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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

Target	Nature of the PCR	References
VP3 gene	RT-PCR	18, 27, 28, 40, 44
VP5 gene	RT-PCR	8
VP6 gene	RT-PCR	29
VP7 gene	RT-PCR	51, 54
NS1 gene	RT-PCR	43, 49
NS1 gene	Multiplex RT-PCR Nested-RT-PCR Multiplex and nested PCR	7 6 57
NS3 gene	RT-PCR Duplex RT-PCR	3, 42 9

The range in detection sensitivity for different RT-PCR assays is between 10 and 100 $TCID_{50}$. 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

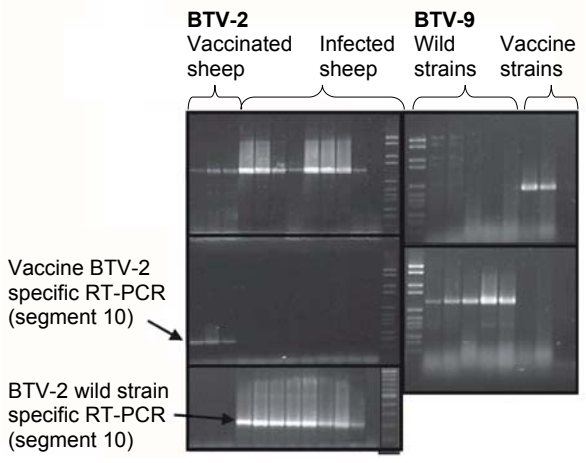


Figure 1
Molecular differentiation between vaccine and wild bluetongue virus serotype 2 (BTV-2) and BTV-9 strains

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.

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