Use of the polymerase chain reaction in differentiating rinderpest field virus and vaccine virus in the same animals

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Summary: In 1991, a disease with clinical signs indicative of rinderpest was reported in yaks in the former Soviet Union, near the border with Mongolia. At the peak of the epizootic, mortality among affected yaks was 32-42% in adults and 65% in animals less than one year old. Pathological samples were examined independently at two institutes in Russia. Both institutes confirmed the presence of rinderpest using complement fixation, agar gel diffusion and immunoassays. Since vaccination had been initiated to control an outbreak of a similar disease several months earlier, the later cases were possibly due to the vaccine and field rinderpest may not have been present. However, the disease had occurred in non-vaccinated animals and these were then vaccinated against the disease. Tissue samples obtained from these animals, which were examined at the Pirbright Laboratory using gel diffusion assays and specific nucleic acid probes, were found to be positive for rinderpest antigen and nucleic acid. Ribonucleic acid derived from the post-mortem tissue samples was amplified using the polymerase chain reaction and rinderpest-specific primers. Sequence analysis of the amplified deoxyribonucleic acid from the samples revealed the presence of two distinct virus strains, one identical to the Plowright rinderpest tissue culture vaccine and the other related to field strains of rinderpest virus circulating in Asia and the Middle East.

KEYWORDS: Polymerase chain reaction – Rinderpest – Russia – Yak.

INTRODUCTION

Rinderpest remains one of the most feared of animal diseases, with some strains of rinderpest virus causing almost 100% mortality in susceptible cattle and wildlife populations. However, in spite of major international attempts to control and eradicate the disease, rinderpest persists in many developing countries and periodically makes damaging excursions into areas considered to be free. Historically, outbreaks of rinderpest have been associated with civil unrest and little has changed in this regard in the late twentieth century. Rinderpest continues to kill cattle in the Middle East and thrives in the disordered communities left in the aftermath of war. In 1992 rinderpest spread, within a few weeks, from the Turkish border with Iraq to the vicinity of Istanbul and threatened to enter Europe. In 1987 the disease spread to Sri Lanka from India, flourished during the military conflict and has still not been eradicated.

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In July 1991, a herd of Russian cattle on summer grazing in Mongolia showed signs of a rinderpest-like disease which affected 20% of the herd, of which 64% died. The herd had been vaccinated against rinderpest three months previously, and nearby Mongolian cattle which had not been vaccinated against rinderpest remained healthy. The disease was diagnosed by two different laboratories as rinderpest and mucosal disease, respectively (12, 13). In December 1991, 389 yaks from a herd of 412 grazing on the Russian side of the Mongolian border were similarly affected with a disease diagnosed as rinderpest, and 265 of these died (11). Some of these yaks and others nearby had been vaccinated with rinderpest vaccine following the outbreak, and it was reported that the disease predominantly affected those animals which had not been vaccinated. In March 1992, an acute infectious disease of unknown aetiology was reported in Mongolian yaks (14). Tissue samples from the Russian animals were received for analysis by the World Reference Laboratory for Foot and Mouth Disease, in Pirbright (United Kingdom), in February 1992.

Molecular techniques have greatly improved the diagnosis of specific pathogens in diseased animals without the need to isolate the infectious agent. This is a great advantage where the agent concerned is difficult to isolate in culture, as is the case for rinderpest virus. Nucleic acid probes have also proved invaluable in discriminating infections caused by antigenically-related viruses, such as rinderpest virus and peste des petits ruminants (PPR) virus (4, 15). Direct sequence analysis of virion ribonucleic acid (RNA) has been used for several years to compare clinical isolates of foot and mouth disease virus. However, this is not possible in cases where the infecting virus does not grow to high titres in tissue culture to allow isolation of sufficient nucleic acid for sequence analysis. In such cases a more sensitive technique, the polymerase chain reaction (PCR), can be used to amplify the virus nucleic acid which can then be used in sequence analysis. This technique has recently been used to analyse strains of rinderpest virus currently circulating in East Africa and Asia (2). The recent cases of mortality in yaks in the former USSR, attributed to rinderpest by the initial Russian investigators, were examined using rinderpest-specific PCR primers and the sequence of the amplified deoxyribonucleic acid (DNA) products determined. This paper describes the analyses carried out on these samples and discusses the relevance of the techniques used to the study of the epidemiology of rinderpest.

**MATERIALS AND METHODS**

Refrigerated tissue samples from diseased yaks from the Tuva region of Russia were sent to Pirbright for diagnosis. The samples included lung, spleen, mediastinal and mesenteric lymph nodes, and abomasum wall with mucous membrane.

**Agar gel immunodiffusion**

Tissue samples were homogenised (10% w/v) in phosphate-buffered saline and analysed in a standard test using hyperimmune rabbit serum and control antigen derived from the spleen of an experimentally-infected animal (7).

**RNA extraction**

RNA was extracted directly from tissue samples, or from control rinderpest- or PPR-infected Vero cell monolayers, using the method described by Chomczynski and Sacchi (3). The final pellet was dissolved in distilled water and the concentration was adjusted to 1 mg/ml, using an optical density reading at 260 nm of 25 for pure RNA.
Nucleic acid hybridisation

Samples of each tissue RNA (5 µg) were mixed with an equal volume of 20X SSC (300 mM NaCl, 30 mM Na₃citrate; pH7), boiled for 5 min and loaded in duplicate on nylon membranes. Hybridisation was then carried out on identical samples using labelled complement DNA (cDNA) probes specific for either rinderpest or PPR as described previously (4).

Polymerase chain reaction

Reverse transcription and polymerase chain reaction amplification were carried out on the RNA as described by Doherty and colleagues (5) and modified by Haas and colleagues (8). Briefly, 5 µg RNA in 5 µl water was first heated to 70°C for 10 min and cooled to room temperature over a 30 min period before the other reactants were added in a final volume of 20 µl. The reverse transcription reaction was carried out at 37°C for 1 h. The reaction was then made up to 100 µl with the following:

- 200 µM of each deoxynucleotide triphosphate
- 10 mM tris-HCl, pH 8.8
- 50 mM KCl
- 1.5 mM MgCl₂
- 0.5% gelatin
- 100 pM each of the upstream and downstream primer.

Taq polymerase (2.5 units) was then added and the reaction overlaid with mineral oil. The thermal cycler was programmed as follows:

- Step 1: 5 min at 95°C
- Step 2: 1 min at 94°C
- Step 3: 1 min at 50°C
- Step 4: 2 min at 70°C.

Steps 2, 3 and 4 were repeated for 30 cycles. A final cycle was included, where the 70°C extension step was extended to 5 min.

The PCR reaction products (5 µl) were first checked for the correct sized amplified DNA on 2% agarose-TBE (0.09 M tris-borate, 0.002 M ethylene diamine tetra-acetic acid; pH 8.0) gels, extracted once with chloroform to remove the mineral oil overlay and then precipitated with 2.5 volumes of ethanol. The products were then purified by electrophoresis on a 0.8% low melting point (LMP) agarose gel.

Cloning and sequencing of DNA

The purified DNA was dissolved in 20 µl distilled water and 2 µl ligated into pT7 vector as recommended by the suppliers. The ligated plasmids were transformed into Escherichia coli cells (strain DH5α) and DNA prepared from at least six white recombinant colonies by the alkaline lysis technique. The plasmid DNA inserts were first checked for the correct size by digestion with EcoRI and XbaI followed by electrophoresis on a 1.0% agarose-TBE gel; those which contained the correct sized DNA insert were sequenced using the dideoxy chain termination method and the M13 universal forward and reverse primers (10). The derived DNA sequences were compared using the DNADIST and KITSCH programmes and the resulting dendrogram plotted using the PLOTGRAM programme of the University of California GCG sequence analysis package (6).
RESULTS

Agar gel diffusion

Samples of spleen and lymph node were positive using agar gel immunodiffusion analysis.

RNA/DNA hybridisation

Total RNA extracted from the tissue samples was hybridised together with positive and negative control samples with a rinderpest-specific cDNA clone (D-74) and a PPR-specific cDNA clone (B2-6). Tissues of spleen and mesenteric lymph node were positive for rinderpest-specific nucleic acid sequences. The sample from the mediastinal lymph node was found to be negative. All samples were negative with the PPR probe.

Polymerase chain reaction

Total RNA extracts from samples of spleen, lung and mesenteric lymph node were copied to DNA using reverse transcriptase and two rinderpest fusion gene-specific primers, each 25 bases long and made to opposite senses of the rinderpest genome RNA. The first primer (5'-GGG ACA GTG CTT CAG CCT ATT AAG G-3') was in the messenger RNA sense and located at nucleotides 814 to 838 on the fusion protein gene. The second primer (5'-CAG CCC TAG CTT CTG ACC CAC GAT A-3') was in the genome sense and located at position 1161 to 1185 on the same gene. The total reverse transcriptase reaction was then amplified using Taq polymerase and the same primer set. The fragments were then cloned and sequenced as described above. When the sequences of the PCR DNA products from the spleen were analysed, it was clear that two unrelated sequences were present (Russia 1 and Russia 2). One sequence (Russia 1) was identical to the RBOK (Rinderpest bovine old Kabete) vaccine strain which the authors believe to be identical to the K37/70 strain used to vaccinate animals at risk during the outbreak. The other (Russia 2) was a totally unrelated sequence which most closely resembled the sequence of rinderpest viruses recently isolated in the Middle East and Asia (2). Computer analysis using the PHYLIP programmes is shown in Figure 1.

DISCUSSION

Rinderpest is often difficult to distinguish clinically from other viral or bacterial infections in bovines. Many isolates of the virus are mild and only cause 40-50% mortality in affected cattle herds while other more virulent isolates can cause 90-100% mortality in susceptible herds. The rinderpest strains currently circulating in East Africa are of the mild type, with mortality of approximately 40% in affected herds (17). Mild strains of rinderpest are clinically very similar to mucosal disease and laboratory confirmation of infection is essential if the causative agent is to be unequivocally identified. However, if animals have been recently vaccinated against rinderpest, the vaccine virus may be re-isolated and the disease wrongly attributed to rinderpest virus. Also, clinically non-relevant viruses which can grow easily in tissue culture can prevent morbillivirus isolation (8).

The tests conducted in Russia and at the Pirbright Laboratory confirmed that both antigen and nucleic acid of rinderpest virus were present in the samples. However, since some of the animals had been vaccinated against rinderpest, the positive results could
Comparisons were performed by assigning an equivalent statistical weight to each base substitution, and a phylogenetic tree was constructed by the Fitch-Margolash and least-squares methods (6) have been due to vaccine virus. The results of the PCR analyses performed at Pirbright clearly showed that two rinderpest virus sequences were present in the samples (the vaccine and an Asian type field strain), confirming the initial diagnosis of rinderpest in the yak population.

PCR has proved to be a valuable tool in epidemiological investigations where it can distinguish different strains and lineages of viruses. It has been widely used in human medicine to analyse variants of human immunodeficiency virus circulating in the population (9) and has been used to show that vaccines and not field virus have been responsible for recent mumps outbreaks (18). PCR has also been used successfully to show that the newly described morbilliviruses in marine mammals are different to the viruses in terrestrial mammals and that two distinct viruses are circulating in pinnipeds (seals) and cetaceans (whales and dolphins) (1). In the case of rinderpest, it has been shown, by using this technique, that the viruses isolated since 1980 fall into two distinct lineages, African and Asian. Recent Kenyan and Sudanese rinderpest virus isolates are closely related to a virus previously found in Egypt and this may represent a mild strain of the disease now circulating in East and North-East Africa. It is also evident that the strains of rinderpest circulating in countries of the Middle East did not have their origin
in Africa but probably in Asia (2). This molecular evidence supports the suggestion based on comparisons of clinical evaluations of virulence by Taylor (16) that these strains originated from India. The non-vaccine Russian sequence is clearly of Asian type and this would be consistent with a source of virus in Central Asia, even though rinderpest has not been reported from the latter region for many years. The use of these molecular techniques will greatly help our understanding of the epidemiology of rinderpest in the future. The sequence of a new rinderpest virus isolate can be compared with those already in the database and this will allow a more precise back-tracing of the source of virus in previously disease-free areas or indicate the emergence of new virus biotypes in enzootic areas.

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LA TECHNIQUE D'AMPLIFICATION EN CHAÎNE PAR POLYMÉRASE APPLIQUÉE À LA DISTINCTION ENTRE LES SOUCHES SAUVAGES ET LES SOUCHES VACCINALES DU VIRUS DE LA PESTE BOVINE CHEZ LES MÊMES ANIMAUX. – T. Barrett, C. Amarel-Doel, R.P. Kitching et A. Gusev.

Résumé : En 1991, une maladie présentant tous les symptômes de la peste bovine a été observée chez des yacks dans l'ancienne Union soviétique, près de la frontière avec la Mongolie. Au plus fort de l'épidémie, la mortalité des yacks était de 32-42 % pour les adultes et de 65 % pour les jeunes de moins d'un an. Des lésions histologiques ont été examinées séparément dans deux instituts russes. Tous deux ont confirmé la présence du virus de la peste bovine à l'aide des méthodes suivantes : épreuve de la fixation du complément, immuno-diffusion en gélose et autres techniques immunologiques. Comme une campagne de vaccination avait été lancée pour lutter contre une maladie similaire apparue quelques mois plus tôt, les derniers cas découverts étaient peut-être dus à une réplication de la souche vaccinale et non au virus sauvage de la peste bovine. Toutefois, la maladie s'est également déclarée chez des sujets non immunisés, qui ont alors été vaccinés. Les prélèvements de tissus effectués sur ces animaux et examinés au laboratoire de Pirbright à l'aide des techniques de diffusion en gélose et de sondes nucléiques spécifiques, ont réagi positivement vis-à-vis de l'antigène de la peste bovine et de l'acide nucléique correspondant. L'acide ribonucléique obtenu à partir des prélèvements de tissus a été amplifié grâce à la technique d'amplification en chaîne par polymérase et à des amorces spécifiques de la peste bovine. L'analyse séquentielle de l'acide désoxyribonucléique amplifié à partir des prélèvements a révélé la présence de deux souches virales distinctes : l'une identique au vaccin de la peste bovine en culture cellulaire de Plowright, l'autre appartenant aux souches sauvages du virus, que l'on rencontre en Asie et au Moyen-Orient.


Resumen: En 1991, una enfermedad que presentaba los síntomas de la peste bovina fue observada en yaques en la ex Unión Soviética, cerca de la frontera con Mongolia. En el estadio de culminación de la epizootia, la mortalidad de los yaques alcanzó a entre 32% y 42% de los adultos y al 65% de los animales jóvenes menores de un año. Se examinaron muestras de animales enfermos en dos institutos rusos, por separado; uno y otro confirmaron la presencia del virus de la peste bovina mediante la prueba de la fijación del complemento, la inmunodifusión en gelosa y otras técnicas inmunológicas. Como se había lanzado una campaña de vacunación para luchar contra una enfermedad similar declarada unos meses antes, se supo que los últimos casos observados podían haber sido causados por una replicación de la cepa vacunal y no por el virus salvaje de la peste bovina. Sin embargo, la enfermedad se declaró asimismo en animales no inmunizados, a los que se vacunó en ese momento. Las muestras de tejidos recogidas de estos animales y examinadas en el laboratorio de Pirbright mediante las técnicas de difusión en gelosa y mediante sondas nucleicas específicas reaccionaron positivamente ante el antígeno de la peste bovina y del ácido nucleico correspondiente. El ácido ribonucleico obtenido a partir de estas muestras fue amplificado según la técnica de amplificación en cadena por polimerasa y con cebadores (primers) específicos de la peste bovina. El análisis secuencial del ácido desoxirribonucleico amplificado a partir de estas muestras reveló la presencia de dos cepas virales distintas: una, idéntica a la vacuna de la peste bovina en cultivo celular de Plowright; la otra, perteneciente a las cepas salvajes del virus tal como se pueden encontrar en Asia y en Medio Oriente.


REFERENCES


