

CHAPTER 2.1.3.

BLUETONGUE AND EPIZOOTIC HAEMORRHAGIC DISEASE

SUMMARY

Bluetongue virus (BTV) infection involves domestic and wild ruminants such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and various other Artiodactyla as vertebrate hosts. This non-contagious, insect-borne viral infection is inapparent in the vast majority of infected animals but causes fatal disease in a proportion of infected sheep, deer and wild ruminants. Infection in cattle mostly has not resulted in clinical signs, with the recent exception of BTV8 infection of cattle in Europe. Cattle are particularly significant in the epidemiology of the disease due to the prolonged viraemia in the absence of clinical disease. Clinical signs range from mild to severe and vary not only between species but between breeds and within the flock or herd. Clinical signs of BT disease are mainly attributable to vascular permeability and include fever, hyperaemia and congestion, facial oedema and haemorrhages, and erosion of the mucous membranes. However in mild cases of the disease, a transitory hyperaemia and slight ocular and nasal discharge may be observed. In very severe cases the tongue may show hyperaemia, become oedematous and protrude from the mouth, or become cyanotic. Hyperaemia may extend to other parts of the body, particularly the coronary band of the hoof, the groin, axilla and perineum. In severe cases there is additionally skeletal and cardiac muscle degeneration. Wool breaks may occur. Sheep may become lame as a result of laminitis and skeletal myopathy. A similar severe disease of wild ruminants is caused by epizootic haemorrhagic disease virus (EHDV), which, like BTV, is a member of the Orbivirus genus, but is classified in a separate species or serogroup.

Identification of the agent: *BTV is a member of the Orbivirus genus of the family Reoviridae, one of 20 recognised species or serogroups in the genus. The BTV species, or serogroup, contains 24 recognised serotypes and EHDV contains at least seven. The orbiviral species are differentiated by immunological tests that detect viral proteins that are conserved within each, and hence are distinguishable by serogrouping tests. However there may be considerable cross-reaction between related species, and this is the case with the BT and EHD serogroups. The serotype of individual viruses in each species is identified on the basis of neutralisation tests and different strains within a serotype are identified by sequence analysis. Complete BTV particles are double-shelled icosahedral double-stranded RNA virus. The outer layer contains two proteins, one of which, VP2, is the major determinant of serotype specificity. The inner shell and core contains two major and three minor proteins and ten double-stranded RNA genetic segments. VP7 located in the inner shell is the major core protein possessing the species or serogroup-specific antigens. Virus identification traditionally requires isolation and amplification of the virus in embryonated hens' eggs, tissue culture or inoculations of susceptible ruminants and the subsequent application of serogroup- and serotype-specific tests. Reverse-transcription polymerase chain reaction (RT-PCR) technology has permitted rapid amplification of BTV and EHDV RNA in clinical samples, and RT-PCR-based procedures are now available. Real-time PCR is allowing development of even more rapid and sensitive tests, and procedures are currently being validated and published. These procedures can augment the classical virological techniques to provide information on virus serogroup, serotype and topotype.*

Serological tests: *Serological responses appear some 7–14 days after BTV infection and are generally long-lasting. Historically, tests such as agar gel immunodiffusion and indirect enzyme-linked immunosorbent assay (ELISA) were used to detect BTV and EHDV species-specific antibodies, but have the major drawback of being unable to consistently distinguish between antibodies to viruses in the two species. A monoclonal antibody-based competitive ELISA has*

solved this problem and competitive ELISAs to specifically detect anti-BTV antibodies are recommended. Procedures to determine the serotype-specificity of antibodies in sera are more complex and time-consuming because they assess whether the sera inhibit the infectivity of panels of known virus serotypes in neutralisation tests.

Requirements for vaccines and diagnostic biologicals: Vaccination is used in several countries to limit direct losses, minimise the circulation of BTV and allow safe movement of animals. However, there is no vaccine to protect against EHDV infection. For many years South Africa has used live, attenuated BT vaccines that are serotype-specific. Live attenuated vaccines are produced by adapting BTV field isolates to growth in vitro through serial passages in tissue culture or in embryonated hens' eggs. Stimulation of a strong antibody response by these vaccines is directly correlated with their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities, they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease. Adverse consequences are depressed milk production in lactating sheep, and abortion/embryonic death and teratogenesis in offspring from pregnant females that are vaccinated during the first half of gestation. Another risk associated with the use of live attenuated vaccines is their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of vaccine virus genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector *Culicoides* in the field has already been documented in Europe. The fact that attenuated viruses are teratogenic makes determination of transmissibility very important. Vaccine efficacy, teratogenic potential and transmissibility should be tested. Hence inactivated or recombinant vaccines would be preferred if effective. The inactivated vaccines are not teratogenic and have been used under government supervision since 2004 in the management of recent European outbreaks.

A. INTRODUCTION

Midges of certain species in the genus *Culicoides* (the insect host) (49) transmit bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) among susceptible ruminants, having become infected by feeding on viraemic animals (the vertebrate host). After a replication period in the insect's salivary glands, that depends upon ambient temperature, the virus can be transmitted to a new vertebrate host during feeding. Infected midges remain infective for life. The central role of the insect in BT and EHD epidemiology ensures that distribution and prevalence of the infection is governed by ecological factors, such as high rainfall, temperature, humidity and soil characteristics, which favour insect survival (9). In many parts of the world therefore, infection has a seasonal occurrence (54). It is accepted that BTV and EHDV do not establish persistent infections in ruminants, and that survival of BTV and EHDV in the environment is associated with insect factors (27, 30). Globally the distributions of BTV and EHDV have been considered on the basis of epidemiological systems (episystems) based on the vector species present and their natural history (50). However, recent information suggests that BTV and EHDV have moved between episystems and with different species of vector midges (23, 36)

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other Artiodactyla such as camels. Although antibodies to BTV, and in some cases virus antigen and/or live virus has been demonstrated in some carnivores, felids, black and white rhinoceroses and elephants, the role of non-ruminant species in the disease in the wild is not known. The outcome of infection ranges from inapparent in the vast majority of infected animals, especially wild Africa ruminants and cattle, to fatal in a proportion of infected sheep, goats, deer and some wild ruminants (54). However a higher incidence of clinical disease has been observed in cattle infected with BTV 8 in Europe. Some breeds of sheep are more susceptible to disease than others, with the result that in some countries BTV infections of livestock can occur unobserved, and be detected only by active surveillance (13). Epizootic haemorrhagic disease virus (EHDV) can produce a disease in wild ruminants with clinical manifestations identical to those observed in response to BTV infection. As EHD has not been studied in detail, there will be less information provided on this virus and disease.

Clinical signs of disease in sheep vary markedly in severity, influenced by the type or strain of the infecting virus, husbandry factors as well as by breed (54). In severe cases there is an acute febrile response characterised by hyperaemia and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and erosions of the mucous membranes. The tongue may show intense hyperaemia and become oedematous, protrude from the mouth and, in severe cases become cyanotic. Hyperaemia may extend to other parts of the body particularly the coronary band of the hoof, the groin, axilla and perineum. There is often severe muscle degeneration. Breaks in the wool may occur associated with pathology in the follicles. A reluctance to move is common and torticollis may

occur in severe cases. In fatal cases the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity and pericardial sac may contain several litres of plasma-like fluid. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (54).

Taxonomically, BTV is classified as a species or serogroup in the *Orbivirus* genus in the family Reoviridae, one of 22 recognised species in the genus that also includes EHDV, equine encephalosis and African horse sickness (AHS) viruses (40). Within species, individual members are differentiated on the basis of neutralisation tests, and currently 24 serotypes of BTV and 7 serotypes of EHD are recognised (3). There is significant immunological cross-reactivity between members of the BT and EHD viruses (40). Recently, a novel BT like virus (Toggenburg orbivirus) was isolated from goats in Switzerland and proposed as a new BTV serotype (22). The broader significance of the finding has yet to be established.

BTV and EHDV particles are composed of three protein layers. The outer layer contains two proteins, VP2 and VP5. VP2 is the major neutralising antigen and determinant of serotype specificity. Removal of the outer VP2/VP5 layer leaves a bi-layered icosahedral core particle that is composed of two major proteins, VP7 and VP3, three minor proteins and the ten species of double-stranded RNA. VP7 is a major determinant of serogroup specificity and the site of epitopes used in competitive enzyme-linked immunosorbent assay (C-ELISA) to detect anti-BTV and EHDV antibodies (39). VP7 can also mediate attachment of BTV to insect cells (57).

Genetic sequencing of BTVs and EHDVs allows differentiation and analysis of strains separately from serotype (20, 34, 45, 56). Even for strains within the one serotype it is possible to identify the likely geographical origin (topotype) (20, 44). Such studies have led to the detection of international movements of BTV strains. Natural movements of vectors by climatic forces are believed to result in some intercontinental movements of BTV and EHDV. Particularly important in recent times as examples of such vector movements have been the incursions of BTV into southern Europe, (37); northern Australia (12) and southern States of the USA (23). However the origins of the recent (2006–2008) incursions of BTV directly into northern Europe remain uncertain. Identification of apparent associations between some genotypes of virus and some vector species has led to the concept of viral-vector ecosystems being proposed (12, 13, 30, 50) Recent movements of several BTV serotypes between vector species and into new geographic regions indicates a more complete understanding of BTV and EHDV epidemiology is required.

B. DIAGNOSTIC TECHNIQUES

1. Identification of the BTV agent (a prescribed test for international trade)

a) Virus isolation

The same diagnostic procedures are used for domestic and wild ruminants. A number of virus isolation systems for BTV are in common use, but the most sensitive method is by inoculation of embryonated hens' eggs (ECE). Inoculation of sheep may also be a useful approach if the titre of virus in the sample blood is very low, as may be the case several weeks after virus infection. Attempts to isolate virus in cultured cells *in vitro* may be more convenient, but the success rate is frequently much lower than that achieved with *in vivo* systems (19). EHDV may be isolated in the same isolation system as for BTV but primary inoculation of cell cultures such as C6/36 cells, deleting the first passage in ECE, has proven to be equally sensitive (19).

- **Isolation in embryonated hens' eggs**

- i) Blood is collected from febrile animals into an anticoagulant such as EDTA (ethylamine diamine tetraacetic acid), heparin or sodium citrate, and the blood cells are washed three times with sterile phosphate buffered saline (PBS). Washed cells are re-suspended in PBS or isotonic sodium chloride and either stored at 4°C or used immediately for attempted virus isolation.
- ii) For long-term storage where refrigeration is not possible blood samples are collected in oxalate–phenol–glycerin. If samples can be frozen, they should be collected in buffered lactose peptone or 10% dimethyl sulphoxide (51) and stored at –70°C or colder. The virus is not stable for long periods at –20°C.
- iii) In fatal cases, spleen and lymph nodes are the preferred organs for virus isolation attempts. Organs and tissues should be kept and transported at 4°C to a laboratory where they are homogenised in PBS or isotonic saline, and used as described below, for blood cells.
- iv) Washed blood cells are re-suspended in distilled water or sonicated in PBS and 0.1 ml amounts inoculated intravascularly into 5–12 ECE that are 9–12 days old. This procedure requires practice. Details are provided by Clavijo *et al.* (10).
- v) The eggs are incubated in a humid chamber at 32–33.5°C and candled daily. Any embryo deaths within the first 24 hours post-inoculation are regarded as nonspecific.

- vi) Embryos that die between days 2 and 7 are retained at 4°C and embryos remaining alive at 7 days are killed. Infected embryos may have a haemorrhagic appearance. Dead embryos and those that live to 7 days are homogenised as two separate pools. Whole embryos, after removal of their heads, or pooled organs such as the liver, heart, spleen, lungs and kidney are homogenised and the debris is removed by centrifugation.
- vii) Virus in the supernatant may be identified either directly by antigen-capture ELISA (21) or reverse transcription polymerase chain reaction (RT-PCR), or indirectly by antigen-detection methods such as immunofluorescence or immunoperoxidase after further amplification in cell culture, as described in the next section.
- viii) If no embryos are killed following inoculation of sample material, an inoculum made from the first egg passage material may be re-passaged in ECE or in cell culture.

- **Isolation in cell culture**

Virus isolation may be attempted in BTV in susceptible cell cultures such as mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero) or *Aedes albopictus* clone C6/36 (AA). The efficiency of isolation is often significantly lower following inoculation of cultured cells with diagnostic samples compared with that achieved in ECE. Highest recovery rates are achieved by primary isolation of virus in ECE, followed by passage in C6/36 (AA) cells for further replication of virus. Additional passages in mammalian cell lines such as BHK-21 or Vero are usually performed. A cytopathic effect (CPE) is not necessarily observed in AA cells but appears in mammalian 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 a number of immunological methods described below, including antigen-capture ELISA, immuno-fluorescence, immunoperoxidase, virus neutralisation (VN) tests, or by RT-PCR.

- **Isolation in sheep**

This procedure is used for isolation of BTV and less sensitive for the isolation of EHDV and not routinely used.

- i) Sheep are inoculated with washed cells from 10 ml to 500 ml of blood, or 10–50 ml tissue suspension. Inocula are administered subcutaneously in 10–20 ml aliquots. Large volumes may aid in the virus isolation attempts and should be administered intravenously.
- ii) The sheep are held for 28 days and checked daily for pyrexia and weekly for antibody response using serological tests such as the C-ELISA as described below. Sheep blood collected at 7 to 14 days post-inoculation will usually contain the isolated virus, which can be stored viable at 4°C or –70°C.

b) Immunological methods

- **Serogrouping of viruses**

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV 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 MAb can be used. A number of laboratories have generated such serogroup-specific reagents (2, 28). Commonly used methods for the identification of viruses to serogroup level are as follows.

- i) *Immunofluorescence*

Monolayers of BHK or Vero cells on chamber slides (glass cover-slips) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific MAbs and standard immunofluorescent procedures.

- ii) *Antigen capture enzyme-linked immunosorbent assay*

Viral antigen in ECE and culture medium harvests (21), infected insects (35) and sheep blood may be detected directly. In this technique, virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb. Serogroup-specific MAbs and polyclonal antibody raised to Baculovirus-expressed core particles have been used successfully to detect captured virus (21).

- iii) *Immunospot test*

Small volumes (2 µl) of infected cell culture supernatant or lysed or sonicated infected cells are adsorbed to nitrocellulose and air-dried. Nonspecific binding sites are blocked by incubation in a

solution containing skim milk protein. After incubation with a BTV serogroup-reactive MAb, bound antibody is detected using horseradish peroxidase-conjugated anti-mouse IgG (18).

- **Serotyping by virus neutralisation**

Neutralisation tests are type specific for the currently recognised 24 BTV and 7 EHDV serotypes and can be used to serotype a virus isolate or can be modified to determine the specificity of antibody in sera. In the case of an untyped isolate, the characteristic regional localisation of BTV and EHDV serotypes can 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 antibodies. Cell lines commonly used are BHK, Vero and L929. Four methods to serotype BTV are outlined briefly below. BTV and EHDV 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.

- i) *Plaque reduction*

The virus to be serotyped is diluted to contain approximately 100 plaque-forming units (PFU), and incubated with either no antiserum or with dilutions of individual standard antisera to a panel of BTV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After adsorption and removal of inoculum, monolayers are overlaid with agarose. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 90%) in the number of PFU. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test, and is similarly neutralised.

- ii) *Plaque inhibition*

Tests are performed in 90 mm diameter Petri dishes containing confluent cell monolayers that are infected with approximately 5×10^4 PFU standard or untyped virus. After adsorption and removal of inoculum, monolayers are overlaid with agarose. Standard anti-BTV antisera are added to individual filter paper discs and placed on the agarose surface. Dishes are incubated for at least 4 days. A zone of virus neutralisation, with concomitant survival of the cell monolayer, will surround the disc containing the homologous antiserum.

- iii) *Microtitre neutralisation*

Approximately 100 TCID₅₀ (50% tissue culture infective dose) of the standard or untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum diluted in tissue culture medium. Approximately 10^4 cells are added per well in a volume of 100 µl, and after incubation for 4–6 days, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show convincing CPE. The unidentified virus is considered to be serologically identical to a standard BTV serotype if both are neutralised in the test to a similar extent.

- iv) *Fluorescence inhibition test*

This rapid and simple neutralisation assay (7) requires varying concentrations of an unknown virus and standard concentrations of reference antisera. Virus isolates grown in cell culture are serially diluted starting and mixed with individual reference antisera in the wells of a Lab-Tek slide for 1 hour prior to addition of cells. After incubation for 16 hours, cells are fixed and probed by an immunofluorescent procedure using a BT serogroup-specific MAb. The serotype of the virus is indicated by the specificity of the antiserum causing the largest reduction in the number of fluorescent cells.

- c) **Reverse-transcription polymerase chain reaction (a prescribed test for international trade)**

Primer-directed amplification of viral nucleic acid has revolutionised BTV and EHDV detection in tested samples (11, 34, 55). RT-PCR techniques have allowed the rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. RT-PCR-based diagnostics should be interpreted with caution. The RT-PCR procedure will detect virus-specific nucleic acid, but this does not necessarily indicate the presence of infectious virus (31). RT-PCR can also be used to 'serogroup' Orbiviruses and may ultimately be possible to 'serotype' BTV within a few days of receipt of a clinical sample, such as infected sheep blood (38). Traditional approaches, which rely on virus isolation followed by virus identification serologically, may require up to 4 weeks to generate information on serogroup and serotype.

Oligonucleotide primers used so far have been derived from RNA 7 (VP7 gene) (55), RNA 6 (NS1 gene) (11), RNA 3 (VP3 gene) (45), RNA 10 (NS3 gene) (6) and RNA 2 (VP2 gene) (34). 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 described in detail below, a 101-nucleotide stretch of RNA 6 is amplified. Primers derived

from the more highly conserved genes, such as VP3, VP7 and NS1, may be used for serogrouping (i.e. will react with all members of the BT serogroup), while primers for which the sequence was determined from VP2 gene sequences provide information on virus serotype. A multiplex RT-PCR assay that depends on the size of the amplified products has been used to identify the five North American BTV serotypes, both alone and in mixtures, in a single reaction (24).

The nucleic acid sequence of cognate BTV genes may differ with the geographical area of virus isolation (20). This has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. Thus, determination of the nucleic acid sequence of portions of RNA may provide information on where the virus came from. It appears likely that sequencing of BTV isolates from other parts of the world may permit finer discrimination of geographical origin. However, the relationship between sequence and geographical origin may not be straightforward. This sequencing information is important and all data regarding BTV segment sequences should be made widely available by submitting the data to officially recognised web sites.

http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ and
http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/btv_sequences.htm.
<http://eubtnet.izs.it/btnet/index.htm>

The web sites provide phylogenetic tree analyses of BTV isolates based on the sequence of RNA segments. These compiled data will provide a resource for epidemiological studies, the identification of new isolates and the design of new primers for further RT-PCR and possibly serotype-specific assays for BTV.

It has been observed that BTV nucleic acid can be detected by RT-PCR from the blood of infected calves and sheep at least 30 days, and sometimes over 90 days, after the virus can be isolated. The presence of virus-specific nucleic acid does not necessarily indicate the presence of infectious virus.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids. The latter may include any primers in use in the laboratory or previously amplified polynucleotides. It is critical therefore to have a 'clean' area containing all equipment necessary for reagent and test preparation and a separate area with its own equipment for amplification. Impervious gloves should be worn and changed frequently at all stages of the procedure, particularly after working with sample RNA or amplified DNA. This will help protect reagents and samples from contamination by ubiquitous RNases and other agents and from cross-contamination by DNA. The possibility of false positives, due to sample contamination, highlights the importance of sequencing RT-PCR products to determine, for example, if the amplified sequence is identical to or different from that of the positive control. False negatives, due for example to poor sample quality or inappropriate primers, may be identified following the failure to amplify both BTV and a host gene, such as globin, from extracts of infected cells. This is covered in more detail in Chapter 1.1.4 Principles and methods of validation of diagnostic assays for infectious diseases.

There are many RT-PCR assays currently in use that use different extraction methods, reverse transcriptases, amplification enzymes, primers and conditions. Technology is changing rapidly. Also the genetic diversity of the BTV genes makes the choice and validation of RT-PCR assays conditional on its application in a regional setting. This is also the case for real-time PCR, which will be developed for use in routine molecular diagnosis of BTV in the future. Therefore the procedures listed below are examples only. Increasingly it will be more important to maintain diagnostic testing under accreditation to an international standard such as ISO/IEC 17025 and participate in proficiency testing. Systems for offering proficiency testing for RT-PCR tests are being developed in a number of countries.

The RT-PCR assay described here involves three separate procedures. In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanate (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available for this purpose and the protocol below describes the use of one such kit, IsoQuick¹. The reagents provided with the kit are numbered and their use is indicated in the protocol below. Other kits are available and one, TRIZOL², 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 to generate cDNA, which is amplified by RT-PCR. In the procedure described below, the SuperscriptTM Pre-amplification System³ is used to transcribe viral RNA, and reagents⁴ are used for the RT-PCR. Equivalent kits and

1 IsoQuick: Orca Research, Bothell, Washington, United States of America (USA).

2 TRIZOL: Life Technologies, Grand Island, New York, USA.

3 SuperscriptTM Pre-amplification System: Life Technologies, Grand Island, New York, USA.

reagents are available from other sources. The final step of the process is the analysis of the RT-PCR product by electrophoresis. Procedures used to determine the sequence of the amplified product are not described here.

- **Extraction of viral RNA**

- Whole blood is collected from test and uninfected control animals in EDTA tubes and centrifuged at 800–1000 **g** for 10 minutes. The plasma is aspirated and the red blood cells (RBCs) are gently resuspended in sterile PBS. RBCs are pelleted by centrifugation at 1000 **g** for 10 minutes and the supernatant is removed.
- Next, 400 μ l of test RBCs is added to each of four 1.7 ml microcentrifuge tubes, and 400 μ l of control RBCs is added to each of two microcentrifuge tubes. An equal volume of RNase-free water is added to each tube and the tubes are vortexed briefly to mix and lyse the cells. Two tubes containing test RBCs are frozen at -70°C for repository purposes and the extraction is continued in duplicate.
- Lysed test and control RBCs are centrifuged at 12,000–16,000 **g** for 10 minutes and the supernatant is discarded. Next, 800 μ l RNase-free water is added and the tubes are vortexed and centrifuged again at the same speed for 10 minutes. The supernatant is removed and the RBC pellet is drained.
- A small volume of BTV (e.g. 5 μ l containing from 10^3 to 10^7 PFU) is added to one of two control RBC pellets. This is the positive control. The other control RBC pellet remains as the negative control.
- Next, 75 μ l of sample buffer (IsoQuick reagent A) is added to each pellet, and the pellets are then vortexed vigorously, followed by the addition of 125 μ l of the GuSCN-containing lysis solution (IsoQuick reagent 1). The mixture is vortexed vigorously for 30 seconds.
- Before use the extraction matrix provided with the kit (IsoQuick reagent 2 plus dye 2A) is shaken vigorously and 500 μ l is added to the sample lysates. Then, 400 μ l extraction buffer (IsoQuick reagent 3) is added and the tubes are vortexed for 10 seconds.
- The tubes are incubated at 65°C for 10 minutes, vortexed briefly after 5 minutes and centrifuged at 12,000 **g** for 5 minutes.
- The aqueous phase (500 μ l) is transferred to a new microcentrifuge tube and an equal volume of extraction matrix (IsoQuick reagent 2) is added. The tubes are vortexed for 10 seconds and centrifuged at 12,000 **g** for 5 minutes.
- The aqueous phase (330 μ l) is transferred to a new microcentrifuge tube and a 10% volume (33 μ l) of sodium acetate (IsoQuick reagent 4) and 365 μ l isopropanol are added. After gentle mixing, the tubes are placed at -20°C for from 20 minutes to 1 hour.
- The RNA is pelleted by centrifugation at 12,000 **g** for 10 minutes. The supernatant is decanted and 1.0 ml 70% ethanol is added and mixed gently. After centrifugation at 12,000 **g** for 5 minutes, the supernatant is decanted and 1.0 ml 100% ethanol is added. The tubes are stored at -70°C until ready for use in the RT-PCR.

- **Reverse-transcription polymerase chain reaction**

- RNA in ethanol is centrifuged at 12,000 **g** for 5 minutes. The ethanol is decanted and the tubes are inverted and allowed to drain. The pellet, which may not be obvious, must not be allowed to dry out because this makes resuspension difficult. A dry pellet is also likely to fall out of the inverted tube.
- Next, 12 μ l RNase-free water is added to each tube, mixed and heated at 65°C for 5–10 minutes. The samples are placed in ice.
- In a 'clean' biohazard hood, stock solutions containing 200 pmol/ μ l of primers A, B, C and D are prepared in RNase-free water and stored at -70°C .

First stage RT-PCR primers (to amplify RNA 6 from nucleotide 11 to 284)

Primer A: 5'-GTT-CTC-TAG-TTG-GCA-ACC-ACC-3'

Primer B: 5'-AAG-CCA-GAC-TGT-TTC-CCG-AT-3'

Nested RT-PCR primers (to amplify RNA 6 from nucleotide 170 to 270)

Primer C: 5'-GCA-GCA-TTT-TGA-GAG-AGC-GA-3'

Primer D: 5'-CCC-GAT-CAT-ACA-TTG-CTT-CCT-3'

- Primer stock solutions are diluted to a concentration of 15–20 pmol/ μ l. Primers for the first stage RT-PCR reaction are prepared by mixing equal volumes of A and B. Primers for the nested RT-PCR reaction are prepared by mixing equal volumes of C and D. Small aliquots of pooled primer mixes are frozen at -20°C .

4 Available from Perkin-Elmer.

- v) RT-PCR reaction tubes are labelled and, for first stage synthesis, 4.0 µl of primer (A + B) mix is added to each tube. The tubes are held on ice.
- vi) In a 'clean' fume hood methylmercuric hydroxide is diluted to 50 mM (1/20 dilution) and 2-mercaptoethanol is diluted to 350 mM (1/40 dilution) in RNase-free water. Methylmercuric hydroxide and 2-mercaptoethanol are considered to be extremely and highly toxic, respectively. Use both chemicals with extreme care and dispose of them and pipette tips as required by safety regulations. Alternative methods using heat denaturation have been described (28, 38).
- vii) Next, 4 µl of test and positive and negative control RNA samples (step ii) are added to 4 µl of the primer mix in RT-PCR tubes (56).
- viii) To each RT-PCR tube 2.0 µl of the 1/20 dilution of methylmercuric hydroxide is added with gentle mixing and allowed to sit at room temperature for 10 minutes prior to adding 2.0 µl of the 1/40 dilution of 2-mercaptoethanol. For safety reasons, some laboratories use formamide instead of methylmercuric hydroxide for double-stranded RNA denaturation. However, for optimum sensitivity, methylmercuric hydroxide is preferred.
- ix) In a 'clean' hood a cDNA mix is prepared containing the following reagents in sufficient volume for the number of samples being tested. The amount given is per sample and the reagents are contained in the kit⁵.
- | | |
|--------------------------------------------------------------------|---------|
| 10 × Superscript™ buffer (200 mM Tris/HCl, pH 8.4, and 500 mM KCl) | 2.0 µl |
| MgCl ₂ (25 mM) | 2.0 µl |
| dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP) | 1.25 µl |
| Dithiothreitol (DTT) (0.1 M) | 2.0 µl |
| Reverse transcriptase (200 units/µl) | 0.75 µl |
- x) Then, 8.0 µl of the mix is added to each RT-PCR tube to a final volume of 20.0 µl.
- xi) The RT-PCR tubes are placed in a thermal cycler, such as GeneAmp™ PCR System 9600, which is programmed for reverse transcription as follows:
- | | |
|-----------|------------|
| Hold 44°C | 50 minutes |
| Hold 4°C | For ever |
- xii) The tubes are removed from the thermal cycler and 1.0 µl RNase H and a wax bead are added to each tube. The cycler is programmed as follows:
- | | |
|-----------|------------|
| Hold 37°C | 20 minutes |
| Hold 98°C | 4 minutes |
| Hold 4°C | For ever |
- xiii) In a 'clean' hood a first stage amplification mix is prepared containing the following reagents and in a volume sufficient for the number of samples being tested. All these reagents except water are available⁶. The amount given is per sample.
- | | |
|------------------------------------------------------------------------|---------|
| RNase-free water | 62.0 µl |
| 10 × PCR Perkin-Elmer buffer (100 mM Tris/HCl, pH 8.3, and 500 mM KCl) | 7.0 µl |
| MgCl ₂ (25 mM) | 7.0 µl |
| dNTP mix (2.5 mM each dATP, dCTP, dGTP, dTTP) | 4.0 µl |
| Taq DNA polymerase (5 units/µl) | 0.85 µl |
- xiv) The first stage mix is removed from the 'clean' area to the thermal cycling area and 80 µl is overlaid in each sample tube. The wax layer must not be pierced. Each tube should now contain 101 µl.
- xv) The tubes are placed in the thermal cycler, which is programmed as follows (correct for GeneAmp PCR System 9600 – programmes for other thermal cyclers would need to be determined) for first stage amplification:
- | | | |
|------------|-----------|------------|
| One cycle: | Hold 95°C | 3 minutes |
| | Hold 58°C | 20 seconds |
| | Hold 72°C | 30 seconds |
| 40 cycles: | Hold 95°C | 20 seconds |
| | Hold 58°C | 20 seconds |
| | Hold 72°C | 20 seconds |
| One cycle: | Hold 95°C | 20 seconds |
| | Hold 58°C | 20 seconds |
| | Hold 72°C | 5 minutes |
| | Hold 4°C | For ever |

5 Superscript™ Pre-amplification System: Life Technologies, Grand Island, New York, USA.

6 Available from Perkin-Elmer.

- xvi) RT-PCR reaction tubes are prepared for the nested reaction in a 'clean' hood 15 minutes before cycling is complete, and held on ice:

Rnase-free water	17 µl per tube
Nested primer mix (C+D)	4.0 µl per tube
Wax bead	

- xvii) When first stage amplification is complete, the tubes are removed from the thermal cycler and placed in a biological safety cabinet (not the 'clean' hood). Then, 1.5 µl of the first stage product is transferred to the corresponding nested RT-PCR tube containing primer, water and a wax bead.

- xviii) The tubes are placed in the thermal cycler, which is programmed as follows for wax layer formation:

Hold 98°C	4 minutes
Hold 4°C	For ever

- xix) In a 'clean' hood the nested mix of the following reagents is prepared in sufficient volume for the number of samples being tested. The reagents used are the same as in the first stage (step xii). The amount given is per sample.

RNase-free water	17.0 µl
10 × PCR buffer	5.0 µl
MgCl ₂	3.5 µl
dNTP mix	4.5 µl
Taq DNA polymerase	0.5 µl

- xx) The nested mix is removed from the 'clean' hood to the thermal cycler and 30 µl is overlaid into each sample tube. Each tube should now contain 52 µl.

- xxi) The tubes are placed in the thermal cycler, which is programmed as follows for nested amplification. After completion, the tubes are held at 4°C or at –20°C until electrophoresis:

One cycle:	Hold 95°C	3 minutes
	Hold 58°C	20 seconds
	Hold 72°C	30 seconds
40 cycles:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	20 seconds
One cycle:	Hold 95°C	20 seconds
	Hold 58°C	20 seconds
	Hold 72°C	5 minutes
	Hold 4°C	For ever

• **Electrophoretic analysis of RT-PCR product**

- i) First, 1 × TBE buffer (0.045 mM Tris/borate, pH 8.6, and 1.5 mM EDTA) is prepared from a ×10 stock solution. For the Bio-Rad Wide Mini-Sub cell system, 700 ml buffer is prepared (100 ml for the gel and 600 ml for the tank buffer).
- ii) A 3% solution of NuSieve 3/1 agarose⁷ or an equivalent is prepared in TBE buffer. The solution is boiled until the agarose is completely dissolved, and then allowed to cool to 40°C. Whereas ethidium bromide was used previously to stain the gel, as it is a mutagen and is toxic, its use has been replaced by SYBR® Safe DNA gel stain⁸ according to the manufacturer's instructions
- iii) The ends of the electrophoresis tray are taped and the agarose solution is poured. The comb is inserted and the agarose is allowed to solidify on a level surface for 30–60 minutes. The comb and the tape are gently removed from the electrophoresis tray.
- iv) Pour the tank buffer into the electrophoresis apparatus and insert the tray with the agarose so that the buffer covers the agarose.
- v) Test and positive and negative control samples are prepared for electrophoresis in 0.65 ml microcentrifuge tubes as follows:

Gel-loading solution ⁹	5.0 µl
Amplified DNA from each of the RT-PCR tubes and an extra tube is set up for a DNA ladder	15.0 µl
Gel-loading solution ⁹	5.0 µl
100 base-pair ladder ¹⁰	1.0 µl

7 NuSieve 3/1 agarose: FMC Bioproducts, Rockland, Maine, USA.

8 SYBR® Safe DNA gel stain: Available from Invitrogen (Cat No. S33102).

9 Gel-loading solution: Cat. G-2526, Sigma, St Louis, Missouri, USA.

- vi) Samples are loaded into the appropriate wells in the gel and run at 65–95 volts for 30-90 minutes or until the dye has travelled about half the length of the gel. The gel is transferred to a transilluminator and photographed for a permanent record. Use protective eye-wear to visualise the gel bands.
- vii) BT-positive samples will have a band of 101 base pairs. For the test to be valid, the positive control must show a band of the correct size, and the negative and 'no RNA' controls show no band. Samples are considered to be positive if there is a band of the same size as the positive control. Duplicate samples should show the same reaction. If there is disparity, the test should be repeated.
- viii) A destaining bag¹¹ is placed in the tank buffer overnight to remove the ethidium bromide. The buffer can then be poured down the drain and the destaining bag, after reuse 10–15 times, should be placed in a properly identified ethidium bromide waste container and ultimately incinerated.

d) Real-time reverse-transcription polymerase chain reaction tests

Real-time RT-PCR is a sensitive method that can be used for the laboratory detection of viral RNA. During the one-step real-time RT-PCR reaction, viral RNA is reverse transcribed into cDNA and subsequently amplified. A fluorogenic signal from dual-labelled probe is released during amplification and recorded in real time. The primers and probe combination for each real-time RT-PCR reaction are designed to amplify and detect a targeted region of the viral genome segment, in this case of BTV.

There are two published real-time assays that have been shown to detect all 24 serotypes (48, 52). Of the two, the assay of Shaw *et al.* (48) has been tested against more serotypes and topotypes to date. So far the real-time RT-PCR assays have not been validated to the level required by the OIE validation template, although ring trials have been conducted (4). A further development is of triplex and duplex assay kits that detect a combination of group, BTV-8, BTV-1, BTV-6 and internal control. Serotype-specific assays against all the other serotypes are in the process of development.

It may be expected that after the validation processes have been completed, the real-time RT-PCR assays will become the tests of choice for diagnosis and in support of international trade.

2. Serological tests

Anti-BTV and anti-EHDV antibodies generated in infected animals can be detected in a variety of ways that vary in sensitivity and specificity. Both serogroup-specific and serotype-specific antibodies are elicited and if the animal was not previously exposed to BTV, the neutralising antibodies generated are specific for the serotype of the infecting virus. Multiple infections with different BTV serotypes lead to the production of antibodies capable of neutralising serotypes to which the animal has not been exposed.

a) Complement fixation

A complement fixation test to detect BTV antibodies was widely used until 1982, when it was largely replaced by the AGID test although the CF test is still used in some countries.

b) Agar gel immunodiffusion (an alternative test for international trade)

The AGID test to detect anti-BTV and anti-EHDV antibodies is simple to perform and the antigen used in the assay relatively easy to generate. Since 1982, the test has been one of the standard testing procedures for international movement of ruminants. However, one of the disadvantages of the AGID used for BT or EHD is its lack of specificity in that it can not differentiate between the BT and EHD serogroup. However, one of the disadvantages of the AGID used for BT or EHD is its lack of specificity in that it can not differentiate between the BT and EHD serogroup. Thus AGID positive sera may have to be retested using a BT or EHD serogroup-specific assay. The lack of specificity and the subjectivity exercised in reading the results have encouraged the development of ELISA-based procedures for the specific detection of anti-BTV antibodies. The preferred format, a C-ELISA is described in the Section B.2.c.

- **Test procedure**

- i) A 2.8 mm thick layer of 0.9% agarose in 0.85% NaCl is prepared and circular wells, 4.0 mm in diameter and 2.4 mm apart, are cut out with six wells arranged around a central well.
- ii) Viral antigen is prepared by generating a crude soluble preparation from BHK or Vero cells infected with a single BTV serotype 24–48 hours previously. Antigen can be concentrated by precipitation or ultrafiltration.

10 100 base-pair ladder: Available from Life Technologies, Grand Island, New York, USA (Cat. 15268-019).

11 Destaining bag: Available from Ameresco, Solon, Ohio, USA.

- iii) A reference positive serum and three test sera are placed in alternate wells in a six-well pattern surrounding antigen in a central well and the plates are incubated at 20–25°C in a humid environment for 24 hours.
- iv) A series of precipitin lines form between the antigen and known positive sera and lines generated by strong positive test sera will join up with those of the positive controls. With weak positive samples the control lines bend toward the antigen and away from the test sample well, but may not form a continuous line between the control test wells. With negative samples, the precipitin lines will continue into the sample wells without bending toward the antigen.
- v) All weak positive samples and other samples that produce questionable results should be repeated using wells that are 5.3 mm in diameter placed 2.4 mm apart or retested using the C-ELISA as described below.

c) Competitive enzyme-linked immunosorbent assay (a prescribed test for international trade)

The BT competitive or blocking ELISA was developed to measure BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (1, 6, 28, 41, 46). The specificity is the result of using one of a number of BT serogroup-reactive MAbs, such as MAb 3-17-A3 (2) or MAb 20E9 (28) or MAb 6C5F4D7 (33). The antibodies were derived in a number of laboratories, and although different, all appear to bind to the amino-terminal region of the major core protein VP7. In the C-ELISA, antibodies in test sera compete with the MAbs for binding to antigen. The following procedure for the C-ELISA has been standardised after comparative studies in a number of international laboratories.

• **Test procedure**

There are several test procedures described; this is an example of one BT ELISA procedure.

- i) First, 96-well microtitre plates are coated at 4°C overnight or at 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen (2) or the major core antigen VP7 expressed in either Baculovirus (42) or yeast (33) and diluted in 0.05 M carbonate buffer, pH 9.6.
- ii) The plates are washed five times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.2).
- iii) Next, 50 µl of test sera is added in duplicate at a single dilution, either 1/5 (1) or 1/10 (28) in PBST containing 3% bovine serum albumin (BSA).
- iv) Immediately, 50 µl of a predetermined dilution of MAb diluted in PBST containing 3% BSA is added to each well. MAb control wells contain diluent buffer in place of test serum.
- v) Plates are incubated for 1 hour at 37°C or 3 hours at 25°C, with continuous shaking.
- vi) After washing as described above, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG (H+L) in PBST containing 2% normal bovine serum.
- vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed five times using PBS or PBST. Wells are filled with 100 µl substrate solution containing 1.0 mM ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]), 4 mM H₂O₂ in 50 mM sodium citrate, pH 4.0, and the plates are shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate length of time to permit colour development.)
- viii) The reaction is stopped by addition of a stopping reagent, such as sodium azide.
- ix) After blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured at 414 nm. Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:
$$\% \text{ inhibition} = 100 - \frac{(\text{Mean absorbance test sample})}{(\text{Mean absorbance MAb control})} \times 100$$

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.
- x) Percentage inhibition values >50% are considered to be positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut-off.
- xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

A number of commercially produced cELISAs based on recombinant VP-7 and anti-VP-7 MAb are now available. These commercial assays are routinely used in many laboratories across the world and have been

proved to be fit for purpose in ring-trials (4). Formal acceptance for trade purposes should depend on adoption of individual kits to the OIE Register.

d) Indirect ELISA

An indirect ELISA for bulk milk samples has been shown to be reliable and useful for surveillance purposes (26). It should be validated for relevant serotypes before use.

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

Both live attenuated and killed BTV vaccines are currently available for use. Vaccines to protect against EHDV infection are not yet readily available. Recombinant BT vaccines based on various approaches are under development but none has been licensed and these vaccines will not be addressed here. In South Africa live attenuated vaccines have been used for over 40 years and are known to induce an effective and lasting immunity (15). Live attenuated vaccines are produced by adapting BTV field isolates to growth *in vitro* through serial passages in tissue culture or in ECEs. Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. Live attenuated vaccines are cheap to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used (14). However, live attenuated BTV vaccines suffer from a variety of documented or potential drawbacks, including under-attenuation, the impact of which may vary with different breeds of sheep. Potential adverse consequences are depressed milk production in lactating ruminants, and abortion/embryonic death and teratogenesis in offspring when used on pregnant females in the first period of pregnancy. Another risk associated with the use of live attenuated vaccines is that of their potential for spread by vectors, with eventual reversion to virulence and/or reassortment of modified live virus (MLV) genes with those of wild-type virus strains. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector midges has already been documented in the USA, South Africa and Europe (17, 32, 40, 53). Therefore inactivated vaccines, if effective, are preferred. Virus inactivation eliminates risks of vector transmission, reversion to virulence, fetal abnormalities and the possibility of viral reassortment.

1. Seed management

a) Characteristics of seed

For live, attenuated vaccines the master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both. Each primary seed virus lot should also be tested for transmissibility and reversion to virulence prior to vaccine manufacture. Samples of vaccine prepared from secondary seed virus at the maximum permitted passage level should be tested in sheep for avirulence, safety and immunogenicity.

For killed vaccines the issues of attenuation do not apply, and the approach adopted has been to use field strains of low passage level with the intent of achieving high antigenicity.

Primary seed virus must be free of contaminating bacteria, viruses, prions, fungi and mycoplasmas, particularly pestivirus contamination. For the latter, particular attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Seed viruses must be shown to have the desired serotype specificity.

BTV seed lot viruses should be sequenced and the data made available to relevant databases (43).

Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot.

b) Method of culture

Although the first attenuated BT vaccines were propagated in ECE subsequently cell cultures have been used for tissue culture adaptation and serial passage. These include primary bovine embryo, lamb and fetal lamb kidney cells, and the continuous BHK cells. Cell cultures must be thoroughly checked for the presence of contaminating viruses.

BTV for inactivated vaccines is produced in large-scale suspension cell systems that have been shown to be susceptible to the virus.

c) Validation as a vaccine

Attenuated BT vaccines must be safe and efficacious, and a brief description of appropriate tests for these parameters is given below. In addition, attenuated viruses should not revert to virulence during replication in

vaccinated animals or be able to be transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to non-immune animals, with the subsequent replicative steps in each host species, increases the possibility of reversion to virulence. Although tests for reversion to virulence and transmissibility are rarely, if ever performed, a brief description of what may be necessary is outlined.

There is a variation in BT susceptibility between breeds of sheep; it is important that sheep that have been proven to be susceptible to infection with BTV be used for vaccine validation.

i) *Safety*

All vaccines must be safety tested. Safety tests for attenuated vaccines do not address the issue of their teratogenicity. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryo death (16, 27).

Demonstration of avirulence is necessary for live, attenuated vaccines. A number of sheep, seronegative by an appropriate, sensitive serological test (that will reliably detect antibodies even in vaccinated animals), are inoculated with the primary seed stock. Temperatures are noted twice daily. The animals are monitored at regular intervals over a period of 28 days for clinical signs and any local or systemic reactions to ensure avirulence and innocuity. Blood samples removed at regular intervals can be used to measure level of viraemia and antibody responses. The test shall be valid if all of the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index is calculated for each animal between days 4 and 14 and must be below a specific standard value.

ii) *Efficacy*

Vaccinated and unvaccinated sheep should be challenged with virulent homologous serotype. It is recommended that the challenge model use virus passaged only in ruminant animals and with no ECE or cell culture passages. Passage in such an isolation system results in viral cultures that might induce clinical BT disease that is milder than the natural disease (16). Animals are monitored for clinical signs of BT, rectal temperatures are taken twice daily and blood samples removed at regular intervals to measure viraemia and antibody responses. Unvaccinated control sheep should show clinical signs of BT and viraemia. However, it is difficult to be certain of the appearance of clinical disease following inoculation of sheep with certain BTV serotypes and isolates, and consequently, evidence of infection of unvaccinated control sheep may rest on the appearance of a temperature rise of at least 1.7°C over the pre-challenge mean and a viraemia. As a further evidence of infection pre- and post-vaccination sera are checked for the presence of neutralising antibody.

iii) *Transmissibility*

Transmissibility is an issue with live attenuated vaccines but not with killed vaccines. Procedures to determine if attenuated virus can be transmitted by insects that feed on vaccinated, viraemic sheep are difficult to perform and analyse statistically, and consequently, this criterion of vaccine validation is rarely sought. Laboratory data indicate that laboratory-adapted viruses can be transmitted by insect vectors (17, 40, 49). A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected *Culicoides*, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. As the titre of attenuated virus in the blood of vaccinated sheep is usually low, very large numbers of *Culicoides* may be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. Although virus titres in blood less than $10^3\text{TCID}_{50}/\text{ml}$ have traditionally been considered a "safe" threshold, authentic instances of insects acquiring BTV from animals with viraemic titres much less than $10^3\text{TCID}_{50}/\text{ml}$ have been reported (8). Given the complex interaction of BTV, *Culicoides* vectors and animal hosts in the life cycle of infection, virus titres induced by live attenuated vaccine should be kept to an absolute minimum especially if field transmission of vaccine strains is a concern.

Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (25, 47). The implications of these observations for virus transmissibility are unclear. A recent study of semen from rams vaccinated with BTV2 live attenuated vaccine showed that even if BTV was not detected in the semen, the vaccine caused a decrease in the quality of the semen (5).

iv) *Reversion to virulence*

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence during a number of sheep-insect replication cycles becomes a distinct prospect. The only appropriate

way to monitor for reversion to virulence under these circumstances is to compare the virulence of the vaccine virus with that which had been subject to several sheep–insect replication cycles as described above. As indicated, this is difficult to achieve. Consequently, the effect of a number of sheep–insect passages on the virulence of attenuated viruses has not been determined. In South Africa, it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small. In Europe, five passages are required.

2. Method of manufacture

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. Because of the concern about transmission of the egg propagated attenuated virus, it has been recommended that animals receiving vaccines produced in ECE should not be moved internationally (43). More recently, it is clear that passage in cultured cells will also result in attenuation of virulence. No studies have been done to precisely relate passage number and extent of attenuation for individual virus isolates or serotypes. To prepare attenuated virus, field isolates are adapted to cell culture and passaged *in vitro* up to 40 times or more. Ideally, a number of plaque-purified viruses are picked at this stage and each is examined to determine the level of viraemia they generate and their ability to elicit a protective immune response in vaccinated sheep. The most suitable virus is one that replicates to low titre but generates a protective immune response, and this may represent the source of vaccine primary seed stock virus.

BTV for killed vaccines is produced in large-scale suspension cell systems under aseptic and controlled conditions. Cell lines adapted for large scale industrial cultures are used and these are proven to be free from contaminating microorganisms. When the viral suspension virus reaches its maximum titre, cell disruption is performed and the culture is clarified and filtered. Subsequently inactivation is performed according to processes adopted by the manufacturer, such as by addition of binary ethyleneimine (BEI) or other inactivants. The process must comply with legislation relevant for the intended market, be validated to ensure complete inactivation and be supported by the appropriate documentation. The inactivation process should not significantly alter the immunogenic properties of the viral antigens. Purification is carried out by chromatography. The inactivated virus is then concentrated by ultrafiltration and stored. The inactivated, chromatography-purified and concentrated BTV antigens are made into vaccine by dilution in a buffer solution and addition of adjuvants.

3. In-process control

All ingredients of animal origin, including serum and cells must be checked for the presence of viable bacteria, viruses, fungi or mycoplasmas.

Virus concentration of attenuated vaccines is assessed by infectivity and ELISAs.

For inactivated vaccines, during inactivation of the virus, timed samples are taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of BHK-21 or other appropriate cell cultures. At the end of the inactivation process, the vaccine is checked to ensure that there is no live virus.

4. Batch control

a) Sterility

Every batch of vaccine should be tested for the presence of contaminant viruses of viable bacterial, fungal or mycoplasmal contamination. For example, in South Africa a pool of ten randomly selected ampoules are inoculated into soya broth and thioglycollate broth, and incubated at room temperature and 37°C, respectively, for 14 days. If contaminated, the batch is disqualified.

b) Safety

Every batch of attenuated vaccine is safety tested in newborn and adult mice, guinea-pigs and sheep. If any adverse reactions or significant signs are noted, the test is repeated. Any increase in the body temperature of the target animal that is above the level expected for the particular strain of attenuated virus under test should be regarded as symptomatic. If the results are unsatisfactory after a second attempt, the batch is disqualified.

Safety testing of inactivated vaccines is conducted in sheep to ensure side effects are not observed.

c) Potency

Each batch is tested by inoculation of susceptible sheep. Pre-vaccination, and 21- and 28-day post-vaccination sera are tested by VN assay to determine neutralising antibody levels. To be passed, the antibody titre must be equal to or higher than a set standard based on international vaccine standards.

d) Duration of immunity

Studies with live attenuated BTV vaccine have shown that antibodies in sheep may appear before day 10 post-vaccination, reach a maximum approximately 4 weeks later and persist for well over a year. There is a temporal relationship between the increase in neutralising antibody titre and clearance of virus from the peripheral circulation. Live attenuated BTV vaccines have been in use for over 40 years and are known to induce an effective and lasting immunity in sheep (54). Many serotypes of BTV may be present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in three polyvalent vaccines that are administered sequentially sometimes means that an effective immune response is not generated to all serotypes, presumably because of the antigenic mass of individual serotype-specific antigens is small. In an attempt to broaden the response, vaccination is repeated annually (15).

Initial studies with inactivated vaccines show that antibody against BTV can be detected by day 7 post-vaccination and increase in titre to days 14–21. A second dose of vaccine boosts the titre. Data to demonstrate the expected duration of immunity are being acquired.

e) Stability

Procedures have been developed for attenuated vaccines. Stability should be tested over a period of 2 years. Vaccines in liquid and lyophilised forms are deemed to have shelf lives of 1 and 2 years, respectively. Each batch of vaccine is subjected to an accelerated shelf-life test by storing it at 37°C for 7 days. It is then titrated and evaluated according to a set standard, as determined in the initial testing of the vaccine.

Inactivated vaccines have been used to the present time in emergency situations where shelf life has not been an issue. Requirements and procedures for routine commercial use have not been developed.

f) Precautions (hazards)

Attenuated vaccines should be used in the cooler months when adult *Culicoides* vector populations are at a minimum. They should not be used in ewes during the first half of pregnancy and in rams 2 months before the breeding season.

5. Tests on the final product

a) Safety

See C.4.b.

b) Potency

See C.4.c.

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NB: There are OIE Reference Laboratories for Bluetongue (see Table in Part 3 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list: www.oie.int).