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

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Summary

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

Keywords

Bluetongue virus – Field strains – *Orbivirus* – Reverse transcriptase-polymerase chain reaction – Segment 2 – Type-specific primers – Vaccine strains – Viral protein 2 – Virus typing.

Introduction

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

The BTV outer capsid is composed of two major structural proteins, VP2 and VP5, which are encoded by genome segments 2 and 6, respectively (12, 13). VP2 is the major neutralisation antigen and consequently plays a major role in the determination of BTV serotype, although VP5 can also play a minor role (11, 13). VP2 is the most variable and VP5 is the second most variable of the BTV proteins, which is reflected in the sequences of

genome segment 2 and to a lesser extent of VP5 (3, 10, 11). The absence of sequence data for segment 2 of many BTV serotypes, has previously made it impossible to design primers for comprehensive reverse transcriptase-polymerase chain reaction (RT-PCR)-based serotyping assays. However, PCR-based assays have been developed for differentiating a limited number of BTV types in Australia and United States (4, 5, 8, 20). These methods were designed for rapid BTV type identification by detection of unique regions of genome segment 2, although the specificity of amplification and detection was not fully validated with multiple isolates all 24 BTV serotypes. Similar RT-PCR assays have also been developed for the identification of African horse sickness virus (AHSV) serotypes (17).

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

Materials and methods

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

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

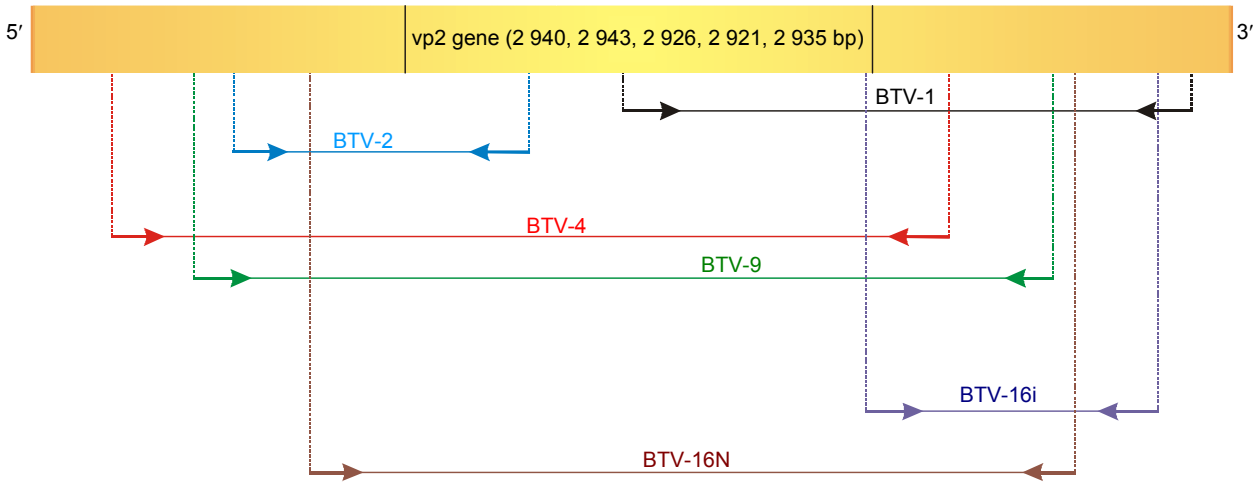
Total RNA was isolated from BTV-infected BHK-21 monolayers using the Trizol™ technique. dsRNA was separated from contaminating single-stranded (ss) RNA by precipitation in 2M lithium chloride (1). Special chemically modified primers (S. Rao, manuscript in preparation) were ligated to the 3′ end of both strands of the RNA; cDNA was synthesised at 37°C for 40 min and then at 42°C for 10 min using a 'reverse transcription system' from Promega without addition of further primers (S. Rao, manuscript in preparation). For PCR amplification, 0.5 -2 µl of full-length cDNA, was prepared as described above and 1 µl of each gene-specific primer (10 pmol/ml) mixed with the following reagents and added: 1 µl 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$), 0.2 µl dNTPs (10 mM each), 2.5 U (0.5 µl) Taq polymerase (Roche, UK). The final volume was made up to 10 µl with nuclease-free water. PCR amplification was performed using a denaturation step of 94°C for 2 min, followed by 30 cycles of melting at 95°C for 15 sec, annealing at 50°C for 30 sec and extension at 72°C for 3 min. The reaction was terminated by a final extension step of 72°C for 5 min. Adjustments to the annealing temperature and extension were made depending on the primers Tm and the size of the amplification product. After amplification, 5 ml of sample was added to 1 ml of 5× loading buffer and analysed by agarose gel electrophoresis in the presence of ethidium bromide.

Reverse transcriptase-polymerase chain reaction using gene specific primers

The protocol used here for each serotype is an adaptation of previously published methods for the transcription and amplification of RNA templates (5, 20). Genome segment-specific primers were chosen for their intratypic conservation and heterotypic variation, by comparing sequences already obtained for the various serotypes (6, 7). These primers were then used in RT-PCR reactions (Fig. 1). For each RT reaction, a denaturation-primer cocktail mix, containing 1 µl of RNA template, 10 mM methyl mercury hydroxide (MMOH) and 1 µM of each primer, was incubated at room temperature for 10 min, after which 1 µl of 350 mM 2-mercaptoethanol was added to each tube to neutralise the MMOH. To this, 10 µl of a reverse transcription mix (50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.5 units/µl RNAse inhibitor, 1 mM dNTPs, 200 units of Moloney murine reverse transcriptase (Promega, UK) was added, giving a final volume of 20 µl. The reactions were incubated at 37°C for 60 min. The PCR reaction steps were performed using the same protocol described above.

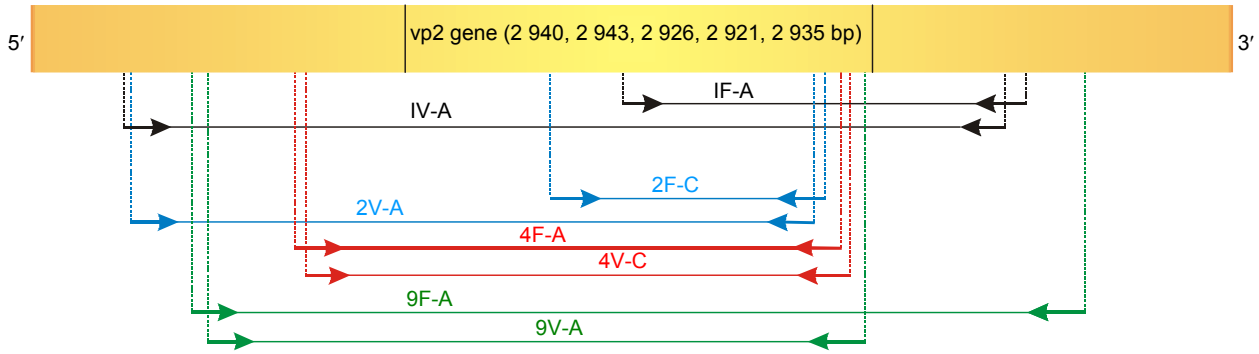
Alternative (one-step) reverse transcriptase-polymerase chain reaction procedure

An alternative one step RT-PCR technique was also used on RNA extracted from virus-infected cell culture cells. This procedure is based on a kit purchased from Amersham Pharmacia Biotech Inc, which consists of ready-to-use 'beads' that include all of the reagents required for the reverse transcription and PCR amplification steps of the reaction. The primer-template mix was heated to 95°C for 3 min, or denatured with methyl mercury hydroxide. This mix was then added to the RT-PCR beads, which



BTV-1 BTV-1/2/p524F & BTV-1/2/p968R=1 334 bp
BTV-4 BTV-4/2/p67F & BTV-4/2/p841R=2 324 bp
BTV-16i BTV-16/2/p670F & BTV-16/2/p924R=762 bp (–Nigerian strain)
BTV-2 BTV-2/2/p198F & BTV-2/2/p416R=653 bp
BTV-9 BTV-9/2/p177F & BTV-9/2/p852R=2 023 bp
BTV-16N BTV-16/2/p273F & BTV-16/2/p792R=1 557 bp (+ Nigerian strain)

Figure 1a
Schematic map showing specific primer positions and expected product sizes with different sets of primers



BTV-1 BTV-1F/2/p543F & BTV-1F/2/p778R=707 bp
BTV-1V/2/p28F & BTV-1V/2/p733R=2 115 bp
BTV-2 BTV-2F/2/p444F & BTV-2F/2/p633R=565 bp
BTV-2V/2/p30F & BTV-2V/2/p632R=1 806 bp
BTV-4 BTV-4F/2/p234F & BTV-4F/2/p645R=1 233 bp
BTV-4V/2/p233F & BTV-4V/2/p650R=1 249 bp
BTV-9 BTV-9F/2/p113F & BTV-9F/2/p890R=2 330 bp
BTV-9V/2/p132F & BTV-9V/2/p713R=1 743 bp

Figure 1b
Schematic map showing primer positions specific for field and vaccine strains and expected product sizes with different sets of primer pairs

had been reconstituted with nuclease-free water, making a final reaction volume of 50 ml. The samples were subjected to reverse transcription at 37°C for 60 min followed by PCR amplification using the following thermal profile: initial denaturation at 95°C for 5 min; 94°C for 30 sec; annealing at 50°C for 30 sec and extension at 72°C for 1 min per kb of fragment to be amplified, for 30 cycles. There was a final, single extension step of 72°C for 10 min.

Results

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

Development of serotype-specific primers

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

Table I
Primers used for serotype specific RT-PCR assays and for differentiation of field and vaccine strains of bluetongue virus serotypes 1, 2, 4, 9 and 16

Primer name (AA)	Primer sequence (5′ to 3′)	Position on genome segment 2 (NT)	Predicted product size (base pair)	Primer pair grading in PCR
Primers for serotype specific amplification of genome segment 2 of European strains of BTV				
BTV-1/2/p524F	TAAGTGGATAATMGCWCCGAT	1572	1 334	+++
BTV-1/2/p968R	TYATACGTTGAGAAGTTTTGT	2906		
BTV-2/2/p198F	TATCAGTTAATAGTGCATT	595	653	+++
BTV-2/2/p416R	TACTAAAKATATACTTCTCCGT	1248		
BTV-4/2/p67F	GCTTAACTATAAACCAACGAGG	200	2 324	+++
BTV-4/2/p841R	AACTTGGACGTC ACAACAGG	2524R		
BTV-9/2/p177F	TCTRATAAATAATGGGTTATAT	533	2 023	+++
BTV-9/2/p852R	CCACATARTGAYGAATGATAGAT	2556		
BTV-16/2/p670F	GAACGTTGTATGATGTCTCG	2010	762	+++
BTV-16/2/p924R	GCCGAAGGTGCGATCTGGCCG	2772		
BTV-16/2/p273F	GGAGGRAACTTCGARCGAA	819	1 557	+++
BTV-16/2/p792R	TCACCAAYCCRAAWCGATT	2376		
Primers for amplification of BTV-1 field strains				
BTV-1F/2/p543F	GCACAACGACGGAATGACG	1629	707 (1F-A)	+++
BTV-1F/2/p778R	GATGACCTTAAGTTCGTGCG	2336		
Primers for amplification of BTV-1 vaccine strains				
BTV-1V/2/p28F	TCACAATAGATGTTGGAACT	84	2 115 (1V-A)	+++
BTV-1V/2/p733R	TTGTACTACGGTAGCGCCACTT	2199		
Primers for amplification of BTV-2 field strains				
BTV-2F/2/p444F	CTAGAGAAAGGCAATCCTTGTGACC	1334	565 (2F-C)	+++
BTV-2F/2/p633R	CAGGATGTATCGTCCAGTC	1898		
Primers for amplification of BTV-2 vaccine strains				
BTV-2V/2/p30F	TAATACTGGTGTAAAATAT	92	1 806 (2V-O)	+++
BTV-2V/2/p632R	TCCAGAATGTATCGCCCGGTT	1898		
Primers for amplification of BTV-4 field strains				
BTV-4F/2/p234F	CTATCATATAACAGATTCATC	701	1 233 (4F-O)	+++
BTV-4F/2/p645R	CGTTTGATGTTCTAAGCCTTC	1934		
Primers for amplification of BTV-4 vaccine strains				
BTV-4V/2/p233F	CTTATCATACAATAGATWTATT	701	1 249 (4V-C)	+++
BTV-4V/2/p650R	CATTTGGTGTTCTAAACCCTCA	1950		
Primers for amplification of BTV-9 field strains				
BTV-9F/2/p113F	GCTATCTGACGCTATGGATG	340	2 330 (9F-O)	+++
BTV-9F/2/p890R	GCCTTGCTGATCCTATGTTG	2670		
Primers for amplification of BTV-9 vaccine strains				
BTV-9V/2/p132F	GCGCGGTCAAGTGTGACTTG	397	1 743 (9V-A)	+++
BTV-9V/2/p713R	GGAATGTGTCAAGTCTATCAG	2140		

NT nucleotide
AA amino acid
PCR polymerase chain reaction
+++ efficient primer pair, which could amplify all available isolates of the homologous type

Table II
Details of virus isolates used for genome segment 2 based RT-PCR assays of bluetongue virus serotypes 1, 2, 4, 9 and 16

Serotype and country of origin	Species	IAH dsRNA virus Reference Collection No.	Accession No.
BTV-1 S. African vaccine strain (OVI)	Not known	RSAvvvv/01	AJ585110
BTV-1 S. African reference strain (OVI)	Not known	RSArrrr/01	AJ585122
BTV-1 India (Rajasthan)	Ovine	IND1992/01	AJ585111
BTV-1 India	Not known	IND1992/02	AJ585112
BTV-1 India	Not known	IND1988/01	AJ585113
BTV-1 India (Haryana)	Ovine	IND1999/01	AJ585114
BTV-1 India (Chennai)	Ovine	IND2001/01	AJ585115
BTV-1 Malaysia (Kuala Lumpur)	Not known	MAY1987/01	AJ585116
BTV-1 Sudan	Not known	SUD1987/01	AJ585117
BTV-1 Nigeria	Not known	NIG1982/01	AJ585118
BTV-1 Cameroon	Ovine	CAR1982/01	AJ585119
BTV-1 Australia	Not known	AUS??/01	AJ585120
BTV-1 Greece	Ovine	GRE2001/01	AJ585121
BTV-2 S. African reference strain (OVI)	Not known	RSArrrr/02	AJ585123
BTV-2 India	Not known	IND1982/01	AJ585152
BTV-2 Nigeria	Not known	NIG1982/02	AJ585153
BTV-2 Corsica	Ovine	FRA2001/03	AJ585154
BTV-2 Sudan	Not known	SUD1985/01	AJ585155
BTV-2 Tunisia	Not known	TUN2000/01	AJ585156
BTV-2 S. African vaccine strain (OVI)	Not known	RSAvvvv/02	AJ585157
BTV-2 Sardinia	Ovine	SAD2001/01	AJ585161
BTV-2 Sardinia	Ovine	SAD2001/02	AJ585162
BTV-2 Sicily	*C. pulicaris*	ITL2002/05	AJ585158
BTV-2 Sicily	*C. pulicaris*	ITL2002/06	AJ585159
BTV-2 Sicily	Ovine	ITL2002/07	AJ585160
BTV-4 S. African reference strain (OVI)	Not known	RSArrrr/04	AJ585125
BTV-4 S. African Vaccine strain (OVI)	Not known	RSAvvvv/04	AJ585163
BTV-4 Turkey vaccine strain	Not known	TURvvvv/04	AJ585164
BTV-4 Turkey	Not known	TUR1978/01	AJ585165
BTV-4 Sudan	Not known	SUD1983/01	AJ585166
BTV-4 Greece	Not known	GRE2000/01	AJ585167
BTV-4 Argentina	Bovines	ARG2002/01	AJ585169
BTV-4 Cyprus	Bovine	CYP1969/01	AJ585180
BTV-9 S. African reference strain (OVI)	Not known	RSArrrr/09	AJ585130
BTV-9 Bulgaria	Not known	BUL1999/01	AJ585170
BTV-9 Greece	Not known	GRE2000/02	AJ585171
BTV-9 Serbia	Ovine	SER2001/01	AJ585172
BTV-9 S. African vaccine strain (OVI)	Not known	RSAvvv1/09	AJ585173
BTV-9 Bosnia	Ovine	BOS2002/02	AJ585174
BTV-9 Turkey	Not known	TUR2000/03	AJ585175
BTV-9 Turkey	Not known	TUR2000/04	AJ585176
BTV-9 Turkey	Not known	TUR2000/05	AJ585177
BTV-16 S. African reference strain (OVI)	Not known	RSArrrr/16	AJ585137
BTV-16 S. African vaccine strain (OVI)	Not known	RSAvvvv/16	AJ585149
BTV-16 Nigeria	Not known	NIG1982/10	AJ585150
BTV-16 Turkey	Not known	TUR2000/01	AJ585146
BTV-16 Turkey	Not known	TUR2000/02	AJ585147
BTV-16 Turkey	Not known	TUR2000/10	AJ585148

IAH Institute for Animal Health, Pirbright
OVI Onderstepoort Veterinary Institute

obtained from the website (iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/viruses-at-iah.htm.)

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

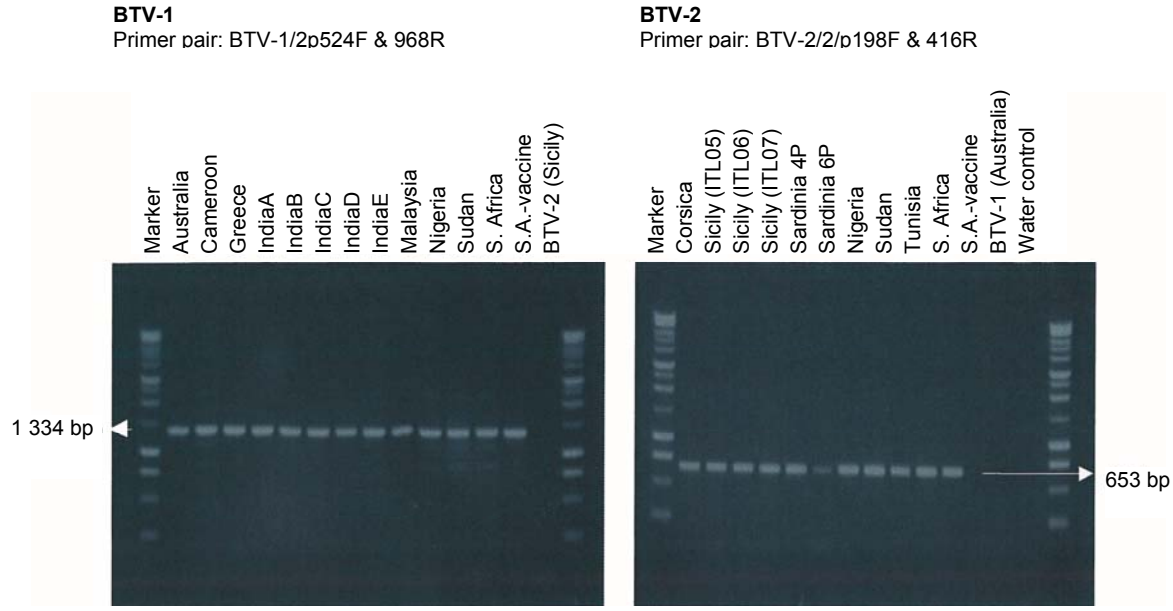


Figure 2
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 1 and 2 using serotype-specific primer pairs, namely BTV-1/2/p524F & BTV-1/2/p968R (1 334 bp) and BTV-2/2/p198F & BTV-1/2/p416R (653 bp)
The new type-specific PCR primers for BTV serotypes 1 and 2 were validated with various isolates from different geographic regions as shown above

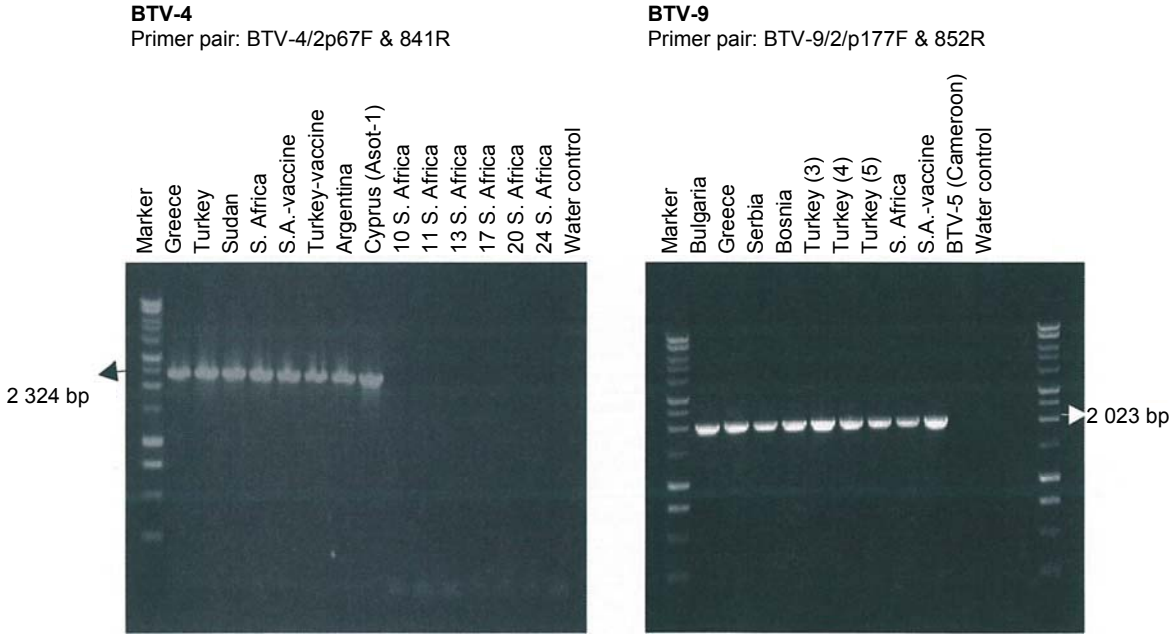


Figure 3
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 4 and 9 using serotype-specific primer pairs, namely BTV-4/2/p67F & BTV-4/2/p841R (2 324 bp) and BTV-9/2/p177F & BTV-9/2/p852R (2 023 bp)
The new type-specific PCR primers for BTV serotypes 4 and 9 were validated with various isolates from different geographic regions as shown above

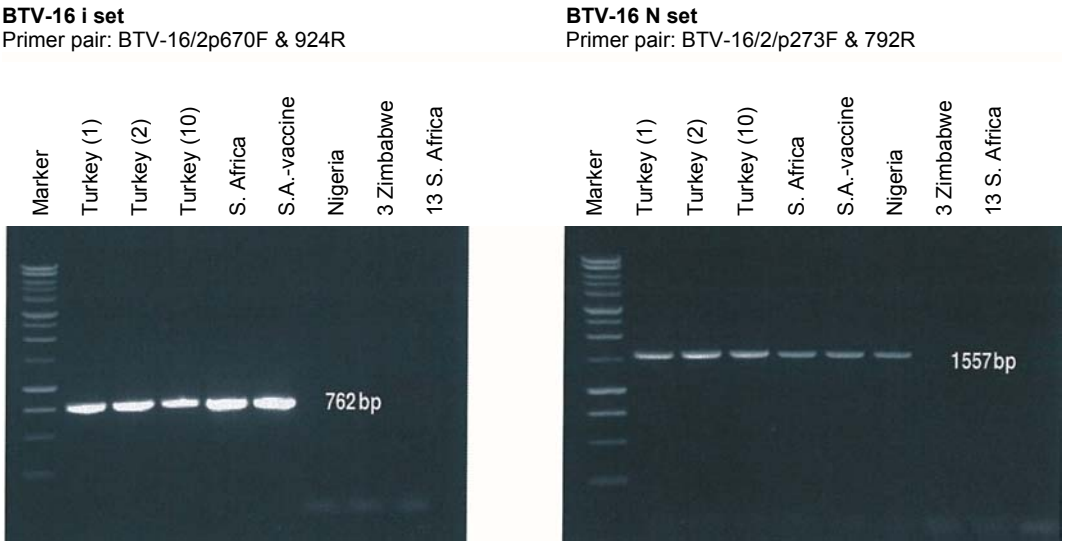


Figure 4
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotype 16 using serotype-specific primer pairs, namely BTV-16/2/p670F & BTV-16/2/p924R (16i pair=762 bp) and BTV-16/2/p273F & BTV-16/2/p792R (16 N pair=1 557 bp)
The new type-specific PCR primers for BTV serotype 16 were validated with various isolates from different geographic regions as shown above
BTV-16i set cannot amplify BTV-16 Nigerian strain while BTV-16 N set can also amplify Nigerian strain of BTV-16

Differentiation of European field and vaccine strains

The aligned VP2 gene sequences were individually compared for each of the five European serotypes, in order to identify specific regions showing variability in sequences between vaccine and field strains. A schematic map (Fig. 1b) shows the positions of primers that were subsequently designed and the product sizes expected with each primer pair. These primers were then used in RT-PCR assays and amplicons of the expected size were obtained (Figs 5 and 6).

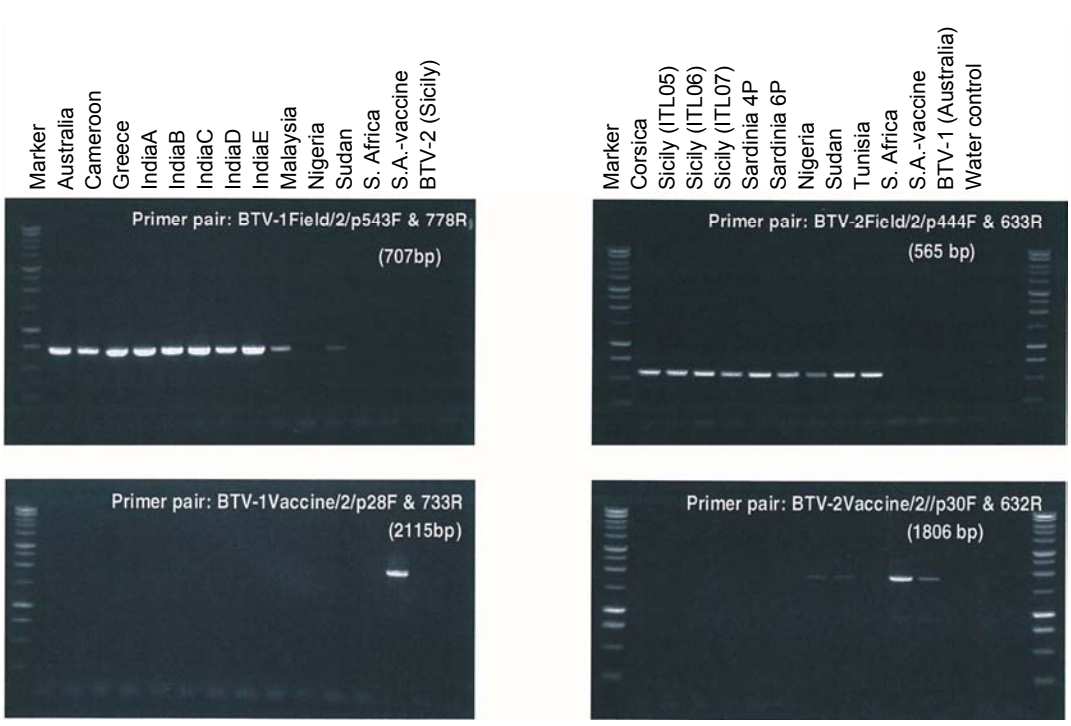


Figure 5
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 1 and 2 using primer pairs specific to field and vaccine strains
The new PCR primers specific to field and vaccine strains for each BTV were validated with various isolates from different geographic regions as shown above

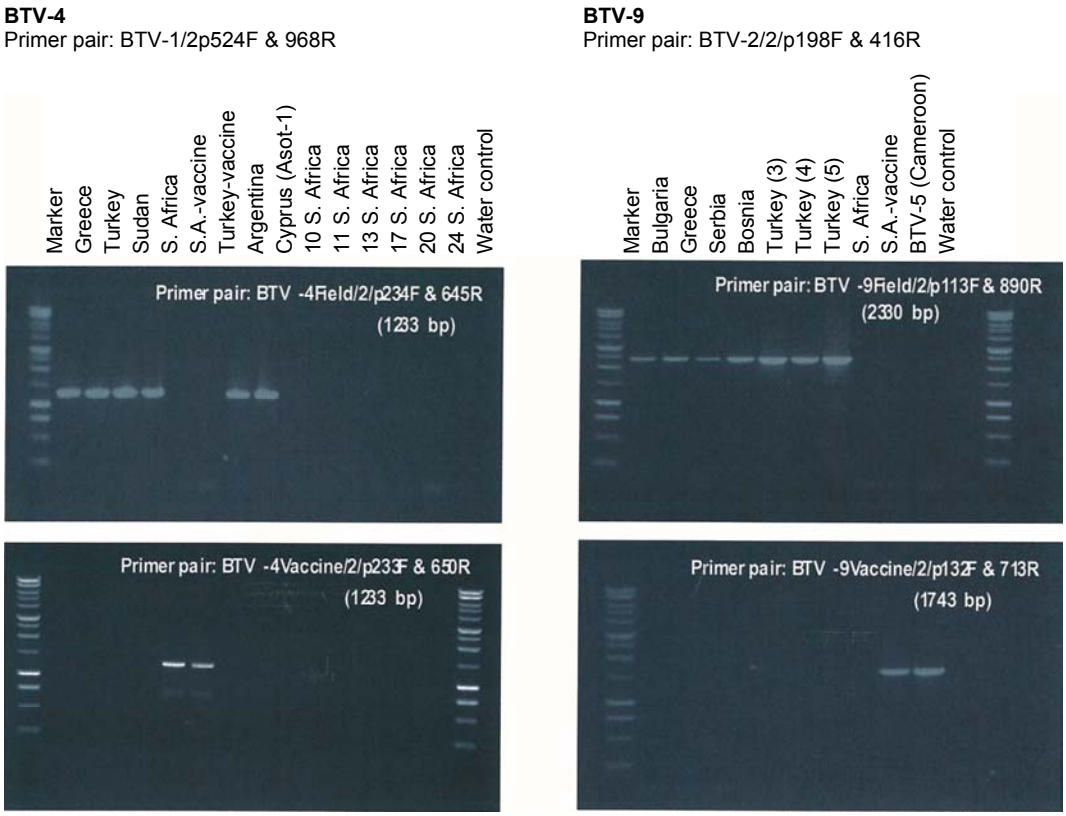


Figure 6
Agarose gel analysis of cDNA products generated from dsRNA genome segment 2 of isolates of bluetongue virus serotypes 4 and 9 using primers specific to field and vaccine strains for each BTV
The new PCR serotype were validated with various isolates from different geographic regions as shown above

The primers designed to amplify field strains of BTV-1 (field primers, namely BTV-1F/2/p543F and 778R) were specific for non-African isolates. The primers designed to amplify vaccine strains of BTV-1 (vaccine primers, namely: BTV-1V/2/p28F and 733R) were specific to the vaccine strain (Fig. 5). BTV-2 (field primers, namely BTV-2F/2/p444F and 633R) were specific to the field isolates tested but the vaccine primers (BTV-2V/2/p30F and 632R) weakly amplified some non-vaccine field strains from Africa (Fig. 5). However, this would not prevent their use to distinguish field and vaccine strains from current European outbreaks.

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

Discussion

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

The ability to rapidly discriminate between viruses which occur as natural outbreaks and those that may be attributable to the vaccine strain, will help to maintain a considered and flexible approach to disease control. The high quality sequence data now

available from our recent studies have supported the design of effective diagnostic primers and protocols for the five European BTV serotypes. It has also been possible to design primers with the potential to distinguish vaccine from field strains of four of the European serotypes (1, 2, 4 and 9), based on topotype variation in genome segment 2, combined with the South African origin of BTV vaccine strains. However, the European strains of BTV-2 originated in sub-Saharan Africa and insufficient nucleotide differences were detected in segment 2 to allow the design of primers that would reliably distinguish vaccine and some of the African field strains. This not only suggests that primers to other regions of the virus genome may be needed to distinguish all vaccine and field strains of BTV-2 (18) but also that other genome segments are likely to be involved in virus attenuation. The close relationship of segment 2 from the field and vaccine strains of BTV-16 also made it difficult to distinguish them by these techniques.

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

References

1. Attoui H., Billoir F., Cantaloube J.F., Biagini P., deMicco P. & de Lamballerie X. (2000). – Strategies for the sequence determination of viral ds RNA genomes. *J. Virol. Methods*, **89**, 147-158.
2. Davies F.G., Mungai J.N. & Pini A. (1992). – A new bluetongue virus serotype isolated in Kenya. *Vet. Microbiol.*, **31**, 25-32.
3. Gould A.R. & Eaton B.T. (1990). – The amino acid sequence of the outer coat protein VP2 of neutralising monoclonal antibody-resistant, virulent and attenuated bluetongue viruses. *Virus Res.*, **17**, 161-172.
4. Gould A.R. & Pritchard L.I. (1990). – Relationships amongst bluetongue viruses revealed by comparisons of capsid and outer coat protein nucleotide sequences. *Virus Res.*, **17**, 31-52.
5. Johnson D.J., Wilson, W. C. & Paul P.S. (2000). – Validation of a reverse transcriptase multiplex PCR test for the serotype determination of US isolates of bluetongue virus. *Vet. Microbiol.*, **76**, 105-115.
6. Maan S., Maan N.S, Samuel A.R., O'Hara R., Meyer A.J, Rao S. & Mertens P.P.C. (2004). – Completion of the sequence analysis and comparisons of genome segment 2 (encoding outer capsid protein VP2) from representative isolates of the 24 bluetongue virus serotypes. *In* Bluetongue, Part II (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (4), 484-488.
7. Maan S., Samuel A.R., Maan N.S., Attoui H., Rao S. & Mertens P.P.C. (2004). – Molecular epidemiology of bluetongue viruses from disease outbreaks in the Mediterranean basin. *In* Bluetongue, Part II (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (4), 489-496.
8. McColl K.A. & Gould A.R. (1991). – Detection and characterisation of bluetongue virus using the polymerase chain reaction. *Virus Res.*, **21**, 19-34.
9. Mellor P.S. & Wittmann E.J. (2002). – Bluetongue virus in the Mediterranean Basin, 1998-2001. *Vet. J.*, **164** (1), 20-37.
10. Mertens P.P.C. (1999). – Orbiviruses and coltiviruses: general features. *In* Encyclopaedia of virology, Vol. 2, 2nd Ed. (R.G. Webster & A. Granoff, eds). Academic Press, London, 1043-1061.
11. Mertens P.P.C., Burroughs J.N. & Anderson J. (1987). – Purification and properties of virus particles, infectious subviral particles, and cores of bluetongue virus serotypes 1 and 4. *Virology*, **157**, 375-386.
12. Mertens P.P.C., Pedley S., Cowley J. & Burroughs J.N. (1987). – A comparison of six different bluetongue virus isolates by cross-hybridisation of the dsRNA genome segments. *Virology*, **161**, 438-447.
13. Mertens P.P.C., Pedley S., Cowley J., Burroughs J.N., Corteyn A.H., Jeggo M.H., Jennings A.M. & Gorman B.M. (1989). – Analysis of the roles of bluetongue virus outer capsid proteins VP2 and VP5 in determination of virus serotype. *Virology*, **170**, 561-565.
14. Mertens P.P.C., Arella M., Attoui H., Belloncik S., Bergoin M., Boccardo G., Booth T.F., Chiu W., Diprose J.M., Duncan R., Estes M.K., Gorziglia M., Gouet P., Gould A.R., Grimes J.M., Hewat E., Hill C., Holmes I.H., Hoshino Y., Joklik W.K., Knowles N., López Ferber M.L., Malby R., Marzachi C., McCrae M.A., Milne R.G., Nibert M., Nunn M., Omura T., Prasad B.V.V., Pritchard I., Samal S.K., Schoehn G., Shikata E., Stoltz D.B., Stuart D.I., Suzuki N., Upadhyaya N., Uyeda I., Waterhouse P., Williams C.F., Winton J.R. & Zhou H.Z. (2000). – Reoviridae. *In* Virus taxonomy. Seventh Report of the International Committee for the Taxonomy of Viruses (M.H.V. Van Regenmortel, C.M. Fauquet, D.H.L. Bishop, C.H. Calisher, E.B. Carsten, M.K. Estes, S.M. Lemon, J. Maniloff, M.A. Mayo, D.J. McGeoch, C.R. Pringle &

R.B. Wickner, eds). Academic Press, London, 395-480.

15. Mertens P.P.C. & Mellor P.S. (2003). – Bluetongue. *State Vet. J.*, **13**, 18-25.
16. Office International des Épizooties (OIE) (2000). – Bluetongue. *In* Manual of standards for diagnostic tests and vaccines. OIE, Paris 109-118.
17. Sailleau C., Hamblin C., Paweska J.T. & Zientara S. (2000). – Identification and differentiation of the nine African horse sickness virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. *J. Gen. Virol.*, **81** (3), 831-837.
18. Singh K.P., Maan S., Samuel A.R., Rao S., Meyer A.J. & Mertens P.P.C. (2004). – Phylogenetic analysis of bluetongue virus genome segment 6 (encoding VP5) from different serotypes. *In* Bluetongue, Part II (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International Symposium, Taormina, 26-29 October 2003. *Vet. Ital.*, **40** (4), 479-483.
19. Tabachnick W.J. (1996). – *Culicoides variipennis* and bluetongue-virus epidemiology in the United States. *Ann. Rev. Entomol.*, **41**, 23-43.
20. Wilson W. C. & Chase C.C.L. (1993). – Nested and multiplex polymerase chain reactions for the identification of bluetongue virus infection in the biting midge, *Culicoides variipennis*. *J. Virol. Methods*, **45**, 39-47.