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

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Summary

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

Keywords

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

Introduction

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

BTV is a small (80 nm in diameter) icosahedral virus with a ten-segmented, double-stranded RNA genome (19). Each of the ten segments codes for at least one of ten distinct viral proteins, seven of which are structural components of the virus particle, and three of which are non-structural (10). The virus particle is arranged as three concentric capsid shells surrounding the viral dsRNA (4, 9, 10). The outermost layer (the outer capsid) is composed of two structural proteins, VP2 and VP5, which are principally involved in virus attachment and penetration of the host cell during the initiation of infection. These are the most variable of the viral proteins and the specificity of their interactions with neutralising antibodies (particularly those of VP2) determines virus serotype (8, 10, 24). The two innermost protein shells that make up the transcriptionally active virus core, are composed of VP3(T2) and VP7(T13), respectively. These are more highly conserved proteins, showing serological cross-reactions within the BTV virus species. VP3 and VP7 surround a central compartment containing the three highly-conserved minor protein components of the transcriptase complex (polymerase, capping enzyme and helicase: VP1(Pol), VP4(CaP) and VP6(Hel), respectively), as well as the ten dsRNA segments of the virus genome (9, 19, 20, 28). Two of the non-structural proteins, NS1and NS2, are also highly conserved, while the smallest viral protein NS3 is more variable.

Historically, BTV has occurred almost worldwide between the latitudes of 35°S and 40°N, although within southern Europe it has only caused periodic and short-lived epizootics involving a single BTV serotype (16, 17, 18). However, since 1998, this range has extended gradually further north (as far as 44°N) into areas of Mediterranean Europe and the Balkans. These outbreaks have also involved five distinct BTV serotypes: 1, 2, 4, 9 and 16, providing evidence of a significant and possibly long-term change in the distribution of both the virus and disease. These changes may result from a combination of several factors, including the ability of the virus to persist from one vector season to the next (overwintering) (27), 'climate change' (which appears to have altered the distribution of the main vector species in the region: *Culicoides imicola*) (30, 31, 32), and a possible involvement of novel vector species that have a more northerly distribution (*C. pulicaris* and *C. obsoletus*) (32).

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

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

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

Methods

Preparation of viral dsRNA

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

Oligonucleotide primers

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

Sample preparation for reverse transcriptase-polymerase chain reaction

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

Nested polymerase chain reaction

Full length cDNA was synthesised with primers TF and R-1, using RT-PCR beads (Amersham Pharmacia) according to the protocol of the manufacturer. The RNA was initially reverse-transcribed at 42°C for 1 h, and then heated to 95°C for 5 min. Thirty PCR cycles were performed, at 94°C for 1 min, 45°C for 1 min and 72°C for 2 min,

Table I
Viruses tested using reverse transcriptase-polymerase chain reaction
Full details of the virus isolates can be seen at the Institute for Animal Health (IAH) website: iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm

Virus serotype	IAH-Pirbright Identification No.	Virus serotype	IAH-Pirbright Identification No.
BTV-1	RSArrrr/01	BTV-8	RSArrrr/08
BTV-1	GRE2001/01	BTV-8	NIG1982/07
BTV-1	GRE2001/02	BTV-8	KEN????/01
BTV-1	IND1992/01	BTV-9	RSArrrr/09
BTV-1	IND1992/02	BTV-9	RSAvvv1/09
BTV-1	IND1998/01	BTV-9	RSAvvv2/09
BTV-1	IND1999/01	BTV-9	BUL1999/01
BTV-1	IND2001/01	BTV-9	GRE2000/02
BTV-1	MAY1987/01	BTV-9	BOS2002/01
BTV-1	SUD1987/01	BTV-9	BOS2002/02
BTV-1	NIG1982/01	BTV-9	BOS2002/03
BTV-1	CAR1982/01	BTV-9	BOS2002/04
BTV-1	AUS1981/01	BTV-9	KOS2001/01
BTV-2	RSArrrr/02	BTV-9	KOS2001/02
BTV-2	ZIM2003/01	BTV-9	KOS2001/03
BTV-2	IND1982/01	BTV-9	KOS2001/04
BTV-2	NIG1982/02	BTV-9	KOS2001/05
BTV-2	FRA2001/01	BTV-9	SER2001/01
BTV-2	SUD1985/01	BTV-9	TUR2000/03
BTV-2	TUN2000/01	BTV-9	TUR2000/04
BTV-2	SAD2001/01	BTV-9	TUR2000/05
BTV-2	SAD2001/02	BTV-10	RSArrrr/10
BTV-2	SAD2001/03	BTV-11	RSArrrr/11
BTV-2	SAD2002/01	BTV-11	NIG1982/08
BTV-2	SAD2001/04	BTV-11	ZIM2003/02
BTV-2	SAD2002/02	BTV-11	ZIM2003/03
BTV-2	SAD2001/05	BTV-12	RSArrrr/12
BTV-2	SAD2002/03	BTV-12	NIG1982/09
BTV-2	SAD2001/06	BTV-12	KEN????/??
BTV-2	SAD2001/07	BTV-13	RSArrrr/13
BTV-3	RSArrrr/03	BTV-14	RSArrrr/14
BTV-3	ZIM2002/01	BTV-14	CAR1982/04
BTV-3	ZIM2002/02	BTV-15	RSArrrr/15
BTV-3	NIG1982/06	BTV-15	ZIM2003/08
BTV-4	RSArrrr/04	BTV-15	ZIM2003/09
BTV-4	TURvvvv/04	BTV-16	RSArrrr/16
BTV-4	GRE1999/01	BTV-16	TUR2000/01
BTV-4	RSAvvv3/04	BTV-16	TUR2000/02
BTV-4	RSAvvv5/04	BTV-16	TUR2000/10
BTV-4	RSAvvv7/07	BTV-16	NIG1982/10
BTV-4	SUD1983/01	BTV-17	RSArrrr/17
BTV-4	ARG2002/01	BTV-18	RSArrrr/18
BTV-4	ARG2002/02	BTV-19	RSArrrr/19
BTV-4	ARG2002/03	BTV-20	RSArrrr/20
BTV-4	ARG2002/04	BTV-20	AUS1978/01
BTV-5	RSArrrr/05	BTV-21	RSArrrr/21
BTV-5	NIG1982/03	BTV-22	RSArrrr/22
BTV-5	NIG1982/04	BTV-22	NIG1982/11
BTV-5	CAR1982/02	BTV-23	RSArrrr/23
BTV-5	NIG1982/05	BTV-23	IND1997/01
BTV-6	RSArrrr/06	BTV-24	RSArrrr/24
BTV-7	RSArrrr/07		

followed by a final extension step at 72°C for 10 min. The second (nested) PCR step was then carried out using puReTaq PCR beads (also from Amersham Pharmacia) with primers IF and R-1, in accordance with the Amersham protocol. The same thermal profile was used (described above).

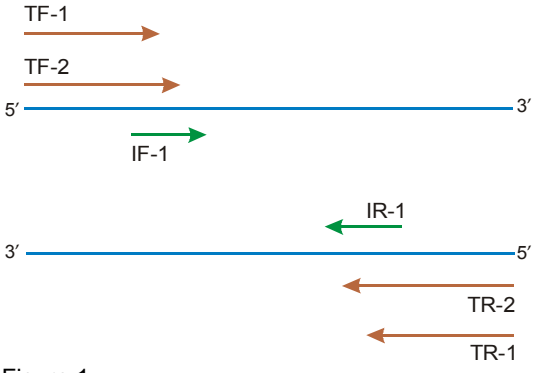

Figure 1
S7 specific primers

Primer	Sequence	Size of amplicon	Ref.
TF-1	GTTAAAAATCTATAGAG	1156bp	29
TR-1	GTAAGTGTAATCTAAGAG	1156bp	29
TF-2	GTTAAAAATCTATAGAGATG	1156bp	7
TR-2	GTAAGTGTAATCTAAGAGA	1156bp	7
IF-1	ACAACTGATGCTGCGAATGA	769bp	29
IR-1	AACCCACACCCGTGCTAAGTGG	769bp	29

Figure 2
S7 primer details

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

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

For the conventional one-step RT-PCR, the RNA was initially reverse-transcribed at 45°C for 30 min. This was followed by an activation step at 94°C for 15 min, to simultaneously activate the DNA polymerases and inactivate the reverse transcriptases.

Forty amplification cycles were then performed at 94°C for 1 min, 45°C for 1 min and 72°C for 2 min. A terminal extension step at 72°C for 10 min completed the thermal protocol.

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

Analysis of polymerase chain reaction products

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

Results

Nested reverse transcriptase-polymerase chain reaction

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

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

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

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

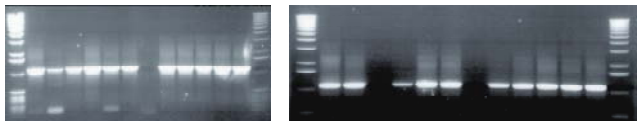

Figure 3
Agarose gel of all 24 reference strains of bluetongue virus
Serotypes 7, 15 and 19 have failed to amplify

Discussion

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

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

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

Segment 7 was chosen as the target gene for PCR because it codes for the major BTV species-specific antigen and is highly conserved. It was therefore reasoned that differences between BTV topotypes (separation of BT virus isolates based on geographic origin) were likely to be relatively low and might

allow detection of all strains of the virus using a single set of primers. However, this was not shown to be possible and variation in segment 7 appears to have prevented the detection of isolates of serotypes 7, 15 and 19 by these relatively simple RT-PCR methods. Analyses of segment 7 from these viruses will be used to determine the nature of sequence variations, compared to other BTV strains. This should permit the design of primers that can detect all 24 serotypes, possibly by introducing a multiplex-PCR approach for BTV-7, BTV-15 and BTV-19. These sequencing studies are in progress.

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

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

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