and plays a greater role in the generation and specificity of neutralising antibodies, VP5 is also involved in the determination of virus serotype, possibly by influencing the conformation of the VP2 molecules in the outer capsid layer (11, 17). The segmented nature of the BTV genome makes it possible for
different strains of the virus that infect the same cell, to exchange genome segments by a process called reassortment. This may play an important role in the emergence of novel virus strains with different combinations of proteins and consequently different serological and/or biological properties.

The present study was undertaken to analyse the nucleotide sequence of genome segment 6 (which encodes VP5) from representative isolates of all 24 BTV serotypes. These studies are providing more information on sequence variation in this gene and its correlation with virus serotype, as well as data that will help (in combination with data for genome segment 2) to assess the relative frequency of genome reassortment that occurs in the field.

Materials and methods

The viruses used in this study were grown in BHK-21 cells and their dsRNA was extracted using Trizol according to the protocol of the manufacturer (Life Technologies), followed by precipitation with 2M LiCl to remove ssRNA. An anchor-oligo was then ligated to the dsRNA segments at 4°C for 12 h. For amplification of genome segment 6, ligated dsRNA segments were separated by 1% agarose gel electrophoresis (AGE). Genome segments 4, 5 and 6 were excised together in one piece of gel and purified using RNAid Kit-BIO 101. Using avian myeloblastosis virus (AMV) enzyme (Promega), dsRNA was heat denatured and reverse transcribed. Amplification of cDNA was then performed using primer 5-15-1 for 30 cycles. The amplified cDNA was analysed by AGE, then segment 6 (1.6 kb) was excised and purified using GeneClean kit-Bio101. The purified PCR products, representing full-length gene segment 6 were cloned in pGEM-T Easy vector (Promega) and transformed into Escherichia coli (ElectroMAX DH10B™) cells. Positive clones were screened by PCR using the 5-15-1 primer, followed by restriction endonuclease digestion (Fig. 2).

Results and discussion

BTV genome segments 4, 5 & 6 were amplified together by PCR from representative isolates of all 24 serotypes (Fig. 1). Gel purified PCR amplicons of genome segment 6 from different serotypes could readily be cloned, as full-length copies, in the T/A cloning vector pGEM-T. Positive colonies containing full-length inserts were screened and identified by PCR using the 5-15-1 primer, followed by restriction endonuclease digestion (Fig. 2).

![Figure 1](image1.png)

**Figure 1**
Polymerase chain reaction amplification of cDNA of bluetongue virus segments 4, 5 and 6, together
Lane M is 1.0 kb DNA marker
Each lane marked by Arabic numerals indicates the different BTV serotypes

![Figure 2](image2.png)

**Figure 2**
Bluetongue virus clones of different serotypes digested by Bam H1, giving right sized product of segment 6 (1.6 kb) and plasmid DNA (3.0 kb)
Lane M is 1.0 kb DNA marker
Each lane marked by Arabic numerals indicates the different BTV serotypes

and gene-specific primers. The sequence data were aligned and analysed using the Bio Edit sequence alignment editor. The segment 6 sequence data from computerised databases were also analysed and compared using the Blast software program (NCBI: National Center for Biotechnology Information).
The asterisk after the isolate number indicates the IAH dsRNA-virus reference-collection number. Additional information is provided on the dsRNA virus website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm).

Segment 6 was found to be 1 635 base pairs long in isolates of BTV serotype 1-Greece (isolate number* GRE2001/01), 2-India (isolate number* IND1982/01), and 23-India (isolate number* IND1997/01); 1 637 base pairs in 3-Zimbabwe (isolate number* Zim2002/01), 5-Cameroon (isolate number* CAR1982/02), 6-South Africa (isolate number* RSArrrr/06), 7-SA (isolate number* RSArrrr/07), 8-Nigeria (isolate number* NIG2002/07), 9-Cameroon (isolate number* SER2001/01), 13-SA (isolate number* RSArrrr/13), 14-Cameroon (isolate number* CAR1982/04), 16-Indonesia (isolate number* ISA1991/01), 18-SA (isolate number* RSArrrr/18), 19-SA (isolate number* RSArrrr/19), and 21-SA (isolate number* RSArrrr/21); 1 638 base pairs in 4-Turkey (isolate number* TUR1978/01), 10-SA (isolate number* RSArrrr/10), 11-SA (isolate number* RSArrrr/11), 17-SA (isolate number* RSArrrr/17), 20-SA (isolate number* RSArrrr/20), and 24-SA (isolate number* RSArrrr/24); 1 639 base pairs in 15-SA (isolate number* RSArrrr/15); 1 645 base pairs in 12-SA (isolate number* RSArrrr/12), and 22-Nigeria (isolate number* NIG1982/11). Similar nucleotide lengths were previously reported for segment 6 of BTV-10-USA strain (15); BTV-11-USA strain (1); BTV-11 and BTV-17-USA strains (19); BTV-13-USA strain (13); BTV-1-Aust. (2) and BTV-2-USA strain (5); BTV-1-South Africa (18). The deduced length of VP5 for isolates of all 24 BTV serotypes was 526 aa, except for BTV-15-SA (isolate number* RSArrrr/15), which was 527 aa; BTV-12-SA (isolate number* RSArrrr/12) and 22-Nigeria (isolate number* Nig1982/11), which were 529 aa.

Phylogenetic comparisons of the segment 6 nucleotide sequences identified two major clusters (Fig. 3), related to BTV-3 (BTV serotypes 3, 5, 6, 9, 13, 14, 16, and 21) or BTV-4 (BTV serotypes 4, 10, 11, 17, 20 and 24), and four minor clusters, related to BTV-7 (BTV serotypes 7 and 19); BTV-8 (BTV serotypes 8 and 18); BTV-12 (BTV serotypes 12 and 22); BTV-1 (BTV serotypes 1, 2 and 23) and BTV-15. Similar groupings of BTV-3 and BTV-4 have previously been reported by Gould and Hyatt, based on segment 2 gene sequences (3). They noted that BTV-15-Australia was also quite divergent (in genome segments 2 and 3) from other BTV serotypes included in their study, which holds true for BTV-15-SA in our study, although it did show some similarity to BTV-12 and BTV-22. Similarities apparent at both the VP6 gene and amino acid level,
Bluetongue virus and disease

demonstrate that some related serotypes have sequences in common, indicating a relatively closer common ancestry. These relationships do not appear to depend on the geographical origin of individual isolates or serotypes.

In spite of their different geographical origins, comparative analysis of deduced amino acid sequences of all the 24 BTV serotypes showed a high level of conservation (70% sequence identity) between different VP5 proteins. Three variable regions were identified and mapped between residues 120-180, 285-345 and 420-475 (Fig. 4). Gould and Pritchard also reported three non-homologous regions in VP5 of BTV-1 Aust strain at amino acid 137-189, 280-333, 410-475 (2). Oldfield et al. observed two variable regions in VP5 of BTV-13-USA strain between residues 130-190, 270-340 (13). Similarly, Yang and Li mapped three variable regions in VP5 of BTV-11 and BTV-17-USA strains between residues 120-180, 273-345 and 435-481 (19).

![Figure 4](image)

**Figure 4**

Variable regions in VP5 (526 aa)

The results of this study have revealed the level of genetic diversity in genome segment 6, between different BTV serotypes, as well as between isolates of the same serotype. The BTV-1 isolate from Greece is genetically closely related in genome segment 6, to strains from India and Australia, but is quite distinct from those from Africa. In contrast, BTV-2 from India is quite different from the European and African isolates, which are themselves closely related (data not shown). These data indicate the origins of the different European BTV strains and have highlighted differences between the vaccine and field isolates of serotypes 1, 2 and 4, which may be sufficient to design RT-PCR primers to distinguish them. Sequence data for BTV genome segment 6 will also help to determine the origins of other virus strains, increasing our understanding of BTV epidemiology and transmission.

This database can also be used to facilitate primer design and RT-PCR conditions suitable for the amplification of genome segment 6 from new BTV isolates. These methods (together with those that have also been generated for BTV genome segment 2) (8), are being used as the basis for serotype-specific RT-PCR assays, to improve the speed and reliability of BTV serotype determination. The sequences that have been generated have been added to those already available for the segments of different BTV serotypes in the international databases and accession numbers are listed on the website of the Institute for Animal Health (6).

**References**


Completion of the sequence analysis and comparisons of genome segment 2 (encoding outer capsid protein VP2) from representative isolates of the 24 bluetongue virus serotypes

Institute for Animal Health, Pirbright Laboratory, Ash Road, Woking, Surrey GU24 0NF, United Kingdom

Summary

Bluetongue (BT) is a non-contagious, arthropod-transmitted viral disease of domestic and wild ruminants. It is caused by bluetongue virus (BTV), a double-stranded (ds) RNA virus that is classified within the genus *Orbivirus*, family *Reoviridae*. There are at least twenty-four serotypes of BTV worldwide, five of which (1, 2, 4, 9 and 16) have been identified recently in Europe. BTV infects ruminants and its distribution throughout temperate and tropical regions of the world is dependent on the activity and abundance of certain vector-competent species of *Culicoides* midge. The outer capsid protein VP2 of BTV is a major protective antigen and the primary determinant of virus serotype. For the first time, the authors have completed the sequence analysis of full-length VP2 genes from the reference strains of each of the 24 BTV serotypes and their amino acid sequences were deduced. Multiple alignment of the VP2 gene (protein) sequences revealed that the level of nucleotide (amino acid) sequence variation between serotypes ranged from 29% (23%) to 59% (73%), confirming that segment 2/VP2 is the most variable BTV gene/protein. Phylogenetic analysis of VP2 grouped together the BTV types that are known to cross-react serologically. Low identity between types was demonstrated for specific regions within the VP2 amino acid sequences that have been shown to be antigenic and play a role in virus neutralisation. The sequence data represent the completion of an important step in the creation of a comprehensive BTV sequence database, which will support more rapid molecular methods for diagnosis and identification of BTV ‘types’, as well as continuing molecular epidemiology and surveillance studies of BTV.

Keywords


Introduction

Bluetongue (BT) is an economically important, infectious, non-contagious and arthropod-borne disease of sheep that is caused by members of the bluetongue virus (BTV) species (genus *Orbivirus*, family *Reoviridae*) (9). BTV has a global distribution between latitudes 50°N and 30°S and is transmitted by biting midges (*Culicoides* spp.). Virus transmission and the incidence of disease are therefore dependent on the seasonal and geographical activity, abundance and distribution of adults of vector-competent *Culicoides* species (4, 6).

The BTV genome is composed of ten segments of dsRNA, packaged within a three-layered icosahedral capsid (90 nm in diameter). The outer capsid layer consists of VP2 and VP5 (encoded by segments 2 and 6, respectively), which can elicit a neutralising and protective antibody response in infected mammalian hosts (7, 12). There are twenty-four distinct serotypes of BTV worldwide (2), five of which (1, 2, 4, 9 and 16) have recently been identified in Europe. VP2 (encoded by genome segment 2) is the most variable viral protein and plays a dominant role in determining serotype (5, 8). Sequence information previously available for BTV genome segment 2 included data (many of which were incomplete) from only 14 of the 24 BTV serotypes.
For the first time, the authors report the completion of full-length sequence analyses of genome segment 2/VP2 from at least one representative isolate of each BTV serotype. This has enabled the construction of phylogenetic trees showing the genetic relatedness of different BTV serotypes. These studies also demonstrate that it is possible to identify and distinguish different BTV serotypes by comparisons of segment 2 sequences. This has the potential to considerably reduce the time taken to identify new BTV isolates. The virus/antibody neutralisation assays that are conventionally used for BTV serotype determination require virus isolation and are therefore time-consuming (up to 6–8 weeks), can generate equivocal results and require standardised and therefore expensive serological reagents. The availability on the Web of a complete set of sequence data of the VP2 genes and proteins from each of the 24 South African reference strains of BTV serotypes will be an important step towards the development of a molecular capability for BTV diagnosis and typing.

Materials and methods

Total RNA was isolated from BTV infected BHK-21 monolayers using Trizol® in accordance with the protocol of the manufacturer; dsRNA was separated from contaminating ssRNA by precipitation in 2M lithium chloride (1). An ‘anchor-primer’ sequence (S. Rao, manuscript in preparation), phosphorylated at the 5’ end, with a C9 spacer connecting two partially complementary halves, was ligated to whole viral dsRNA (S. Rao, personal communication). The 10 µl ligation reactions, containing 1 µg of viral dsRNA, 10 units of RNA ligase (New England Biolabs) and 1.1 µg of anchor-primer (Integrated DNA technology, USA), were incubated at 10°C for 12 h. Ligated product was separated from unligated anchor-primer by 1% agarose gel electrophoresis and segments 2 and 3 were excised and purified using RNaid kit (Bio 101, Vista, California, USA). The RNA was precipitated using the Pellet Paint method. First strand cDNA was synthesised at 37°C for 40 min then at 42°C for 10 min using a ‘reverse transcription system’ (Promega, UK) without further addition of primers.

For amplification of fragments to be cloned and sequenced, PCRs were performed using a ‘Triple Master PCR system’ (Eppendorf AG, Cambridge, UK) using primer (5-15-1) which is partially complementary to the anchor spacer (S. Rao, manuscript in preparation). Gel slices containing full-length amplicons of segment 2 were excised, purified and prepared for cloning, or used directly in sequence reactions. PCR products were subjected to cycle sequencing using a Beckman capillary sequencer/ALF express DNA sequencer (Pharmacia Biotech Sweden). Sequence analysis software, BioEdit (version 5.0.9.1), was used to align all sequences. Ambiguities were resolved by manually checking chromatograms and by re-sequencing from both directions using multiple primers. Sequence and phylogenetic analyses were performed using Orf Finder and ClustalX (version 1.8.1).

Results

Complete sequence analysis and comparison of genome segment 2 from the 24 BTV serotypes showed significant levels of variation, which correlate with virus serotype. The length of BTV genome segment 2 varies from 2 904 bp (BTV-12) to 2 947 bp (BTV-19). The different BTV serotypes also show some variation in the length of the single VP2 open reading frame (ORF) and non-coding regions (Table I). The plus-strand conserved-terminal hexanucleotide sequences of genome segment 2 from each of the 24 BTV serotypes, agree with those previously described for BTV and EHDV by Mertens et al. (11).

A neighbour-joining tree was drawn using the deduced amino acid sequences and compiled from multiple alignments of the VP2 amino acid sequences (Fig. 1). This tree demonstrates that the 24 BTV serotypes can all be differentiated from each other. However, there are groups of serotypes which show closer relationships, possibly indicating a more recent common ancestry (e.g. BTV-5 and BTV-9 and similarly BTV-4, BTV-10, BTV-11, BTV-17, BTV-20 and BTV-24). Some BTV serotypes are generally acknowledged to have closer serological relationships (3, 14) (Fig. 2), which are clearly reflected in higher levels of amino acid/nucleotide sequence identity, which therefore suggest closer phylogenetic relationships (Fig. 1). Several discrete groups of highly conserved amino acids were also found during these comparisons.

Genome segment 2 nucleotide sequences were shown to vary by up to 32% within a single serotype (within BTV-9), while VP2 amino acid sequences varied by up to 16% (within BTV-16). The level of nucleotide sequence variation between serotypes ranged from a minimum of 29% (BTV-8 and BTV-18) to a maximum of 59% (BTV-16 and BTV-22) while the level of VP2 amino acid differences between serotypes ranged between 23% minimum (BTV-6 and BTV-14) and 73% maximum (BTV-14 and BTV-15) confirming that segment 2/VP2 as the most variable BTV gene/protein.
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<th>Accession number</th>
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<th>Open reading frame (nt)</th>
<th>G+C content (%)</th>
<th>A+T content (%)</th>
<th>Size of protein (aa)</th>
<th>Predicted protein molecular mass (Da)</th>
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</table>

IAH Institute for Animal Health
BTV bluetongue virus

* More details on virus isolates can be obtained from
  iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm
Bluetongue virus and disease

Veterinaria Italiana, 40 (4), 2004

Figure 1
Phylogenetic tree (unrooted neighbour-joining) constructed (using ClustalX version 1.8.1) from amino acid sequence data of the full-length VP2 gene of 24 reference strains of bluetongue virus. The branch lengths reflect genetic distances. Sequences obtained from GenBank (Accession Nos X06464, M21946, U04200, X55801, D00153, S72158, M17437, L29027).

Discussion
The analysis of full-length sequence data of VP2 genes from all 24 serotypes of BTV is described. This represents the first report of the sequence analysis of a single BTV genome segment and protein from all 24 serotypes. These data suggest that BTV serotypes originated from a common ancestor but have evolved differently, some as closely related groups, others independently. The 24 BTV serotypes are currently identified by their ability to cross-neutralise in serological tests, a process that involves virus isolation, tissue culture passage and serological assays, which may take up to eight weeks. This study was conducted to develop molecular methods for BTV serotype identification that are more reliable and rapid than the current serological assays (potentially within 24 h). These techniques will not only support more rapid design and implementation of appropriate vaccination strategies, they also provide epidemiological information that cannot be generated by the conventional serotyping assays. These data will help identify the origins of individual strains and help...
understand the mechanisms and routes by which they have spread. This will facilitate the design and refinement of appropriate disease control strategies.

Although sequences of more isolates will inevitably generate a clearer picture of inter-relationships, the strains of different BTV serotypes that were compared to generate the amino acid tree appear to cluster as ten evolutionary lineages. Earlier studies of reassortant BTV viruses that were generated by co-infections using parental strains of two different types, demonstrated that both VP2 and VP5 help to determine BTV serotype. These sequencing studies indicate that some BTV ‘types’ are serological variants within a single genetic lineage of genome segment 2/VP2. However, this may reflect variations in genome segment 6 (encoding the smaller outer coat protein VP5) and its usually minor role in determination of serotype (10). It has previously been observed that some serotypes cross-react as indicated in Figure 2, although it is again uncertain in each case what contribution, if any, VP5 makes to this cross-reactivity. Further sequencing studies of VP5 (13), combined with serological analyses using a panel of high quality sera against all 24 BTV serotypes may help to resolve these questions.

Many serotypes analysed are represented by only single isolates and many of the isolates are from Africa. A current evaluation of the genetic diversity and relationships within and between the serotypes may be biased by the isolates that are available. However, the authors believe that this database of segment 2/VP2 sequences represents a significant resource for the comparison and identification of BTV types that will be strengthened by addition of further data for additional BTV isolates.

References

Molecular epidemiology of bluetongue viruses from disease outbreaks in the Mediterranean Basin

Department of Molecular Biology, Institute for Animal Health, Pirbright Laboratory Ash Road, Pirbright, Surrey GU24 ONF, United Kingdom

Summary
Bluetongue virus (BTV) serotype is primarily controlled by the variable outer coat protein VP2, encoded by genome segment 2. Phylogenetic analyses of segment 2 show that recent Mediterranean isolates of BTV-2 have a similar genetic lineage to those from sub-Saharan Africa and North America but are distinct from Asian strains. In contrast, isolates of BTV-9, from the eastern Mediterranean, are related to a genetic lineage from Asia. BTV-1 from Greece 2001 is also more closely related to Indian isolates, suggesting (in both cases) virus movement from east to west. Recent BTV-4 field isolates from Greece and Turkey are similar to each other, but differ from the Turkish type 4 vaccine strain. These sequencing studies are being used to establish a database for molecular epidemiological studies which is available on the website of the Institute for Animal Health. This resource will support and improve BTV serotype identification methods, by using sequence comparisons (via the Web) rather than by conventional serological techniques that require standardised (and therefore expensive) serological reagents. Phylogenetic trees for BTV genome segment 2 are available on the website.

Keywords
Bluetongue virus – Orbivirus – Phylogenetic analysis – Segment 2 – Serotype – Viral protein 2 – Virus.

Introduction
Prior to 1998, Europe had been largely free of bluetongue (BT) disease, apart from sporadic and limited epizootics caused by single bluetongue virus (BTV) types that were restricted to areas of the Iberian Peninsula (below 40°N), or to some of the Mediterranean islands (27, 28, 29). The distribution of disease outbreaks in southern Europe (both BT and African horse sickness) mirrored the distribution and abundance of the major vector species Culicoides imicola (27).

However, since 1998, five BTV types (1, 2, 4, 9 and 16) have caused outbreaks across much of southern Europe, collectively representing the largest epizootic of BT ever recorded. Morbidity and mortality levels are estimated in some areas at 18% and 3.4%, respectively, and overall losses already exceed 500,000 sheep. The virus and disease have spread northwards into new areas of Europe (Bulgaria, Serbia and Croatia) that are beyond the range of C. imicola, suggesting the involvement of novel insect vector species (26). Indeed, the virus has recently been isolated in several areas, from both C. obsoletus and C. pulicaris (species that are abundant across much of northern Europe). These observations, together with the projected effect of global climate change on the distribution and activity of vector species, and the existence of an overwintering mechanism for BTV (33, 34), suggest that the virus will persist and may continue to spread northwards. Additional areas of Europe must therefore also be considered to be ‘at-risk’ from the disease.

BTV serotype is primarily controlled by the variable outer coat protein VP2 (encoded by segment 2 of the virus genome), which determines the specificity of interactions between neutralising antibodies and the virus particle. Sequence variations in genome segment 2 consequently reflect variation in virus ‘type’. The results from sequence analyses of BTV genome segment 2 are being used to establish a
database for molecular epidemiological studies (22, 25). This will provide a resource to support and improve BTV serotype identification by sequence comparisons (via the Web), rather than by conventional serological techniques that require standardised (scarce and expensive) serological reagents. More rapid and accurate BTV typing and strain identification techniques will also provide valuable information concerning the route and direction of virus spread, which cannot be obtained from conventional serological typing assays. It is anticipated that such information will be of considerable importance to achieve a full understanding of the disease and to combat transmission that may occur as a result of ‘climate change’ and during the increasingly complex movement of animals in the livestock trade.

This study presents the results of a phylogenetic analysis of the five BTV types that have caused outbreaks in the Mediterranean Basin in recent years. Field strains of each type were compared to viruses of the same type, isolated at different times and from different geographical origins.

Materials and methods

Strains used in this study are shown in Table I. More information concerning the strains used and those stored in the reference collection at the Institute for Animal Health in Pirbright can be found on the website (23).

Oligonucleotide primers for sequencing used in the study are shown in Table II. A list of primers (which will be periodically updated) that can be used to amplify genome segment 2 of BTV can also be found on the IAH website (24).

Total RNA was isolated from BTV-infected BHK-21 monolayers using Trizol®, in accordance with the protocol of the manufacturer; dsRNA was separated from contaminating ssRNA by precipitation in 2M lithium chloride (17). An anchor-primer sequence, phosphorylated at the 5´ end, with a C9 spacer connecting two partially complementary halves (S. Rao, manuscript in preparation), was ligated to whole viral dsRNA. The 10 µl ligation reaction had 1 µg of viral dsRNA, 20 units of RNA ligase (New England Biolabs) and 1.1 µg of anchor-primer (Integrated DNA technology, USA) were incubated at 10°C for 12 h. Ligated product was separated from unligated primer by 1% agarose gel electrophoresis and segments 2 and 3 excised and purified using RNaid kit (Bio 101, Vista, California, USA). The RNA was precipitated using the Pellet Paint method. First strand cDNA was synthesised at 37°C for 40 min then at 42°C for 10 min using a ‘reverse transcription system’ (Promega, UK) without further addition of primers.

For amplification of fragments to be cloned and sequenced, PCRs were carried out using a ‘Triple Master PCR system’ (Eppendorf AG, Cambridge, UK) using primer (5-15-1), which is partially complementary to the anchor spacer (S. Rao, manuscript in preparation). Gel slices containing full-length amplicons of segment 2 were excised, purified and prepared for cloning or to be used directly in sequence reactions. PCR products were subjected to cycle sequencing using a Beckman capillary sequencer/ALF express DNA sequencer (Pharmacia Biotech Sweden). Sequence analysis software, BioEdit (version 5.0.9.1), was used to align all sequences. Ambiguities were resolved by manually checking chromatograms and by re-sequencing from both directions using multiple primers. Sequence and phylogenetic analysis were performed using Orf Finder and Clustal X (version 1.81).

Results

Comparisons of genome segment 2 sequences from different field and vaccine strains belonging to the five BTV types examined (1, 2, 4, 9 and 16) demonstrate that they cluster into five distinct and type-specific groups (Fig. 1). Although significant variation was detected between isolates of the same type within each of these clusters, some isolates also showed significantly closer relationships. For example, within the BTV-1 cluster, the strains that were analysed from India all appear to be closely related, suggesting a recent common ancestry. These isolates were also more closely related to the BTV-1 strains from Australia and Malaysia, than to those from Africa (Nigeria, Sudan and Cameroon), indicating diversity between ‘eastern’ and ‘western’ isolates. The BTV-9 isolates from outbreaks in Europe are more closely related to strains from the East (Australia and Indonesia) than to South African reference strains, as are the BTV-16 isolates. BTV-4 isolates from Greece and Turkey are clustered (within the serotype) with African reference strains and are distinct from the published sequence of a Chinese isolate. This probably reflects the western group of viruses. The same effect is noticeable for BTV-2, the European field isolates are more closely related to the Western group represented by isolates from Africa.

Discussion

In October 1998, there were outbreaks of bluetongue (caused by BTV-9) on four Greek islands close to the Anatolian coast of Turkey (Rhodes,
Table I
Details of virus isolates used for sequencing studies of segment 2 of bluetongue virus serotypes 1, 2, 4, 9 and 16

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<td>L.J. Preischl, personal communication**</td>
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</table>

N/A not available

* published sequences used for sequence comparisons are shown in italics

OVI Onderstepoort Veterinary Institute
P partial sequence

** personal communication
### Table II
Primer designations used for sequencing segment 2 of bluetongue virus serotypes 1, 2, 4, 9 and 16

<table>
<thead>
<tr>
<th>Primer designation</th>
<th>Orientation</th>
<th>Primer sequence (5' - 3')</th>
<th>Effectiveness for sequencing</th>
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<td>BTV-1/2/p368R</td>
<td>Antisense</td>
<td>GATATGGRTTYGACCACCA</td>
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<tr>
<td>BTV-1/2/p207F</td>
<td>Sense</td>
<td>AGGAGAGATCGAGTCA</td>
<td>+++</td>
</tr>
<tr>
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<td>TGAATCTGAGTCTTCTCTCT</td>
<td>+++</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>+++</td>
</tr>
<tr>
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<td>+++</td>
</tr>
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<tr>
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<td>Sense</td>
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<td>CTTGAGTGGTGCAGGACTCAG</td>
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Figure 1
Neighbour-joining (unrooted) tree showing relationships between full length genome segment 2 (VP2 gene) of the five European serotypes of bluetongue virus
The tree was generated using Clustal X (version 1.81) with the default parameters
Published sequences used for sequence comparisons are shown in italics in Table I

Leros, Kos and Samos) (1, 2). This was the first time that BTV-9 had been recorded in Europe, although serological evidence had previously identified the serotype in Turkey (35). After December 1998, no further outbreaks of BT were recorded until June 1999 when BTV-9 was again identified as the cause of outbreaks in south-east Bulgaria, which persisted until October 1999 (3, 4). However, in July 1999 the Turkish authorities also reported a BT incursion into European Turkey involving animals in those provinces bordering mainland Greece and Bulgaria (4). The Turkish veterinary authorities vaccinated some 60 000 sheep with a BTV-4 vaccine (19) although it was later confirmed that the outbreaks were caused by BTV-9. In August 2000, BTV-16 was also reported from outbreaks in the Province of Izmir, Turkey (8).

In August 1999 the Greek authorities reported BTV on mainland Greece, initially adjacent to the Bulgarian and Turkish borders, although the virus subsequently spread in a westerly direction across northern Greece, as far as Thessaloniki and Larisa (4). Outbreaks were reported until December 1999 and again in the summer of 2000. Three serotypes were found to be responsible for the Greek outbreaks of 1999-2000, BTV-4, BTV-9 and BTV-16. BTV-4 was initially reported near the Turkish border but was later recorded more widely. Initially the BTV-4 outbreaks were viewed with some suspicion because of the use of the live-attenuated BTV-4 vaccine in Turkey. However, BTV-4 has previously been recorded in a number of countries further to the east (e.g. Syria, Jordan, Israel and Turkey) (35) and sequence comparisons indicate that the Turkish vaccine strain was not the cause of the European outbreak. In September 2001, BT was again confirmed in north-western Greece, close to the Albanian border and subsequently in central Greece (16). There were also reports from Serbia, Montenegro, Kosovo, Macedonia, Bulgaria and Croatia (10, 13, 14, 15, 16).

Outbreaks of bluetongue were also reported from the south-western Mediterranean Basin during 2000. Tunisia reported BT for the first time in January
Bluetongue virus and disease

2000 (6). Further outbreaks were reported in Tunisia in June and in Algeria during July of 2000 (7, 9). Most of the Algerian outbreaks were close to the Tunisian border, until September 2000, when BTV was confirmed in areas as far as 250 km further west (8). In late 2000, BTV-specific antibodies were found in samples from animals in Algerians and northern Morocco (27). In late August 2000, BTV-2 was found on the Italian island of Sardinia (8) and by October, it had been confirmed on Sicily and in Calabria (southern mainland Italy) (5). The BT outbreaks in Sardinia were severe and 90,000 sheep died, either as a result of disease or control measures (5). BTV-2 was reported on the French island of Corsica during October 2000 and the Spanish islands of Menorca and Mallorca up until December 2000 (8, 11). In September 2001 BT was reported in Corsica, Sardinia and Calabria (southern Italy) (12) and also from north of Rome in Lazio and Tuscany. The outbreaks in Corsica were caused by BTV-2 but those in Calabria were BTV-9.

In 2002, outbreaks continued around the Mediterranean Basin. During the period from June to August there were outbreaks on mainland Italy attributable to BTV-2, BTV-9 and BTV-16. Sicily had outbreaks of BTV-2 and BTV-9. Sardinia had outbreaks due to BTV-2 and Calabria had BTV-16. In the Balkan states between August and December, there were outbreaks of BTV-9 in Bosnia, Bulgaria, Montenegro, Yugoslavia and Albania and an unconfirmed report of BTV in Kosovo. During October 2003, BTV-4 was confirmed in Sardinia then suspected in Corsica, and confirmed in the Balearic islands.

Phylogenetic analysis of genome segment 2 from several different isolates of BTV types, that were currently causing disease within Europe, demonstrated that they could be separated into five distinct groups, which accurately reflect virus serotype (Fig. 1). Significant but lower levels of sequence variation were also apparent between individual isolates of the same type, within each of these groups. These differences were greatest between isolates from geographically distant locations, and there was clear evidence of an East-West divide, particularly for isolates of types 1, 2 and 9. The BTV-1 isolate from Greece (GRE2001/01) was shown to be more closely related to a group of eastern BTV-1 strains, particularly those from India, indicating that it may have eastern origins. It is considered unlikely that this European BTV-1 was introduced directly from India, and although there was no evidence of BTV-1 infection in Turkey during a serological survey in the early 1980s (35), it is considered likely that it originated from eastern Turkey or the Middle East.

The cluster of BTV-4 isolates contained strains representing Africa, South America, Turkey and China. The overall diversity between most of these BTV-4 isolates was quite low, suggesting that they represent a western grouping and may even reflect the spread of a strain related to, but not identical to, the South African reference or vaccine strain. However, the Chinese isolates showed higher levels of diversity, again suggesting that it belongs to a distinct Far Eastern group.

Sequence comparisons of S2 of BTV-9 strains showed that all of the isolates from recent BTV-9 outbreaks in Europe are grouped together and are almost identical but quite diverse from South African reference or vaccine strains. They belong to the same genetic lineage as those from Australia and Indonesia suggesting an ‘eastern’ origin. BTV-9 have been reported in Anatolian Turkey, Syria, Jordan and Israel over a number of years (35). It seems likely that these strains have spread in a westerly direction moving into Europe, causing the current outbreaks of BTV-9. However, sequence data for genome segment 2 of other viruses from this and other regions, would be required to fully verify this hypothesis.

The studies of BTV-16 did not include a sufficiently wide selection of virus isolates to exhibit the same phenomena (an east-west split). The BTV-16 isolates from Turkey form a closely related group, as would be expected from isolates from the same year that may all be directly related to each other. They also show a very close relationship to the South African reference and vaccine strains. The strain from Nigeria is less closely related to other isolates of BTV-16, showing a level of divergence similar to that observed between the eastern/western strains of BTV-1, 2 and 9. However, due to the small numbers of isolates available for study, the significance of this difference is still to be confirmed.

Nucleotide sequence comparisons show that BTV-2 isolates from recent outbreaks in the Mediterranean Basin are more closely related to viruses from Nigeria, Sudan and South Africa and this may reflect a sub-Saharan origin. Although the Sahara represents an effective physical barrier to the movement of both insect and mammalian species, and therefore the spread of disease, foot and mouth disease virus (FMDV) isolated form outbreaks of disease in Algeria, Morocco and Tunisia during 1999, was shown to have a sub-Saharan origin (32). It was suggested that transportation of FMDV-infected Zebu cattle across the Sahara in late 1999 may have provided an entry route to North Africa. BTV appeared in Tunisia at around the same time and it is possible that it was transported as a sub-clinical
infection of the same cattle. However, FMD epidemics have also been caused in North Africa by movement of sheep from the Middle East, during periods of religious festivals such as Aid-el-Kebir (31, 35) types 2, 4, 6, 9 and 13 in Syria and Jordan and types 2, 4, 9, and 13 were recorded in Turkey during the early 1980s. Although BTV isolates of Middle Eastern origin were not available for this study, it is also possible that BTV-2 could have spread from the Middle East (Syria, Jordan, Israel) across North Africa to Tunisia. Previous studies have also recorded the movement of BTV as the result of wind-borne spread of infected midges. It is not possible to rule out this route as an important factor in the origin and spread of the current European outbreaks.

Once an arthropod-borne disease, like BT, is established within the mammalian host-population and competent vector species are present (as in the Mediterranean Basin) outbreaks may occur in successive years. This is particularly true, if a mechanism exists for virus survival from one ‘vector-season’ to the next (BTV-overwintering) (33, 36). At present, only live-attenuated vaccines are commercially available for disease control measures. A monovalent, live-attenuated BTV-2 vaccine was used successfully in Corsica and the Balearic islands. However, pentavalent vaccines were also used in Bulgaria but did not stop the spread of BTV-9. In Italy, a BTV-2 monovalent vaccine and subsequently bivalent BTV-2 and BTV-9 vaccines have so far failed to prevent re-occurrence of the disease. The use of live vaccines poses an as yet unquantified risk of transmission of the vaccine strain. Vaccination in the face of an outbreak may also allow genome segment reassortment to occur between the vaccine and field virus, increasing the genetic diversity within the virus population, and potentially generate a virus strain that has novel biological properties.

The introduction of five serotypes of BTV into the Mediterranean region and the occurrence of outbreaks at latitudes further north than previously described, is a stark reminder that the epidemiology of exotic diseases needs to be closely monitored. The absence of the major European vector species (C. imicola) from the Balkan region indicates the involvement of novel vector species, possibly C. obsoletus and/or C. pulicaris. If future control measures for BTV and other related viruses are to be effective it is essential that the way in which they enter the region is fully understood. Phylogenetic analysis of sequence data is a powerful tool that can be used to identify virus strains accurately and pinpoint where these introductions come from. Access to an adequate collection of contemporary virus isolates from different regions and an open exchange of data is essential for the continuation and success of these studies. Completed sequence data will therefore be added to the international sequence databases as soon as possible. Other data will be disseminated via the dsRNA virus website of the IAH.

References

Bluetongue virus and disease


Excretion of bluetongue virus in cattle semen:
a feature of laboratory-adapted virus

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Summary

A series of experiments was conducted over a period of four years and involved both young (2-4 years) and old bulls (5-15 years) that were both naturally and experimentally infected with bluetongue virus (BTV). Several different virus serotypes were studied. In the Northern Territory, young bulls were exposed to natural infection with BTV over three wet seasons. During this time, bulls were infected with BTV-1, BTV-3, BTV-16 and BTV-20. In New South Wales, semen samples were examined from a large group of bulls of mixed ages that were naturally infected with BTV-1. Experimental infections in both young and old bulls (5-8 animals per group) employed both ‘wild-type’ and laboratory-adapted viruses from serotypes 1 and 23. A total of 41 bulls were included in the studies of natural BTV infection and 52 bulls in experimental infections.

There was no evidence of BTV in any of the semen samples collected from naturally infected bulls or experimentally infected young bulls. BTV was detected intermittently in semen from a number of old bulls infected with both laboratory-adapted BTV-1 and BTV-23. These detections occurred during or immediately after the period of detectable viraemia. Virus was also detected in a few semen samples from very old bulls infected with ‘wild-type’ BTV-23. These samples were collected during the period of viraemia and there was usually evidence of blood in the semen. Viraemia varied in duration between 17 and 38 days. Following immunosuppression, there was no evidence of resurgence of viraemia, or excretion of virus in semen, even in animals in which virus had been previously detected in semen. When the bulls were slaughtered, virus was not detected in any tissues.

Keywords


Introduction

Reports of the excretion of bluetongue virus (BTV) in the semen of bulls have influenced international policy for the movement of both live animals and semen between countries, and sometimes, within countries. In the United States of America (USA), Bowen et al. (1) were able to isolate BTV from the semen of bulls experimentally infected with US serotypes and suggested that this phenomenon may be related to the age of the bull. Trials in Australia (Darwin) with naturally infected bulls failed to detect any evidence of BTV in semen, even while bulls were viraemic (2). This project was conducted to investigate the effect of laboratory passage of virus, age of bulls and BTV serotype on the excretion of BTV virus in bovine semen. The study also investigated the possibility of persistence of virus after natural and experimental infection.

Methods

Experiments were conducted to monitor the duration of viraemia and excretion of virus in semen in both naturally and experimentally infected bulls. For both blood and semen, a large volume of samples was examined to maximise virus detection.
Natural infection

Each year in January, a group of 10 seronegative young bulls was introduced to Beatrice Hill, approximately 50 km south-east of Darwin in the Northern Territory of Australia (Fig. 1). Bulls were bled and ejaculated twice weekly from January to June. Whole blood and semen samples were collected for virus isolation and sheep inoculation. Sera from these bulls were checked monthly for seroconversion to BTV.

Following inoculation, whole blood and semen samples for virus isolation and serum for serology were collected twice weekly for 4 to 6 weeks then weekly for up to 8 weeks. Samples were held at 4°C, –80°C or in liquid nitrogen.

Persistent infection

To examine bulls for latent infection with BTV, nine seropositive bulls were immunosuppressed by corticosteroid administration. Blood and semen samples were collected for a further four weeks. Seropositive bulls were also slaughtered and a wide range of tissues was collected and checked for the presence of virus. Tissues included right and left testicles (upper and lower portion), head and tail of right and left epididymis, right and left seminal vesicle, right and left ampulla, prostate and spleen.

Virus isolation

Whole blood (2-5 ml) and undiluted semen samples (2-2.5 ml) were inoculated into separate sheep. The sheep were bled at the time of inoculation and four weeks later for serology. The presence of virus in a sample was indicated by seroconversion of the recipient sheep. Blood and semen samples were also inoculated intravenously into 11-day-old embryonated chicken eggs. The embryos were homogenised and the clarified supernatant inoculated onto Aedes albopictus cell cultures with up to three further passages on BHK-21 monolayers to detect virus (2).

Serology

Serum samples from all bulls (naturally and experimentally infected) and recipient sheep were tested for antibodies to BTV group antigens by agar gel immunodiffusion (AGID) and competitive enzyme-linked immunosorbent assay (c-ELISA) and for type-specific antibody by virus neutralisation (VN) tests. The AGID, c-ELISA and VN tests were performed using standard methods (3).

Results

Natural infection

At Beatrice Hill, 22 bulls were naturally infected during the study period as follows: 10 with BTV-1, 1 with BTV-3, 10 with BTV-16 and 1 with BTV-20. All sheep receiving blood from infected bulls seroconverted to the corresponding virus, but no sheep receiving semen seroconverted. In New South Wales, there was no evidence of BTV in any of the 130 batches of semen collected from 19 BTV-1 infected bulls around the period of viraemia.
Experimental infection: laboratory-adapted virus

BTV-1 in mature bulls (5-6 years)

All eight bulls became viraemic with the longest duration of viraemia being 27 days (Table I). BTV was identified in 22 semen samples from five bulls (1 to 6 ejaculates). In three (B, F and H) of these five bulls, virus was only detected in the semen during the period of viraemia (Fig. 2). Virus was detected in the semen of the other two bulls for up to three collections over a period of 10 days beyond the period of detectable viraemia. Although semen collections from the five bulls continued for a further 12 weeks, no further virus isolations were made. Titrations of the virus in semen and blood showed that the levels of virus were similar (Fig. 3).

<table>
<thead>
<tr>
<th>Bull</th>
<th>Age (years)</th>
<th>Duration of viraemia (days)</th>
<th>Virus</th>
<th>Ejaculates with BTV-1/ ejaculates tested</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>6</td>
<td>13</td>
<td>Lab* BTV-1</td>
<td>5/20</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>20</td>
<td>Lab* BTV-1</td>
<td>5/20</td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>23</td>
<td>Lab* BTV-1</td>
<td>0/12</td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>13</td>
<td>Lab* BTV-1</td>
<td>0/12</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>16</td>
<td>Lab* BTV-1</td>
<td>0/12</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>27</td>
<td>Lab* BTV-1</td>
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</tr>
<tr>
<td>G</td>
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<td>H</td>
<td>6</td>
<td>27</td>
<td>Lab* BTV-1</td>
<td>1/12</td>
</tr>
<tr>
<td>I</td>
<td>6</td>
<td>17</td>
<td>Wild BTV-1</td>
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<td>J</td>
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</tr>
<tr>
<td>P</td>
<td>5</td>
<td>23</td>
<td>Wild BTV-1</td>
<td>0/12</td>
</tr>
</tbody>
</table>

* Laboratory-adapted virus

BTV-23 in mature bulls (14-15 years)

All five bulls became viraemic with the maximum period of viraemia being 27 days (Table II). Virus was detected in six semen samples from three bulls during the period of viraemia (1 to 3 ejaculates) but never after the cessation of viraemia (Fig. 4). Three bulls died during the observation period from unrelated causes.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Age (years)</th>
<th>Duration of viraemia (days)</th>
<th>Virus</th>
<th>Ejaculates with BTV-23/ ejaculates tested</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>14</td>
<td>16</td>
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<tr>
<td>2</td>
<td>14</td>
<td>16(b)</td>
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<tr>
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<td>6</td>
<td>12</td>
<td>27</td>
<td>Wild BTV-23</td>
<td>1/10</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>21</td>
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<td>8</td>
<td>12</td>
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<td>0/10</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>21</td>
<td>Wild BTV-23</td>
<td>2/10</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>24</td>
<td>Wild BTV-23</td>
<td>2/10</td>
</tr>
</tbody>
</table>

a) Laboratory-adapted virus

b) Died from unrelated causes
Bluetongue virus and disease

Figure 4
Comparison of titres of bluetongue virus in the blood and semen of Bull G (Table I), a mature bull infected with laboratory-adapted BTV-1

BTV-1 and BTV-23 in young bulls (2-3 years)
Each of the 13 young bulls inoculated with laboratory-adapted virus became infected, with patterns of viraemia similar to the old bulls (up to 27 days for BTV-1 and 38 days for BTV-23). Virus was not identified in the semen of any of these bulls.

Experimental infection: ‘wild’ virus

BTV-1 in mature bulls (5-6 years)
All eight bulls became viraemic with the longest duration of viraemia being 28 days. No virus was detected in any of the semen samples (Table I).

BTV-23 in mature bulls (6-12 years)
All five bulls became viraemic with the longest duration of viraemia being 27 days (Table II). Virus was detected in five semen samples from three bulls during the period of viraemia (1 to 2 ejaculates) (Fig. 5). Each of these infected semen samples was collected at the peak of viraemia and four showed visible contamination with blood.

BTV-1 and BTV-23 in young bulls (2-3 years)
Each of the 13 young bulls inoculated with ‘wild’ type virus became infected, with patterns of viraemia similar to the old bulls. Virus was not identified in the semen of any of these bulls.

Latent infection

BTV was not isolated from any tissues of the genital tract or spleen when five of the bulls inoculated with laboratory-adapted virus (not treated with corticosteroids) were slaughtered 56 days post inoculation (Bulls C, D, E, F and H) (Table II). Nine bulls were immunosuppressed with dexamethasone. Three of these bulls had previously been infected with laboratory-adapted BTV-1 in New South Wales (Bulls A, B and G). Four of the bulls were naturally infected with BTV-1 and two were experimentally infected with ‘wild’ BTV-23 in the Northern Territory. There was no evidence of a resurgence of viraemia and no evidence of BTV in the semen. BTV was not isolated from any tissues of the reproductive tract or spleen when these bulls were slaughtered.

Figure 5
Duration of viraemia and virus detection in semen of mature bulls infected with wild-type bluetongue virus serotype 23

Discussion

These studies suggest that the use of laboratory-adapted BTV may be a major factor contributing to the contamination of bovine semen observed experimentally. More than half of the old bulls infected with laboratory-adapted BTV-1 and BTV-23, were found to excrete virus in their semen. These detections occurred during or immediately after the period of detectable viraemia. There was no evidence of virus in the semen or blood of these bulls during the subsequent 12 weeks of observation. The maximum duration of viraemia in any animal in these studies was 38 days.

When bulls were experimentally infected with ‘wild’ BTV, the results were more variable. Virus was detected in a few semen samples from very old bulls infected with ‘wild’ BTV-23. Each of these semen collections was made during the period of viraemia, in the first week, and probably near the peak of viraemia. There was usually evidence of blood in the semen. Virus was not found in the semen of any of the bulls infected with ‘wild’ BTV-1. The extreme age of the bulls infected with ‘wild’ BTV-23 was likely to have been a contributing factor to the detection of virus in their semen. Bulls aged between 10 and 12 years would not usually be used as donors for commercial collections of semen. It is believed that virus may be present in the semen of old bulls as
a result of inflammatory changes that occur in older animals (1).

There was no evidence of BTV in the semen of any of the 41 naturally infected bulls or 26 experimentally infected young bulls. The younger age of these bulls may have been a factor contributing to the lack of contamination of semen with virus. These findings are consistent with other data for the testing of semen for evidence of BTV contamination (4, 5). To date, there has been no published report of the isolation of BTV from the semen of a naturally infected bull.

Immunosuppression did not result in a resurgence of viraemia or excretion of BTV in semen, even in animals in which virus had previously been detected in semen. Virus was not detected in any tissue when bulls were slaughtered.

These studies indicated that unless semen is contaminated with blood, the excretion of BTV is confined to old bulls infected with laboratory-adapted virus. This may have implications for countries where live BTV vaccines are used because it is possible that vaccine virus may be spread in the field. There was no evidence of long-term persistence of the virus, even with laboratory-adapted strains.

Acknowledgements

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References

Bluetongue virus does not persist in naturally infected cattle

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Summary

Studies were designed to test if observations by Takamatsu et al. in 2003 were applicable to natural infection of cattle with bluetongue virus (BTV). These observations suggested that ovine gamma delta T-cells could become persistently infected and subsequent midge feeding could induce virus replication. Skin biopsies and blood were collected from 28 cattle naturally infected with BTV-1. Blood samples were processed for virus isolation by embryonated chicken egg inoculation and for serology by BTV competitive enzyme-linked immunosorbent assay and BTV-1 virus neutralisation. BTV-1 was isolated from the blood of all animals and serology confirmed infection with BTV-1. A total of 288 skin biopsies were collected and cultured in the presence of interleukin 2 and epidermal growth factor. Sampling commenced as soon as either serology or virus isolation indicated infection with BTV and continued at weekly intervals for at least eight weeks then monthly for another two months. The natural viraemias in this experiment ranged from one to five weeks. BTV-1 was isolated from only one skin biopsy sample. This sample was collected during the week in which the animal was viraemic. These findings provide compelling evidence that BTV does not persist in gamma delta T-cells in the skin of naturally infected cattle.

Keywords

Australia – Bluetongue virus – Bovine skin fibroblasts – Cattle – Natural infection – Persistence.

Introduction

Recent studies in the United Kingdom have reported that infectious bluetongue virus (BTV) could be recovered from ovine skin biopsies more than nine weeks post infection (9). This experimental work was conducted using laboratory-adapted BTV-1.

Adaptation of field isolates of BTV to growth in tissue culture is known to alter the biological properties of the virus (1). Experiments were therefore designed to assess if the observations reported by Takamatsu et al. (9) were applicable to natural BTV infection of cattle. These experiments were conducted at a site of known high arbovirus activity where cattle are continuously exposed to insect attack. Regular monitoring at this site also allows periods of natural BTV activity to be identified.

Materials and methods

Sampling collection and frequency

Two sentinel cattle herds at Beatrice Hill Farm (12°39'S, 131°20'E), approximately 50 km south-east of Darwin, Northern Territory, Australia were sampled weekly. Group 1 was sampled from 6 September 2002 until 10 April 2003 and Group 2 was sampled from 10 April 2003 until 7 August 2003. Each group contained 24 animals.

Ten ml of blood for serum and 10 ml of lithium heparin anticoagulant treated blood were collected weekly from the sentinel cattle for the period of the experiment. This included all days on which skin biopsy samples were collected. Skin biopsy samples were collected from a total of 28 animals beginning as soon as either serology or virus isolation indicated possible infection with BTV. Biopsy sampling continued weekly for eight weeks from initial sampling and then monthly for another two months.

Skin biopsy collection

Duplicate 6 mm skin biopsies were taken weekly from the backline of each selected sentinel animal for the duration of the experiment. The site was clipped, then washed with 70% ethanol and the biopsy taken. The biopsies were placed in 5 ml of heart brain infusion broth containing antibiotics. The samples were kept at 4°C for 2 h before processing.
Cattle skin fibroblast cultures
The skin biopsies were processed for culture using a method adapted from Takamatsu and Jeggo (8). The outer hair and skin layer were removed and discarded and the samples were cut into small sections, treated with trypsin ethylenediaminetetraacetic acid (EDTA) for 45 min at room temperature and stirred continuously. The processed cells were recovered by centrifugation and washing and each sample cultured in a single well of a 24-well plate. The medium used was minimum essential medium (MEM) supplemented with 20% foetal bovine serum, 1 mM/ml of sodium pyruvate, 10 ng/ml epidermal growth factor, 10 IU/ml of recombinant human interleukin 2 and antibiotics. The cultures were maintained at 37°C/5% CO₂ and the cultures harvested when they reached >80% confluency, usually 8 to 11 days post seeding. Cells were removed by scraping and combined with the culture supernatant and stored at –70°C until processed for virus isolation.

Co-culture of peripheral blood mononuclear cells and skin fibroblast culture
Five animals from Group 2 were selected for peripheral blood mononuclear cells (PBMC) skin fibroblast culture. A primary bovine skin fibroblast cell line was developed from a healthy non-infected animal. Using the method described above and then weekly passage, the cell line could be maintained for at least 20 passages. The cell line was shown to be able to be infected with BTV-1 (data not shown). PBMC were isolated from anticoagulant treated blood by gradient centrifugation (Nycoprep, Norway) and co-cultured with confluent bovine skin fibroblast cultures (medium as described above) for 7 days. The co-cultures were then harvested and stored at –70°C until processed for virus isolation.

Virus isolation
The anticoagulant treated blood samples and skin fibroblast culture samples were processed for virus isolation through embryonated chicken eggs (ECE) as described by Gard et al. (2), with the final cell passages through microtitre plates rather than cell culture tubes. The microtitre cell culture plate method for virus isolation is adapted from Lindsay et al. (5). In this method, duplicate wells of a 96-well microtitre plate containing C6/36 mosquito cell cultures were used as a first passage. The second passage used C6/36, BSR (7) and porcine stable equine kidney (PSEK) mammalian cell cultures, followed by a third passage that comprised BSR (a clone of baby hamster kidney cell line) and PSEK cell cultures.

The ECE homogenates were inoculated for first passage in C6/36 mosquito cell cultures and the plates were incubated at room temperature (25°C) in a humidified container for 7 days. The first passage supernatant was then inoculated to identical wells of three plates containing C6/36, BSR or PSEK cells. The plates were examined for the presence of CPE from day three post inoculation. The type and extent of CPE was recorded and at 80-100% CPE, the supernatant was aseptically removed from the CPE-positive wells and inoculated to 25 cm² BSR tissue culture flasks for the production of seed stock virus for identification. Inoculation of the third cell culture passage used the C6/36 second passage plates. The second passage C6/36 supernatant was inoculated to identical wells of two plates containing either BSR or PSEK cells and examined as described above.

Virus identification
Virus isolates were identified by a combination of BTV antigen capture enzyme-linked immunosorbent assay (ELISA) (4) and virus neutralisation (VN) (3).

Serology
Bluetongue group and serotype-specific antibody was monitored by competitive ELISA (c-ELISA) (6) and VN (3).

Results
During the observation period, 14 cattle in each group of 24 were naturally infected with BTV-1. Infection was confirmed by virus isolation from blood samples and by serology. The periods of viraemia varied from one to five weeks.

Duplicate skin biopsy samples were taken from individual animals for between 16 and 24 weeks post initial detection of viraemia. Between 7 and 13 biopsies were taken from each animal. A total of 288 skin biopsies were examined from the two groups of cattle. Results are shown in Tables I and II.

Only one skin biopsy yielded BTV-1 following fibroblast culture and virus isolation. This biopsy, from animal number 80, was taken in the week when the animal was viraemic. Infectious BTV was not isolated from any the remaining 287 skin biopsies.
## Table I
Blood, serum and skin biopsies processed from cattle infected with BTV-1: Group 1

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NT: not tested

* antibody c-ELISA or BTV-1 neutralising antibody
Table II
Blood, serum and skin biopsies processed from cattle infected with BTV-1: Group 2

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<tr>
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<tr>
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</tr>
</tbody>
</table>

NT not tested  * antibody c-ELISA or BTV-1 neutralising antibody
Bluetongue virus and disease

Lymphocytes were harvested from blood samples collected up to 7 weeks after the first isolation of BTV-1 from five of the infected cattle in Group 2. In three of these animals, sampling commenced during viraemia. In the remaining two animals, sampling commenced two weeks after the first isolation of BTV-1 when the animals were no longer viraemic. A total of 30 PBMC skin fibroblast co-cultures were performed. Infectious BTV-1 was not isolated from any of these cultures (Table III).

### Table III
Peripheral blood mononuclear cells co-cultured with skin fibroblast

<table>
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<th>Animal</th>
<th>Test</th>
<th>Weeks from first isolation of BTV-1</th>
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</thead>
<tbody>
<tr>
<td>68</td>
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<td></td>
<td>Virus PBMC:fibroblast</td>
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<td>Virus PBMC:fibroblast</td>
<td>0 1 2 3 4 5</td>
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<tr>
<td>78</td>
<td>Virus blood</td>
<td>2 3 4 5 6 7</td>
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<td>Virus PBMC:fibroblast</td>
<td>0 1 2 3 4 5</td>
</tr>
<tr>
<td>79</td>
<td>Virus blood</td>
<td>2 3 4 5 6 7</td>
</tr>
<tr>
<td></td>
<td>Virus PBMC:fibroblast</td>
<td>0 1 2 3 4 5</td>
</tr>
</tbody>
</table>

PBMC, peripheral blood mononuclear cells

Additional tests were used to confirm the presence of lymphocytes in the skin fibroblast cultures. A skin biopsy from each animal was taken in formalin for histopathology. Examination of haematoxylin and eosin stained sections confirmed the presence of lymphocytes in the skin biopsies. Smears were also made of centrifuged deposits from the skin fibroblast cultures. These were stained with ‘Diff Quik’ (Lab Aids Pty Ltd) and examined for the presence of lymphocytes. Lymphocytes were identified in these smears.

A primary skin fibroblast cell line was developed from a healthy non-infected bovine. This cell line was maintained for at least 20 passages at weekly intervals. The ability of this cell line to support the growth of BTV was confirmed by inoculation of known BTV-1. The cell line showed cytopathic effect three days after inoculation and the identification of BTV-1 was confirmed by VN (3).

Exposure of the cattle to insect attack was confirmed using a mechanical aspirator. Six animals were used for midge collection and six collections each of 5 min were made. The total area of skin over which collections were made was approximately 3.5 m². Collections were made each week during the period of observation and Culicoides vector species sorted and counted. Culicoides actoni, C. brevitarsis and C. fulvus were all collected from the cattle with C. actoni being the dominant species. An average of 863 midge vector species were collected during a total collection time of 30 min between 5.15 pm and 7.45 pm.

### Discussion

Takamatsu et al. (9) suggested that BTV could persist in γδ T-cells in the skin of infected sheep. Subsequent midge feeding could then induce virus replication. This finding was proposed as a mechanism for ‘overwintering’ survival of BTV. It does however also raise concerns about the movement of sheep and cattle with antibodies to BTV and the possible reintroduction of international restrictions on movement of such animals.

The experimental work conducted by Takamatsu et al. used laboratory-adapted virus. Adaptation of field isolates of BTV to growth in tissue culture has been shown to alter the biological properties of BTV (1). The studies described in this paper were conducted to determine if natural BTV infections in cattle could result in persistent infection of γδ T-cells and if infectious BTV could be recovered from skin biopsies from these cattle. The cattle were exposed to biting midges throughout the observation period.

The work was performed over 11 months and involved 288 individual skin biopsies from cattle naturally infected with BTV-1. Only one skin sample yielded infectious BTV-1 and that was taken when the animal was viraemic. As many of the skin biopsies were blood contaminated it is probable this was virus contamination from blood at the peak of viraemia. Lymphocyte co-cultures with a skin fibroblast cell line also failed to yield any infectious BTV. This work also showed a maximum viraemia of five weeks, confirming that viraemia with BTV-1 in cattle is limited. Isolation of BTV-1 from the blood of these cattle and from one cultured skin biopsy indicated that the systems used were sensitive enough to detect virus if present. These findings provide compelling evidence that BTV does not persist in γδ T-cells in the skin of naturally infected cattle.

Experimental work is currently underway at two locations in Australia to see if wild or laboratory-adapted virus can persist in sheep. Confirmation of
BTV persistence in the skin of sheep should be obtained before this finding is accepted as the mechanism for ‘overwintering’. This work confirms there is no need to restrict the movement of seropositive cattle post viraemia, as such animals are not persistently infected.

Acknowledgements

Jason Stevens and Robert Kelly assisted with skin biopsies at Beatrice Hill. Margaret Harmsen and Lynne Chambers assisted with the serology and virus isolations.

References


Characterisation and monitoring of neutralisation-resistant VP2 phenotypes in BTV-1 isolates from northern Australia collected over a twenty-year period

J.R. White(1), V. Boyd(1), J.K. Brangwyn(1), C.J. Duch(1), L.I. Pritchard(1), L.F. Melville(2), T.D. St George(3) & B.T. Eaton(1)

(1) Australian Animal Health Laboratory, CSIRO, Division of Livestock Industries, Private Mail Bag 24, Geelong, VA 3220, Australia
(2) Department of Primary Industry and Fisheries, Berrimah Veterinary Laboratory, Post Office Box 990, Darwin, NT 0801, Australia
(3) 16 Tamarix Street, Chapel Hill, QLD 4069, Australia

Summary

Phenotypic profiles of the VP2 protein of isolates of bluetongue virus serotype 1 (BTV-1) collected from Queensland and the Northern Territory, Australia, between 1979 and 1986 were analysed using neutralising monoclonal antibodies (MAbs) raised to the prototype isolate of Australian BTV-1 collected in the Northern Territory in 1979. Two distinct profiles were found. Northern Territory isolates exhibited the prototype profile, yet those from Queensland had a significantly different (‘resistant’) profile. Nucleotide sequencing of gene segment 2 from both groups of isolates was undertaken. When the nucleotide sequences of isolates from a later period in each State were analysed (1997-2001), all exhibited the ‘resistant’ profile. Thus, a novel VP2 phenotype, already in existence in Queensland, had supplanted a pre-existing VP2 phenotype in the Northern Territory between the two periods. Furthermore, amino acid differences between the resistant and prototype VP2 proteins were analogous to amino acid substitutions known to be associated with neutralisation resistance. The host immune response may therefore have contributed to selection of the ‘resistant’ phenotype.

Keywords


Introduction

Earlier cross-neutralisation data (10, 14) and subsequent phylogenetic analysis of the gene segment 2 sequence of various bluetongue viruses (BTV) has provided a good understanding of the level of genetic relatedness between different serotypes of BTV and between isolates of the same serotype from well separated geographic regions (9, 15, 18, 19). To determine whether isolates of the same BTV serotype from within a restricted geographic region show significant phenotypic variation, neutralising monoclonal antibodies (MAbs) were used to demonstrate the co-existence of different VP2 antigenic profiles (1, 12, 13, 16).

Phylogenetic analysis has further confirmed the coexistence of multiple VP2 phenotypes in geographically restricted field locations for both BTV (3, 4) and a related orbivirus (11).

We were interested to investigate the extent of phenotypic variation in the VP2 protein of field isolates of Australian BTV-1 from several isolated geographic locations in Northern Australia collected over a substantial time period. Of particular interest was the potential for changes in phenotypic expression over time and whether any associations could be made between those amino acid sites found to differ between isolates and sites known to be related to virus neutralisation (8, 17).
Bluetongue virus and disease

Materials and methods

Virus isolation and propagation

Field isolates of BTV-1 were initially propagated in chick embryos from infected bovine blood and then plaque-picked and passed in cell culture (Vero and/or BHK-21 cells). In addition to the prototype isolate CSIRO156 (CS156) initially isolated from Beatrice Hill in the Northern Territory, the following isolates were used in this study: CS786, CS787, CS1564, CS1566, V1240 and V1241 (Queensland) and CS160, CS418, CS420, CS421, CS445, V1240, V4045, V4234, V4907, V4942, V4959, V4961, V4962, V4968 and V4977 (Northern Territory). The attenuated vaccine strain of South African BTV-1 was obtained from CSL, Melbourne, Australia. Attenuation was achieved by prior extensive passage in chick embryos at the Onderstepoort Veterinary Research Laboratory in South Africa.

Monoclonal antibodies

Neutralising murine MAbs E3/F4, D8/A1, J3/G6, A2/D2, E6/A4, E10/A11 and D8/A12 were raised to a purified, infected cell lysate of CS156 as previously described (7, 17).

Serum neutralisation test

Approximately 200 TCID50 units of virus in 25 µl of Eagle’s minimum essential medium (EMEM) were mixed in triplicate within the wells of a 96-well microtitre tray with MAbs appropriately diluted in EMEM. The plate was incubated for 1 h at 37°C, then 200 µl of EMEM maintenance medium (with 2% [v/v] foetal calf serum) containing approximately 10^5 Vero cells was added to each well and the plate incubated for 4 days at 37°C under 4.5% CO2. Following incubation, neutralisation titres were estimated by determining the highest dilution of MAb that prevented cytopathic effect (CPE) in at least 50% of the monolayer. Neutralisation titres were determined by the addition of 0.75 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the growth medium followed by dissolving the resultant dye in 100 µl of isopropanol/well and determination of the absorbance at 560 nm.

Nucleotide sequencing

Viral RNA was isolated from clarified, virus-infected cell culture supernatant using the QiAamp viral RNA mini kit (Qiagen, Australia). Following denaturation of dsRNA with deionised formamide, reverse transcriptase (RT) and polymerase chain reaction (PCR) amplification was achieved using a Qiagen one step RT-PCR kit incorporating the appropriate primer sets. Eighteen to 22-mer primer sets were designed to amplify five separate 600-800 nucleotide regions along the gene 2 segment. Amplified DNA was purified using Qiaquick (Qiagen) then transferred to a sequencing reaction using the ABI Prism BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster, California). Product sequence was read on an automated DNA sequencer (ABI Prism model 377). Sequence homologies were determined using the Lasergene software package (DNASTAR Inc., Madison, Wisconsin).

Results

Six isolates of BTV-1 from four collection sites in the Northern Territory (Beatrice Hill, Tortilla Flat Coastal Plains and Berrimah) and four isolates from two well separated sites in Queensland (Kairi and Peachester) (Fig. 1) were tested for their susceptibility to neutralisation by a panel of seven VP2-specific MAbs raised to the prototype isolate of BTV-1 Australia (CS156) (Table I). All five of the Northern Territory isolates collected from 1979 to 1980 from Beatrice Hill, Tortilla Flats and Coastal Plains (CS160, CS418, CS420, CS421, CS445) essentially displayed the same susceptibility to neutralisation as the prototype virus. The single Northern Territory isolate from Berrimah in 1986 (V930) showed resistance to neutralisation by two of the MAb panel. However all four of the isolates from Queensland (CS786, CS787, CS1564, CS1566) collected from 1981 to 1983, showed substantial
resistance to up to five MAbs, indicating that significant phenotypic variation existed in the VP2 protein of BTV-1 populations in each State. To assess the extent and nature of this variation, gene segment 2 of all ten isolates was completely sequenced and the degree of homology between the isolates determined. All six Northern Territory isolates were essentially identical to the prototype virus (>99.7% protein sequence homology), whereas the four Queensland isolates all exhibited a second phenotypic profile which varied from 2.6% to 2.9% from the prototype virus, depending on the particular isolates compared. It was presumed the individual amino acid differences between the two phenotypes may, at least in part, relate to the ability of the relevant isolates to resist neutralisation by particular MAbs.

When gene segment 2 of nine Northern Territory isolates (from Berrimah, Katherine, Douglas Daly and Victoria River) and two Queensland isolates (from Maryborough) (Fig. 1) collected between 1997 and 2001 was fully sequenced, all isolates were found to have essentially the same genotypic profile as the neutralisation resistant phenotype present in Queensland in the earlier collection period (between 99.3% and 99.9% protein sequence homology). These results indicated a unique VP2 phenotype, present in Queensland, had been introduced into the Northern Territory at some time prior to 1997 and became the dominant form due to some selective advantage.

To investigate whether the immune response of host animals represented a potential for selective pressure on the VP2 phenotypes, we examined the individual amino acid differences between the prototype and resistant forms, isolated in each of the two collection periods (Table II). In all, 25 amino acid differences existed between the prototype phenotype and all isolates displaying the resistant phenotype from the first collection period. Fourteen of these involved significant amino acid changes. The substitution at aa219 (Ser-Gly) was only seen in isolate CS787 and the substitution at aa586 (Gly-Glu) only occurred in isolates CS786 and CS1566. In addition to the 14 shared aa substitutions, three further significant amino acid differences existed between the resistant phenotype from the earlier collection period and that from the later period. Of the total of 19 significant changes identified, 10 sites were identical to amino acid differences existing between the prototype sequence and an attenuated vaccine strain of an isolate of South African BTV-1 (Table II). However, despite these similarities, the overall amino acid homology between the resistant phenotype and the vaccine strain was the same as that previously observed between the later sequence and the prototype strain (CS156) (81%) (8). Eight sites showed analogous changes to those amino acid substitutions induced in variant viruses by selection with individual neutralising MAbs. Five of these sites were included in the 10 sites of significant difference present in the vaccine strain. The vaccine strain strongly resisted neutralisation by five MAbs of the total panel (8, 17) (Table I). All variant viruses and the vaccine strain had previously been shown to be resistant to neutralisation by a bovine reference antiserum to CS156, indicating the relevance of these amino acid sites to the immune response of a common host animal for BTV in Australia (17).

Table I
The susceptibility of the prototype isolate, other field isolates and the South African vaccine strain of bluetongue virus serotype 1 to neutralisation by a panel of monoclonal antibodies

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Table II
Significant amino acid differences observed between the prototype VP2 sequence of bluetongue virus serotype 1 (BTV-1) (Aust.) and the sequences of the resistant phenotype, neutralisation-escape variants and the vaccine strain of BTV-1 (South Africa)

<table>
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<tr>
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<td>892 (Leu-Phe)</td>
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a) Gould and Eaton (8)
b) Unpublished data
c) Arg-Pro in CS786
d) Not seen in CS786, CS1564 and CS1566
e) Only in isolates collected after 1997
f) Only in CS786 and CS1566

The Northern Territory isolate (V930) from the first collection period (isolated in 1986) which exhibited some resistance to MAb neutralisation possessed three significant amino acid changes compared to the prototype virus, i.e. aa158 (Ser-Pro), aa314 (Asn-Asp) and aa628 (Arg-Cys).

Discussion

It is likely that phenotypic variants of individual BTV-specified proteins are constantly generated in the field (2, 5, 6) such that at any given time and possibly despite the selection pressure of a host immune system (6, 13), a particular viral protein within a BTV population actually exists as a heterologous collection, comprising major stable protein phenotypes along with a series of minor phenotypic variants (2, 3). The unique characteristics of the VP2 sequence of the Northern Territory isolate V930, analysed in this study, might represent a manifestation of this phenomenon. The selection pressures arising from vector/host interactions and the immune response of host animals are likely factors in specific phenotypes being maintained or ‘fitter’ phenotypes becoming established (2, 6).

In this study, by producing MAb-defined antigenic profiles and sequence data from BTV-1 isolates collected over a significant time period, we have demonstrated that at some point within the last two decades at least two distinct and stable phenotypic forms of the VP2 protein co-existed in Northern Australia. The maintenance of separate phenotypes in relatively close, yet distinct geographic regions has been observed for a related orbivirus (11). At some point in the approximately twenty-year period between virus isolations, a VP2 phenotype already in existence in Queensland, was introduced into the Northern Territory and became dominant at all collection sites. A temporal involvement in the establishment of new and dominant phenotypic forms of VP2 has been documented by others (4). We have also shown that a strong association exists between the significant amino acid changes present in the dominant neutralisation resistant phenotype (compared to the prototype VP2 sequence) and changes known to be associated with experimentally generated neutralisation-escape variants and from a highly resistant isolate of BTV-1 from a geographic region remote from Australia (Table II). The data generated in this study thus suggest the incursion into the Northern Territory and eventual dominance of the ‘resistant’ phenotype may be due to the combined effects of time and selection pressure imposed by the immune response of host animals.

Acknowledgements

The authors wish to thank Ross Lunt and Kim Newberry for cultivation of some of the virus isolates used in this study and Tony Pye for operation of the DNA sequencer.

References


Lack of detectable bluetongue virus in skin of seropositive cattle: implications for vertebrate overwintering of bluetongue virus

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Summary

The overwintering mechanism of bluetongue virus (BTV) has eluded researchers for many years. It was recently proposed that ovine gamma delta T-cells may become persistently infected with BTV, and serve as a reservoir for infection of naive vectors in the next transmission season. Since cattle are more numerous than sheep in the western United States (where BTV is endemic), this hypothesis was tested in bovines. In the winter of 2002-2003, 54 cattle from an endemic site in northern Colorado were age-selected to ensure that possible BTV exposure must have occurred in the summer of 2002. These cattle were tested for the presence of anti-BTV antibody by ELISA; 53 were seropositive, and one was seronegative. Naive Culicoides sonorensis colony insects were fed on skin sites of four seropositive and one seronegative cattle at day 1 (135 days after the first frost), then sequentially on separate sites for three days. Virus isolation and/or reverse transcriptase-nested polymerase chain reaction from engorged insects and 6 mm skin biopsy samples were performed for detection of viable BTV or BTV nucleic acid; all were negative. These data suggest that cattle are not a reservoir host for BTV overwintering in the western United States. The role of sheep in the trans-seasonality of BTV still remains to be determined.

Keywords


Introduction

Bluetongue virus (BTV) is the prototype of the genus Orbivirus, family Reoviridae. BTV is an arthropod-borne virus that is maintained in the United States of America (USA) in a natural transmission cycle involving the primary haematophagous insect vector Culicoides sonorensis and sheep, cattle and wild ruminant amplification hosts (21, 41). The BTV genome is composed of ten double-stranded RNA segments designated large (L1-3), medium (M4-6), and small (S7-10) (41). In the mature virion, the segments are encapsidated in two concentric protein shells. The outer capsid is composed of virion proteins (VP) 2 and 5. The inner capsid is composed of VP7 trimers that ‘float’ dynamically on VP3 pentamers, which provide the scaffolding for viral assembly (20) and bind to the dsRNA. Genome segments S7 and L3 encode VP7 and VP3, respectively. VP7 is a group specific antigen (10), and other work has demonstrated that segments from multiple serotypes can be detected with a single primer set (51).

BTV overwintering/trans-seasonality

BTV may be maintained in year-round transmission cycles in tropical parts of the world, and even possibly in mild temperate winters (16; F. Holbrook, personal communication). However, such cycles are impossible in some regions due to environmental conditions (18). The virus must have a mechanism(s) that allows it to survive periods of inclement climatic conditions and vector diapause in temperate regions of the world, as well as in tropical regions that experience a significant dry season. Three principal hypotheses have been proposed for BTV trans-seasonal maintenance, as follows:

1) high altitude, air-current based, reintroduction of infected insects from year-round cycles to
endemic foci on a yearly basis

2) vertical transmission from the infected insect to its progeny

3) survival in the vertebrate host, either through persistent infection of adults or transplacental transmission to foetuses.

**Annual reintroduction of bluetongue virus-infected culicoids to endemic foci in temperate climates**

The reintroduction of infected insects from continuous, tropical transmission cycles to endemic foci in temperate zones is unlikely to account for the observed epidemiology of BTV. Several areas of the world do have low-level BTV activity year-round (16, 31), and introduction of BTV-infected culicoids into previously BTV-free, local areas on air currents can be an important mode of transmission on a small geographic scale (37). However, previous attempts to support this hypothesis (7, 42, 43, 44, 45) have failed to account for the random nature of aerial dispersal of pathogens (9) versus the epidemiological stability of BTV within endemic foci. In the absence of a consistent and positive link between weather patterns and the natural state of BTV in temperate zones, this hypothesis must be considered to be the least likely of the possibilities.

**Overwintering of bluetongue virus in the invertebrate vector**

The hypothesis that BTV overwinters through vertical transmission of the virus by infected adult *C. sonorensis* to their overwintering larval progeny is supported by our previous studies (14, 38, 51). BTV has been isolated from adult midges (reared in the laboratory from field-collected larvae) from an endemic focus in northern Colorado near the commencement of the transmission season (14), and BTV dsRNA and antigen have been detected in *C. sonorensis* larvae collected from the same focus (38, 51). Although experimental evidence indicated that transovarial transmission (TOT) of BTV does not occur in colonised *C. sonorensis* (25, 33), it is possible that outbred insects may be capable of vertical transmission, especially in light of the studies cited above, as well as the isolation of another orbivirus (Orungo virus) from wild-caught male mosquitoes in the Côte d’Ivoire (11). In addition, BTV antigen was detected in the vitelline membrane of infected, adult females and proteoid yolk bodies of their oocytes, but not in the ovarian tissue itself. This demonstration of ‘dense’ BTV antigen in the reproductive structures in which it was found (33) would suggest that vertical transmission could occur. Vertical transmission of flaviviruses in mosquitoes occurs via the micropyle as the egg passes through the oviducts during oviposition (40). A similar mechanism could function for vertical transmission of BTV in *C. sonorensis*.

**Overwintering of bluetongue virus in vertebrate hosts**

For many years it was assumed that vertebrate animals exhibiting a long-term viraemia following acute infection was the most likely mechanism for BTV trans-seasonality. However, infectious virus is >99% likely to be eliminated from infected cattle after 63 days (46), and viral RNA cannot be detected by reverse transcriptase-nested polymerase chain reaction (RT-nPCR) after 222 days (4). Between those times, samples are RT-nPCR-positive but virus isolation (VI) negative, and non-infectious virus or viral RNA probably exists in close association with (or inside) erythrocytes (32). Studies were conducted to determine if the bite of an uninfected insect could rescue virus that had eluded attempts at virus isolation. Naive *Culicoides sonorensis* were intrathoracically inoculated with cattle blood samples positive by RT-PCR for viral RNA, but negative for BTV by VI. Not only did those insects not become infected, they also did not transmit BTV to uninfected sheep upon taking blood-meals after an appropriate extrinsic incubation period (48).

Assuming that animals with a long-term ‘viraemia’ (as above) cannot directly infect vectors, they would then have to be able to infect other vertebrates that could then serve as the reservoir for naive vectors. Venereal transmission of BTV in the semen of infected males (bulls or rams) is unlikely because virus can only be detected in semen shortly after peak viraemia (3 to 10 days post infection) and is limited to that time alone (30). In addition, excretion of BTV in semen of naturally infected bulls appears to be an extremely rare event, if it occurs at all (15, 47). Finally, heifers impregnated with BTV-infected semen seroconverted in only 4 of 9 experimental exposures, and none of the foetuses showed any pathology or sign of infection (5, 6). Viraemias in infected calves last as long as 20 weeks (by RT-PCR detection), but they were shown to be infective for naive *Culicoides sonorensis* for only two weeks (27). Thus, venereal and/or vertical infection of vertebrate hosts with BTV represents a theoretical trans-seasonal maintenance mechanism; however, it would not appear to be a mechanism that is active in the natural cycle of the virus.

Immunosuppression induced by drugs (hormones or other), pathogens (i.e. ovine lentivirus), or stress could be responsible for the establishment of persistent BTV infection in sheep (8) and as such could have implications in the trans-seasonal
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maintenance of BTV. While this is an intriguing possibility, its importance in the year-to-year maintenance of the virus cannot be fully assessed with the available data. We do know that naive vectors cannot recover latent virus from RT-nPCR-positive but VI negative cattle blood (48). However, those cattle may not adequately represent a truly ‘persistently infected’, stressed animal. Other immunosuppressive factors in addition to the stress of the bite(s) and the immunosuppressive qualities of culicoid saliva (35, 36) could increase the time that a cow or bull was infectious to a naive, competent vector. Further research in this area may be warranted.

Finally, persistent infection of immunotolerant cattle by BTV was reported in 1975 (22, 26), but this could be an artefact of laboratory-passaged BTV (2, 17, 19, 28, 50; A.J. Luedke and T.E. Walton, personal communications). Recent evidence has suggested that ovine γδ T-cell lines may become persistently infected with a laboratory-adapted strain of BTV, and that virus production is increased in response to cell-cycle arrest or activation (49). It was hypothesised that these T-cells could become persistently infected in vivo, migrate to the intraepithelial space, and become activated in response to the bite of a naive vector. They would then shed virus that would be available to infect either that vector or subsequently biting vectors. However, in a test of this hypothesis, virus was not detected by standard techniques in skin samples, blood samples, or naive culicoids fed on these ‘persistently infected’ sheep. The only way virus was detected was by culturing the skin samples in the presence of human IL-2 for 7 days. These findings are very provocative, and prompted us to test this hypothesis in cattle, which are much more prevalent than sheep in the western USA and therefore are more likely to play a pivotal role in the trans-seasonality of BTV in the country. Additionally, the authors themselves suggest that cattle are more likely to become persistently infected than sheep (49).

Materials and methods

Fifty-four cattle, approximately 8 to 10 months of age, were identified from a long-term endemic site in northern Colorado in January 2003. Blood samples were collected from these animals and assayed for the presence of serum antibody to BTV by a competitive enzyme-linked immunosorbent assay (c-ELISA) developed at the United States Department of Agriculture-Agricultural Research Service-Arthropod-Borne Animal Diseases Research Laboratory (ABADRL) (29). Fifty-three of the tested cattle were positive for antibody to BTV (>75% inhibition by c-ELISA), indicating exposure in the previous transmission season (summer 2002). Four seropositive animals and the one seronegative animal were brought into the Large Animal Isolation Building at the ABADRL in Laramie, Wyoming, for exposure to naive Culicoides sonorensis from our colony and sample collection. Four sites were shaved on each side of the animal and assigned a letter identifier (Fig. 1). All sites were exposed to insects on day 1, which was 135 days after the first significant frost in Adams County (14 October, 2002). Then, two 6 mm punch biopsies were taken from each site (A-H) after a subcutaneous lidocaine block was performed. One biopsy was placed in formalin, and the other was split for virus isolation (into cell culture medium) and RNA extraction (snap frozen in liquid nitrogen). Sites B, C, and D were fed upon by naive colony insects on days 2, 3, and 4, respectively, and skin samples were taken as above each day.

Figure 1
Experimental sites
Cattle were clipped in a large area, then closely shaved in the four areas where insects would be fed and skin samples would be collected (A-D). The pattern was repeated on the opposite side of the animal as a negative control (no insect treatment, E-H).
Sites A-D were fed upon by naïve Culicoides sonorensis on day 1, then sequentially on days 2 to 4 (i.e. site B was exposed to insects on day 2, site C on day 3, and site D on day 4). Skin samples were taken with 6 mm punch biopsy instruments (arrow), and processed for virus isolation and detection of BTV nucleic acid by RT-nPCR.

Skin samples for virus isolation were sonicated for 1 min in a cup horn sonifier (Ultrasonics Inc. Cell disruptor, Duty Cycle 60%, Output 6.5) in 0.5 ml of cell culture medium containing antibiotics (199-E, 10% foetal bovine serum, 400 IU/ml penicillin, 400 µg/ml streptomycin, 200 µg/ml gentamicin, 5 µg/ml amphotericin B) and directly plated onto Vero-MARU cells. Cultures were maintained for 10 days (3 to 4 days after positive controls showed 100% cytopathic effect). Cultures showing signs of bacterial or fungal infection were scraped and passed...
through a 0.2 µm non-protein binding filter and placed onto a new flask of cells.

Insects were anaesthetised with carbon dioxide, and insects that had taken a blood-meal were removed for further processing (Fig. 2). Insects were pooled in 0.5 ml of cell culture medium containing antibiotics (as above) in groups of approximately 20 and ground using sterile microcentrifuge tube pestles. The ground insects were added to cells as above, held for 10 days, and processed in the same way as the skin samples.

**Results and discussion**

All of the skin samples and blood-fed insects were negative for viable BTV isolation and/or BTV nucleic acid detection. RT-nPCR is the most sensitive detection method for bluetongue virus nucleic acid used today (3), and recovery of live virus by naive vector insects is recognised as the most sensitive and biologically relevant method of detecting latent BTV infections (48). The lack of detection of BTV by either method gives a high degree of confidence that the virus was not present in the skin of any of the animals.

Persistent infection of immunotolerant cattle by BTV was suggested as a virus overwintering mechanism (22, 26), but these initial studies must be questioned because numerous attempts to replicate them have failed (2, 17, 19, 28, 34, 39, 50; T.D. St George and N.J. MacLachlan, personal communications). Perhaps the initial study was an artefact of cell culture passage of BTV (1; A.J. Luedke and T.E. Walton, personal communications). Other studies have suggested that the biological behaviour of laboratory-passaged BTV is different from virus that exists in nature. Laboratory-passaged and vaccine strains of BTV were shown to cause abortions in susceptible sheep, whereas wild virus did not (24, 47; T.D. St George, personal communication). Additionally, bulls with naturally acquired infections do not shed BTV in their semen, while only older bulls experimentally infected with laboratory-passaged virus rarely shed virus in the peri-viraemic period (15, 30, 47). Therefore, it would seem that any examination of the behaviour of bluetongue virus relevant to its natural epidemiology would require significant steps to ensure the ‘wildness’ of the virus.

The hypothesis proposed by Takamatsu *et al.* (49) is intriguing. However, their results could be an

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**Table I**

<table>
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<th>Primer name</th>
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* All primers were designed based on published sequences
artefact of virus ‘...passaged several times in BHK cells...’, and have little relevance to the natural epidemiology of BTV. This view is supported by their failure to recover live virus from their persistently infected sheep by naive vector insect bite, as well as the failure to isolate viable BTV from the blood or skin samples that were processed normally. Only by culturing skin samples with human interleukin-2 for one week could virus be isolated. Other studies have indicated that IL-2 levels are either down-regulated (12) or not changed (13) in the inflammatory lesions generated by insect bites. However, other factors noted by the authors could be responsible for the activation of persistently infected γδ T-cells in the inflammatory lesions generated by the bite of vector insects (49).

As stated earlier (49), cattle, instead of sheep, are the more likely host in which one would anticipate the persistence of BTV. However, viable BTV and BTV-specific nucleic acids were not detected in BTV-exposed animals from an endemic focus in northern Colorado subsequent to feeding by naive vector insects. The role of ovinities in the overwintering of BTV still remains to be resolved.

References

Bluetongue virus and disease


Replication of epizootic haemorrhagic disease and bluetongue viruses in DH82 cells

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Summary

Bluetongue viruses (BTV) and epizootic haemorrhagic disease viruses (EHDV) can be propagated in a variety of mammalian cell lines, but in the ruminant host these viruses infect and replicate in endothelium and monocytes/macrophages. In vitro studies of virus-monocyte interactions typically rely on primary ruminant macrophage cultures that cannot be propagated and a continuous cell line of monocytes would be useful in the study of these interactions. The objective of this study was to determine if DH82 cells, a continuous canine macrophage-monocyte cell line, are susceptible to BTV and EHDV infection. We found DH82 cells susceptible to both BTV and EHDV infection resulting in high titres and the development of massive numbers of viral-associated tubules. This system could be useful for viral-monocyte receptor and viral-associated cytokine expression studies, and the production of large quantities of viral-associated tubules for structural or functional studies of these tubules.

Keywords


Introduction

Bluetongue viruses (BTV) and epizootic haemorrhagic disease viruses (EHDV) can be propagated in a variety of mammalian cell lines ranging from green monkey fibroblasts (Vero cells) to bovine nasal turbinate cells (13). However, in the ruminant host, these viruses infect and replicate in endothelium and monocytes/macrophages (5, 7). Primary ruminant endothelial cell cultures that can be used to study virus-endothelial interactions are available and can be propagated and passed numerous times (14). On the other hand, continuous ruminant macrophage-monocyte cultures are not available and in vitro studies of virus-monocyte interactions rely on primary macrophage-monocyte cultures that cannot be propagated. Dogs were shown to be susceptible to BTV infection when pregnant bitches died of bluetongue following inoculation of a BTV-contaminated vaccine (3). We hypothesised that DH82 cells, a commercially available continuous canine macrophage-monocyte cell line, would be susceptible to these viruses and could prove useful in the study of virus-monocyte interactions (15). The objective of this study was to determine if DH82 cells are susceptible to BTV and EHDV infection.

Materials and methods

Cells

DH82 cells (American type culture collection CRL-10389) were propagated in minimum essential medium (MEM) with non-essential amino acids, 2 mM L-glutamine, and 10% foetal calf serum (MEM growth media). Baby hamster kidney (BHK-21) cells were propagated as described previously (8).

Viral inoculums

Flasks monolayered with BHK-21 cells were inoculated with EHDV serotype 2 (EHDV-2) or
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BTV serotype 10 (BTV-10). At 80% cytopathic effect, the flasks were scraped and infected cells and supernatant were collected and centrifuged. The resulting pellet was resuspended in Dulbecco's phosphate-buffered saline, sonicated, recentrifuged and aliquots frozen at –70°C. The viral titre was determined by endpoint titration in BHK-21 cells. The EHDV-2 was originally isolated from a white-tailed deer lymph node in BHK-21 cells, and the BTV-10 was from stock originally obtained from the National Veterinary Services Laboratories, USDA, in Ames, Iowa.

Experimental design

DH82 cells scraped from a 25 cm² flask were resuspended in 14 ml of MEM growth media and fourteen wells of a 24-well plate were each seeded with 1 ml of resuspended cells. Five wells were inoculated directly with $10^{2.5}$TCID$_{50}$ of EHDV-2 and five wells were inoculated directly with $10^{2.5}$TCID$_{50}$ of BTV-10. The other four wells containing cells served as uninoculated negative control wells to compare cytopathic effects and for immunocytochemistry. The remaining ten wells contained MEM growth media, but no cells, and served as virus controls: five were inoculated with $10^{2.5}$TCID$_{50}$ of EHDV-2 and five with $10^{2.5}$TCID$_{50}$ of BTV-10. On days 1 to 5 post inoculation (pi), one DH82 and one cell-free virus inoculated well for each virus was harvested. The DH82 wells were scraped and the cells resuspended in the media and 500 µl was removed for virus endpoint titration. The remaining contents of the virus-inoculated DH82 wells were cytocentrifuged onto glass slides, fixed by immersion in cold acetone for 10 min, and stained for EHDV or BTV by immunocytochemistry. On days 2 to 5 pi, one uninoculated DH82 well was scraped and the contents prepared for immunocytochemistry by cytocentrifugation.

Virus endpoint titrations

Viral titres were quantified by endpoint titration in 96-well tissue culture plates using BHK-21 cells. Six ten-fold dilutions were made and eight replicate wells were used for each dilution; titres were determined as described by Reed and Muench (9).

Immunocytochemistry

Fixed slides were hydrated in phosphate-buffered saline for 15 min and then treated with 1.0 mM EDTA solution, pH 6.8 for 30 min at 37°C. Slides were rinsed, blocked with 0.05% casein solution for 30 sec, rinsed, and then either rabbit anti-BTV-10 (1:1 000) or rabbit anti-EHDV-2 (1:2 000) was applied for 2 h at room temperature. After rinsing, antibody was detected using a commercially available avidin-biotin alkaline phosphatase technique (BioGenex) with fast red as the chromagen (BioGenex). In duplicate slides, rabbit anti-Helicobacter antibody replaced the primary antibody as a negative control.

Transmission electron microscopy

A 25 cm² flask of DH82 cells was inoculated with $10^{4.4}$TCID$_{50}$ of EHDV-2. On day 1 pi, 50% of the flask was scraped and the cells suspended in the media of the flask. The media was then harvested and centrifuged and the resulting pellet was fixed in 2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.1 M Cacoclyte-HCl buffer. Media was replaced in the flask and the process repeated on days 2 and 3 pi. In addition, an aliquot of media was removed for virus titration on days 2 and 3 pi. After fixation, cells were pelleted, enrobed in agar, post-fixed in 1% osmium tetroxide and embedded in Epon-Araldite. Thin sections were post-stained with uranyl acetate and lead citrate.

Results

Viral titres in DH82 cells inoculated with both EHDV and BTV were greatly increased over controls (virus and media without cells) by day 2 pi (Table I). Titres peaked slightly on day 3 pi, but remained high until termination on day 5 pi. Titres were similar for both viruses, varying by less than 0.5 logs. Virus was detected in the control (no-cell) wells until day 5 pi, but never exceeded the titre of the original inoculum. Discrete plaques were not appreciated microscopically. By immunocytochemistry, rare DH82 cells in cytocentrifuge preparations were positive for either BTV or EHDV on day 1 pi, but by day 2 pi, and up to day 5 pi, approximately 10% of the cells were immunopositive for BTV or EHDV.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Replication of bluetongue virus serotype 10 (BTV-10) and epizootic haemorrhagic disease virus serotype 2 (EHDV-2) in DH82 cells</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>Virus titre (log$<em>{10}$TCID$</em>{50}$/ml) Day post inoculation</td>
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<td>---------------------------------------------------------------</td>
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<tr>
<td></td>
<td>1                 2                3                4                5</td>
</tr>
<tr>
<td>BTV-10 and DH82 &amp;</td>
<td>2.97              5.26              5.60              5.60              5.39</td>
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<tr>
<td>EHDV-2 and DH82 &amp;</td>
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<td>BTV-10 + media &amp;</td>
<td>2.26              2.20              2.20              2.20              2.20</td>
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<tr>
<td>EHDV-2 + media &amp;</td>
<td>2.20              2.20              2.20              2.20              2.20</td>
</tr>
<tr>
<td>a) DH82 cells inoculated with $10^{2.5}$TCID$_{50}$ BTV-10</td>
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<tr>
<td>b) DH82 cells inoculated with $10^{2.5}$TCID$_{50}$ EHDV-2</td>
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<tr>
<td>c) Media (no cells) inoculated with $10^{2.5}$TCID$_{50}$ BTV-10</td>
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<tr>
<td>d) Media (no cells) inoculated with $10^{2.5}$TCID$_{50}$ EHDV-2</td>
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Ultrastructurally, day 1 pi, EHDV-infected cells had multiple coated pits on their surface, some of which contained extracellular viral particles, and a few viral particles and viral-associated tubules were present in the cytoplasm of some cells (Fig. 1). By day 2 pi, some cells were either swollen and lysed (necrotic) or shrunken and dark with condensed nuclei (apoptotic). Intact cells contained viral matrices, sometimes containing viral particles and viral tubules (Fig. 2). Groups of viral particles, matrix, and tubules were associated with the cytoplasmic remnants of lysed cells (Fig. 3). The shrunken dark cells did not appear to be infected. By day 3 pi, no lysed or shrunken dark cells were observed; however, many cells were infected and contained large viral matrices and significant numbers of viral-associated tubules (Fig. 4). Viral particles were scarce and, when observed, were near the plasma membrane, often in a cytoplasmic protrusion. Virus titres were $10^{7.03}$TCID$_{50}$/ml and $10^{5.80}$TCID$_{50}$/ml on days 2 and 3 pi, respectively.

Discussion

DH82 cells were susceptible to both EHDV and BTV infection resulting in relatively high viral titres of similar magnitude. DH82 cells were derived from a canine malignant histiocytoma and were originally characterised as histiocytic by positive staining reactions for alpha naphthyl acetate esterase and acid phosphates, presence of Fc receptors, phagocytosis of latex beads and plastic adherence in culture (15). As EHDV and BTV can infect a variety of cell types, and have been shown to replicate in cow (16) and...
deer (11) peripheral blood monocytes in vitro, it is not surprising that DH82 cells were susceptible to infection. Unlike peripheral blood monocytes, DH82 macrophage-monocytic cells can be continually propagated in culture facilitating in vitro studies. Infection of large numbers of monocytic cells with these viruses, as seen in this system, would assist viral-monocyte receptor studies. DH82 cell surface antigens have been partially characterised and the cells are known to express CD14, and partially express CD5 and CD45 (1).

Cytopathic effects were difficult to appreciate in infected DH82 cell cultures despite the relatively high viral titres. This may be partially explained by the immunocytochemistry results, where, despite high titres, only about 10% of the cells appeared to be infected.

Curiously, in the ultrastructural study, evidence of viral induced DH82 cell death was present on day 2 pi, but had disappeared by day 3 pi. This is unlike infection in other cell systems, such as BHK-21 and cow pulmonary artery endothelial cells, where cell death is progressive (E.W. Howerth, personal observation), and the possibility of persistent infection of DH82 cells needs to be addressed. Also intriguing in the ultrastructural study was the presence of cell death by necrosis (lysis) in obviously infected cells and death by apoptosis in apparently uninfected cells. It is possible that the apoptosis was induced by cytokines released from infected cells rather than by direct viral induced damage. This is similar to what has been observed in BTV-infected endothelial cultures where cell death by apoptosis appears to be, at least partially, mediated by interleukin-1 (IL-1) rather than direct viral replication (2). DH82 cells are known to produce tumour necrosis factor alpha (TNF-α), IL-1, IL-6, IL-5, IL-8, and IL-10, so these cells could be used to study cytokine expression by BTV or EHDV infected monocytes (1).

Although viral-associated tubules normally develop in BTV and EHDV infected cells, both in vitro and in vivo (4, 10, 12), massive quantities of these tubules developed in EHDV-infected DH82 cells in this study. These tubules are formed from the virus coded non-structural protein, NS1, but their function is poorly understood (10). The production of large quantities of these viral-associated tubules in DH82 cells might prove useful for structural or functional studies of these tubules.

DH82 cells are routinely used for the isolation of ehrlichial agents from deer, cattle and sheep (6, 17). Isolation of these agents could be confounded by the concurrent isolation of either EHDV or BTV from animals co-infected with both types of agents.

References


Developing new orbivirus diagnostic platforms

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Summary

Traditionally, successful orbivirus identification and characterisation has been dependent upon the development and application of techniques for virus isolation. In recent years however polymerase chain reaction (PCR)-based molecular detection systems have revolutionised medical infectious disease diagnosis and in some instances have removed the requirement to isolate pathogens for confirmation of clinical diagnoses. In multiplexing formats, PCR-based methods also have the capacity to detect novel pathogens and variants of existing pathogens. Detection and characterisation of veterinary pathogens such as bluetongue virus will follow the same evolutionary path. Work is underway in a number of laboratories to develop the infrastructure and databases required to permit the use of DNA-based molecular systems for orbivirus detection and characterisation. Novel multiplexed protein analysis platforms also offer opportunities to not only enhance the speed and sensitivity of serological assays but also permit the development of serological procedures that a few years ago were not technically feasible.

Keywords

Array technologies – Bluetongue virus – Diagnosis – Embryonated chicken eggs – Orbivirus – Polymerase chain reaction – Multiplex platforms – Virus isolation.

Virus isolation

A number of methods for the isolation of bluetongue virus (BTV) have been developed over the past fifty years in an attempt to increase the efficiency with which virus in field material can be amplified to facilitate identification. Favoured methods include replication in embryonated chicken eggs (ECE), sheep, suckling mouse brain and a wide variety of cultured cells (7, 12, 13, 17, 33). A number of the more popular approaches will be summarised briefly, prior to discussing the impact of polymerase chain reaction (PCR) and multiplexing technologies on the continuing need for virus isolation. The potential application of multiplexed platforms for other aspects of orbivirus diagnosis will also be discussed.

Embryonated chicken eggs

In a letter to Nature in 1940, Mason, Coles and Alexander first reported the growth of BTV in chicken embryos following inoculation into the yolk sac of ECE (25). Over a quarter of a century later, Goldsmitt and Barzilai (14) and Foster and Luedke (11) showed that in vivo inoculation of ECE was 100-1 000 times more sensitive than yolk-sac administration. Since then, intravenous inoculation of 10-13-day-old ECE has been widely used as the method of choice in the isolation of BTV from clinical samples. A detailed procedure is described by Clavijo et al. (7). The preferred tissues for isolation include washed, unseparated blood cells, spleen, lung and lymph nodes (28). Preparation of washed blood cells for inoculation into ECE is straightforward, whereas tissues must be homogenised by grinding with sand in a mortar and pestle, in tissue grinders or more recently in ‘BeadBeaters’ with zirconia/silica beads. The number of ECE inoculated per sample varies but is usually 10, the incubation temperature 33-34°C and the inoculum dose 0.01 ml. Although dead embryos are usually the source of virus for the next step in the isolation process, embryo deaths are neither an indication of BTV replication nor are surviving embryos indicative of virus absence. Following death of the embryos (only a proportion of embryos may die even at the highest sample concentrations), the tissues are emulsified and the virus subjected to a second passage in either cultured cells or in ECE via the yolk sac or intravenous routes of administration.
Sheep inoculation

Sheep have been variously described to be as efficient as ECE (11, 15), less efficient than ECE (4) and more efficient than ECE (23, 27). The latter authors suggested that the larger sample volume that can be administered to sheep might account for the enhanced efficiency of isolation compared with ECE. However, sheep inoculation is often an impracticable option for many laboratories because of the requirement to maintain the sheep in holding facilities for at least 30 days after inoculation to permit development of the antibody response that provides evidence of virus infection.

Cultured cells

The first successful attempt to grow BTV in cultured cells was in 1956. BTV adapted for growth in eggs by serial passage in ECE was shown to replicate in primary lamb kidneys (18). This observation was reproduced several years later and a variety of continuous cell lines were shown to support the replication of BTV (10, 22, 29). The first successful isolation in tissue culture of wild-type non-egg adapted virus from the blood of infected sheep was in 1959 (10). Shortly thereafter Livingston and Moore (22) and Pini et al. (29) confirmed the feasibility of direct isolation in cultured cells.

With the development of an increasing number of continuous cell lines from vertebrates and invertebrates, many investigators sought to identify cultured cells with increased susceptibility to BTV. Methods to enhance virus adsorption by modifying either the sample, increasing the efficiency of the infection process or altering the method used to detect infected cells were investigated. Among the large number of mammalian cell lines that have been evaluated for their sensitivity to BTV and the maximal titres of virus generated, baby hamster kidney (BHK), African green monkey (Vero) and calf pulmonary artery endothelium (CPAE) are most frequently used or cited (28). Of two continuous invertebrate cell lines examined, the C6/36 cloned line derived from *Aedes albopictus* (20) manifests greater sensitivity than the cells derived from the primary insect vector of BTV in the United States, *Culicoides tarsi* (34). It is worth noting that whereas mammalian cells exhibit a cytopathic effect (CPE) after BTV infection, invertebrate cells routinely become persistently infected after peak production of virus in the absence of a CPE.

The search for the optimal cell line for routine isolation of BTV is made difficult by the large number of serotypes that need to be tested and compounded by additional isolates that defy unambiguous classification into one of the 24 currently defined serotypes. Thus few studies have examined more than the serotypes available in a specific country or geographic area and more often than not, ECE were not included in the comparison and the viruses examined were tissue culture- or ECE-adapted rather than wild-type virus isolates. The fact that tissue culture-adapted and non-adapted viruses have different biological properties is now recognised. Consequently, there is no standard procedure with international credentials for the isolation of all BTV serotypes in cell culture. However, a generalisation can be made. Primary isolation of BTV in tissue culture, even in ‘susceptible’ BHK or Vero cells, is significantly less efficient than in ECE (12). Although a number of techniques have been developed to identify BTV in ECE (6), primary isolation in ECE and subsequent passage in tissue culture results in the selection of a virus population amenable to investigation using traditional virus identification processes in cultured cells. These include immunofluorescence and immunoperoxidase assays using BTV-specific monoclonal antibodies (17). Neutralisation assays with serotype specific antisera are also conducted in cultured cells but it must be said that one of the major drawbacks of the current orbivirus diagnostic procedures is the time it takes – potentially a minimum of three weeks – to isolate a virus in ECE and provide a serotype diagnosis following virus neutralisation assays.

Virus isolation and identification or virus identification and isolation?

Traditionally, orbiviruses have been isolated in cultured cells prior to their identification and biochemical, antigenic and biological characterisation. However, in the past decade, traditional procedures for virus characterisation, such as enzyme-linked immunosorbent assay (ELISA) and serum neutralisation with serotype-specific antisera, have been supplemented by PCR and sequencing. In another paper in this section, Zientara et al. summarise current PCR technologies and their importance in differentiating wild-type and vaccine strains during the current European outbreaks (36). Hamblin discusses the application of the serological procedures currently used to detect BTV antigens and antibody (19). He also describes some of the practical problems that diagnostic laboratories face when using these tests in different epidemiological situations.

When the diagnostic technologies discussed at the Second International Symposium on bluetongue, African horse sickness and related orbiviruses in Paris in 1991 are compared with those in operation
around the world in 2003, not surprisingly there have been major advances, especially in the area of PCR-based diagnosis. I would like to look forward and consider the direction that improvements in orbivirus diagnostics might take in the next decade. In recent years, nucleic acid-based technologies have revolutionised medical infectious disease diagnosis and epidemiology and significantly decreased the time taken to confirm the presence of, or identify, pathogens in human clinical material. Molecular systems have, in many instances, removed the requirement to isolate pathogens as a mandatory step towards pathogen identification and for the confirmation of human clinical diagnoses. Detection and characterisation of veterinary pathogens will follow the same evolutionary path. Work is underway in a number of laboratories, particularly in the laboratory of Peter Mertens in Pirbright, to develop the infrastructure and databases required to underpin the use of DNA-based molecular systems for the detection and characterisation of orbiviruses.

**Multiplexing platforms**

In addition to PCR, the other generic technology platform that reduces the need to isolate virus prior to identification, is ‘multiplexing’, the ability to quantify multiple reactions occurring simultaneously in a single reaction vessel. There are a number of technologies that make multiplexing possible. Some, such as macro- and micro-arrays, can be used for the simultaneous screening of amplified DNA against multiple targets and will be an integral part of nucleic acid-based diagnostic platforms. For orbiviruses, such systems based on RNA 2 and perhaps RNA 5 will provide information on virus serotype and those based on other segments such as RNA 3, 6 and 7 will simultaneously provide geographic and topotypic data and identify the genetic origin of RNA segments in naturally occurring reassortants. Application of PCR-based methods in multiplexing formats also have the capacity to detect novel pathogens and variants of existing pathogens.

Array technologies are not the only multiplexing systems under investigation. Other approaches, for example particle-based flow cytometric assays like those developed by the Luminex Corporation, can be used for screening against 100, and potentially a greater number, of DNA and protein targets. Whilst multiplexed DNA array detection systems are becoming widespread in many areas of biological science where there is a need to look at ‘whole organism’ transcriptional responses, the concept of multiplexed protein-based assays is relatively new and offers the capacity to quantify the final products of the transcription and translation processes.

I would like to look at how the next generation of protein-based multiplexing assays may impact on the evolution of new diagnostic technologies for orbiviruses using Luminex® as the technology platform (www.luminex.com). As mentioned below, this technology can also be used as a DNA-based detection system, and in fact may be a more readily accessible platform for this purpose than macro- or micro-array techniques.

In Luminex®, as in other bead technologies, the reactions take place on the surface of microspheres. Each bead has a unique colour-code generated by the relative content of red and orange fluorescent dyes. Protein (antigen, antibody, receptor, etc) or nucleic acid molecules are conjugated to the surface of specific beads, via a number of different chemistries, with the bead colour-code being used to identify the reagent on the surface of the bead. Once coupled, microspheres coated with individual viral antigens for example can be pooled to create a bead set that can be used to determine if an individual animal has been exposed to one or more of the viral antigens in the set. Serum is added to the bead set and secondary antibody or protein-A labelled with a fluorescent reporter dye such as phycoerythrin, measures reactions occurring on the surface of individual beads. Beads are aligned in single file by a microfluidics system and individually interrogated by two lasers. One laser illuminates the colours inside the bead thereby identifying the reagent on the bead surface. The second laser illuminates reporter molecules and provides information on the extent of the reaction on the bead surface. The optical signals for each bead are captured and digital signal processing translates them into real-time, quantitative data.

Multiplex reactions save on labour and consumables and the multi-analyte format supports faster decision-making than multi-stage screens. Current data on test sensitivity indicate that the fluorescent read-out is more direct, stable and sensitive than the colorimetric readout of the ELISA. As the ELISA requires enzyme amplification, it is prone to variability. Furthermore, reducing the number of beads per test can enhance sensitivity by increasing the number of fluorescent signals per bead. A single instrument can be used to assay nucleic acids, antigen-antibody binding, enzymes, and receptor-ligands interactions. The rapid reaction kinetics and the homogeneous format reduce incubation times and the throughput of 20,000 microspheres per second shortens analysis time. The technology has found increasing application for the simultaneous measurement of antibodies to multiple pathogens (3, 26), cytokines (9, 31), immunoglobulins (8, 16) and antibodies to multiple variable regions of the same
protein (21). Suspension arrays have also been used for multiplexed detection of viral nucleic acids (30) and high throughput multiplexed single nucleotide polymorphism genotyping (2, 5).

In addition to the use of microsphere bead sets in the detection and discrimination of orbivirus nucleic acids, as described above for array technology, I can envisage a number of additional ways in which such a system might be used to enhance orbivirus diagnostics.

**Competitive ELISA**

Although the current competitive ELISA (c-ELISA) assays utilise monoclonal antibodies that target the same region of the BTV core protein VP7 (32), they do not detect antibodies to all BTV serotypes with equal efficiency (1, 24). This may be due to antigenic variation in some virus serotypes that lead to the removal or modification of the epitope defined by the monoclonal antibody used in the test (or of neighbouring epitopes). Antibodies generated in response to such serotypes may not react efficiently with the test epitope and thus may not prevent monoclonal antibody binding and may therefore not be detected in the test. The Luminex<sup>®</sup> platform enables the combination of a number of c-ELISA assays, thereby significantly decreasing the possibility of obtaining false negative results. This could be done by coupling the monoclonal antibodies in each c-ELISA to different beads and using a baculovirus-expressed biotin-labelled VP7 antigen to which the monoclonal antibodies bind with approximately equal efficiency. The capacity of antibodies in test serum to block binding of biotin-labelled VP7 to the beads can be measured using streptavidin-phycocerythrin. Because the beads used for each c-ELISA in the multiplex reaction can be differentiated, a single test run will simultaneously provide the results of different c-ELISA formats for the one serum.

**Identification of antibody specificity**

This can be a challenging and time-consuming task using currently available technology. In this application of the Luminex<sup>®</sup> platform, the bead set is composed of microspheres containing purified viruses and/or a range of expressed portions of each VP2 molecule representing the serotypes of interest. Addition of test serum to the bead set will result in the preferential binding of antibodies to epitopes from specific serotypes and closely related serotypes. Antibody binding may be detected using a fluorescent-labelled secondary antibody or labelled protein-A. The conformational and antigenic structure of VP2 is complex, as shown by the ability of monoclonal antibodies that neutralise one serotype to bind to, but not neutralise, other serotypes (35). However, it is the capacity to simultaneously determine the extent of antibody binding to a large number of different viruses, expressed proteins or peptides and the generation of patterns of reactivity that provide the possibility of identifying the virus against which the antibody response was mounted. In individual geographic areas, bead sets may be generated that contain a representative collection of all the viruses in the area.

**Virus detection**

Virus in ECE lysates, tissue culture medium and potentially blood may be trapped by polyclonal antiserum (or a mixture of sera) coupled to a single bead type and bound virus detected using biotinylated polyclonal anti-viral antibodies and streptavidin-phycocerythrin. This application does not make use of the multiplex capacity of Luminex<sup>®</sup> technology but the increased sensitivity and speed of the assay over current ELISA approaches may make this a viable alternative.

**New platforms notwithstanding**

Many reasons to isolate pathogenic and non-pathogenic BTVs remain. While rapid detection and identification of virus RNA in clinical or surveillance samples and characterisation of the virus to family, genus, serotype and topotype level on the basis of nucleic acid sequence are very powerful adjuncts to clinical and epidemiological studies, we do not yet know enough about the molecular basis of pathogenesis and virulence, or the molecular foundations of the complex antigenic basis of serotype definition to warrant relying on nucleic acid sequences solely for virus characterisation. There are still many situations where it will be necessary to use live virus in cell culture, animal or vaccine studies. In addition, the speed and multiplexing capacity of PCR-based technologies must not blind us to the fact that these techniques do not detect live virus per se. While nucleic acid and protein-based multiplexed procedures will clearly have a significant impact, for the foreseeable future, virus isolation will remain an important component of BTV diagnosis and research.

**References**


Bluetongue diagnosis by reverse transcriptase-polymerase chain reaction

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Summary

Bluetongue virus (BTV) is the prototype member of the genus Orbivirus, family Reoviridae. The BTV serogroup contains 24 serotypes. Diagnostic tests currently used for the detection of BTV involve the isolation and growth of virus isolates in eggs or mice, followed by passaging in tissue culture. The virus is subsequently characterised using serological tests to detect reaction with reference antisera, such as the agar gel immunodiffusion test or serum neutralisation test. These procedures are time-consuming and may fail to detect low levels of infectious virus or strains of BTV that do not replicate in eggs, mice or cell culture. The use of the enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to BTV in infected animals is faster but does not necessarily confirm recent infection. Similarly, ELISA may be used to detect virus directly in tissue samples but the sensitivity is relatively low. A number of procedures have been developed to detect the presence of BTV antigens or nucleic acids. The polymerase chain reaction (PCR) technique has proved to be a powerful tool for BTV diagnosis. PCR techniques may be used not only to detect the presence of viral nucleic acid but also to ‘serogroup’ orbiviruses and provide information on the serotype and possible geographic source (topotype or genotype) of BTV isolates within a few days of receipt of clinical samples, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least three to four weeks to generate information on serogroup and serotype and yield no data on the possible origin of the virus isolated. Moreover, PCR enables differentiation between field isolates and vaccine strains.

Keywords

Bluetongue – Diagnosis – Polymerase chain reaction – Reverse transcriptase – Virus.

Introduction

Bluetongue (BT) is a non-contagious, infectious viral disease of domestic and wild ruminants, although clinical signs are usually only seen in sheep and certain species of deer. The BT viruses (BTV) are arthropod-borne and constitute the type-species of the genus Orbivirus within the family Reoviridae. The genome is composed of ten double-stranded RNA segments (52), which encode at least ten viral proteins. Seven of the viral proteins are structural and form the double-shelled virus particle. At least three non-structural proteins (NS1, NS2 and NS3) have been identified (20, 34, 46, 47). The inner capsid of BTV is composed of five polypeptides: three minor proteins (VP1, VP4 and VP6) and two major proteins (VP3 and VP7) (21, 46, 53). The outer capsid is composed of two major viral proteins, VP2 and VP5, which determine the antigenic variability of the BTV (13, 14, 21, 53). To date, 24 antigenically distinct serotypes of the virus have been identified.

BTV is transmitted almost entirely by the bites of certain species of Culicoides biting midges, and as a result is restricted to areas in which these vectors are present (30). Transmission only occurs when the adult insects are active. Culicoides imicola is considered to be the most efficient vector in Europe, although other species of Culicoides, of lesser importance, have been identified in these areas. The global distribution of BTV lies approximately between latitudes 35°S and 40°N, although in parts of western North America and in the People’s Republic of China, it may extend up to almost 50°N (12, 45). Lundervold et al. (25) report the results of a serological survey for BTV in Kazakhstan. BT disease has never been reported in Kazakhstan but this study suggests that
the virus may be endemic in this region of the world as far as 50°N.

Routine diagnosis of BTV infection is based primarily on serological methods that detect virus-specific antibodies in serum (39, 41). A number of other procedures are also currently used to detect BTV from the blood or tissues of infected animals. These include direct inoculation of cultured mammalian or insect cells, or intravenous inoculation into 10-12 day-old embryonated chicken eggs (ECE), followed by one passage in insect cell cultures and up to three passages in mammalian cell cultures (9). In particular, the inoculation of ECE and passaging through cell culture is the generally accepted method for testing of animals for export and other regulatory purposes (9). This is, however, a laborious and time-consuming protocol that may take between three and four weeks to complete. Consequently, alternative methods of virus detection have been sought. These include antigen capture enzyme-linked immunosorbent assay (ELISA), dot immunobinding assay (DIA), immunoelectron microscopy and polymerase chain reaction (PCR) (1, 2, 19, 22, 24, 26, 27, 31, 32, 33, 37, 38, 49). The use of antigen capture ELISA for the detection of BTV in the blood of infected ruminants has either been unsuccessful (31), has detected antigen only in animals with high viraemias (50), or was not consistent enough to provide a reliable diagnosis of BTV infection (19).

To avoid these problems, PCR-based assays were developed and evaluated for the detection of BTV serotypes based on nucleotide sequences of different genome segments (3, 4, 6, 11, 28, 40, 49, 54, 56, 57). The PCR, first described in 1985, is a highly sensitive and specific technique used for the detection of nucleic acids (48). The inventor of this technology earned a Nobel Prize for his achievement (35, 36), which has revolutionised research and diagnosis. As far as BTV diagnosis is concerned, the application of PCR technology has led to very rapid amplification of BTV RNA in clinical samples and PCR-based procedures are now available to provide information on virus serogroup and serotype. In PubMed (ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed), more than 60 international publications deal with ‘bluetongue and PCR’ and more than 30 are related to the use of ‘PCR in BTV diagnosis’. An overview of the uses of PCR for the detection of BTV, for BTV typing and for the differentiation between wild and vaccine strains will be presented.

(Reverse transcriptase-) Polymerase chain reaction

The PCR is a method for in vitro amplification of DNA. It has substantially accelerated the pace of research in many fields of biology by reducing the time required to perform routine manipulations of DNA and by making new manipulations possible. In essence, PCR is a series of multiple rounds of primer extension reactions in which complementary strands of a defined region of a DNA molecule are simultaneously synthesised by a thermostable DNA polymerase. During repeated rounds of these reactions, the number of newly synthesised DNA strands increases exponentially so that after 20 to 30 reaction cycles, the initial DNA template will have been replicated several million-fold. This power to ‘faithfully’ amplify the DNA, together with the low cost and simplicity of the method, have made PCR an indispensable tool. With RNA viruses, like BTV, a complementary DNA (cDNA) copy of the RNA must first be made using reverse transcriptase (RT), then the PCR can be used for amplification. This technique, referred to as RT-PCR, is used to detect RNA viruses.

The remarkable ability of PCR to amplify specific DNA sequences has, along with its obvious benefits, some practical pitfalls that require careful attention. First among these is the ability of PCR to amplify DNA inadvertently introduced into the reaction. Precautions against contamination are especially important in clinical applications and must be considered in every laboratory using the technique. Some principles used in sterile culture of micro-organisms are applicable, but additional precautions (such as strict segregation of sample preparation, reaction assembly, thermocycler, analysis work areas and the use of positive displacement pipettes or aerosol preventive tips) may also be necessary. The use of ultraviolet light and chemical decontamination procedures as well as of enzymatic methods to prevent the amplification of ‘carry-over’ templates should also be employed in some situations.

Sequencing

By determining the nucleotide sequences of the PCR amplified products, it is now possible to rapidly confirm the specificity of the PCR reaction with ease. Since the nucleic acid sequences of known BTV genes appears to differ in distinct geographic areas, the comparison of nucleotide sequences can give useful data to complement BTV epidemiology (16, 27).
Group-specific reverse transcriptase-polymerase chain reaction

The high level of conservation of the nucleic acid sequences of some BTV genome segments among the different BTV serotypes has enabled the selection of primers that allow specific amplification of BTV genes. To date, oligonucleotide sequences have been derived from the genes encoding several viral proteins (VP7, NS1, VP3 and NS3) (6, 11, 18, 23, 27) (Table I).

Table I
Group-specific reverse transcriptase-polymerase chain reaction described in the literature

<table>
<thead>
<tr>
<th>Target</th>
<th>Nature of the PCR</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>VP3 gene</td>
<td>RT-PCR</td>
<td>18, 27, 28, 40, 44</td>
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<tr>
<td>VP5 gene</td>
<td>RT-PCR</td>
<td>8</td>
</tr>
<tr>
<td>VP6 gene</td>
<td>RT-PCR</td>
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<tr>
<td>VP7 gene</td>
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<td>NS1 gene</td>
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<td>NS1 gene</td>
<td>Multiplex RT-PCR</td>
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<td>NS3 gene</td>
<td>Duplex RT-PCR</td>
<td>9</td>
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</table>

The range in detection sensitivity for different RT-PCR assays is between 10 and 100 TCID<sub>50</sub>. Hence, the diagnostic sensitivity of group-specific RT-PCR is greater than virus isolation in ECE or assays of cytopathic effects on cultured cells. The conditions of stringency, the choice of primers and the different PCR parameters (e.g. denaturation, annealing and extension conditions, number of cycles performed, RT and Taq polymerase concentrations) have been studied by different laboratories to optimise the parameters that influence DNA amplification efficiency and specificity. As a result, pairs of primers are now available that do not cross-react with other orbivirus genomes (e.g. EHDV) (49, 55).

Determination of the nucleic acid sequence of specific regions of the genome segment 3 (VP3) gene may provide information on the geographic origin of the virus (15, 16, 39). Nested-PCRs have also been developed, for example, the nested-PCR based on NS1 gene (57) is very sensitive, detecting the equivalent of 1 plaque-forming unit of virus. Although the reverse-transcriptase multiplex PCR has been developed, it has not yet been validated (23).

The group-specific RT-PCR described here is rapid and specific for the detection of BTV and may be used with confidence as an alternative to the time-consuming, costly and cumbersome conventional procedures. However, virus isolation is still required for confirmation of active BTV infection.

Type-specific reverse transcriptase-polymerase chain reaction

The low level of conservation of the nucleic acid sequences of some BTV genome segments (e.g. segment 2) among the different BTV serotypes has enabled the selection of primers that allow specific amplification of these viral genes. Segment 2 has a single open reading frame encoding the VP2 protein, which comprises 962 amino acid residues. Oligonucleotide primers have been designed for the amplification and sequence analysis of genome segment 2 (VP2 gene) of BTV serotypes 1, 2, 4, 9 and 16 (S. Zientara, personal findings). These pairs of primers do not amplify segment 2 of the other 23 serotypes. These can be used in a multiplex RT-PCR reaction to generate different size products from each of the five serotypes that are currently present in Europe and thereby identify their serotype.

Specific primers, based on the published nucleotide sequence of the genome S2 of BTV-2 (GenBank accession number M21946 (58, 60) were designed. A BTV-9 specific RT-PCR is also available using oligonucleotide primers designed and published by McColl and Gould (27). These BTV-9 serotype specific primers amplified a 805 bp fragment of the BTV-9 vaccine strain.

Differentiation of wild and vaccine BTV strains

BTV-2 was first suspected in Tunisia in December 1999. Virus was isolated and confirmed as BTV-2 early in 2000. The virus spread westwards to Algeria and northwards into southern Italy. In October 2000, BTV-2 was confirmed on the Spanish islands of Majorca and Minorca and on the French island of Corsica (59). Following the isolation and confirmation of BTV-2 in Corsica 2000, the French veterinary authorities decided to systematically vaccinate all sheep on the island during the winter of 2000-2001 (January to April 2001) using the South African attenuated BTV-2 vaccine virus (Batch 7, Onderstepoort Biological Products, South Africa) (59). Approximately 80% of sheep (non-pregnant or over the age of three months) were vaccinated on the island (60).

Coincidentally, outbreaks of a second BTV serotype, BTV-9, were being reported and confirmed on mainland Italy at this time and as a result a bivalent
attenuated vaccine against BTV-2 and BTV-9 was used in some regions of Italy.

Alignment of the S2, S7 and S10 of the BTV-2 vaccine and Corsican virus isolates showed that the nucleotide sequence of the S10 gene presented the highest degree of divergence between these strains. Primers that incorporated nucleotide sequence differences in genome S10 of the vaccine and wild-type strains of BTV-2 were designed and used to help differentiate between vaccination and infection with field strains of BTV (10). The S10 gene has two in-phase and overlapping open reading frames, which encode the NS3 and NS3A non-structural proteins, containing 229 and 216 amino acids, respectively. The nucleotide sequence of the S10 gene from the BTV-2 vaccine strain showed 82.1% homology with that of the S10 from the wild-type strains isolated in 2000 and 2001. The predicted amino acid sequence of the NS3 protein of the BTV-2 vaccine strain showed 94.3% homology with the wild-type BTV-2 NS3 protein.

Primers showing a high level of nucleotide substitution within the S10 gene of the wild-type and vaccine viruses were selected. The specific vaccine or wild strain primers were tested and validated on RNAs extracted from red blood cells (RBC), spleen, lymph nodes, samples from experimentally vaccinated lambs, blood of vaccinated Corsican sheep, from infected Corsican sheep in 2001, or from field isolates of BTV-2 (17). The results shown in Figure 1 demonstrate that the group-specific primers for S10 amplify viral nucleic acid from infected and vaccinated sheep whereas primers specific for the S10 gene of the vaccine virus only amplified virus from vaccinated sheep. Similar observations have been reported in the Balearic islands by Agüero et al. (5). These authors developed a PCR method (based on the use of an RT-PCR, followed by restriction endonuclease analysis) to differentiate between the NS1 genes of BTV-2 and that of the Onderstepoort commercially available live-attenuated BTV-2 vaccine virus. Moreover, primers designed from the nucleotide sequence of the BTV-9 segment 2 amplified genome segment 2 of the wild BTV-9 strain (that was present in Italy and Greece) but did not detect the vaccine virus (S. Zientara, personal findings). The primers designed by McColl and Gould (27) only amplified the BTV-9 vaccine strain (Fig. 1). However, it is still necessary to validate the designed primers for BTV-2 and BTV-9 and to select primers for the other 22 serotypes.

**Conclusion**

Traditional approaches that rely on virus isolation followed by virus identification may require at least three to four weeks to generate information on serogroup and serotype and yield no data on the possible geographic origin of the virus isolated. On the other hand, the BTV RT-PCR can provide rapid, sensitive and specific viral identification for BTV infections. However the interpretation of positive BTV PCR results must be analysed carefully, particularly in areas that are BTV-free. Before officially reporting BTV cases in the absence of epidemiological data, virus isolation is strongly recommended to confirm molecular diagnosis.

The nucleotide sequence divergence between field and vaccine strains has led to the design of differential diagnostic primers. These tools can also provide information on the disappearance of a wild virus in a vaccinated population. Implementing such assays is important and will help in our understanding of the epidemiology and spread of BTV in vaccination areas. The information obtained will also prove invaluable in control and eradication programmes.

**References**


Diagnostics


Bluetongue virus antigen and antibody detection, and the application of laboratory diagnostic techniques

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Summary

Despite the significant advances that have been made in molecular techniques, the traditional approach using biology-based test procedures is still the mainstay for the laboratory confirmation of clinical diagnoses. The serological and virological techniques available for the detection and identification of bluetongue virus and antibody fall into two categories; those that are serotype-specific and those that are serogroup-specific. Although several assay methodologies have been described and used, thought should always be given to their use in different epidemiological situations and to the interpretation of results obtained therein.

Keywords


Introduction

Identification of bluetongue virus (BTV) and antibody is an essential part of the laboratory confirmation of BTV infection. This may be achieved in three different ways, as follows:

a) identification of antibody by serological assay
b) identification of the virus antigen by virological assay
c) identification of the specificity of nucleic acids by reverse transcriptase-polymerase chain reaction (RT-PCR) and sequence analysis (44), which are discussed elsewhere. Both serological and virological assays rely on the fact that BTV-specific antibody ‘complexes’ or ‘binds’ with BTV.

Pearson gave a clear and concise overview of some the most frequently used antibody detection tests at the Second International Symposium on bluetongue (35). Details of some of the traditional virological assays currently used are outlined in the OIE Manual of standards for diagnostic tests and vaccines (33). This paper discusses the application of some of these frequently used techniques, in different epidemiological situations.

Serological techniques

BTV has a 10-segmented genome and each segment encodes for a different viral protein, seven of which are structural and three non-structural. The outer capsid, structural viral proteins VP2 and VP5 are the serotype determinants and are responsible for generation of serotype-specific neutralising antibody. Serogroup specificity on the other hand is generally considered to be a measurement of the immunodominant VP7, a major, structural core viral protein, although antibody against other structural and non-structural viral proteins undoubtedly contribute (38).

It is essential that diagnostic laboratories use the most appropriate test methods available to achieve the desired result and therefore they must have a clear understanding of the test uses and limitations. Testing sera for the presence of BTV antibody may be required for the following purposes:

a) to facilitate safe international trade in live animals, animal products and germplasm
b) for serological surveillance
c) for monitoring vaccination campaigns
d) for serotype identification of field strains.
The OIE Manual (33) outlines two prescribed tests for international trade, namely, the agar gel immunodiffusion (AGID) (34) and competitive enzyme-linked immunosorbent assay (c-ELISA) (24). Both of these tests are serogroup-specific. Other methodologies employed to measure serogroup-specific antibody include the complement fixation test (CFT) (6), the indirect or blocking ELISA (2, 25), fluorescent antibody (37) and dot immunoblotting (13). Although use of the haemagglutination inhibition (HI) test (42) has been reported for serotype identification, serum neutralisation (18, 28) is probably the most frequently used type-specific assay.

**Agar gel immunodiffusion (AGID): group-specific test**

The AGID test (34) is well documented as a serogroup-specific test for the detection of BTV antibody. The 2000 edition of the OIE Manual includes the AGID test as a prescribed test for international trade but goes on to say that the c-ELISA is the preferred test. Although the AGID test may still be used in some laboratories, the lack of sensitivity (14, 35) and documented cross-reactions that can occur with other Orbivirus serogroups, particularly epizootic haemorrhagic disease virus (35), makes the continued use of this assay questionable when more rapid, sensitive and specific tests are readily available.

**Enzyme-linked immunosorbent assay (ELISA): group-specific test**

The ELISA has been used for approximately 40 years (43) and has provided a valuable means of studying numerous antigens and their antibodies. Several different formats have since been applied to the detection of BTV antibody that include the indirect (25), the antibody blocking (2, 29), the competitive (1, 14) and IgM capture ELISA (45). Of significance is the fact that these ELISAs are all serogroup-specific, identifying primarily the highly conserved BTV VP7 of all 24 known serotypes. The OIE Manual (33) describes the competitive c-ELISA as a prescribed test for international trade and as a result the c-ELISA is probably the most widely used and validated method (24). Provided the test has been fully validated and fulfills the requirements in terms of sensitivity and specificity, some modification to the procedure and/or reagents used e.g. cell extracted, yeast (24) and baculovirus (20) expressed antigens, seem to have little effect. More important is when, where and how the assays are applied. For example, the slope of the antibody curve can vary considerably depending when (after infection or vaccination) the sera were collected.

Using the c-ELISA as a spot test will only provide a qualitative measurement of positivity.

**International trade**

Diagnostic laboratories are frequently required to assay sera from apparently normal animals, for example, the import/export of llama, alpaca and deer, and sheep used to raise therapeutic antibodies such as anti-rattlesnake venom. Usually these types of samples are expected to be negative and therefore testing at a single dilution using a prescribed c-ELISA is recommended. Regardless of the modifications to the test protocol used in different laboratories, this rapid assay has proven to be sensitive, specific and reliable.

If a positive reaction is recorded in the spot test then a second blood sample may be requested and both sera can be titrated by c-ELISA. A significant rise in antibody titre recorded in sequential samples would indicate a recent infection and therefore present a potential risk to the importing country.

**Incursion of virus in the absence of vaccination**

As the ELISA is rapid and reliable, it is ideally suited for confirmation of exposure to a single BTV serotype and thereafter for serological surveillance to help determine the transmission and spread of BTV, particularly in the absence of disease. If the virus incursion involves more than one serotype and infection is prevalent, testing young and/or sentinel animals rapidly by ELISA may provide information on the presence and distribution of BTV and allow virus isolation to be attempted at an early stage of the infection.

**Epizootic/enzootic and vaccinated countries/regions**

The c-ELISA cannot differentiate between infection and vaccination with a live-attenuated vaccine, but being more rapid and economic, it is the preferred method of testing animals (non-vaccinated cattle and sheep) to help determine the distribution of BTV in a country/area, and for monitoring the efficiency of a vaccination campaign in non-infected sheep. If, as expected, inactivated and/or recombinant BTV vaccines become commercially available then new and improved ELISA methodologies that will differentiate between infection and vaccination can be devised, validated and introduced into routine use. Previously developed assays for the detection of BTV NS1 (3) and African horse sickness virus (AHSV) NS3 (27) have shown this to be feasible.

**Serum neutralisation tests: type-specific test**

Serologists often refer to the method used for identifying antibody to type as a serum neutralisation...
test (SNT). This is to help differentiate this method from the virus neutralisation test (VNT) which, as the name implies, is used to identify a virus to type. The SNT is serotype-specific and can be used to differentiate between the antibody produced against each of the 24 antigenically distinct serotypes of BTV. The methodologies used can vary considerably but the principles remain the same; that is, test sera are reacted separately with a constant amount of each BTV serotype. The amount of neutralisation of virus, relative to a homologous virus control in the absence of any serum, is measured biologically using mammalian cells as an indicator of virus infectivity.

The SNT is considered to be highly sensitive and specific in that it does not cross-react with other Orbivirus serogroups. The assay is not usually used for routine testing because it is time-consuming, expensive of reagents and the quality of the test sera may affect the cells. Other techniques such as the ELISA are often more rapid and reliable.

Incursion of virus in the absence of vaccination

Following infection by a single BTV serotype, animals develop homologous, neutralising antibody to the infecting agent and in some cases lower levels of heterologous antibody (23). In the absence of disease and/or isolation of virus, the SNT can be used to identify the dominant antibody serotype and thereby help in the selection of the most appropriate vaccine at the commencement of an outbreak. As these assays are relatively expensive in terms of time and reagents, the advantages and disadvantages should be considered before they are used for routine surveillance.

When an incursion of BTV involves more than one serotype, the interpretation of results can be more difficult; however, if the outbreaks are sporadic then it is possible to identify and map the location and spread of different virus serotypes (Table I).

Incursion of virus and after vaccination

The currently available BTV vaccines are live and contain attenuated strains of the virus and the antibodies they induce are indistinguishable by SNT from those produced after natural infection. However, provided only sheep are vaccinated, as is the case in some countries, it is possible to derive important epidemiological information about the circulating virus serotypes by testing cattle. The SNT also has value for monitoring the efficiency of sheep vaccination campaigns in buffer zones.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Species</th>
<th>BTV serotype</th>
<th>4</th>
<th>9</th>
<th>16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mainland</td>
<td>Drama</td>
<td>Cattle</td>
<td>–</td>
<td>120*</td>
<td>–</td>
</tr>
<tr>
<td>Rodopi</td>
<td>Cattle</td>
<td>–</td>
<td>160</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Islands</td>
<td>Rhodes</td>
<td>Sheep</td>
<td>–</td>
<td>20</td>
<td>–</td>
</tr>
<tr>
<td>Rhodes</td>
<td>Cattle</td>
<td>–</td>
<td>–</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>Rhodes</td>
<td>Goat</td>
<td>–</td>
<td>–</td>
<td>640</td>
<td></td>
</tr>
<tr>
<td>Kos</td>
<td>Sheep</td>
<td>–</td>
<td>–</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Kos</td>
<td>Sheep</td>
<td>–</td>
<td>–</td>
<td>480</td>
<td></td>
</tr>
<tr>
<td>Larisa</td>
<td>Sheep</td>
<td>–</td>
<td>30</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Evia</td>
<td>Sheep</td>
<td>–</td>
<td>30</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Lesvos</td>
<td>Sheep</td>
<td>–</td>
<td>&lt;60</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Samos</td>
<td>Goat</td>
<td>–</td>
<td>–</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Samos</td>
<td>Goat</td>
<td>–</td>
<td>–</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Samos</td>
<td>Cattle</td>
<td>–</td>
<td>80</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Samos</td>
<td>Cattle</td>
<td>–</td>
<td>240</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

* Reciprocal, arithmetic antibody titre
Sera supplied by Olga Mangana and Kiki Nomikou, Ministry of Agriculture, Athens

Multiply infected epizootic/enzootic/vaccinated regions

In 1986, Jeggo and others demonstrated experimentally that sheep serially inoculated with two or more different BTV serotypes over a period of time developed a broad, heterotypic antibody response against multiple BTV serotypes (22, 23). If this is indeed the case with only two BTV serotypes, then the situation will be considerably more complex in BTV epizootic and enzootic regions where multiple serotypes are circulating (41). Testing sera from older animals with an unknown history from these areas is of limited value but testing non-vaccinated susceptible animals between the age of six months, after waning of maternal antibody, and one year of age by SNT can provide valuable information about the BTV serotypes currently circulating (12). This was achieved using a technique often referred to as ‘clustering’. By testing several younger animals against each of the known BTV serotypes it became apparent that certain serotypes predominated in antibody titre and occurrence. High frequencies (clusters) of antibody against specific BTV serotypes are believed to correspond to the BTV serotypes that are currently circulating in an area. From the results, these authors (12) suggested that at least three different BTV serotypes, namely: BTV-6, BTV-14
and BTV-17 were likely to have been circulating in some of the Caribbean and South American countries between 1981 and 1982.

**Virological techniques**

Isolation of virus and identification of serotype are the preferred and most certain methods of determining a BTV infection but the entire process of virus isolation, adaptation, amplification and finally identification can be expensive, particularly in terms of time. Application of a fully validated, serotype-specific PCR, together with relevant sequence data (44) would be considerably faster and will be a welcome and valuable addition to the bank of assays currently available.

As mentioned earlier, the serotype determinants of BTV, VP2 and VP5, are located on the outer capsid. Although the 24 BTV serotypes are antigenically distinct, they all belong to the same *Orbivirus* serogroup and are therefore related. The degree of relatedness between 23 of the 24 BTV serotypes as determined by plaque reduction was originally reported by Erasmus (11). Figure 1 includes the results obtained at the Institute for Animal Health (IAH) in Pirbright by SNT for the 24th BTV serotype. Remarkably the phylogenetic sequence analysis of genome segment 2 of the 24 BTVs shows a very similar relatedness between the serotypes (30).

In the face of an outbreak of disease, it is of paramount importance to provide a rapid identification of BTV type so that appropriate control measures can be implemented without delay. Thereafter confirmatory serotyping of isolates should be made at intervals throughout the outbreak to ensure that incursions by new serotypes would be detected. Whole blood, animal tissue and, occasionally, field isolates are the most common samples submitted to the diagnostic laboratory for confirmation of a clinical diagnosis. Cultured isolates may be received although these are often for retrospective BTV serotyping.

Several virus/antibody-based methodologies for the identification of BTV have been described, which include fluorescence (5, 26, 36), immunoperoxidase (7), ELISA (10, 15), electron microscopy (21, 32), and plaque (9, 19), and virus neutralisation (33) techniques. As with the serological assays, they fall into two categories, being either serotype- or serogroup-specific. Not surprisingly, the serological and virological tests within each category complement one another and therefore the principles of the different assays are very similar. This paper discusses some of the applications of the two assays which are used at the IAH in Pirbright viz. the serotype-specific VNT and the serogroup-specific indirect ELISA (i-ELISA), also known as the antigen-capture or sandwich ELISA.

**Enzyme-linked immunosorbent assay: serogroup-specific test**

Mortality following infection with BTV can be as high as 70% in individual sheep flocks but it is usually much less than this. Studies have shown the i-ELISA to be capable of detecting approximately $3.0 \log_{10}\text{TCID}_{50}/\text{ml}$ of infectious virus (15, 40). Based on the work of Crafford (8), this equates to approximately 9 ng/ml of purified BTV antigen. Although infectious BTV can be isolated from the blood of some sheep up to at least 47 days after infection, and virus titres above $7.0 \log_{10}\text{TCID}_{50}/\text{ml}$ have been recorded at the peak of viraemia (16), the efficiency of ELISA for detecting BTV in blood remains questionable (17, 31, 39). The ELISA cannot therefore be relied upon as a diagnostic tool for assaying blood directly.

Most animals that die do so from sequelae after a chronic infection. Data recorded in studies performed on tissues collected from sheep killed sequentially after infection show that although infectious virus may persist in certain tissues for at least 12 days, the amount of antigen necessary to ensure a positive ELISA reaction does not (Table II). This may be due to clearance of antigen from the...
animal but is more likely to be a direct result of interference from developing antibodies, particularly against the highly conserved VP7.

A similar observation was noted during the AHSV serotype 7 outbreak that occurred in the surveillance zone of the Western Cape Province of South Africa in 1999 (4). The i-ELISA proved very efficient at identifying AHSV in tissues during the early stages of the outbreak (A.J. Guthrie and P.G. Howell, personal communication). The decision to initiate vaccination was made prior to the confirmation of the infecting serotype. Polyvalent vaccine against seven of the nine AHSV serotypes is available in two vials, each vial being inoculated three weeks apart. Unfortunately the first vaccines inoculated did not contain the correct AHSV serotype. Shortly after administering this vaccine, the efficiency of the ELISA started to decrease. It is believed that this decrease was primarily due to the heterologous VP7 binding to (but not the neutralisation of) the infecting virus. Thus samples from susceptible animals that were infected and became sick and died after administration of the initial vaccine were recorded negative by i-ELISA.

### Virus neutralisation tests: serotype-specific test

Like the SNT, the VNT is a serotype-specific test, which can be used to identify the 24 antigenically distinct serotypes of BTV. Knowledge of the spatial and temporal distribution of the different BTV serotypes can be an advantage to a diagnostician and with experience can obviate the need to use all 24 serotypes (33). The sensitivity of the assay used at Pirbright is dependent on the titre of virus in the test sample being sufficiently high to give a 2 log10 or greater reduction against the homologous serum when compared to the virus control in the absence of any BTV antisera.

Two problems may be encountered when attempting to serotype a BTV. First, the apparent relatedness and/or cross-reactivity that may be observed with some BTV isolates could make it difficult to confirm the actual virus designation. This may relate to the specificity of the antisera and/or the assay being used. Ideally, the diagnostician should know the reactivity of each of the 24 virus serotypes against each of the 24 currently used typing antisera in his/her laboratory because individual animals used to raise specific antibody may exhibit a slightly different antibody profile in different assays (9). Obviously this is a laborious task and such information is probably not available in most laboratories. Fortunately, many of the BTV serotypes can be excluded after the initial screening of a virus because they will not show any cross-reactivity. The information obtained each time a new BTV is serotyped can form the basis for a comprehensive database of the BTV cross-reactivities. Sometimes it may be sufficient to either test another virus isolate from the same outbreak or to use typing antisera produced in different animals.

### Table II

Direct detection of bluetongue virus and viral antigen in sheep samples by ELISA and following egg inoculation

<table>
<thead>
<tr>
<th>Sample</th>
<th>Euthanised ELISA</th>
<th>Bluetongue virus ELD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>7 dpi</th>
<th>12 dpi</th>
<th>ELD&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>NT</td>
<td>6.5</td>
<td>NT</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Buccal salivary gland&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>+</td>
<td>2.7</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Parotid salivary gland</td>
<td>+</td>
<td>2.6</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Mandibular salivary gland</td>
<td>+</td>
<td>2.3</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Parotid lymph node</td>
<td>+</td>
<td>3.6</td>
<td>–</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Retro pharyngeal lymph node</td>
<td>+</td>
<td>3.2</td>
<td>–</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>Axillary lymph node</td>
<td>+</td>
<td>3.7</td>
<td>–</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>Mediastinal lymph node</td>
<td>+</td>
<td>3.2</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Mesenteric lymph node</td>
<td>+</td>
<td>3.2</td>
<td>–</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>4.4</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
<td>NVD</td>
<td>–</td>
<td>NVD</td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>5.0</td>
<td>+</td>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>a) blood: log&lt;sub&gt;10&lt;/sub&gt; titre/ml</th>
<th>ELD&lt;sub&gt;50&lt;/sub&gt;</th>
<th>b) tissues: log&lt;sub&gt;10&lt;/sub&gt; titre/g</th>
<th>dpi</th>
<th>days post infection</th>
<th>NT not tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>egg lethal dose at 50% end point</td>
<td>dpi</td>
<td>days post infection</td>
<td>NVD no virus detected</td>
<td></td>
</tr>
</tbody>
</table>
Secondly, some isolates/serotypes that are readily isolated in eggs do not easily adapt to cell culture, therefore making it impossible to perform a ‘normal’ VNT. However, by using eggs as the indicator of virus neutralisation, rather than mammalian cells, it is possible to obtain a clean and reliable typing without trying to adapt the virus.

Finally, although the methods described by the OIE for the micro-VNT (33) suggest that test plates should be incubated at 37°C for between 3 and 7 days, it is possible to make a clear typing after as little as 24 h, providing the cells have grown adequately and the virus has replicated to a sufficiently high titre.

**Conclusion**

Regardless of the development and application of new and improved molecular-based diagnostic techniques there will, at least for the foreseeable future, be a requirement to demonstrate the presence of actively growing virus before full control measures (vaccination and slaughter) are introduced. Modifications and adaptations to the existing techniques continue to be made and reported in an attempt to develop super-sensitive tests that retain a high degree of specificity. In general, the traditional tests have withstood this barrage and have proven to satisfy the requirements for international trade, laboratory diagnosis and epidemiological investigation.

**References**


Diagnoses


Differential diagnosis of bluetongue virus using a reverse transcriptase-polymerase chain reaction for genome segment 7

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Summary
Bluetongue virus (BTV) has persisted within Europe for the past five years, highlighting a need for rapid and reliable virus detection and identification methods. Various RT-PCR protocols and strategies, which target genome segment 7, were evaluated for their ability to detect all members of the BTV species (serogroup), with the aim of developing a fully validated reverse transcriptase-polymerase chain reaction- (RT-PCR) based diagnostic assay. A nested PCR strategy, using near terminal and internal segment 7 primers, detected all 24 BTV serotypes, but also cross-reacted with some other related Orbivirus species. In an attempt to circumvent these problems, conventional PCR and touch-down PCR methods, using similar primers were also investigated. Both methods were able to amplify cDNA from only 21 of the 24 BTV types. Further sequence analyses of the VP7 gene from the remaining isolates (types 7, 15 and 19) will permit the design of additional and more effective virus-species specific primers and RT-PCR-based assays. This may include the introduction of a multiplex PCR system.

Keywords
Bluetongue – Diagnosis – Europe – Orbivirus – Polymerase chain reaction – Virus.

Introduction
Bluetongue (BT) is an infectious, non-contagious arthropod-borne disease of ruminants. It is caused by BT virus (BTV), the prototype species of the genus Orbivirus within the family Reoviridae (21). Twenty-four BTV serotypes have been identified to date; they are transmitted between their vertebrate hosts via the bites of certain ‘vector-competent’ species of biting midges (Culicoides species) (15). Orbiviruses are responsible for several economically important diseases of cattle, sheep, horses and some wild ruminants and can infect many animal species (21, 22, 23). It has been estimated that BTV alone causes losses to international livestock trade in excess of US$3 billion a year (26) and is classified as a ‘List A’ pathogen by the Office International des Épizooties (OIE).

BTV is a small (80 nm in diameter) icosahedral virus with a ten-segmented, double-stranded RNA genome (19). Each of the ten segments codes for at least one of ten distinct viral proteins, seven of which are structural components of the virus particle, and three of which are non-structural (10). The virus particle is arranged as three concentric capsid shells surrounding the viral dsRNA (4, 9, 10). The outermost layer (the outer capsid) is composed of two structural proteins, VP2 and VP5, which are principally involved in virus attachment and penetration of the host cell during the initiation of infection. These are the most variable of the viral proteins and the specificity of their interactions with neutralising antibodies (particularly those of VP2) determines virus serotype (8, 10, 24). The two innermost protein shells that make up the transcriptionally active virus core, are composed of VP3(T2) and VP7(T13), respectively. These are more highly conserved proteins, showing serological cross-reactions within the BTV virus species. VP3 and VP7 surround a central compartment containing the three highly-conserved minor protein components of the transcriptase complex (polymerase, capping enzyme and helicase: VP1(Pol), VP4(CaP) and VP6(Hel), respectively), as well as the ten dsRNA segments of the virus genome (9, 19, 20, 28). Two of the non-structural proteins, NS1 and NS2, are also highly conserved, while the smallest viral protein NS3 is more variable.
Historically, BTV has occurred almost worldwide between the latitudes of 35°S and 40°N, although within southern Europe it has only caused periodic and short-lived epizootics involving a single BTV serotype (16, 17, 18). However, since 1998, this range has extended gradually further north (as far as 44°N) into areas of Mediterranean Europe and the Balkans. These outbreaks have also involved five distinct BTV serotypes: 1, 2, 4, 9 and 16, providing evidence of a significant and possibly long-term change in the distribution of both the virus and disease. These changes may result from a combination of several factors, including the ability of the virus to persist from one vector season to the next (overwintering) (27), ‘climate change’ (which appears to have altered the distribution of the main vector species in the region: Culicoides imicola) (30, 31, 32), and a possible involvement of novel vector species that have a more northerly distribution (C. pulicaris and C. obsoletus) (32).

The persistence of BTV within Europe has highlighted a need for quick, sensitive and reliable methods of virus identification and diagnosis. Current BTV detection methods, which include the enzyme-linked immunosorbent assay (ELISA) and serum neutralisation assays, can be time-consuming, taking several weeks to confirm initial clinical diagnoses (1, 25, 29). Clinical samples received for diagnostic assays (e.g. blood or other tissues) are often from animals that have died after the levels of BTV antigen have dropped below a detectable threshold. Such samples are often unsuitable for ELISA, and the virus needs to be amplified and isolated by passage through embryonated chicken eggs and cell culture, providing material that can be tested for the presence of BTV. The highly specific and sensitive nature of reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays makes them ideal for the detection of BTV-specific RNAs directly from tissue samples, potentially removing the current requirements for virus isolation. Indeed, it has already been shown that assays based on an RT-PCR for BTV segment 7 can detect as few as 6 molecules of BTV dsRNA (29).

Protocols that have already been published for detection of BTV RNA vary considerably in both the genome segments that they target, and the serotypes that they can detect (1, 2, 3, 5, 7, 11, 13, 14, 25, 29, 33). However, few if any of these assays have been validated using all 24 BTV types, or representative topotypes from different geographic origins around the world. Consequently, a fully validated and therefore reliable RT-PCR assay is still needed for routine detection and identification of BTV. The purpose of this study was to evaluate current BTV genome segment 7-based RT-PCR primers/protocols and, if possible, develop and validate a virus species-specific assay method.

Genome segment 7 was chosen because it encodes VP7, the main group-specific antigen of the virus and is therefore thought likely to vary in a manner that reflects the group-specific serological properties of the virus. The conserved nature of this genome segment should therefore allow the detection of all BTV topotypes and serotypes.

Methods

Preparation of viral dsRNA

Virus isolates for all 24 serotypes of BTV (including reference strains and different geographic topotypes) (Table I) were grown in BHK-21 cell monolayers and harvested when 100% cytopathic effect (CPE) was observed. (Full details of the viruses available in the collection of the Insitute for Animal Health (IAH) in Pirbright can be seen at the following web address: iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm.) RNA was then extracted from the cell-free supernatant fluid, using the QIAamp Viral RNA Mini Kit from QIAGEN, according to the protocol of the manufacturer.

Oligonucleotide primers

Three sets of primers were evaluated, as shown in Figures 1 and 2. TF/R-1 and IF/R-1 (29) were tested in a nested RT-PCR, which used the terminal primers (TF/R-1) in an initial round of amplification to generate full-length cDNA. This was followed by a second PCR using internal primers (IF/R-1) to raise a smaller 769 base pair (bp) product. TF/R-2 (7) were evaluated separately for their ability to amplify full length S7 cDNA in a conventional one-step RT-PCR.

Sample preparation for reverse transcriptase-polymerase chain reaction

The test sample of potential BTV RNA was denatured in 5 µl of 0.1 mM methyl mercury hydroxide (MMOH), as described by Wade-Evans et al. (29). After 10 min incubation at room temperature, the reaction was neutralised by adding 1 µl of 0.7M 2-mercaptoethanol.

Nested polymerase chain reaction

Full length cDNA was synthesised with primers TF and R-1, using RT-PCR beads (Amersham Pharmacia) according to the protocol of the manufacturer. The RNA was initially reverse-transcribed at 42°C for 1 h, and then heated to 95°C for 5 min. Thirty PCR cycles were performed, at 94°C for 1 min, 45°C for 1 min and 72°C for 2 min,
Table I
Viruses tested using reverse transcriptase-polymerase chain reaction
Full details of the virus isolates can be seen at the Institute for Animal Health (IAH) website: iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm

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Figure 1
S7 specific primers

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Figure 2
S7 primer details

Conventional one-step reverse transcriptase-polymerase chain reaction and touch-down polymerase chain reaction strategies

Both strategies were tested using the Qiagen one-step RT PCR kit and primers TF/R-2. A reaction master mix was prepared that contained 29 µl of RNase free water, 10 µl of 5 X Qiagen one-step RT-PCR buffer, 2 µl of dNTP mix, 0.6 µM of both the forward and reverse primers and 2 µl of the enzyme mix (containing RT and PCR reaction enzymes). Then 5 µl of denatured RNA was added to the master mix.

For the conventional one-step RT-PCR, the RNA was initially reverse-transcribed at 45°C for 30 min. This was followed by an activation step at 94°C for 15 min, to simultaneously activate the DNA polymerases and inactivate the reverse transcriptases.
Forty amplification cycles were then performed at 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. A terminal extension step at 72°C for 10 min completed the thermal protocol.

For the touch-down RT-PCR protocol, RNA was initially reverse-transcribed and activated in the same way as described for conventional one-step RT-PCR. This was followed by 10 cycles of 94°C for 1 min, 60°C for 1 min (which was decreased by 2°C per cycle, so that it touched-down at 40°C after 10 cycles) and 72°C for 2 min. A further 30 cycles of 94°C for 1 min, 40°C for 1 min and 72°C for 2 min then followed.

Analysis of polymerase chain reaction products

All PCR products were analysed by 0.9% agarose gel electrophoresis (AGE), and visualised using ethidium bromide staining and UV light.

Results

Nested reverse transcriptase-polymerase chain reaction

Using the nested RT-PCR system, cDNA was amplified from segment 7 of all 24 BTV types, but was not consistent for BTV-7 (less efficient approximately 60% of the time, using different samples of the same strain). The assay was able to detect all of the topotypic variants of BTV that were tested from the reference collection at the IAH in Pirbright. However, a significant level of cross-reaction was also seen when the method was tested against related orbiviruses, epizootic haemorrhagic disease virus (EHDV), African horse sickness virus (AHSV), and equine encephalosis virus (EEV).

Conventional one-step reverse transcriptase-polymerase chain reaction and touch-down reverse transcriptase-polymerase chain reaction

In an attempt to alleviate problems of cross reaction, RT-PCR strategies were evaluated using higher stringency conditions. A touch-down PCR (which exhibits high levels of specificity), was compared in terms of its sensitivity and specificity with a conventional one-step PCR (as developed by Bréard et al. (7)).

The results were broadly similar to both the conventional one-step and touch-down RT-PCR protocols detecting 21 of the 24 serotypes (including all of the available topotypes) (Fig. 3). Neither protocol was capable of detecting BTV serotypes 7, 15 or 19. When tested against all of the serotypes of EHDV, AHSV and EEV, neither method showed any cross-reaction.

Discussion

Various RT-PCR assays were evaluated in this study. The nested PCR is sensitive enough to detect all of the serotypes and topotypes of BTV tested, but also detects cross-reactions with other orbiviruses and is therefore unsuitable as a diagnostic assay for BTV. Both the conventional one-step PCR, and the touch-down PCR strategies detected 21 of the 24 serotypes of BTV, and neither showed any cross-reaction with related orbiviruses.

Using the conventional one-step RT-PCR and touch-down PCR strategies, neither BTV-7 or BTV-19 could be amplified. Analysis of the VP2 sequences of all 24 serotypes of BTV, have shown types 7 and 19 to form a distinct genetic cluster (12). The failure to detect these BTV serotypes using an RT-PCR designed to target a conserved gene encoding a group-specific antigen (VP7) suggests that they may also display some variations in their VP7 sequences. While unlikely to mirror the extent of variation in segment 2/VP2, they may also form distinct phylogenetic clusters based on VP7 sequences. Further sequencing studies of BTV genome segment 7 will answer this question.

Both PCR protocols have also failed to amplify cDNA for BTV-15. Based on VP2 sequences, BTV-15 (like BTV-7 and BTV-19) is genetically distinct from other serotypes of the virus. Results of the RT-PCR assays described here suggest that the sequence of VP7 from BTV-15 may also be distinct. Available sequence data and phylogenetic analysis of segment 7 from both Chinese and Australian isolates, appear to confirm that BTV-15 has a high level of genetic divergence from other BTV strains (6).

Segment 7 was chosen as the target gene for PCR because it codes for the major BTV species-specific antigen and is highly conserved. It was therefore reasoned that differences between BTV topotypes (separation of BT virus isolates based on geographic origin) were likely to be relatively low and might
allow detection of all strains of the virus using a single set of primers. However, this was not shown to be possible and variation in segment 7 appears to have prevented the detection of isolates of serotypes 7, 15 and 19 by these relatively simple RT-PCR methods. Analyses of segment 7 from these viruses will be used to determine the nature of sequence variations, compared to other BTV strains. This should permit the design of primers that can detect all 24 serotypes, possibly by introducing a multiplex-PCR approach for BTV-7, BTV-15 and BTV-19. These sequencing studies are in progress.

However, even in their current stage of development, the conventional and touch-down RT-PCR methods tested can be used to identify BTV of all serotypes that are currently circulating in Europe and North and South America. As such, it could already provide a valuable tool for the monitoring of BTV epidemiology in these areas.

There were no significant differences in the ability of either the touch-down PCR, or the conventional one-step PCR, to detect BTV serotypes. Future development will concentrate on a conventional PCR protocol (7) because its simplicity will make it more accessible to potential users.

References


Development of reverse transcriptase-polymerase chain reaction-based assays and sequencing for typing European strains of bluetongue virus and differential diagnosis of field and vaccine strains

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Summary
Bluetongue virus (BTV) is a double-stranded (ds) RNA virus, classified within the genus Orbivirus, family Reoviridae, which causes bluetongue (BT), an infectious, non-contagious disease of ruminants. The virus exists as 24 distinct serotypes, which are currently identified by virus isolation and serum neutralisation assays. The most variable outer capsid protein VP2 (encoded by genome segment 2), is the primary determinant of BTV serotype. Reverse transcriptase-polymerase chain reaction (RT-PCR) assays, based on amplification of segment 2, have been developed for identification of the five European BTV types (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16). Primer pairs were designed that are specific for each BTV serotype. The resulting RT-PCR assay was both sensitive and specific, providing BTV typing within 24 h. Perfect agreement was recorded between the RT-PCR and virus neutralisation assays. The primer s for each serotype could successfully amplify the BTV isolates of that serotype from different regions and showed no cross-amplification of the most closely related BTV serotypes. RT-PCR primers were also developed for the discrimination of field and vaccine strains of BTV serotypes currently circulating in Europe. The primer pairs which could amplify field and vaccine strains of BTV-1, BTV-2, BTV-4 and BTV-9 were validated with several isolates of each serotype from various geographic origins around the world and their type specificity was again tested with the most closely related serotypes. Overall, these RT-PCR assays provide a rapid and reliable method for the identification and differentiation of field and vaccine strains of different BTV types. The primers used in this study are listed on the website of the Institute for Animal Health, Pirbright.

Keywords

Introduction
Bluetongue (BT) virus (BTV) infects most ruminant species but causes BT (an economically important disease) primarily in sheep (10, 15). It has been estimated that BTV causes losses of US$3 billion a year worldwide (19) and BT is included in OIE disease ‘List A’. BTVs have a ten-segmented double-stranded (ds) RNA genome and are classified within the species BTV, the prototype of 21 different species of the genus Orbivirus within the family Reoviridae. Twenty-four distinct serotypes of BTV are currently recognised (2, 14), which can be identified by serum neutralisation assays (16). Since 1998, BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16 have caused disease in southern Europe, with a gradual movement northward, reflected by outbreaks in Bulgaria, Serbia and Croatia (9).

The BTV outer capsid is composed of two major structural proteins, VP2 and VP5, which are encoded by genome segments 2 and 6, respectively (12, 13). VP2 is the major neutralisation antigen and consequently plays a major role in the determination of BTV serotype, although VP5 can also play a minor role (11, 13). VP2 is the most variable and VP5 is the second most variable of the BTV proteins, which is reflected in the sequences of
genome segment 2 and to a lesser extent of VP5 (3, 10, 11). The absence of sequence data for segment 2 of many BTV serotypes, has previously made it impossible to design primers for comprehensive reverse transcriptase-polymerase chain reaction (RT-PCR)-based serotyping assays. However, PCR-based assays have been developed for differentiating a limited number of BTV types in Australia and United States (4, 5, 8, 20). These methods were designed for rapid BTV type identification by detection of unique regions of genome segment 2, although the specificity of amplification and detection was not fully validated with multiple isolates all 24 BTV serotypes. Similar RT-PCR assays have also been developed for the identification of African horse sickness virus (AHSV) serotypes (17).

Full-length sequences for genome segment 2 of representative isolates of all 24 BTV serotypes are now available, as well as sequence data for multiple European isolates of BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16 (6, 7). This has facilitated the design of ‘serotype-specific’ primers, to identify isolates of different serotypes, which can now be achieved in less than 24 h. The efficiency of the type-specific primers initially designed for types 1 and 2, varied with isolates from different geographic regions. Additional primers were therefore designed and tested. Primers were also designed to differentiate field and vaccine strains of the European BTV serotypes. These PCR procedures, which detect unique regions on the serotype-specific segment 2, have the potential of providing rapid and reliable serotype and strain identification. The specificity of amplification and detection was validated in every case with isolates of the most closely related BTV types. The results from these assays are presented.

Materials and methods

Three RT-PCR protocols were evaluated by the methods described below.

Reverse transcriptase-polymerase chain reaction using full-length cDNA products

Total RNA was isolated from BTV-infected BHK-21 monolayers using the Trizol™ technique. dsRNA was separated from contaminating single-stranded (ss) RNA by precipitation in 2M lithium chloride (1). Special chemically modified primers (S. Rao, manuscript in preparation) were ligated to the 3’ end of both strands of the RNA; cDNA was synthesised at 37°C for 40 min and then at 42°C for 10 min using a ‘reverse transcription system’ from Promega without addition of further primers (S. Rao, manuscript in preparation). For PCR amplification, 0.5-2 µl of full-length cDNA, was prepared as described above and 1 µl of each gene-specific primer (10 pmol/µl) mixed with the following reagents and added: 1 µl 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2), 0.2 µl dNTPs (10 mM each), 2.5 U (0.5 µl) Taq polymerase (Roche, UK). The final volume was made up to 10 µl with nuclease-free water. PCR amplification was performed using a denaturation step of 94°C for 2 min, followed by 30 cycles of melting at 95°C for 15 sec, annealing at 50°C for 30 sec and extension at 72°C for 3 min. The reaction was terminated by a final extension step of 72°C for 5 min. Adjustments to the annealing temperature and extension were made depending on the primers Tm and the size of the amplification product. After amplification, 5 µl of sample was added to 1 µl of 5X loading buffer and analysed by agarose gel electrophoresis in the presence of ethidium bromide.

Reverse transcriptase-polymerase chain reaction using gene specific primers

The protocol used here for each serotype is an adaptation of previously published methods for the transcription and amplification of RNA templates (5, 20). Genome segment-specific primers were chosen for their intratypic conservation and heterotypic variation, by comparing sequences already obtained for the various serotypes (6, 7). These primers were then used in RT-PCR reactions (Fig. 1). For each RT reaction, a denaturation-primer cocktail mix, containing 1 µl of RNA template, 10 mM methyl mercury hydroxide (MMOH) and 1 µM of each primer, was incubated at room temperature for 10 min, after which 1 µl of 350 mM 2-mercaptoethanol was added to each tube to neutralise the MMOH. To this, 10 µl of a reverse transcription mix (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl2, 0.5 units/µl RNase inhibitor, 1 mM dNTPs, 200 units of Moloney murine reverse transcriptase (Promega, UK) was added, giving a final volume of 20 µl. The reactions were incubated at 37°C for 60 min. The PCR reaction steps were performed using the same protocol described above.

Alternative (one-step) reverse transcriptase-polymerase chain reaction procedure

An alternative one step RT-PCR technique was also used on RNA extracted from virus-infected cell culture cells. This procedure is based on a kit purchased from Amersham Pharmacia Biotech Inc, which consists of ready-to-use ‘beads’ that include all of the reagents required for the reverse transcription and PCR amplification steps of the reaction. The primer-template mix was heated to 95°C for 3 min, or denatured with methyl mercury hydroxide. This mix was then added to the RT-PCR beads, which
had been reconstituted with nuclease-free water, making a final reaction volume of 50 ml. The samples were subjected to reverse transcription at 37°C for 60 min followed by PCR amplification using the following thermal profile: initial denaturation at 95°C for 5 min; 94°C for 30 sec; annealing at 50°C for 30 sec and extension at 72°C for 1 min per kb of fragment to be amplified, for 30 cycles. There was a final, single extension step of 72°C for 10 min.

**Results**

The oligonucleotide primers used in these studies to amplify BTV genome segment 2 are listed in Table I and can be found at iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-S2-primers.htm. This website is periodically updated as further research and primer development is still underway.

**Development of serotype-specific primers**

Primer sets were designed for each of the five European serotypes and were used to generate PCR amplicons of the expected size, from multiple isolates of the homologous BTV serotype isolated in different geographic areas around the world. The virus strains used in these assays are listed in Table II and are stored in the reference collection at the Institute for Animal Health (IAH) Pirbright. More information about the origins of each isolate can be
Table I
Primer sequences used for serotype specific RT-PCR assays and for differentiation of field and vaccine strains of bluetongue virus serotypes 1, 2, 4, 9 and 16

<table>
<thead>
<tr>
<th>Primer name (AA)</th>
<th>Primer sequence (5′ to 3′)</th>
<th>Position on genome segment 2 (NT)</th>
<th>Predicted product size (base pair)</th>
<th>Primer pair grading in PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for serotype specific amplification of genome segment 2 of European strains of BTV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTV-1/2/2/p524F</td>
<td>TAAGTGGGATAATMGCWCCGAT</td>
<td>1572</td>
<td>1 334</td>
<td>+++</td>
</tr>
<tr>
<td>BTV-1/2/p968R</td>
<td>TYATACGTTGAGAAGTTTTTG</td>
<td>2906</td>
<td></td>
<td></td>
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<tr>
<td>BTV-2/2/p198F</td>
<td>TATCGATTAATAGTGCTATT</td>
<td>595</td>
<td>653</td>
<td>+++</td>
</tr>
<tr>
<td>BTV-2/2/p416R</td>
<td>TACTAAATATATACTCTCGGT</td>
<td>1248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTV-4/2/p67F</td>
<td>GCTTAACTATAAACCAACGAGG</td>
<td>200</td>
<td>2 324</td>
<td>+++</td>
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<td>BTV-4/2/p841R</td>
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<td></td>
<td></td>
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<tr>
<td>BTV-9/2/p177F</td>
<td>TCTRATAAATAATGGGTTATAT</td>
<td>533</td>
<td>2 023</td>
<td>+++</td>
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<td>BTV-9/2/p852R</td>
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<td>BTV-16/2/p670F</td>
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<td>2010</td>
<td>762</td>
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<tr>
<td>BTV-16/2/p924R</td>
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<tr>
<td>BTV-16/2/p273F</td>
<td>GGAGGCAACTTCCGACGGGAA</td>
<td>819</td>
<td>1 557</td>
<td>+++</td>
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<tr>
<td>BTV-16/2/p792R</td>
<td>TCACCAAYCRAAWCGATT</td>
<td>2376</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| **Primers for amplification of BTV-1 field strains** | | | | |
| BTV-1F/2/p543F | GCACAACGAGCGGAAATGACG | 1629 | 707 (1F-A) | +++ |
| BTV-1F/2/p778R | GATGACCTTAAGTTCGTGCG | 2336 | | |

| **Primers for amplification of BTV-1 vaccine strains** | | | | |
| BTV-1V/2/p733R | TTCATACCTACGTTGACG | | | |

| **Primers for amplification of BTV-2 field strains** | | | | |
| BTV-2F/2/p444F | CTAGAGAAAGGCAATCCTTGACCC | 1334 | 565 (2F-C) | +++ |
| BTV-2F/2/p633R | CAGGGATATGCCTGGCAGTC | 1898 | | |

| **Primers for amplification of BTV-2 vaccine strains** | | | | |
| BTV-2V/2/p503F | TAAATCCTGGTGAAATATAT | 92 | 1 806 (2V-O) | +++ |
| BTV-2V/2/p632R | TCCAGAATGTATGCGCCCCTT | 1898 | | |

| **Primers for amplification of BTV-4 field strains** | | | | |
| BTV-4F/2/p234F | CTATACATATAACAGATTCATC | 701 | 1 233 (4F-O) | +++ |
| BTV-4F/2/p465R | CGTTTGTATGTCGCTAGGCT | 1934 | | |

| **Primers for amplification of BTV-4 vaccine strains** | | | | |
| BTV-4V/2/p233F | CTATACATATAACAGATTCATC | 701 | 1 249 (4V-C) | +++ |
| BTV-4V/2/p650R | CATTGTTGCTTCTAACACCTCA | 1950 | | |

| **Primers for amplification of BTV-9 field strains** | | | | |
| BTV-9F/2/p113F | GCTATCTGACGCTATGGATG | 340 | 2 330 (9F-O) | +++ |
| BTV-9F/2/p890R | GCCCTGTGATCAGCTATGTTG | 2670 | | |

| **Primers for amplification of BTV-9 vaccine strains** | | | | |
| BTV-9V/2/p132F | GCGCCGTCAAGTGACTTGG | 397 | 1 743 (9V-A) | +++ |
| BTV-9V/2/p713R | GGAATGTGTCATACG | 2140 | | |

NT nucleotide
AA amino acid
PCR polymerase chain reaction
+++ efficient primer pair, which could amplify all available isolates of the homologous type
Table II
Details of virus isolates used for genome segment 2 based RT-PCR assays of bluetongue virus serotypes 1, 2, 4, 9 and 16

<table>
<thead>
<tr>
<th>Serotype and country of origin</th>
<th>Species</th>
<th>IAH dsRNA virus Reference Collection No.</th>
<th>Accession No.</th>
</tr>
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<tbody>
<tr>
<td>BTV-1 S. African vaccine strain (OVI)</td>
<td>Not known</td>
<td>RSAvvv/01</td>
<td>AJ585110</td>
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<tr>
<td>BTV-1 S. African reference strain (OVI)</td>
<td>Not known</td>
<td>RSArrr/01</td>
<td>AJ585122</td>
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<tr>
<td>BTV-1 India (Rajasthan)</td>
<td>Ovine</td>
<td>IND1992/01</td>
<td>AJ585111</td>
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<td>BTV-1 India</td>
<td>Not known</td>
<td>IND1992/02</td>
<td>AJ585112</td>
</tr>
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<td>BTV-1 India</td>
<td>Not known</td>
<td>IND1988/01</td>
<td>AJ585113</td>
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<td>BTV-1 India (Haryana)</td>
<td>Ovine</td>
<td>IND1999/01</td>
<td>AJ585114</td>
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<tr>
<td>BTV-1 India (Chennai)</td>
<td>Ovine</td>
<td>IND2001/01</td>
<td>AJ585115</td>
</tr>
<tr>
<td>BTV-1 Malaysia (Kuala Lumpur)</td>
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<td>MAY1987/01</td>
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<td>BTV-1 Sudan</td>
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<td>SUD1987/01</td>
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<td>BTV-1 Cameroun</td>
<td>Ovine</td>
<td>CAR1982/01</td>
<td>AJ585119</td>
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<td>AJ585121</td>
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<td>BTV-2 India</td>
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<td>IND1982/01</td>
<td>AJ585152</td>
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<td>AJ585160</td>
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<td>RSArrr/04</td>
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<td>TURvvv/04</td>
<td>AJ585164</td>
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<td>BTV-4 Turkey</td>
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<td>AJ585165</td>
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<td>BTV-4 Sudan</td>
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<td>AJ585166</td>
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<tr>
<td>BTV-4 Argentina</td>
<td>Bovines</td>
<td>ARG2002/01</td>
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<td>AJ585130</td>
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<td>BTV-16 S. African vaccine strain (OVI)</td>
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<td>TUR2000/10</td>
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</table>

IAH Institute for Animal Health, Pirbright
OVI Onderstepoort Veterinary Institute
obtained from the website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm.)

The primers were also tested for specificity with the most closely related BTV types (as identified by Maan et al.) (6) and no cross-reactions were apparent. Figures 2, 3 and 4 are images of representative gels showing serotype-specific PCR products using the primers illustrated in Figure 1a.

**Figure 2**
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 1 and 2 using serotype-specific primer pairs, namely BTV-1/2/p524F & BTV-1/2/p968R (1 334 bp) and BTV-2/1/p198F & BTV-1/2/p416R (653 bp)

The new type-specific PCR primers for BTV serotypes 1 and 2 were validated with various isolates from different geographic regions as shown above

**Figure 3**
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 4 and 9 using serotype-specific primer pairs, namely BTV-4/2/p67F & BTV-4/2/p841R (2 324 bp) and BTV-9/2/p177F & BTV-9/2/p852R (2 023 bp)

The new type-specific PCR primers for BTV serotypes 4 and 9 were validated with various isolates from different geographic regions as shown above

**BTV-1**
Primer pair: BTV-1/2/p524F & 968R

**BTV-2**
Primer pair: BTV-2/2/p198F & 416R

**BTV-4**
Primer pair: BTV-4/2/p67F & 841R

**BTV-9**
Primer pair: BTV-9/2/p177F & 852R
**BTV-16 i set**
Primer pair: BTV-16/2/p670F & 924R

**BTV-16 N set**
Primer pair: BTV-16/2/p273F & 792R

---

**Figure 4**
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotype 16 using serotype-specific primer pairs, namely BTV-16/2/p670F & BTV-16/2/p924R (16i pair=762 bp) and BTV-16/2/p273F & BTV-16/2/p792R (16 N pair=1 557 bp)

The new type-specific PCR primers for BTV serotype 16 were validated with various isolates from different geographic regions as shown above

BTV-16i set cannot amplify BTV-16 Nigerian strain while BTV-16 N set can also amplify Nigerian strain of BTV-16

---

**Differentiation of European field and vaccine strains**

The aligned VP2 gene sequences were individually compared for each of the five European serotypes, in order to identify specific regions showing variability in sequences between vaccine and field strains. A schematic map (Fig. 1b) shows the positions of primers that were subsequently designed and the product sizes expected with each primer pair. These primers were then used in RT-PCR assays and amplicons of the expected size were obtained (Figs 5 and 6).
The primers designed to amplify field strains of BTV-1 (field primers, namely BTV-1F/2/p543F and 778R) were specific for non-African isolates. The primers designed to amplify vaccine strains of BTV-1 (vaccine primers, namely: BTV-1V/2/p28F and 733R) were specific to the vaccine strain (Fig. 5). BTV-2 (field primers, namely BTV-2F/2/p444F and 633R) were specific to the field isolates tested but the vaccine primers (BTV-2V/2/p30F and 632R) weakly amplified some non-vaccine field strains from Africa (Fig. 5). However, this would not prevent their use to distinguish field and vaccine strains from current European outbreaks.

The primers designed for BTV-4 (BTV-4F/2/p234F and 645R for field strains and BTV-4V/2/p233F and 650R for vaccine strains) and BTV-9 (BTV-9F/2/p113F and 890R and BTV-9V/2/p132F and 713R for field and vaccine strains, respectively) specifically amplified the relevant field and vaccine strains (Fig. 6). Primers for BTV-16 were not able to differentiate field from vaccine strains (data not shown) due to insufficient nucleotide differences in the field and vaccine strains of BTV-16. This reflects the very close relationship between segment 2 from these viruses and may suggest a relatively recent common ancestry.

**Discussion**

The ability to rapidly diagnose the serotype of BTV causing outbreaks is of major importance for disease control strategies, particularly if vaccination is one of the options. Indeed, delays in virus identification (which can take in excess of six weeks by conventional virus isolation and serum neutralisation assays) have, on at least one occasion resulted in use of a vaccine from a heterologous BTV type. Rapid typing methods are of particular importance in areas such as Europe where outbreaks of disease caused by five different BTV serotypes have occurred recently. Some of the countries involved have used vaccination to control disease (e.g. Italy, France, and Turkey). However, the use of live-attenuated vaccines in itself may pose some risk due to the ability of the virus (in at least some cases) to be transmitted in the field and its ability to reassort (exchange) genome segments during mixed infections.

The ability to rapidly discriminate between viruses which occur as natural outbreaks and those that may be attributable to the vaccine strain, will help to maintain a considered and flexible approach to disease control. The high quality sequence data now
available from our recent studies have supported the design of effective diagnostic primers and protocols for the five European BTV serotypes. It has also been possible to design primers with the potential to distinguish vaccine from field strains of four of the European serotypes (1, 2, 4 and 9), based on topotype variation in genome segment 2, combined with the South African origin of BTV vaccine strains. However, the European strains of BTV-2 originated in sub-Saharan Africa and insufficient nucleotide differences were detected in segment 2 to allow the design of primers that would reliably distinguish vaccine and some of the African field strains. This not only suggests that primers to other regions of the virus genome may be needed to distinguish all vaccine and field strains of BTV-2 (18) but also that other genome segments are likely to be involved in virus attenuation. The close relationship of segment 2 from the field and vaccine strains of BTV-16 also made it difficult to distinguish them by these techniques.

The results obtained identify a need for continuing appraisal and refinement of these techniques, to take into account the changing epidemiological situation, involving both novel virus strains and the geographic movement of the viruses. All of the PCR methods used were effective with the primer sets described; however, the one-step PCR procedure had some benefits. The main advantages being better standardisation of the reagents, a reduction in pipetting steps and therefore less potential for error or contamination, a combination of the RT and PCR steps in one tube and a combined programme on the thermocycler, making the entire technique more suitable for the design of ‘standard operating procedures’.

References


Bluetongue in Bosnia: comparisons of competitive enzyme-linked immunosorbent assay and standard agar gel immunodiffusion tests

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Summary

At the end of August 2002, clinical symptoms of bluetongue (BT) (fever between 39°C and 41°C, muco-purulent or bloody nasal discharge, oedema of the lips and the intramandibular space, foot lesions including laminitis and coronitis in some cases, diarrhoea and dysentery) were recorded in Pramenka sheep flocks in north-east Bosnia in August 2002. A total of 9,599 serum samples (ovine: 8,967; bovine: 632) from 40 communities of Bosnia and Herzegovina were tested for the presence of anti-bluetongue virus (BTV) antibodies using competitive enzyme-linked immunosorbent assay (c-ELISA) and the standard agar gel immunodiffusion (AGID) test. The c-ELISA revealed BTV-seropositive reactions in 187 (1.94%) samples and the AGID test detected 141 (1.53%) cases. Complete agreement was recorded between the c-ELISA and AGID test results for bovine sera. These results indicate that the ability of c-ELISA to detect anti-bluetongue virus antibodies in ovine sera was superior to that of the AGID. All positive sera were collected from animals in the river areas of Bosnia and Herzegovina.

Keywords

Agar gel immunodiffusion test – Bluetongue – Bosnia – Culicoides – Enzyme-linked immunosorbent assay – Herzegovina.

Introduction

Bluetongue (BT) virus (BTV) is an arthropod-borne pathogen, transmitted by species of the genus Culicoides. BTV can infect several species of domestic and wild ruminants, but sheep are most susceptible (3). BTV occurs in the Americas, Africa, Asia and Australia. At least 24 serotypes of BTV have been identified worldwide. The virus can cause an acute, sub-acute, mild or inapparent disease (4). The agar gel immunodiffusion test (AGID) and competitive enzyme-linked immunosorbent assay (c-ELISA) are the most common tests for the detection of group-specific anti-BTV antibodies (1, 2, 5).

Between 1998 and 2001 incursions of BTV were recorded in countries around the Mediterranean Basin that are usually free from infection, but no occurrence was reported in Bosnia. Like many of the neighbouring countries, Bosnia had no previous record of the disease. At the end of August 2002, clinical symptoms of BT were reported for the first time in local Pramenka sheep flocks in north-east Bosnia, close to the border with Serbia. The virus spread across Bosnia between September and October, travelling through river areas.

Materials and methods

A total 9,599 field sera from 8,967 sheep and 632 cattle in 40 communities of Bosnia and Herzegovina were collected between August and December 2002. All the sera were transported on ice and were stored at –20°C until tested. Diagnosis was based on clinical signs and laboratory confirmation. For the detection of anti-BTV antibodies, all sera were tested using the c-ELISA and standard AGID tests.

Results

In August 2002, BTV was confirmed for the first time ever in Bosnia and Herzegovina, initially in Kalesija (north-east Bosnia, close to the Serbian border). During September and October, the virus...
spread across the country, travelling through river areas. BT antibody was tested in sheep flocks in 40 communities (Fig. 1).

**Figure 1**
Communities of Bosnia and Herzegovina with seropositive reactors of bluetongue in 2002

Clinical signs were observed in 5-10% of the animals in flocks. The most common signs were fever of between 39°C and 41°C, muco-purulent or bloody nasal discharge, oedema of the lips and the intramandibular space, foot lesions, including laminitis and coronitis in some cases, diarrhoea, dysentery and death.

The comparative results of the c-ELISA and standard AGID test on 8,967 sheep and 632 cattle sera are presented in Table I.

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of positive reactors</th>
<th>Cattle No.</th>
<th>Percentage</th>
<th>Sheep No.</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-ELISA</td>
<td>6</td>
<td>100</td>
<td>187</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>AGID</td>
<td>6</td>
<td>100</td>
<td>141</td>
<td>77.9</td>
<td></td>
</tr>
</tbody>
</table>

Forty-six of the sheep sera were negative using the AGID test but positive with the c-ELISA. Complete agreement was recorded between the c-ELISA and AGID test results for cattle sera.

The Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’ in Teramo confirmed the presence of BTV serotype 9.

**Discussion**

Currently, the most common serodiagnostic tests for BTV are the AGID test and c-ELISA that detect the VP7 group-specific antibodies (5). Although the AGID test is simple to perform and rapid, it is not highly sensitive or quantitative and has limitations regarding specificity. Sera containing antibodies to other groups of Orbiviruses (e.g. epizootic haemorrhagic disease) may give a non-specific reaction in the AGID test. Among several ELISAs that have recently been developed, the c-ELISA, in which a group-specific MAb to BTV is used, has proved to be the most sensitive and specific assay for detection of BTV antibodies. Following extensive national and international validation, the c-ELISA is gradually replacing the AGID test (1, 2). The results of our study indicate that the ability of the c-ELISA to detect anti-BTV antibodies in sheep sera was superior to that of the AGID test.

**Acknowledgements**

Grateful thanks are extended to F.G. Santini from the Istituto Zooprofilattico Sperimentale dell’Abruzzo e del Molise ‘G. Caporale’, Nihadu Fejzicu from the State Veterinary Office in Bosnia and Herzegovina and colleagues from the Veterinary Station in Kalesija for their suggestions and help.

**References**

Bluetongue: an overview of recent trends in diagnostics

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Summary

Bluetongue (BT) virus (BTV) is a species in the genus Orbivirus of the family Reoviridae; it causes a viral disease of sheep and occasionally cattle, and is transmitted by biting midges of the genus Culicoides. BTV replicates in the haemopoietic and endothelial cells of the blood vessels. Rarely, and only when a bull is viraemic, may BTV be recovered from semen. Serological techniques, most notably enzyme immunoassays, based on the detection of a group antigen, have been used where required in the certification of animals as ‘bluetongue-free’. The various techniques used for diagnosis are viral isolation, the enzyme-linked immunosorbent assay and the polymerase chain reaction.

Keywords

Bluetongue – Diagnosis – Sheep – Viral disease.

Introduction

Bluetongue (BT) is a viral disease of sheep and occasionally cattle, and is transmitted by biting midges of the genus Culicoides. Bluetongue virus (BTV) is a species in the genus Orbivirus of the family Reoviridae. There is considerable genetic variability within the serogroup of BTV which has at least 24 serotypes worldwide. This arises by genetic drift of individual gene segments when ruminants or the vectors are infected with more than one strain. Until the 1940s, this disease was recognised only in Africa, then following a major epidemic in 1956-1957 in Portugal and Spain, the disease was recognised in the United States of America (USA), the Middle East, Asia and later in Australia (1).

BTV replicates in the haemopoietic and endothelial cells of the blood vessels. Rarely, and only when a bull is viraemic, BTV may be recovered from semen.

BTV is often difficult to isolate in the laboratory. The success of virus isolation is enhanced if blood is collected from animals showing clinical signs and at an early stage of the disease. Viraemia is primarily associated with red blood cells and leucocytes and the virus can coexist in infected animals with high concentrations of neutralising antibody. Serological techniques, most notably enzyme immunoassays, based on the detection of a group antigen have been used where required to certify animals as ‘bluetongue-free’. However, intermediate serological reactions have been a major problem. For accuracy in diagnosis, more sensitive and specific assays, such as those based on antigens produced by recombinant DNA technologies and the polymerase chain reaction (PCR) should prove useful (2).

Diagnostic techniques for bluetongue: identification of the agent

Virus isolation

The same diagnostic procedures are used for domestic and wild ruminants. A number of virus isolation systems are in common use, but two of the most efficient are embryonated chicken eggs (ECE) and sheep. Identification of BTV following inoculation of sheep may still be a useful approach if the titre of virus in the sample blood is very low, as may be the case several weeks after infection. Attempts to isolate virus in cultured cells in vitro may be more convenient.

Isolation in cell culture

Virus may also be isolated in mouse L, baby hamster kidney (BHK)-21, African green monkey kidney (Vero) or Aedes albopictus (AA) cells in culture. The efficiency of isolation is often significantly lower following direct addition to cultured cells compared with that achieved in ECE. Greatest efficiency of isolation in cell culture is achieved by first passaging ECE homogenates in AA cells, followed by either
antigen detection procedures or additional passages in mammalian cell lines, such as BHK-21 or Vero. A cytopathic effect (CPE) is not necessarily observed in AA cells. Cell monolayers are monitored for the appearance of a CPE for 5 days at 37°C in 5% CO₂ with humidity. If no CPE appears, a second passage is made in cell culture. The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by antigen-capture ELISA, immunofluorescence, immunoperoxidase or virus neutralisation (VN) tests.

**Immunological methods**

**Serogrouping of BTV**

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP 7, that are conserved within each serogroup. The cross-reactivity between BT and epizootic haemorrhagic disease (EHD) viruses raises the possibility that an isolate of EHD virus could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific monoclonal antibody (MAb) can be used. A number of laboratories have generated such serogroup-specific reagents (3). In contrast to serogrouping, the usual method of serotyping is by VN testing using methods described below. Commonly used methods for the identification of virus to serogroup level are as follows:

a) immunofluorescence
b) antigen-capture enzyme-linked immunosorbent assay (ELISA)
c) immunospot test
d) indirect peroxidase/antiperoxidase identification.

**Serotyping by virus neutralisation**

Neutralisation tests are type-specific for the 24 BTV serotypes currently recognised and can be used to serotype a virus isolate, or can be modified to determine serotype of antibody. In the case of an untyped isolate, the characteristic regional localisation of BTV serotypes should generally obviate the need to attempt neutralisation by all 24 antisera, particularly when endemic serotypes have been identified.

There is a variety of tissue-culture-based methods available to detect the presence of neutralising anti-BTV antibody. Cell lines commonly used are BHK, Vero and L 929. BTV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included to ensure that an effective level of standard antiserum is used against comparable and standardised titres of standard and untyped virus. Four methods to serotype BTV are outlined briefly below:

a) plaque reduction
b) plaque inhibition
c) microtitre neutralisation
d) fluorescent inhibition test.

**Polymerase chain reaction**

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis. Results to date indicate that PCR techniques may be used, not only to detect the presence of viral nucleic acid, but also to ‘serogroup’ orbiviruses and provide information on the serotype and possible geographic source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least three to four weeks to generate information on serogroup and serotype and yield no data on the possible geographic origin of the isolated virus.

Oligonucleotide primers used to date have been derived from RNA 7 (VP7 gene), RNA 6 (NS1 gene), RNA 3 (VP3 gene) and RNA 2 (VP2 gene). The size of the amplified transcripts is usually small (in the order of several hundred nucleotides) but can also be a full-length gene. In the procedure, a 101 nucleotide stretch of RNA 6 is amplified. Primers derived from the highly conserved genes, such as VP3, VP6, VP7, NS1 and NS3, may be used for serogrouping (i.e. they will react with all members of the BT serogroup) and topotyping (i.e. they will react with BTV isolates from the same geographic area), while primers whose sequence was determined from VP2 gene sequences provide information on virus serotype.

The capacity of PCR assays to detect single molecules of nucleic acid means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids.

The PCR assay involves three separate procedures. In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanates (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available for this purpose and the protocol describes the use of one such kit: IsoQuick (Orca Research, Bothell, Washington, USA). The reagents provided with the kit are numbered and their use is indicated in the protocol. Other kits are available and one, TrizoT™ (Life Technologies, Grand Island, New York), is particularly useful for the extraction of viral nucleic
acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription (RT) to generate DNA, which is amplified by PCR. In the procedure, the Superscript™ Preamplification System (Life Technologies) is used to transcribe viral RNA and reagents from Perkin-Elmer are used for the PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the PCR product by electrophoresis (3).

References

Application of diagnostic procedures to epidemiological situations

with special reference to arboviral infections

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Summary

The rationale behind the methodology employed to investigate a new disease that appears in an area hitherto unaffected is fundamentally different from that applied in an endemic disease situation. Special consideration must also be given to disease agents that appear and reappear at cyclical intervals.

The authors present three separate approaches applicable in three different epidemiological situations. Each one may be used initially to determine the identity of the causal agent and the nature of the disease. These approaches, although described in a stepwise (flowchart) manner, are not meant to be applied rigidly, but rather should serve as a guideline for investigators. Special consideration is given to situations in which disease appears intermittently, using a sentinel model. Although this latter approach is expensive and time-consuming, it can yield excellent and reliable results when applied correctly.

Keywords


Introduction

The primary step in planning an investigation of an outbreak is to establish a goal, and in the case of an infectious (arboviral) disease, this is to identify the causative agent. The entire investigation, planning, gathering of data and sample collection must be designed to minimise economic losses to the farmer on one hand but must not tarnish the reputation of the National Veterinary Authority on the other hand. This activity embraces a multitude of disciplines since it inherently depends on expertise in epidemiology, microbiology, pathology, entomology and meteorology (in the case of a suspected incursion of an arbovirus) among others.

The preparedness of a state system, such as a national veterinary authority and its field and diagnostic laboratories, depends very much on its expertise in mounting a campaign against a national epidemic.

A methodical approach to the investigation of an outbreak is the best way to achieve the objectives of containment or eradication of an exotic disease, the control of an endemic disease and to prevent the reappearance of an eradicated disease. This approach is further justified if the causative agent is an Office International des Épizooties (OIE) List B disease and even more importantly, if it is an OIE List A disease.

The rationale behind the methodology (Table I) employed to investigate an emerging disease (Fig. 1) is fundamentally different from that applied to an endemic disease (Fig. 2). Special consideration must also be given to pathogenic agents that appear and reappear at cyclical intervals (Fig. 3). Akabane virus (AKAV) is known to cause outbreaks of abortions, stillbirths and foetal abnormalities in cattle and is designated as the congenital arthrogryposis-hydranencephaly syndrome of the musculo-skeletal and nervous systems (4). AKAV was isolated originally from mosquitoes and later
from the midge *Culicoides imicola*. *Culicoides* is now considered to be the major vector (3). AKAV is in the Simbu serogroup of the family *Bunyaviridae* (3).

### Table I

**Awareness and system preparedness and the types of intervention according to the epidemiological model**

<table>
<thead>
<tr>
<th>Awareness/preparedness</th>
<th>Epidemiological presentation</th>
<th>Systems awareness</th>
<th>Intellectual and personnel preparedness</th>
<th>Laboratory and diagnostic means/preparedness</th>
<th>Specific intervention</th>
<th>Expected time interval between the onset and definitive diagnosis</th>
<th>Type of intervention</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endemic zone</td>
<td>High</td>
<td>Low (if any)</td>
<td>Low (if any)</td>
<td>Highly needed</td>
<td>Very short</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Emerging disease</td>
<td>Low</td>
<td>Low (if any)</td>
<td>Low (if any)</td>
<td>Highly needed</td>
<td>Very long</td>
<td>Preferably, eradication and a vaccination belt</td>
</tr>
<tr>
<td></td>
<td>Transitional (cyclic intervals)</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Moderate</td>
<td>Intermediate</td>
<td>Vaccination is weighted (cost-effective decision)</td>
<td></td>
</tr>
</tbody>
</table>

In this study, we present three separate approaches applicable to each epidemiological situation (Figs 1, 2 and 3). Each of these can be applied initially to determine both the identity of the causal agent and the extent of the infection. Special consideration is also given to situations where the disease appears intermittently using the sentinel model (Fig. 4). Although this latter approach is expensive and time-consuming, it yields excellent and reliable results when applied correctly.

**Figure 1**

Proposed applications of diagnostic procedures giving the rationale behind the methodology employed to investigate different epidemiological situations with special reference to arboviral infections: emerging disease.
Proposed applications of diagnostic procedures giving the rationale behind the methodology employed to investigate different epidemiological situations with special reference to arboviral infections: endemic disease

Methodological background

Independently of the point of origin within the diagnostic chain, i.e. whether it starts with a serum sample or from diseased or dead animals or from the harvested insect vector, it always assumes the same direction (Figs 1, 2 and 3).

Serology

It is advisable that IgG/IgM-specific ELISA tests will follow group-specific ELISA screening on serum samples collected during and/or after the epidemic. The first recommended method is the group-specific ELISA test for investigating the different groups of the possible causative virus. Each serological test is directed at one group of viruses that shares the same group antigen (group cross-reactivity). After identification of the suspected ‘group’, it is recommended to pass to the second phase to focus on the causative agent responsible for the epidemic. For this purpose, monospecific and monoclonal antibodies are used to screen the causative agent amongst those sharing the same seroreactivity. The antibody that reacts strongest is probably directed against the causative agent of the epidemic. To obtain a definitive diagnosis, the SNT with a known pathogen or with the suspected pathogen that has been isolated from pathological samples should fulfil this aim. Convalescent sera are the best choice for the SNT. The use of precolostral serum sample taken from the affected offspring (or the aborted foetus), and a serum sample taken at the same time from the dam, is useful for providing serological evidence of in utero infection by viruses belonging to the Simbu serogroup (4, 10).

Vector harvesting

Two informative tests can be performed with harvested insects: To save time and resources, PCR is the method of choice (assuming there is a panel of primers available), whereas virus isolation would take much more time. Both tests, when supplemented with virus genome sequencing, will provide valuable molecular epidemiological data. The isolated virus can be used in the SNT with the serum samples collected from convalescent animals during the
ongoing outbreak. This SNT will definitely confirm the identity of the causative agent and therefore is of high priority.

ELISA enzyme-linked immunosorbent assay
PCR polymerase chain reaction
SNT serum neutralisation

Figure 4
Proposed applications of diagnostic procedures giving the rationale behind the methodology employed to investigate different epidemiological situations with special reference to arboviral infections: sentinel model

Animals
Animals are the best source of serum (specific antibodies) and antigens (causative agents). Moreover, the diseased animal itself presents a spectrum of clinical signs, some of which are pathognomonic and are of incalculable diagnostic value.

The pristine sentinels are the first to be infected by the causative agent, and if infected demonstrate characteristic clinical signs. They may develop a high virus titre during the viraemic phase and produce specific IgM (primary) and IgG (secondary) antibodies if the animals enter the convalescent phase. When the sentinel and other animals die, their organs are optimal sources of antigen. The gross and histopathological lesions observed provide excellent clinical-pathological aids that help the investigator identify the causative virus.

Epidemiological data
While this is not within the scope of this presentation, epidemiological tools are essential in investigating any disease and in particular an emerging disease. We would like to mention the most important features of collecting data:

Firstly, it is crucial to determine the frequency. Thereafter, the investigator will answer ‘the three W’s’ – when (season, year), where (the geographic and topographical area affected) and which (description of the population affected).

The discovery of even one (exotic) case might be considered as an outbreak, such as the discovery for the first time of a single case of an OIE List A disease in a previously unaffected area.

Sentinel model
There are two main types of sentinels. The first is an animal that is left unvaccinated while all others in the herd or flock are immunised routinely. For this purpose, a sentinel must be pristine and susceptible to the pathogen to be monitored, and be old enough to have no specific maternal antibodies. Sentinel animals provide valuable epidemiological data, such as whether and when the causative agent is present and whether the agent alternates its activity with periods of quiescence and the interval between these periods. Sentinels might also be a source of viable pathogens that can be used for antigenic typing and genome sequencing. This information is crucial for evaluating possible antigenic changes, which might lead to potential vaccination failure due to changes in antigenicity. Sentinels can be monitored periodically for eventual seroconversion with IgM-specific ELISA that can detect a recent infection. Sentinels scattered in different geographic or topographical areas would provide data about the extent of the infected area and the duration of each attack. Cohort animals are the second type of sentinel. They are suitable for such purposes from the time when the maternal antibodies disappear to the time of seroconversion. By testing these animals periodically it is possible to obtain information similar to that described above. In order to ‘capture’ the pathogenic agent, we must rely on good fortune. If bleeding is performed during the acute phase of the disease there is a good possibility of detecting viraemia. It is recommended to take blood with an anticoagulant and attempt to isolate the virus from the blood cells or perform the PCR on the cells. There is a good chance that the blood cells collected close to the seroconversion event will contain the causative agent.

During the epidemic of AKAV infection in 2002/2003, the Israeli Veterinary Services (IVS) employed most of the diagnostic tools shown in the flowchart; the chart depicts how to reach a conclusion in the case of cyclical reappearance of a disease (Fig. 3). The IVS gave a definitive diagnostic
answer as to which arbovirus of the Simbu serogroup was responsible for the epidemic within a relatively short time (1).

We succeeded in working with diagnostic tools that had been dormant since the last epidemic and succeeded in detecting the causative agent, defining its geographical boundaries and its spread from the primary focus to other geographic areas. Another achievement was the use of PCR (nested and real-time), to study the molecular properties of AKAV (9). To achieve all these goals we adopted the algorithm of a transitional zone where a disease reappears cyclically (Fig. 3).

The charts presented here might aid investigators in their pursuit of a causative agent in various epidemiological situations.

References


Molecular differentiation of field and vaccine strains of bluetongue virus serotype 2 using the real-time polymerase chain reaction and fluorescent resonance energy transfer hybridisation probes

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Summary

As a consequence of the recent outbreaks of bluetongue (BT) disease amongst sheep in the Mediterranean Basin, and following the subsequent vaccination campaign to control further spread of the disease and its long-term maintenance, it has become most important to develop rapid and sensitive methods that can reliably differentiate between field and vaccine strains of the causative virus. The authors describe a new method to differentiate bluetongue virus serotype 2 (BTV-2) field and vaccine strains, using the VP2 gene sequence differences between the South African vaccine and the Italian field wild-type strains. The method is based on the principle that the melting temperature of a DNA duplex gives information on the sequence, which enables the identification of even single-base alterations in the amplicon. The real-time polymerase chain reaction the generation of melting curves and fluorescence detection were all performed using the light cycler system (Roche). Primers and probes were designed using VP2 gene sequences. After RT-PCR, the melting curves analysis, derived by the fluorescence resonance energy transfer (FRET) real-time PCR, was performed using the light cycler data analysis program (Roche). To assess the diagnostic value of the method, a BTV-2 vaccine strain (Onderstepoort Biological Products, South Africa) was first compared against a field strain of BTV-2 (isolated during an outbreak in 2000 in Sardinia). The ability of the method to reliably identify all the BTV-2 strains was tested using an array of eleven BTV-2 field strains isolated during outbreaks in various Italian regions between 2000 and 2002 and other serotypes (BTV-1, BTV-4, BTV-9 and BTV-16) that had been isolated during recent outbreaks of BT in the Mediterranean Basin. The method was clearly able to differentiate BTV-2 strains of vaccine virus from all wild-type strains of the same serotype tested. The resultant melting curves distinctly reveal the two strains to have differing peak values of 47.8°C ± 0.6°C and 60.5°C ± 0.6°C, respectively.

Keywords


Introduction

Bluetongue (BT) virus (BTV) is the prototype species of the genus Orbivirus, family Reoviridae. BTV is the causative agent of BT disease in sheep, an infectious and non-contagious disease transmitted by haematophagous insects of the genus Culicoides, family Ceratopogonidae. The distribution of the disease extends between the 35°S and 50°N parallels. Twenty-four immunologically distinct serotypes of BTV are known, of which only a few have been reported to occur in various parts of the Mediterranean Basin (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16). In Italy, following the first
occurrence of the disease in Sardinia in 2000, three BTV serotypes have been isolated to date: BTV-2, BTV-9 and BTV-16. There is evidence that two additional serotypes (BTV-1 and BTV-4) are also circulating. The disease is clinically apparent in domesticated breeds of sheep, of which the European breeds seem to be particularly susceptible. The most common symptoms include fever, catarrhal stomatitis, rhinitis, enteritis and lameness. The mortality rate can vary from 0% to 30%, but can reach 75% (4) in highly susceptible animals but is dependent upon the serotype involved. The real significance of BT lies in the indirect losses sustained; these include abortions in pregnant ewes and severe loss of condition during prolonged convalescence (9). Laboratory diagnosis includes the detection of antibodies using the agar gel immunodiffusion (AGID) test or enzyme-linked immunosorbent assay (ELISA), virus isolation and RT-PCR methods for the detection of BT virus or nucleic acid in clinical samples and virus neutralisation (VN) test for virus identification (6). Ultimately, the control of the disease is still best achieved through prophylactic vaccination, and not through the housing of sheep in insect-proof buildings (which is not applicable in the Mediterranean region due to transhumance and roaming shepherding), nor through the elimination of the insect vector (2). As a consequence of the vaccination campaign which was implemented to limit further spread and long-term maintenance of BT in Italy, it became important to develop rapid and sensitive methods that can reliably differentiate between field and vaccine strains of the causative virus. Although direct sequencing is the gold standard for characterising known gene regions and for mutation detection, it remains impractical for routine purposes. A previously untried PCR real-time method was used here for the differentiation of BTV-2 field and vaccine strains, based on the use of FRET (fluorescence resonance energy transfer) probes using segment 2 of the BTV genome.

Materials and methods

Recently developed instruments which couple the PCR method to fluorescent hybridisation probes now allow target amplification and analysis without sample handling. The method used here is based on the principle that the melting temperature of a DNA duplex gives information on the sequence and identifies even single-base alterations in the amplicon (3). The RT-PCR, the generation of melting curves, and fluorescence detection, were all performed using the Light Cycler System (Roche Mannheim, Germany). The primers (OG174: 5’-GCTGCTTCCGACACTTACAAT 3’; OG175: 5’-ACTAAATATATACTTCTCCGTTTCCCG-3’) were designed using VP2 gene sequences available on the Internet, to flank a region of about 192 base pairs (bp) where appropriate sequence differences were identified by alignment using the ClustalW program. The FRET method was created using separate 3’ and 5’ labelled probes (OG178-fluoresceina; OG177-BODIPY 630/640, red) which hybridised adjacent to the unlabelled complementary PCR strand (Fig. 1). The primers and probes were designed using the Oligo 6.0 program (MedProbe, Oslo, Norway). After RT-PCR, the reaction is cooled automatically and the subsequent slow heating (0.1°C-0.2°C per second) from 40°C to 95°C, as an addendum to PCR, causes denaturation of the amplicon/probe heteroduplex (melting curve), and so allows for the identification of the sequence differences. The melting temperature (Tm) is measured at the point at which 50% of the probe is denatured away from the amplicon causing a change in fluorescence. For the real-time RT-PCR, the master hybridisation probes reaction mix (Roche Mannheim, Germany), RNA template, primers and probes were used in a final volume of 20 µl. The analysis of the melting curves derived by the FRET real-time PCR, was performed using the light cyc ler data analysis program (Roche).

Bluetongue virus strains used

To assess the diagnostic value of the method, a vaccine strain of BTV-2 (Onderstepoort Biological
Products [OBP], South Africa) was first compared against a field strain of BTV-2 (BT28340) isolated in Sardinia during one of the first outbreaks of BT in 2000. The resultant melting Tms, visualised by taking the negative first derivative (–dF/dT) of the melting curve, distinctly revealed the two strains to have differing Tm values of 47.8°C ± 0.6°C and 60.5°C ± 0.6°C, respectively. The ability of the method to reliably identify all the BTV-2 strains was tested using an array of eleven BTV-2 strains, isolated during outbreaks in various Italian regions between 2000 and 2002. The sensitivity of the method was calculated using BTV-2 of known titre and a lower limit of detection (LOD) of 10^2 copies of RNA was determined. The specificity was tested against other serotypes (i.e. one strain of BTV-1, one strain of BTV-4, five strains of BTV-9 and two strains of BTV-16) that had also been isolated during the recent outbreaks of BT in the Mediterranean Basin. The strains used to validate the method were assigned to the specific serotype and geographic origin, using the VN test and gene sequencing of segments 2 and 10 of the BTV genome. The results of segment 2 sequencing are published elsewhere in these proceedings (8). The segment 10 analysis was performed in collaboration with U. Balasuriya and N.J. MacLachlan (University of California, Davis) following published methods (1, 7). The results are shown in Figure 2. To investigate how the method could be used in field conditions, 47 additional viral strains samples from sheep, goats and bovines during outbreaks of BT, were also tested. Twenty-five blood samples from sheep collected in ethylene-diaminetetra-acetic acid (EDTA) were also processed. Ten blood samples were taken from sheep a month after vaccination, while fifteen samples were from sheep suspected of being infected with BT.

**RNA extraction**

All samples were treated for RNA extraction using Trizol™ reagent (Life Technologies Inc, Gaithersburg) following the instructions of the manufacturer. All RNAs were re-suspended in 30 µl of nuclease-free H2O and kept at –80°C until use.

**Fluorescence resonance energy transfer real-time polymerase chain reaction**

The real-time PCR reaction was performed using the light cycler system in a final volume of 20 µl, at 2.5 mM MgCl2, 5 pmoles/primer and 10 pmoles of each probe and 2 µl of RNA. The real-time PCR reaction was performed as follows:

- **a)** synthesis: 55°C for 10 min
- **b)** heating cycle: 95°C for 30 sec (1 cycle)
- **c)** PCR cycles: 95°C for 0 sec, 51°C for 10 sec, 72°C for 8 sec (35 cycles)
- **d)** melting cycle: from 40°C to 95°C with a ramping increase of 0.1°C/sec. The results were read in the F2 channel (640 nm) using the ‘compensation colour’ mode.

**Results**

The FRET real-time PCR, as evaluated here, had an LOD of about 100 copies of the RNA target (Fig. 3). Of the eleven BTV-2 strains examined, nine had the wild-type profile with a Tm of 60.5°C and two had the vaccine profile with a Tm of 47.8°C. The difference in Tm of 12.7°C allows for easy differentiation of the two profiles. No cross-reaction was detected with the strains of BTV-1, BTV-4, BTV-9 and BTV-16, indicating the specificity of the method to be 100% (Table I and Fig. 4). The forty-seven BTV field isolates resulted in eleven BTV-2 wild-type, one BTV-2 vaccine type, thirty non-reactive (identified as BTV-9 by VN test), whilst five showed a mixed profile with the simultaneous presence of Tm 47.8°C and 60.5°C. This suggests the presence of both wild-type and vaccine strains in the same samples (Table II). To verify the applicability of the method, twenty-five blood samples taken in the field were also examined. Fifteen samples were from sheep suspected of being infected with BT, and ten were from sheep vaccinated with the BTV-2 monovalent vaccine (OBP). Ten samples from the suspected sheep had a Tm equal to 60.5°C, four had a Tm of 47.8°C, while one presented both profiles, indicating the
circulation of both the wild-type and the vaccine strain in the specific outbreak. The ten vaccinated sheep presented only the Tm 47.8°C profile (Table III).

Table I
Twenty bluetongue virus strains processed using fluorescence resonance energy transfer real-time polymerase chain reaction compared to results of the virus neutralisation tests

<table>
<thead>
<tr>
<th>Virus neutralisation</th>
<th>FRET real-time PCR for BTV-2 wild-type/vaccine strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV serotype No.</td>
<td>Tm 60.5°C wild-type Tm 47.8°C vaccine</td>
</tr>
<tr>
<td>BTV-1</td>
<td>1 0 0</td>
</tr>
<tr>
<td>BTV-2</td>
<td>11 9 2</td>
</tr>
<tr>
<td>BTV-4</td>
<td>1 0 0</td>
</tr>
<tr>
<td>BTV-19</td>
<td>5 0 0</td>
</tr>
<tr>
<td>BTV-16</td>
<td>2 0 0</td>
</tr>
</tbody>
</table>

BTV bluetongue virus
FRET fluorescence resonance energy transfer
PCR polymerase chain reaction
Tm temperature

Table II
Results of the fluorescence resonance energy transfer real-time polymerase chain reaction, applied to 47 field isolates of bluetongue virus

<table>
<thead>
<tr>
<th>Virus neutralisation</th>
<th>FRET real-time PCR for BTV-2 wild-type/vaccine strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV serotype No.</td>
<td>Tm 60.5°C wild-type Tm 47.8°C vaccine Tm wild-type and vaccine</td>
</tr>
<tr>
<td>BTV-2</td>
<td>17 11 1 5</td>
</tr>
<tr>
<td>BTV-9</td>
<td>30 0 0 0</td>
</tr>
</tbody>
</table>

BTV bluetongue virus
FRET fluorescence resonance energy transfer
PCR polymerase chain reaction
Tm temperature

Conclusions

In all cases, the FRET real-time PCR was able to identify the BTV-2 serotype as previously demonstrated by VN test, and further confirmed by sequencing of the VP2 gene. This FRET real-time PCR system was also able to differentiate (within the same serotype) between the BTV-2 wild-type and vaccine strains.

The NS3 gene sequence analyses, conducted on the same samples, showed that all the BTV-2 strains isolated from Italian outbreaks, and carrying the wild-type profile as revealed by FRET real-time PCR, cluster together, but separately from African strains of the same serotype. One strain that had the BTV-2 vaccine profile falls into the African cluster, although it was more closely related to the various BTV-9 strains than to the BTV-2 strains. This could indicate gene reassortment in the field, as has been recently suggested (5), and is a possibility that needs to be investigated further.
In a few instances this method was also useful to reveal the presence of wild-type and of vaccine virus in the BTV isolates and clinical samples. This would not be possible using serological techniques or the VN test.

Table III
Results of the fluorescence resonance energy transfer real-time polymerase chain reaction applied to 25 blood samples from vaccinated and infected sheep

<table>
<thead>
<tr>
<th>Blood sample</th>
<th>FRET real-time PCR for BTV-2 wild-type/vaccine strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tm 60.5°C</td>
</tr>
<tr>
<td>Sheep No.</td>
<td></td>
</tr>
<tr>
<td>Suspected infection</td>
<td>15</td>
</tr>
<tr>
<td>Vaccinated</td>
<td>10</td>
</tr>
</tbody>
</table>

BTV  bluetongue virus
FRET fluorescence resonance energy transfer
PCR  polymerase chain reaction
Tm  temperature

An added value of the FRET real-time PCR is the possibility of establishing automated high-throughput testing procedures and thus reducing time-consuming post-PCR steps.

References

Bluetongue laboratory diagnosis: a ring test to evaluate serological results using a competitive ELISA kit

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(2) Laboratory of Virology, Centre of Athens Veterinary Institutions, Ministry of Agriculture, 25 Neopoleos Street, 15310 Athens, Greece

Summary

The occurrence of bluetongue (BT) in Italy prompted an increase in disease surveillance. Thus a competitive enzyme-linked immunosorbent assay (c-ELISA) to detect immunoglobulins to BT virus (BTV) was developed and distributed amongst 27 laboratories comprising the Italian veterinary diagnostic laboratories network to screen field sera. This ring test enabled comparison of the results and the evaluation of the reproducibility of the method. The c-ELISA developed by the National Reference Centre for Exotic Diseases (c-ELISA-IZSA&M) was compared also against a commercially available c-ELISA. In addition, results obtained by the Centre of Athens Veterinary Institutions are presented.

Keywords

Antibody detection – Bluetongue – Competitive enzyme-linked immunosorbent assay – Diagnosis – Ring test.

Introduction

Bluetongue (BT) disease occurred in Italy for the first time in 2000 and spread to a large area of the country (1). Surveillance plans and vaccination campaigns were implemented in regions affected and at risk. The serological surveillance plan stipulated that sentinel animals had to be tested fortnightly for early detection of virus circulation (2). Testing was performed using a competitive enzyme-linked immunosorbent assay (c-ELISA) kit produced by a private company. The National Reference Centre for Exotic Diseases developed a c-ELISA method referred to as the c-ELISA-IZSA&M (Istituto Zooprofilattico Sperimentale, dell’Abruzzo e del Molise ‘G. Caporale’, Teramo) which had high levels of sensitivity and specificity (6). This kit was distributed to all national laboratories involved in the BT serological plan. To verify laboratory performance, an inter-laboratory ring test was designed and implemented (3, 4, 6), whereby a panel of sera had to be tested simultaneously using the commercial kit and the c-ELISA-IZSA&M kit. The ring test enabled an evaluation to be made of laboratory performance in terms of accuracy of results and also provided an opportunity to evaluate the reproducibility of the c-ELISA-IZSA&M kit, adding new information to its validation (5). The c-ELISA-IZSA&M method was also supplied to the Laboratory of Virology of the Centre of Athens Veterinary Institutions in Greece. A panel of 162 sera was tested using both methods and the results compared using Cohen’s K agreement index.

Materials and methods

Reference sera

A test panel comprised three sera: a strong-positive serum (from an infected animal), a negative serum (from an uninfected animal) and a weak-positive serum obtained by blending the positive and negative sera. Sera had been filtered through glass filters and 0.22 µm durapore® membranes. Sera (0.5 ml) were then distributed into vials and freeze-
dried. Homogeneity and stability of the product were then evaluated.

**Inter-laboratory testing scheme**

An identification number was assigned to all participating laboratories, each of which received 30 blind samples: 14 negatives, 10 weak-positives and 6 strong-positives. Each sample was identified using a unique code for each participant and was tested using both the c-ELISA-IZSA&M and a commercial kit. Results were entered on a standardised form and returned within 15 days of the despatch of samples.

**Statistical evaluation of results**

Results were analysed using a Bayesian approach (8). The beta distribution, based on the results from each laboratory, was calculated and used to express the probability of each laboratory to give a correct result and the uncertainty of this estimate, using the following formula:

\[ \text{Beta}(\alpha_1, \alpha_2) = \frac{1}{B(\alpha_1, \alpha_2)} \int_0^1 t^{\alpha_1-1} (1-t)^{\alpha_2-1} \, dt \]

Where

- \( \alpha_1 = \text{correct results} + 1 \)
- \( \alpha_2 = \text{tested samples} - \text{correct results} + 1 \)

The number of samples to be tested was chosen taking into account the statistical significance of the results. For this reason, 30 samples were despatched since a laboratory giving 30 correct results out of 30 has a 95% probability of providing correct results at least for 90.7% of tested sera. The comparison between the commercial c-ELISA and the c-ELISA-IZSA&M was performed using the McNemar \( \chi^2 \) test and Cohen’s K agreement index (7).

**Results**

Figures 1 and 2 show the estimates of the percentage of correct results of participants using both kits. Using the c-ELISA-IZSA&M, 24 of 27 laboratories obtained 100% correct results, whereas two laboratories obtained 29/30 correct results and one laboratory 28/30 correct results. Using the c-ELISA commercial kit, the results obtained ranged from 20 to 30 correct results. Results obtained using both assay methods are reported in Table I. The test results compared with the true status of samples are shown in Tables II and III. Reproducibility distributions are shown in Figure 3.

![Figure 1](image1.png)

**Figure 1**

Distribution of correct results of participating laboratories using the c-ELISA-IZSA&M kit

![Figure 2](image2.png)

**Figure 2**

Distribution of correct results of participating laboratories using the c-ELISA commercial kit

<table>
<thead>
<tr>
<th>Table I</th>
<th>Contingency table: c-ELISA commercial kit and c-ELISA-IZSA&amp;M kit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c-ELISA-IZSA&amp;M</td>
</tr>
<tr>
<td>c-ELISA</td>
<td>Negative</td>
</tr>
<tr>
<td>commercial kit</td>
<td>Positive</td>
</tr>
<tr>
<td>Total</td>
<td>379</td>
</tr>
</tbody>
</table>

c-ELISA competitive enzyme-linked immunosorbent assay  
IZAM Istituto Zooprofylattico Sperimentale, dell’Abruzzo e del Molise  
\( \chi^2 \) McNemar = 40.5 p<0.0001  
Cohen’s K agreement index = 0.88 p<0.0001
Table II
Contingency table: c-ELISA commercial kit and the true status of samples

<table>
<thead>
<tr>
<th>True status of samples</th>
<th>c-ELISA commercial kit</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>375</td>
<td>425</td>
</tr>
<tr>
<td>Positive</td>
<td>2</td>
<td>384</td>
</tr>
<tr>
<td>Total</td>
<td>377</td>
<td>809</td>
</tr>
</tbody>
</table>

c-ELISA competitive enzyme-linked immunosorbent assay

Table III
Contingency table c-ELISA-IZSA&M kit and the true status of samples

<table>
<thead>
<tr>
<th>True status of samples</th>
<th>c-ELISA-IZSA&amp;M kit</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>376</td>
<td>379</td>
</tr>
<tr>
<td>Positive</td>
<td>1</td>
<td>430</td>
</tr>
<tr>
<td>Total</td>
<td>377</td>
<td>809</td>
</tr>
</tbody>
</table>

c-ELISA competitive enzyme-linked immunosorbent assay
IZAM Istituto Zooprofilattico Sperimentale, dell’Abruzzo e del Molise

Discussion and conclusion

As shown in Figure 1, the performance of each of the participants was satisfactory when using the c-ELISA-IZSA&M kit. Due to sample size, the uncertainty of the estimate of correct results will be reduced further when other proficiency tests, analysing at least 120-150 serum samples, are performed. Laboratories failing one test result have a 83-99% probability of obtaining a correct result with a 95% confidence level. Therefore, a more appropriate evaluation of these laboratories will be possible only after a greater number of samples have been screened. The opportunity of performing repetitions of the same panel of samples meant that the reproducibility distribution of the c-ELISA-IZSA&M could be evaluated, thereby completing verification of the characteristics of the method and the sensitivity and specificity values calculated previously (100% and 99.1%, respectively) (5). In a given serology laboratory, proficiency testing and evaluation of results are usually performed by analysis of a single serum sample. In this study, the results obtained using a panel of different serum samples were combined so as to obtain an overall evaluation of participating laboratories. As far as the comparison between the two methods is concerned, although Cohen’s K agreement index shows almost perfect agreement between the two methods (K = 0.81), the McNemar test highlights a significant difference in the results (Table I).

The variability of results was higher when using the commercial c-ELISA kit as shown in Figure 2 and also the reproducibility of this test was less precise than that of the c-ELISA-IZSA&M kit (Fig. 3).

The results obtained by the Laboratory of Virology in Athens indicate that the c-ELISA-IZSA&M kit is sensitive. However, it is not possible to evaluate its specificity since the serum neutralisation test was not...
performed and the true sanitary status of donor animals was unknown.

**Acknowledgements**

The authors wish to thank all those who participated in this study, namely: Giuseppe Addis (IZS Cagliari), Nicola Cavaliere (IZS Foggia), Francesco Casalinuovo (IZS Catanzaro), Rita Catanzariti (IZS Taranto), Giuseppina Ciccarese (IZS Lecce), Renato Ugo Condoleo (IZS Latina), Pasquale Cossu (IZS Nuoro), Luigi De Grossi (IZS Viterbo), Gian Mario De Mia (IZS Perugia), Dario Deni (IZS Arezzo) Annalisa Guercio (IZS Palermo), Maria Guidoni (IZS Grosseto), Riccardo Forletta (IZS Pisa), Giuseppe Lucifora (IZS Cosenza), Luisa Marati (IZS Portici), Loretta Masoero (IZS Torino), Stefano Nardelli (IZS Padova), Annalisa Oggiario (IZS Sassari), Antonio Parisi (IZS Putignano), Vincenzo Quaranta (IZS Potenza), Elena Rocchegiani (IZS Ancona), Angelo Ruui (IZS Oristano), Giovanni Sala (IZS Brescia), Gianfranco Santagada (IZS Matera), Maria Teresa Scicluna (IZS Roma) and Elena Teneggi (IZS La Spezia).

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**References**


The first bluetongue virus isolation in Yugoslavia

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(2) Veterinary Scientific Institute, Vojvode Toze 10, Belgrade, Serbia and Montenegro
(3) VSI ‘Kraljevo’, Zicka 32, Kraljevo, Serbia and Montenegro

Summary

Catarrhal fever in sheep or bluetongue (BT) has not been recorded in Yugoslavia until recently. During the first incidence of BT disease in Serbia and Montenegro in 2001, the authors conducted field studies on suspected cases of the disease and collected samples for laboratory diagnosis. BT virus (BTV) was isolated and identified as serotype 9 by the Institute for Animal Health in Pirbright, United Kingdom (the Office International des Épizooties BT reference laboratory).

Keywords


Introduction

Catarrhal fever in sheep, or bluetongue (BT) is a disease that has only recently been detected in Yugoslavia. It is a viral disease of sheep, goats and cattle, to which deer are also susceptible. It appears to be enzootic, taking the form of infections with natural foci, and is transmitted by biting midges of the genus Culicoides (Diptera: Ceratopogonidae). The world-wide incidence of the disease is connected with the movements and spread of infected insects. Infected cattle rarely develop clinical signs and present viraemia for two to six weeks; consequently, they play a role in viral amplification. Immunoprophylaxis is an unpopular method of protection, prevention and eradication of the disease, so attention is focused on general measures, namely detection and removal of reactors, as well as attempting to control Culicoides. Correct diagnosis and timely detection is of vital importance in the control of the disease. During the first ever recorded occurrence of BT in Serbia and Montenegro in 2001, field studies were conducted to identify the disease and samples were collected for laboratory diagnosis. The virus was isolated and the results verified as BT serotype 9 by the Institute for Animal Health (Office International des Épizooties BT reference laboratory) in Pirbright, United Kingdom.

Materials and methods

Serum samples were collected from cattle and sheep. In addition, blood in heparin, spleen, lymph nodes, lungs, liver and kidney were collected from sick sheep (Figs 1 and 2).

Figure 1
Positive skin test for bluetongue

Blood sera were used for serological tests and washed blood cells were used for virus isolation. Parts of organs were used for virus isolation and preparation of frozen sections for fluorescent antibody examination. Virus isolation was carried out in 11-day-old embryonating chicken eggs, Vero cell cultures and newborn mice. Serum samples were tested using the competitive ELISA (c-ELISA) and immunodiffusion test.
Results

Serologically positive test results matched the clinical appearance of the disease in the outbreaks, as well as matching the results of the fluorescent antibody tissue section technique, and the appearance of cytopathic effect in Vero cell cultures (Fig. 3).

Conclusions

Standard virological and serological methods can be used successfully for the laboratory diagnosis of BTV. For a quick presumptive diagnosis, the c-ELISA method can be used for the detection of antibody against BTV. Diagnosis can be verified by virus isolation and fluorescent antibody tissue section techniques.

Additional reading

Frequency of serological cross-reactions between Ibaraki and bluetongue viruses using the agar gel immunodiffusion test

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(1) National Institute of Animal Health Japan, 3-1-4 Kan-non-dai, Tsukuba, Ibaraki 305-0856, Japan
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(3) Animal Hygiene Service Station of Saga Prefecture, 2-7-4 Wakakusu, Saga, Saga 849-0928, Japan

Summary

The frequency of serological cross-reactions between Ibaraki (IB), and bluetongue (BT) viruses using the agar gel immunodiffusion (AGID) test was investigated. The percentage of IB neutralisation-positive bovine serum samples that were positive to the BT AGID test was 42.5%; 12.2% of the BT AGID-positive serum samples and 2.5% of the BT AGID-negative serum samples were positive to the IB AGID test. When the BT competitive ELISA (c-ELISA) was used, these cross-reactions disappeared. These results indicate that serum samples from areas in which IB is epidemic are often positive against the BT AGID test, but negative against the BT virus neutralisation test (VNT). To obtain specific BT surveillance results in these IB endemic areas, the AGID-positive results should be confirmed using the c-ELISA or VNT.

Keywords


Introduction

Ibaraki (IB), a member of the epizootic haemorrhagic disease (EHD) serogroup, is endemic in Japan (5, 7). The agar gel immunodiffusion test (AGID) (13) is performed to survey for BT antibody in Japan. BT AGID-positive results are sometimes observed in areas of Japan in which IB is endemic and this makes interpretation of BT surveillance results difficult. Cross-reactions between bluetongue (BT) and EHD virus serogroups have been reported (9, 10, 11, 12, 14, 15, 16, 17). These cross-reactions were apparent in the serum samples from experimentally immunised animals (9, 10, 11, 14, 16). However, the frequency of cross-reactions of BT virus (BTV) or IB virus (IBV)-positive field samples against the IB and BT AGID tests is not clear (11, 17). The authors attempted to clarify the significance of BT and IB AGID test cross-reactions. Furthermore, we evaluated the cross-reaction between IB and BT in the competitive enzyme-linked immunosorbent assay (c-ELISA) (8, 14).

Materials and methods

Serum samples

Forty serum samples from serologically positive IB cattle were collected in 1997 from the Hyogo Prefecture in Japan to evaluate the serological cross-reactions with BT. In 1994, 202 serum samples were collected from serologically positive BT herds in the Tochigi Prefecture to check serological cross-reactions with IBV. IB was not epidemic in the Tochigi Prefecture. Of the 202 samples, 125 were from cattle and 77 from sheep. Of the cattle sera, 65 were positive for BTV neutralising antibody and 60 negative; of the sheep sera, 58 were positive for BTV neutralising antibody and 19 negative.

Serological tests

Sera from animals that were suspected of being infected with IBV were tested to determine the IBV and BTV neutralising antibody titres and the reactions to the BT and IB AGID tests. In addition, the percentage inhibition of the samples to the BT
c-ELISA was determined. Serum from animals that were suspected of being infected with BTV were tested to determine the BTV neutralising antibody titres, the reactions to the BT and IB AGID tests and the percentage inhibition of samples to the BT c-ELISA. The serological test procedures were as described in the OIE Manual of standards for diagnostic tests and vaccines (13).

**Virus**

BT virus serotype 21 or IB virus (EHDV type 2) were used in the virus neutralisation test (VNT).

**Results**

**Cross-reaction of Ibaraki-positive serum using the bluetongue agar gel immunodiffusion test**

Serum samples that were positive for IBV neutralising antibody had the following reactions:

a) IB AGID test: 90% (36/40)
b) BT AGID test: 42.5% (17/40)
c) BT c-ELISA: 2.5% (1/40)
d) BT VNT: 0% (0/40) (Fig. 1).

Thus, 42.5% of Ibaraki-positive serum samples, which were negative to the BT VNT, were positive to the BT AGID; only one of the IB-positive serum samples was positive to the BT c-ELISA.

As shown in Figure 2, only a few samples with low IBV neutralising antibody titres had cross-reactions on the BT AGID test; however, all of the samples that had NT titres of more that a 1:256 were BT AGID positive.

**Cross-reaction of bluetongue virus neutralisation-positive serum using the Ibaraki immunodiffusion test**

Of the 125 bovine serum samples, 60 were negative (<1:2) to the BT VNT and 65 were positive (>=1:2). One BT VNT-negative bovine serum sample and five BT VNT-positive bovine serum samples were positive to the IB AGID. The rates of cross-reactions were: 4.8% (6/125) in total; 7.7% (5/65) of the bovine BT VNT-positive samples were IB AGID-negative and 1.7% (1/60) BT VNT-negative serum samples were positive to the IB AGID (Table I).

![Graph showing cross-reaction rates](image)

**Table I**

Cross-reaction of bovine sera from bluetongue-infected herds using the Ibaraki agar gel immunodiffusion test

<table>
<thead>
<tr>
<th>BTV-negative serum 60</th>
<th>BTV-NT positive serum 65</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibaraki AGID Positive</td>
<td>Ibaraki AGID Positive</td>
</tr>
<tr>
<td>1</td>
<td>59</td>
</tr>
<tr>
<td>(1.7%)</td>
<td>(98.3)</td>
</tr>
</tbody>
</table>

BTV bluetongue virus
NT neutralisation
AGID agar gel immunodiffusion
Of the 77 sheep serum samples, 19 were BT VNT-negative (<1:2) and 58 were positive (>1:2). One BT VNT-negative ovine serum sample and ten BT VNT-positive ovine serum samples were positive to the IB AGID. The rates of cross-reaction of the ovine samples between BT VNT and IB AGID were 14.3% (11/77) in total; 5.3% (1/19) of the BT VNT-negative samples were positive and 17.2% (10/58) of ovine BT VNT-positive serum samples were positive to IB AGID (Table II).

Table II
Cross-reaction of ovine sera from bluetongue-infected herds using the Ibaraki agar gel immunodiffusion test

<table>
<thead>
<tr>
<th>BTV-negative serum 19</th>
<th>BTV-NT positive serum 58</th>
<th>Ibaraki AGID</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV AGID Positive</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Negative</td>
<td>(5.3%)</td>
<td>(17.2%)</td>
</tr>
<tr>
<td>IB AGID Positive</td>
<td>18</td>
<td>48</td>
</tr>
<tr>
<td>Negative</td>
<td>(94.7%)</td>
<td></td>
</tr>
</tbody>
</table>

BTV bluetongue virus  
NT neutralisation  
AGID agar gel immunodiffusion

Table II
Cross-reaction of ovine sera from bluetongue-infected herds using the Ibaraki agar gel immunodiffusion test

The agreement between BT VNT and both the BT c-ELISA and BT AGID test was 88.1%, and the agreement between BT c-ELISA and BT AGID was 98.0% (Fig. 3).

Figure 3
Agreement rate between bluetongue c-ELISA and bluetongue agar gel immunodiffusion classified by bluetongue virus neutralisation titre

Discussion

Cross-reactions between the BTV and EHDV serological tests have been documented for serum samples from immunised animals. A high incidence of BT AGID-positive serum samples from North American wild ruminants has been reported (12, 17); these positive results may be due to EHDV infection (9). However, the cross-reactions of field samples to the IB and BT AGID tests is unclear (11). Our results indicated that serum from IB VNT-positive animals were frequently positive to the BT AGID. Furthermore, serum samples with low IBV neutralising antibody titres showed cross-reactions to the BT AGID test. IB-positive serum samples were rarely positive to the BT c-ELISA. These results indicate that a more specific test, such as c-ELISA, should be used for BT surveys in EHDV-endemic areas. As the serum samples with high neutralising antibody titres (more than 1:256) cross reacted with the BT AGID, more cross-reactions can be expected following an epidemic of IB when the antibody titres would be expected to be higher.

BT-positive serum samples quite frequently were positive to the IB AGID but there were fewer IB-positive samples using the BT AGID test. These results indicate that cross-reactions between IB and BT on AGID do occur. As previously reported (1, 2, 3, 4, 6, 14), results of the BT c-ELISA were very similar to the results of the BT VNT and the results of the BT c-ELISA were more precise than the BT AGID (1, 2, 3, 4, 6). Although the agreement between c-ELISA and BT AGID was 98%, BT c-ELISA is still recommended for serological surveys to detect BTV infection in EHDV-endemic areas.

Conclusion

Specific serodiagnostic techniques, such as c-ELISA or BT VNT, should be used for BT surveillance in IBV-epidemic areas because IB-positive serum samples may result in false-positive BT AGID test reactions.

References


Field-deployable real-time polymerase chain reaction detection of bluetongue and epizootic haemorrhagic disease viral ribonucleic acid

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Summary

Nucleic acid sequence information from molecular evolution studies of bluetongue virus (BTV) and related epizootic haemorrhagic disease virus (EHDV) strains has resulted in a large database of genomic information. Published sequence data and sequence data from our laboratory were used to design real-time field-deployable reverse transcriptase-polymerase chain reaction assays for the detection of BTV or EHDV viral RNA. The assays used standard RNA extraction and TaqMan chemistries and the entire process was completed in \( \leq 3 \) h. The reaction conditions have been adapted to run on a field-deployable ruggedised advanced pathogen identification device (RAPID) instrument from Idaho Technologies, Inc. This instrument consists of a 50-lb (22.68 kg) backpack containing everything needed to run the assays. The current assays are specific for United States serotypes of BTV and EHDV; however, new designs based on new sequencing information are being evaluated to improve specificity and sensitivity for additional serotypes. This new technology greatly enhances the speed of virus detection and the ability to monitor disease outbreaks.

Keywords


Introduction

Rapid detection of bluetongue (BT) and epizootic haemorrhagic disease (EHD) viruses is important in disease outbreak situations as well as for determining disease-free status for animal exports. Many diagnostic tests have been developed for BT virus (BTV) and EHD virus (EHDV) including antibody, antigen and nucleic acid detection methods (14). Antibody detection indicates that an animal has been previously exposed to the virus and is not necessarily an indicator of viraemia. Antigen and nucleic acid detection methods (14). Antigen stimulation detection indicates that an animal has been previously exposed to the virus and is not necessarily an indicator of viraemia. Antigen and nucleic acid detection assays are more indicative of viraemia, but can detect residual non-infectious molecules from a recent infection. None of the available antigen or nucleic acid detection assays has been validated for all 24 serotypes of BTV or 8 serotypes of EHDV. Although antigen detections assays are very sensitive, inexpensive and reliable, they take longer to run and reagents are more difficult to develop than nucleic acid detection tests. Therefore, the development of a nucleic amplification-based assay for all serotypes of BTV and EHDV was necessary. Several nucleic acid-based assays have been reported (3, 4, 6, 11, 18, 22). The primary gene target for group-specific amplification has been M6 that encodes the non-structural protein 1 (NS1) because this gene is highly conserved within the serogroups (Table I). Detection of two gene targets is recommended because of the possibility of genetic variation in a single target gene. An alternative target is S10 that encodes NS3 because it is also highly conserved within the serogroups (10) (Table I). Serotype-specific polymerase chain reaction (PCR) assays are directed toward the L2 gene segment.

Differential detection of BTV and EHDV is required because of reports of serological cross-reaction between these viruses and because bluetongue-like disease in cattle has been associated with EHDV (Ibaraki virus) (16). Experimentally, United States strains of EHDV have not been
shown to cause disease in cattle (1); however, BTV does cause haemorrhagic disease in white-tailed deer (*Odocoileus virginianus*) (8, 9). In this report, we discuss progress in developing a single PCR-based assay to detect and differentiate the BTV and EHDV serogroups.

**Materials and methods**

**Sample collection and extraction of total/viral RNA**

Heparinised or ethylene-diaminetetra-acetic acid (EDTA)-treated sheep whole blood samples and frozen tissue samples from animals of varying age, gender, breeds and species were provided by veterinary animal hospitals located across the United States. When whole blood samples were used, 0.5 ml of whole blood was lysed with 1 ml of sterile water on ice for 10 min and then centrifuged for 5 min at 4°C at 12 000 rpm. The supernatant was removed and the pellet resuspended in lysis buffer provided in an RNAeasy mini kit (QiaGen, Inc.). The total RNA extraction was then completed as described in the instructions of the manufacturer.

**Multiplex-nested polymerase chain reaction**

The reverse transcriptase-PCR (RT-PCR) was completed as previously described, except methyl mercury hydroxide was used as a denaturant and the primer mixes contained four primers (20 pmoles each): two primers specific to the M6 gene of BTV and two primers specific to the M6 gene of EHDV (18, 19).

**Real-time polymerase chain reaction**

The real-time RT/PCR reactions were run using the one-step RT/TaqMan kit (Applied Biosystems, Inc.) as described by the manufacturer. The RT reactions were allowed to incubate for 30 min prior to a 10 min 95°C activation and PCR amplification (95°C for 15 sec and 60°C for 1 min) for 40 cycles. The BTV amplification primers were BTVM6+209F (GGCGAAAGCATACAAATTGGTT) and BTVM6-287R (TTTGAAACATTGAAGCCAGACTGT) and the probe was BTVM6-235P (6-FAM-CCCGATCATACATTGCTTCCTTTGCCA-TAMRA). The EHDV amplification primers were EHDM6-580F (TTTACCCAATGAGAAGGCCTAGAAT) and EHDM6-710R (GTATGGTAACATTTGCATTGGGAAA) and the probe was EHDM6-615C (6-FAM-CCACCGCTTCCCTTCAGCTTCCGACA-TAMRA). The

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**Table I**

Published polymerase chain reaction methods that are available for the detection of bluetongue virus RNA

<table>
<thead>
<tr>
<th>Type</th>
<th>Gene</th>
<th>No. of cycles</th>
<th>Detection method</th>
<th>Serotype</th>
<th>Source</th>
<th>Sensitivity</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>Single</td>
<td>2, 3</td>
<td>25-40</td>
<td>Gel, RE, hybridisation</td>
<td>1, 3, 9, 15, 16, 20, 21, 23</td>
<td>Blood, cell-culture</td>
<td>1 pg, 1 fg</td>
<td>12</td>
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<tr>
<td>Single</td>
<td>10</td>
<td>40</td>
<td>Gel, slot hybridisation</td>
<td>2, 4, 6, 10, 11, 13, 16, 17</td>
<td>Blood, semen, spleen, cell-culture</td>
<td>1 pg, 1 fg</td>
<td>2</td>
</tr>
<tr>
<td>Multiplex, single</td>
<td>2, 3</td>
<td>35</td>
<td>Gel</td>
<td>2, 10, 11, 13, 17</td>
<td>Insects Individual</td>
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<td>21</td>
</tr>
<tr>
<td>Nested</td>
<td>6</td>
<td>45, 45</td>
<td>ELISA</td>
<td>All 24</td>
<td>Blood, cell-culture</td>
<td>&gt;ECE</td>
<td>11</td>
</tr>
<tr>
<td>Nested</td>
<td>2, 6</td>
<td>35, 30, 30</td>
<td>Gel</td>
<td>1, 2, 3, 4, 6, 8, 10, 11, 12, 13, 17</td>
<td>Insects, cell-culture</td>
<td>≤1 pfu</td>
<td>22</td>
</tr>
<tr>
<td>Nested</td>
<td>3</td>
<td>40, 25</td>
<td>Gel</td>
<td>1, 11, 17, 23</td>
<td>Blood, frozen and fixed tissues</td>
<td>1 pg, 1 fg</td>
<td>17</td>
</tr>
<tr>
<td>NA</td>
<td></td>
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<td>ECE</td>
<td></td>
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</tr>
<tr>
<td>Nested</td>
<td>6</td>
<td>40, 40</td>
<td>Gel, hybridisation</td>
<td>2, 10, 11, 13, 17</td>
<td>Blood, cell-culture</td>
<td>0.1 fg ~5 particles</td>
<td>3</td>
</tr>
<tr>
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<td>6</td>
<td></td>
<td></td>
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<td></td>
<td>5</td>
</tr>
<tr>
<td>Nested</td>
<td>6</td>
<td>40, 25</td>
<td>Gel, tagged</td>
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<td>18</td>
</tr>
<tr>
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<td>40, 25</td>
<td>Gel</td>
<td>2, 10, 11, 13, 17</td>
<td>Semen</td>
<td>1 CCID 50/ml</td>
<td>20</td>
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RE restriction endonuclease digest
pg picogram
fg femtogram
ELISA enzyme-linked immunosorbent assay
ECE embryonated chicken eggs
pfu plaque-forming unit
NA not available
CCID cell culture infective dose
primers as probes were used at 5 µM for the EHDV assay.

**Virus isolation and antigen-capture enzyme-linked immunosorbent assay**

Virus isolation (VI) was conducted using standard cell-culture procedures. If there was no detectable cytopathic effect (CPE), some samples were passed up to four times prior to antigen detection. Antigen-capture enzyme-linked immunosorbent assay (ELISA) was used to verify the presence of BTV and/or EHDV on cell culture supernatant samples as described (13).

**Results and discussion**

Several diagnostic laboratories have successfully used the nested-PCR (nPCR) assays that we developed (18, 19). The nPCR was also found to be useful in the post-mortem detection of EHDV from white-tailed deer during an outbreak in Alabama (L. Li et al., unpublished data). In our original published nPCR procedures, we utilised formamide as a denaturant because it is a safe chemical denaturant. Methyl mercury hydroxide (MeHgOH), although hazardous, provides a 10-fold increase in sensitivity in the nPCR (data not shown). This denaturant can be safely used as long as a chemical fume hood is available. Our BTV and EHDV nPCR assays were designed to have different-sized final products. The sizes for a BTV-positive and an EHDV-positive product are 218 bp (base pairs) and 138 bp, respectively. We utilised MeHgOH as a denaturant and combined the BTV and EHDV primer pairs for a one-tube BTV/EHDV nPCR. The reaction conditions were identical to that described previously for this multiplex-nPCR (18).

The initial evaluation of this test was run on blood samples obtained from farmed deer in a BTV- and EHDV-endemic area (Table II). This was reflected by the high incidence of seropositive animals determined by competitive ELISA (c-ELISA) that was performed as described previously (15). Several animals were found to be positive for both BTV and EHDV RNA. Unfortunately, there was not sufficient sample to perform VI or to confirm the PCR results. Additional samples were obtained and subjected to the multiplex-PCR and serial passage VI (Table III). There was a good correlation between the PCR and VI; however, there were several samples that were positive by only one test. PCR-positive/VI-negative samples probably reflect the presence of residual RNA after virus has been cleared or improper storage of the sample prior to testing. The PCR-negative/VI-positive may be due to primer interactions reducing the sensitivity of the assay or the presence of PCR inhibitors in these RNA samples. Sequence variation could also account for the negative PCR result but this was not investigated. Overall the test appeared to be useful for rapid detection of United States BTV and EHDV serotypes; however, it was found to produce unreliable results with serotypes that are exotic to the United States.

The lack of detection of exotic serotypes and concerns of potential contamination problems with nPCR assays prompted investigations of real-time RT-PCR methodology. New primers for the detection of both BTV and EHDV M6 genes using TaqMan chemistries were designed using Primer Express (Applied Biosystems, Inc.). TaqMan chemistry is advantageous because it uses a single amplification and an internal fluorescent probe that enhances specificity and sensitivity. In addition, different detection dyes can be used so that the assay can be easily multiplexed. Once the primer and probe concentrations were optimised, a re-evaluation of the dsRNA denaturising procedure was done. As with the nPCR, the use of MeHgOH as a denaturant increased sensitivity 10-fold (Table IV). Therefore, the sensitivity of the assays were then determined using MeHgOH as a denaturant. The initial evaluation was performed on an ABI 5700 block-type sequence detection system and easily detected 1 pg of BTV RNA. The test was then optimised for a field-deployable capillary type sequence detection system. This system called ruggedised advanced pathogen identification device (RAPID) was designed to contain everything needed to run real-time PCR pathogen detection in a backpack (Idaho Technologies, Inc.). The sensitivity with this system was identical to the ABI 5700 (Fig. 1). The BTV real-time PCR was evaluated using total RNA from indigenous and exotic BTV serotypes. Only 58% of the serotypes were detected (Table V). Although preliminary real-time assays did detect all United States serotypes, BTV-2 occasionally was below the threshold. This test may be useful as a quantitative test for experimental purposes, but new primers need to be selected for a useful diagnostic test.

Similarly, the EHDV real-time PCR detected both United States serotypes, but only detected two of the six EHDV serotypes exotic to United States. The sensitivity of the EHD real-time PCR was 5 pg of RNA. When tested against a panel of United States BTV and EHDV field isolates, the EHDV real-time assay detected all but two of 43 EHDV strains and did not detect the BTV strains. Therefore, the EHDV real-time PCR could be used in the United States, but re-design is needed for a truly group-specific test for all serotypes. To that end, we have sequenced the M6 and S10 gene of 22 of the
Table II  
Results of the multiplex nested-polymerase chain reaction for bluetongue virus and epizootic haemorrhagic disease virus RNA detection compared to c-ELISA results

<table>
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<th>Sample</th>
<th>Species</th>
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<th>BTV ELISA</th>
<th>EHD ELISA</th>
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<td></td>
<td></td>
<td>BTV</td>
<td>EHDV</td>
<td>Inhibition (%)</td>
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<td>1</td>
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<td>+</td>
<td>96.6</td>
<td>0.3</td>
</tr>
<tr>
<td>2</td>
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<td></td>
<td>98.1</td>
<td>0.4</td>
</tr>
<tr>
<td>3</td>
<td>White-tailed deer</td>
<td>+</td>
<td>87.4</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
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<td>+</td>
<td>97.7</td>
<td>1.3</td>
</tr>
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<td>5</td>
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<td>0.8</td>
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<td>8</td>
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<td>4.9</td>
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<td>41</td>
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<td>74.0</td>
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PCR: polymerase chain reaction  
BTV: bluetongue virus  
ELISA: enzyme-linked immunosorbent assay  
EHD: epizootic haemorrhagic disease  
SD: standard deviation  
+ visible RT/PCR product by agarose gel electrophoresis  
% inhibition is the percent blocking of virus-specific monoclonal antibody to a bound antigen  
NA: not available
<table>
<thead>
<tr>
<th>Sample</th>
<th>Species</th>
<th>Tissue</th>
<th>State</th>
<th>BTV</th>
<th>EHDV</th>
<th>Virus isolation</th>
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<td>W9473</td>
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<td>W9501</td>
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<td>Wyoming</td>
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<td>–</td>
<td>+</td>
<td>+</td>
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<td>–</td>
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<td>+</td>
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<td>+</td>
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<td>–</td>
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<td>ABADRL-13</td>
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<td>+</td>
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<td>CC194-98</td>
<td>White-tailed deer</td>
<td>Blood</td>
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<tr>
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<td>Lung</td>
<td>Tennessee</td>
<td>–</td>
<td>–</td>
<td>EHDV-2</td>
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</tbody>
</table>

**BTV**  bluetongue virus  
**EHDV**  epizootic haemorrhagic disease virus  
+ visible reverse transcriptase-polymerase chain reaction product by agarose gel electrophoresis  
? faint
Table IV
Denaturant effect on the real-time polymerase chain reaction sensitivity for bluetongue virus RNA detection from inoculated blood

<table>
<thead>
<tr>
<th>Bluetongue virus titre</th>
<th>Methyl mercury hydroxide denaturant</th>
<th>Formamide denaturant</th>
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<tr>
<td>10² pfu/ml</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>10³ pfu/ml</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>No template control</td>
<td>Negative</td>
<td>Negative</td>
</tr>
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</table>

pfu plaque-forming units

Table V
Detection of bluetongue virus RNA by real-time polymerase chain reaction

<table>
<thead>
<tr>
<th>Serotype</th>
<th>CT</th>
<th>Serotype</th>
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<tr>
<td>BTV+</td>
<td>21</td>
<td>NTC</td>
<td>Negative</td>
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<tr>
<td>BTV-1</td>
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<td>BTV-15</td>
<td>Negative</td>
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<tr>
<td>BTV-2</td>
<td>Negative</td>
<td>BTV-16</td>
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<tr>
<td>BTV-14</td>
<td>Negative</td>
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</table>

CT cycle threshold
BTV bluetongue virus
NTC no template control
EHD epizootic haemorrhagic disease
Positive detection denoted by the cycle threshold value

Figure 1
The sensitivity of the real-time polymerase chain reaction for bluetongue virus RNA determined using the ruggedised advanced pathogen identification device instrument

The line colour and samples are designated in the left hand panel
Line 1 is the no template control and is not shown because it was below the light green threshold line
Purified BTV serotype 10 dsRNA was used and -# indicates the ng of dsRNA in each sample
BT10NS1 is a BTV-10 NS1 cDNA
The cycle times where the samples crossed the threshold line are noted

24 serotypes of BTV and are currently sequencing those genes for all the EHDV serotypes. The intent is to use two gene targets because of the potential of genetic variation in a primer-binding region. The BTV real-time PCR primer design has been completed but has not been evaluated.

The real-time PCR assays can be run in 2-3 h and can provide quantitative as well as qualitative information. The presence of viral RNA does not necessarily indicate active viraemia so positive results should be confirmed by VI or antigen detection. Real-time PCR should be very useful in monitoring the movement of virus during disease outbreak situations and as a pre-screening tool before using more labour-intensive and time-consuming assays.

Acknowledgements
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References


Genetically engineered structure-based vaccine for bluetongue disease

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Summary

At present the only vaccines used against bluetongue (BT) disease are live-attenuated virus vaccines. Since viruses with RNA genomes such as BT virus (BTV) have a high frequency of mutations, live virus vaccines could have breakthroughs (vaccine failures) and mutate into virulent strains. In addition, multiple BTV serotypes exist in nature which could potentially cause additional problems with live virus vaccines. The BTV genome is made up of 10 segments and therefore potentially could exchange these segments (or genes) randomly between different viruses including vaccine strains, generating novel viruses with mixed genes. Hence it is necessary to develop BTV vaccines that pose no such threat. Ideally BTV vaccines should be completely devoid of harmful genes. Recent protein expression technology has provided novel approaches for the development of intrinsically safe vaccines. The technology involves the synthesis of immunogenic proteins and particles that elicit highly protective immune responses. We have generated such vaccines, termed virus-like particle (VLP) vaccines. These vaccines (which do not carry either BTV or foreign genes) give the immune system information about viral structures so that it can generate a complete defence against the real virus infection very efficiently. A series of vaccine trials were undertaken outdoors under natural UV light using 50-200 BTV-susceptible sheep per trial. Vaccination trials of sheep showed that the VLPs were highly immunogenic, and protected sheep when animals were challenged with virulent virus even 15 months after the first immunisation. Moreover, a cocktail of five VLPs afforded protection against not only each of the homologous BTV serotypes but also against certain heterologous serotypes that are genetically related to some of these vaccine strains. VLPs representing a number of serotypes are currently available and can be produced fairly quickly if there is such a need. Based on our sequence data it can be predicted that a mixture of seven or eight types of VLPs (that are already available) will provide protection against at least 10 or more serotypes depending on their phylogenetic relationships. BTV VLPs offer particular advantages as potential vaccines over other systems. Large quantities of VLPs can be produced and easily purified using a one-step protocol based on the physical proprieties of the particle. More importantly, these particles are devoid of any nucleic acid and thus pose no threat by re-assortment or the re-emergence of virulence that attenuated vaccines can cause.

Keywords

arthropod and vertebrate cells, often causing severe disease and high mortality. BTV is transmitted by *Culicoides* spp., causing disease in ruminants in many parts of the world. To date, some 24 different BTV serotypes have been identified (BTV-1, BTV-2, etc.) from different parts of the world (6). In sheep, the disease is acute and mortality may be high, whereas in cattle and goats the disease is usually milder (6). In a typical case in sheep, the onset of the disease is marked by high fever lasting about 5-7 days. By 7-10 days, distinctive lesions appear in the mouth and the tongue can be severely affected, turning blue. In contrast to sheep, infected cattle experience prolonged viraemia and infection during pregnancy can cause teratogenic defects in calves and abortion of the foetus.

Other orbiviruses infect a variety of animals and also cause significant diseases, for example the recent outbreak of African horse sickness virus (AHSV) in Spain and Portugal. The spread of *Culicoides* insects from endemic to non-BTV and non-AHSV regions in the past highlights the concern that these viruses are a threat to areas that are presently free from viral infection. As a result of its economic significance, BTV has been the subject of extensive molecular, genetic and structural studies and now represents one of the most well characterised viruses (1). BTV virions are architecturally complex structures composed of seven discrete proteins (Fig. 1) in a specific but non-equimolar ratio that are organised into two shells, the inner core and outer capsid (1). The virion contains a genome of ten double-stranded (ds) RNA segments. The outer capsid is composed of two major structural viral proteins (VP2 and VP5) and is involved in cell attachment and entry of virus during the initial stages of infection (8). Shortly after infection, BTV is uncoated (VP2 and VP5 are removed) to yield a transcriptionally active core particle that is composed of two major proteins (VP3 and VP7), three minor proteins (VP1, VP4 and VP6) and the dsRNA genome (1). While the four major proteins (VP2, VP3, VP5 and VP7) form the bulk of the virus capsid, the three minor proteins, together with genomic RNA, form the virus replication complex. In addition to the structural proteins, non-structural (NS) proteins NS1, NS2, NS3 and NS3A are made in BTV-infected cells which are involved in virus replication and assembly (1).

Of the ten BTV proteins, only the two outer capsid proteins, VP2 and VP5 that are responsible for virus entry into susceptible host cells (8, 9), are variable from serotype to serotype (24 serotypes), although close phylogenetic relationships are easily detectable indicating that the genome mutations may have played a major role in generating multiple serotypes (17). In contrast to VP2 and VP5, all five core proteins and the three non-structural proteins are highly conserved (17). Both by *in vitro* (tissue culture) and *in vivo* (animal) studies it has been shown that BTV is highly capable of reassorting the RNA segments between different serotypes (1). Therefore, potentially, attenuated virus vaccines might play a significant role in the generation of endemic strains.

In the past, live-attenuated orbiviruses have been employed as vaccines in those regions of the world where BTV or AHSV cause epidemics of disease in livestock. Although reasonably effective, since vaccine strains are replication competent and since orbiviruses have segmented genomes, there is some concern over the use of live-attenuated virus vaccines. Live virus vaccines may aid virus maintenance in nature, and facilitate genome segment reassortment and the generation of new viral genotypes. Moreover, since there are multiple serotypes of BTV and AHSV, it is likely that not all vaccine strains would have the required level of attenuation. In order to develop rationally designed BTV vaccines, over the past few years an understanding has been developed of the structural and functional relationships of the BTV genes and gene products and the assembly pathway for the formation of virions. Recent advances in gene manipulation have made it possible to express foreign genes in heterologous systems. The productivity and flexibility of insect baculovirus expression vectors and the ability of the baculovirus genome to incorporate (and express) large amounts of foreign DNA in *Spodoptera frugiperda* insect cells have permitted this system to be used for the expression of not only a single gene, but also for the simultaneous expression of dual and multiple genes. To accomplish this, several expression vectors have...
been developed based on the resident promoters of the nuclear polyhedrosis virus of *Autographa californica* (AcNPV) (1, 2, 3). Using these various expression vectors, all ten genes of BTV have been expressed either individually or in various combinations using single, dual, triple and quadruple expression vectors and the structure-function of each gene and gene-product analysed (17). Information gained from these studies has served as platform for rationally designed safe vaccines for BTV and AHSV. Extensive clinical trials have been conducted using these proteins and protein structures, some of which are discussed below.

**Three-dimensional structures of BTV virion and core**

To design the virus-like particle (VLP) vaccine it is necessary to understand the structural organisation of the BTV capsid. Therefore, the three-dimensional structure of cores and virions were determined by cryoelectron microscopy (Cryo-EM) and computer image reconstruction methods which allow visualising the individual protein organisation in large particles such as viruses. The major advantage of Cryo-EM over conventional electron microscopic techniques is that the biological molecules are observed in a frozen hydrated state in amorphous ice, which closely resembles the native aqueous state. The problems associated with heavy metal stains, fixatives and dehydration are thus avoided.

The surface of the core serves as a foundation for deposits of the two outer capsid proteins, VP2 and VP5. Cryoelectron micrographs analysis has revealed a well-ordered morphology of the virion outer capsid. This is in contrast to the morphology deduced by negative-staining methods, which indicated that the outer capsid of the complete BTV particle has a fuzzy appearance (1). The capsid has an icosahedral configuration and the two proteins of the capsid have distinctive shapes, one globular and almost spherical, the other sail-shaped (Fig. 2a) (10, 11). The globular proteins, 120 in number, sit neatly in the channels formed by each of the six-membered rings of VP7 trimers. The sail-shaped spikes, which project 4 nm beyond the globular proteins, are located above 180 of the VP7 trimers and form 60 triskelion-type motifs that cover nearly all the VP7 molecules. These spikes are trimers of the viral haemagglutinating protein VP2, which also contains the virus-neutralising epitope, and the globular proteins are the trimers of VP5. The two proteins are attached to the surface of the VP7 layer and together they form a continuous layer (outer capsid) around the core except for holes on the fivefold axis. The structures of these two outer capsid proteins indicate that the formation of VP7 layer is essential for deposition of the outer capsid.

It was therefore necessary to examine the core structure. Cryo-EM analysis of BTV cores revealed that the core has a diameter of 69 nm and that the surface exhibits icosahedral symmetry with a triangulation number of 13 (Fig. 2b) (7). The core structure is divided into two concentric layers of protein enclosing the RNA and minor proteins. The surface layer of the core is made up of clusters of VP7 trimers, which bear prominent knob-like protrusions and which are organised into pentameric and hexameric units with channels between them (Fig. 2b, panel A). There are a total of 780 VP7 molecules per particle, 132 channels and 260 trimers or knobs at all the threefold axes. The smooth scaffold for the VP7 trimers is made up by 120 molecules of the second major protein VP3 (Fig. 2b, panel B) which is closely associated with the VP7 trimers. The remaining three minor enzymatic proteins, VP1, VP4, VP6 that are responsible for replication of the viral genome occupy the innermost component genomic RNA (Fig. 2b, panel C).

![An icosahedral complex whole virus particle viewed along a two-fold axis, showing the topography of the two outer capsid proteins, one globular-shaped (VP5) and the other sail-shaped (VP2) protruding 4 nm above the surface of the particles](image1)

![BTV core viewed along the icosahedral three-fold axis](image2)

![Surface representation of a cryoelectron micrograph of bluetongue virus](image3)

**Figure 2**

*Surface representation of a cryoelectron micrograph of bluetongue virus*
Assembly of bluetongue core-like and virus-like particles by baculovirus expression systems

Since VP2 and VP5 together induced a protective immune response in sheep, it is likely that the immunity would be enhanced if these proteins could be presented in a similar manner as in native virion particles. It would be rewarding if virus capsid structures without the genetic materials could be synthesised. The flexibility of baculovirus expression vectors and the capacity of the baculovirus genome to accommodate large amounts of foreign DNA enabled exploitation of the system for the simultaneous expression of multiple BTV genes in a single insect cell. Since 3D studies indicated that it might be possible to obtain a stable scaffolding core structure consisting of only the VP3 and VP7 which may allow eventually assembling VP2 and VP5 on the surface, dual and multigene baculovirus vector systems were prepared.

To assemble the VP3 and VP7, a dual baculovirus expression vector was utilised to express the coding sequences of the L3 (VP3) and S7 (VP7) genes of BTV (5). Recombinant baculoviruses synthesising both proteins were isolated and indeed core-like particles (CLPs) were produced and distributed throughout the infected insect Spodoptera cells. Gradient-purified CLPs were similar in size and appearance to cores prepared from BTV (Fig. 3). VP3 and VP7 were the only protein components identified in the expressed particles and the molar ratios of these two proteins were similar to those of VP3 and VP7 derived from infectious BTV. The CLPs appeared to lack nucleic acids when analysed by phenol-chloroform extraction and alcohol precipitation.

Subsequently baculovirus multigene vectors were constructed to co-synthesise up to four BTV proteins in the same cell (2). Two different expression cassettes were generated; one that expressed VP2 and VP5 simultaneously and the other that expressed VP2, VP3, VP4 and VP7 proteins in a single cell (1, 4). The expressed proteins from the quadruple vector assembled into virtually homogenous double-capsid particles (Fig. 3). Co-infections with two dual expression vectors (namely VP3/VP7 and VP2/VP5) gave VLPs that contained different amounts of the outer capsid proteins, depending on the experiment (4). The formation of complete VLPs in the absence of non-structural proteins or the internal minor proteins implies that these proteins are not necessary for the assembly of these double-capsid particles or for CLPs. VLPs express high levels of hemagglutination activity, similar to that of BTV virions. Furthermore, antibodies raised to the expressed particles gave high titres of neutralising activity against the homologous BTV serotype (4). When the 3D structure of CLPs and VLPs were analysed by Cryo-EM, both types of particles were clearly comparable to that of authentic cores and virions and exhibited essentially the same basic features and full complement of the two or four proteins (11, 14). VLPs synthesised by recombinant baculoviruses were also characterised further at the biological and immunological levels and compared to those of the native virion. VLPs exhibited high levels of haemagglutination activity similar to those of authentic BTV. Further antibodies raised to the expressed particles contained high titres of neutralising activity against the homologous BTV serotype emphasising their authenticity at a functional level (4).

Virus-like particles as vaccine

Since recombinant baculovirus-derived VLPs elicited strong neutralising antibodies in guinea-pigs, it can be anticipated that VLPs should elicit protective responses in sheep against BTV infection. Consequently, a number of experiments were performed to examine the protective efficacy of VLPs in sheep. In each experiment, BTV-susceptible, one-year old Merino sheep (BTV-free) were divided into groups, and each group was immunised subcutaneously with purified VLPs in saline containing various amounts of protein suspended in 50% Montanide Incomplete Seppic Adjuvant (ISA-50, Seppic, Paris). Each animal received 2 ml of the mixture. For each concentration of protein, a minimum of two sheep was used. For control experiments, one group of sheep received only saline. All vaccinated animals were boosted with the same amounts of protein on day 21. From the day of challenge to day 21, serum was collected from each animal at intervals and virus neutralisation tests were performed by plaque reduction neutralisation assay. Sheep that received VLPs developed demonstrable neutralising antibodies, albeit to different levels (15, 16). The levels of neutralising
antibodies depended on the amount of VLPs administered (Fig. 4). Significant levels of neutralising antibodies were elicited with all concentrations of VLPs and persisted throughout the study. The control sheep inoculated with saline remained seronegative. All sheep were challenged by subcutaneous inoculation of 1 ml of infective sheep blood containing virulent BTV-10 (South African strain) at day 117 (Fig. 4). The clinical reaction index (CRI) of the animals and viraemia were monitored from 3 to 14 days post-challenge (13). The challenged sheep neither developed clinical signs nor viraemias, indicating suppressed replication of BTV. The post-challenge blood samples of the sheep that only received saline were viraemic and these sheep developed high neutralising antibody titres indicative of a primary infection. In summary, protective immunity to BTV disease was obtained by vaccinating sheep with doses of 10 µg or more of BTV VLPs. The duration of protection obtained with only 10 µg VLPs was much higher than was obtained with the high doses (100 µg or more) of single (e.g. VP2) or dual antigens (e.g. VP2 and VP5) (data not shown) (17).

![Figure 4](image)

**Figure 4**
Vaccination trials of sheep with bluetongue virus serotype 10 virus-like particles
2-4 sheep were vaccinated with various doses of VLPs (1 ml) in the presence of 50% ISA-50 (1 ml)
Neutralising antibodies of sheep at various intervals after vaccination with bluetongue are indicated
VLPs and their protective responses following challenge (after 4 months) with homologous virulent bluetongue viruses

To analyse further the protective effects and duration of VLP vaccination, a similar protocol was employed for VLPs (10 µg or 50 µg per sheep) representing BTV-10 and BTV-17 (16). The neutralising antibody titres of the vaccinated sheep were determined at weekly intervals and over a sixty-week period after the booster. Both types of VLP elicited (to various levels) antibodies that neutralised the homologous virus. In almost all cases these neutralising titres remained high throughout the sixty-week period. The neutralising antibody titres for the animals that received 50 µg doses of VLPs were not significantly higher than those that received the 10 µg doses (Fig. 5). Sheep vaccinated with the mixture of the two types of VLPs induced antibodies that neutralised both types of virus as well as some related heterologous viruses (e.g. BTV-4) when tested by plaque reduction assays. As expected, the control sheep that were inoculated with saline remained seronegative. All the sheep were challenged 14 months after the booster vaccination by the subcutaneous injection of virulent BTV. The animals that were challenged with the homologous viruses (BTV-10, BTV-17) were completely protected and showed no clinical reactions, even those that received 10 µg doses of VLP (Fig. 5). Also, no viraemias were detected in these animals after challenge. In addition, some animals inoculated with 50 µg VLPs were also partially protected when challenged with heterologous virus. By comparison, the control animals developed high or moderate signs of disease (BTV-10, CRI: 7.1-8.0; BTV-17, CRI: 1.6-2.7) and produced viraemias. Similar vaccination trials with a cocktail of five different BTV VLPs (Fig. 6) representing five different serotypes were also undertaken. When these vaccinated animals were challenged with heterologous types that were not included in the cocktail, very encouraging data was obtained. There were clear indications of cross protection and such protection was dependent on both the amount of VLP in the vaccine dose as well as the sequence variation of the outer capsid proteins.

In summary, the data showed that long-lasting protection against homologous BTV challenge was
provided by vaccination with VLPs. Some preliminary evidence was obtained for cross-protection, depending on the challenge virus and the amounts of antigen used for vaccination (16).

Figure 6
Virus-like particle vaccination trials with a cocktail of bluetongue virus serotypes 1, 2, 10, 13 and 17 VLPs (10 µg each) in 50% ISA-50 given in each step, boosted after 21 days
The protection against various virulent virus challenges were determined as in Figures 2 and 4

Protection afforded by cores
BTV cores are conserved across the 24 serotypes. Therefore, it would be highly beneficial if synthetic CLPs could afford any protection against BTV infection. The question of whether CLPs containing the two conserved proteins, VP3 and VP7, would provide a measure of homologous and heterologous BTV protection by cell-mediated mechanisms was therefore investigated. For initial studies, two groups of five sheep each were used. One group of five sheep inoculated with 100 µg BTV-10 CLP in ISA-50 boosted on day 21 was challenged with BTV-10 two weeks later. All sheep developed viraeasias and neutralising antibodies after challenge (18). However, with the exception of fever, the vaccinated sheep developed only slight clinical reactions whereas controls showed characteristic mouth and feet lesions in addition to fever. The average CRI of the vaccinated sheep was 3.5 whereas that of the control sheep was 9.0 (Fig. 7). In summary, partial protection against BTV challenge was afforded by CLP vaccination.

Discussion
BT has been known to be associated with disease and mortality in sheep and cattle for decades. Despite the fact that this can have serious economic impacts, not only in terms of animal health but for some countries import and export regulations for sheep and cattle, only live-attenuated vaccines have been developed in South Africa and in the United States. In South Africa, sheep are vaccinated with three different pentavalent live-attenuated virus vaccines at three-week intervals. Conventional live-attenuated virus vaccines have certain inherent disadvantages. In the case of BT, such virus vaccines can cause infection in the foetus with teratological consequences. When used as a polyvalent vaccine, interference occurs between the component BTV serotypes, resulting in the development of incomplete immunity. Moreover, live-attenuated vaccine strains may be neutralised passively by the antibody in maternal colostrum.

Recent developments in biotechnology have made it possible to synthesise double-shelled BTV-like particles, mimicking authentic virions but lacking the harmful genetic material and viral replicating machinery. Therefore, these particles are as safe as subunit vaccines, and potentially as effective as ‘whole’ virus vaccines. A number of vaccination trials in BTV-susceptible sheep were undertaken. The results clearly demonstrated that VLP vaccines are highly efficacious. A very small amount (10 µg) of VLPs (10-20% of the VLP mass is VP2, i.e. 1-2 µg of VP2 per dose), in the presence of appropriate adjuvant, protected the sheep against the disease. There are several possible explanations. Firstly, the conformational presentations of the relevant epitopes on VP2 probably mimic those on the authentic virus. Secondly, both VP2 and VP5 are present. Thirdly, VP3 and VP7 may provide a necessary scaffold for VP2 and VP5 antigen presentation. Fourthly, any of the four BTV proteins might have a direct role in eliciting cell-mediated immunity induced by the BTV VLPs. It can also be anticipated from the results obtained that this technology has much to offer for development of vaccines for both veterinary and human diseases.
Vaccines

VLPs offer particular advantages as potential vaccines over other systems. An additional advantage is that large quantities of these particles (BTV proteins) can be produced due to the high expression capabilities of baculovirus vectors (produced in serum-free medium), and purified using a one-step generic protocol based on the physical proprieties of the particle.

Complete sequence analysis of cDNA clones of viral RNA species, have demonstrated that both outer capsid proteins VP2 and VP5 are among the most variable proteins of different BTV serotypes. Depending on the serotype, they exhibit sequence relationships to other BTV serotypes (17). Data indicating that antigens of one BTV serotype (12) could neutralise other BTV serotypes and a mixture of two or five VLPs demonstrated no interference but protected against both homologous and heterologous virulent virus challenge. There is every reason to believe that VLPs representing only a few serotypes would afford protection against other serotypes in addition to the vaccine types and that not all 24 types would be needed to be present in the vaccine cocktail. This is an exciting prospect for the future.

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References

Bluetongue vaccination in Europe: the Italian experience

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Summary

The incursion of bluetongue (BT) into Italy in August 2000 caused heavy economic losses, partly due to the disease itself, but mostly because of disruption caused to the national animal trade structure. To limit direct losses and the circulation of BT virus (BTV), the Italian Ministry of Health ordered, on 11 May 2001, the vaccination of all susceptible domestic ruminant species (i.e. sheep, goats, cattle and water buffalo) in both infected and surrounding areas. The vaccination strategy was based on a risk assessment that suggested it would prevent direct economic losses and significantly reduce virus circulation. Vaccination of the target animal populations commenced in January 2002, prior to the epidemic peak of BT that began in July 2002. The proportion of vaccinated animals differed between the various regions and the varying levels of vaccination of these populations had clear consequences on the occurrence of clinical disease and the spread of BTV infection. In those regions where more than 80% of the target population were properly vaccinated, the disease disappeared almost completely and virus circulation was reduced significantly. The importance of this reduced circulation of BTV (i.e. infection did not spread from affected areas) was immediately obvious in areas affected by the less virulent BTV serotype 9 where, despite the virtual absence of clinical disease, trade of animals to other areas was prohibited. The areas affected by the highly virulent BTV-2 also benefited from vaccination because it eliminated clinical disease while animal movements were prohibited. The main consequence of the reduction of virus circulation after vaccination, as documented by serological surveillance, was a significantly reduced expansion of the areas that were subjected to animal movement restrictions. Subsequently, analysis of surveillance data, coupled with specific risk assessments, led to a progressive relaxation of movement restrictions even in areas where the infection was still present but where most of the population had been adequately vaccinated.

The effectiveness of the strategy used in Italy (i.e. vaccination of all domestic ruminants) was reinforced by extensive experimental and field studies. The aim of these studies was to:

a) evaluate levels of individual and herd immunity and resistance to challenge conferred by vaccination, and

b) quantify the frequency and severity of the adverse effects of vaccination on domestic ruminants.

Ongoing research has focused on the ability of vaccination to suppress or reduce viraemia in ruminants following natural challenge by a virulent BTV strain. These studies address the issue of safety of the trade and movement of vaccinated animals that originate from areas in which BTV continues to circulate and could justify the reversal in current policy that restricts the international trade of animals vaccinated against BT.

Keywords

Bluetongue– Cattle – Italy – Sheep – Vaccine – Vaccination.
In November 2000, after the incursion of bluetongue into Sardinia on 18 August (10), the Balearic islands on 29 September (2) and Corsica on 18 October (1), the European Union issued Directive 2000/75/CE (9) that fixed specific rules for the control and the eradication of BT. Specifically, the Directive stipulated the following:

a) demarcation of a protection zone with a radius of 100 km around outbreaks of BT or around any farm on which virus circulation was confirmed
b) establishment of a surveillance zone that extended 50 km around the protection zone
c) slaughter of animals to prevent the spread of the epidemic and to destroy, eliminate, burn and bury their carcasses
d) implementation of serological and entomological surveillance programmes in the protection and surveillance zones
e) ban on animal movements from protection to surveillance zones.

To complement these measures, the Directive foresaw the possibility of conducting a vaccination programme in the protection zone but, since the Directive contained no specific criteria on serological and entomological surveillance, each country could freely propose its own programme to the European Commission, taking into account specific national needs and geographical or livestock breeding conditions.

The application of Directive 2000/75/CE (9), through the adoption of Decision 2001/138/CE (4), disrupted animal trade in at least a third of Italy and, if sustained, would possibly have caused an irreversible decline in the cattle and smaller ruminants production sector.

Italy is a relatively small country (301 230 km²), smaller than other industrialised countries (such as the United States, Australia or South Africa) that have experience of BT or incursions of BTV. The small size of Italy makes it difficult to apply the movement restrictions prescribed by the Directive without extensive changes to the structure of the Italian livestock industry, which is characterised by different levels of specialisation, with individual phases of the production cycle performed in different regions. It rapidly became evident after demarcation of the protection and surveillance zones, which involved about one third of the country, that this designation would have prevented the slaughter of cows and the fattening of calves concentrated in the free zone in the north of Italy. In addition, it would have been impossible to continue the practice of transhumance, because the traditional pathways along which livestock are moved cross the surveillance zones. Transhumance has been practised since pre-Roman times and is therefore embedded deeply in the livestock culture of Italy.

Potential vaccination strategies for BT include (30):

a) vaccination of cattle only
b) vaccination of both sheep and cattle
c) vaccination of sheep only.

Vaccination clearly reduces the number of susceptible animals; therefore, fewer animals will become viraemic following infection. Vaccination of cattle may be a more effective control measure than the vaccination of sheep, as viraemic cattle are more common than viraemic sheep and viraemic cattle are frequently the source of BTV. Vaccination of sheep can be effective in reducing the number of cases of BT; indeed, the large-scale use of attenuated vaccines in South Africa and Israel has made sheep farming possible in areas where it was previously uneconomical.

The three control strategies adopted in Europe and in the rest of the world can be summarised as follows:

1) direct, rather than preventive, control measures, such as those adopted in Greece
2) vaccination of sheep only (adopted in the Balearic islands and in Corsica); this reduces the impact of the disease but has no impact on virus circulation between cattle and vectors
3) vaccination of all susceptible ruminant livestock (cattle, buffalo, sheep and goats); this is the approach adopted by the Italian authorities and is designed to interrupt the natural cycle of BTV infection.

The vaccination strategy adopted in Italy was based on a risk assessment which suggested that direct economic losses would be reduced or eliminated and virus circulation would be reduced significantly (15). The risk assessment showed that when at least 80% of the susceptible populations has been immunised, the number of secondary cases should be less than 1% of the number expected to occur in the absence of vaccination. Thus, it was predicted that vaccination of all ruminants would not only reduce virus circulation and consequently reduce the size of the restricted areas, but also reduce the duration of movement restrictions. Existing literature on the use of BT vaccines is exclusively related to sheep; no studies address the vaccination of cattle against BT. Therefore, the application of the strategy suggested by the risk assessment required preliminary experimental field trials.
This paper describes research conducted in Italy prior to the commencement of the BT vaccination campaign and provides the results of the campaign itself.

Preliminary studies and field trials

A number of preliminary studies were conducted prior to the commencement of the vaccination campaign and during the initial BT vaccination campaign itself. The objective was to investigate potential adverse side-effects of BT vaccination in cattle and sheep, the kinetics of the antibody response in vaccinated cattle and sheep, and the effect of vaccination on clinical BT and viraemia in animals exposed to wild-type BTV in the field. Some of these studies were performed in controlled conditions and others in the field. The field studies were performed only in infected areas so as to limit the risk of virus dissemination connected with field trials.

A phylogenetic tree was compiled from the VP2 gene sequences of the Italian, Greek, Israeli and South African BTV serotypes 2, 4, 9 and 16 (BTV-2, BTV-4, BTV-9 and BTV-16), reference and field isolates of BTV, as well as of VP2 gene sequences currently available on GenBank. The Italian isolates were obtained from different regions, species (cattle, goats, sheep and deer) over different years (2000-2002). Phylogenetic analysis showed that all the Italian BTV-2 isolates grouped together and they were also identical to the strain of BTV-2 isolated in Corsica. There was 96% identity between the European BTV-2 isolates and the BTV-2 South African reference and vaccine strains. The analysis of Italian isolates of BTV-9 showed clearly that these isolates were almost all identical to the BTV-9 isolates from 2001, and they were highly homologous (99%) to BTV-9 isolate from Greece. In contrast, the VP2 genes of Australian and European isolates of BTV-9 had only 89% identity and the two groups of isolates shared only 67% identity to the reference BTV-9 isolate from South Africa (26).

The modified live-attenuated monovalent vaccine against BTV-2 elicited complete protection against challenge with $10^{5.5} \text{TCID}_{50}$/ml of virulent homologous virus in cattle inoculated seven months after vaccination, without any detectable viraemia (23). The duration and titre of viraemia were also reduced in animals challenged 14 months after vaccination and viraemia in vaccinated animals was considered of insufficient titre to infect vector insects (G. Savini, personal communication). Despite the lower homology between vaccine and wild strains of BTV-9, vaccination protected sheep against challenge with $10^{5.5} \text{TCID}_{50}$/ml of virulent homologous virus of Italian origin, three months after vaccination. There was no detectable viraemia in vaccinated sheep after challenge (G. Savini, personal communication).

Median antibody titres in cattle two months after vaccination with monovalent vaccine BTV-2 were 1:160 (22). Median antibody titres in sheep, at 42 days post vaccination with BTV-2 monovalent vaccine were 1:42.5 (27) whereas titres at 42 days after vaccination with BTV-9 monovalent vaccine were as low as 1:5 (27). Cattle vaccinated with bivalent vaccine (BTV-2 and BTV-9) had median antibody titres of 1:160 against BTV-2, with 13% negative animals, and median antibody titres of 1:20 against BTV-9 with 23% negative animals (20).

Concerning the possible adverse effects of vaccination on reproduction, neither abortion nor teratogenic defects were observed in cattle immunised with the monovalent BTV-2 vaccine, either in controlled or in field conditions (18). Similarly, in a field trial in cattle, no adverse effect on reproduction was observed after vaccination with the bivalent BTV-2 and BTV-9 vaccine (16). The administration of monovalent BTV-2 vaccine (14) or bivalent BTV-2 and BTV-9 vaccine (19) to cattle in field conditions did not affect the quantity and quality (somatic cell count, protein and fat content) in milk. Similarly, the administration of monovalent BTV-2 vaccine to sheep in controlled conditions did not affect milk production (11); however, the administration of bivalent BTV-2 and BTV-9 vaccine to sheep caused a transient 30% decrease in production that persisted for about one week (24, 25).

Results of vaccination campaigns

Due to limited knowledge on the distribution of vectors and the epidemiology of BT in northern Mediterranean countries during the first epidemic of 18 August 2000-14 May 2001, coupled with a lack of the necessary vaccine doses, the Italian authorities adopted actions that mainly addressed monitoring of the disease and development of a surveillance system. A total of 6 869 outbreaks of BT were reported during this first epidemic. On 11 May 2001, following the collection of data and a risk assessment, the Italian Ministry of Health ordered the vaccination of all domestic ruminants susceptible to the infection (sheep, goats, cattle and buffalo) in infected and in areas at risk (17). Despite the Ministerial Order, virtually no ruminants were vaccinated during 2001. The result was another
6 807 outbreaks and 250 662 affected sheep, according to the risk assessment.

Vaccination of susceptible populations began in autumn 2002. However, in the majority of the regions and provinces involved, activity actually began in January 2002 (12) (Fig. 1). Vaccination in Italy was implemented using two different vaccines, according to the BTV types observed in the various zones: monovalent BTV-2 vaccine was used in Sardinia, Tuscany, and Latiun and bivalent vaccine with serotypes BTV-2 and BTV-9 was used in the regions of southern Italy. Zones in which vaccination was practised were modified according to the spread of infection during 2002 (Fig. 2).

When the new epidemic commenced in July 2002, the level of vaccination in susceptible populations varied greatly in the different regions. In July 2002, when the new epidemic peak commenced, 57% of the eligible animals had already been vaccinated (Fig. 1) but vaccination coverage in the various regions varied greatly (Figs 3-9). Approximately 90% of susceptible animals were vaccinated in Sardinia (Fig. 3) and Tuscany (Fig. 4) (97% in Sardinia and 87% in Tuscany) before the start of the new epidemic peak. In Basilicata, on the other side of Italy (Fig. 5) only 2% of the population was vaccinated before the start of the new epidemic peak, but 84% of the eligible population was vaccinated by the end of the year. In the other regions (Sicily, Latium, Calabria and Campania) (Figs 6, 7, 8 and 9), less than two-thirds of the population had been vaccinated by the end of 2002 (12).
Figure 3
Percentage of population vaccinated against bluetongue and monthly number of outbreaks in Sardinia

Figure 4
Percentage of population vaccinated against bluetongue and monthly number of outbreaks in Tuscany

Figure 5
Percentage of population vaccinated against bluetongue and monthly number of outbreaks in Basilicata

Figure 6
Percent vaccinated population and monthly number of outbreaks in Sicily

Figure 7
Percent vaccinated population and monthly number of outbreaks in Latium

Figure 8
Percentage of population vaccinated against bluetongue and monthly number of outbreaks in Calabria
The third BT epidemic commenced on 15 April 2002 and ended on 14 April 2003. During the 2002-2003 epidemic, infection due to both serotypes BTV-2 and BTV-9 spread to the province of Avellino (Campania) in July and to the provinces of Benevento and Caserta (Campania), Foggia and Bari (Apulia), L’Aquila (Abruzzo) and Isernia (Molise) in September. The only spread of BTV-2 to previously unaffected areas occurred in Massa (Tuscany) in September. The total number of outbreaks detected in the third epidemic was 427 in eight regions (Table I). The geographic distribution of the infection is presented in Fig. 10.

The different levels of vaccination had clear consequences on the occurrence of disease. In the two regions where approximately 90% of susceptible ruminants were vaccinated, clinical disease either disappeared (Tuscany, 158 outbreaks and 693 diseased animals in the 2001-2002 epidemic, 0 outbreaks in 2002-2003) (Fig. 4) or was reduced by a factor of 1/100 (Sardinia, 6 090 outbreaks and 239 178 diseased animals in the 2001-2002 epidemic, 10 outbreaks and 28 diseased animals in 2002) (Fig. 5). In the same regions, the spread of the infection was also substantially reduced by the vaccination campaign (Fig. 11). A clear demonstration of the efficacy of vaccination was shown in Sardinia where, in August 2003, a new epidemic due to BTV-4 occurred, causing

![Figure 9](image.png)

**Figure 9**
Percentage of population vaccinated against bluetongue and monthly number of outbreaks in Campania

![Figure 10](image.png)

**Figure 10**
Geographical distribution of the infection in Italy, 15 April 2002-14 April 2003

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of outbreaks</th>
<th>Total number of animals in infected flocks</th>
<th>Number of diseased animals</th>
<th>Number of dead animals</th>
<th>Number of slaughtered animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basilicata</td>
<td>59</td>
<td>5 145</td>
<td>223</td>
<td>96</td>
<td>–</td>
</tr>
<tr>
<td>Calabria</td>
<td>15</td>
<td>797</td>
<td>87</td>
<td>–</td>
<td>87</td>
</tr>
<tr>
<td>Campania</td>
<td>251</td>
<td>20 918</td>
<td>1 951</td>
<td>1 495</td>
<td>213</td>
</tr>
<tr>
<td>Lazio</td>
<td>14</td>
<td>1 702</td>
<td>44</td>
<td>37</td>
<td>–</td>
</tr>
<tr>
<td>Molise</td>
<td>13</td>
<td>2 781</td>
<td>5</td>
<td>8</td>
<td>–</td>
</tr>
<tr>
<td>Puglia</td>
<td>17</td>
<td>2 484</td>
<td>284</td>
<td>245</td>
<td>1</td>
</tr>
<tr>
<td>Sardegna</td>
<td>10</td>
<td>2 120</td>
<td>28</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Sicilia</td>
<td>53</td>
<td>12 304</td>
<td>1 076</td>
<td>1 092</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>427</td>
<td>47 404</td>
<td>3 531</td>
<td>2 953</td>
<td>305</td>
</tr>
</tbody>
</table>

Table I
Clinical outbreaks of bluetongue in Italy during the third epidemic, 15 April 2002-14 April 2003
850 outbreaks of BT in six weeks. This showed that the reduction in the circulation of BTV-2 was due to the immunity induced by the vaccine and not to the disappearance of the conditions conducive to spread of the virus.

Vaccination apparently did not significantly reduce the spread of either disease or infection in the five regions of central-southern Italy (Basilicata, Calabria, Campania, Latium and Sicily) where variable proportions of the eligible population were vaccinated before the beginning of the new epidemic. A total of 559 outbreaks were recorded in central and southern Italy prior to vaccination. In the 2001-2002 epidemic (after the introduction of vaccination), infection spread to two additional regions (Molise and Puglia) causing a total of 417 outbreaks. However, vaccination did limit direct losses in these regions, despite the spread of infection to neighbouring regions and a total number of outbreaks that was similar to the previous year. The number of outbreaks recorded in the 2002-2003 epidemic in the five regions of central and southern Italy was, in fact, significantly correlated to the level of vaccination achieved by each region at the end of July 2002 (Spearman’s \( \rho = -0.9150, p<0.0001 \)) (28).

It is concluded, therefore, that the vaccination of ruminants led to a progressive reduction of virus circulation and consequently of the zones in which movement restrictions were applied. In Sardinia, for example, a decrease in monthly seroconversion rates began in May 2002, when more than 90% of the susceptible population had been vaccinated (Fig. 12). The seroconversion rate was 6.8% in April 2002 and decreased to 4.8% by May, stabilising at approximately 4% in the following months, through to December 2002. The period between July and October when the monthly seroconversion rate fluctuated at around 4% corresponded to the epidemic peak of previous years. A further decrease in seroconversion rates occurred after January 2003 when the temperature was unfavourable to Culicoides activity and the second vaccination campaign commenced, with the monthly rate declining to 1% without any increase in April, the month in which the maximum seroconversion rate was recorded the previous year.

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The arrival and spread of BTV-4 to most of the island in August 2003 again halted exports from Sardinia.

![Figure 13](image)

**Animals exported from Sardinia to continental Italy, January 2002-June 2003**

Monitoring of the possible side-effects of the BTV vaccine (death, abortion, stillbirth), was based on the following:

a) sampling of animals to determine the presence of the vaccine virus

b) collection of information concerning the vaccination itself (type of vaccine damage and dates of vaccination on the farm).

Samples were submitted for laboratory tests for the detection of vaccine and field strains of BTV. During the first vaccination campaign, there were few notifications of undesired vaccine side-effects (i.e. number of holdings requiring veterinary intervention and the collection of samples); only 312 of 87 245 holdings on which vaccination was applied were affected, representing 0.16% of cattle herds and 0.50% of small ruminant flocks vaccinated in Italy. Even fewer were confirmed by laboratory diagnosis (47 holdings, which is equivalent to 0.01% of vaccinated cattle herds and 0.09% of vaccinated sheep and goats flocks) (3). These field results were later confirmed by experimental studies on abortion and the teratogenic effects of the vaccine (16, 18).

A comparison can be made with data collected between 1991 and 2001 in the United States by the Vaccine Adverse Event Reporting System (VAERS). During the study period, 1.9 billion doses of 27 different types of human vaccines were administered and the prevalence of adverse events was 0.01% (29).

**Discussion**

Data collected during the successful vaccination campaign against BT in Italy led to the amendment of European Union legislation and ultimately to the adoption of the following measures:

1) From 10 January 2003 (Decision 2003/14/CE) (6), the despatch of slaughter animals from infected to free areas was allowed, provided that vaccination coverage included at least 80% of susceptible animals in the province of origin and that a risk assessment had been made.

2) Decision 2003/218/CE (7) of 27 March 2003 introduced the concept of ‘risk’ into the European provisions and subdivided regions into areas of higher and lower epidemiological risk for BT. The decision, therefore, allows the despatch of live animals from the ‘lower risk areas’ where viral circulation has not been detected to all of the European Union, and the despatch of slaughter animals from ‘lower risk areas’ even those with active infection and from ‘higher risk areas’ where viral circulation has not been detected to free areas in the national territory. The latter is allowed only if the animals have been vaccinated at least 30 days prior to movement, they belong to a herd in which all the animals have been vaccinated, and transport occurs during daylight hours only. According to the Decision 2003/218/CE, the member state is free to demarcate ‘epidemiological relevant areas of origin’. In other words, on the basis of surveillance results, it can reduce or increase the protection zone to a radius of greater or less than 20 km and can evaluate the possibility of demarcating lower risk areas in higher risk territories.

3) All existing European Union legislation regarding the compensation of farmers was developed in relation to contagious diseases of OIE ‘List A’, mainly foot and mouth disease and hog cholera (classical swine fever). In the case of an outbreak of such diseases, the control strategy in Europe relies on the stamping-out of infected and in-contact animals and, since 1990, vaccination is only an ancillary control measure. The principal economic losses in such a scenario are direct, and result from the slaughter of infected and in-contact animals; any compensation for indirect losses would represent, according to the European legislation, a disturbance of the market. In the case of vector-borne disease, especially when vaccination is the principal control measure, direct losses are virtually negligible but indirect losses due to movement restrictions become substantial. Moreover, losses incurred as a result of movement restrictions also have an
impact on farmers in free areas who are dependent on animal movement for their livelihoods as well. This has only very recently been recognised (July 2003) by the European Commission with the enactment of Decision C(2003)2519fin (8), authorising Sardinia to compensate farmers who suffered indirect losses due to movement restrictions between 6 September 2000 and 31 December 2001.

Based on the results of trials that evaluated the levels of viraemia after natural BTV infection of vaccinated animals, as well as results of the vaccination campaign (conducted between January and May 2003), a further risk assessment will be conducted to evaluate the possibility of trading vaccinated animals from areas in which virus circulation is still active. This risk assessment will also be relevant to international animal trade and might serve as the basis for a revision of existing international standards on BT.

References


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Bluetongue control using vaccines: experience of the Mediterranean islands

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Summary

Following the emergence of bluetongue (BT) virus serotype 2 on the island of Corsica in 2000, annual monovalent vaccination campaigns of the ovine population were conducted between 2001 and 2003. Despite vaccination, outbreaks were reported from several areas in 2001, but since November 2001, the absence of clinical cases in Corsica proves that vaccination is effective. This experience in Corsica is examined and, using available data, compared to the situation and the vaccination strategies on other Mediterranean islands. In light of the expansion of a new serotype of BTV onto these islands, a Mediterranean information network for BT and other emerging diseases is proposed.

Keywords


As the ability to control vector populations of insects is limited, vaccination against bluetongue (BT) remains a very useful disease control tool. Four objectives can be sought when using this tool, as follows:
1) prevention of the establishment of BT virus (BTV) in an area
2) reduction of the number of clinical BT cases
3) eradication of BTV infection
4) immunisation of animals that will be introduced into the affected region.

After defining the objectives of BT vaccination, the following questions remain:
1) What level of vaccination should be reached?
2) Which species should be immunised?
3) Is the objective of eradication feasible?
4) When should vaccination cease?
5) How can the arrival of new serotypes be prevented?

In this review we will first present the example of BT in Corsica between 2000 and 2002. After a summary of the epidemic, the use of vaccination is evaluated and then compared with experience in the other islands of the western Mediterranean (the Balearic islands, Sardinia and Sicily) (Fig. 1).

Figure 1
The larger islands of the western Mediterranean
History of bluetongue in Corsica

2000 and 2001 bluetongue epidemic

Following the first description of the principal vector (*Culicoides imicola*) in Corsica at the beginning of October 2000 (6), 49 outbreaks of BT (involving approximately 12,000 sheep) were later recorded on the island, whereas BT did not occur in continental France (Fig. 2). Serological surveys showed that BTV spread throughout the island. Corse-du-Sud was more infected with a 41% morbidity rate and twice the seropositivity rate of Haute-Corse (24% vs 40% in cattle, 16% vs 38% in sheep). Sheep were vaccinated with homologous vaccine against BTV-2 during the winter of 2000-2001. On average, 78% of the sheep flocks were vaccinated. Nevertheless, 335 outbreaks of BT were recorded between July and November 2001 and Haute-Corse was more affected than Corse-du-Sud (7). The last outbreak of BT in Corsica with serotype 2 was recorded on 8 November 2001. The 2000 and 2001 data confirm that BT commenced late in 2000 and ceased after the onset of cooler weather in November. A relatively mild winter allowed the persistence of infection and outbreaks began again the following July.

![Figure 2](image1)

**Figure 2**
Spatial distribution of bluetongue cases in Corsica, 2000 and 2001
*Source: National Food Directorate (Direction générale de l’Alimentation: DGAL)*

Serosolectural prevalence in non-vaccinated cattle and sheep

A total of 113 cattle herds were sampled in spring 2002, and 48% of the animals were seropositive. Corse-du-Sud has a significantly higher prevalence (68%) than Haute-Corse (39%). The serological rate of infection increased in cattle between the winters of 2000-2001 and 2001-2002 (increase of 63% in Haute-Corse and 70% in Corse-du-Sud) with a stable difference between the two regions. The geographic distribution of samples influenced these results, as the seropositivity rates (>75%) were higher in coastal areas and lower (<25%) in the mountains (Fig. 3). At this stage of the epidemic, a substantial portion of the cattle population (32% in Corse-du-Sud and 61% in Haute-Corse) was still susceptible (seronegative) and so could play a role in virus multiplication and dissemination. Nevertheless, the highest proportion of seronegative cattle occurred in the central areas of Corsica where the altitude is high and vector abundance is limited. The more affected areas in 2000 and 2001 were those with more seropositive cattle. The amplifying role of cattle in high altitude areas would, therefore, seem to be limited.

![Figure 3](image2)

**Figure 3**
Seropositivity against bluetongue virus in cattle in Corsica in 2002
*Source: National Food Directorate (Direction générale de l’Alimentation: DGAL)*

Assessment of bluetongue virus circulation through serological analyses in sentinel cattle

Despite the vaccination campaign, circulation of BTV was observed in sentinel herds of cattle in 2002. Although the percentage of seroconversion at herd level is similar, the raw numbers show that the circulation of BTV was higher in Haute-Corse (Table I). This has to be compared with the presence of *C. imicola* during eight months from May to December 2002. The principal vector of BT is now permanently established in Corsica. To summarise
the situation in France in September 2003, neither \textit{C. imicola} nor cases of BT have been found in mainland France and clinical BT due to BTV-2 has not been observed in Corsica since 8 November 2001. Vaccination was very effective in reducing clinical symptoms but BTV still circulates at a low level.

**Table I**

<table>
<thead>
<tr>
<th>Herd level</th>
<th>Corse-du-Sud</th>
<th>Haute-Corse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of herds</td>
<td>35</td>
<td>48</td>
</tr>
<tr>
<td>Percentage of herds with at least one seroconversion</td>
<td>37.1% (13/35)</td>
<td>64.6% (31/48)</td>
</tr>
</tbody>
</table>

**Evaluation of vaccination in Corsica**

The possible use of vaccination against BT was discussed by the European Union scientific committee on animal health and welfare on 27 June 2000, prior to the initial report of BT in Corsica. At the national level, the case was submitted to the official risk assessment institution, the French Agency for Food Safety (AFSSA: Agence française de Sécurité sanitaire des Aliments). The choice to vaccinate was largely based on the assumption that \textit{C. imicola}, which had been captured for the first time in Corsica, would survive the winter of 2000-2001. It was decided to vaccinate sheep, but not other species, with the South African attenuated monovalent BTV-2 vaccine as only BTV-2 had been isolated in the outbreaks of 2000 and 2001.

**Impact of vaccination on serological status of ruminants**

Serological status after the first vaccination campaign (2000-2001)

Assuming that there was no contact with vectors because of altitude, a sample of 13 herds (1 461 animals) vaccinated in February 2001 and 9 non-vaccinated herds (306 animals) was selected (4). These flocks went to summer pastures in 2001. Seven months after vaccination they were tested using the competitive immunosorbent assay (c-ELISA). The seropositivity rate was significantly different ($\chi^2 = 36.88, p<0.05$) (Table II) between the two populations. Some positive results in the vaccinated population were due to previous infection. The non-vaccinated population was taken as a control population to calculate the effect of vaccination. From the 1 461 animals vaccinated, 43\% were positive before vaccination and 275 became positive after vaccination (out of 831 negative animals before vaccination). Vaccination caused seroconversion of only 33\% of the seronegative animals (Fig. 4). This very poor result has been explained by the delay between vaccination and serology (7 months), the vaccination protocol (single vaccination of all sheep, regardless of age) and vaccination failure (i.e. excessive delay between vaccine reconstitution and injection). These results fully explained the poor protection of sheep herds during the 2001 epidemic.

**Table II**

<table>
<thead>
<tr>
<th>Sheep population</th>
<th>c-ELISA</th>
<th>Seropositivity rate</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinated transhumant population</td>
<td>905</td>
<td>556</td>
<td>62%</td>
</tr>
<tr>
<td>Non vaccinated transhumant population</td>
<td>132</td>
<td>174</td>
<td>43%</td>
</tr>
</tbody>
</table>

**Figure 4**

Effect of vaccination in transhumant sheep

Serological status after the second vaccination campaign (2001-2002)

The proportion of BT-seropositive animals is 91\% on average. The northern and southern areas of Corsica show the same results (Table III). The serological prevalence in 2002 is obviously very high and is the result of a more efficient vaccination campaign during the 2001-2002 winter, and also because of the very high levels of virus circulating during the 2001 epizootic. These data are consistent with the absence of clinical BT amongst ruminants in Corsica in 2002.
Effect of vaccination

A comparison of mortality and morbidity rates between vaccinated and non-vaccinated animals shows that non-vaccinated animals are more affected, which validates the decision to undertake massive vaccination in 2000 (Table IV).

Table III
Serological status of vaccinated sheep flocks in Corsica

<table>
<thead>
<tr>
<th>Flocks</th>
<th>Corse-du-Sud</th>
<th>Haute-Corse</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of flocks</td>
<td>57</td>
<td>50</td>
<td>107</td>
</tr>
<tr>
<td>Number of samples</td>
<td>1 118</td>
<td>1 058</td>
<td>2 176</td>
</tr>
<tr>
<td>Positive</td>
<td>91%</td>
<td>91%</td>
<td>91%</td>
</tr>
<tr>
<td>Negative</td>
<td>7%</td>
<td>7%</td>
<td>7%</td>
</tr>
<tr>
<td>Doubtful</td>
<td>2%</td>
<td>2%</td>
<td>2%</td>
</tr>
</tbody>
</table>

Table IV
Bluetongue outbreaks in Corsica in 2001

<table>
<thead>
<tr>
<th>Flocks</th>
<th>Haute-Corse</th>
<th>Corse-du-Sud</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected flocks</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total number</td>
<td>211</td>
<td>124</td>
<td>335</td>
</tr>
<tr>
<td>Sheep belonging to infected flocks</td>
<td>63 274</td>
<td>21 664</td>
<td>84 938</td>
</tr>
<tr>
<td>Vaccinated sheep in infected flocks</td>
<td>48 833</td>
<td>17 678</td>
<td>66 511</td>
</tr>
<tr>
<td>Morbidity rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected flocks</td>
<td>16.67%</td>
<td>14.18%</td>
<td>16.03%</td>
</tr>
<tr>
<td>Vaccinated animals belonging to infected flocks</td>
<td>6.06%</td>
<td>6.99%</td>
<td>6.31%</td>
</tr>
<tr>
<td>Non vaccinated animals belonging to infected flocks</td>
<td>52.52%</td>
<td>46.04%</td>
<td>51.12%</td>
</tr>
<tr>
<td>Mortality rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infected flocks</td>
<td>12.77%</td>
<td>12.43%</td>
<td>12.68%</td>
</tr>
<tr>
<td>Vaccinated animals belonging to infected flocks</td>
<td>4.65%</td>
<td>6.41%</td>
<td>5.12%</td>
</tr>
<tr>
<td>Non vaccinated animals belonging to infected flocks</td>
<td>40.22%</td>
<td>39.11%</td>
<td>39.98%</td>
</tr>
</tbody>
</table>

Source: National Food Directorate (Direction générale de l’Alimentation: DGAL)

Comparison with the other Mediterranean islands

The situation in Corsica is best compared to that of the other islands in the western Mediterranean where BT outbreaks due to BTV-2 were recorded, namely: Sardinia, Sicily and the Balearic islands (Fig. 5). The presence of C. imicola was confirmed in all of these islands except Ibiza (5). After one annual vaccination campaign from October 2000 to spring 2001, no clinical cases of BT were recorded in the Balearic islands (1). Only two annual vaccinations of sheep were necessary to obtain the same results in Corsica. In Sardinia, the vaccination coverage was between 94.58-99.97% and in Sicily between 22.93-93.90% in 2002 (2). In Sicily and Sardinia, clinical cases of BT were still recorded during 2002-2003 despite annual vaccination (10 and 53 outbreaks compared to 6 090 and 6 in 2001-2002, respectively) using the same attenuated vaccine against BTV-2. Three important factors may explain this contrast: First, the sheep population is much greater on Sardinia and Sicily than on the other islands (Table V). It is also possible that an ‘island effect’ played a role in the extinction of BTV infection in the Balearic islands as the susceptible population may not have been sufficient to maintain the infection; similar findings have been observed with other contagious diseases like measles (3). Second, the geographic position of the different islands has to be taken into account. The Balearic islands are located 200 km from continental Spain but 350 km from Sardinia, whereas Corsica and Sardinia are separated by only 12 km, and Sicily and continental Italy are almost connected. It is then probable that only the Balearic islands can be considered an independent entity from an epidemiological perspective. The close interaction between Sicily and continental Italy is illustrated by the presence of the same serotypes of BTV in Sicily and Calabria (BTV serotypes 2 and 9). Third, vaccination in the Balearic islands was implemented very rapidly whereas it took three months in France.

Figure 5
Bluetongue in the larger islands of the Mediterranean, September 2003

It is very important to evaluate the level of vaccination coverage that should be attained to eradicate disease, specifically the herd immunity threshold (HIT). An empirical evaluation gives 80% (Charles Nicolle’s law) but it has been shown that HIT calculation should be based on the evaluation
of the strength of spread of the disease measured by the basic reproduction ratio R0. Basically, if R0<1, the disease cannot persist in a population. Let R0 be the reproduction ratio in a population; to decrease R0 to a value below 1, a simple calculation indicates that the proportion of vaccinated animals should be more than 1-1/R0. In vector-borne diseases, several factors attributable to the vector will change the approach (biting rate, trophic preferences, survival rate, incubation period and vectorial competency). Consequently, even with high vaccination coverage, it is still possible that the virus will persist in small areas or in reservoir animals. There is then a risk of re-emergence when coverage declines. For instance, the very high coverage in Sardinia in 2002 (from 95% to 99%) did not prevent all outbreaks.

Table V
Bluetongue in the larger islands of the Mediterranean

<table>
<thead>
<tr>
<th>Island</th>
<th>Area (km²)</th>
<th>Sheep</th>
<th>Goats</th>
<th>Cattle</th>
<th>First</th>
<th>Last</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sardinia</td>
<td>24,090</td>
<td>3,283,000</td>
<td>283,000</td>
<td>230,000</td>
<td>18 August 2000</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Sicily</td>
<td>25,700</td>
<td>1,032,000</td>
<td>197,000</td>
<td>465,000</td>
<td>10 October 2000</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Corsica</td>
<td>8,682</td>
<td>137,000</td>
<td>24,800</td>
<td>55,860</td>
<td>18 September 2000</td>
<td>08 November 2001</td>
</tr>
<tr>
<td>Ibiza</td>
<td>572</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No outbreak</td>
</tr>
<tr>
<td>Majorca</td>
<td>3,639</td>
<td>334,775</td>
<td>19,228</td>
<td>46,934</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minorca</td>
<td>702</td>
<td></td>
<td></td>
<td></td>
<td>9 September 2000</td>
<td>27 November 2000</td>
</tr>
</tbody>
</table>

Conclusion
The experience in the Balearic islands, Corsica, Sicily and Sardinia showed that attenuated vaccine can safely be used to reduce the direct costs of BTV infection, i.e. morbidity and mortality in sheep. Even when side effects were reported by farmers, investigations did not confirm them. In September 2003, the question for the French authorities was whether to stop vaccination after two years without any outbreaks of BT. However, this question has been negated by the occurrence of new outbreaks of BT due to serotype 4 in Sardinia. On 25 August 2003, outbreaks were reported in Sardinia and serotype 4 was confirmed on 15 September 2003. It was then decided to vaccinate animals in Corsica against serotypes 2 and 4. In this case, the vaccination strategy was to prevent the establishment of the new serotype in Corsica. This experience illustrates the need for regional collaboration to define optimal vaccination strategies. The importance of information about circulating serotypes has also to be stressed. For this purpose, it is proposed that a Mediterranean information network be established for BT and other emerging diseases.

References
The use of vaccination in the control of bluetongue in southern Africa

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Summary

The eradication of bluetongue virus (BTV) from endemic regions of Africa is virtually impossible due to the role played by widely distributed Culicoides spp. midge vectors and the ubiquitous distribution of vertebrate reservoir species. In endemic areas, attempts can only be made to limit the occurrence of bluetongue (BT) disease and its economic impact through vaccination. Despite several potential problems (teratogenicity, risk of reassortment, and reversion to virulence of the attenuated viral strains), the current live-attenuated vaccine, produced by Onderstepoort Biological Products (OBP), South Africa, has been used for decades in enzootic regions, and has been shown to provide a safe and efficacious means to control the disease in regions of southern Africa, as well as other areas of the world.

Keywords


Bluetongue (BT) virus (BTV) is the prototype species of the genus Orbivirus in the family Reoviridae. The viral genome consists of 10 double-stranded RNA segments that encode four non-structural (NS1, 2, 3 and 3A) and seven structural (VP1-VP7) proteins (31, 39). Currently, there are 24 known serotypes of BTV worldwide (25). BT is an Office International des Épizooties (OIE) ‘List A’ disease, and is thus of serious socio-economic concern and of major importance in the international trade of animals and animal products. BTV has been recognised as an important aetiological agent of disease in sheep in South Africa for over a century, and for many years was believed to be restricted to Africa south of the Sahara (14). However, since 1943, BTV has been identified in several countries outside Africa, such as Argentina, Australia, Bulgaria, China, Cyprus, France, India, Israel, Italy, Malaysia, Pakistan, Portugal, Spain, the United States of America (16) and, most recently, Kazakhstan (24). BTV commonly occurs between latitudes 35°S and 40°N, but the virus has also been detected further north beyond 48°N in Xinjiang, China, western North America and Kazakhstan (12, 24, 29).

Possible factors that have contributed to the spread of BTV include animal migration and importation, extension in the distribution of its major vector, Culicoides spp., involvement of newly identified or as yet unidentified vector(s), the apparent ability of the virus to overwinter in the absence of adult vectors, and its occurrence in healthy reservoir hosts, such as cattle and some wild ruminants. On account of the wide host range of BTV and its biological transmission by insects, control of BT in an endemic region is based primarily on the active immunisation of susceptible animals, as well as on the prevention or limitation of contact between the susceptible host and insect vectors.

Bluetongue endemicity in southern Africa

The enzootic nature of BTV in large regions of the African continent and more specifically southern Africa is supported by climatic factors that favour the maintenance and recirculation of the virus in its vertebrate and non-vertebrate hosts. Reservoir and amplifying hosts, such as game, cattle and goats, compounded by the ubiquitous distribution of suitable midge species, contribute to the persistence and transmission of BTV. In areas where the winter is mild, BTV may be transmitted throughout the year.
Most African indigenous sheep breeds are resistant to or show only mild clinical symptoms of BT, which is generally not considered serious in many sheep-rearing communities. This has resulted in the limited use of effective control measures, including vaccination, in many African countries despite evidence of BTV infection through serology and virus isolation (23). In South Africa, however, where the majority of the sheep population consists of originally exotic wool breeds, outbreaks of clinical disease are common and result in economical losses, either through direct mortality, or indirectly as a result of the loss in condition, compromised breeding efficiency and reduction in wool quality. Factors such as sheep breed susceptibility, variation within the breed, virulence of the virus strains, and year-to-year variation in climatic conditions, such as rainfall, make it difficult to envisage control measures other than vaccination. Due to the large number of circulating serotypes, it is generally impossible to predict the serotype for a specific season or area. Furthermore, several serotypes tend to circulate simultaneously (40).

In a surveillance programme initiated in 1979, BTV was detected in Culicoides midges from 12 different sites throughout South Africa (3). This study revealed that the total number of BTV serotypes isolated per season varied from 11 to 17, and varied in prevalence. In each season 2 to 5 serotypes dominated, but were replaced by different, highly prevalent serotypes the next year. These serotypes, which included BTV serotypes 1-8, 11, 12, 16, 19, and 24, generally had a high transmission potential. However not all the serotypes were highly pathogenic for sheep. It was speculated that serious outbreaks of BT were possibly caused by those serotypes possessing a high transmission potential as well as a high pathogenic index for sheep (3). In 1996, there were several outbreaks of BT and epizootic haemorrhagic disease (EHD) in South Africa, following heavy rainfalls. BTV serotypes 1-9 and 12 were isolated from sheep (G. Gerdes, personal communication) and serotypes 2, 3, 6, and 8 were isolated from cattle (4) in the same year, supporting the previous findings of the co-circulation of several serotypes during any BT season, and the long-term persistence of these serotypes in susceptible and reservoir hosts in an endemic area.

At present, 17 of the 24 known serotypes of BTV have been detected in South Africa. However, serotype 15 has only been isolated from sheep during an outbreak in 1976. Serotypes 1, 2, 3, 4, 6 and 10 are known to have a high pathogenic index and high epidemic potential.

**Historical background to the current Onderstepoort Biological Products bluetongue vaccine**

As it was clear at the turn of the last century that BTV was enzootic to South Africa, vaccination was recognised as a suitable means of controlling the disease. The attenuated blood vaccine developed by Theiler in 1906 (38) was used over almost 40 years, and was based on a virulent BTV strain (now known as serotype 4) that had been passaged until it lost its virulence. The realisation of the plurality of different BT serotypes that were involved in outbreaks, and safety concerns, brought Alexander to develop an embryonated egg-passaged quadrivalent lyophilised BT vaccine (2). Isolates of BTV were subsequently attenuated by 100 passages in eggs, and showed a reduction in the severity of temperature reactions and incidence of post-vaccination clinical disease in sheep. Immunogenicity of these isolates was further improved by using plaque selection or purification to select strains at lower egg-passage levels. Ten plaques were selected at random and screened in sheep for low pathogenicity and good immunogenicity. Seventeen of the then 20 known serotypes of BTV were shown to be present in South Africa. Since it was also known that several serotypes could be involved in an outbreak, the use of a polyvalent vaccine was imperative. A single vaccine containing 14 serotypes was then developed and used for a time (3, 13, 14). Around 1977/1978, serotype 19 was added to the vaccine due to an outbreak in 1976 in the Orange Free State caused primarily by serotypes 18 and 19. However, this single dose, multivalent vaccine did not induce adequate protection in sheep to all serotypes (14). Later, the attenuation of the strains was further modified by a reduction in the number of egg passages, followed by plaque selection and further cell culture passage (3).

Since BTV is an RNA virus and exists as a quasispecies, there is a variation in the presence of virulent and avirulent strains present at any one time. Passaging of the virus allows this ratio to change and thereby creates the opportunity for the selection of avirulent or attenuated virus. On the basis of the febrile reaction and incubation period in sheep, which correlated with the rate of replication of the viral serotypes, the current Onderstepoort Biological Products (OBP) vaccine was developed. The present OBP vaccine (Reg. No. G 358 Act No. 36/1947) comprises three bottles (vaccines A, B, and C) comprised of five serotypes each (Fig. 1) administered separately at three-week intervals. The selection of the serotypes included in the current vaccine was based on the prevalence and pathogenic
Vaccines

Figure 1
Serological cross-neutralisation of bluetongue virus serotypes
The serological cross-neutralisation between 23 of the 24 known BTV serotypes is represented graphically, as well as the component serotypes of the three vaccine bottles (bottles A, B and C) of the live-attenuated BT vaccine from Onderstepoort Biological Products, South Africa.

index of the prevailing serotypes in South Africa at the time, as well as the ability of these serotypes to provide adequate cross-protection to other less dominant serotypes, as assessed by cross-neutralisation and cross-challenge studies (B.J. Erasmus, personal communication). The specific combination of the serotypes in each vaccine bottle is based on the replication rate of the different serotypes, which correlates with the degree of attenuation. The slower replicating serotypes are given first. The vaccine strains presently used by OBP were originally obtained from clinical cases of BT, and only serotype 10 of the original vaccine developed by Howell is still contained in the current vaccine.

**Immune response to bluetongue vaccine**

Studies have demonstrated that both the humoral and cellular immune responses play a role in immunity to BTV (20, 22). Both homologous and heterologous neutralising antibodies have been demonstrated, depending on whether the animals were experimentally inoculated simultaneously or sequentially with BTV. Cellular immune responses mediated by cytotoxic T lymphocytes (CTLs) generally give heterotypic protection, which is relatively short-lived. Although CTLs do not prevent virus infection, they act to clear the virus from an infected host, and have been shown to effectively clear homologous and heterologous serotypes of BTV (21). In addition, it has been shown that BTV-specific ovine CTLs are cross-reactive (37). More recently, it was shown that CTL recognition patterns in sheep are quite diverse, possibly due to the different distribution of CTL epitopes on different viral proteins (19). The NS1 and VP2 proteins are most frequently recognised by CTLs, and each contains more than one CTL epitope. The use of a multivalent whole live vaccine allows for a greater and more extensive induction of a CTL response due to the presence of multiple CTL epitopes. The heterologous cross-neutralisation between certain BTV serotypes that has been demonstrated by serological studies (Fig. 1), is further supported by partial sequence analysis of the VP2 (Fig. 2) and VP5 genes (C. Potgieter, personal communication), which shows a similar cross-relationship, and lends support.
to the broad-spectrum coverage that the existing vaccine components contribute to BTV serotypes occurring in South Africa.

**Bluetongue vaccine use and production in South Africa**

An average of eight million doses of the OBP tri-pentavalent vaccine is sold and used annually in South Africa. However, this only affords protection to approximately one third of the commercial sheep population in the country (Fig. 3). The Eastern Cape Province is the biggest consumer of the vaccine as it is home to the largest number of wool-producing sheep. A further one million doses are sold to some of the neighbouring countries annually. In southern Africa, sheep should be vaccinated from August to October with 1 ml of each of the three vaccine

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**Figure 2**

Phylogenetic relatedness of bluetongue virus serotypes

Phylogenetic tree of the cloned partial VP2 genes from reference and vaccine strains of BTV serotypes indicating a similar grouping as seen by cross-neutralisation (Fig. 1).

Courtesy: C. Potgieter, Onderstepoort Veterinary Institute
bottles at three-weekly intervals. The immunisation of ewes should commence 9-12 weeks before mating. However, it is not advisable to immunise pregnant ewes during the first half of pregnancy. Rams should be inoculated after the mating season. Lambs born of vaccinated ewes should be vaccinated at 6 months or older, or if administered earlier in heavily infected areas, they should be re-vaccinated at 6 months of age. A reasonable protection against most of the serotypes is achieved within 3 to 4 weeks after the last vaccine bottle is administered, but cannot be guaranteed in all vaccinated animals. Thus sheep must be vaccinated annually, to ensure adequate immunity to all serotypes.

Safety aspects of the Onderstepoort Biological Products bluetongue vaccine

All batches of vaccines are produced according to national and international guidelines, and extensively tested to comply with purity, safety, efficacy and potency standards. Nonetheless, there are several concerns regarding the use of live-attenuated vaccines for the immunisation of sheep against BT. One potential safety problem relating to the use of live vaccines is the release and transmission of attenuated virus strains into the environment, which may result in a reversion to virulence through reassortment with a wild-type strain. However, vaccine strains, which produce less than $1 \times 10^3$ pfu/ml of blood at the height of viraemia in test animals, and elicit neutralising antibodies, are selected for vaccine production. Viraemias below $1 \times 10^3$ pfu/ml are thus considered to be safe and will ensure that the virus is not transmitted by vector midges. Preliminary studies conducted at the OBP to determine the level of viraemia in sheep post vaccination have shown that for serotypes 1, 2 and 4, no clinical reactions, elevated temperatures or virus were detected. Animals vaccinated with serotypes 10 and 16 demonstrated no clinical reactions but had a mild and brief fever that persisted for 5-6 days. However, viraemia levels in sheep vaccinated with serotypes 10 and 16 ($1.25 \times 10^2 - 7.5 \times 10^2$ pfu/ml) peaked below the desired minimum of $1 \times 10^3$ pfu/ml of blood, and declined as the febrile reaction waned.

Wild-type BTV does not appear to be able to cross the placenta to cause teratogenicity, or the production of physical defects in offspring in utero. Previous cases of teratogenic defects in sheep attributable to BTV were related to the use of chick-embryo propagated BTV vaccine, and foetuses were shown to be most susceptible at 5 to 6 weeks in utero (34). Current vaccine strains are derived from virus propagated first on chick embryos, then plaque purified and adapted on cell culture. A study performed with Australian BTV serotype 23 has shown that BTV adapted to cell culture is capable of crossing the placenta and inducing teratogenesis (15). Live BTV vaccine strains may thus be responsible for spontaneous cases of BTV-induced malformation in both sheep and cattle. However, the Australian study was conducted using only serotype 23, which is not highly pathogenic or prevalent in South Africa. Different serotypes of BTV differ in their pathogenesis, transmissibility and growth characteristics. Although no study has yet been conducted, there is a possibility that different attenuated serotypes with different passage history will vary in their teratogenicity; this should be further investigated in the field. However, for safety reasons and as a precaution, it is thus advised that pregnant ewes are not vaccinated in the first half of pregnancy. If an annual vaccination programme is implemented, as is advised, then both ewes and foetuses should be adequately protected during pregnancy. All lambs should be vaccinated at six months of age.

The 24 BTV serotypes have been shown to have considerable strain variation in the different gene segments within each serotype (5, 6, 7, 8, 9, 10, 17, 28, 30, 41). The reassortment of gene segments has been reported among strains of BTV (9, 18, 27, 32, 33). Recently it was demonstrated that variation of gene segments encoding the VP2 and NS3/NS3A proteins also occurred through genetic drift (7). As BTV is an RNA virus, it is likely to exist as a heterogeneous population of closely related variants characterised by one or more dominant nucleotide sequence(s) (quasispecies) (11). Arthropod-borne RNA viruses generally evolve more slowly than do non-arthropod-borne RNA viruses, most probably because of the restrictive pressures imposed during
alternating passing in their vertebrate and invertebrate hosts (35). Nonetheless, the quasispecies trait bestows significant adaptive ability on RNA viruses, through the selection of mutants with highest fitness in a new environment, allowing for rapid evolution (26).

Although the reassortment of BTV genes has been demonstrated in the laboratory, only rarely has it been reported to occur in the field (36). Despite being an RNA virus, BTV is relatively stable and thus reassortment events are likely to be rare – unless driven by a specific or environmental factor. The risk of reassortment in the field is minimised by the long interval between the recommended vaccination period (late winter, early spring) and the BT season (summer), which would make the incidence of co-circulating vaccine and virulent wild-type viruses highly unlikely. During reassortment, progeny viruses receive one of each of the genome segments, but probably not from a single parent. Thus in the case of BTV which has 10 segments, in a mixed infection of only two serotypes there could be \(2^{10} = 1024\) possible progeny viruses. Where more serotypes are present, the possible reassortment combinations rapidly increase. Due to the high recombination possibilities, the mixing of a wild-type virulent and an attenuated vaccine strain is highly unlikely to result in the generation, and persistence, of a new virulent strain, or its subsequent effective transmission via the insect vector, and persistence and survival particularly where it competes with existing virulent wild-type strains.

In an epidemiological surveillance study conducted in the Balearic islands, BTV from cattle and goats was specifically found by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis to be a field virus, while that from vaccinated sheep was a vaccine strain (1). This finding supports the highly unlikely possibility of midges transmitting vaccine viruses from vaccinated to unvaccinated animals under field conditions.

**Conclusion**

The eradication of BTV from endemic regions of Africa and certain parts of the world is impossible due to its ubiquity, broad host range, the multiplicity of serotypes that may be circulating at any point in time, and the role played by the widely distributed Culicoides vectors.

The vaccination of sheep with the OBP live-attenuated polyvalent vaccine is presently still the most effective and practical control measure against BTV in South Africa, as has been demonstrated by laboratory and field trials, and the extensive use of the vaccine over many years.

**References**

Vaccines


Bluetongue control using vaccines: the experience of Emilia Romagna, Italy

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Summary

In 2003, thirty municipalities of the provinces of Parma, Reggio Emilia and Modena in the Emilia Romagna region of Italy, bordering the region of Tuscany, were included in the national bluetongue (BT) vaccination programme, using monovalent live-attenuated type 2 vaccine. The purpose of the study was to evaluate the organisation of a vaccination programme designed by the Regional Veterinary Service and the relative cost of the campaign, as a large number of animals were involved. To better evaluate the real cost of the campaign, costs sustained by the Reggio Emilia Local Sanitary Unit were specifically analysed. BT vaccination of all domestic ruminants is a very expensive operation (€9.20 per vaccinated animal). Consequently, to evaluate the need for a vaccination campaign in a new area, the risk of disease spread, as well as the cost of the operation, should be considered.

Keywords


Introduction

A national vaccination programme against bluetongue (BT) commenced in 2001 and involved several regions of Italy. The third annual vaccination campaign has now been completed. The objectives were to protect sheep and goats from clinical illness and to create an immune population of domestic ruminants to stop further circulation of BT virus (BTV). Vaccination of at least 80% of the susceptible population was considered necessary. The region of Emilia Romagna became involved in the national vaccination programme in 2003: thirty municipalities in three provinces (Parma, Reggio Emilia and Modena) bordering the region of Tuscany were included, using monovalent BTV-2 live-attenuated vaccine. These prophylactic measures were designed to create a barrier to the possible spread of BTV to northern Italy, currently still free from infection. The results of the regional vaccination campaign are presented in Table I. By the end of the campaign (1 January-30 April 2003), over 95% of susceptible animals (about 40 000 cattle and 8 000 sheep and goats) had been vaccinated. The Local Health Units involved devoted most of their available resources to this activity.

The Regional Veterinary Service led the study which was to evaluate the organisation of the vaccination programme and to determine the costs of the campaign, taking into account the large number of animals involved. To better evaluate the expense of such a vaccination campaign, the costs sustained by the Reggio Emilia Local Sanitary Unit (RELSU) are analysed specifically.

Results

Territory

Figure 1 shows the region of Emilia Romagna and highlights the eight municipalities of the Reggio Emilia Province in which vaccination was compulsory. The area is mountainous and has a low...
Table I
Animals vaccinated in Emilia Romagna by the end of the bluetongue vaccination campaign, 30 April 2003

<table>
<thead>
<tr>
<th>Species</th>
<th>Province</th>
<th>Farms</th>
<th>Animals</th>
<th>No. of vaccinated animals</th>
<th>Percentage of vaccinated animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>Modena</td>
<td>379</td>
<td>9 383</td>
<td>8 991</td>
<td>95.8%</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td>Modena</td>
<td>44</td>
<td>1 455</td>
<td>1 412</td>
<td>97.0%</td>
</tr>
<tr>
<td>Cattle</td>
<td>Reggio Emilia</td>
<td>455</td>
<td>16 812</td>
<td>16 170</td>
<td>96.2%</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td>Reggio Emilia</td>
<td>63</td>
<td>3 358</td>
<td>2 884</td>
<td>85.9%</td>
</tr>
<tr>
<td>Cattle</td>
<td>Parma</td>
<td>589</td>
<td>14 471</td>
<td>13 982</td>
<td>96.6%</td>
</tr>
<tr>
<td>Sheep and goats</td>
<td>Parma</td>
<td>98</td>
<td>2 949</td>
<td>2 620</td>
<td>88.8%</td>
</tr>
<tr>
<td>Total Cattle</td>
<td></td>
<td>1 423</td>
<td>40 666</td>
<td>39 143</td>
<td>96.3%</td>
</tr>
<tr>
<td>Total Sheep and goats</td>
<td></td>
<td>205</td>
<td>7 762</td>
<td>6 916</td>
<td>89.1%</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1 628</td>
<td>48 428</td>
<td>46 059</td>
<td>95.1%</td>
</tr>
</tbody>
</table>

Figure 1
Emilia Romagna councils (yellow) subjected to compulsory vaccination

animal density (0.8 farm/km²; 31.3 animals/km²).
The results of the vaccination campaign in the eight municipalities are presented in Table II. By the end of the campaign, the percentage of susceptible animals vaccinated was over 80% (minimum level) in all municipalities; in seven of them coverage was over 90%.

Vaccination costs
The costs of the vaccination campaign were analysed taking into consideration human resources (veterinarians, accountants, etc.), transport costs (kilometres), disposables and vaccine, as described in greater detail below.
Human resources

The area of this study is under the authority of the Area Sud District, one of the three districts that form the RELSU. The RELSU decided to use available resources, organising the veterinary staff of the entire province without employing practitioners. Twenty-eight veterinarians (12 from Area Sud District and 16 from the other districts of the RELSU) were employed. To complete the entire vaccination programme, they worked 2,677 hours, of which 300 hours were spent organising the campaign (contacts with farmers, recording and reporting) and 2,377 hours were spent in the field. The agreement between farmers and veterinarians was fundamental to obtaining the necessary co-operation during the vaccination campaign. Media reports contributed to the increase of fear of vaccination amongst farmers. The announcement of negative effects on the reproductive system in cattle and in sheep represented a serious problem and a lot of time was spent convincing farmers of the need to vaccinate. Considering that a veterinarian costs €60.35 per hour, the RELSU sustained a total cost of €161,556. One accountant was employed for administrative and other activities, working 80 hours at €25.00 per hour, representing a total cost of €2,000. Thus, the total costs for staff were €163,556.

Transport costs

Most of the farms were small (less than 50 animals) scattered in mountainous territory; veterinarians had to cover a total of about 21,300 km to reach all the farms. The cost was approximately €4,515.

Disposable equipment and vaccine

Multi-dose syringes (€1,500), needles (one for each animal vaccinated, €1,000), tattooing forceps, disposable boots, gloves and overalls (about €2,500) cost approximately €5,000. Vaccine was supplied by the Reggio Emilia diagnostic laboratory of the Istituto Zooprofilattico Sperimentale della Lombardia e dell’Emilia Romagna, which was supplied by the National Reference Centre for Exotic Diseases, Teramo, Italy (CESME: Centro Studi Malattie Esotiche) in Teramo. Considering European Union Decision 2002/545/CE that contributed €700,000 to Italy for the purchase of 6,500,000 doses of vaccine, we estimated the cost per dose of vaccine to be €0.11 (1).

Vaccination in the Reggio Emilia (19,054 animals) required about 19,500 doses (195 bottles 100 ml) of BTV-2 vaccine. Vaccination on farms was planned to ensure that the entire bottle of vaccine was used before the expiry date to avoid wastage.

By the end of the campaign, over 95% of the susceptible animals in the area had been vaccinated, as shown in Table II. A few abortions in cattle and sheep were reported (Table III), but BTV was never isolated from either the foetus or the placenta.

Table II
Municipalities in the Reggio Emilia Province involved in the bluetongue vaccination campaign and vaccine coverage attained by the end of the campaign on 30 April 2003

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Farms</th>
<th>Vaccinated</th>
<th>Sheep and goats</th>
<th>Ruminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Busana</td>
<td>100.0%</td>
<td>91.5%</td>
<td>67.2%</td>
<td>85.2%</td>
</tr>
<tr>
<td>Castelnuovo ne’ Monti</td>
<td>100.0%</td>
<td>96.0%</td>
<td>89.8%</td>
<td>95.5%</td>
</tr>
<tr>
<td>Collagna</td>
<td>100.0%</td>
<td>96.0%</td>
<td>89.0%</td>
<td>92.0%</td>
</tr>
<tr>
<td>Ligonchio</td>
<td>100.0%</td>
<td>96.2%</td>
<td>71.4%</td>
<td>93.9%</td>
</tr>
<tr>
<td>Ramiseto</td>
<td>100.0%</td>
<td>98.3%</td>
<td>82.7%</td>
<td>91.5%</td>
</tr>
<tr>
<td>Toano</td>
<td>100.0%</td>
<td>98.5%</td>
<td>82.7%</td>
<td>98.2%</td>
</tr>
<tr>
<td>Vetro</td>
<td>98.5%</td>
<td>97.1%</td>
<td>89.4%</td>
<td>95.9%</td>
</tr>
<tr>
<td>Villa Minuzzo</td>
<td>100.0%</td>
<td>95.2%</td>
<td>86.2%</td>
<td>92.4%</td>
</tr>
<tr>
<td>Total</td>
<td>99.8%</td>
<td>96.9%</td>
<td>85.9%</td>
<td>95.0%</td>
</tr>
</tbody>
</table>

Table III
Abortions recorded in vaccinated cattle in the Reggio Emilia Province and the results of virological tests

<table>
<thead>
<tr>
<th>Event</th>
<th>Days after vaccination</th>
<th>Virological test (PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abortion at 12th week of pregnancy</td>
<td>9</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion at 20th week of pregnancy</td>
<td>10</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion at 21st week of pregnancy</td>
<td>18</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion at 25th week of pregnancy</td>
<td>19</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion</td>
<td>1</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion</td>
<td>1</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion</td>
<td>1</td>
<td>Negative</td>
</tr>
<tr>
<td>Abortion at 24th week of pregnancy</td>
<td>15</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table IV summarises the costs sustained by the RELSU during the vaccination campaign in Reggio Emilia at approximately €9.20 per vaccinated animal.

Conclusions

BT vaccination of all domestic ruminants is very expensive and informing and convincing farmers of the necessity of vaccination required a lot of time. Nevertheless, the vaccination programme was
completed with the co-operation of farmers and without requesting additional funding. It is important to note that farmers attributed abortions, stillbirths, neonatal mortality and weak and slow development amongst the newborn animals to BT vaccination.

Table IV
Bluetongue vaccination campaign costs sustained by the Local Sanitary Unit of the Regio Emilia Province

<table>
<thead>
<tr>
<th>Description</th>
<th>No.</th>
<th>Total hours</th>
<th>Unit cost</th>
<th>Total (euros)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veterinarians</td>
<td>28</td>
<td>2 677</td>
<td>60.35</td>
<td>161 556.95</td>
</tr>
<tr>
<td>Accountant</td>
<td>1</td>
<td>80</td>
<td>25.00</td>
<td>2 000.00</td>
</tr>
<tr>
<td>Transport (km)</td>
<td>21</td>
<td>300</td>
<td>0.21</td>
<td>4 515.00</td>
</tr>
<tr>
<td>Disposable equipment (syringes, needles, etc.)</td>
<td>5</td>
<td></td>
<td></td>
<td>5 000.00</td>
</tr>
<tr>
<td>Vaccine (doses)</td>
<td>19</td>
<td>500</td>
<td>0.11</td>
<td>2 145.00</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>175 216.95</td>
</tr>
</tbody>
</table>

Cost per vaccinated animal (19 054 animals) 9.20

After the risk period (August to November 2003), it would be useful to establish the most cost-effective outcomes of the campaign. An evaluation of the prevention of the spread of BT infection to the entire country and limiting the next vaccination campaign to regions at high risk must also be made.

Reference

Field vaccination of sheep with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9: effect on milk production

G. Savini(1), F. Monaco(1), A. Facchinei(1), C. Pinoni(1), S. Salucci(1), F. Cofini(2) & M. Di Ventura(1)

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(2) Cofini’s farm, Via Umberto I No. 2, Massa d’Albe (AQ), Italy

Summary

In response to complaints of the potential side-effects of the bivalent live-modified vaccine used to control the spread of bluetongue (BT) virus (BTV) serotypes 2 and 9 in Italy, a study was conducted to determine the effects of immunisation on milk production. Thirty-four Comisana cross-bred sheep were vaccinated with the bivalent BTV-2/BTV-9 modified-live vaccine produced by Onderstepoort Biological Products in South Africa; six animals served as unvaccinated controls. All animals were bled twice a week for two months and the presence and titres of BTV in the blood determined. The somatic cell count, pH, fat, protein and lactose content of the milk, as well as the quantity of the milk produced, were also measured. Vaccine virus was isolated from vaccinated animals between day 3 and day 20 post vaccination (pv) with peak titres observed on days 3 and 6 pv for BTV-2 and BTV-9, respectively. Milk production declined in the vaccinated group between days 8 and 14 pv, with the greatest decrease on day 9 pv. No differences were observed in the somatic cell count and pH, or in the milk fat, protein and lactose content.

Keywords


Introduction

Since the first isolation of bluetongue (BT) virus (BTV) in Italy in 2000, four serotypes (BTV-2, BTV-4, BTV-9 and BTV-16) have been reported to occur in the country; of these, BTV-2 and BTV-9 were the most widespread (Fig. 1). To control the spread of these two serotypes and to ease the pressure created by livestock movement restrictions, the Italian government implemented a compulsory vaccination campaign in May 2001. In those areas where both viruses were circulating, the bivalent modified-live vaccine against BTV-2 and BTV-9 was administered and in 2001 and 2002, almost all domestic ruminants were vaccinated. It was the first time that a vaccine with this combination of BTV serotypes had been used in the field and therefore no data were available on its potential side-effects. This study reports on the effects this bivalent modified-live vaccine has on the milk production of dairy sheep.

Material and methods

Vaccine

A combination of BTV-2 and BTV-9 monovalent modified-live vaccines produced by Onderstepoort Biological Products in South Africa was used in this study. Before inoculation, both serotypes were suspended in 100 ml of appropriate diluent. A dose of vaccine contained \(10^{4.37} \text{TCID}_{50}/\text{ml}\) BTV-2 and \(10^{4.24} \text{TCID}_{50}/\text{ml}\) BTV-9.

Animals

This study was conducted between May and July 2002 in the province of L’Aquila (at an altitude of 1100 m) in a flock comprising nearly 1 000 Comisana cross-bred sheep raised for both milk and meat. A group of 34 BT seronegative sheep was selected and vaccinated subcutaneously with a single dose of the vaccine. Another group comprising six seronegative sheep served as unvaccinated controls. To avoid any potential
management bias, both groups were maintained under similar conditions. Temperatures were recorded daily and the animals observed for clinical signs. Total morning milk production per group was recorded daily and individual milk samples were collected four days before commencement of vaccination. The total farm milk production was recorded for twenty days. Ethylene-diaminetetra-acetic acid (EDTA) and plain blood samples were collected twice a week from each animal for the following two months.

Virological and serological tests
EDTA blood samples were screened for the presence of BTVs and their titres measured. The competitive enzyme-linked immunosorbent assay(c-ELISA) (3) and the serum-neutralisation (SN) test (2) were used to detect BTV antibodies. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV from EDTA blood samples according to the method described by Savini et al. (5). The serotype of each virus isolated from the blood of viraemic animals (6), and the viral titres, were determined.

Milk production
Milk samples were analysed for protein and lactose content using the Milkoscan system 4000 and for somatic cell count (SCC) the Fossomatic 400. The pH was measured using a Crison Micro TT250 electrode probe.

Statistical analysis
Differences between the mean milk production per week of the vaccinated and unvaccinated groups of sheep were analysed using the nonparametric Mann-Whitney test for independent groups. Similarly, milk quality data were grouped and for each group the mean weekly value calculated. Statistical differences between weekly data of vaccinated and unvaccinated groups were determined also using the Mann-Whitney test.

Results
The vaccinated animals showed an increase in temperature and exhibited facial oedema between days 7 and 10 post vaccination (pv). Vaccine virus was isolated from the blood of vaccinated animals from day 3 to day 20 pv, with peak titres observed on days 3 and 6 pv for BTV-2 and BTV-9, respectively. BTV-9 viraemia titres were much higher than those for BTV-2 (Fig. 2). No significant differences were observed between the somatic cell count, pH, milk fat, protein and lactose content of the vaccinated and unvaccinated groups. However, milk production in the vaccinated group dropped significantly between days 8 to 14 pv (p<0.05); the lowest production was recorded on day 9 pv (Fig. 3).
Discussion and conclusions

This study demonstrated that vaccination with a bivalent BTV-2/BTV-9 modified-live vaccine had an impact on total milk production but not on the milk quality. Losses commenced two weeks following vaccination and lasted for one week. On day 9 pv, milk production decreased to less than 30% of normal production levels. This drop was also seen in the total milk production of the farm with a 21% decline between days 9 and 12 pv. The decrease was significant and occurred just after peak viraemia of BTV-9. The relationship between the two events was clearly demonstrated (Fig. 4). BTV-9 showed the highest viraemia levels and probably accounted for the drop in milk production. This argument is supported by results obtained in an earlier study on dairy sheep where the use of monovalent BTV-2 vaccine did not affect either milk quantity or quality (1). In another study, no effect on milk yield or on total production was observed when cows were injected with the same BTV-2/BTV-9 vaccine combination; similarly, a higher BTV-9 viraemia titre was also recorded in cattle (4). In sheep, peak viraemia coincided with or occurred just before the appearance of clinical symptoms; in cattle, no clinical signs were observed after vaccination. Thus, it would appear that BTV did not interfere directly with the mammary tissue and that the decrease in milk production was due to BT disease that the vaccine had initiated in the sheep but not in the cattle; the fact that there were no changes in the quality of the various components of the milk would tend to confirm this.

References


Virological and serological response of sheep following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9

G. Savini, F. Monaco, A. Conte, P. Migliaccio, C. Casaccia, S. Salucci & M. Di Ventura

Istituto Zooprofilattico Sperimentale, dell’Abruzzo e del Molise ‘G. Caporale’, via Campo Boario, 64100 Teramo, Italy

Summary

A group of 44 sheep was vaccinated with the bivalent modified-live vaccine against bluetongue virus (BTV) serotype 2 (BTV-2) and BTV-9 to evaluate viraemia and antibody kinetics. Blood samples were taken from the sheep three times a week for two months and screened for the presence of BTV and for antibody using the competitive enzyme-linked immunosorbent assay (c-ELISA) and the virus neutralisation (VN) test. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV-2 and BTV-9 from the ethylene-diaminetetra-acetic acid (EDTA) blood samples; virus titres were also determined in the viraemic animals. BTV was detected in the blood of 39 sheep between day 3 and day 24 post vaccination (pv). Viraemia peaked on day 7 pv with average titres of $10^{5.3}$ TCID$_{50}$/ml. Antibodies were first detected in the c-ELISA on day 6 pv and by day 16, all sheep were seropositive. Only 36 of the 44 inoculated sheep developed virus-neutralising antibodies against both BTV-2 and BTV-9 while 4 were positive to BTV-2 only; neutralising antibodies were not detected in the 4 remaining animals. Antibody titres were very low and unstable and often bordered on the negative/positive threshold.

Keywords


Introduction

The first isolation of bluetongue (BT) virus (BTV) in Italy led to the implementation of an intensive surveillance and monitoring programme, which eventually revealed the presence of four serotypes (BTV-2, BTV-4, BTV-9 and BTV-16) in the country. To control the spread of BTV-2 and of BTV-9 infection and to ease the pressure caused by livestock movement restrictions, a vaccination campaign, using monovalent BTV-2 or a combination of BTV-2 and BTV-9 attenuated vaccines, was initiated in 2001. The criterion for vaccine selection was based on serotype presence in the area to be vaccinated. Vaccines consisting of attenuated virus strains are highly effective, especially in epidemic situations where only one serotype of BTV is involved. Conversely, in those areas where multiple serotypes exist, the situation is more complicated. The Italian scenario required, at least in some areas, the use of bivalent vaccines despite the potential problems associated with interference between virus strains, differences in immunogenicity and growth rates between various strains, as well as differences in the response of individual animals to the components of such vaccines. Furthermore, although the vaccine viruses are attenuated, they are able to replicate and induce viraemia in the host. The purpose of this study was to evaluate viraemia and antibody kinetics in sheep after immunisation using a bivalent modified-live BTV-2 and BTV-9 vaccine. The duration of viraemia and the virus titres after immunisation were also determined. This may provide information on the importance of vaccinated sheep as a source of BTV vaccine virus.
Materials and methods

Vaccine

A combination of a BTV-2 and a BTV-9 monovalent modified-live vaccine produced by Onderstepoort Biological Products in South Africa was used in this study. Before inoculation, both serotypes were suspended in 100 ml of the appropriate diluent. A single dose of vaccine containing $10^{4.37}$ TCID$_{50}$/ml of BTV-2 and $10^{4.25}$ TCID$_{50}$/ml of BTV-9 was administered.

Animals

This study was conducted between May and August 2002 in the Province of L’Aquila (altitude: 1 100 m) in a flock comprising nearly 1 000 Comisana cross-bred sheep raised for both milk and meat. A group of 44 seronegative sheep were selected and vaccinated subcutaneously with a single dose of vaccine. Ethylene-diaminetetra-acetic acid (EDTA) and plain blood samples were collected from each animal three times a week for the following two months.

Virological and serological studies

EDTA blood samples were screened for the presence of BTVs and their titres measured. The competitive enzyme-linked immunosorbent assay (c-ELISA) (4) and the virus neutralisation test (1) were used to detect BTV antibodies. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV from EDTA blood samples according to the method described by Savini et al. (6). The virus titres of BTV-2 and BTV-9 were determined in viraemic animals by neutralising the samples with specific antisera. Each viraemic sample was divided into two aliquots of 1 ml each; one aliquot was mixed with an equal amount of a 1:10 dilution of BTV-2-specific antisera while the second aliquot was mixed with an equal amount of a 1:10 dilution of BTV-9-specific antisera. The samples were incubated at 37°C for 1 h and the virus titres determined as described previously (6). Virus-typing assays were also employed to verify whether specific virus neutralisation occurred.

Statistical analysis

The extent of viraemia was analysed using the Kaplan-Meier survival technique (SppS® 11.0.1) in which the event is the absence of detectable BTV-2 and/or BTV-9 viraemia. The survival probability at each time interval (i.e. the probability of an animal being viraemic at time t) was interpolated using the exponential function in SppS® 11.0.1, and the date on which the probability of being viraemic decreases below 0.01, calculated.

Results

BTV was detected in the blood of 39 animals (88.6%) from day 3 to day 24 post vaccination (pv). For both serotypes, the highest peak of viraemia was reached on day 6 pv. BTV-9 viraemic titres were much higher than those of BTV-2 (Fig. 1). Antibody was first detected using the c-ELISA on day 6 pv and by day 16 all the animals were positive. Of the 44 inoculated animals, 40 developed virus-neutralising antibodies against BTV-2 (90.9%), while only 36 developed antibody against both BTV-2 and BTV-9 (81.8%) (Figs 2 and 3). Viraemia was not detected in three of the four sheep that did not show neutralising antibodies. Moreover, in those animals with virus-neutralising antibodies, titres were very low and unstable, and were often on the borderline between the negative and positive threshold for serotype 9 (Fig. 4). Figure 5 gives the interpolation of cumulative probability of animals being viraemic after X days following vaccination. The exponential equation is as follows:

$$y = 4.5695 \times e^{-0.2203 \times x}$$

According to this formula, the probability of finding an animal without viraemia 28 days after immunisation is 0.99.

Discussion

Modified-live virus vaccines are produced by adapting BTV field isolates in vitro through serial passages in tissue culture or in embryonating chicken eggs. This process selects viruses with an increased aptitude to grow in vitro but possess a reduced capacity to replicate in vivo and to cause disease. Following serial passages, viruses are plaque purified with individually cloned viruses being selected for inoculation into sheep.
The virus which elicits only a mild febrile reaction, and which generates titres of less than $10^3$ plaque-forming units per ml (pfu/ml) at the height of viraemia, and elicits neutralising antibody, is selected for vaccine production (5). According to the results from this study, the monovalent BTV-2 and the monovalent BTV-9 modified-live vaccines, when combined, do not perform as expected. On average, titres higher than $10^5$TCID$_{50}$/ml were observed for BTV-9; although lower titres were observed for BTV-2, these were still higher than $10^4$TCID$_{50}$/ml. It is not known whether the higher virus titres in vaccinated sheep is a consequence of the two serotypes used in combination, or if it is due to the viruses being insufficiently attenuated during vaccine production. This study marks the first instance in which BTV-2 and BTV-9 monovalent vaccines were combined into a single inoculum for use in sheep. The fact that in a previous report (3) BTV-2 attenuated virus, when administered as a monovalent vaccine, never induced titres higher than $10^3$TCID$_{50}$/ml would suggest that the high titres observed in this study might be the result of interference between strains. However, the possibility that BTV-9 had been insufficiently attenuated cannot be excluded. BT disease is caused by an arthropod-borne virus of the genus *Orbivirus* (family: *Reoviridae*) and, in Europe is transmitted by at
least three species of biting midges of the genus *Culicoides* (6). Being an arthropod-borne disease, transmission relies upon the presence of the insect vectors, and of virus circulating in the bloodstream of the animal hosts. It is believed that virus titres lower than 10^3 pfu/ml in vaccinated animals will ensure that the virus is not imbibed by blood-feeding insects (5). The duration of viraemia within the animal host is also crucial to transmit the virus. An understanding of the levels and duration of viraemia in the animal host that are infective to vector *Culicoides* would enable the development of improved low-risk trade policies for BT. In this study, sheep vaccinated with BTV-2 and BTV-9 vaccine showed virus titres higher then 10^3 pfu/ml for 15 days (from 6 to 21 days pv). Previous studies suggest that during this period the elevated virus titres in the vaccinated sheep would have been able to infect the insect vector. The Kaplan-Meier survival technique helped to determine the probability of moving viraemic animals following immunisation. This technique showed that sheep could be moved safely 28 days following immunisation with the BTV-2/BTV-9 bivalent modified-live vaccine. After this interval, the risk of spreading vaccine viruses falls below 0.01%.

Another feature that is characteristic of a good vaccine is its ability to elicit a protective immune response. No challenge studies were conducted in this study and therefore, the levels of immunity had to be calculated on the serological data. Most sheep (90.9%) produced neutralising antibodies following immunisation; protective immunity has been shown to be linked to the levels of neutralising antibody (2). However, in most of the sheep, titres remained low and it appeared that they would not persist for long. Antibody titres to both serotypes peaked 24 days pv and showed a second peak 50 days pv. This study also provided evidence that the strain of BTV-9 used in the vaccine combination was of low immunogenicity, there being a clear discrepancy between viraemia and neutralising antibody titres.

References

Effect of levamisole administration on bluetongue vaccination in sheep

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Summary

Levamisole is an anthelmintic drug with immunostimulant properties when administered at repeated doses of 2.5 mg/kg prior to a vaccine being administered. In order to assess the effect of levamisole administration on bluetongue (BT) vaccination in sheep, four groups of unvaccinated pregnant sheep (8 sheep per group) were used. Group A received vaccine only; Group B received levamisole + vaccine; Group C received Levamisole only; Group D was a non-treated control. Levamisole (Citarin L – 10%) was administered three times weekly at an initial dose of 5.0 mg/kg of body weight and subsequently at 2.5 mg/kg of body weight. There was a significant decrease in faecal egg count of gastrointestinal strongyles in Groups B and C. At the beginning of the trial, all animals were serologically negative for BT antibodies; after vaccination, there was a difference in antibody response in animals in the treated groups. Significantly, more animals in Group B developed BT antibodies following vaccination than those in Group A. In conclusion, levamisole appeared to have an immunostimulating effect on the response of sheep to BT vaccination.

Keywords


Introduction

Levamisole is an anthelmintic drug that stimulates the parasympathetic and sympathetic ganglia in susceptible worms. It is also an immunomodulator and exerts an immunostimulant action in different animal species when administered at repeated doses of 2.5 mg/kg prior to vaccine being administered. Immunostimulating effects are not well understood. It is believed that an immunomodulator restores cell-mediated immune function in peripheral T-lymphocytes and phagocytosis by monocytes (7, 8). Furthermore, an immunomodulator appears to stimulate the production of interleukin-2 (IL-2) and lysozyme, to enhance lymphocyte blastogenesis and to increase the level of specific immunoglobulin in the colostrum of vaccinated animals (1, 2, 3). Bluetongue (BT) is an arthropod-borne disease of domestic and wild ruminants. Its causative agent is an Orbivirus in the family Reoviridae. Clinical disease is usually mild or absent in cattle, camels and goats, but sheep can be severely affected with mortality rates varying from 1% to 30% (5). To date, 24 different bluetongue virus serotypes have been identified in several countries in tropical and temperate areas that support the survival of biting midges (Culicoides spp.), the vectors responsible for transmission of the disease. One of the control methods for the disease is the use of a vaccine containing live-attenuated BT virus (BTV). The response to vaccination is directly linked to the immunological condition of the vaccinated animals. The aim of the present clinical trial was to determine if levamisole administration can exert an immunostimulating effect on sheep when vaccinated against BT.
Materials and methods

Animals

Four groups of sheep in the last month of gestation (8 Sardinian ewes in each group) were used: Group A received only vaccine, Group B received levamisole and vaccine, Group C received only levamisole and Group D was the untreated control group. Before the trial was initiated, the animals were examined and found to be clinically healthy and had not previously received BT vaccine. The ewes came from a typical, reasonably productive sheep farm and had all been subjected to the same environmental and nutritional conditions.

Levamisole and vaccine administration

Both Groups B and C were given three subcutaneous injections of levamisole (Citarin® L 10%, Bayer) at seven-day intervals, at an initial dose of 5 mg/kg of body weight and subsequently at 2.5 mg/kg of body weight. In Group B, the last dose was administered in conjunction with vaccination. Groups A and B received vaccine containing live-attenuated BTV-2 virus (Onderstepoort Biological Products, South Africa).

Sampling and laboratory analysis

Blood and faecal samplings were performed four times on all sheep: at the first administration of levamisole (sample 1); at vaccination (sample 2), 48 h after vaccination (sample 3) and three weeks after vaccination (sample 4). Faecal samples were analysed using the McMaster technique with a minimum sensitivity of 50 epg/opg (eggs per gram/oocysts per gram). Blood samples were taken in duplicate (clot tubes and tubes containing ethylene-diaminetetraacetic acid [EDTA]). Serum samples were screened using a competitive enzyme-linked immunosorbent assay (c-ELISA) to detect antibodies against BTV (BTV antibody test kit, c-ELISA, VMRD, Inc.). EDTA tubes were placed in refrigerated containers and used for haematological parameter determinations. A complete blood count was performed: red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean red blood cell volume (MCV), mean cellular haemoglobin content by mass (MCH), mean cellular haemoglobin concentration (MCHC) and the white blood cell, neutrophil, lymphocyte, monocyte, eosinophil and basophil cell count using an automated haematology analyser (Advia 120 Haematology System).

Statistical analysis

Anova double factors analysis (time of sampling and group) was used comparing groups and the response to the treatment by each group using procedure GLM of software SigmaStat 2.03.

Results

White blood cells and leukogram data are presented in Table I; all animals had leukocyte counts higher than the reference range for sheep (4-12×10³/µl) (6). Neutrophilia were not present (Table I) and all values were normal (reference range 0.7-6×10³/µl); therefore, the other leukogram data influenced the total number of leucocytes (e.g. lymphocytes or eosinophils with reference ranges of 2.9×10³/µl and 0.1×10³/µl, respectively). In response to the treatments, the absolute number of monocytes significantly increased (P<0.05) after vaccination from 0.57 ± 0.1×10³/µl to 0.94 ± 0.3×10³/µl in Group B. The absolute number of eosinophils increased in all groups in the fourth sample with a significant difference (P<0.05) for Groups B and C (Table I). The erythron data (Table II) showed slight anaemia with RBC, HGB and HCT lower than the reference range (9-15×10⁶/µl, 9-15 g/dl and 27%-45%, respectively). The MCHC value decreased significantly for all groups. Anaemia could have been caused by heavy helminthic infestation. Treatment with levamisole (Groups B and C) demonstrated a significant decrease in the helminthic populations that are usually sensitive to the action of this anthelmintic drug (Table III).

Regarding vaccination, it was clear that the administration of levamisole affected seroconversion (Table IV); the mean antibody response, measured by optical density, of Group B was higher than that of Group A (0.323 ± 0.0320 vs 0.346 ± 0.0339). Moreover, with the serological technique used (c-ELISA) a value lower than 0.325 is considered positive. Therefore, the mean value of Group A could be indicative of vaccination failure; in addition, it was important to evaluate the total number of animals within each group that had an optical density lower than the positive cut-off. In fact only four animals (50%) from Group A seroconverted (<0.325) while seven animals (87.5%) from Group B showed a strong seroconversion.

Discussion

Vaccination is a means to reduce losses caused by a disease and so maintain the normal profit margins of the farmer. These clinical trials demonstrated that animals considered immunologically competent and under favourable environmental conditions could experience a vaccination failure. Anaemia found in
### Table I
Values of leukogram data (mean ± standard error means) obtained in the clinical trial

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample</th>
<th>White blood cells (x 10^3/µl)</th>
<th>Neutrophils (x 10^3/µl)</th>
<th>Lymphocytes (x 10^3/µl)</th>
<th>Monocytes (x 10^3/µl)</th>
<th>Eosinophils (x 10^3/µl)</th>
<th>Basophils (x 10^3/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>19.48 ± 1.6</td>
<td>5.75 ± 0.8</td>
<td>11.95 ± 1.2</td>
<td>0.78 ± 0.1</td>
<td>0.69 ± 0.2</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16.54 ± 1.6</td>
<td>3.97 ± 0.8</td>
<td>10.77 ± 1.2</td>
<td>0.58 ± 0.1</td>
<td>0.84 ± 0.2</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.93 ± 0.9</td>
<td>3.66 ± 0.2</td>
<td>10.63 ± 0.7</td>
<td>0.48 ± 0.1</td>
<td>0.76 ± 0.2</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>17.93 ± 1.6</td>
<td>3.88 ± 0.8</td>
<td>11.45 ± 1.2</td>
<td>0.58 ± 0.1</td>
<td>1.65 ± 0.2</td>
<td>0.26 ± 0.03</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>16.47 ± 1.5</td>
<td>3.73 ± 0.8</td>
<td>11.09 ± 1.1</td>
<td>0.48 ± 0.1</td>
<td>0.91 ± 0.2</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17.04 ± 1.6</td>
<td>4.48 ± 0.8</td>
<td>11.16 ± 1.2</td>
<td>0.57 ± 0.1</td>
<td>0.46 ± 0.2</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>15.14 ± 1.6</td>
<td>3.69 ± 0.4</td>
<td>9.81 ± 1.3</td>
<td>0.94 ± 0.1</td>
<td>0.43 ± 0.05</td>
<td>0.17 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>17.87 ± 1.5</td>
<td>4.01 ± 0.8</td>
<td>10.95 ± 1.1</td>
<td>0.40 ± 0.1</td>
<td>2.19 ± 0.2</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>16.98 ± 1.5</td>
<td>5.38 ± 0.8</td>
<td>9.87 ± 1.1</td>
<td>0.49 ± 0.1</td>
<td>0.92 ± 0.2</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>14.99 ± 1.5</td>
<td>4.85 ± 0.8</td>
<td>8.76 ± 1.1</td>
<td>0.47 ± 0.1</td>
<td>0.62 ± 0.2</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.68 ± 1.5</td>
<td>4.97 ± 0.8</td>
<td>7.98 ± 1.1</td>
<td>0.49 ± 0.1</td>
<td>0.79 ± 0.2</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>15.05 ± 1.5</td>
<td>3.61 ± 0.8</td>
<td>8.78 ± 1.1</td>
<td>0.42 ± 0.1</td>
<td>1.93 ± 0.2</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>14.29 ± 1.7</td>
<td>3.87 ± 0.9</td>
<td>8.49 ± 1.3</td>
<td>0.78 ± 0.1</td>
<td>0.83 ± 0.3</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>15.39 ± 1.6</td>
<td>3.7 ± 0.8</td>
<td>10.04 ± 1.2</td>
<td>0.42 ± 0.1</td>
<td>0.93 ± 0.2</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>13.79 ± 2.5</td>
<td>4.47 ± 0.4</td>
<td>7.66 ± 1.9</td>
<td>0.51 ± 0.2</td>
<td>0.86 ± 0.3</td>
<td>0.19 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16.27 ± 1.6</td>
<td>4.23 ± 0.8</td>
<td>9.42 ± 1.1</td>
<td>0.53 ± 0.1</td>
<td>1.83 ± 0.2</td>
<td>0.21 ± 0.03</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b) within each group show a significant difference among samples: P<0.05

### Table II
Values of erythron data (mean ± standard error means) obtained in the clinical trial

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample</th>
<th>RBC (x 10^6/l)</th>
<th>HGB (g/dl)</th>
<th>HCT (%)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>7.57 ± 0.4</td>
<td>8.91 ± 0.4</td>
<td>27.45 ± 1.5</td>
<td>36.27 ± 0.8</td>
<td>11.76 ± 0.3</td>
<td>32.47 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.35 ± 0.4</td>
<td>8.76 ± 0.4</td>
<td>26.71 ± 1.5</td>
<td>36.34 ± 0.8</td>
<td>11.94 ± 0.3</td>
<td>32.87 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.13 ± 0.2</td>
<td>8.51 ± 0.3</td>
<td>25.81 ± 0.8</td>
<td>36.24 ± 0.7</td>
<td>11.98 ± 0.2</td>
<td>33.07 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.06 ± 0.4</td>
<td>8.14 ± 0.4</td>
<td>26.11 ± 1.5</td>
<td>37.04 ± 0.8</td>
<td>11.57 ± 0.3</td>
<td>31.22 ± 0.4</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>7.26 ± 0.4</td>
<td>8.80 ± 0.4</td>
<td>27.32 ± 1.4</td>
<td>37.73 ± 0.7</td>
<td>12.11 ± 0.2</td>
<td>32.14 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.04 ± 0.4</td>
<td>8.76 ± 0.4</td>
<td>26.66 ± 1.5</td>
<td>37.95 ± 0.8</td>
<td>12.45 ± 0.3</td>
<td>32.85 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.41 ± 0.3</td>
<td>9.21 ± 0.4</td>
<td>27.97 ± 1.3</td>
<td>37.72 ± 0.6</td>
<td>12.42 ± 0.2</td>
<td>32.96 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.97 ± 0.4</td>
<td>8.22 ± 0.4</td>
<td>26.30 ± 1.4</td>
<td>37.89 ± 0.7</td>
<td>11.86 ± 0.2</td>
<td>31.29 ± 0.3</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
<td>7.65 ± 0.4</td>
<td>9.20 ± 0.4</td>
<td>28.66 ± 1.4</td>
<td>37.74 ± 0.7</td>
<td>12.13 ± 0.2</td>
<td>32.14 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>7.30 ± 0.4</td>
<td>8.99 ± 0.4</td>
<td>27.34 ± 1.4</td>
<td>37.70 ± 0.7</td>
<td>12.41 ± 0.2</td>
<td>32.92 ± 0.3</td>
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<tr>
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<td>3</td>
<td>7.36 ± 0.4</td>
<td>8.76 ± 0.4</td>
<td>27.56 ± 1.4</td>
<td>37.90 ± 0.8</td>
<td>12.23 ± 0.2</td>
<td>32.59 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.40 ± 0.4</td>
<td>8.74 ± 0.4</td>
<td>27.98 ± 1.4</td>
<td>38.02 ± 0.8</td>
<td>11.88 ± 0.2</td>
<td>31.27 ± 0.3</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>6.83 ± 0.5</td>
<td>8.30 ± 0.5</td>
<td>25.63 ± 1.6</td>
<td>37.81 ± 0.8</td>
<td>12.31 ± 0.3</td>
<td>32.56 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.54 ± 0.4</td>
<td>7.86 ± 0.4</td>
<td>24.07 ± 1.5</td>
<td>36.99 ± 0.8</td>
<td>12.07 ± 0.3</td>
<td>32.72 ± 0.4</td>
</tr>
<tr>
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<td>3</td>
<td>6.58 ± 0.5</td>
<td>7.98 ± 0.4</td>
<td>24.66 ± 1.7</td>
<td>37.80 ± 1.6</td>
<td>12.34 ± 0.5</td>
<td>32.62 ± 0.5</td>
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<td>6.60 ± 0.4</td>
<td>7.87 ± 0.4</td>
<td>24.84 ± 1.5</td>
<td>37.95 ± 0.8</td>
<td>12.07 ± 0.3</td>
<td>31.76 ± 0.4</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b) within each group show a significant difference among samples: P<0.05

- **RBC**: red blood cells
- **HGB**: haemoglobin
- **HCT**: hematocrit
- **MCV**: mean red blood cell volume
- **MCH**: mean cellular haemoglobin content by mass
- **MCHC**: mean cellular haemoglobin concentration
Table III
Faecal egg/oocyst counts in treated (B and C) and untreated (A and D) groups with levamisole

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Gastrointestinal strongyles(^{(a)})</th>
<th>Other species</th>
<th>Trichuris sp.(^{(b)})</th>
<th>Tapeworms(^{(a)})</th>
<th>Eimeria sp.(^{(b)})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nematomirus sp.</td>
<td>Strongylodes papillosus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Before</td>
<td>0</td>
<td>450 ± 96.8</td>
<td>352.5 ± 111.1</td>
<td>6.2 ± 6.9</td>
<td>125 ± 29.2</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>6.2 ± 7.1</td>
<td>468.7 ± 96.8(^{a})</td>
<td>518.7 ± 111.1(^{a})</td>
<td>18.7 ± 6.9</td>
<td>31.2 ± 29.2</td>
</tr>
<tr>
<td>B</td>
<td>Before</td>
<td>11.1 ± 6.7</td>
<td>122.2 ± 91.3</td>
<td>130 ± 104.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0</td>
<td>22.2 ± 91.3(^{a})</td>
<td>0</td>
<td>0</td>
<td>22.2 ± 27.5</td>
</tr>
<tr>
<td>C</td>
<td>Before</td>
<td>5.5 ± 6.7</td>
<td>100 ± 91.3</td>
<td>238.9 ± 104.8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>0</td>
<td>0 b</td>
<td>22.2 ± 104.8(^{a})</td>
<td>5.5 ± 6.5</td>
<td>5.5 ± 27.5</td>
</tr>
<tr>
<td>D</td>
<td>Before</td>
<td>12.5 ± 7.1</td>
<td>218.7 ± 96.8</td>
<td>425 ± 111.1</td>
<td>0</td>
<td>18.7 ± 29.2</td>
</tr>
<tr>
<td></td>
<td>After</td>
<td>6.2 ± 7.1</td>
<td>318.7 ± 96.8</td>
<td>393.7 ± 111.1(^{a})</td>
<td>12.5 ± 6.9</td>
<td>43.7 ± 29.2</td>
</tr>
</tbody>
</table>

\(^{(a)}\) eggs per gram of faeces (mean ± standard error)
\(^{(b)}\) oocysts per gram of faeces (mean ± standard error)

Different superscript letters (c, d) among groups in the treatment rows show a significant difference: P<0.05

Table IV
Bluetongue virus antibody response as measured by optical density (mean ± s.e.m.), c-ELISA method

Optical densities of less than 0.325 are considered positive

<table>
<thead>
<tr>
<th>Sample</th>
<th>A (vaccine)</th>
<th>B (vaccine + levamisole)</th>
<th>C (levamisole)</th>
<th>D (control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.720 ± 0.0339</td>
<td>0.730 ± 0.0320</td>
<td>0.716 ± 0.0320</td>
<td>0.670 ± 0.0363</td>
</tr>
<tr>
<td>2</td>
<td>0.677 ± 0.0339</td>
<td>0.700 ± 0.0339</td>
<td>0.675 ± 0.0320</td>
<td>0.660 ± 0.0339</td>
</tr>
<tr>
<td>3</td>
<td>0.387 ± 0.0297</td>
<td>0.681 ± 0.0274</td>
<td>0.656 ± 0.0320</td>
<td>0.611 ± 0.051</td>
</tr>
<tr>
<td>4</td>
<td>0.346 ± 0.0339</td>
<td>0.323 ± 0.0320(^{a})</td>
<td>0.449 ± 0.0320(^{a})</td>
<td>0.467 ± 0.0339(^{a})</td>
</tr>
</tbody>
</table>

Different superscript letters (a, b) within each sample show a significant difference among groups: P<0.05

All animals was probably due to excessive parasitism caused by the absence of pasture rotation. The decrease of MCHC in all animals, but more especially in Groups B and C, could be indicative of reticulocytosis (erythroid regeneration). Therefore, improvement in health was due to a decrease of parasitosis following levamisole treatment. Eosinophilia present in the fourth sample in all groups could be ascribed to the activation of hypobiotic larvae after parturition.

The blood monocytes, together with tissue macrophages, constitute the mononuclear phagocyte system which has a microbiocidal action against bacteria, viruses, fungi and protozoa. Moreover, the function of monocytes (macrophages) includes regulation of the immune response when exposed to bacteria, antigens or tissue injury; they produce cytokines (IL-1 and tumour-necrosis factor). In Group B (vaccination combined with levamisole), there was a clear increase in the absolute number of monocytes 48 h post vaccination, probably due to the action of levamisole treatment. Indeed, the anthelmintic drug seems to stimulate the production of IL-2 and lysozyme. It has been hypothesised that levamisole restores the cell-mediated immune function in peripheral T-lymphocytes and phagocytosis by monocytes (4, 8). Furthermore, Group B showed a higher seroconversion rate when compared to Group A. This result is similar to other trials demonstrating an increase in specific immunoglobulin levels after treatment (3). In conclusion, this clinical trial demonstrated the immunostimulating effect of levamisole on BT vaccination in sheep due to an improvement in their general condition with a decrease of helminthic infestation and a direct effect on immunocompetent cells.

References


Efficacy and safety studies on an inactivated vaccine against bluetongue virus serotype 2

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Summary
An inactivated vaccine was produced from an Italian field isolate of bluetongue virus serotype 2 (BTV-2) with a titre of $10^{7.8}$ TCID$_{50}$/ml. The virus was purified through a molecular cut cassette membrane, inactivated with ß-propirolactone and emulsified with ISA 206 (Seppic) adjuvant. The vaccine was then tested for sterility, toxicity and safety in laboratory and target animals according to European Pharmacopoeia standards. Immunogenicity was assessed by inoculating subcutaneously 10 sheep and 10 goats each with 2 ml of the vaccine and 10 bovines each with 5 ml of the vaccine. A booster dose was inoculated after 14 days and no side-effects were reported following vaccination. Fourteen days after the booster dose, all vaccinated animals developed virus neutralising (VN) bluetongue (BT) antibody titres that on day 60 post vaccination ranged between 1/20 and 1/1280. After one year, goats still had high VN antibody titres. Sheep were challenged 138 days after vaccination by subcutaneously inoculating 1 ml of $10^{5.6}$TCID$_{50}$/ml of an Italian field isolate of BTV serotype 2; four unvaccinated animals were also inoculated and used as controls. Starting from day 6 post challenge, control animals developed a fever, with temperature ranging from 39.9°C to 40.6°C and lasting 48 h on average. BTV-2 was also isolated from the blood of control animals between days 4 and 20 post challenge. Conversely, neither fever nor viraemia were detected in the vaccinated animals that were challenged. A new trial with a larger number of animals, including all target species, has been planned and is in progress.

Keywords

Introduction
Bluetongue (BT) is an infectious disease caused by a virus belonging to the family Reoviridae, genus Orbivirus; 24 serotypes with differing pathogenicity have been reported. The infection is transmitted by haematophagous insects belonging to the family Ceratopogonidae, genus Culicoides. The infection affects domestic and wild ruminants causing significant losses mainly in sheep and goats. Disease is rare in cattle and, when present, clinical signs are much milder. In those areas where the virus is present, losses in cattle are mainly due to the ban on animal transport; because of the effect on international trade, the economic repercussions of this disease in cattle can be severe. BT is included in ‘List A’ of the Office International des Épizooties (OIE) (10, 15, 16).

BT virus vaccines presently used in Europe are prepared with attenuated bluetongue virus (BTV) strains. These vaccines are usually safe and effective and are currently the most effective tool available to control BT outbreaks (1, 6). They stimulate good immune response and are quite inexpensive; however, the use of such vaccines in areas where the disease is considered exotic may give rise to difficulties with zoo-sanitary regulations and with the international trade of animals. Furthermore, they can produce undesirable side-effects in animals in poor health and are not recommended for use in ewes during the first three months of pregnancy, as they have been reported to cause abortions and foetal
malformations. Inactivated vaccines have been developed previously (9, 10, 11, 12); however, the safety and efficacy of these vaccines has been reported to be marginal. This report summarises the results achieved when an experimental inactivated vaccine against BTV-2, prepared according to European Pharmacopoeia guidelines, was administered to sheep, goats and cattle.

Materials and methods

Viral strain

The virus used in this study was BTV-2, strain 1486/A/00; the virus was obtained from the National Reference Centre for Exotic Diseases, Teramo, Italy (CESME: Centro Studi Malattie Esotiche). Before the strain was used for seed production, it was passaged three times in specific pathogen-free (SPF) embryonating chicken eggs inoculated intravenously, and then passaged three times in baby hamster kidney-21 (BHK-21) cell cultures. The virus was then plaque-purified according to the method described by Dulbecco (17) and the seed virus then lyophilised. The seed virus was checked for the presence of contaminating bacteria, viruses, fungi and mycoplasmas (8). Virus identity and titre were also confirmed.

Vaccine production and in-process control

The seed virus was passaged five times in cell culture and was harvested when the characteristic cytopathic effect (CPE) was fully developed. The viral suspension (VS) was aseptically harvested, purified through a molecular cut cassette membrane and inactivated with β-propriolactone at a final concentration of 0.2% (v/v) (14). Two aliquots of the inactivated VS were subsequently prepared and checked in vitro and in vivo for inactivation according to the protocol below.

In vitro inactivation test

Three 175 cm² tissue culture flasks containing confluent monolayers of BHK-21 cells were each inoculated with 10 ml of inactivated VS (first aliquot). After three passages, no viral CPE or BTV immunofluorescence was observed.

In vivo inactivation test

A second aliquot of VS was injected into four BT seronegative sheep (20 ml/sheep). Two further unvaccinated sheep were used as negative controls. Temperatures were recorded daily for 21 days and animals were observed for signs of abnormality. Furthermore, every second day, for 21 days, ethylene-diaminetetra-acetic acid (EDTA) blood samples were collected and tested for the presence of BTV.

Once the inactivation was verified, the VS was emulsified with an equal volume of adjuvant Montanide® ISA 206. The vaccine was then tested in accordance with European Union (EU) legislation (5, 6, 7) and European Pharmacopoeia guidelines (8).

Safety test

The vaccine underwent safety tests by administering single and double doses to sheep, goats and cattle. The experiment was performed at the Centro Zootechnico e Caseario di Bonassai in Sassari, Italy, on unvaccinated and BT-seronegative animals.

Efficacy test

Vaccine efficacy tests were performed by screening the animals for neutralising antibodies and by monitoring them for clinical signs of BT. This was done on the same animals that had been used in the safety tests and which were inoculated with a single vaccine dose.

a) Antibody response

Plain blood samples were collected from sheep and goats to determine the neutralising antibody curve. Samples were collected from the sheep at the time (t) of vaccination (t=0) and 14, 60 and 137 days post vaccination (pv); samples were collected from goats at t=0, 14, 60, 137, 285 and 365 days pv. In this study, cattle were included only for the safety test; therefore, antibody levels in cattle were only determined at t=0, 14 and 60 days pv. No further testing was conducted on the cattle. Collected samples were tested using a competitive-enzyme linked immunosorbent assay (c-ELISA) (11) and the virus neutralisation (VN) test (13).

b) Challenge study

As sheep usually develop clinical signs following infection, they were chosen for the challenge studies. The experimental procedures were conducted according to current animal welfare provisions (2, 3, 4).

Vaccinated sheep and the respective control group, were infected with $10^{5.8}$TCID$_{50}$ of BTV-2 137 days pv. Temperatures were recorded daily for 24 days and animals were observed for clinical signs. In addition, EDTA and plain blood samples were collected from each animal every second day for 24 days.
Results

Vaccine production and in-process control

Control of materials

Materials used during vaccine production fulfilled all the EU and European Pharmacopia guidelines (8); controls for sterility were also found to be satisfactory.

Primary seed virus and viral suspension

Identity and sterility were confirmed as well as the absence of viral contaminants. Following the purification process, the BTV titre of the VS was $10^{7.8}$ TCID$_{50}$/ml. Inactivation tests, verified both in vitro and in vivo, were satisfactory. No fever or side-effects were observed after injection of one or two doses. Virus was not isolated from the pv EDTA blood samples.

Tests on the final product

The final product was checked according to European Pharmacopoeia and EU provisions and was found to be free of contaminating bacteria, fungi and mycoplasmas.

Safety tests

Virus was not isolated from the blood samples collected from vaccinated animals. Temperatures were within the normal range. None of the animals in the trial developed any type of clinical signs. Only four animals, which were inoculated with a large dose of vaccine (10 ml/animal subcutaneously in the area of the neck), showed a slight phlogistic reaction at the inoculation site but this disappeared within 9 days.

Potency test

Following immunisation, all vaccinated animals developed virus neutralising and c-ELISA antibodies, confirming the vaccine to be immunogenic (Figs 1, 2 and 3). In goats, neutralising antibody levels remained constant throughout the observation period of more than one year (Fig. 2).

Challenge test

After challenge, vaccinated sheep did not show any clinical signs of BT disease and their temperatures remained normal; BT virus was not isolated from any of the challenged vaccinated animals. Control animals developed fever and clinical signs commencing on day 6 post challenge (Fig. 4). Viraemia was also detected from day 2 to day 20 post challenge (Fig. 5).
Discussion and conclusions

The present study demonstrated that the experimental inactivated vaccine prepared from purified, inactivated and adjuvanted BTV-2 was safe and effective and met the requirements of the European Pharmacopoeia and the EU. Neither pyrexia nor other significant signs were observed in animals. The vaccine was safe and the capacity of β-propiolactone, at a final concentration of 0.2% (v/v), to inactivate BTV, was confirmed.

In all species tested, 100% seroconversion was observed following the first injection, and confirms the findings of Murray et al. (12), Parker et al. (14) and Lefèvre and Desoutter (9). At 137 days, the animals that had been immunised with one or two doses of the vaccine were protected against challenge. Neutralising antibody titres in goats were still high after one year.

The results of this study have been compared with those from the Italian BTV vaccination campaign where an attenuated virus vaccine, produced by Onderstepoort Biological Products, was used.

Results of the vaccine comparison are as follows:

a) the inactivated vaccine had no side-effects or limitations in its use in terms of safety

b) all animals vaccinated with the inactivated vaccine developed neutralising antibodies, whereas only 92% of those vaccinated with the live-attenuated vaccine showed neutralising antibodies.

It would appear that the inactivated vaccine can be used in all epidemiological and production conditions requiring maximum security and effectiveness, including the need to discriminate between vaccinated and infected animals. To fulfil this final condition, a diagnostic kit that can distinguish between vaccinated and naturally infected animals is being developed. To test further these promising results and to evaluate the minimum protective vaccinal dose, a new trial with a larger number of animals, and including all target species, has been planned and is in progress.

References


Serological response in cattle and sheep following infection or vaccination with bluetongue virus

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Summary

Data from various experimental and field studies were compiled and analysed to evaluate the serological response in sheep and cattle against different bluetongue (BT) virus (BTV) vaccine combinations (Onderstepoort Biological Products, South Africa); the accuracy of diagnostic procedures commonly used for detecting BTV antibodies was also assessed. Using the competitive enzyme-linked immunosorbent assay (c-ELISA) (IZSA&M, Teramo, Italy) and the virus neutralisation (VN) test, antibody responses were evaluated under the following vaccination regimes: monovalent modified-live vaccine against BTV-2 in cattle and sheep, monovalent modified-live vaccine against BTV-9 in sheep, and bivalent modified-live vaccine against BTV-2 and BTV-9 in cattle and sheep. The data were compared to serological results observed in cattle and sheep infected with Italian field strains of BTV-2 or BTV-9. The c-ELISA consistently detected antibodies earlier than the VN test in both livestock species and against all BTV serotypes. The highest and most rapid antibody responses were observed in sheep infected in the field. In cattle and in sheep, high VN titres were detected using monovalent vaccines, while bivalent vaccines initiated lower antibody titres that developed more slowly.

Keywords


Introduction

The outbreak of bluetongue (BT) and subsequent vaccination using monovalent and bivalent modified-live vaccines have resulted in the widespread immunisation of domestic ruminants across Italy. In the first three seasons of the outbreak, animals were exposed to bluetongue virus (BTV) serotype 2 (BTV-2) and/or BTV-9 field strains, monovalent BTV-2 modified-live vaccine and bivalent BTV-2 and BTV-9 modified-live vaccine. Various vaccination scenarios were studied under controlled conditions and serological responses, following immunisation with different BTV vaccine combinations and following infection with field strains, were evaluated. These data were also used to assess the accuracy of diagnostic procedures commonly used to detect BTV antibodies.

Materials and methods

The BTV-2 and BTV-9 monovalent modified-live vaccines used in this study were produced by Onderstepoort Biological Products in South Africa. A single dose of the monovalent modified-live vaccine against BTV-2 contained $10^{5.15}$ TCID$_{50}$/ml of virus, while that of the BTV-9 monovalent vaccine contained $10^{5.8}$ TCID$_{50}$/ml of virus. A single dose of the combined BTV-2/BTV-9 monovalent modified-live vaccine contained $10^{4.37}$ TCID$_{50}$/ml of BTV-2 and $10^{4.24}$ TCID$_{50}$/ml of BTV-9. BTV-2 challenge material was prepared from the spleen of a naturally infected sheep that died during the 2000 BT outbreak in Sardinia; the BTV-9 challenge material was prepared from blood sampled from a naturally infected cow in Calabria as described by Savini et al. (5).
To study the serological responses to field isolates of BTV-2 and BTV-9 in sheep, two groups of five ewes each were randomly selected; one group was challenged by subcutaneous injection of 1 ml of $10^{5.8}$ TCID$_{50}$ of BTV-2 and the other group challenged with $10^{5.8}$ TCID$_{50}$ of BTV-9. The same dose of the BTV-2 field isolate was used to subcutaneously infect four cattle. All challenged animals were kept in an insect-free stable for the duration of the study. As described previously, a further 44 sheep and 30 cows were selected (3, 6) and the bivalent BTV-2/BTV-9 modified-live vaccine administered subcutaneously. Also, monovalent BTV-2 modified-live vaccine was administered subcutaneously to seven sheep and five cows, whilst monovalent BTV-9 modified-live vaccine was administered to five sheep. Serum samples were tested for the presence of BTV antibodies using a competitive enzyme-linked immunosorbent assay (c-ELISA) (2) and the virus neutralisation (VN) test (1). Positive and negative controls for the VN were kindly provided by the OIE reference laboratory of the Onderstepoort Veterinary Institute (OVI) in South Africa.

**Results**

Apart from four sheep inoculated with the bivalent vaccine, all the remaining animals (96.2%) developed c-ELISA and virus neutralising antibody titres. Table I indicates the date at which antibody was first detected using the BT c-ELISA and the date at which all animals developed BTV c-ELISA antibodies. In general, higher neutralising antibody titres were observed after animals had been infected with field isolates of the two virus serotypes.

<table>
<thead>
<tr>
<th>BTV serotypes</th>
<th>Species</th>
<th>Days following vaccination or experimental infection</th>
<th>First positive animals</th>
<th>100% positive animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTV-2 field isolate</td>
<td>Bovine</td>
<td>14</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ovine</td>
<td>7</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>BTV-9 field isolate</td>
<td>Bovine</td>
<td>Not done</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ovine</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>BTV-2 modified-live</td>
<td>Bovine</td>
<td>10</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>vaccine</td>
<td>Ovine</td>
<td>14</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>BTV-9 modified-live</td>
<td>Bovine</td>
<td>Not done</td>
<td>Not done</td>
<td></td>
</tr>
<tr>
<td>vaccine</td>
<td>Ovine</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>BTV-2 and BTV-9</td>
<td>Bovine</td>
<td>9</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>bivalent modified-live vaccine</td>
<td>Ovine</td>
<td>6</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

However, in both species VN titres were also obtained after immunisation using a monovalent vaccine while animals vaccinated using a bivalent vaccine developed antibody later and had lower antibody titres (Figs 1, 2, 3 and 4). The most rapid response, with the highest titres, occurred in sheep infected with field isolates of BTV (Fig. 4).
Discussion and conclusions

One of the principal measures used to control the 2000-2003 outbreaks of BTV in Italy was the vaccination of all susceptible ruminants using monovalent BTV-2 and bivalent BTV-2 and BTV-9 modified-live vaccines. The evaluation of the immune responses generated by these vaccines provided useful information not only on the probable immune status of the animals after vaccination but also on the value of the diagnostic tests. As observed previously (4), the c-ELISA consistently detected antibodies earlier than the VN test and against both serotypes of BTV. As expected, the field isolates of BTV-2 and BTV-9 elicited higher VN titres than the modified-live vaccine strains. Although this discrepancy was evident for both serotypes in sheep, it was substantially greater for BTV-9, suggesting that the South African vaccine against this strain had a reduced immunogenic capacity.

References


Effects of vaccination against bluetongue on milk production and quality in cattle vaccinated with live-attenuated monovalent type 2 vaccine

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Summary

The first epidemic of bluetongue (BT) to affect the three regions of Sardinia, Sicily and Calabria (Italy) in 2000 induced high economic losses caused by the disease itself and by the cessation of ruminant movements both within, and out of, the infected areas. In order to reduce virus circulation, and to create a resistant livestock population, the Italian Ministry of Health ruled, in May 2001, that all sheep, cattle, goats and water buffalo, in infected and in neighbouring regions, be vaccinated. The live-attenuated BTV-2 monovalent vaccine produced by Onderstepoort Biological Products in South Africa was to be used. Accordingly, in 2002, 98.6% of the sheep and goats, and 88.1% of the cow, on Sardinia were vaccinated. Included was the vaccination of >70% of the cattle in the province of Oristano where >18 000 dairy cows in >220 herds are concentrated in the municipality of Arborea (Oristano) and which account for 65-70% of the milk produced in Sardinia. Using data collected at the centralised dairy co-operative since 1999 the quantity and quality of milk produced before vaccination against bluetongue was compared to that produced after vaccination. The following variables were analysed: average milk production/cow/month, monthly average fat content (%), monthly average protein content (%), average monthly somatic cell count and average monthly platelet count. The findings indicate that vaccination against BTV-2 in Sardinian dairy cattle did not impact negatively upon milk quantity nor milk quality.

Keywords

Bluetongue – Cattle – Italy – Milk production – Sardinia – Vaccine.

Introduction

Since 2000, Italy has been affected by the largest bluetongue (BT) epidemic ever to be recorded in Europe. The infection was first reported in Sardinia in August 2000 and then spread to large areas in central and southern Italy (2, 12). In May 2001, the Minister of Health ruled that all domestic ruminants were to be vaccinated not only in the infected regions but also in neighbouring regions to which infection could spread. Selection of the vaccination strategy was based on a risk assessment which indicated that viral circulation within Italy could only be interrupted by creating a large population of infection-resistant ruminants (11).

The use of a live-attenuated vaccine may induce a rise in temperature ranging from 39.4°C to 39.8°C and lasting one to three days with transient viraemia. It has also been reported that, under experimental conditions, sheep vaccinated during the first four months of pregnancy may show teratogenic effects (10) but this is a phenomenon that has not been observed in the field. However, after the commencement of the vaccination campaign in Italy, vaccine-related problems were recorded in several regions, particularly in the south. In addition to the
problems described in the literature, a decrease in milk production was also reported. Existing studies on the effect of BT vaccination have only been conducted in sheep; no studies have been performed on dairy cattle.

An earlier study on the Sardinian sheep breed examined the effect of the vaccine on milk production under controlled conditions. Two groups of sheep studied, the first was vaccinated with BTV-2 vaccine while the second acted as the non-vaccinated control. No significant differences in the quantity and quality (fat, protein and lactose content, somatic cell count) of the milk produced by the two groups, before and after vaccination, were reported (4). A later study conducted in Sardinia to assess the effects of infection and vaccination on milk production (13) showed that infection significantly decreases milk production but is influenced by poor flock management. No effects on milk quality were reported. In most flocks, vaccination did not have any significant statistical effects on the lactation curve but even when the lactation curve was significantly affected, the effect was negligible from a practical viewpoint (maximum recorded loss: 10.5 g milk/animal/day).

The aim of this study was to establish whether the use of a monovalent BTV-2 significantly affects the quality and quantity of milk of dairy cattle.

Materials and methods

Population involved in the study

Dairy cattle in this study were located in the municipality of Arborea (Oristano Province, Sardinia). Two-thirds of the total bovine milk production of Sardinia is concentrated in the Arborea municipality, which has more than 18 000 cows distributed in about 220 holdings with an average monthly milk production of 12 700 tons. All milk produced in this municipality is collected by the ‘3A Assegnatari Associati Arborea’ Co-operative, where the milk is checked for both quality and quantity.

The dairy cows of the Arborea Municipality were selected for the following reasons:

a) they are genetically homogenous, almost all animals being Italian Friesian 
b) almost all the farms in the municipality apply the same nutritional uni-feed schemes 
c) the health status of the population is relatively homogenous 
d) similar management practices are applied throughout the area.

Data collected are standardised and are therefore comparable, as all the milk produced is delivered to a single point.

Vaccination

The first vaccination campaign in Sardinia took place in 2002. In total, 98% of the sheep and goats and 88.1% of the cattle were vaccinated. The first cattle vaccination campaign in the Arborea municipality was conducted from May to September 2002, after the vaccination of sheep and goats had been completed during the first few months of 2002 (Fig. 1). A live-attenuated monovalent BTV-2 vaccine, produced by Onderstepoort Biological Products (OBP) in South Africa, was used.

Variables considered in the study

The qualitative and quantitative data on the milk collected prior to vaccination (April 1999-April 2002) by the Co-operative ‘3A’ and were analysed and compared to the data collected after vaccination (May 2002-April 2003). The following variables were considered:

• number of farms to deliver milk 
• average number of cows per farm 
• total number of cows on the farms that delivered milk 
• total quantity of milk delivered 
• average monthly production per animal 
• average monthly fat and protein percentage in the milk delivered 
• average monthly plate count in the milk delivered
• average monthly somatic cell count in milk delivered.

The following laboratory methods were used:
• fat and protein content: FIL/IDF 141C:2000 standard procedure ‘Determination of milk fat, protein and lactose content (guide for the operation of mid-infrared instruments)’

The average number of animals in lactation for each herd was derived from the annual declaration made by farmers (L1 form), in compliance with the Commission Regulation (EC) 1392/2001 of 9 July 2001, laying down detailed rules for applying Council Regulation (EEC) 3950/92 for establishing an additional levy on milk and milk products (7, 8).

Statistical analysis
To verify whether increasing or decreasing trends existed, linear regressions were used (1, 3). Variables used were: average milk production per animal per month, somatic cell count, plate count, fat and protein content. Time was the independent variable for all regressions. The monthly numbers were transformed into the corresponding serial numbers of the days that had elapsed since 1 January 1900, up to the fifteenth day of every month.

Paired samples Student’s t-test with one tail significance (3) was used to compare the average monthly values of each variable before and after vaccination. The following hypotheses (H₀) were tested:
• individual milk production: average values, May 2002/April 2003 ≥ April 1999/April 2002
• somatic cell count: average values, May 2002/April 2003 ≤ April 1999/April 2002

Table I
Results of regression between time and average monthly values of production per animal, somatic cell count, plate count, fat and protein content

<table>
<thead>
<tr>
<th>Variable</th>
<th>Average milk production per animal</th>
<th>Somatic cell count</th>
<th>Plate count</th>
<th>Fat content</th>
<th>Protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted R²</td>
<td>0.00</td>
<td>0.45</td>
<td>0.40</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>F</td>
<td>0.95</td>
<td>40.48</td>
<td>33.48</td>
<td>2.73</td>
<td>7.41</td>
</tr>
<tr>
<td>P</td>
<td>0.33</td>
<td>7.6 ×10⁻⁸</td>
<td>5.7 ×10⁻⁷</td>
<td>0.11</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Results
The production data show that from 1999 to 2003:
• The average monthly production/animal ranged from 590 kg to 760 kg, depending on the season (Fig. 2); the slight increase recorded over time is not statistically significant \((R²=0; p=0.33)\) (Table I); the production between May 2002 and April 2003 (during the vaccination campaign) was not significantly lower than that between April 1999 and April 2002 (before vaccination) \((t=5.55; p=0.99)\) (Table II)
Table II
Results of comparison between average milk production per animal per month, somatic cell count, plate count, fat and protein content, April 1999-April 2002 and May 2002-April 2003

<table>
<thead>
<tr>
<th>Compared periods and tested hypothesis</th>
<th>Average milk production per animal</th>
<th>Somatic cell count</th>
<th>Plate count</th>
<th>Fat content</th>
<th>Protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 02-Apr. 03</td>
<td>May 02-Apr. 03</td>
<td>≤</td>
<td>≤</td>
<td>≥</td>
<td>≥</td>
</tr>
<tr>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
</tr>
<tr>
<td>Apr. 99-Apr. 02: 651.2</td>
<td>Apr. 99-Apr. 02: 299.7</td>
<td>Apr. 99-Apr. 02: 266.5</td>
<td>Apr. 99-Apr. 02: 40.9</td>
<td>Apr. 99-Apr. 02: 3.67</td>
<td>Apr. 99-Apr. 02: 3.32</td>
</tr>
<tr>
<td>May 02-Apr. 03: 676.8</td>
<td>May 02-Apr. 03: 266.5</td>
<td>May 02-Apr. 03: 26</td>
<td>May 02-Apr. 03: 36.5</td>
<td>May 02-Apr. 03: 3.33</td>
<td>May 02-Apr. 03: 3.33</td>
</tr>
</tbody>
</table>

Student's t | p
--- | ---
5.55 | 0.99
10.01 | 1
4.44 | 0.99
0.81 | 0.22
0.99 | 0.83

- The average monthly somatic cell count improved significantly during the entire study period ($R^2=0.45; p=7.6 \times 10^{-8}$) (Table I) from 300 cells/ml in 1999 to about 250 in 2003 (Fig. 3); the cell count between May 2002 and April 2003 (during the vaccination campaign) was not significantly higher than that between April 1999 and April 2002 (before vaccination) ($t=10.01; p=1$) (Table II); the same result was obtained even when the trend effect was taken into account ($t=1.01; p=0.83$) (Table III)
- The average monthly plate count decreased significantly ($R^2=0.40; p=5.7 \times 10^{-7}$) (Table I), from 40-100 cfu/ml in 1999 to less than 40 cfu/ml in 2003 (Fig. 4); the plate count between May 2002 and April 2003 was not significantly higher than that between April 1999 and April 2002 ($t=4.44; p=0.99$) (Table II); the same result was obtained even when the trend effect was taken into account ($t=1.77; p=0.052$) (Table III)
- The average monthly fat content remained steady and only showed seasonal fluctuations (Fig. 5); the slight increase recorded over time was not statistically significant ($R^2=0.03; p=0.11$) (Table I); the fat content between May 2002 and April 2003 was not significantly lower than that recorded between April 1999 and April 2002 ($t=0.81; p=0.22$) (Table II)

Figure 3
Dairy cattle in Italy: somatic cell count and vaccine coverage during the first vaccination campaign against bluetongue

Table III
Results of comparison between regression residuals of average monthly values of somatic cell count, plate count and protein content, April 1999-April 2002 and May 2002-April 2003

<table>
<thead>
<tr>
<th>Compared periods and tested hypothesis</th>
<th>Somatic cell count</th>
<th>Plate count</th>
<th>Protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>May 02-Apr. 03</td>
<td>≤</td>
<td>≤</td>
<td>≥</td>
</tr>
<tr>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
<td>Apr. 99-Apr. 02</td>
</tr>
<tr>
<td>Apr. 99-Apr. 02: 1.59</td>
<td>Apr. 99-Apr. 02: -1.01</td>
<td>Apr. 99-Apr. 02: 0.01</td>
<td></td>
</tr>
<tr>
<td>May 02-Apr. 03: -1.775</td>
<td>May 02-Apr. 03: 4.94</td>
<td>May 02-Apr. 03: -0.03</td>
<td></td>
</tr>
</tbody>
</table>

Student's t | p
--- | ---
1.01 | 0.83
1.77 | 0.052
3.40 | 0.003
• The average monthly protein content increased significantly ($R^2=0.12$; $p=0.009$) (Table I) from an average 3.28% in 1999 to 3.33% in 2003 (Fig. 6); the protein content between May 2002 and April 2003 was not significantly lower than between April 1999 and April 2002 ($t=0.99$; $p=0.83$) (Table II); since a significant increase in protein content was observed over time, this trend had to be removed; therefore, the residuals of regression during the vaccination period were significantly lower than the residuals before the vaccination campaign ($t=3.40$; $p=0.003$) (Table III), indicating an increase in protein content over time that was lower than expected according to the trend.

Discussion

The goal of the study was to verify, through field data, whether BT vaccination affects the quality and quantity of milk produced by cattle vaccinated with a live-attenuated BTV-2 vaccine. The cattle population selected were characterised by similar genetic, health and management features (especially the type of nutrition) and also single-source standardised data. The data assessment showed that milk production in the Arborea municipality over the past four years has generally improved, especially milk quality (a statistically significant reduction in the average somatic cell and plate counts), probably due to compliance with the Council Directives 92/46/EEC, 92/47/EEC and the national laws on milk quality (5, 6). The comparison of variables between April 1999 and April 2002 and between May 2002 and April 2003, i.e. before and after vaccination, shows clearly that vaccination did not have a negative effect on the quantity and quality of milk produced in the municipality (Fig. 2 and Table II). The only significant difference to be noted was a decreased milk protein content. However, no pathogenic mechanism is known, whereby BT vaccination could decrease milk protein content, especially when no other variables are affected. Since such a difference was recorded only after removal of the trend in the data (compare Table II with Table III), the association detected between vaccination and protein content could be due to non-linearity of the trend itself, characterised by a horizontal asymptote. This trend was not considered in the analysis, due to the data fluctuations observed (Fig. 6). The residuals of regression in the horizontal asymptote area being
deviated towards low values, thus leading to a significant, but artificial, difference between the two study periods.

References


Effects of bivalent bluetongue virus serotypes 2 and 9 vaccine on reproductive performance of cattle: a case study in Calabria, Italy

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Summary

Following the occurrence of bluetongue (BT) in Italy in the summer and autumn of 2000, the Italian Ministry of Health decreed (in May 2001) that all sheep, goats and cattle in infected, and in neighbouring areas, be vaccinated. The principal aim of the vaccination campaign was to create a resistant animal population, thereby reducing overall virus circulation. Accordingly, during 2002, the live-attenuated bivalent vaccine against BT virus (BTV) serotypes 2 and 9, produced by Onderstepoort Biological Products in South Africa, was administered in Calabria. A large herd of cattle (over 900 animals, including 390 cows) was monitored for four months after vaccination to establish whether any abnormalities (such as stillbirth and placental retention) occurred during parturition. During the study, 111 cows (89 vaccinated and 22 unvaccinated) gave birth; in 26 cows, abnormalities were observed but no association was found to occur between the vaccination of cows against BT and the birthing abnormalities observed (Pearson’s chi-square = 0.517, P>0.05). The animals were divided into four groups according to the number of days between vaccination and parturition (less or equal to 31 days, 31-60 days, 61-90 days, more than 90 days); again no link was found between vaccination and the abnormalities observed.

Keywords


Introduction

Bluetongue (BT) is an infectious, non-contagious, arthropod-borne disease that primarily affects sheep but occurs also in cattle, goats and wild ruminants. The causative agent of the disease is a virus of the genus Orbivirus, family Reoviridae, and comprises 24 immunologically distinct serotypes. In Italy, BT infection was first reported in Sardinia in August 2000 and then spread to large areas of central and southern Italy (2, 4). The first case of BT in Calabria was reported on 11 October 2000 in a flock in the province of Reggio Calabria. Virus isolation confirmed the presence of BT virus (BTV) serotype 2. The disease spread along the Ionian coast of Calabria and in November was diagnosed in the provinces of Crotone and Cosenza. The presence of BTV-9 was confirmed at the end of 2000 in five holdings in the province of Cosenza (2). In the summer and autumn of 2001, the disease occurred again along the Tyrrenian coast in the provinces of Catanzaro, Cosenza and Vibo Valentia (2). In May 2001, the Minister of Health announced that all domestic ruminants reared in the infected regions were to be vaccinated. Based on the virus isolations of the previous year, the vaccine used in Calabria was a live-attenuated bivalent BTV-2/BTV-9 vaccine produced by Onderstepoort Biological Products (OBP) in South Africa. The live-attenuated vaccine used may cause transient viraemia and a rise in temperature ranging from 39.4°C to 39.8°C and lasting for one to three days. Vaccine side-effects have been studied primarily in sheep; data on side-effects in cattle are very limited. Studies had been conducted on the effects of ‘wild-type’ BTV infection on the reproductive performance of cattle (7, 8, 9).

The aim of the present study was to verify whether the use of a bivalent BTV-2/BTV-9 vaccine
significantly affected the reproductive performance of a large bovine herd.

**Materials and methods**

**Population involved in the study**

A large bovine herd (of more than 900 animals, including 390 cows) was monitored for four months after vaccination to verify if any abnormalities (stillbirth, placental retention, etc.) were recorded during parturition. The farm under study, La Favella, is located in a low plain in the municipality of Corigliano Calabro in the province of Cosenza (Fig. 1) where approximately 90 cattle herds and 30 sheep and goats flocks are located, totalling 6,800 ruminants.

All cows in the herd are registered in the Italian Freisian Herd Book and are part of a genetic improvement programme in which only artificial insemination is used. The farm consists of a roofed holding area with straw on the ground and an open yard area for feeding and exercise surrounded by fences (Fig. 2). Cows are divided into different groups according to reproductive stages and productive performances. On 11, 13, 14 and 18 March 2002, the Veterinary Service vaccinated 514 animals in the herd, 392 of which were cows. Cows that were in the first half of pregnancy were not vaccinated. During the four-month study period, 111 cows (89 vaccinated and 22 unvaccinated) gave birth. The following data was recorded for each animal: ID code, date of vaccination, date of parturition, and any problems observed. Five cows died one to two months before the expected calving date. The cause of death was recorded, together with the indication of the possible relationship between the cause of death and problems during parturition. During the study, blood samples were collected every two weeks from 10 non-vaccinated animals.

Antibodies against BTV were not detected in any of the samples; these negative sentinel animals indicated that no ‘wild type’ BTV virus was circulating during the study.

**Vaccine**

A live-attenuated bivalent BTV-2/BTV-9 vaccine, produced by OBP, was used.

**Statistical analyses**

The possible link between vaccination and abnormal parturition was verified. Pearson’s chi-square test was used to evaluate the data and, in cases where one or more expected values were less than 5, the exact Fisher probability test was used (1, 3).

**Results**

Between 20 March and 28 July 2002, 111 cows (89 vaccinated and 22 unvaccinated) gave birth. In 26 cases, abnormalities were observed during or following parturition, as follows:

- 23 presented placental retention
- one cow had a mummified fetus
- gangrenous mastitis occurred in one cow 2 weeks after calving
- one cow died from abomasal dislocation 12 days after parturition.

All abnormalities correlated directly or indirectly with any hypothetical alteration during pregnancy or parturition were considered in the study. No association, however, was found between vaccination of cows and the abnormalities observed (Pearson’s chi-square = 0.517, P > 0.05) (Table I). In addition, the data was grouped into four classes according to the number of days between vaccination and parturition (less than or equal to 30 days, 31-60 days, 61-90 days, more than 90 days);
in all cases the exact probability Fisher test did not reveal any significant association (P >0.05) between vaccination and abnormalities (Table I).

### Table I
Comparison between the number of parturitions, with and without abnormalities, in vaccinated and in unvaccinated cows (bivalent bluetongue virus serotypes 2 and 9 live-attenuated vaccine)

<table>
<thead>
<tr>
<th>Days between vaccination and calving</th>
<th>Abnormal parturition</th>
<th>Animals</th>
<th>Vaccinated</th>
<th>Not vaccinated</th>
<th>Association between pathologies and vaccination (probability)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;31 days</td>
<td>Pat+</td>
<td>9</td>
<td>2</td>
<td>&gt;0.05*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pat–</td>
<td>22</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31-60 days</td>
<td>Pat+</td>
<td>5</td>
<td>1</td>
<td>&gt;0.05*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pat–</td>
<td>21</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>61-90 days</td>
<td>Pat+</td>
<td>2</td>
<td>0</td>
<td>&gt;0.05*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pat–</td>
<td>8</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;90 days</td>
<td>Pat+</td>
<td>6</td>
<td>1</td>
<td>&gt;0.05 *</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pat–</td>
<td>16</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>Pat–</td>
<td>67</td>
<td>18</td>
<td>&gt;0.05**</td>
<td></td>
</tr>
</tbody>
</table>

Pat+ – parturition with abnormality (stillbirth, placental retention, etc.)
Pat– – parturition without abnormality (stillbirth, placental retention, etc.)

* exact probability Fisher test
** Pearson’s Chi-square test

### Discussion

The vaccination strategy adopted against BT in Italy aimed to reduce virus circulation so as to diminish direct losses due to mortality and indirect losses linked to the prolonged ban on animal movements. Accordingly, all domestic ruminants susceptible to infection (sheep, goats, cattle and buffalo) in infected and at-risk areas were vaccinated. The use of a live-attenuated BT vaccine on a large cattle population is a unique BT control measure, and has provided new information on the efficacy and safety of the vaccine in this species (6). The effect of vaccination on bovine milk production has been provided new information on the efficacy and safety of the vaccine in this species (6). The effect of vaccination on bovine milk production has been reported elsewhere (5); its effect on the reproductive performance of cattle is reported in the present study. The target population in this study was 111 cows forming part of a large cattle herd located in the province of Cosenza. No association was found between vaccination using live-attenuated bivalent BTV-2/BTV-9 vaccine and problems encountered during parturition. The small number of animals studied and the fact that a single herd was investigated could lead to the conclusion that the present results are not representative of the entire vaccinated cattle population of Italy. However, the data, derived from a genetically homogeneous and consistently husbanded herd, indicate that the vaccination of cattle against BT does not have a negative impact on their reproductive performance.

### References
Virological and serological response of cattle following field vaccination with bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9

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Summary
Following the bluetongue (BT) epidemic in Italy, the government initiated a vaccination campaign involving all domestic ruminants (cattle, sheep and goats) in the affected and adjacent areas to create a resistant population and to reduce virus circulation. Based on the serotypes circulating in the affected areas, monovalent BT virus (BTV) serotype 2 (BTV-2), or bivalent BTV-2 and BTV-9, modified-live vaccines were used. These are manufactured by Onderstepoort Biological Products in South Africa and, because they are recommended for use in sheep only, very little data exists on their use in cattle under field conditions. To evaluate duration and levels of viraemia and the antibody response following vaccination, 30 cattle in various stages of pregnancy were selected and vaccinated with a bivalent BTV-2/BTV-9 vaccine. Blood samples were taken from the animals three times a week for two months and screened using the competitive enzyme-linked immunosorbent assay (c-ELISA) and the virus neutralisation (VN) test. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV-2 and BTV-9 from ethylene-diaminetetra-acetic acid (EDTA) blood samples, and virus titres determined in viraemic animals. Titres against BTV were detected in 27 animals between days 4 and 35 post vaccination (pv). Viraemia peaked on day 9 pv with average viral titres of $10^{4.5}$ TCID$_{50}$/ml. From day 9 pv, the c-ELISA detected antibodies in all animals while low VN titres were observed commencing on day 18 pv. Furthermore, VN antibody to BTV-2 was detected in only 17 of the animals vaccinated and to BTV-9 in 27 animals.

Keywords

Introduction
Bluetongue (BT) virus (BTV) is the etiological agent of BT, a non-contagious, arthropod-borne disease of both domestic and wild ruminants. Throughout tropical and temperate regions of the world (3, 5, 8) its distribution coincides with the presence of competent insect vectors, haematophagous biting midges of the genus Culicoides (3, 15). BT can produce an acute disease in sheep with a mortality rate reaching 10% or more, incurring major production losses. However, in cattle, the infection is typically asymptomatic (2, 5) and is characterised by viraemia lasting 30 to 45 days (5, 6, 9, 10, 14). Due to its ability to spread rapidly under suitable circumstances, BT is classified a ‘List A’ disease by the Office International des Épizooties (OIE). This means that in infected areas, trade bans will be imposed on the susceptible species, often with serious socio-economic consequences. Vaccination is one of the most effective preventive tools against viral diseases and especially for those transmitted by insects. Since 2000, Italy has experienced the most severe outbreaks of BT ever to be recorded. In an
attempt to reduce direct losses due to disease and indirect losses due to virus circulation, the Italian government has implemented a compulsory BTV vaccination campaign involving all susceptible domestic ruminants since May 2001. Based on the serotype/s present in a given area, a monovalent BTV-2 or a bivalent BTV-2/BTV-9 modified-live vaccine, produced by Ondersteepoort Biological Products (OBP) in South Africa, was used. The vaccine was produced by the serial passage of virulent field strains of BTV in embryonating chicken eggs and in cell cultures. This vaccine is recommended for use in sheep only. The purpose of this study was to evaluate duration and levels of viraemia and antibody kinetics in cattle after immunisation with the bivalent modified-live BTV-2/BTV-9 vaccine to determine whether vaccinated cattle serve as a source of BT vaccine virus to bloodsucking arthropods.

Materials and methods

Sixty cattle at various stages of pregnancy were selected, 30 of which were vaccinated with the bivalent BTV-2/BTV-9 vaccine. All animals tested negative against the most common reproductive diseases (bovine virus diarrhoea, infectious bovine rhinotracheitis, bovine herpesvirus-4, salmonellosis, chlamydiosis, neosporosis and brucellosis). Antibodies to BT were not detected in their blood prior to vaccination. Each vaccine serotype was tested for contamination and titrated on Vero cells. Animals were inoculated with 1 ml of vaccine in the region of the neck. Vaccinated animals and negative controls were kept under field conditions and stabled on six different farms. All animals were checked daily for clinical signs and body temperatures were recorded daily for 15 days post vaccination (pv). Ethylene-diaminetetra-acetic acid (EDTA) and plain blood samples were collected from each animal three times a week for 30 days and then twice a week for the following month. EDTA blood samples were screened for the presence of BTV and if virus was detected, the virus titre was determined for each of the serotypes isolated as described previously (13). The competitive enzyme-linked immunosorbent assay (c-ELISA) (7) and the virus neutralisation (VN) test (4) were used to detect the presence of BTV antibodies. Intravenous egg inoculation followed by two blind passages in Vero cells was used to isolate BTV from EDTA blood samples according to the method described by Savini et al. (12). The duration of viraemia was analysed using the Kaplan-Meier Survival technique (SPSS® 11.0.1) in which the event is the absence of detectable BTV-2 and/or BTV-9 viraemia. The survival probability at each time interval (i.e. the probability of an animal being viraemic at time t) was interpolated through the linear regression function in SPSS® 11.0.1, and the date on which the probability of being viraemic decreases below 0.01, was calculated.

Results

No viral or bacterial contaminants were detected in the vaccine. A dose of vaccine contained $10^{4.37}$TCID$_{50}$/ml BTV-2 and $10^{4.24}$TCID$_{50}$/ml BTV-9. The vaccinated animals did not show any clinical signs of BT infection. The calves born from the vaccinated cows during the experiment were healthy and no abortions or reproductive diseases were observed.

BTV-2 titres were detected in the circulating blood of 22 (73.3%) vaccinated animals between day 8 and day 28 pv. Viraemia peaked on day 11 pv with average viral titres of $10^{3.8}$TCID$_{50}$/ml. BTV-9 caused detectable viraemia in 25 (83.3%) cattle commencing on day 4 pv and lasting 24 days; maximum viral titre was detected on day 9 pv with an average titre of $10^{4.7}$TCID$_{50}$/ml (Fig. 1). None of the negative controls developed detectable viraemia. Commencing on day 9 pv, c-ELISA antibodies were detected in all vaccinated animals while low VN titres were observed, which commenced on day 18 pv.

![Figure 1](https://example.com/figure1.png)

**Figure 1** Bluetongue virus titres in cattle following vaccination with a bivalent BTV-2/BTV-9 modified-live vaccine

Antibodies against BTV-2 were detected in 29 animals commencing on day 16 pv with the highest titre on day 42 pv. All animals except one developed an immune response to BTV-9; VN antibody was detected on day 18 pv, two days later than BTV-2, and BTV-9 antibody titre peaked on
day 35 pv (Fig. 2). None of the control animals developed antibody against BTV-2 or BTV-9. Figure 3 gives the interpolation of cumulative probability of viraemic animals after X days. The linear equation was $y = 1.3036 - 0.0404x$. The probability of an animal being viraemic after 32 days is less than 0.99.

Discussion

Following the incursion of BT into Italy, the vaccination of all domestic ruminants (cattle, sheep and goats) was implemented in affected and in adjacent areas to create a resistant population and to reduce virus circulation. Based on the serotype/s circulating in the affected areas, a monovalent BTV-2 or bivalent BTV-2/BTV-9 modified-live vaccine was used. These vaccines are manufactured by OBP in South Africa and, because they are recommended for use in sheep only, no data exists on the vaccination of cattle under field conditions. Modified-live vaccine virus has to replicate in order to stimulate a protective immunity; in the process, they may induce a mild or subclinical illness in the immunised hosts. In this study, the vaccine did not induce any clinical signs of the disease in the vaccinated animals.

One of the potential drawbacks which might occur following immunisation with a modified-live vaccine is its subsequent potential transmission by insects. Previous studies have demonstrated that, in cattle, BTV persists in the erythrocytes, allowing the virus to remain in the blood despite the presence of neutralising antibodies (1, 2, 6, 14). In this study, viraemia persisted for 28 days and neutralising antibodies and virus were present from days 18 to 28 pv. It is critically important to understand whether the levels of vaccine viral titres in vaccinated cattle are high enough to infect locally active Culicoides because in this way the virus can be maintained in the environment. In this study, both BTV-2 and BTV-9 virus titres exceeded the value of $10^3$TCID$_{50}$/ml for 6 and 12 days respectively; this titre is considered the threshold for possible Culicoides infection (11) (Fig. 1). Based on this hypothesis, the Kaplan-Meier survival technique was applied to assess the risk of moving viraemic animals after immunisation. According to this formula, cattle could be moved safely 32 days following immunisation with bivalent BTV-2/BTV-9 modified-live vaccine. After this time lapse, the risk of spreading vaccine viruses through bloodsucking insects is less than 0.01%.

Even though the bivalent vaccine used in this study was capable of inducing an immune response in almost all the vaccinated animals, seroconversion was not correlated with the titre of virus in the blood. In fact, although BTV-9 replication induced viraemic titres ($10^{4.7}$TCID$_{50}$/ml) higher than BTV-2 ($10^{3.8}$TCID$_{50}$/ml), the average BTV-9 neutralising antibody titres were lower than those observed for BTV-2.

References


Field vaccination of cattle using a bivalent modified-live vaccine against bluetongue virus serotypes 2 and 9: effect on milk production

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Summary

To evaluate the effect of bluetongue (BT) vaccination on milk production in cattle, 30 cows at various stages of gestation were vaccinated using a bivalent bluetongue virus serotype 2 (BTV-2) and BTV-9 modified-live vaccine produced by Onderstepoort Biological Products in South Africa. A second group of 30 pregnant cows was used as unvaccinated controls. Blood samples were taken from all animals three times a week for two months. Virus titres were determined and the daily milk production of each cow was quantitatively and qualitatively evaluated. From 27 of the 30 vaccinated cows, BTV was isolated from day 4 to day 28 post vaccination. BTV vaccination had no effect on milk production, somatic cell count, pH, milk fat, protein and lactose content. It is concluded that the bivalent BTV-2/BTV-9 modified-live vaccine does not affect milk production in cows.

Keywords


Introduction

The occurrence of bluetongue (BT) can result in the imposition of a trade ban on susceptible ruminant species and can have serious socio-economic effects on the livestock industry in infected countries. Since August 2000, when the first outbreak of BT was reported in Italy, four serotypes of the BT virus (BTV) (BTV-2, BTV-4, BTV-9 and BTV-16), have circulated in the country. Of these serotypes, BTV-2 and BTV-9 were by far the most widespread (Sicily and numerous central and southern peninsular regions). In an attempt to reduce direct losses due to disease and indirect losses due to virus circulation, the Italian Ministry of Health implemented a compulsory vaccination campaign using the bivalent BTV-2 and BTV-9 modified-live vaccine in areas where both serotypes prevailed. In 2001 and 2002 almost all domestic ruminants were vaccinated. As it was the first time that the combined BTV-2 and BTV-9 vaccine was used in the field, no data were available on its potential side-effects. This study evaluated its effect on milk production in dairy cattle.

Material and methods

Vaccine

A bivalent BTV-2/BTV-9 modified-live vaccine produced by Onderstepoort Biological Products in South Africa was used. Before inoculation, both serotypes were suspended in 100 ml of appropriate diluent. A single dose of vaccine contained $10^{4.37}$ TCID$_{50}$/ml BTV-2 and $10^{4.24}$ TCID$_{50}$/ml BTV-9.

Animals

Sixty cows, at various stages of pregnancy, were selected and thirty were vaccinated with the bivalent vaccine. All animals tested negative against the most common reproductive diseases (bovine viral diarrhoea, infectious bovine rhinotracheitis, bovine herpesvirus-4, salmonellosis, chlamydiosis, neosporosis and brucellosis) and no antibodies against BTV were detected. Temperatures were recorded daily and clinical signs monitored. Blood samples were taken from all animals three times a week for two months. Virus titres were determined.
and the daily milk production from each animal was evaluated both quantitatively and qualitatively.

Virological and serological tests
Ethylene-diaminetetra-acetic acid (EDTA) blood samples were examined for the presence of BTV and, in viraemic animals, virus titres determined. The competitive enzyme-linked immunosorbent assay (c-ELISA) (2) and the virus neutralisation (VN) test (1) were used to detect BT antibodies. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV from EDTA blood samples according to the method described by Savini et al. (4). BTV isolation was attempted from the blood of viraemic animals and the serotype determined (5).

Milk production study
Milk samples were analysed for fat, protein and lactose content using the Milkoscan system 4000 and the somatic cell count determined using a Fossomatic 400. The pH was recorded using a Crison Micro TT250 electrode probe.

Statistical analysis
Differences between the weekly mean milk production of vaccinated and unvaccinated groups were analysed using the nonparametric Mann-Whitney test for independent groups.

Similarly, milk quality data were grouped and for each group the weekly mean value was calculated. Statistical differences between weekly quality data of vaccinated and unvaccinated groups were also determined using the Mann-Whitney test.

Results
No clinical signs were observed in vaccinated animals. Vaccine virus was isolated from the blood of vaccinated animals commencing on day 4 and continuing to day 28 post vaccination (pv); peak titres were observed on days 11 and 9 pv for BTV-2 and BTV-9, respectively. BTV-9 viraemia titres were much higher than those for BTV-2 and are shown in Figure 1. None of the negative controls developed detectable viraemia. No significant differences were observed in somatic cell count, pH, milk fat, protein and lactose content between vaccinated and unvaccinated groups. Similarly, no significant differences were observed in milk production between vaccinated and unvaccinated groups (Fig 2).

Discussion and conclusions
This study demonstrates that vaccination using a bivalent BTV-2/BTV-9 modified-live vaccine did not interfere with milk production in dairy cows; the results were consistent with those observed elsewhere (3). However, they differed from those observed in sheep when the same vaccine combination was used; in sheep, a drop in milk production was observed shortly after peak BTV-9 virus titres were attained. The decrease in production commenced one week pv and lasted for seven days. In this study, the BTV-9 virus titre was similar to that observed in sheep but no losses in milk production were recorded (Fig. 3).
A possible explanation for this discrepancy is that sheep developed clinical signs and so BT disease may have caused the drop in milk production. The vaccinated cattle exhibited no disease and no loss in milk production.

References


Monovalent modified-live vaccine against bluetongue virus serotype 2: immunity studies in cows

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Summary

A challenge study was conducted to determine the efficacy of vaccination against bluetongue (BT) virus (BTV) serotype 2 in protecting cattle against infection. A group of 30 cows, vaccinated seven months previously with monovalent BTV-2 modified-live vaccine produced by Onderstepoort Biological Products in South Africa, were challenged subcutaneously with $2 \times 10^{5.8}$ TCID$_{50}$/ml of BTV-2 field isolate. All cattle originated from the same population in the Sardinian province of Oristano. Eight unvaccinated calves from a BTV-free herd also participated in this study; four were inoculated with BTV-2 and used as positive controls whilst the remaining four were used as negative controls to confirm that no BTV was circulating locally. Blood samples were taken from all animals three times a week for two months. Serum samples were tested for antibody against BTV using the competitive enzyme-linked immunosorbent assay (c-ELISA) and the virus neutralisation (VN) test. Virus isolation was attempted on the blood samples by intravenous egg inoculation followed by two blind passages in Vero cells. Virus titres following challenge were determined also. Of the 30 cows vaccinated, 29 were positive in the c-ELISA and demonstrated neutralising antibodies. At the time of challenge, 11 cows had no virus neutralising antibody while the remainder had low titres ranging from 1:10 (11 cows) to 1:20 (6 cows); two cows showed titres of 1:40 and 1:80, respectively. None of the cows showed signs of disease after challenge and no BTV was isolated from the blood of the 29 cows that had developed antibodies after vaccination. Commencing on day 9 post challenge, BTV-2 was isolated from the blood of the single cow that had not seroconverted following vaccination and from the blood of the unvaccinated controls. Viraemia lasted until day 21 post challenge. Neither BTV nor antibody was detected in the blood samples taken from the negative control group. These observations indicate that the monovalent BTV-2 modified-live vaccine protects most animals when challenged with field virus seven months post vaccination.

Keywords


Introduction

Bluetongue (BT) is an arthropod-borne, viral disease which affects mainly sheep. Due to its ability to spread rapidly under suitable circumstances, BT is classified as a ‘List A’ disease by the Office International des Épizooties (OIE) and occurrence of disease gives rise to trade bans being imposed on susceptible ruminant livestock species in infected areas. Such movement restrictions have had serious socio-economic repercussions on the industry in infected countries. In an attempt to reduce direct losses due to disease and indirect losses due to virus circulation, the Italian Ministry of Health declared vaccination against BT compulsory for all susceptible ruminants in May 2001. During the first year of the campaign, Sardinia and Tuscany vaccinated more than 80% of their cattle populations using a monovalent modified-live vaccine against BT virus (BTV) serotype 2 manufactured by Onderstepoort.
Biological Products (OBP) in South Africa. Since it was the first time that monovalent BTV-2 vaccine had been used in cattle, no data existed on the safety and efficacy of the vaccine in cattle. Consequently, a challenge experiment was conducted to determine the level of homologous protection induced by the vaccine in cattle.

**Materials and methods**

**Virus**
Challenge virus was prepared from the spleen of a naturally infected sheep that had died in the 2000 BT outbreak in Sardinia. The spleen was disrupted using sterile quartz powder, the product suspended with lactose phosphate buffer containing antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml gentamycin and 50 IU/ml nystatin) and sonicated. A 0.1 ml volume of the spleen suspension was inoculated intravenously into embryonating chicken eggs and subsequently passaged onto confluent Vero cell monolayers as described by Savini et al. (4). At maximal cytopathic effect (CPE), the material was harvested, aliquoted and stored at –80°C. Presence and identity of BTV were determined as previously described (4). The highest dilution of the virus to produce CPE in half of the Vero cells inoculated (TCID₅₀) was the assay used to calculate the titre of the isolate. For the CPE assay, 50 µl of several virus dilutions, from 10⁻¹ to 10⁻⁸, were placed into 96 flat-bottomed microtitre plate wells. Approximately 10⁴ Vero cells were added per well in a volume of 100 µl of minimum essential medium (MEM) containing antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml gentamycin and 50 IU/ml nystatin) and 3% foetal calf serum (FCS). The test was read after incubating the plates for six days at 37°C under 5% CO₂. The endpoint of the assay was determined using the method of Reed and Muench.

**Animals**
Between July and August 2002, a group of 1 005 cattle of various breeds and ages were selected at random from 10 herds in the Sardinian province of Oristano. These animals were vaccinated subcutaneously with 1 ml of 10⁶TCID₅₀ BTV-2 modified-live vaccine. Blood samples from all animals were taken monthly for three months post vaccination (pv) as described by Savini et al. (5). From these, a group of 30 vaccinated cows was selected and divided further into three groups of ten cows each, based on their virus neutralisation (VN) titres. One group included animals which developed VN titres of 1:80 or less, the second group included those with titres ranging between 1:160 and 1:320, whilst the third group included those with VN titres above 1:320. All 30 vaccinated cows were then challenged seven months after immunisation by subcutaneous injection of 1 ml sonicated spleen, containing 10⁵.8TCID₅₀/ml of BTV-2 field isolate. Of eight unvaccinated calves from a BTV-free population, four were similarly inoculated with BTV-2 and used as positive controls; the remaining four calves were used as negative controls to detect possible local circulation of wild BTV in the experimental groups. Ethylene-diaminetetra-acetic acid (EDTA) blood and serum samples were taken from all animals three times a week for two months and tested for the presence of BTV-2 antibodies using the c-ELISA and VN test.

**Virological tests**
Intravenous egg inoculation followed by two blind passages in Vero cells was used to isolate BTV-2 from EDTA blood samples, according to the method described by Savini et al. (4). Virus titres were determined in the blood of viraemic animals as follows: the blood cells were washed three times in phosphate-buffered saline (PBS) containing antibiotics. After the last washing, the sample was resuspended in MEM with antibiotics (1/10 v/v) and sonicated. Four tenfold dilutions of each sample suspension (from 1:10 to 1:10 000) were inoculated into 96 flat-bottomed microtitre plate wells, following the method described in the OIE Manual of standards for diagnostic tests and vaccines (3). Four replicates were made for each dilution. Approximately 10⁴ cells, in a volume of 100 µl of MEM plus antibiotics and 3% FCS, were added per well and the plates incubated at 37°C under 5% CO₂. The plates were examined after six days and the TCID₅₀ calculated.

**Serological tests**
The antibody response was monitored using both the c-ELISA (2) and the VN test. The VN was performed as described by Savini et al. (4). Reference virus and serum for BTV-2 were supplied by the OIE Reference Laboratory for bluetongue in Onderstepoort. Those sera neutralising BTV-2 at a dilution of 1:10 were considered positive. The antibody titre of the test serum was the highest dilution capable of neutralising 50% of the virus activity.

**Statistical analysis**
The BT viraemia data in animals were analysed using the Beta (ρ+1, n-ρ+1) distribution where ρ, the number of successes, is the total number of viraemic animals and n, the number of trials, is the total number of animals tested. The peak of the distribution represents the most probable value of
the percentage of viraemic animals and the extent of deviation gives information about the uncertainty of the estimates due to sample size. However, from an epidemiological point of view, it is far more interesting to know the percentage of vaccinated animals that are protected when challenged using a homologous BTV serotype, 1-beta (s+1, n-s+1), which represents the probability that more than a certain percentage of animals that are protected when challenged, was calculated.

**Results**

Of the 30 vaccinated cows, 29 developed ELISA and virus neutralising antibodies after immunisation; one animal was negative. Of these 29 cows, 8 had VN titres below 1:80, 15 had neutralising antibodies ranging between 1:160 and 1:320 while the remaining 6 had titres above 1:320. At the time of challenge, 11 cows had no VN antibodies, while the rest had low levels of circulating VN antibody titres of 1:10 (11 cows) and 1:20 (6 cows), while one cow had a titre of 1:40 and another a titre of 1:80 (Fig. 1). No animal showed signs of disease either after vaccination or after challenge. In addition, no BTV was isolated from the blood of the 29 cows that had developed antibodies pv. Commencing on day 9 post challenge (pc), BTV-2 was isolated from the blood of the single vaccinated cow which had not seroconverted after immunisation.

Levels and duration of viraemia in this animal were similar to those observed in the positive control group and lasted until day 21 pc (Fig. 2). Neither BTV nor antibody was detected in the blood samples collected from the negative control group. Figure 3 shows the curve of the probability that vaccinated cattle are protected, not showing any detectable viraemia against homologous challenge.

![Figure 1](image1)

Neutralising antibody titres in cattle vaccinated with monovalent bluetongue virus serotype 2 modified-live vaccine

![Figure 2](image2)

Circulating virus titres in cattle challenged with bluetongue virus serotype 2 field isolate

![Figure 3](image3)

Probability that cattle vaccinated with monovalent bluetongue virus serotype 2 modified-live vaccine are protected against homologous challenge

The P value is at least equal to the x-axis percentages

**Discussion**

Subclinical BT infection is common in cattle in infected zones and is characterised by prolonged viraemia, making cattle amplifying hosts and reservoirs for the virus (1). As a consequence, the movement of cattle from infected zones into areas where Culicoides and susceptible ruminants co-exist is one of the ways of spreading BT infection. The
Italian strategy to combat BT includes the vaccination of cattle to reduce virus circulation and to eventually ease the ban on cattle movements. This study demonstrated that the monovalent BTV-2 modified-live vaccine has an appropriate balance between attenuation of virulence and ability to replicate in cattle. More importantly, the antigenic stimulus provided by its replication elicits complete protection against challenge using a virulent homologous virus and does not cause any detectable viraemia in serologically positive vaccinated animals. These results show that at least 90.5% of vaccinated cattle will not develop a BT viraemia when challenged with virulent homologous virus seven months after immunisation. Furthermore, it would appear that the risk of spreading BTV-2 infection through the movement of vaccinated cattle will be very low. This study also confirmed that protective immunity against BT is associated with the presence of type-specific neutralising antibodies (6). However, it is worth noting that the level of circulating antibody, at the time of challenge, in animals that had previously seroconverted, does not seem to play an important role in preventing infection. In fact 10 of the 11 animals which had no detectable neutralising antibodies at the time of challenge were protected from homologous BTV challenge; the only animal that showed pc viraemia was the one in which neutralising antibodies had never been observed. According to this observation, it could be hypothesised that circulating antibody is a significant indicator of immunity but is not essential to prevent the development of viraemia in infected animals.

References

Neutralising antibody response in cattle after vaccination with monovalent modified-live vaccine against bluetongue virus serotype 2

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Summary

The antibody response following bluetongue (BT) vaccination under both field and experimental conditions, and the duration of colostral antibodies in calves born from vaccinated dams, were evaluated. To this end, 1,005 animals of various breeds and ages were selected at random from 10 herds in the Sardinian province of Oristano. During the first year of the vaccination campaign, the animals selected were vaccinated against BT virus (BTV) serotype 2 between July and August 2002. Blood samples were taken from all animals monthly for three months after vaccination and tested for the presence of BT antibodies using the competitive enzyme-linked immunosorbent assay (c-ELISA) and the virus neutralisation (VN) test. Serological results from field vaccinated animals were compared with those obtained following the vaccination of five animals under experimental conditions. Out of 1,005 animals, 994 (98.1%) developed BT antibody following vaccination whereas antibody was detected in all cows vaccinated under experimental conditions. Both groups showed the highest median titres of 1:160 after two months. To assess the duration of colostral antibodies in calves born from vaccinated dams, the sera of 47 calves were screened using the c-ELISA and VN test. Calves were divided into three age groups: Group A included 22 calves aged 1 to 25 days, Group B 13 calves aged 26 to 39 days and Group C 12 calves aged 40 to 60 days. Antibody was detected in calves in Groups A and B (68.2% and 46.1%, respectively) whereas the calves in Group C were serologically negative.

Keywords

Bluetongue – Cattle – Colostral antibody – Competitive enzyme-linked immunosorbent assay – Vaccine – Virus neutralisation – Virus.

Introduction

In areas infected with bluetongue (BT) virus (BTV), mortality and livestock movement restrictions cause considerable economic losses. The Italian Ministry of Health has implemented a compulsory BT vaccination campaign since May 2001. In an attempt to reduce direct losses due to disease and indirect losses due to virus circulation, the campaign has been conducted in infected and in adjacent areas and includes all susceptible domestic ruminants. During the first year of the campaign, Sardinia vaccinated 98.6% of the total sheep and goat population and 88.1% of the cattle population using a monovalent modified-live vaccine against BTV serotype 2 (BTV-2), manufactured by Onderstepoort Biological Products (OBP) in South Africa. It was the first time that the monovalent BTV-2 vaccine had been used in cattle and thus no information on its use in this species was available. Therefore, the primary aim of this study was to monitor the antibody response in cattle following vaccination under both field and controlled conditions. Acquired colostral immunity in calves is an outcome of vaccination; to design the vaccination campaign programme effectively, it is thus fundamental to know how long this immunity persists in the calves. Determination of the duration of colostral antibodies in calves born from vaccinated dams was the second objective of this study.
Materials and methods

The monovalent BTV-2 modified-live vaccine was produced by OBP in South Africa. Two groups of animals were used in this study. To monitor antibody response under field conditions, 1 005 cattle of various breeds and ages were selected at random from 10 herds in the Sardinian province of Oristano. Five cows were selected and kept in the security unit of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSA&M) to monitor their serological response to vaccination in a controlled environment. Between July and August 2002, both groups were vaccinated subcutaneously with 1 ml of $10^4$TCID$_{50}$ BTV-2 modified-live vaccine. Blood samples were taken from all animals monthly for three months thereafter. To assess duration of colostral antibodies, blood samples from 47 calves born from vaccinated dams were taken periodically. Based on their age at the time they were bled, the calves were divided into three groups: Group A included 22 calves 1 to 25 days old, Group B comprised 13 calves aged 26 to 39 days and Group C had 12 calves aged 40 to 60 days. Serum samples were tested for the presence of antibodies against BTV using a competitive enzyme-linked immunosorbent assay (c-ELISA) (VMRD, USA) and a virus neutralisation (VN) test (1). Positive and negative controls for the VN test were kindly provided by the Onderstepoort Veterinary Institute (OVI) (OIE Reference Laboratory), South Africa.

The serological data were analysed using the Beta (s+1, n−s+1) distribution where s, the number of successes, is the total number of positives and n, the number of trials, is the total number of tested animals. The peak of the distribution represents the most probable value of the percentage of positive animals and the distribution provides information about the uncertainty of the estimates due to sample size.

Results

Of the 1 005 animals vaccinated with monovalent BTV-2 modified-live vaccine under field conditions, 994 (98.1%) were positive for antibody against BTV using the VN test and c-ELISA, while 11 (1.09%) did not develop measurable BT antibody levels. BTV-2 antibodies were detected in all cows vaccinated under experimental conditions. High VN titres were observed in both groups. The experimental group had the highest peak antibody response one month post vaccination (pv), whereas in the field group, the highest titres were observed two months pv (Fig. 1). VN antibody titres detected in the animals vaccinated under field conditions are shown in Figure 2 while the distribution of the percentages of positive animals is displayed in Figure 3. When re-vaccinated after a year, the 11 animals that had previously been found serologically negative, developed neutralising antibody titres.

BT antibody was detected in calves in Groups A and B (68.2% and 46.1%, respectively), whereas the calves in Group C were serologically negative. Figure 4 illustrates the distribution of the percentage of positive calves according to age at the time blood was taken.
Discussion

As previously mentioned, most, if not all, of the information available concerning the use of the BT modified-live vaccine discusses only the application in sheep. Modified-live virus vaccines are produced by adapting BTV field isolates in vitro through serial passages in tissue culture or in embryonating chicken eggs. This process selects viruses that have a predilection for in vitro propagation and a reduced capacity to replicate in vivo and to cause disease. According to the literature, modified-live vaccines are effective in controlling clinical outbreaks of BT in endemic areas and in the face of outbreaks. If the vaccine virus retains an appropriate balance between attenuation of virulence and ability to replicate, the antigenic stimulus provided by its replication will elicit complete protection against challenge with virulent homologous virus and no clinical disease will develop. In this study, all animals exhibited an antibody response after being vaccinated twice with the BTV-2 modified-live vaccine; indeed a very high percentage (98.9%) developed antibody after a single vaccination. The neutralising antibody titres in the vaccinated cattle were similar to those observed in sheep (3). According to the beta distribution, based on the positive animals in this study, one injection of the BTV-2 modified-live vaccine in cattle is capable of producing VN antibodies in at least 98.2% of animals, with a confidence level of 95%. Since it has been demonstrated that protective immunity in BT is generally associated with the presence of type-specific neutralising antibodies (4), it can be concluded that the use of a monovalent BTV-2 modified-live vaccine in cattle will stimulate protection in almost all animals.

The second objective of this study addressed another important topic which has practical consequences. Knowing how long colostral immunity lasts in calves is crucial for livestock movement purposes in order to establish the age at which calves should be vaccinated. In lambs born to immune ewes, colostral immunity may last six months, during which time they are refractory to immunisation (2). In this study, no neutralising antibodies were found in calves older than 40 days. Due to the small number of animals tested, the probability curves were very wide and, for the oldest group of animals, the lower and upper prevalence levels were 0.2% and 21% (95% confidence level), respectively. Consequently, in calves older than 40 days, the prevalence of BT antibody-positive animals is lower than 21% with a confidence level of 95%.

References

Vaccination of cattle using monovalent modified-live vaccine against bluetongue virus serotype 2: innocuity, immunogenicity and effect on pregnancy

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Summary

The immunogenicity, innocuity and possible teratogenic effects of the monovalent modified-live vaccine against bluetongue (BT) virus (BTV) serotype 2, manufactured by Onderstepoort Biological Products in South Africa, was evaluated in cows. Twenty-one cows, 14 of which were at different stages of gestation, were vaccinated with 2 ml of monovalent vaccine; two served as unvaccinated controls. After immunisation, 16 vaccinated and the 2 unvaccinated controls were kept in the field; the remaining 5 pregnant cows were maintained in an insect-proof stable with a controlled environment. Blood samples were taken from field cattle once a week for two months and from the stable cattle three times a week. All samples were screened for the presence of BTV and for BT antibody using the competitive enzyme-linked immunosorbent assay (c-ELISA) and the virus neutralisation (VN) test. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV-2 from ethylene-diaminetetra-acetic acid (EDTA) blood samples and virus titres in viraemic animals were determined. After immunisation, 9 of the cows developed a viraemia which commenced on day 7 post vaccination (pv) and lasted for three weeks. The virus titres were never higher than $10^{2.8}$TCID$_{50}$/ml with the highest titre observed on day 14 pv. None of the vaccinated animals developed clinical symptoms that could be attributed to BTV; after three weeks all animals showed a serological response to BTV-2. In the c-ELISA, antibodies were detected from day 7 pv while in the VN test, antibodies were observed from day 21 pv. All pregnant cows completed their gestation: 13 gave birth to healthy calves, while one of those in the field group, vaccinated at the six months gestation, delivered a calf with prosencephalic hypoplasia, possibly developed during foetal organogenesis prior to vaccination. Fourteen months after immunisation the stabled cows were challenged subcutaneously by administering $2 \times 10^{6.8}$TCID$_{50}$ BTV-2 Italian isolate. A third group of 4 cows was also inoculated with the BTV-2 Italian field isolate, as described for the second group and was used as the unvaccinated positive control group. Vaccinated cows had a detectable viraemia only on day 14 pv and virus titres were very low. Virus titres never exceeded $10^{2.3}$TCID$_{50}$/ml, while the unvaccinated group developed a long and intense viraemia, peaking on day 14 pv with a titre of $1.18 \times 10^4$. It is concluded that the BTV-2 modified-live vaccine used in this study was a harmless and effective immunogen that did not cross the placental membrane.

Keywords

Introduction

Bluetongue (BT) is a viral disease of domestic and wild ruminants caused by an Orbivirus belonging to the family Reoviridae (14). Bluetongue virus (BTV) is transmitted by arthropod vectors of the genus Culicoides (17). The disease in sheep can produce severe clinical symptoms (4) while in cattle and wild ruminants few or no clinical signs are observed (2). BT has been designated by the Office International des Epizooties (OIE) as a ‘List A’ disease; this has prompted restrictions on trade of susceptible animals from a BT-infected country (15). After the outbreaks of BT in Italy in 2000, the Italian Ministry of Health implemented a campaign whereby all domestic ruminants were to be vaccinated against BT to reduce direct losses due to disease and indirect losses due to virus circulation. The modified-live vaccine against BTV-2 manufactured by Onderstepoort Biological Products (OBP) in South Africa was used; this was the only BTV-2 vaccine commercially available. Use of this vaccine was recommended only in sheep and since it can be teratogenic and can induce abortions, administration was not recommended during the first half of pregnancy. Since little or no data was available on the vaccination of cattle, the present study was conducted to evaluate the immunogenicity, innocuity, efficacy and possible teratogenic effect of monovalent BTV-2 modified-live vaccine in cattle. The virus titre and the duration of viraemia following immunisation were also determined.

Materials and methods

Vaccine

Before use, the modified-live virus (MLV) vaccine was reconstituted in phosphate-buffered saline (PBS) pH 7.2 and titrated and checked for contaminants. Innocuity was tested by inoculating six adult mice and two guinea-pigs intraperitoneally (0.25 ml) and four sheep subcutaneously (1 ml). All animals were monitored daily for two weeks and any clinical signs recorded.

Animals

Eighteen cows virologically and serologically negative for BTV were selected from two different farms located in the provinces of Palermo and Trapani. In both areas, BTV had been reported previously. However, no outbreaks or viral circulation had been observed during the period of the trial (March to July 2001).

Of the 18 animals, 9 were pregnant. A total of 16 were vaccinated in the region of the neck with 2 ml of vaccine and 2 non-pregnant cows were inoculated with a placebo and kept as negative controls.

A second group of 5 pregnant cows was similarly vaccinated and kept in an insect-proof stable with a controlled environment. Fourteen months after vaccination, the 5 cows were challenged by subcutaneously administering a BTV-2 Italian field isolate; the titre of the inoculum was $2 \times 10^{6.8}$ TCID$_{50}$. A third group of 4 cows was also inoculated with the BTV-2 Italian field isolate, as described for the second group, and used as unvaccinated positive controls. Table I provides additional details on the cows used in this study.

The temperatures of all cows were recorded daily and clinical signs monitored. All newborn calves were examined for possible malformations and, when possible, blood was taken from each calf before colostrum was consumed. Ethylenediaminetetra-acetic acid (EDTA) blood and serum samples were taken once a week from all field cows for two months, and three times a week for the stabled cows. The EDTA blood samples were screened for the presence of BTV while sera were examined for c-ELISA and virus neutralising (VN) antibodies. Intravenous egg inoculation, followed by two blind passages in Vero cells, was used to isolate BTV according to the method described by Savini et al. (17); virus titres were also determined (17).

Results

The vaccine was found to be free of bacterial and viral contaminants; the virus titre of a single dose was found to be $2 \times 10^{2.2}$ TCID$_{50}$ of modified-live BTV-2. No clinical signs were observed in vaccinated or in challenged animals. After immunisation, 14 animals developed viraemia with titres of less than $10^{2.8}$ TCID$_{50}$/ml. Virus was first isolated 7 days post vaccination (pv) and viraemia lasted for three weeks reaching a peak on day 14 pv (Fig. 1).

When the vaccinated group was challenged, only two cows developed detectable viraemia ($10^{2.3}$ TCID$_{50}$/ml) on day 14 post challenge (pc), whereas in the unvaccinated group, viraemia lasted for five weeks and reached higher titres (Fig. 1).

All vaccinated animals developed BTV-2 antibodies; c-ELISA antibodies were detected from day 7 pv while VN antibodies were observed from day 21 pv and peaked four weeks later (Fig. 2). Negative controls remained serologically and virologically negative against BTV throughout the study.
Table I
Gestation status of 27 cows vaccinated using a bluetongue virus serotype 2 live-modified vaccine, Teramo, Palermo and Trapani Provinces, Italy, 2001-2002

<table>
<thead>
<tr>
<th>No.</th>
<th>Tag No.</th>
<th>Farm</th>
<th>Artificial insemination date</th>
<th>First pregnancy test results</th>
<th>Second pregnancy test results</th>
<th>Pregnancy stage when vaccinated</th>
<th>Delivery date</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>C 279</td>
<td>Farm 1</td>
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<td>6 months</td>
<td>7 months</td>
<td>6 months</td>
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</tr>
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<td>2</td>
<td>C 280</td>
<td>Farm 1</td>
<td>February</td>
<td>Doubtful</td>
<td>Negative</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>C 283</td>
<td>Farm 1</td>
<td>October</td>
<td>5 months</td>
<td>6 months</td>
<td>5 months</td>
<td>July</td>
</tr>
<tr>
<td>4</td>
<td>C 270</td>
<td>Farm 1</td>
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<td>5.5 months</td>
<td>6.5 months</td>
<td>5.5 months</td>
<td>July</td>
</tr>
<tr>
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<td>C 261</td>
<td>Farm 1</td>
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<td>7.5 months</td>
<td>6.5 months</td>
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</tr>
<tr>
<td>6</td>
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<td>–</td>
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<td>7</td>
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<td>–</td>
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<td>8</td>
<td>Control</td>
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<td>–</td>
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<td>–</td>
<td>–</td>
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<tr>
<td>10</td>
<td>C 001</td>
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<td>Negative</td>
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<td>–</td>
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<tr>
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<td>February</td>
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<td>Negative</td>
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<td>–</td>
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<tr>
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<td>Farm 2</td>
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<td>2.5 months</td>
<td>1.5 months</td>
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<td>–</td>
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<td>6.5 months</td>
<td>5.5 months</td>
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</tr>
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<td>2875</td>
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<td>October</td>
<td>5 months</td>
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Figure 1
Bluetongue virus serotype 2 titres in cattle following vaccination and/or experimental infection

Figure 2
Bluetongue virus serotype 2 mean neutralising antibody titres in cattle following vaccination and/or experimental infection
All pregnant cows completed their gestation: 13 gave birth to healthy calves, while one cow from the field group that had been vaccinated during the sixth month of gestation, delivered a seriously malformed calf. Autopsy revealed an internal deviation in the left foreleg and in the neck. The upper part of the head was flattened and had a hole that opened into the cranial cavity. Post-mortem examination of the skull revealed a thickening of the bone, hyperaemia of the meninx, and hypoplasia of the brain and of the dura madre. A gelatinous subcutaneous oedema and a deviation of the atlas and epistropheus joint were clearly visible in the area of the neck. Heart degeneration and pericardial haemorrhagic petechiae were also detected in the thoracic cavity. Punctiform haemorrhages were observed on the surface of the thymus. The abdominal cavity showed haemoperitoneum, soft kidney, liver degeneration and haemorrhagic foci on the surfaces of the liver and the spleen. Bacterial culture of the organs of the stillborn calf revealed the presence of Pseudomonas spp. and of Escherichia coli; the polymerase chain reaction (PCR) was negative for bacterial and virological abortigenic agents. Of the 13 calves born to vaccinated dams, four had both VN and ELISA antibodies.

At the time of challenge, animals of the second group were still c-ELISA positive and had average neutralising antibody titres of 1:128. The titres increased rapidly pc and peaked (1:360) at three weeks.

The group of unvaccinated animals developed c-ELISA antibodies from day 14 pv while VN antibodies were observed from day 21 pv, and peaked three weeks later (Fig. 2).

**Discussion**

The current vaccination strategy to prevent BTV infection in ruminants relies on MLV vaccines such as that used in this trial. The vaccine was developed using field strains isolated from infected animals and then attenuated by serial passage in embryonating chicken eggs and in cell culture. MLV vaccines have to replicate in the vaccinated animal in order to stimulate protective immunity and, in the process, they might induce a mild, or subclinical, illness (6). MLV has several potential drawbacks, including reversion to virulence, insect transmission, impaired reproductive performance and foetal malformations. The latter have been reported in vaccinated ewes, with malformations observed primarily during the first half of gestation (6, 7, 8, 18). In South Africa, the vaccine is used in sheep only and little information is available on its use in cattle. Cases of hydroanencephaly were described in calves born to cows experimentally inoculated by the intrauterine route in the first half of gestation with tissue culture-adapted BTV (1, 10, 11, 20). BT infection (with malformations) was confirmed by the presence of BT antibodies in calves that had not received colostrum (9). It has been demonstrated that field strains of BTV rarely cross the placental barrier; therefore, abortions and foetal malformations should be infrequent under field conditions (11, 12). However, MLVs have been capable of crossing the placental barrier with foetal malformations in sheep (19). In this study, the vaccine appeared to be innocuous despite the administration of a double dose: no animals developed clinical symptoms of BT, and no abortions, or impairment of reproductive function, were observed. The teratogenic malformations reported in the stillborn calf probably commenced prior to vaccination of the dam as the malformation was not typical of BTV-induced hydroanencephaly but was of a prosencephalic hypoplasia, characterised by a small opening in the skull (3). Furthermore, BTV was not isolated from the calf and the haemorrhagic lesions could have been the consequence of a septicaemic form of E. coli. Further proof that the MLV did not cross the placental barrier was the absence of BT antibodies in calves that had not received colostrum. Titres were observed in four calves, but blood had been drawn only after they had received colostrum. The vaccine was strongly immunogenic and all vaccinated cattle seroconverted. In line with previous work (16), the c-ELISA proved to be more sensitive than the VN test; c-ELISA antibody was detected during the second week pv. Interestingly, neutralising titres were still present 14 months pv in animals which had no evidence of detectable viraemia after vaccination. Protective immunity against BT has been associated with the presence of type-specific neutralising antibodies (5). In this study, BTV-2 modified-live vaccine was able to protect cattle from infection with the homologous BTV serotype 14 months after immunisation. Vaccinated cows had detectable viraemia only on day 14 pv and the virus titres were very low. Virus titres were never higher than 10^2 TCID50/ml while the unvaccinated group developed a long and intense viraemia, peaking on day 14 pv with a titre of 1.18 × 10^4. The duration of viraemia and the high virus titres are of great epidemiological importance as they significantly influence the persistence of BTV in the environment. It has been shown that titres below 10^3 TCID50/ml are not able to infect Culicoides (13). In this study, the virus titres pv and pc in vaccinated animals were below 10^3 TCID50/ml, indicating that vaccine virus would not have been spread by locally active and competent Culicoides vectors (Fig. 1).
In conclusion, these experimental trials demonstrated the BTV-2 MLV vaccine to be both safe and effective, and that it did not cross the placental barrier.

References


Office International des Épizooties international standards for bluetongue

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Summary

Preventing the spread of disease through international trade is one of the primary objectives of the Office International des Épizooties (OIE), the World Organisation for Animal Health. This is accomplished by establishing international standards that facilitate trade while minimising the risk of introducing diseases such as bluetongue (BT). The OIE standards for BT are contained in the Terrestrial animal health code (Code) and the Manual of diagnostic tests and vaccines for terrestrial animals (Manual). These standards include procedures for prompt reporting of BT outbreaks; requirements that should be met for a country or zone to be defined as free of bluetongue virus (BTV); recommendations for the safe importation of live animals, semen and embryos into a BTV-free country or zone; and the general provisions that countries should meet to reduce the risk of spread of BTV through trade. The Manual describes in detail the various tests for the diagnosis of BT. It provides a list of prescribed tests; these are the tests that are required by the Code for the testing of animals in connection with international trade.

There are 24 serotypes of BTV and infected countries have the right to restrict imports from countries that have different types of BTV. However, this should only be done if a surveillance and monitoring programme has confirmed that the other types are not present. Zoning for an arbovirus is difficult to apply but zoning for vectors is practicable. Some countries have demonstrated that there is no evidence of infection in their country or parts of their country even though there has been unrestricted animal movement between endemic zones and free zones. This freedom is due to the absence of vectors in the free zone. Based on this observation, free countries and zones can be established if an appropriate surveillance and monitoring programme is in place to define their boundaries. Consequently, there have been extensive changes in the Code to allow the establishment of BTV-free countries and zones and seasonally free countries and zones to provide the basis for safe trade, while minimising the risk of the introduction of BTV.

Keywords

international standards by the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) of the World Trade Organization (WTO) (4).

Developing BT standards that allow the safe trade of animals and animal products, has been very difficult as much of the world between latitudes of approximately 40°N and 35°S is infected or has the potential of being infected. These standards have taken on new importance as the infection has moved north and west in Europe. Another factor that has complicated the development and application of these standards is that there are 24 serotypes of BTV and many known and potential vectors of different competencies.

The OIE standards that have been and are being developed to promote safe trade in live animals, semen and embryos will be discussed.

The World Trade Organization Sanitary and Phytosanitary Agreement

The OIE was identified by the WTO SPS Agreement as the competent international organisation for developing international standards, guidelines, and recommendations relating to animal diseases and zoonoses. In order to harmonise health measures, the Agreement states that governments should use these international standards, guidelines and recommendations. The goal of the Agreement is to remove unjustifiable sanitary (health) restrictions on international trade. The Agreement states that it is the sovereign right of a country to provide an appropriate level of animal health protection against pest or disease entry. However, this sovereign right is not to be misused for protectionist purposes and import sanitary measures can only be enforced if a similar level of protection against the disease is applied to all imports, and internally, by the importing country. Member countries can introduce standards providing a higher level of protection than provided by the OIE standards if there is a scientific justification but these national standards must be based on a risk analysis. With regard to a disease such as BT, a member country which is infected with one or several types of BTV has the right to adopt sanitary measures to prevent the entry of other types of BTV from which it is free. A monitoring and surveillance programme must be in place to demonstrate that the other types are not present.

OIE standards for bluetongue

The OIE standards are contained in the Terrestrial animal health code (Code) (2) and the Manual of diagnostic tests and vaccines for terrestrial animals (Manual) (3). The Code provides the chief veterinary officers of the OIE member countries with recommendations for establishing national or regional sanitary measures or rules applicable to the importation of animals and animal products. The Manual describes the diagnostic methods that are to be used and the methods for the production and control of biological products. The Code and Manual are developed by the OIE specialist commissions made up of experts elected by the OIE member countries. Proposed new or revised standards are submitted to the member countries for review and comment. These comments are included as appropriate in the proposed standards which are then submitted to the international committee of these member countries for approval. A revised version of the Code is published annually and a revised Manual is published every four years; the current edition of the Manual was published in 2004.

Terrestrial animal health code on bluetongue

Chapter 2.1.9 of the Code outlines the requirements that should be met for a country or zone to be defined as free of BTV and the sanitary measures that should be applied when importing live animals, semen and embryos into a BTV-free country or zone. Prior to 1999, this chapter of the Code stated that BTV-susceptible species could only be imported from an infected country into a free country if they were negative for antibodies and were held in quarantine for 40 days. Requests from member countries to modify the bluetongue chapter of the Code resulted in the formation of an ad hoc group which developed a new chapter which was approved by the international committee in 1999. Refinements to the chapter have been approved each subsequent year. Some of the key components of the chapter as published in the 2003 version of the Code (2) are summarised here.

The first article of the chapter includes some general, very important information. It states that for the purposes of the Code, the infective period for BTV shall be 100 days. This article specifies that the BTV distribution historically has been between latitudes of approximately 40°N and 35°S. It also outlines the surveillance and monitoring requirements that are to supplement the general provisions of the Code, which will be described later. It states that, if a country or zone lies between 40°N and 35°S and does not have confirmed clinical BTV infection, it should establish a surveillance and monitoring programme to demonstrate its BTV status. This programme should be adjusted for local conditions such as historical, geographical and climatic factors, ruminant and Culicoides population data, or proximity to enzootic or incursional zones. Random and targeted
serological surveillance should provide at least a 95% level of confidence of detecting an annual seroconversion incidence of 2% in cattle (or other ruminant species if sufficient cattle are not available).

It continues to state that countries or zones located outside this part of the world but adjacent to a country or zone not having free status should be subjected to similar surveillance; this surveillance should be carried out over a distance of at least 100 km from the border with that country or zone. An appendix to the Code is being drafted that will provide a more detailed standard for BTV surveillance and monitoring. The chapter defines a free country or zone as:

1) a country or zone that lies wholly north of 40°N or south of 35°S, and is not adjacent to a country or zone not having free status; or

2) a country or zone that has a surveillance and monitoring programme, as outlined above, which has demonstrated that there is no evidence of BTV in the country or zone during the past two years and there has been no vaccination against BT during the previous twelve months; or

3) a country or zone that has a surveillance and monitoring programme that demonstrated no evidence of Culicoides.

The chapter provides three approaches for moving live animals, based on the epidemiology of BT. It states that:

1) animals kept in a BTV-free country or zone or protected from Culicoides since birth or for at least the 100 days prior to shipment can move without further restriction into a free country or zone

2) animals that have been in a BTV-free country or zone or protected from Culicoides for 28 days should be tested for BTV antibody using the agar gel immunodiffusion (AGID) test or the enzyme-linked immunosorbent assay (ELISA) and, if negative, can be imported into a free country or zone

3) after 7 days in the free country or zone the animal can be tested for BTV nucleic acid using the polymerase chain reaction (PCR) or virus isolation and, if negative by one of these procedures, can be imported into a free country; in the case of import from infected countries or zones, the animals must be protected from Culicoides for the 14 days prior to shipment and subjected twice to such testing.

In addition, the chapter states that the importation of animals from an infected country or zone will not affect the status of a free country or zone in which surveillance and monitoring have found no evidence of BTV vectors; this was a significant change that was added in the 1999 revision.

The 1999 revision of the chapter added an article describing a BTV seasonally free zone. This is an infected country or zone in which, for part of a year, surveillance and monitoring demonstrate no evidence either of BTV transmission or of adult Culicoides. The seasonally free status is applicable up to 28 days before the earliest date when historical data indicate that virus activity would recommence. Animals can be imported into a free country after 100 days in a seasonally free zone; however, in many countries or zones this is not feasible as they do not have a free period of 100 days and the Code states that animals in the seasonally free zone during the free period can be imported if they have had two negative AGID or ELISA tests seven days apart after 21 days of residence or two negative PCR tests seven days apart after 7 days of residence.

A BTV-infected country or zone is a country or zone that does not meet the requirements to be free or seasonally free. The 1999 revision provided more methods to import from an infected country or zone to a free country. It states that, if the animals were protected from Culicoides attack, they can be imported to a free country using about the same three approaches as outlined above for importing from a seasonally free zone.

Semen can be imported into a free country or zone using a similar approach. Semen from animals that have been in a free country for 100 days at the time of collection can be imported without further restriction. Semen from other animals can be imported into a free country after two negative AGID or ELISA tests on blood samples from donors taken 28 and 60 days after collection, or one negative PCR or virus isolation test on a blood sample taken at the time of collection.

In accordance with International Embryo Transfer Society (IETS) recommendations, in vivo derived bovine embryos are considered not to present a risk of BTV transmission. Embryos from other susceptible species should meet criteria similar to those for the importation of semen.

Terrestrial animal health code: general provisions

The chapters in part 1 of the Code, general provisions provide the basic standards underpinning the disease specific chapters (2). Some of the more significant points included in these chapters are given below.
Diseases in ‘Lists A and B’
The OIE has designated 15 diseases, including BT, as being ‘List A’ diseases. List A diseases are transmissible diseases which have the potential for very serious and rapid spread, irrespective of national borders, and consequently are of major importance in the international trade of animals and animal products. There are also 67 List B diseases, which are considered to be less significant in international trade. A single list of diseases, replacing Lists A and B, will become operational in January 2005.

Notification and epidemiological information
Member countries of the OIE are obliged to make available to other countries through the OIE whatever information is available that will help prevent the spread of important animal diseases. Member countries must report outbreaks of List A diseases, including BT, to the OIE within 24 hours if they were previously considered free. They also need to report a provisional diagnosis of BT if this represents important new information of epidemiological significance to other countries. Following the initial report, monthly reports need to be provided. The OIE forwards this information to member countries. Several countries have reported to the OIE every year that BT is endemic in cattle, sheep, goats, wildlife and camels; as they are not considered free, there is no requirement to report each new case within 24 hours. New notification requirements, based on the single list of diseases, will also become operational in January 2005.

Evaluation of veterinary services
This chapter provides guidelines for the evaluation of veterinary services, which is an important element in the risk analysis process of an importing country. The results of this evaluation can help provide the importing country with the assurance that information on the animal health situation provided by the veterinary services of an exporting country is objective, meaningful and correct.

Obligations and ethics in international trade
The obligations of the importing and exporting countries are described. As stated in the SPS Agreement, commodities imported should comply with the national level of protection that the importing country has established. The requirements applying to pathogens or diseases subject to official control programmes in a country or zone should not provide a higher level of protection on imports than that provided for the same pathogens or diseases within that country or zone. For most diseases, a country that has endemic infection and no programme to monitor and perhaps control the disease cannot apply import restrictions. However, since there are 24 types of BTV that do not cross-protect, restrictions on exotic types can be justified. Before these restrictions are put in place, surveillance and monitoring programmes should be conducted to determine what types of BT are present in the country.

Surveillance and monitoring of animal health
This chapter outlines the minimum requirements for a surveillance and monitoring programme that will substantiate elements of a report from a country on its animal health situation, and is the basis for a country to be able to claim a certain status for a disease. Information provided by the surveillance and monitoring programme of the exporting country is a key component of the application of OIE standards and of the risk analysis conducted by an importing country. As outlined above, obtaining freedom of BT requires documented evidence that an effective system of surveillance for BTV infection is in operation. The OIE is developing general guidelines on surveillance for diseases and a Code appendix on surveillance for BTV.

Zoning and regionalisation
The procedure for designating a zone is provided. Zoning provides a country that has the disease in one portion of the country a method of establishing a disease-free zone in another portion of the country. The size, location and delineation of a zone will depend on the epidemiology of the disease, environmental factors, the surveillance conducted and applicable control measures. The extent of zones and their limits should be established by the veterinary administration on the basis of natural, artificial, or legal boundaries and made public through official channels. The chapter encourages importing countries to recognise the zones that an exporting country develops. Several countries have succeeded in defining a BTV-free zone from which animals can be exported with fewer restrictions.

Import risk analysis
Detailed procedures for conducting an import risk analysis are provided. The components of risk analysis are: hazard identification, BTV in this case; risk assessment, which is the evaluation of the likelihood and consequences of entry, establishment, and spread of BTV and includes release assessment, exposure assessment, consequence assessment, and risk estimation; risk management, which describes the determination of the measures necessary to reduce the level of risk to a level acceptable for the importing country; and risk communication, which is the exchange of information with stakeholders during the risk analysis.
Manual of diagnostic tests and vaccines for terrestrial animals

The Manual is a companion volume to the Code and provides a uniform approach to the diagnosis of BT. The purpose is to facilitate international trade in animals and animal products by describing internationally agreed laboratory methods for diagnosis and requirements for the production and control of BT vaccines. The methods described also form the basis for effective BTV surveillance and monitoring. The Manual describes in detail the various tests for the diagnosis of BT. It provides a list of prescribed tests; these are the tests that are required by the Code for the testing of animals in connection with international trade. The 2004 edition of the Manual specifies that agent identification, AGID, ELISA and PCR tests are the prescribed tests and these tests are described in detail. The agent identification procedures described are intravenous inoculation of embryonating chicken eggs and sheep inoculation. The Manual states that isolation can be attempted in cell culture but the success rate is often much lower than in eggs or sheep. These procedures require 3 to 5 weeks; consequently, they are usually not practical to meet the import requirements outlined above. The 1999 changes in the Code allowed the use of PCR to qualify animals for importation. The following is a quote from the Manual concerning the use of PCR for BTV. ‘Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis. Results to date indicate that polymerase chain reaction techniques may be used, not only to detect the presence of viral nucleic acid, but also to ‘serogroup’ orbiviruses and provide information on the serotype and possible geographical source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample such as infected sheep blood.’ It goes on to provide complete details for conducting the test, including the suggested primers for a conserved region of the gene.

The AGID and ELISA are prescribed serological tests for BT and are described in detail in the Manual. Both tests are group-specific and can detect all types of BTV with one test. The AGID was described about 30 years ago but is still used by many countries. The lack of specificity of this test is a limitation as it can detect antibodies to other orbiviruses, particularly those in the epizootic haemorrhagic disease (EHD) serogroup; however, the sensitivity is adequate. A competitive or blocking ELISA (c-ELISA) procedure is described. The monoclonal antibodies that can be used for this test have been derived in a number of laboratories, and appear to bind to the major core protein VP7. In the c-ELISA, antibodies in test sera compete with the monoclonal antibody for binding to antigen. The c-ELISA has been standardised after comparative studies in a number of international laboratories. Advantages of the ELISA over AGID are its adaptability to automation and less subjectivity exercised in reading the results. Virus neutralisation is designated as an alternative test. An alternative test is one that is suitable for the diagnosis of disease within a local setting, and can also be used in the import/export of animals after bilateral agreement. The virus neutralisation test is specific for each type of virus; consequently each virus type must be included in the test. This makes the test difficult and usually impractical for use as an export test.

Discussion and conclusions

The 2003 edition of the BT chapter of the Code and the 2004 edition of the Manual have attempted to address the need to establish methods for safe importation from BTV-infected, seasonally free, as well as free countries or zones. These changes take into account the fact that much of the world is infected or potentially infected with some of the 24 serotypes of BTV and should not be restricted unnecessarily from trading BTV-susceptible animals. Another issue that is addressed in the revised Code is the need for surveillance to determine the distribution of BTV. BTV often does not produce clinical disease in many susceptible animals; consequently, surveillance must be conducted to determine the extent of the distribution of the virus in the potentially infected countries or zones. The continuing spread of the virus north and west in Europe will require increased surveillance in the region. BTV surveillance and monitoring procedures are described in the Code and will be expanded upon in the new chapter that is being developed as an appendix to the Code. Zoning for an arbovirus is difficult to apply but zoning for vectors is practicable. Two countries (Australia and the United States of America) have demonstrated that there is no evidence of infection in part of their countries due to the absence of vectors in the free zone even though there has been unrestricted animal movement between the endemic and free zones. Based on this observation, free zones can be established if an appropriate surveillance and monitoring programme is in place to define their boundaries.

References

2. Office International des Épizooties (2003). – Bluetongue. In Terrestrial animal health code,


European Union policy for the control and eradication of bluetongue

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Summary

When bluetongue (BT) reappeared in Europe in 1998, the rules that applied to the control of this vector-borne disease had to be reassessed. Accordingly, a draft directive submitted to the European Council as early as 1999 was adopted in 2000, just in time to face the unprecedented situation which occurred in the late summer of that year. Although based on the existing rules applicable to African horse sickness, there is greater flexibility in the establishment of restricted BT zones and in the movement of livestock in order to deal more adequately with specific local situations. Later, as the outbreak evolved, a number of provisions were adopted within the framework of this new directive in regard to both the protection and surveillance zones and to the movement of animals into and out of these zones. Based upon a risk assessment conducted by the scientific committee, the European Commission (EC) also considered the option of a vaccination policy using the live-attenuated vaccine available. On request from the EC, studies were conducted to test the safety and the efficacy of this vaccine in sheep, cattle and goats. By July 2000, a vaccine bank had been established which facilitated rapid and successful intervention in the Balearic islands. The EC later supported the vaccination option whenever national authorities wished to adopt this policy. In addition, the EC modified the rules regarding financial contributions by the Community to cover not only emergency situations but also the long-term surveillance of BT and control actions.

Keywords

Bluetongue viruses and trade issues: a North American perspective

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Summary

The geographic distribution of bluetongue (BT) viruses (BTVs) is governed by definable virus-vector-ecological/environmental relationships. The infection can only be transmitted by competent vectors. In the United States of America (USA), the New England States (Maine, Vermont, New Hampshire, Massachusetts, Rhode Island and Connecticut) and the northern tier of states from Maine to Montana are free of BTV because they are vector-free. Likewise, the eastern provinces of Canada are free of both the vector and the viruses. In Mexico, different virus-vector ecosystems exist in the northern and southern regions of the country. Historically, significant trade in cattle has occurred between Canada and the USA and the USA and Mexico. Although unrestricted year-round movement of cattle from BTV-endemic areas to vector-free and BTV-free areas occurs, BTVs have never been isolated from resident cattle in such virus-free areas in the USA. The authors discuss current BT-related requirements for trade within North America and elsewhere.

Keywords


Bluetongue virus serotypes and vector relationships in mainland North America

The geographic distribution of bluetongue (BT) viruses (BTVs) is governed by definable virus-vector-ecological/environmental relationships. BTVs can only be transmitted by competent vectors. Within the United States of America (USA), the New England States and the northern tier of states from Maine to Montana are essentially BTV-free because they are vector-free. Despite the fact that Montana, like the prairie provinces of Canada, is not free of *Culicoides sonorensis*, there is little evidence that these populations are competent vectors. Moreover, while Canada has concerns about the competence of *C. sonorensis* populations in Montana, there is limited serological evidence of the presence of BTV antibodies in cattle. No virological or clinical evidence has been detected that suggests that BTV transmission occurs or that competent vectors exist in Montana. Regardless, Canada recently has recognised that Montana has a low risk of virus transmission.

Five serotypes of BTV, 2, 10, 11, 13 and 17, occur in the continental United States. Serotypes 10, 11, 13 and 17 are associated with *C. sonorensis*, which is prevalent in much of the middle, southern and western USA. This vector does not persist in the north-east USA, perhaps due to competition with *C. variipennis*, the probable vector of epizootic haemorrhagic disease viruses. The other virus-vector association which has been reported is with BTV-2 and *C. insignis* in southern Florida. A similar vector-virus relationship exists with BTV serotypes 1, 3, 4, 6, 8, 12, 14 and 17 in the Caribbean islands and the territories of Puerto Rico and the United States Virgin Islands, again associated with *C. insignis*.

Canada conducts triennial serological surveys for BTV exposure and has occasionally found serological evidence of infection in the Okanagan Valley of British Columbia, which extends into the State of Washington. In addition, *C. sonorensis* has been found in southern portions of the western provinces.

Mexico, on the other hand, has both *C. sonorensis*-BTV serotype associations typical of those in the
mainland USA and *C. insignis*-BTV associations common to the rest of the tropical regions of the Americas.

Mainland USA has no BT requirements on cattle from Puerto Rico or the United States Virgin Islands, despite the different virus-vector ecosystems in the Caribbean Basin. The same is not true regarding trade between other Caribbean islands and the USA. Regardless, Caribbean BTV serotypes have not been introduced by livestock movement into the continental USA.

**Cattle trade between the United States and Canada**

The USA has been the primary destination for Canadian beef exports; the estimated value to industry is US$1.2 billion annually. In 2002, the USA imported just under 1.7 million head of live cattle from Canada, mostly for slaughter. During the same year, the USA exported 134,220 head of live cattle to Canada, with a trade value estimated at US$50 million. Most of these cattle were feeder cattle imported into Canada under the ‘restricted feeder programme’. This programme mitigates the principal disease concerns of Canada, namely BT and anaplasmosis, and does so by allowing the controlled importation of cattle during winter months only (October to March) from certain low-risk states and with a treatment protocol for anaplasmosis.

The volume of cattle trade involved in the restricted feeder programme from 2000 to 2002 ranges from approximately 209,000 to 119,000. During 2003, trade was reduced significantly due to drought and the finding of a single case of bovine spongiform encephalopathy (BSE) in Canada, which led to a cessation of live cattle exports from Canada to the USA.

The USA and Canadian cattle industries have been working to find ways to increase exports to Canada, while continuing to protect the health status of the Canadian cattle herd. Increasing scientific evidence has indicated that the risk of BT and anaplasmosis spread and disease establishment in Canada through live cattle trade may not be as great as previously thought; risk may be mitigated through simple, science-based measures.

The eastern provinces of Canada, the northern tier of states from Minnesota to Montana and the New England states, extending as far south as Maryland and Pennsylvania, are BT-free because they are vector-free. While populations of a known BTV vector species, *C. sonorensis*, occur in Montana and the southern parts of the prairie provinces of Canada, there is no virological or clinical evidence to suggest that these populations are competent vectors. The scant serological data may reflect the limits of the serological tests. Environmental and ecological conditions in the northern states and Canadian provinces may not permit the phenotypic expression of the genetically controlled oral susceptibility of these populations to BTV. Recognising this, Canada has taken action to lift their restrictions for the movement of feeder cattle from all parts of the USA to the eastern provinces.

Under a pilot programme, Canada has expanded the restricted feeder programme for cattle from Montana and North Dakota into the summer months because the proposed importation into defined feedlots with vector control programmes was judged to present a low risk for the introduction of BTV. While vector competence and vector capacity studies are being conducted on populations of *C. sonorensis* from Alberta and Montana by scientists in Lethbridge (Alberta) and Laramie (Wyoming), respectively, the four basic criteria to prove vectorship must be satisfied and evaluated with the considerable body of historic virological and clinical evidence that BTV transmission does not occur in these areas.

As Canada is vector-free (except for occasional incursions into the Okanagan Valley of British Columbia) and BT-free, the perspectives for Canada are significantly different from those for the USA and Mexico. Due to its classification as ‘free’ from BT, Canada must immediately report any case of this Office International des Épizooties (OIE) ‘List A’ disease. By contrast, BTV is endemic in many parts of the USA and in Mexico. As such, it is notifiable annually to the OIE. Canada has committed to work with the USA to address the classification matter at the international level.

Trade in live animals during the vector-free period, recognising the incubation and infective period of the disease, has been facilitated. The experience gained suggests that amendments to the existing OIE *Terrestrial animal health code* provisions could be accommodated without jeopardising the health status of the receiving country. The vector-free principle can be applied equally to the country of origin as well as to the country of destination.

One of the first international success stories for the use of regionalisation was achieved in Canada in 1988 through the use of ongoing surveillance and sentinel programmes and movement controls, as well as recognition of vector dynamics. Consequently, the international community has recognised Canada to be free of BTV infection with a small geographically defined exclusion zone in the Okanagan Valley of...
the Province of British Columbia and provided a science-based standard for trade.

The current ban on the importation of live cattle and other ruminants from Canada into the USA is due to the finding in May 2003 of a single case of BSE in Canada. The Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) continues working to resolve the impediments to live cattle exports to Canada where this is technically possible and as resources allow. APHIS has fostered continued collaboration with states, representatives of the cattle industry, universities, USDA Agricultural Research Service scientists, and the Canadian Food Inspection Agency (CFIA). APHIS has provided available information to CFIA and will continue to foster the sharing of data. Furthermore, APHIS is actively consulting with CFIA on anaplasmosis and BT, and intends to continue submitting requests for risk evaluation by CFIA to further reduce Canadian import requirements for BT, giving consideration to existing and emerging scientific knowledge.

APHIS is actively collaborating in various studies concerning BT and anaplasmosis. Several of these studies include official Canadian participation, as follows:

1) the joint Montana-Alberta feeder cattle study is examining the status of feeder cattle from various regions in Montana relative to BTV and Anaplasma exposure over a three-year period.

2) A BTV surveillance pilot project was conducted in three states (Nebraska, North Dakota and South Dakota). Samples were tested for antibodies to BTV and Anaplasma. Data collection is complete and a series of reports are being developed. Preliminary results of the study showing the prevalence of antibodies to BTV in each of the states and the vector trapping results were discussed at the 2002 United States Animal Health Association (USAHA) meeting at the General Session. Ongoing analysis will evaluate animal and operation level factors as well as climatic factors related to BTV exposure. This information should help to explain the observed and expected distribution of BTV exposure.

3) The biennial national BTV surveillance project has increased the number of states represented to a total of 24 (six new states in 2003) in 16 groupings, and is looking at anaplasmosis for the first time in many years. Results of the testing for BTV were shared at the 2003 USAHA meeting. The analysis of the Anaplasma test results is underway.

4) Culicoides trapping was conducted in Montana in the summer of 2002 and continued during the summer of 2003. In addition, serum samples collected in the autumn of 2003 on operations where trapping occurred are being tested for BTV antibodies.

On 15 July 2002, CFIA notified APHIS that Montana is now considered a ‘low incidence state’ for BTV. This change allows Montana breeding cattle, bison, sheep and goats to enter Canada with a single BTV test during the vector season. CFIA also informed the USA in 2003 that imports of feeder cattle from Hawaii are now permitted year-round without restriction for anaplasmosis and BT.

In October 2002, APHIS convened an expert panel to develop a USDA strategy on anaplasmosis, tick and wildlife issues, treatment mitigation, the use of tetracyclines and to address any research needs. APHIS has shared the document that emanated from this meeting with CFIA and is in the process of implementing the strategy set forth in that document. The United States scientists who participated in that panel expressed support for the industry proposal for a pilot feeder programme during the summer months.

In response to United States and Canadian industry requests, Canadian officials indicated in March 2003 that CFIA would implement a pilot project to permit the summer importation of United States feeder cattle into a single quarantine feedlot in Alberta and that additional feedlots could be added. The project will cover animals originating in Montana and North Dakota, with the main target period for import being August or September. A number of monitoring provisions are being developed. Moreover, an assessment of the project will be performed to determine if it can be continued or expanded. The implementation of the summer programme has been delayed due to the BSE crisis, as resources are diverted from the establishment of the necessary administrative requirements and animal health safeguards and because of market uncertainty for the slaughter cattle product. Cattle prices in Canada are currently very low.

Cattle trade between the United States and Mexico

In Mexico, different virus-vector ecosystems exist in the northern and in the southern regions of the country. The USA has no BT requirements for cattle and other ruminants from Mexico, despite the existence of a virus-vector ecosystem in southern Mexico and Central America that differs from the vector-virus ecosystem found in the United States and northern Mexico. In large part, this is because the USA has not recognised any disease risk from BTVs during the long livestock trading history.
between the two countries. Similarly, Mexico, with its two virus-vector ecosystems, does not impose regulatory controls for BT with its neighbouring countries, the USA to the north, and Belize and Guatemala to the south.

**Cattle trade between Canada and Mexico**

Traditionally, there has been no export of Mexican cattle to Canada because of a lack of Canadian demand for Mexican cattle. However, prior to the diagnosis of BSE in Canada, Mexico was an important market for Canadian dairy cattle. Mexico recognises Canada as BT-free based upon the geographic and ecological characteristics that make it vector-free. Mexico believes there is no BTV risk associated with the movement of Canadian cattle to Mexico. However, there has been a temporary disruption of trade due to the diagnosis of BSE in Canada.

**Cattle trade between the United States and Europe**

Exports of live cattle to the European Union (EU) have ceased since 1980 due to BT restrictions on United States cattle. The EU has adopted regionalisation policies that permit the importation of live cattle from countries not entirely free of the disease and/or virus (notably Canada). The scientific community recognises that live cattle can be exported from or imported to regionalised areas of infected countries when following recognised testing and quarantine procedures.

APHIS has continued to negotiate with the EU to open the market to breeders and exporters of United States cattle. In June 2003, APHIS presented a proposal to the European Commission (EC) for the export of live cattle from the USA to the EU. The meeting represented the first assembly of an Animal Health Technical Working Group, a new format for resolution of outstanding animal health issues between the USA and the EU, as recommended by the Joint Management Committee of the Veterinary Equivalence Agreement.

APHIS provided alternative equivalent mitigation measures to those found in the then-approved EU animal health certificate. Equivalent mitigation measures were agreed upon for BT.

As the EC requires compliance with the standards of a new draft animal health certificate concerning the feeding of proteins derived from mammals, a final protocol remains pending. At present, the USA cannot meet these requirements as a complete mammalian to ruminant feed ban is not currently enforced. An updated proposal was forwarded to the EC in August 2003 with the alternative mitigation measures agreed upon in the meeting for BT. This proposal is under review by the EC.

**Cattle trade between Canada and Europe**

Traditionally, several European countries were important export markets for breeding cattle from Canada. Cattle exports to Europe ceased in 2001 when the EU implemented the ban on the feeding of mammalian proteins to ruminants and restricted imports to source countries with similar feed standards.

Prior to that time, the sanitary certification for BT for Canadian cattle exports varied from country to country. Several countries recognised the BT-free status of Canada outside of the Okanagan Valley of British Columbia and permitted the importation of Canadian animals without mitigating requirements for BT. The United Kingdom (UK), on the other hand, had traditionally required the animals being imported to be subjected to a serological test for BTV and to arrive in the UK during a defined calendar window, annually.

Canada has not imported breeding cattle from Europe in more than a decade. When cattle were imported from Europe during the 1980s and from the UK before the BSE outbreak occurred, Canada recognised the status of exporting countries for BT and did not require BT certification to accompany the imported animals.

**Cattle trade between Mexico and Europe**

Historically, Mexico had not imported live cattle and other ruminants from Europe due to foot and mouth disease (FMD). Since the 1980s, however, imports have been restricted because of the diagnosis of BSE in many European countries.

Mexico has conducted negotiations with the EU to export fighting bulls to Spain and France. Mexico has provided surveillance information on BTV and vesicular stomatitis viruses to access this market. Serological and virological evidence of the presence of BTV in the absence of clinical disease has been found in Mexico.

**Cattle trade between the United States, Australia and South America**

The USA has relaxed BT test requirements/restrictions on importation of cattle from Australia. The USA does not require BTV testing of cattle after 60 days in vector-free isolation.

The USA has not imported any significant numbers of live cattle from South America, in large part due to the presence of FMD in many countries there. During the 1980s and before, when the high-security
Harry S. Truman Animal Import Center was functioning and imports of cattle and other ruminants were permitted, the USA applied isolation and testing requirements for BT. During a period when Uruguay was considered free, importers in both the USA and Mexico expressed interest in cattle from Uruguay and a protocol was under development by the USA. This work is not currently progressing.

**United States bluetongue position**

APHIS believes that BT is not an emerging disease. Wind-borne infected vectors, rather than viraemic livestock, have been demonstrated as the cause for virus movement and the establishment of virus in new regions. Ecosystem expansion and contraction is related to prevalent vector species, climatic events, ecology, and environment rather than infected livestock movement. The USA does not consider the unlikely possibility that seropositive cattle may be viraemic to be a disease or trade risk.

Despite unrestricted year-round movement of cattle from the western BTV-endemic areas in the USA to the vector-free northern and north-eastern BTV-free areas of the USA, there has never been a case of BT in, or virus isolated from, resident cattle.

The USA is receptive to considering importation of livestock from other BT-affected countries. Currently, the USA does require some mitigation for BT for animals imported from the Caribbean (outside the USA Territories) and regions with serotypes that are not common to North America.

**Conclusions**

The USA believes that BT is a non-tariff trade barrier to the unrestricted movement of cattle. The preponderance of scientific evidence indicates that the movement of infected insects, not infected livestock, is responsible for the movement of BTVs.

Historically, there has been significant trade in cattle between Canada, the USA and Mexico. Although unrestricted year-round movement of cattle from BTV-endemic areas to vector-free and BTV-free areas occurs, the virus has never been isolated from resident cattle in such BTV-free areas in the USA.

**Additional reading**


Bluetongue trade issues – an Australian perspective

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Summary

Although bluetongue (BT) causes large numbers of sheep mortalities in some parts of the world, the main economic global impact is due to restricted trade. Australia supports a risk-based approach using current scientific knowledge to determine animal health requirements for international trade. It is important that import health conditions for bluetongue virus (BTV) are based on science and are consistent with international guidelines. The Sanitary and Phytosanitary (SPS) Agreement of the World Trade Organization (WTO) specifies basic rights and obligations of importing and exporting member countries. The Terrestrial animal health code of the Office International des Épizooties (OIE) provides specific guidelines for BTV and general guidelines for many trade-related matters, including surveillance and zoning. The combined effect of relevant WTO-related measures and the OIE guidelines is to both encourage and require countries to apply import health requirements that are based on sound science and which afford justifiable protection without being unnecessarily trade restrictive.

Keywords


Australia has developed excellent bluetongue (BT) virus (BTV) surveillance systems and expertise over the past two decades. The objective of this work is to provide early warning of any change in the national BTV situation, to provide expert advice to producers and exporters and to support trade. Australia is a net exporter of agricultural products and very large numbers of sheep and cattle are exported from Australia each year.

BT disease is very rare in Australia because only a few hundred sheep of the national flock of 100 million graze in areas where pathogenic BTV serotypes are present. Australia has monitored the distribution of BTVs since they were first identified in Australia in 1977. Consequently, the distribution and epidemiology of the viruses and vectors in Australia is well understood. The ongoing surveillance programme, in combination with ad hoc research, continues to provide the scientific support for the export of ruminants to BTV-sensitive markets.

Although under certain circumstances BT causes large numbers of sheep mortalities in some parts of the world, the main economic global impact is due to trade restriction. Trade in sheep, cattle and goats is often prevented, or made very expensive, because of test and/or other requirements used to manage perceived BTV risk.

New trade guidelines and rules

Since the most recent BT Symposium in Paris in June 1991, there have been two major developments that influence the effect of BTVs on trade: the WTO Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) and the revision of the Office International des Épizooties (OIE) guidelines on BT. These outline certain rights and obligations for WTO member countries when implementing trade restrictive measures, and provide scientifically based guidance on managing BT risks in international trade.

World Trade Organization developments

The SPS Agreement elaborates rules for the application of SPS measures with respect to the provisions of the General Agreement on Tariffs and Trade (1). The SPS Agreement states that although members have the right to take quarantine measures necessary for the protection of animal, human and plant health, these measures should be consistent
with the provisions of the Agreement and should not constitute a disguised restriction on trade. It specifies basic rights and obligations of both importing and exporting countries that are members of the WTO, through the use of such concepts as harmonisation, equivalence, appropriate levels of protection, and the use of relevant international guidelines in international trade. Article 6 of the Agreement includes special reference to ‘disease free areas’.

The SPS Agreement recognises the OIE as the body responsible for establishing animal health guidelines and standards for international trade in animals and animal products. By expecting member countries to comply with relevant international guidelines, the SPS Agreement links the OIE Terrestrial animal health code (Code) (2) with the rights and obligations of members under the WTO.

Revised bluetongue chapter of the Terrestrial animal health code

On the request of the OIE Code Commission, an OIE ad hoc group examined current scientific information on BT, including the infective period in susceptible animals, the geographical distribution of BTVs, the competence of Culicoides spp. as vectors, the possibility of recognising seasonally free periods and the effectiveness of various methods of surveillance and monitoring.

The OIE international committee adopted the latest revised chapter at the 70th General Session of the OIE in May 2002. The recommendations in this chapter are based on current scientific opinion and, if applied, facilitate trade in ruminants and genetic material with negligible risk. The revised chapter includes the following:

a) guidance on acceptable surveillance programmes necessary to establish a BTV-free country or zone
b) recognition that BTVs occur in a broad geographic belt around the planet from 40°N to 35°S (countries or zones located outside this belt, but adjacent to areas that do not have free status, need similar surveillance to demonstrate freedom; ongoing surveillance is required to demonstrate continuing freedom; the corollary to this is that unless a country has a suitable BTV surveillance programme, or are separated from the global ‘BT belt’ by a zone that does, then that country cannot claim freedom from BTV)

c) a requirement that free areas that adjoin infected ones should include a clearly defined surveillance zone (with continuous surveillance).
d) recognition of, and guidance for establishing, seasonally free zones
e) recognition that animals do not present any risk of transmitting BTV providing they have been held in a free zone for a defined period prior to export, regardless of antibody status
f) recognition of the concept of protecting animals from vector attack during transportation to the place of shipment.

Other relevant revisions to the Code

Chapters on zoning and regionalisation (chapter 1.3.5.) and surveillance and monitoring (chapter 1.3.6.) have been added to the Code (the latter is currently under review). These chapters provide further guidance relevant to the establishment of BTV-free zones. They emphasise that the size, location and delineation of a zone will depend on the epidemiology of the disease in question, environmental factors and surveillance measures used.

It is clear that there are differences in the epidemiology of BTV in various regions of the world. There are different vectors with varying ecological needs and characteristics. There are also differences in the species and breed of ruminants, and husbandry practices. Housing animals during the winter may enable vectors to cycle BTV over the winter months. Some regions are geographically uniform, with gradual changes in climate and ecosystems, and others variegated, with opportunity for isolated pockets of vectors to survive. The possibility of re-assortment of laboratory-adapted genes derived from live vaccines needs to be considered – resultant viruses might behave differently in vectors, have longer viraemic periods and other variant characteristics.

An Australian viewpoint

There is a growing suite of international guidelines that relate to trade between countries. As a major exporter of agricultural products, Australia welcomes the introduction and development of these tools to assist in the conduct of safe and fair trade. Australia will continue to actively contribute to the development of international guidelines and encourage the adoption of current scientific information, knowledge and methods.

Australia supports a risked-based approach using current science to determine health requirements for international trade. Australia complies with the international guidelines and rules in national BTV requirements for the import of ruminants and ruminant genetics, and advocates their use for export from Australia. Surveillance and research on BTV and its vectors have been conducted in Australia for
many years, providing scientific support for the export of ruminants and their genetic material from Australia, and has assisted in our contributions to the development of scientifically based international standards for BTV. Australian authorities have determined a BTV-free zone based on surveillance information, knowledge of the epidemiology of BTV and its vectors in Australia, and relevant international guidelines.

The BTV situation in Australia appears relatively stable. No new BTV serotypes have been identified since 1985. Research continues on such matters as the behaviour of vectors in Australia, *Culicoides* repellents, the development of improved insect traps and diagnostic tests, infective periods, genetic sequencing, and modelling the distribution of BTVs in Australia. The well developed arbovirus monitoring systems in Australia provide temporal and spatial information on the distribution of BTVs and their vectors in the country and serve as an early warning mechanism for the possible introduction of new BTVs or vectors.

The emergency disease response arrangements made in Australia (1) include detailed plans for BT. These are documented at the Animal Health Australia website (aahc.com.au/ausvetplan). Vaccination (with live-attenuated virus) is not included as a management option in the face of an outbreak. This is due to concerns relating to possible genetic re-assortment of vaccinal strains with wild strains (which could introduce undesirable characteristics into circulating BTVs), and the logistics of rapidly procuring sufficient serotype-specific vaccine that satisfies national import health requirements and other regulatory controls for vaccines for farm animals.

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References

Trade implications of bluetongue in Africa

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Summary
Bluetongue virus is endemic in most parts of Africa and control measures are influenced by a number of factors including climatic conditions, susceptibility of animals, presence of reservoir hosts, occurrence of Culicoides and the serotype incriminated in a particular locality. To export animals and animal products from endemically infected countries is a major challenge, as all these factors have to be taken into consideration. Attempts aimed at control or eradication of such a disease must therefore consider these factors very carefully.

Keywords
Africa – Bluetongue – Breed – Climate – Culicoides – Reservoir – Trade.

Introduction
Bluetongue (BT) has been recognised as a disease entity in South Africa for more than a century, since Merino sheep were introduced into the Cape colony (2). For many years, the disease was thought to be restricted to Africa, particularly south of the Sahara but, since 1943, BT has been reported in countries outside Africa such as Cyprus, India, Israel, Pakistan, Portugal and the United States of America. By the end of 2001, Bulgaria, France, Italy, Macedonia, Tunisia and Yugoslavia experienced outbreaks of BT. The disease has a history of periodic incursions into southern Europe where its impact can be devastating.

BT is primarily a disease of sheep although all ruminant breeds are susceptible to varying degrees. Indigenous South African breeds are less susceptible than the Merino, while most exotic breeds such as the European mutton breeds are more susceptible than the Merino. Susceptibility may also vary among individuals of a breed. Goats are susceptible to infection although clinical disease is rarely encountered. Cattle are frequently infected with BT virus (BTV), but clinical disease is rare (1). The absence of BT in sheep does not necessarily imply the absence of BTV or viral activity in a particular region or country at a time. Sheep could therefore be regarded as merely an indicator of the presence of the disease.

BT is transmitted by midges of the genus Culicoides. To date, a number of Culicoides are known to harbour the virus and have been listed (5). Given the present inability to eradicate the vectors responsible, attention should be given to measures by which the susceptible host is protected from contact with them.

In an endemically infected country such as South Africa, prophylactic vaccination of sheep in undoubtedly the most practical and effective control measure. The most serious obstacle to effective immunisation is the existence of a multitude of serologically distinct virus serotypes. To date 21 of the 24 known BTV serotypes have been isolated from sheep in South Africa (3). It has been argued that to obtain effective immunity against so many serotypes poses a formidable challenge to any vaccine. Despite some degree of cross-immunity between heterologous serotypes, effective protection of sheep is dependent upon the presence of antibodies homologous to the challenge virus.

Climatic factors play a role in the epidemiology of the disease; nevertheless, BT occurs within relatively stable ecosystems (4). Recent climatic changes might presently be having an impact on the further spread of the disease (7). It is important to note that these climatic changes may now be enabling Culicoides to spread into territories that were previously less suitable.

The foregoing remarks indicate that it is virtually impossible to control bluetongue. In practice, this means living with the disease while attempting to minimise the risks and losses as far as possible.
Bluetongue is one of the diseases in ‘List A’ of the Office International des Épizooties (OIE) and has become a barrier to trade in animals and animal products. In some countries, the requirements of the OIE Terrestrial animal health code (6) have made it virtually impossible to trade in animals and animal products. Lack of knowledge of the epidemiology of the disease creates unnecessary requirements when trading in animals and animal products. The current process within the OIE to review Lists A and B diseases will offer an opportunity to update the bluetongue chapter in the Code (6).

When exporting live animals from endemic countries, importing countries require double sampling to reveal any possible rise in virus titres. Virus isolation is also a requirement. In certain instances, importing countries lack sufficient information about the disease or do not have diagnostic tools to confirm diagnosis. In such countries, additional measures are enforced to minimise risk of transmission of disease. Some countries require the presence of a surveillance programme, which in most instances is expensive for developing countries.

The direct losses due to mortality may be high in naive populations but in most countries in Africa certain breeds have adapted to local conditions. In such cases there is marked loss in condition of animals and thus marketing of slaughter animals may be delayed.

Conclusions

The abundance of Culicoides species, the presence of the reservoir hosts and the climatic conditions suitable for the spread of the disease in Africa, makes it impossible for African countries to control bluetongue. Any control measures or guidelines recommended to guide trade should be scientifically justified and should not be introduced to cause unnecessary barriers to trade. More research needs to be conducted to determine the climatic implications of the spread of BT and the presence of Culicoides in areas where they have previously never occurred.

The current status on the spread of animal diseases especially those which are transmitted by vectors, will place a challenge on the certification of products for export. The OIE, as an international standard-setting body, should look into a mechanism of certifying products free of diseases instead of countries putting all resources in for declaring disease freedom.

Finally, is it justified to include bluetongue as an OIE List A disease, i.e. have we not learned enough about the virus to recognise that we can live with it?

References

The impact of current and proposed changes to general guidelines on bluetongue surveillance of the Office International des Épizooties

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Summary

New draft guidelines for surveillance have been prepared for possible submission to the Office International des Épizooties (OIE) General Session for adoption in 2005. These guidelines are non-prescriptive and output-oriented, but also identify a series of critical elements required to effectively implement and evaluate surveillance systems. The guidelines allow for the use of a range of approaches to surveillance, including the use of non-random data sources and the combination of multiple sources of evidence. They also require transparency and the presence of basic quality assurance systems. The guidelines deal with surveillance to demonstrate freedom from infection as well as surveillance to determine the distribution and occurrence of infection. If the draft guidelines are adopted, a range of novel approaches to surveillance of bluetongue virus (BTV) may become formally acknowledged and accepted under the OIE Terrestrial Animal Health Code. This may enable different countries to tailor their BTV surveillance systems more closely to their own needs and capabilities while maintaining equivalence in the outputs of the systems.

Keywords


Surveillance for bluetongue (BT) virus (BTV) infection poses a number of challenges. Complicating factors include seasonal, climatic and environmental variations in vector and virus distribution, and the persistence of antibodies in animals moving from one area to another. If surveillance is being undertaken to estimate the risk of infection, traditional tools such as randomised cross-sectional surveys are less useful, as they are generally only able to provide estimates of seroprevalence. Estimates of incidence are more likely to be of value, but are often more difficult to measure with reasonable levels of precision. This is particularly the case in areas where transmission occurs at low levels, such as at the margins of endemic areas. If, on the other hand, the purpose of surveillance is to substantiate zone or national claims of disease freedom, traditional cross-sectional survey approaches used in isolation are now recognised as often being expensive and inefficient. Combination of survey data with other existing sources of evidence may be able to generate the same level of confidence at lower cost. Surveillance systems need to be flexible enough to take into account available sources of evidence, as well as differences in production systems and environmental conditions.

Over the last four years, the Office International des Épizooties (OIE), through ad hoc groups, has been involved in a process of revising its guidelines for general surveillance. Separate guidelines have been developed for aquatic animals and terrestrial animals, both based on the same set of principles. At the time of writing, the revised aquatic animal guidelines had been endorsed by the 2003 General Session of the OIE and incorporated into the diagnostic manual, and the draft terrestrial animal guidelines were under consideration for possible submission to the 2005 General Session. If endorsed by OIE members, these terrestrial guidelines will have an impact on the approach to surveillance for all OIE listed diseases, including BT. When planning long-term approaches to BT surveillance, it may therefore be useful for member countries to be aware of some of the principles in the current draft guidelines.
Draft Office International des Épizooties guidelines

Background

The philosophy behind the draft guidelines is important to understand. It is consistent with the approach taken in the chapter on risk analysis. In order to demonstrate disease status, or to determine the distribution or impact of disease or infection in a country or zone, it is necessary to have an effective surveillance system. However, if the outputs of a surveillance system are to be used with confidence, the reliability of the surveillance system must be able to be evaluated – hence the need for international standards.

In the past, OIE standards have often been relatively prescriptive. This is more evident in the Aquatic animal health code where in many cases sample sizes, sampling frequencies, and methods of analysis have all been specified. This prescriptive approach has the advantage of providing detailed guidance in the planning of surveillance activities, and allowing simple objective assessment of compliance with the standards. The disadvantage of this approach lies in the assumption that there is only one valid way to conduct surveillance activities, and that this one approach will be valid in all countries of the world. In the last decade or two, there has been a dramatic increase in research on epidemiological techniques for disease surveillance, and the analysis of surveillance data. As a result, approaches that may have seemed adequate several years ago can now be recognised as being either technically incorrect due to false assumptions, or inefficient. There is no reason to assume that the advances in the field of surveillance techniques will cease in the near future. The range of more precise, practical and efficient approaches to surveillance that have appeared offer countries the opportunity to adopt different techniques, selecting the ones that are best suited to their own situation, be it economic, cultural, climatic, geographic or biological. This opportunity has brought with it two problems for international trade: first, the new techniques are not formally recognised as valid approaches to surveillance in the existing Code standards (1); and second, it is much more difficult to assess the validity and compare the outputs of a range of different surveillance systems in order to establish equivalence.

The draft guidelines were formulated to address these issues, with the somewhat contradictory aims of:

1) being non-prescriptive to allow the application of the most appropriate surveillance techniques to a particular situation

2) providing objective standards by which all such surveillance systems could be judged.

The approach taken was to assume that any current or future approach to surveillance should be considered acceptable, as long as it is able to meet a certain set of criteria. These criteria, referred to in the draft as ‘critical elements’, either determine standards or identify factors, which must be taken into consideration. For example, the first critical element identified is the population. In order to be valid, the target and study populations must be identified and differences between them identified.

A second aspect of the philosophy behind the draft chapter is the creation of a mechanism to enable application of general guidelines to a specific disease. Continuing the example of populations, the draft chapter provides general guidelines as to the best way to select appropriate populations for surveillance. However, they indicate that the appropriate populations defined in the disease chapters of the Code should be used, where such definitions exist. This pattern is repeated in other areas, so that the guidelines provide the framework for designing and evaluating a surveillance system, as well as advice on the selection of appropriate values, while the disease-specific chapters provide detailed parameters suitable for the particular disease. This removes the need for any surveillance guidelines in many of the disease chapters, but requires the same chapters to be revised to provide appropriate parameters to be applied to the general surveillance chapter.

Another example of this is given by the choice of the value of design prevalence (also called threshold prevalence or minimum expected prevalence). A definition for design prevalence, and explanation of the importance of specifying the value selected is contained in the surveillance guidelines. However, appropriate values will vary for each disease, depending on a number of factors including the speed of transmission.

The third philosophical basis for the chapter is that it aims, as far as possible, to be output-oriented, rather than input-oriented. In other words, it aims to define what a surveillance system should be able to achieve, rather than specify what is required in order to achieve this. This approach is consistent with the aim of being non-prescriptive and recognising that there may be a number of different ways to achieve the same outcomes. While allowing considerable flexibility in the surveillance methodologies used, the guidelines are much more specific, for instance, about the level of confidence required to demonstrate freedom.
Contents

In brief, the contents of the draft guidelines are as follows.

In Section 1, there is a statement of the objectives of the document, namely to provide:

a) guidance to the type of outputs that a surveillance system should generate

b) guidelines to assess the quality of disease surveillance systems

c) guidelines for the outputs needed from surveillance systems for the risk analysis process

This is followed in section 2 by definitions of terms used in the chapter.

Section 3 deals with general principles of surveillance. A distinction is drawn between structured population-based surveys and non-random data sources that may be used for surveillance purposes. Critical elements applicable to all surveillance activities are identified followed by special considerations for surveys and for the use of non-random data. These ‘critical elements’ provide the mechanism by which standards are set in the draft chapter. Notes on the combination of data from multiple data sources are also included.

The critical elements identified include definitions of the population, cases and outbreaks, consideration of any tests used (including guidelines to documenting the performance of the test especially with regard to precision, sensitivity and specificity), sampling methods, sample size calculation and data analysis methods. In all cases, full transparency should be achieved through appropriate documentation. A relatively new inclusion in the area of surveillance is the requirement for demonstrable quality control systems. These may be relatively simple, but should document both the established protocols for surveillance, and be able to detect and document any departures from these protocols.

Section 4 deals specifically with surveillance to demonstrate freedom from infection, starting with general guidelines for declaring freedom, including historical freedom. It then lists general critical elements required for demonstrating freedom, and specific issues for surveys and for the analysis of non-random data sources. Section 5 is concerned with surveillance to determine the distribution and occurrence of infections, providing general guidelines, as well as guidelines for the use of surveys and non-random data sources.

The final section 6 highlights the relationship between surveillance and risk analysis, identifying the range of surveillance outputs and their role in the different components of risk analysis.

Implications for bluetongue virus surveillance

The guidelines for surveillance contained in the current Code chapter on bluetongue (under review) are relatively flexible, but contain a number of statements that make their interpretation and practical implementation somewhat problematic. If the draft general guidelines for surveillance are ultimately accepted, most disease chapters will need to be progressively updated to reflect the changed guidelines. This means that general statements regarding the approach to surveillance may be removed from the disease chapters, while specific information required for effective surveillance needs to be included. For instance, the requirement for both random and targeted surveillance could be removed, while the output confidence level of 95% would be retained. Design prevalence values, currently specified simply as 2%, may need to be expanded to capture the concept of clustered populations, and include both animal- and herd-level design prevalence values. More specific information may be required on the appropriate way to identify populations for targeted sampling (e.g. those adjacent to any zone of possible BTV activity).

While these and further similar changes may be required in the BT chapter, the effect of these changes on the practical implementation of surveillance will be far greater. Under the guidelines, there is no limit to the variety of approaches that may be used for surveillance, as long as they meet the requirements of the chapter (e.g. are scientifically valid and recognised), and the specified outputs (achieve a 95% confidence level). Some of the alternatives that may be possible include the following:

1) Surveillance not based on serology: the current chapter specifies that serology should be used. Newer techniques may mean that in the future other approaches to surveillance become more efficient or more practical, such as the use of polymerase chain reaction (PCR) on trapped Culicoides to detect BTV.

2) Quantitative combination of multiple sources of evidence: instead of depending on a single source of evidence, such as serological surveys, to provide all the confidence required for a free zone, it may be possible to combine a number of different sources of evidence (sentinel sites, cross sectional surveys, vector trapping data, etc.) to
produce a single, quantitative estimate of the combined confidence achieved.

3) The use of quantitative approaches to the analysis of data from targeted surveillance: most sentinel herd systems (a commonly used approach to surveillance for BTV) represent a form of targeted rather than random surveillance, due to the targeted placement of the herds in areas of particular interest (e.g. high risk areas). This approach is acknowledged in the current chapter, but traditional analytical techniques make it impossible to quantitatively evaluate the confidence that can be gained from non-random data. Newer approaches currently under development may enable data from sentinel sites to provide valid quantitative input into the overall assessment of confidence of freedom.

4) The use of other existing non-random data sources to supplement surveillance data: an example may be provided by data from routine testing of export animals from the free zones.

These are just a few examples of different approaches that may be taken to achieve equivalent outcomes under the draft guidelines. It is important to note that, under the draft guidelines, whatever approach is used, any potential biases in the data and imperfections in diagnostic system sensitivity and specificity must be taken into account and the methods used for data analysis must be valid and internationally accepted.

Conclusion

If adopted, these guidelines are likely to have two major effects. Firstly, there is the opportunity to develop more effective and more affordable approaches to surveillance, closely matched to the differing needs and practical constraints of different member countries. On the other hand, without prescriptive guidelines, there will be a requirement for greater skilled input into the design, documentation and assessment of surveillance systems. For BT, a range of different approaches to surveillance may be available to produce equally acceptable outputs.

Acknowledgements

The ideas presented in this paper were jointly developed by the author and other members of the OIE ad hoc group, including Alejandro Lopez, Cristobal Zepeda, Preben Willeberg, Steve Weber, Gideon Brückner, and Armando Giovannini, and by previous ad hoc groups, supported by the OIE.

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Reference

Risk analysis on the introduction into free territories of vaccinated animals from restricted zones

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Summary

Between August 2000 and 14 May 2001 (defined as the 2000-2001 epidemic) bluetongue (BT) was reported in three regions of Italy: Sardinia, Sicily and Calabria. During the 2001-2002 epidemic (between 15 May and 14 April 2002), the disease spread to five additional regions (Puglia, Basilicata, Campania, Latium and Tuscany). In May 2001 the Italian Ministry of Health decided to restrict animal movements and to vaccinate all susceptible domestic ruminant populations in infected and in neighbouring regions. This action was taken to reduce virus circulation with the aim of decreasing direct losses in sheep and goats due to the disease, and indirect losses in cattle populations due to movement restrictions. Furthermore, the Italian authorities implemented an epidemiological surveillance system to monitor the spread of the virus and to provide more effective movement controls. In 2002, the vaccination campaign reached the set goal of vaccinating more than 80% of susceptible domestic ruminants in Abruzzo, Sardinia and Tuscany. The vaccination campaign successfully reduced clinical disease in Sardinia and Tuscany. Before the advent of BT, cattle had always been moved from Sardinia, Sicily and the southern regions for fattening and slaughter in northern Italy. During the tracing of animals that had left infected areas in 2000 it was found that 10,957 cattle had been exported from Sardinia between June and August 2000 and were scattered throughout continental Italy. In addition, most cows selected for culling from the southern regions and the islands were sent to northern Italy for slaughter. However, since August 2000 the animal trade between infected and free areas has come to a complete standstill. Sardinia, in particular, due to the climatic and epidemiological conditions (vectors survive almost throughout the year), was no longer able to export any ruminants to the mainland. Long-term standstill therefore led to heavy economic losses and had even greater social consequences. As farmers are not compensated, it is impossible to enforce these restrictions indefinitely. The Italian authorities and the European Commission thus decided to adopt a policy of risk management allowing some animal movement. This paper presents an analysis that assesses the risk associated with animal movement from restricted areas, according to the level of immunity of susceptible animal populations due to vaccination in the same areas. Results of the analysis indicate that when more than 80% of the susceptible population in the territory of origin is vaccinated, the risk associated with the movement of vaccinated animals to free areas appears acceptable and can be mitigated further by adopting ancillary control measures.

Keywords


Introduction

From August 2000 to 14 May 2001 bluetongue (BT) was reported in three regions of Italy: Sardinia, Sicily and Calabria (1). During the second BT epidemic (from 15 May to 14 April 2002) the disease spread to five additional regions (Puglia, Basilicata, Campania, Latium and Tuscany) (3). The high economic losses due to movement restrictions in infected areas and the need to reduce virus circulation as far as possible persuaded the Ministry of Health to vaccinate susceptible domestic ruminants; vaccination involved sheep, goats and cattle. This was done to create a resistant population to either reduce or interrupt BT virus (BTV) circulation, at least in those zones with
low levels of virus and vector pressure. The selection of the vaccination strategy was based on a risk assessment which indicated that the viral circulation throughout Italy could be significantly reduced by obtaining a large population of ruminants resistant to infection (2).

The vaccination campaign commenced in Sicily in October 2001, in Sardinia and in Calabria in January 2002, and in Latium and Tuscany in March 2002. In 2002, the goal of vaccinating more than 80% of susceptible domestic ruminant populations, set by the Ministry of Health (4), was achieved only in Abruzzo, Sardinia and Tuscany. The vaccination campaign successfully reduced clinical disease. In Sardinia, the number of clinical outbreaks declined from 6,090 in the 2001-2002 epidemic to 10 outbreaks in 2002-2003. In Tuscany, after 158 outbreaks in the 2001-2002 epidemic, the clinical disease disappeared altogether.

Since August 2000, animal trade between infected and free areas has come to a complete standstill. Due to the climatic and epidemiological conditions specific to Sardinia, vectors survive throughout the year. For this reason, all movements of ruminants to the mainland was prohibited. Long-term standstill leads to economic losses and negative social consequences which are sometimes greater than those due to the disease. As farmers did not receive compensation, the standstill measures could not be enforced indefinitely. The aim of this paper is to assess the risk associated with animal movement from restricted areas, according to the level of immunity induced by vaccination of susceptible animal populations in the same areas.

Materials and methods

Risk assessment on introduction of viraemic animals into free areas through animal trade

A simulation model was developed to assess the expected number of viraemic animals introduced into free areas from infected areas. The following assumptions were considered in the risk assessment:

a) besides vaccination, no other epidemiologically relevant factor (e.g. abundance of vectors, vector activity and climatic variables) differed significantly compared to previous years

b) the incidence of infection was estimated assuming a decrease in a vaccinated population compared to a non-vaccinated population proportional to the product of infected multiplied by susceptible animals

c) duration of viraemia that could result in the transmission of infection, 60 days (conservative assumption)

d) random selection of animals to be moved to

uninfected areas.

The following input variables were used in the model (the source of the data is given in brackets):

a) population of susceptible species (local Veterinary Services)

b) number of vaccinated animals, by species (local Veterinary Services)

c) average monthly incidence of infected cattle in previous years (archives of the national BT information system)

d) average monthly incidence of infected sheep during previous years (archives of the national BT information system)

e) frequency distribution of serum neutralising (SN) antibody titres (archives of the national BT information system).

The output variables of the model were:

a) number of new cases in the vaccinated population each month (simulated on the basis of the binomial distribution, the total number of susceptible animals and the incidence of infection in the vaccinated population)

b) incidence of infection in free areas that surround infected areas which was calculated on the basis of:

i) the mean delay from the onset of a new case of infection and the adoption of restrictions

ii) the population within a radius of 20 km from the new case

iii) number of new cases in the vaccinated population monthly

the choice of a 20-km radius is based on the definition of infected area around any new evidence of BTV circulation, stated by the law (5)

c) prevalence of viraemic animals in the vaccinated population (calculated on the basis of the number of new cases per month and the assumed duration of viraemia)

d) number of viraemic animals moved to a free area (simulated on the basis of the prevalence of viraemic animals and of eight different scenarios according to animal species, number of animals moved and presence/absence of virus circulation in the territories of origin.

The model was implemented using @Risk © software (Palisade Corporation) (7). The expected number of viraemic animals moved to a free area was simulated through 10,000 iterations, with Latin hypercube sampling. Three scenarios were considered, describing different types of territory that were chosen as paradigmatic examples, as follows:

a) scenario A, a territory in which >80% of the total animal population has been vaccinated and the incidence of infection in previous years was high
b) scenario B, a territory in which about 50% of the total animal population has been vaccinated
c) scenario C, a territory in which a negligible fraction of the total population has been vaccinated.

Validation of the model

The model was validated using data from Sardinia and Tuscany, derived from the national BT surveillance system. In particular, the expected prevalence of viraemic animals in Sardinia and Tuscany, simulated by the model, was compared with the number of sentinel animals that seroconverted in a period extending 100 days. In relation to the value of minimal protective antibody titres, two different scenarios were considered in the model validation, as follows:

1) all vaccinated animals are protected against the infection, irrespective of antibody titre
2) only animals with an SN antibody titre of at least 1:10 are protected against infection.

Results

Validation of the model

Results of the two scenarios were compared with surveillance data. Figure 1 presents the probability distributions of the number of positive sentinels in Tuscany and Sardinia, respectively. The number of positive sentinels observed during the previous 100 days in Tuscany was 6, which is compatible with both scenarios under consideration, while in Sardinia the number of positive sentinels observed was 28, which is compatible with immune protection of animals, irrespective of circulating antibody. The number of positive sentinels foreseen by the model varied from 26 to 34, with a median value of 30. Consequently, the model was considered capable of predicting the expected monthly number of new cases. In subsequent simulations, therefore, all vaccinated animals were considered protected against infection.

Risk assessment on introduction of viraemic animals in free areas through animal trade

The results of the risk assessment varied according to the different scenarios considered, as follows:

a) if 5 000 cattle are selected at random from a population of domestic ruminants in which vaccinated animals represent less than 80% of the total (scenarios B or C), irrespective of the fact that they are selected in either infected or uninfected areas, the expected number of viraemic animals among them varies between 384 and 938 (Figs 2 and 3); if 5 000 sheep are chosen at random from the same areas, the expected number of viraemic animals among them varies between 28 and 141 (Figs 5 and 6).

b) if 5 000 cattle are chosen at random from a population of domestic ruminants in which vaccinated animals represent more than 80% of the total (scenario A), irrespective of the fact that they are selected in either infected or uninfected areas, the expected number of viraemic animals among them varies between 10 and 56 (Fig. 4); if 5 000 sheep are selected at random from the same areas, the expected number of viraemic animals among them varies between 0 and 12 (Fig. 4).

c) if 5 000 cattle are selected at random from a population of domestic ruminants in which vaccinated animals represent less than 80% of the total (scenarios B or C) and originate in uninfected areas, the expected number of viraemic animals sent to free areas varies between 28 and 141 (Figs 5 and 6); if 5 000 sheep are chosen at random from the same areas, the expected number of viraemic animals among them varies between 0 and 69 (Figs 5 and 6).

d) if 5 000 cattle are chosen at random from a population of domestic ruminants in which vaccinated animals represent more than 80% of the total (scenario A) and originate in uninfected areas, the expected number of viraemic animals sent to free areas varies between 28 and 141 (Figs 5 and 6); if 5 000 sheep are selected at random from the same territores, the expected number of viraemic animals among them varies between 0 and 3 (Fig. 7).
Figure 2
Expected number of viraemic cattle and sheep moved to areas free of bluetongue in the case of random selection of animals from the entire regional population (irrespective of infected or uninfected areas): Scenario B

Figure 3
Expected number of viraemic cattle and sheep moved to areas free of bluetongue in the case of random selection of animals from the entire regional population (irrespective of infected or uninfected areas): Scenario C

Figure 4
Expected number of viraemic cattle and sheep moved to areas free of bluetongue in the case of random selection of animals from the entire regional population (irrespective of infected or uninfected areas): Scenario A

Figure 5
Expected number of viraemic cattle and sheep moved from uninfected free areas of Scenario B to areas free of bluetongue

Figure 6
Expected number of viraemic cattle and sheep moved from uninfected free areas of Scenario C to areas free of bluetongue

Figure 7
Expected number of viraemic cattle and sheep moved from uninfected free areas of Scenario A to areas free of bluetongue
Discussion

Risk analysis is recognised worldwide as a tool to assess risk associated with the international movement of animals (6). The same approach was applied in this study to the internal movement of domestic ruminants from areas in which movement restrictions were enforced due to the presence of bluetongue to areas free from infection. The results of the risk assessment described here were taken into account by the Italian General Directorate of Veterinary Services, which modified national veterinary legislation on movement bans from restricted zones.

The expected number of viraemic ruminants among animals from areas subjected to movement restrictions varies according to the level of vaccination in the population of the area of origin. When less than 80% of the domestic ruminant population of the area of origin is vaccinated, the risk of transferring a number of viraemic cattle sufficient to cause the spread of BTV to free areas is not negligible, even if other risk mitigation measures are applied. On the contrary, when more than 80% of the domestic ruminant population in the area of origin is vaccinated, the risk of spreading the infection by moving the animals to free areas is significantly lower. In the case of vaccinated cattle sent directly to slaughter, in particular if transfer occurs during daylight, the risk of infection spreading to the receiving free areas can be considered negligible. When the animals come from an area in which over 80% of the domestic ruminant population is vaccinated and there is no evidence of BTV circulation, the risk of BTV spread, due to cattle or sheep movement, can be considered absolutely negligible.

In the case of vaccinated sheep being moved from areas with no evidence of BTV circulation, irrespective of whether the 80% vaccination level in the domestic ruminant populations has been attained, the risk of spread of infection in the receiving free areas is virtually nil. This is especially true if the animals are sent directly to slaughter, preferably during daylight hours. The difference observed in the expected number of viraemic cattle compared to sheep is a consequence of the difference between the average monthly incidence of infection observed in previous years (input variables c and d), namely: 8.7% in cattle and 3.6% sheep.

A summary of the different modes of shipment of animals from areas in which vaccination is practised to infection-free unvaccinated areas, accompanied by suggested risk mitigation measures derived from the results of the risk assessment is given in Table I.

Table I
Possible trade patterns of animals from vaccinated areas to bluetongue-free areas and suggested risk mitigation measures

<table>
<thead>
<tr>
<th>Origin of animals</th>
<th>Possibility of shipment of animals to free areas</th>
<th>Suggested risk mitigating measure(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected and uninfected areas of regions where more than 80% of the susceptible population is vaccinated</td>
<td>Yes</td>
<td>Movement of vaccinated animals only directly to slaughterhouse, preferably during daylight</td>
</tr>
<tr>
<td>Infected and uninfected areas of regions where less than 80% of the susceptible population is vaccinated</td>
<td>No</td>
<td>Not applicable</td>
</tr>
<tr>
<td>Uninfected areas only of regions where more than 80% of the susceptible population is vaccinated</td>
<td>Yes</td>
<td>Movement of vaccinated animals only</td>
</tr>
<tr>
<td>Uninfected areas only of regions where less than 80% of the susceptible population is vaccinated</td>
<td>Yes</td>
<td>Movement of vaccinated sheep only directly to slaughterhouse, preferably during daylight</td>
</tr>
</tbody>
</table>

References

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obbligatoria contro la febbre catarrale degli ovini (Blue-tongue), parte IV, allegato I (izs.it/bluetongue/ministero/hpage_file/spostamento_all_I_parte_V_rev2.pdf accessed on 12 September 2004).


Implementation of a new contingency plan for bluetongue disease in

Italy

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Summary

Since the first appearance of bluetongue (BT) in Italy in late August 2000, the ecology of vectors and the environmental conditions affecting their distribution and survival proved to be the most difficult factors to monitor, and represented a serious challenge to the effectiveness of the National Contingency Plan promulgated in 1991. Moreover, the Italian Ministry of Health considered the national management plan of BT inadequate to prevent further spread of the disease. The authors describe the implementation of a new BT contingency plan, integrating an operations manual and an ad hoc information system, which operated also as a decision-support system both at local and central levels. The plan describes the national capacity for dealing with BT outbreaks, the composition and duties of the National Disease Control Centre and Local Disease Control Centres, the chain of command and the strategies adopted.

Keywords


Commission conducted an inspection in Italy to monitor the implementation of the foot and mouth disease, classical swine fever (hog cholera) and bluetongue (BT) contingency plans.

Following the inspection, a number of recommendations were submitted to the Italian Ministry of Health. The recommendations included the need to:

a) integrate the provisions of Directive 2000/75/EC (7) into national legislation
b) better define a chain of command and to establish a national disease control centre (NDCC)
c) immediately implement a contingency plan for the management of BT outbreaks (6).

On receipt of these recommendations, the Italian Ministry of Health decided to implement a new contingency plan for BT disease having regard to Directive 2000/75/EC and its national transposition law (1), to Commission Decision 91/42/EEC (4) and to Commission guidelines on contingency planning (3, 5). The plan meant that technical memoranda drawn up after the first appearance of the disease in 2000 were revised and transformed into a set of complete instructions. The contingency plan drafted by the National Reference Centre for Exotic Diseases in Teramo (Centro Studi Malattie Esotiche) was developed through an ad hoc information system and was supported by an operations manual, in accordance with Commission guidelines. The operations manual gives details of the actions to be taken at all institutional levels in response to suspected and confirmed outbreaks, and is aimed at improving the decision-making capabilities of veterinary units.

Activities and results

The following activities were conducted when implementing the integrated system for the management of BT epidemics:

- drafting of BT contingency plan and operations manual
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- simplifying technical procedures for the updating of existing regulations to react promptly to changes in the epidemiological patterns of the disease, and of the European regulation dealing with the disease in Italy
- supplying regional veterinary offices with the necessary software for the management of veterinary activities
- implementation of a computer network, linking veterinary services and the National Reference Centre for Epidemiology
- implementation of a geographic information system (GIS) connected to a domestic animal identification database
- setting up an experimental telematic support system for emergency management
- staff training
- supplying all the necessary tools, equipment and materials to veterinary personnel.

Legal powers and chain of command

Regionalisation, recently strengthened by Constitutional Law No. 3 of 18 October 2001 (2), implies a substantial levelling of authority between central and local authorities. Difficulties exist in the interactions between these two authorities and in identifying roles and responsibilities both at strategic decision-making and operational levels. The BT contingency plan is a tangible example of the way to solve the aforementioned difficulties. The plan identifies an intervention strategy that attributes an essential role to the central level, with regard to the control of Office International des Épizooties (OIE) ‘List A’ disease outbreaks.

The adoption of this strategy is backed by the definition of OIE List A diseases that necessarily imposes outbreak control and management at a central level: ‘transmissible diseases which have the potential for very serious and rapid spread, irrespective of national borders, which are of serious socio-economic or public health consequence and which are of major importance in the international trade of animals and animal products’ (8). Within this framework, regional control authorities are responsible for the monitoring of the correct implementation of control actions in compliance with directives given by the Italian Ministry of Health and have a supervisory role over local authorities. The structure of the chain of command is presented in Figure 1.

Following the outbreaks of BT in 2000, the Italian Ministry of Health decided to implement a surveillance programme with the following objectives:

- conduct surveys for the presence/absence of virus circulation throughout the country
- monitor the status of free areas
- identify BT seasonally free zones.

The surveillance programme was adopted by ministerial decree of the Italian Ministry of Health. The measures that form the programme are listed in the ministerial decree and include activities to be implemented to control disease outbreaks (i.e. vaccination programme, instructions for animal movement and transhumance, serological and entomological surveillance).

The ministerial decree authorises the Chief Veterinary Officer (CVO) to amend the enclosures of the ministerial decree with individual measures. When needed, this strategy enables the amendment of the operative component of the national regulation without delay.

In the event of changes to existing regulations or changes in the epidemiological pattern of the disease, the CVO may resort to delivering technical memoranda in which instructions are provided on how certain activities should be conducted. In this way, all control measures are adopted, managed and monitored at the central level. This approach is an effective method to control a disease transmitted by vectors which spread infection well beyond regional boundaries.
Disease control centres and other operational institutions

The plan describes the structures, duties and functions of the NDCC, Regional Disease Control Centres (RDCCs), LDCCs and other operational institutions. The management of disease surveillance and control is subdivided into ‘peace-time’ and emergency management for didactic reasons. During ‘peace-time’ management, the NDCC, RDCCs and the LDCCs promote all activities necessary to maintain and develop expertise to ensure disease preparedness while, during emergency management, the NDCC, under the provisions of the competent central authority and with the contribution of expert groups, co-ordinates the adoption and implementation of surveillance and control measures adopted by the regional authority and implemented by the LDCC.

The regional control authority plays an important role in the adoption and co-ordination of control measures emanating from strategic options made by decision-making authorities. The BT emergency communications flowchart is illustrated in Figure 2. The competent institutions are divided into political-administrative and technical-operational institutions due to the fact that all veterinary and zootechnical provisions must be adopted within legal-administrative acts, including financial backing for the activities implemented.

Figure 2
Flowchart showing the bluetongue emergency communications network
Problems to be solved

The principal political-institutional problems concerning the BT control and contingency plan still to be solved are:

1) The change to the overall institutional structure of Italy together with the reinforcement of the regional legislative power. Regions can adopt self-governing regulations that have been transposed from EU legislation. In the future, more effort will probably be required from the Ministry of Health to co-ordinate disease control strategies across Italy.

2) The need to create additional legal-administrative tools that will allow the involvement of other institutions (i.e. army and civil protection unit) to facilitate staff recruitment, purchase of equipment and funding.

3) The need to revise criteria for the allocation of financial resources and the amount of financial resources to be made available by the Italian National Health Fund. Funds should be available not only to face an emergency but also for all strategic programming activities.

The main technical problems to be solved are as follows:

• complete the telematic support system for emergency management;

• extend the GIS application to include all animal holdings nationwide and to have staff and resources available to better define the control measures that should be adopted.

References


Health management of large transhumant animal populations and risk of bluetongue spread to disease-free areas

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Summary

Transhumance, or seasonal grazing, in central Italy is a husbandry practice that is over two thousand years old. It involves the seasonal movement of sheep, goats and cattle from the southern lowlands of mainly the Puglia and Lazio regions, to summer pastures in the mountains of Abruzzo and Molise. Bluetongue (BT) made its appearance in Italy in 2000. In the early summer of 2001, disease was present in three regions: Sardinia, Sicily and Calabria. Neither an effective surveillance system nor a vaccination campaign had been implemented. Movement of ruminants to the disease-free regions of Abruzzo and Molise was therefore banned. The Italian Veterinary Services had to meet the challenge of the movement of ruminants from surveillance to disease-free zones, given the impossibility of stopping transhumance. The General Directorate of Veterinary Public Health, Food and Nutrition of the Ministry of Health developed a plan for both the Puglia and Abruzzo regions based on serological, virological and entomological surveillance. The plan was implemented between May and June 2001 when 7000 animals moved from the Puglia surveillance zone to the infection-free summer pastures.

In the early summer of 2002, eight regions were infected (Sardinia, Sicily, Calabria, Basilicata, Puglia, Campania, Lazio and Tuscany). Simultaneously, a nationwide surveillance system and a vaccination campaign, were implemented in infected regions. In the provinces where vaccination was compulsory, deviation from the animal movement ban was allowed if at least 80% of susceptible stock had been vaccinated. However, this objective was not achieved in the provinces of Rome and Viterbo (Lazio) where a large transhumant population was present and where sporadic virus circulation had been detected. A specific control plan to allow transhumance from Lazio to Abruzzo, Marche and Umbria was designed and implemented to increase the number of animals that could be moved. Between May and June 2002, authorisation was granted to move 28,000 head, whereas prohibition of movement was ordered for 12,000 sheep (belonging to 21 flocks). Regional authorities financed feeding, watering and housing for these animals. Transhumance did not spread infection to disease-free areas either in 2001 or in 2002.

Keywords


Transhumance, involving the seasonal movement of livestock between summer and winter pastures has existed in Italy for the past 2000 years or more and has become a social and cultural event of some economic importance. At the end of spring, ruminant livestock and their attendants leave their winter residences (often situated near the coast or in the plains of the south and centre of the country) and migrate to inland mountain pastures where they stay until the beginning of autumn. The bluetongue
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(BT) epidemic that affected Italy in the summer of 2000 (2), soon spread to the regions of Puglia and Lazio that have practised transhumance since Roman times (Fig. 1). The problem of animal survival and welfare in areas in which feed and water resources diminish during the summer, and where pastures are either leased or used by the owners for summer cultivation, makes the prohibition of transhumance impossible. Indeed, strong objections were voiced in regard to the limitation of movement from surveillance zones where no infection was present. The prohibition of animal movement from the BT surveillance and protection zones of Puglia and Lazio to the disease-free regions of Abruzzo, Molise, Marche and Umbria, together with the practical impossibility of applying the movement ban to transhumant animals, encouraged the veterinary authorities to formulate and implement specific controls to reduce the risks linked to the movement of transhumant animals to summer pastures. The strategy adopted during the two epidemics in June 2001 and June 2002 is described here.

First scenario

In June 2001 European Commission Decision 2001/138/EC (3), in accordance with European Council Directive 2000/75/EC (4), established protection zones of a radius of 100 km around outbreaks or in areas in which virus circulation had been observed. Surveillance zones of a radius of 50 km radius were established around protection zones. Prohibition of ruminant animal movement from protection and surveillance zones to disease-free areas was strictly enforced. The surveillance zones included all the provinces of Puglia, in particular Foggia (Fig. 2), from which transhumant animals had to be moved to the disease-free regions of Abruzzo and Molise. At that time, the surveillance system was not fully operational nationwide (6) and compulsory vaccination, established by the Ministry Order of 11 May 2001, had not commenced (5, 9).

Second scenario

In February 2002, compulsory vaccination implemented in Sardinia, Calabria, Basilicata, three provinces of Sicily, three provinces of Puglia and one province of Campania, was extended to all provinces of Sicily and Puglia and to six provinces of Tuscany and four of Lazio. In the latter two regions, vaccination was limited to a band extending 20 km around disease outbreaks or from the Tyrrhenian

Disease scenarios and health strategies

The first case of BT was reported in Italy in August 2000. By the early summer of 2001, the disease was present in Sardinia, Sicily and Calabria (2). Between November 2000 and May 2001, surveys were conducted on the bovine population in the neighbouring protection and surveillance zones and the demonstrated the circulation of BT virus in two additional regions: Campania and Basilicata. By the early summer of 2002, BT had occurred in Sardinia, Sicily, Calabria, Basilicata, Puglia, Campania, Lazio and Tuscany (2).
coast (Fig. 3). In April 2002, national regulations stipulated that the movement of animals from restricted zones (where vaccination was compulsory) to free areas could be authorised only if the animals had been vaccinated more than 30 days previously and from zones in which at least 80% of the susceptible population had been vaccinated. In June 2002, when the third epidemic started in the Viterbo and Roma provinces (5), only 47.1% and 47.3% of animals, respectively, had been vaccinated and surveillance data were insufficient to exclude virus circulation (6). The two provinces had to move animals to the disease-free regions of Abruzzo, Umbria and Marche (Figs 1 and 3).

Figure 3
Vaccination areas in Italy, June 2002

**General provisions**

To enable the movement of animals to summer grazing pastures, serological testing had to be performed in winter grazing areas to verify presence of virus circulation (first operative phase) and in the summer grazing areas of destination to exclude the introduction of the virus (second operative phase). In the areas of origin (Figs 4 and 5), the introduction and export of animals was banned for the entire period in which serological testing was conducted. Single herds could be moved to summer pastures only on specific authorisation issued by the Ministry of Health and was dependent upon serological, entomological and virological results. Movement had to take place in escorted sealed lorries. The local municipal veterinary services, in areas of both origin and destination, and the police, had to verify that the regulations were being implemented correctly. Animal movement was restricted to the pastures of initial destination until results of serological tests, performed 21 days after arrival, had been received.

Figure 4
Winter residence municipalities of transhumant flocks in Foggia Province, Italy

Figure 5
Winter residence of transhumant flocks in the Viterbo and Roma Provinces, Italy

**Laboratory testing: first scenario**

**First phase (7-29 June 2001): winter pastures**

The province of Foggia was subdivided into 20 ‘cells’, 16 of which were 400 km² and four 200 km². These cells included all 43 bovine herds and 19 ovine or caprine flocks that had applied for authorisation to move to summer grazing areas (Fig. 4). In addition to the serological testing of all animals to be moved, the control plan included the capture of insects using blacklight traps, these positioned every night at different sites in each cell.
The objective was to collect insects that would be representative of the entire cell. Captures had to be made until all the animals had been moved. *Culicoides* spp. (parous and engorged) were tested using polymerase chain reaction (PCR) assay to detect or exclude the presence of viral antigen. A total of 3 433 cattle and 9 865 sheep and goats were tested using the competitive enzyme linked immunosorbent assay (c-ELISA) and gave negative results (Table I).

A total of 305 insects were captured, 246 of which were tested by PCR. *C. imicola* was found in only one catch in the municipality of San Marco in Lamis (Table II), where *C. imicola* was detected on 28 June 2001 (Fig. 4). The finding of *C. imicola* blocked the transfer of animals towards summer pastures. Of the 13 973 animals for which authorisation had been requested, 9 824 (70.3%) were authorised, 2 890 (20.7%) were refused permission, and 1 150 (8.2%) were not moved for other reasons.

**Second phase (30 June-30 July 2001): summer pastures**

Commencing on the 21st day after arrival at summer pastures (Fig. 1), a total of 2 647 cattle and 6 865 sheep and goats were tested by c-ELISA, giving negative results (Table I); 49 and 30 insect catches were tested for the presence of *C. imicola* and for virus using PCR, respectively, and also gave negative results (Table II).

**Second scenario**

**First phase (26 June-15 July 2002): winter pastures**

The provinces of Viterbo and Roma were divided into 23 cells, 22 of which were 400 km² and one, on the coast, 200 km². These cells included all 17 municipalities that had requested to move herds (Fig. 5). All animals had to be vaccinated at least 30 days before movement. The following additional control measures were applied:

- all sentinel cattle located in the 23 cells were tested serologically; one seroconversion was detected and a buffer zone of 20 km in radius was established around the positive herd immediately
- unvaccinated sentinel animals (usually lambs less than three months of age) were selected in each transhumant flock and tested by c-ELISA twice after 7-15 days; seroconversion was observed in two flocks and a buffer zone of 20 km in radius was established
- a sample of vaccinated animals was tested using c-ELISA to verify the immune status of herds and flocks
- *C. imicola* were found in eight holdings
- PCR was performed on engorged and parous *Culicoides* spp. specimens captured and positive results were obtained on catches from eight holdings; an insect catch was made for each transhumant flock within a radius of 20 km of the eight *C. imicola*-positive holdings (Table II); no viral antigen was detected by PCR in catches from transhumant flocks/herds.

**Table II**

Entomological control under the transhumance plan in winter pastures, 2001 and 2002

<table>
<thead>
<tr>
<th>Scenario</th>
<th>No. of catches</th>
<th>No. of PCR tests</th>
<th>No. of herds/flocks positive for <em>C. imicola</em></th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>305</td>
<td>246</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>174</td>
<td>150</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table I**

Results of the first transhumance scenario in Italy, 2001

<table>
<thead>
<tr>
<th>Scenario 1</th>
<th>Flocks/herds</th>
<th>Head</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine</td>
<td>Ovine/caprine</td>
<td>Bovine</td>
</tr>
<tr>
<td>Authorisation requested</td>
<td>24</td>
<td>19</td>
<td>3 743</td>
</tr>
<tr>
<td>Application withdrawn</td>
<td>1</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>Authorisation denied</td>
<td>8</td>
<td>6</td>
<td>656</td>
</tr>
<tr>
<td>Authorisation granted</td>
<td>15</td>
<td>11</td>
<td>2 891</td>
</tr>
<tr>
<td>Serological controls on winter pastures</td>
<td>23</td>
<td>17</td>
<td>3 433</td>
</tr>
<tr>
<td>Serological controls on summer pastures</td>
<td>15</td>
<td>11</td>
<td>2 647</td>
</tr>
</tbody>
</table>

The different numbers of animals in the subsequent phases described are consistent with the normal fluctuations of flock/herd sizes. The total difference is 109 animals (equivalent to 0.8%).
The creation of the buffer zone around seroconverted animals led to the prohibition of movement of all herds/flocks within the buffer zone, with the exception of three PCR-negative flocks that were moved a day before the zone was established. Of 44,776 animals, 28,052 (62.6%) were authorised to move, 12,575 (28.1%) were refused permission while 4,149 (9.3%) decided not to move for other reasons (Table III). Authorisation was denied to 19 holdings located within the buffer zone around seroconverted animals and authorisation was not given to two flocks that refused to apply the control measures required (Fig. 6). The percentage of immunised cattle and sheep/goats was 86% and 87%, respectively.

Table III
Results of the second transhumance scenario in Italy, 2002

<table>
<thead>
<tr>
<th>Scenario 2</th>
<th>Flocks/herds</th>
<th>Head</th>
<th>Flocks/herds</th>
<th>Head</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine</td>
<td>Ovine/caprine</td>
<td>Bovine</td>
<td>Ovine/caprine</td>
</tr>
<tr>
<td>Authorisation requested</td>
<td>7</td>
<td>66</td>
<td>680</td>
<td>44,096</td>
</tr>
<tr>
<td>Application withdrawn</td>
<td>–</td>
<td>8</td>
<td>–</td>
<td>4,149</td>
</tr>
<tr>
<td>Authorisation denied</td>
<td>4</td>
<td>17</td>
<td>575</td>
<td>12,000</td>
</tr>
<tr>
<td>Authorisation granted</td>
<td>3</td>
<td>41</td>
<td>105</td>
<td>27,947</td>
</tr>
</tbody>
</table>

Winter pastures

| On sentinel cattle | 124 | 1,384 | 1 | 1 |
| First control on sentinel sheep | 64 | 679 | 13 | 27 |
| Second control on sentinel sheep | 58 | 604 | 2 | 2 |
| On vaccinated animals | 26 | 73   | 485 | 1,508 |
| Summer pastures |
| On sentinel sheep | 11 | 1,158 | 0 | 0 |

Figure 6
Results of the plan for allowing movement of transhumant flocks from the Viterbo and Roma Provinces, Italy
Control and trade

Second phase (16 July-25 September 2002): summer pastures

On arrival in the summer grazing areas, the 41 herds were grouped in 11 pastures and were considered epidemiological units. All sentinel sheep in transhumant herds were serologically tested 21 days after arrival and found negative for BT antibodies (Table III).

Conclusions

Despite the movement of 9,824 unvaccinated ruminants in 2001 and 28,052 vaccinated ruminants in 2002, the movement of transhumant animals did not spread BT into disease-free grazing areas in 2001 or 2002. The spread of infection to the southern area of Abruzzo on 30 August 2002 (5) was not related to transhumant animals. Those animals moved to summer pastures in the northern part of the region, were serologically retested 21 days after their arrival and gave negative results. Furthermore, no clinical disease was observed in the unvaccinated local populations living in summer pastures and with which the transhumant animals had mixed. It seems, therefore, that the risk of infection spread through animal movement can be minimised by adopting appropriate sanitary control measures.

References


Group 1

Monitoring and surveillance

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Working Group charge

To consider which monitoring and surveillance practices should be developed to address all of the animal, vector and virus factors associated with the potential risk of spread of BTV, and how these practices should be interfaced with the current OIE Terrestrial animal health code. Also, consider innovative ways to evaluate risk pertaining to the movement of animals from BTV-endemic areas, including the risk associated with the movement of immune versus non-immune animals.

Prior to consideration of a review of the requirements for surveillance and monitoring for BTV, the Working Group was briefed on a planned OIE chapter on General guidelines for surveillance and monitoring. The key features of the draft of the proposed General surveillance and monitoring chapter are as follows:

a) Compared to the surveillance guidelines in the current bluetongue chapter, the proposed chapter on surveillance and monitoring is not prescriptive. If adopted, it would be acceptable to use a number of different sources of data and the merits of each different source could be taken into account. Data sources also could be derived on a random or non-random (structured/planned) basis.

b) The analysis of data must be scientifically sound. The proposed chapter recognises the merits of merging data from different sources. Though different data sets may be complex, they may enhance each other.

c) The aim of surveillance and monitoring is to generate data for use in risk-based assessments to support trade and usually aims to demonstrate freedom from infection, or the presence of an agent, and define areas of low risk. The approach in the proposed chapter is intended to be output-oriented, not method-oriented.

Recommendations

The Working Group recommends that the OIE convene an ad hoc group to review the current bluetongue chapter which is too prescriptive and confusing. A number of issues require particular attention; they are listed in the order in which they appear in the Code and are not in any order of priority, as follows:

1) The infective period is currently defined as 100 days, but there is no data to support a period of longer than 60 days. Consideration could be given to risk assessments based on probabilities determined from the distribution of the duration of viraemias.

2) Reference to northern and southern limits in terms of latitude. In view of the changing
distribution of BTV, specifying actual northern or southern latitudes is not appropriate. In the absence of confirmed disease, when a country lies within the latitude of the current distribution of BTV, or is adjacent to an infected country or region, a surveillance and monitoring programme should be conducted.

3) Use of the term ‘*Culicoides*’ on its own is misleading because most countries have one or more species of midges from this genus. The taxonomic term should be clarified to indicate actual species from the genus *Culicoides* that have been shown to be, or are suspected to be, vectors of BTV.

4) Methods of surveillance and levels of sampling needed to achieve the required degree of confidence need not be specified; rather, surveillance should comply with the provisions of the proposed general chapter. Nevertheless, some examples of appropriate surveillance systems that provide guidance to the intensity and frequency of surveillance could be of benefit.

5) The extent of a surveillance programme in countries adjacent to a country that does not have free status needs to be clarified. A distance of 100 km is specified but a lesser distance could be acceptable if there are relevant geographical features that interrupt the transmission of BTV.

6) When a country is proven to be free, consideration should be given to less frequent surveillance but only if the country is not immediately adjacent to a bluetongue zone where the situation is unstable.

7) The term ‘surveillance zone’ is confusing because surveillance also occurs within the free zone. The purpose of this zone is to acknowledge a degree of uncertainty in the exact limits of BTV activity and to increase confidence in the status of the free zone. The term ‘buffer zone’ is more appropriate though it is acknowledged that this term is defined in the *Code* as a zone that is used to prevent spread of a disease or agent into a free zone. Depending on geographical features, this zone may not actually prevent spread of BTV, though it does provide additional assurance for the safety of the free zone. While the width of such a zone has been suggested as 50 km, this may need to be narrower or wider, depending on local circumstances that are relevant to BTV transmission.

8) It would be of benefit if, in future, the *Manual of diagnostic tests and vaccines for terrestrial animals* specify measures of sensitivity and specificity to assist in the design of surveillance programmes. In the absence of these measures in the *Manual* or when different tests are used, when a surveillance programme is designed the performance characteristics of the test should be described.

9) When surveillance is conducted, the animal species, and the ages of the animals, need to be considered to ensure that there is appropriate sensitivity for that surveillance. While cattle are usually more readily infected, other species may be used if they have been shown to be infected at a higher incidence.

10) The presence of ecological zones for BTV in different parts of the world warrants recognition. Factors pertaining to vectors and hosts in one system may not be relevant to another.

11) In consideration of the movement of live animals and germplasm between countries or zones within a country, it is suggested that a risk-based approach be adopted. Persistent infection with BTV does not occur. Furthermore, the occurrence of virus in semen is rare and confined to the early period of viraemia. Consequently, appropriate strategies can be developed to allow the safe movement of animals (including those that are seropositive either as a result of natural infection or vaccination) and of semen from animals in zones where BTV infection may occur. These movement controls should reflect the finite period of viraemia in both natural infections and after vaccination with live vaccines.

### Research needs

The following research activities would be of benefit to surveillance and monitoring activities:

a) tests that distinguish between vaccinated and naturally infected animals

b) detailed studies of viruses and vectors and their inter-relationships at the boundaries of continental epysystems

c) improved type-specific serology

d) enhanced methods for antigenic and genetic analyses of viruses.

The Working Group also endorses the recommendations for research on vectors.
Working Group 2

Vectors

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Working Group charge

To develop specific recommendations that address issues pertaining to assessment of:

a) vector speciation and systematics
b) vector competence
c) vector capacity
d) vector ecology and control.

Vector systematics and taxonomy

A clear understanding of Culicoides systematics and taxonomy is crucial to virtually all bluetongue virus (BTV) vector studies. Most important Culicoides vectors exist as species complexes and the members of these complexes may occur together or in different regions. Since individual members may differ widely in vector capacity it is vital that they are able to be distinguished.

Recommendation 1

Better tools to identify and distinguish members of these complexes are urgently required. Tools to be developed should be both morphological and molecular, with the one informing the other.

At least one important Culicoides vector, C. imicola, appears to be spreading rapidly in Europe. The pattern of spread is not known. There is evidence that C. imicola in Europe occurs as several haplotypes.

Recommendation 2

Molecular tools to identify haplotypes and other specific traits should continue to be developed as a priority to enable vector population movement to be identified and monitored.

In many parts of the world, especially Europe, Asia and South America, the systematics and taxonomy of Culicoides are in need of revision. Identification of related species may facilitate the discovery of novel vectors and should significantly improve our ability to assess disease risk.

Recommendation 3

The systematics and taxonomy of Culicoides in Europe, Asia, South America and other parts of the world should be addressed. Phylogenetic
Conclusions

analysis of the sequences of multiple genes should be used to identify the relationships between known and novel vector species.

Worldwide, there are few competent *Culicoides* taxonomists.

**Recommendation 4**

Consideration should be given to capacity building in the systematics and taxonomy of *Culicoides*.

**Vector competence**

Vector competence is under genetic and environmental control, and varies inter- and intra-specifically. In refractory species or individuals, barriers to infection may occur at several steps in the infection and transmission processes. These barriers are poorly understood, and consequently, no methods currently exist for predicting whether species or populations are competent.

**Recommendation 5**

Barriers to the infection and dissemination of BTV within individual *Culicoides* should be characterised, and molecular genetic tools developed that permit prediction of vector competence.

Vector competence is difficult to measure, as field-caught *Culicoides* do not survive well in captivity and rarely feed. Consequently, transmission from field-caught *Culicoides* to hosts can rarely be demonstrated. There is some recent preliminary evidence suggesting that vertical transmission of BTV might occur in vector *Culicoides* species.

**Recommendation 6**

Methods to improve laboratory survival and feeding of field-caught *Culicoides* should be investigated. Direct and indirect methods of recording transmission, or transmission potential, should be evaluated. Possible vertical transmission of BTV in vector *Culicoides* should be further investigated.

Relatively little is known about the competence of *Culicoides* vectors in many parts of the world, especially Europe, Asia and South America. Work to date indicates complex relationships between vector species and their competence for different orbiviruses and/or viral genotypes as well as intraspecific variability in vector competence.

**Recommendation 7**

The vector competence of *Culicoides* species and populations should be measured, where possible using field viruses. Candidate species can be prioritised on the basis of epidemiological evidence, feeding preference for hosts and level of abundance.

Epidemiological analysis (serological surveys, vector surveys, ecological analysis, study of outbreaks) can provide guidance for the selection of candidate species for vector competence studies, and can be used to assess the likely significance of results.

**Recommendation 8**

Future and historical data sets should be analysed to investigate the possible role played by different vector species in the transmission of BTV.

**Vectorial capacity**

Vectorial capacity provides a measure of disease risk, incorporating vector competence, abundance, biting rates, survival rates and extrinsic incubation period. Many of these remain to be determined. Methods and tools for measuring some components remain to be developed, particularly in a field context. Interactions of these variables with the environment remain to be characterised.

**Recommendation 9**

Standard techniques for measuring the variables of vectorial capacity should be developed and adopted, to facilitate comparison of data and data sharing.

Trapping methods should be evaluated against a ‘gold standard’ (e.g. drop-trap over animal, and the Onderstepoort-type light trap).

Biases in trapping methods should be measured.

Improved methods for reliably aging *Culicoides* should be developed.

Improved methods for recording host preferences should be developed.

The effects of the environment, host demography and climate on vectorial capacity should be investigated.

Measures of vectorial capacity should be correlated with other indicators of disease risk, such as host disease status.

**Ecology**

The ecology of the major and minor *Culicoides* vectors is poorly understood and their breeding sites are largely uncharacterised. Means and rates of adult
dispersal, both local and long distance, are unknown. The comparative value of sentinel herds or wild-caught \textit{Culicoides} as an aid to the early detection of virus activity has not been fully investigated. Adult overwintering in temperate zones has been little studied, but could play a part in the persistence of BT.

**Recommendation 10**

Larval microhabitats and diets should be characterised as an aid to colonisation and to the identification of breeding sites. Means and rates of dispersal of adult \textit{Culicoides}, both local and long distance, need to be defined. Rates and times of virus or viral RNA detection in sentinel herds and vector surveillance systems should be compared. The possibility of adult overwintering in temperate and cool zones needs to be investigated. Development of vector population-simulation models is a long-term goal.

**Control**

Vector control methods are often used in the event of BT disease outbreaks, but there has been little quantitative work on short and long-term efficacy. Other means of reducing virus transmission that have lower environmental impact (e.g. physical and chemical barriers, husbandry modification), have received little attention.

**Recommendation 11**

Specific methods for the long and short-term suppression of \textit{Culicoides} populations (adults and immatures) should be evaluated and quantified, and clear recommendations given to veterinary authorities. Alternative methods of interrupting the transmission cycle, such as the use of repellents, housing, breeding site destruction or modification, should be investigated. These measures should be evaluated in the context of existing arthropod control efforts. Control success should be judged in terms of disease reduction and/or seroconversion.
Working Group 3

Diagnostics

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Working Group charge

To develop specific recommendations that address issues pertaining to the perceived advantages and disadvantages of existing and new virological and serological diagnostic procedures for detection of BTV infection of insects and animals and how these interface with the OIE Manual of diagnostic tests and vaccines for terrestrial animals.

Specifically address the issue of the role of the polymerase chain reaction (PCR) assay in the regulation of animal movement.

Existing procedures in the Manual

Virus isolation

Intravenous inoculation of embryonated chicken eggs (ECE) is the most sensitive technique for isolation of BTV. However, it is a slow procedure, compounded by the need for subsequent virus identification steps. Some ECE-propagated viruses may not readily replicate in cell culture.

Virus identification

Serogrouping

A number of techniques such as anti-antigen capture enzyme-linked immunosorbent assay (ELISA) and immunofluorescence that take advantage of the availability of serogroup-specific monoclonal antibodies work well.

The use of serogroup-reactive polymerase chain reaction (PCR) increases the speed of identification. Precautions must be taken to prevent cross-contamination while performing PCR.

Serotyping

The neutralisation test is biologically relevant and has a number of successful formats, such as plaque reduction and microtitre neutralisation. Virus cross-relatedness may make interpretation of results difficult. Maintaining serotyping reagent uniformity is difficult, particularly on a world-wide basis. Such reagents are also costly to make.

‘Typing’ by PCR-sequencing is a novel and welcome addition to the repertoire of typing tests. It is very rapid and highly informative (see new procedures).
Conclusions

Serological tests

The agar gel immunodiffusion (AGID) assay, while easy and cheap to perform, lacks sensitivity and manifests cross-reactions with epizootic haemorrhagic disease virus (EHDV). The competitive ELISA (c-ELISA) is now standard technology.

New procedures

Typing instead of serotyping

PCR sequencing provides information on ‘type’, genotype and topotype very rapidly. Segments coding for VP2, VP5, VP3, NS1 and NS3 are currently relevant.

Successful identification of BTV around the world depends on availability of relevant sequence data for primer development

Every effort should be made to send viruses or PCR products to all OIE reference laboratories or other competent laboratories to be sequenced and primer information made available (via the web) to facilitate characterisation at the source laboratory

An excellent start has been made in the process of collecting relevant sequence data: iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and iah.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv_sequences.htm provide phylogenetic tree analysis of BTV isolates based on RNA2

Real-time versus nested PCR?

Real-time PCR technology is faster and more expensive than traditional PCR methods but is less susceptible to contamination problems. There may be problems attempting to identify new isolates with already-existing ‘real-time’ probes. The technology requires expensive equipment.

IgM ELISA

An IgM ELISA would provide information on recent infection status and offer an opportunity to determine if the presence of IgM antibodies was correlated with the duration of viraemia.

Future trends

Possibilities include multiplexed flat and bead DNA and protein technologies and biosensing technologies.

Recommendations

The Working Group recommends that:

1) the AGID test remain in the Manual but not be a prescribed test for international trade.
2) research into novel diagnostic methods continue with tests showing promise being subject to validation by collaborating OIE laboratories and other competent national laboratories
3) the genetic characterisation of BTV isolates from diverse regions of the world continue, with the aim of:
   a) compiling sequence data and identifying new viruses and their genetic relationships
   b) sharing sequence information thereby increasing the size of the data bases
   c) facilitating establishment of PCR technology and use of appropriate primers in the submitting country
   d) validating the technology by reference to the ‘gold standard’ neutralisation test
4) following extensive validation by collaborating laboratories, the current neutralisation-based virus serotyping system be replaced by a genetic typing system
5) an IgM ELISA or similar test be investigated to determine if they would provide a simple test that correlates with viraemia in infected animals and could be used to facilitate trade.
6) use of the PCR to differentiate between wild-type and vaccine virus continue.
Working Group 4

Vaccines and vaccination

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Working Group charge

To address issues pertaining to both safety and efficacy of currently available and new (future) BTV vaccines.

Recommendations

Specific recommendations in regard to vaccines and vaccination strategy:

1) encourage the development and transfer of complementary and alternative vaccine materials and strategies that provide safe and efficacious inactivated or subunit BTV vaccines, and further encourage that vaccine companies adopt these products and make them available to producers

2) vaccine strains should be fully sequenced and the data made available to the FAO/OIE Reference database as well as other databases such as the European Molecular Biology Laboratory (EMBL) database

3) encourage the development and validation of technologies that will distinguish vaccinated from infected animals, both for current vaccines and the vaccines that are likely to be available in the foreseeable future

4) encourage countries applying current or future vaccine technologies and strategies to make all data on monitoring of vaccination programmes, and the surveillance of control programmes, available to the OIE for addressing future disease outbreaks

5) animals receiving vaccines produced by culture in embryonated chicken eggs shall not be moved internationally

6) update and keep current the OIE Manual of diagnostic tests and vaccines for terrestrial animals on research information and data on the efficacy of both subunit and inactivated BT vaccines.
**Working Group 5**

**Impact of interventional strategies on virus spread, disease and regulation**

**Group members**

Toby St George (Chair), Australia  
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**Working Group charge**

To address issues pertaining to the impact of interventional strategies on monitoring and surveillance practices and the risk of spread of bluetongue virus.

**Recommendations**

Considering the potential movement of bluetongue seropositive animals from an infected to a free zone or country:

a) animals may move at any time without posing a risk of virus spread if they have been vaccinated with a licensed or authorised attenuated, inactivated, sub-unit or genetically manipulated vaccine at least one month prior to movement, provided that the vaccine used covers all serotypes which would be expected from adequate surveillance to be present at origin and that the animals are identified as vaccinates in the accompanying certification

b) in the case of healthy, non-vaccinated animals, animals (whether seropositive from natural infection or seronegative) may move at any time without posing a risk of virus spread provided that an adequate surveillance system has been in place in the source population for a period of 60 days immediately prior to despatch without detecting evidence of bluetongue virus circulation

Pursuant to the above recommendations, the Working Group invites the OIE to review the relevant chapters of the *Terrestrial animal health code* to bring them in line.

The Working Group recommends the OIE to back up safe trade in bluetongue seropositive animals by ensuring the existence of an adequate network of reference laboratories which will *inter alia* ensure the archiving of viral strains and derived sequence data to provide a comprehensive database to be made available for research, surveillance and trade purposes.

The Working Group recommends that animals vaccinated with attenuated vaccines produced by culture in embryonated eggs shall not be moved.
Working Group 6

Control and trade

Group members

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Working Group charge

To address the potential impact of issues raised by the five other working groups on international trade and movement of animals; specifically, to address issues pertaining to the movement of seropositive as well as potentially viraemic animals.

Recommendations

Specific conclusions of the Working Group:

1) In considering the potential movement of BTV seropositive animals from an infected to a free zone or country, the Working Group concludes that animals may move at any time without posing a risk of virus spread if they have been vaccinated with a licensed or authorised attenuated, inactivated, subunit, or genetically manipulated vaccine at least one month prior to movement provided that the vaccine used covers all serotypes which would be expected to be present at origin from adequate surveillance and that the animals are identified as vaccinates.

2) In the case of healthy, non-vaccinated animals, animals (whether seropositive from natural infection or seronegative) may move at any time without posing a risk of virus spread provided that an adequate surveillance system has been in place in the source population for a period of 60 days immediately prior to dispatch without detecting evidence of bluetongue virus circulation.

3) The Working Group endorses the recommendations of Working Group 5 (Impact of interventional strategies on virus spread, disease and regulation) that the OIE should re-evaluate the Terrestrial animal health code in light of conclusions of the Third Symposium. In addition, that the OIE can further ensure the continued safe movement of ruminants that are seropositive to BTV by supporting the network of reference laboratories that will archive BTV strains and derived sequence data to ensure that a comprehensive database is available for research, surveillance and trade purposes.

4) The Working Group encourages the OIE to ensure that periodic surveillance for BTV occurs in zones with no previous evidence of virus activity; and, that any new evidence of virus activity in these zones be immediately reported to the OIE.

5) The Working Group considers that the agar gel immunodiffusion (AGID) test assay lacks the requisite sensitivity and specificity (because of
potential cross reactions with other viruses, particularly epizootic haemorrhagic disease virus). The competitive enzyme-linked immunosorbent assay (c-ELISA) is now considered the standard and appropriate technology for serological diagnosis of previous exposure to animals to BTV.

6) The Working Group endorses the use of polymerase chain reaction (PCR)-based technologies for detection of BTV nucleic acid in animals and insects. The ‘real-time’ PCR technology is faster than traditional PCR methods, and is less susceptible to the problems of contamination that compromise nested PCR assays in particular. However, further validation is required as there may be problems in the identification of new strains of BTV with existing “real-time” probes.

7) The Working Group recommends that the OIE convene an ad hoc working group to address the current bluetongue chapter and the guidelines for bluetongue surveillance and monitoring, as it is agreed that the current chapter is both prescriptive and confusing.

8) Issues to be addressed, as detailed by Working Group 1:

a) The infective period should be based on current scientific information and technologies, i.e., vector capabilities and competence, cell culture and PCR information, etc.

b) The recent information on the distribution of BTV makes the current BTV limits based on latitudes obsolete. Consider that BTV distribution is based on continental ecological zones or episystems with associated defined parameters. Adjacent zones should have surveillance and monitoring practices for BTV presence. Evidence of BTV in the adjacent zone should be immediately reported to the OIE.

c) Reconsider the broad use of the term ‘Culicoides’ to indicate midges from the genus Culicoides that have been shown or are suspected to be probable vectors of BTV. In other words, be specific as to the species involved.

d) Consider broad guidelines addressing the intensity and frequency of surveillance, which will complement the provisions of the general chapter.

e) The extent of a surveillance programme in countries (zones) adjacent to a country (zone) that does not have free status (as currently specified in the Code)

f) When a surveillance programme is designed, the predictive value of the tests used in the programme should be described as part of the study.

g) When surveillance is conducted, the species and age of animals need to be considered to ensure that there is appropriate sensitivity for that surveillance.

h) The presence of ecological zones for BTV in different parts of the world warrants recognition. Factors pertaining to vectors and hosts in one system may not be relevant to another.

i) Tests that distinguish between vaccinated and naturally infected animals will be of value to surveillance programmes.

9) Specific recommendations in regard to vaccines and vaccination strategy:

a) encourage the development and transfer of complementary and alternative vaccine materials and strategies that provide safe and efficacious inactivated or subunit BTV vaccines, and further encourage that vaccine companies adopt these products and make them available to producers

b) vaccine strains should be fully sequenced and the data made available to a reference database(s)

c) encourage the development of technologies that will distinguish vaccinated from infected animals

d) encourage countries applying current or future vaccine technologies and strategies to make all data on monitoring and surveillance of control programmes available to the OIE for addressing future disease outbreaks

e) animals receiving vaccines produced by culture in embryonated chicken eggs shall not be moved internationally

f) update and keep current the OIE Manual of diagnostic tests and vaccines for terrestrial animals on research information and data on the efficacy of both subunit and inactivated bluetongue vaccines.
Overview

Third International Symposium on bluetongue

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Scientists, regulatory officials and livestock producers met at the Third International Symposium on bluetongue (BT) to discuss current scientific advances, issues and policies as well as to identify areas needing additional research related to policy matters.

The symposium addressed the following:
1) epidemiology and global distribution
2) monitoring and surveillance
3) biology of BT and its vectors
4) diagnostics
5) vaccines
6) strategies for intervention.

Epidemiology and global distribution

Significant changes in our understanding of BT became evident during the course of the symposium when we learned of the changes in the global distribution of the disease. As recently as the previous symposium, the distribution was thought to occur between the latitudes of 40°N and 35°S. Since 2000, BT appears to have become established at 45°N to 50°N. These new observations of distribution have expanded our perceptions of BT.

At the Second International Symposium on BT, the epidemiology of BT viruses (BTV) was categorised into three zones, namely: endemic, epidemic and incursion zones. The endemic zone lies in tropical climates where competent Culicoides spp. are actively spreading BTVs all year. The disease is rarely observed in this zone. The epidemic zone is located in temperate climates where competent Culicoides spp. appear during the warm season, and some disease is observed seasonally. The incursion zones are those where BT appears every decade or so, associated with climatic changes. The competent Culicoides spp. appear for one to two years and disease outbreaks occur as long as competent vectors are in the area.

Maps depicting the distribution of BT are historic records of the occurrence of BT. Boundaries move with the vectors, which do not respect political boundaries. Instead, vector distribution is based on climatic and environmental conditions. We realised that we must now approach BT, not as a disease of countries, but one of continents.

Monitoring and surveillance

The symposium highlighted the critical role of vectors as the principal means of spreading BTVs. Not all Culicoides spp. transmit BTV. When seeking to determine potential distribution of BTVs, regulatory agencies need only consider those Culicoides spp. that are competent for transmission of BTV.
In the absence of competent *Culicoides* spp. vectors, BTV will not survive in an area. There is no evidence that BTV persist in cattle, a clear indication that ruminants are of no importance in the movement of BTV from one region to another. Symposium participants acknowledged the importance of competent *Culicoides* spp. vectors in the distribution of BTV in Europe.

**Biology of bluetongue and its vectors**

BTVs are gastrointestinal viruses of *Culicoides* spp. Domestic and wild ruminants are the amplifying hosts for the insect vectors of BTV. One gene controls BTV competency in *Culicoides* spp. The phenotypic expression of the gene is influenced by temperature, rainfall, soil pH, and other factors. The role of these factors in overwintering of BTV in *Culicoides* spp. appears to be based on temperature. If the environmental temperature is not sufficient for complete viral protein assembly, incomplete virus will remain in the intestinal cells of the vector until the critical temperature for virus assembly is reached.

Identifying the *Culicoides* spp. vectors in Europe and Central Asia will provide a better understanding the distribution of BTV. The genotyping of viruses based on non-structural protein 3 (NS3) has led to the concept of topotyping and topotyping makes a significant difference in determining the limitations of the virus serotypes in various locations around the world. For example, BTV serotypes 2, 10, 11, 13 and 17 occur in North America. BTV-2 is only described in Florida and adjacent states in the United States of America (USA). The vector for BTV-2 is *Culicoides insignis*, whereas the other North American serotypes are transmitted by *C. sonorensis*. BTV-2 has not adapted to *C. sonorensis*, even though this vector is present in Florida.

Scientists have also made remarkable progress in characterising the BTV structure and function since the Second International Symposium on BT. Phenomenal advances have taken place with the BTV model, which has helped define serology, virulence, cell biology and viral assembly.

Topotyping strategies have led to important advances in our understanding of the biology of BTV. The topotyping procedures of BTVs in Australia, South-East Asia, and South-Central Asia have led to the recognition of regionally distinct viral groupings classified as Australia A, Java A, Java C and Malaysia A. Classifying these viral isolates is important for evaluating whether new groupings will move into defined geographical areas. Experimental evidence was presented to demonstrate that BTV is a quasi-species virus.

Understanding the pathogenesis of BTV infection in ruminants helps define the pathogenic characteristics of these viruses in sheep and cattle. BTV infection is capable of causing haemorrhagic lesions. BTV in sheep causes vascular damage resulting in disseminated intravascular coagulopathy with secondary effects that include haemorrhage, oedema and vascular thrombi leading to skeletal and cardiac muscle necrosis. Endothelial damage does not occur in cattle and therefore clinical disease is rare.

Studies undertaken to follow viraemias in experimentally infected cattle revealed that the virus that can be recovered by virus isolation techniques for as long as 45 to 50 days. In contrast, viral ribonucleic acid (RNA) can be detected by polymerase chain reaction (PCR) for as long as 220 days after infection. The significance of this observation is that careful consideration of the clinical signs and PCR results is critical for appropriate diagnosis.

**Diagnostics**

Researchers have also developed improved viral diagnostics by applying molecular techniques to PCR assays for the identification of viral RNA in tissues of infected animals. The potential for application of new sophisticated technologies could greatly enhance diagnostic capabilities for virus identification and differentiation in the near future. Serological tests can be used in a variety of ways to evaluate BTV infections and epidemiology.
Vaccines

Information derived from molecular studies of viral assembly have led to the development of subunit viral proteins that can be recombined to create efficacious and safe vaccines. These newer vaccine types may ultimately replace attenuated and inactivated vaccine products which have been associated with foetal malformation and contamination of semen.

The South African attenuated virus vaccine strategies used on ruminants in Corsica and Italy were described. The sophisticated epidemiological studies will provide the relevant information on the effectiveness of the vaccines in controlling infection, mortalities and distribution of BTV in Southern Europe. The vaccine strategies used in South Africa were described where three different vaccinations containing five serotypes of virus are administered over a three-week period. This strategy has proven to be an effective means of controlling disease in ruminants in South Africa.

Control and trade issues

A review of the OIE International Standards for BT set the stage for reports of regulatory procedures in North America, South America and the European Union. The movement of animals in North America bridges all of the epizones in which BT is known to occur. Cattle movement from Mexico with similar and different serotypes of virus found in the USA was confined by the vector species. Cattle movement did not influence the distribution of virus beyond the vector boundaries. Similarly, the movement of cattle from the epizootic and incursion zones of the USA into the non-BT north-eastern USA and Canada has not resulted in the establishment of BTV infection in those zones. Again, C. sonorensis is not present in north-eastern USA or Canada, thereby limiting the distribution of BTV to those areas. BTV infection has been described in Argentina, Brazil and Chile. The virus was confined to the more temperate climates of these South American countries.
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