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Molecular differentiation of field and vaccine strains of bluetongue virus serotype 2 using the real-time polymerase chain reaction and fluorescene resonance energy transfer hybridisation probes

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Summary

As a consequence of the recent outbreaks of bluetongue (BT) disease amongst sheep in the Mediterranean Basin, and following the subsequent vaccination campaign to control further spread of the disease and its long-term maintenance, it has become most important to develop rapid and sensitive methods that can reliably differentiate between field and vaccine strains of the causative virus. The authors describe a new method to differentiate bluetongue virus serotype 2 (BTV-2) field and vaccine strains, using the VP2 gene sequence differences between the South African vaccine and the Italian field wild-type strains. The method is based on the principle that the melting temperature of a DNA duplex gives information on the sequence, which enables the identification of even single-base alterations in the amplicon. The real-time polymerase chain reaction the generation of melting curves and fluorescence detection were all performed using the light cycler system (Roche). Primers and probes were designed using VP2 gene sequences. After RT-PCR, the melting curves analysis, derived by the fluorescence resonance energy transfer (FRET) real-time PCR, was performed using the light cycler data analysis program (Roche). To assess the diagnostic value of the method, a BTV-2 vaccine strain (Onderstepoort Biological Products, South Africa) was first compared against a field strain of BTV-2 (isolated during an outbreak in 2000 in Sardinia). The ability of the method to reliably identify all the BTV-2 strains was tested using an array of eleven BTV-2 field strains isolated during outbreaks in various Italian regions between 2000 and 2002 and other serotypes (BTV-1, BTV-4, BTV-9 and BTV-16) that had been isolated during recent outbreaks of BT in the Mediterranean Basin. The method was clearly able to differentiate BTV-2 strains of vaccine virus from all wild-type strains of the same serotype tested. The resultant melting curves distinctly reveal the two strains to have differing peak values of 47.8°C ± 0.6°C and 60.5°C ± 0.6°C, respectively.

Keywords

Bluetongue – *Culicoides* – Energy transfer – Fluorescence resonance – Molecular differentiation – Polymerase chain reaction.

Introduction

Bluetongue (BT) virus (BTV) is the prototype species of the genus *Orbivirus*, family *Reoviridae*. BTV is the causative agent of BT disease in sheep, an infectious and non-contagious disease transmitted by haematophagous insects of the genus *Culicoides*, family Ceratopogonidae. The distribution of the disease extends between the 35°S and 50°N parallels. Twenty-four immunologically distinct serotypes of BTV are known, of which only a few have been reported to occur in various parts of the Mediterranean Basin (BTV-1, BTV-2, BTV-4, BTV-9 and BTV-16). In Italy, following the first

occurrence of the disease in Sardinia in 2000, three BTV serotypes have been isolated to date: BTV-2, BTV-9 and BTV-16. There is evidence that two additional serotypes (BTV-1 and BTV-4) are also circulating. The disease is clinically apparent in domesticated breeds of sheep, of which the European breeds seem to be particularly susceptible. The most common symptoms include fever, catarrhal stomatitis, rhinitis, enteritis and lameness. The mortality rate can vary from 0% to 30%, but can reach 75% (4) in highly susceptible animals but is dependent upon the serotype involved. The real significance of BT lies in the indirect losses sustained; these include abortions in pregnant ewes and severe loss of condition during prolonged convalescence (9). Laboratory diagnosis includes the detection of antibodies using the agar gel immunodiffusion (AGID) test or enzyme-linked immunosorbent assay (ELISA), virus isolation and RT-PCR methods for the detection of BT virus or nucleic acid in clinical samples and virus neutralisation (VN) test for virus identification (6). Ultimately, the control of the disease is still best achieved through prophylactic vaccination, and not through the housing of sheep in insect-proof buildings (which is not applicable in the Mediterranean region due to transhumance and roaming shepherding), nor through the elimination of the insect vector (2). As a consequence of the vaccination campaign which was implemented to limit further spread and long-term maintenance of BT in Italy, it became important to develop rapid and sensitive methods that can reliably differentiate between field and vaccine strains of the causative virus. Although direct sequencing is the gold standard for characterising known gene regions and for mutation detection, it remains impractical for routine purposes. A previously untried PCR real-time method was used here for the differentiation of BTV-2 field and vaccine strains, based on the use of FRET (fluorescence resonance energy transfer) probes using segment 2 of the BTV genome.

Materials and methods

Recently developed instruments which couple the PCR method to fluorescent hybridisation probes now allow target amplification and analysis without sample handling. The method used here is based on the principle that the melting temperature of a DNA duplex gives information on the sequence and identifies even single-base alterations in the amplicon (3). The RT-PCR, the generation of melting curves, and fluorescence detection, were all performed using the Light Cycler System (Roche Mannheim, Germany). The primers (OG174: 5′-GCTGCTTCCGACACTTACAAT 3′; OG175: 5′-ACTAAATATATACTTCTCCGTTTCCCG-3′) were designed using VP2 gene sequences available on the Internet, to flank a region of about 192 base pairs (bp) where appropriate sequence differences were identified by alignment using the ClustalW program. The FRET method was created using separate 3′ and 5′ labelled probes (OG178-fluoresceina; OG177-BODIPY 630/640, red) which hybridised adjacent to the unlabelled complementary PCR strand (Fig. 1). The primers and probes were designed using the Oligo 6.0 program (MedProbe, Oslo, Norway). After RT-PCR, the reaction is cooled automatically and the subsequent slow heating (0.1°C-0.2°C per second) from 40°C to 95°C, as an addendum to PCR, causes denaturation of the amplicon/probe heteroduplex (melting curve), and so allows for the identification of the sequence differences. The melting temperature (Tm) is measured at the point at which 50% of the probe is denatured away from the amplicon causing a change in fluorescence. For the real-time RT-PCR, the master hybridisation probes reaction mix (Roche Mannheim, Germany), RNA template, primers and probes were used in a final volume of 20 µl. The analysis of the melting curves derived by the FRET real-time PCR, was performed using the light cycler data analysis program (Roche).

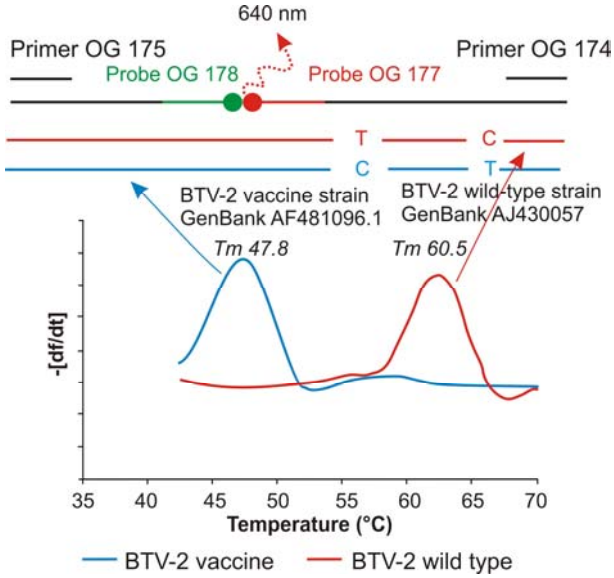

Figure 1
Schematic representation of the primers and fluorescence resonance energy transfer hybridisation probe positions
a) Mismatch positions between the BTV-2 wild-type and vaccine strains
b) Melting curves of the wild-type and vaccine strain BTV-2

Bluetongue virus strains used

To assess the diagnostic value of the method, a vaccine strain of BTV-2 (Onderstepoort Biological

Products [OBP], South Africa) was first compared against a field strain of BTV-2 (BT28340) isolated in Sardinia during one of the first outbreaks of BT in 2000. The resultant melting Tms, visualised by taking the negative first derivative (–dF/dT) of the melting curve, distinctly revealed the two strains to have differing Tm values of 47.8°C ± 0.6°C and 60.5°C ± 0.6°C, respectively. The ability of the method to reliably identify all the BTV-2 strains was tested using an array of eleven BTV-2 strains, isolated during outbreaks in various Italian regions between 2000 and 2002. The sensitivity of the method was calculated using BTV-2 of known titre and a lower limit of detection (LOD) of 10^2 copies of RNA was determined. The specificity was tested against other serotypes (i.e. one strain of BTV-1, one strain of BTV-4, five strains of BTV-9 and two strains of BTV-16) that had also been isolated during the recent outbreaks of BT in the Mediterranean Basin. The strains used to validate the method were assigned to the specific serotype and geographic origin, using the VN test and gene sequencing of segments 2 and 10 of the BTV genome. The results of segment 2 sequencing are published elsewhere in these proceedings (8). The segment 10 analysis was performed in collaboration with U. Balasuriya and N.J. MacLachlan (University of California, Davis) following published methods (1, 7). The results are shown in Figure 2. To investigate how the method could be used in field conditions, 47 additional viral strains isolated from sheep, goats and bovines during outbreaks of BT, were also tested. Twenty-five blood samples from sheep collected in ethylene-diaminetetra-acetic acid (EDTA) were also processed. Ten blood samples were taken from

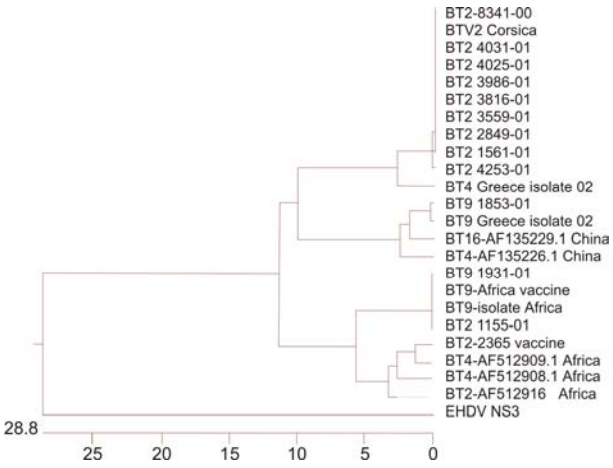

Figure 2
Nucleotide sequence phylogeny of bluetongue virus genome segment 10, from various field strains isolated in Italy, and compared with strains from Greece, Corsica, Africa and People's Republic of China; the latter three from GenBank

sheep a month after vaccination, while fifteen samples were from sheep suspected of being infected with BT.

RNA extraction

All samples were treated for RNA extraction using Trizol™ reagent (Life Technologies Inc, Gaithersburg) following the instructions of the manufacturer. All RNAs were re-suspended in 30 µl of nuclease-free H_2O and kept at –80°C until use.

Fluorescence resonance energy transfer real-time polymerase chain reaction

The real-time PCR reaction was performed using the light cycler system in a final volume of 20 µl, at 2.5 mM MgCl, 5 pmoles/primer and 10 pmoles of each probe and 2 µl of RNA. The real-time PCR reaction was performed as follows:

a) synthesis: 55°C for 10 min
   followed by:
b) heating cycle: 95°C for 30 sec (1 cycle)
c) PCR cycles: 95°C for 0 sec, 51°C for 10 sec, 72°C for 8 sec (35 cycles)
d) melting cycle: from 40°C to 95°C with a ramping increase of 0.1°C/sec. The results were read in the F2 channel (640 nm) using the 'compensation colour' mode.

Results

The FRET real-time PCR, as evaluated here, had an LOD of about 100 copies of the RNA target (Fig. 3). Of the eleven BTV-2 strains examined, nine had the wild-type profile with a Tm of 60.5°C and two had the vaccine profile with a Tm of 47.8°C. The difference in Tm of 12.7°C allows for easy differentiation of the two profiles. No cross-reaction was detected with the strains of BTV-1, BTV-4, BTV-9 and BTV-16, indicating the specificity of the method to be 100% (Table I and Fig. 4). The forty-seven BTV field isolates resulted in eleven BTV-2 wild-type, one BTV-2 vaccine type, thirty non-reactive (identified as BTV-9 by VN test), whilst five showed a mixed profile with the simultaneous presence of Tm 47.8°C and 60.5°C. This suggests the presence of both wild-type and vaccine strains in the same samples (Table II). To verify the applicability of the method, twenty-five blood samples taken in the field were also examined. Fifteen samples were from sheep suspected of being infected with BT, and ten were from sheep vaccinated with the BTV-2 monovalent vaccine (OBP). Ten samples from the suspected sheep had a Tm equal to 60.5°C, four had a Tm of 47.8°C, while one presented both profiles, indicating the

circulation of both the wild-type and the vaccine strain in the specific outbreak. The ten vaccinated sheep presented only the Tm 47.8°C profile (Table III).

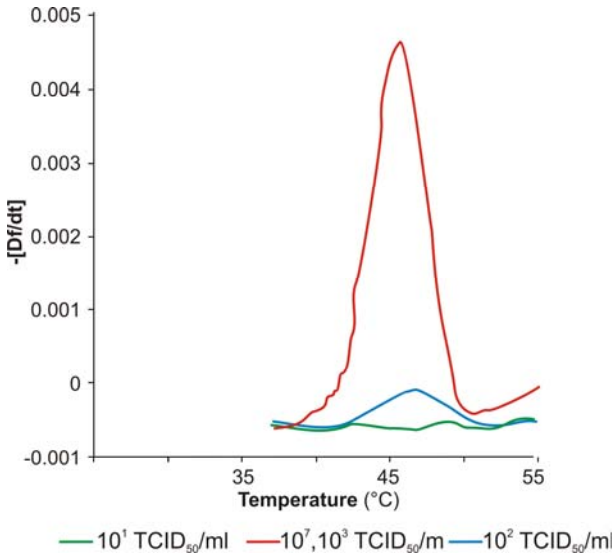

Figure 3
Melting curve obtained using extracted RNA from a bluetongue virus serotype 2 vaccine strain with a starting titre of 10^7 (df/dt = negative first derivative)

Table I
Twenty bluetongue virus strains processed using fluorescence resonance energy transfer real-time polymerase chain reaction compared to resuts of the virus neutralisation tests

Virus neutralisation	FRET real-time PCR for BTV-2 wild-type/vaccine strains		
BTV serotype	No.	Tm 60.5°C wild-type	Tm 47.8°C vaccine
BTV-1	1	0	0
BTV-2	11	9	2
BTV-4	1	0	0
BTV-19	5	0	0
BTV-16	2	0	0

BTV bluetongue virus
FRET fluorescence resonance energy transfer
PCR polymerase chain reaction
Tm temperature

Conclusions

In all cases, the FRET real-time PCR was able to identify the BTV-2 serotype as previously demonstrated by VN test, and further confirmed by sequencing of the VP2 gene. This FRET real-time PCR system was also able to differentiate (within the same serotype) between the BTV-2 wild-type and vaccine strains.

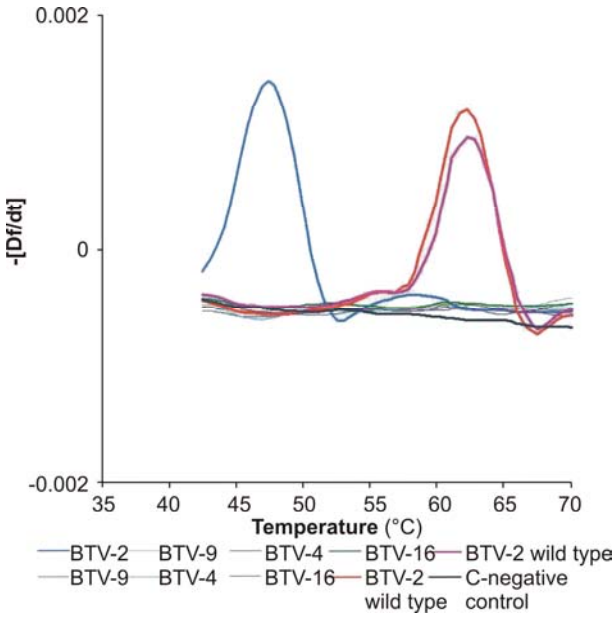

Figure 4
Melting curves obtained for the various bluetongue virus serotypes using the resonance energy transfer real-time polymerase chain reaction
Results show the melting curves of BTV-2 vaccine (Tm = 47.8°C) and BTV-2 wild-type (Tm = 60.5°C)
No reaction is apparent using BTV-1, BTV-4, BTV-9 and BTV-16 strains

Table II
Results of the fluorescence resonance energy transfer real-time polymerase chain reaction, applied to 47 field isolates of bluetongue virus

Virus neutralisation	FRET real-time PCR for BTV-2 wild-type/vaccine strains			
BTV serotype	No.	Tm 60.5°C wild-type	Tm 47.8°C vaccine	Tm wild-type and vaccine
BTV-2	17	11	1	5
BTV-9	30	0	0	0

BTV bluetongue virus
FRET fluorescence resonance energy transfer
PCR polymerase chain reaction
Tm temperature

The NS3 gene sequence analyses, conducted on the same samples, showed that all the BTV-2 strains isolated from Italian outbreaks, and carrying the wild-type profile as revealed by FRET real-time PCR, cluster together, but separately from African strains of the same serotype. One strain that had the BTV-2 vaccine profile falls into the African cluster, although it was more closely related to the various BTV-9 strains than to the BTV-2 strains. This could indicate gene reassortment in the field, as has been recently suggested (5), and is a possibility that needs to be investigated further.

In a few instances this method was also useful to reveal the presence of wild-type and of vaccine virus in the BTV isolates and clinical samples. This would not be possible using serological techniques or the VN test.

Table III
Results of the fluorescence resonance energy transfer real-time polymerase chain reaction applied to 25 blood samples from vaccinated and infected sheep

Blood sample		FRET real-time PCR for BTV-2 wild-type/vaccine strains		
Sheep	No.	Tm 60.5°C wild-type	Tm 47.8°C vaccine	Tm wild-type and vaccine
Suspected infection	15	10	4	1
Vaccinated	10	0	10	0

BTV bluetongue virus
FRET fluorescence resonance energy transfer
PCR polymerase chain reaction
Tm temperature

An added value of the FRET real-time PCR is the possibility of establishing automated high-throughput testing procedures and thus reducing time-consuming post-PCR steps.

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