

Protocol

Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay

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Abstract

Bluetongue virus (BTV) is the causative agent of Bluetongue (BT) disease in ruminant livestock and occurs almost worldwide between latitudes 35°S and 50°N; 24 serotypes of BTV are known of which 8 circulate periodically within parts of the Mediterranean Region. A fast (about 3.5 h) and versatile diagnostic procedure able to detect and quantify BTV-RNA, has been developed using a Molecular Beacon (MB) fluorescent probe; PCR primers were designed to target 91 bp within the NS3 conserved region of the viral RNA segment 10 (S10) and bracketed the MB fluorescence probe hybridisation site. The MB fluorescent probe was used to develop two Bluetongue serogroup-specific assays: a quantitative real time reverse transcriptase polymerase chain reaction (RT-PCR) and a traditional RT-PCR. These were tested using BTV-RNAs extracted from the blood and organs of BT-affected animals, and from virus isolate suspensions. The samples included ten serotypes (BTV-1–BTV-9 and BTV-16); of these, BTV serotypes -1, -2, -4, -9 and -16 have since 1998 been involved in the extensive outbreaks of BT across the Mediterranean Region. To evaluate the specificity and sensitivity of the MB probe, all positive samples (and negative controls) were tested using the developed quantitative real time RT-PCR and traditional RT-PCR assays. The former test had a detection limit of 10³ cDNA molecules per reaction with a log-linear quantification range of up to 10¹¹ ($R^2 = 0.98$), while the latter test was able to detect 500 cDNA-BTV molecules/PCR. The results show that the MB fluorescent probe is both rapid and versatile for the laboratory diagnosis of Bluetongue and for quantifying levels of *viraemia* in BTV-affected animals. An “*in silico*” comparison of the primers and MB fluorescent probe used in this study showed that it is possible to detect all 24 serotypes of BTV.

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1. Type of research

Bluetongue (BT) is an insect-borne disease of ruminants caused by an arbovirus and transmitted by haematophagous midges of the genus *Culicoides*, family *Ceratopogonidae* (Du Toit, 1944; Price and Hardy, 1954; Bowne and Jones, 1966; MacLachlan et al., 1994). The BT virus (BTV) is the prototype species of the genus *Orbivirus*, family *Reoviridae* and occurs

almost globally between latitudes 35°S and 50°N. In infected animals the disease is characterised by various clinical forms, with symptoms ranging from acute to subacute, mild or inapparent. At least 24 immunologically distinct serotypes of the virus are known (Gorman, 1990; Davies et al., 1992; Mertens and Diprose, 2004); the serotypes present in the Mediterranean basin are BTV-1, BTV-2, BTV-3, BTV-4, BTV-6, BTV-9, BTV-10 and BTV-16 (Mellor and Wittmann, 2002). Of these BTV-2, BTV-4, BTV-9 and BTV-16 have most recently been isolated in Italy after the disease first appeared in Sardinia in the year 2000.

The BT viral genome comprises 10 double-stranded (ds) RNA segments varying in size from 0.5 to 2.7 kDa and which

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encode for seven structural proteins (VP1–VP7) and four non-structural proteins (NS1–NS3 and NS3A) (Roy, 1989; Roy et al., 1990). The non-structural proteins NS3 and NS3A are encoded by genes inside the segment S10 (822 nucleotides) via two in-frame initiation codons. NS3 corresponds to a 229—aminoacid protein with initiation codon nucleotides at the 20–22 nucleotide position; NS3A is a 216—aminoacid protein in which synthesis is initiated by an in-frame downstream AUG codon located at the 59–61 nucleotide position (Wade-Evans, 1990; Roy, 1992). These proteins facilitate the egress of virus particles from the infected cells (Han and Harty, 2004). The phylogenetic study of S10 and of its constituent protein sequence, has enabled the identification of three different genetic groups within BTV and which represent monophyletic geographical clusters irrespective of serotype (Bonneau et al., 1999). Nucleotide identities between the S10 gene sequences range from 81.8 to 100% with intra-isolate variation being only 7% (Pierce et al., 1998). This indicates that S10 could be of value for developing refined molecular diagnostic methods for BT; this was confirmed when the aminoacid sequences of the serotypes BTV-1, BTV-2, BTV-10, BTV-11, BTV-13 and BTV-17 were compared revealing a high degree of sequence similarity and conservation (Hwang et al., 1992; Pierce et al., 1998). The genetic variability of the NS3/NS3A sequence in field strains is likely the consequence of genetic drift with the co-circulation of related genetic variants in vector insects (Bonneau et al., 2001); the apparent conservation of NS3/NS3A amongst different BTV serotypes and strains likely reflects functional constraints on the protein tolerating little variation.

The laboratory diagnosis of BT and the identification of the BTV serotype involved, is today done using various antibody detection methods including antigen-capture, agar-gel immunodiffusion (AGID), enzyme-linked immunoadsorbent assay (ELISA) (Singer et al., 1998; Hawkes et al., 2000; Zhou et al., 2001) and virus neutralisation (VN) for which serotype-specific BTV hyperimmune sera are employed (Koumbati et al., 1999; Clavijo et al., 2000). Detecting BTV in clinical samples is done by virus isolation in cell cultures (BHK-21, Vero) or in embryonated chicken eggs (ECE) (Howell and Verwoerd, 1971; Erasmus, 1975), while tests for its nucleic acid employ a variety of molecular methods (McCull and Gould, 1991; Pearson et al., 1991; Akita et al., 1992; Katz et al., 1993; Stanislawek et al., 1996; Aradaib et al., 1998; Orrù et al., 2004). Various RT-PCR assays have been developed for detecting BTV serotypes such as those for BTV-2, BTV-10, BTV-11, BTV-13 and BTV-17 in North America (Pearson et al., 1991; Aradaib et al., 1998, 2003). A laborious molecular method, based on the sequence of the gene encoding for the NS1 protein and able to identify all 24 serotypes of BTV, was developed by Brown et al. (1993) using a digoxigenin-labeled RNA probe. Other authors used colorimetric PCR-based techniques (Katz et al., 1993) and virus titration in cell culture or in ECE, to study the kinetics of BT viraemia and the site of replication in ruminants (Stanislawek et al., 1996; Koumbati et al., 1999; Clavijo et al., 2000). More recently a real time RT-PCR method was developed in Italy for the identification of BTV-2 and for differentiating the wild type from the vaccine strain (Orrù et al., 2004). Another method,

but using real time fluorogenic RT-PCR method, was developed also recently (Jiménez-Clavero et al., 2006). However, all these molecular methods were not suited to the simultaneous detection and quantitation of BTV in a given sample. In an effort to resolve this we describe a simple, rapid and versatile system for the simultaneous recognition and quantitation of all the BTV serotypes directly from clinical samples. The system utilises a particular stem-loop probe known as the Molecular Beacon (MB). The MB is a circular oligonucleotide characterized by a high specificity for the chosen target and by its ability to develop a colour-forming reaction when used in a conventional RT-PCR test, thereby rendering the agarose electrophoresis detection step unnecessary (Tyagi et al., 1998; Marras et al., 1999; Poddar, 1989; Tan et al., 2000; Landry et al., 2005).

2. Time required

RNA extraction was done in about 2.5 h using Trizol reagent protocol (Life Technologies Inc., Gaithersburg). The conventional RT-PCR required three hours. Agarose gel staining was not performed because the MB probe allowed direct visualization of the reacting samples. The quantitative real time RT-PCR, performed with the LightCycler (Roche Diagnostics, Mannheim, Germany), required 30 min.

3. Materials

3.1. Special equipment

Biological safety cabinet, class II, type B2, externally vented (KW, Monteriggioni, Siena, Italy).
LightCycler Instrument (Roche Diagnostics).
LightCycler Software, version 3.5.3 (Roche Diagnostics).
Centrifuge 13,000 rpm (model 5410, Eppendorf, Hamburg, Germany).
Oligo software version 4 or 6 (MedProbe, Oslo, Norway).

3.2. Chemicals and reagents

Trizol reagent (Life Technologies Inc.).
LightCycler RNA Amplification kit Hybridisation Probe (Roche Diagnostics).
LightCycler Capillaries (Roche Diagnostics).
GeneAmp EZ r Tth RNA PCR Kit (Applied Biosystems).
Uracil-DNA Glycosylase, heat labile (Roche Diagnostics).
The oligonucleotides were custom-synthesised by MWG Biotech (Ebersberg, Germany) (<http://www.mwg-biotech.com/html/all/index.php>).

4. Detailed procedure

4.1. Positive controls

The RNAs extracted from the supernatant of infected cell cultures, and containing 10 different BTV serotypes (BTV-

1–BTV-9 and BTV-16) were used as positive controls. These strains were assigned to the specific serotype using the VN test (Singer et al., 1998); their geographical origins are shown in Table 2.

4.2. Clinical samples

A total of 77 clinical samples (blood and organs) from ovine and bovine (47 samples from BT- affected animals and 30 samples from disease-free animals) were processed also (Table 2).

4.3. Viral nucleic acid extraction

The RNA was extracted from each specimen (positive and negative controls and clinical samples) using the Trizol procedure (Chomczynski and Sacchi, 1987). An aliquot of 250 μ l from each cell culture supernatant and blood sample or 1 cm³ from organ tissue, was sampled and used for RNA extraction using Trizol (Life Technologies Inc.) following the manufacturer's instructions. All the RNAs were re-suspended in 30 μ l of nuclease-free H₂O and kept in a deep freeze at -80°C until use.

4.4. Oligonucleotide primers and "mismatch-tolerant" Molecular Beacon fluorescent probe design

The oligonucleotide primers and the Molecular Beacon (MB) fluorescent probe used to perform both the quantitative real time RT-PCR, and the conventional RT-PCR, were designed from BTV S10 gene sequences available in GenBank. The theoretical melting temperatures of the primers (T_m s), the formation of oligonucleotide dimers, and self-complementarity, were evaluated using Oligo Program version 6 (MedProbe, Oslo, Norway). The PCR primers OG265—5'-AYAAAGCGATGTCAAA-3' and OG266—5'-TCATCACGAAACGCTTC-3' were designed to flank a region of 91 bp of the BTV S10 gene sequence from positions 172–263 (GenBank accession number AY438034). This area of was chosen for its high similarity among all BTV serotypes. The positions of the PCR primers, and the various BTV serotype sequences used, are shown in Table 1. Of the many MB probes scrutinised, the one named LF1 MB (5'-FAM-CCG GTG CTA CGC AAA CAC AGG-DABCYL-3') showed the most thermodynamically stable target-loop sequence (Tyagi et al., 1998; Marras et al., 1999). This LF1 MB sequence was complementary to a region bracketed between the two primers OG265 and OG266. The MB fluorescent probe arm sequences formed a stable stem hybrid at the PCR annealing temperature calculated with the aid of the *mfold* program (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna>) and was selected despite the presence of a single nucleotide polymorphism (SNP) at the sixth position (marked in bold above). The accidental presence of this unwanted SNP was solved utilising a "mismatch-tolerant" MB fluorescent probe carrying a bigger loop and therefore able to tolerate a mismatch in the target-loop sequence (Table 1). The T_m folding conditions were Na⁺ 1 mol/l, Mg²⁺ 2.75 mol/l at the hybridization temperature of 47 $^{\circ}\text{C}$. Fluoresceine (FAM) was used to label the

probe at position 5', while DABCYL was used as a quencher at position 3'.

4.5. Synthetic quantitative oligonucleotide targets

Two 91 bp cDNA fragments representative of the two allelic variations within the MB probe target region (Table 1) were used as quantitative standards and were named, respectively, LF4 (5'-TCA TCA CGA AAC GCT TCT GCG TAC GAT GCG AAT GCA GCC TTC TCC GCC TTC T**GTGTT** TGC **GTT** GCA CC) A GTT GTG TTT GAC ATC GCT TTG T-3') and LF5 (5'-TCA TCA CGA AAC GCT TCT GCG TAC GAT GCG AAT GCA GCC TTC TCC GCC TTC **TGT** GTT**TGC** **GT**A GCA CC) A GTT GTG TTT GAC ATC GCT TTG T-3'). These cDNAs were custom synthesized by the MWG Biotech company. Both LF4 and LF5 were serially 10-fold diluted to contain molecule numbers ranging from 10¹¹ to 10¹ per 2 μ l volume for use as standards in the quantitative real time RT-PCR assay. These standards were suspended in ultra-pure RNase- and DNase-free distilled water and stored at -80°C until use.

4.6. Synthetic qualitative oligonucleotide targets

The two synthetic cDNA fragments described above were used to perform the melting curve analysis (Fig. 1). Two short oligonucleotides (LF2 5'-CCA CGT TGC GTT TGT G-3' and LF3 5'-CCA CGA TGC GTT TGT G-3') were designed within the LF4 and the LF5 sequences, respectively (as shown in the sequence frame boxes above) and were representative, in this region, of all 17 serotypes/alleles available in GenBank. They differed only in a single nucleotide polymorphism at the sixth position (A or T).

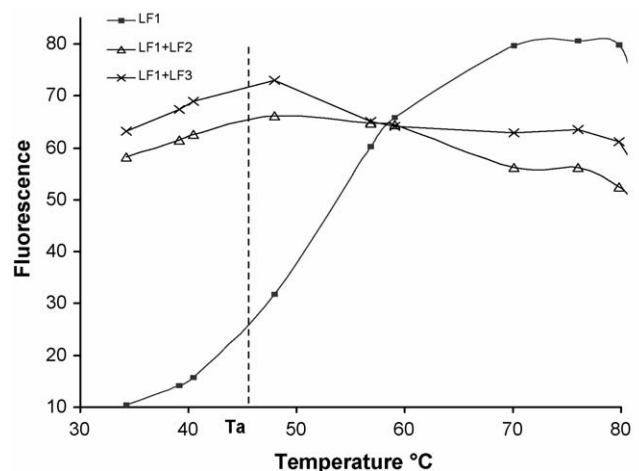


Fig. 1. Molecular Beacon fluorescent probe (LF1 MB) melting curve analysis using two alleles (LF2 and LF3) in the targeted region of the S10 gene; LF1 = Molecular Beacon without target, LF1 + LF3 = Molecular Beacon with perfectly complementary target (BTV serotypes 1, -2, -3, -6, -9, -12, -18 and BTV-23); LF1 + LF2 = Molecular Beacon with target allele containing the A/T mismatch (BTV-4, BTV-8, BTV-10, BTV-11, BTV-13, BTV-16, BTV-17, BTV-20 and BTV-21). The two alleles showed a low difference in fluorescence (of about 5 units) at the annealing step ($T_a = 47^{\circ}\text{C}$).

Table 1

Alignment of the 17 BTV NS3 GenBank sequences; the sequence positions of the primers (OG265 and OG266) and the Molecular Beacon (LF1) used are indicated

Accession numbers- Serotype	Upper primer	M. Beacon	Position	Lower primer	Position
AY42 6598 -1	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCATACGCAGAAGCGTTTCGTGATGATGTAAGAC	290
AY43 8033 -2	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		221	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	271
AY42 6601 -3	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AY42 6602 -4	GATAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AY42 6603 -6	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AY42 6604 -8	GACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAC	290
AY43 8034 -9	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAGAAGCGCGA		221	GAAGGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAT	271
AF04 4385 -10	GACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AF51 2923 -11	GACAAAGCGATGTCAAACACAACGGGTGCTACGCAAACACAAAAACGCGA		221	GAAGGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAT	271
AY42 6595 -12	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AF04 4713 -13	GACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AF13 5229 -16	GATAAAGCGATGTCAAACACTACTGGTGTGCTACGCAAACACAGAAGCGCGA		221	GAAAGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAC	271
AY42 6597 -17	GACAAAGCGATGTCAAATACAACGGGTGCTACGCAAACACAAAAACGCGA		240	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	290
AF51 2915 -18	GACAAAGCGATGTCAAACACAACCTGGTGCAACGCAAACACAAAAACGCGA		221	GAAGGCTGCATTGCGATCGTACGC GGAAGCGTTTCGTGATGATGTAAGAT	271
AF52 9056 -20	GATAAAGCGATGTCAAACACTACTGGTGTGCTACGCAAACACAGAAGCGCGA		221	GAAAGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAC	271
AF52 9058 -21	GATAAAGCGATGTCAAACACTACTGGTGTGCTACGCAAACACAAAAACGCGA		221	AAAAGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAC	271
AF52 9059 -23	GATAAAGCGATGTCAAACACTACTGGTGTGCAACGCAAACACAGAAGCGCGA		221	GAAAGCTGCATTGCGATCGTACGCAGAAGCGTTTCGTGATGATGTAAGAC	271
Aligned sequences					
5'	3'	5' OG 265	LF1 MB	OG 266	

4.7. “Mismatch-tolerant” Molecular Beacon thermal denaturation profiles

To determine the capacity of the LF1 MB to detect all the BTV allelic sequences, its thermal denaturation profiles were studied. The resulting melting curves proved reliable ensuring correct real time RT-PCR conditions such as maximum specificity of annealing temperature (T_a). The reaction, using the “LightCycler RNA Amplification Kit Hybridisation Probe” in the LightCycler Instrument, was performed in a final volume of 20 μ l solution containing 10 μ M of the LF1 MB only, and, subsequently, with the addition of the single-stranded synthetic oligonucleotide targets LF2 and LF3 separately (also at concentrations of 10 μ M per reaction). The changes in fluorescence during the real-time assay were measured. The LF1 MB temperature denaturation profiles were obtained using the LightCycler melting curve procedure, i.e. initial heating at 95 °C and subsequent cooling at 45 °C. A final heating step at 95 °C was achieved with a controlled temperature transition rate of 0.1 °C/s. The resulting fluorescence was recorded starting at the final 95 °C heating step using the parameter “continuous mode” and the F1 channel (Fig. 1).

4.8. Real time RT-PCR conditions

In order to evaluate the applicability of the method described here as a diagnostic tool, BTV strains and clinical samples (listed in Table 1) were processed both with conventional RT-PCR (described below) and real time RT-PCR employing LF1 MB. The real time RT-PCR, including the preliminary reverse transcription step, was performed in a one-step reaction using the “LightCycler RNA Amplification Kit Hybridisation Probes” according to the manufacturer’s instructions. The reaction mixture at the final volume of 20 μ l, contained 5 mM MgCl₂, 0.25 μ M of each primer, 10 μ M of LF1 MB and 2 μ l of extracted RNA. The PCR program used had the following parameters: (i) initial denaturation of dsRNA at 95 °C for 30 s; (ii) reverse transcription step at 55 °C for 10 min; (iii) denaturation step at 95 °C for 30 s, and (iv) 40 cycles each of 0 s at 95 °C, 10 s at 47 °C and 4 s at 72 °C. The fluorescence signal was detected at the end of the 47 °C reaction segment, in the annealing step, in “single mode” setting. To initially evaluate the specificity of this procedure a 10 μ l aliquot of each real time RT-PCR reaction was also analysed in horizontal electrophoresis using a 2% agarose gel stained with a solution of 0.5 mg/ml ethidium bromide.

4.9. LF1 MB conventional RT-PCR

Conventional RT-PCR was performed in 50 μ l reaction volume using GeneAmp EZ rTth RNA PCR Kit (Applied Biosystems) and according to the manufacturer’s instructions. The 50 μ l of mixture used contained 0.45 μ M of each primer (OG265 and OG266), 10 μ M of the LF1 MB, and 2 μ l of extracted RNA. The RT-PCR conditions used were the following: (i) initial denaturation of dsRNA at 95 °C for 2 min; (ii) reverse transcription at 60 °C for 60 min; (iii) denaturation at 95 °C for 2 min,

followed by (iv) 40 cycles each of 1 min at 95 °C, 1 min at 47 °C and 1 min at 72 °C. The thermal reactions were performed using a Primus 96 Plus thermocycler (MWG AG, Biotech). A 10 μ l aliquot of each PCR product was electrophoretically analysed in a 2% agarose gel stained with ethidium bromide (0.5 mg/ml).

5. Results

5.1. Melting curve analysis

The synthetic LF2 and LF3 oligonucleotides represent the two allelic variations of the target sequence corresponding to the LF1 MB hybridisation site. They have been used to verify the ability of the chosen LF1 MB to generate the expected fluorescence signal despite the presence of an A/T SNP within the targeted region. The melting curve analysis was performed to confirm that despite the presence of this SNP, the real time RT-PCR could be used to equally detect both allelic forms. In fact the fluorescent signals using the LF1 MB separately with each allelic form (LF2 and LF3) differed by only 5 units (Fig. 1). The analysis of the melting curves performed using real time RT-PCR, showed that in the presence of these two alleles, the fluorescence signal increased significantly, while no fluorescence was detected in the case of target absence. The melting curve analyses revealed that the best hybridisation reaction temperature range lay between 45 and 50 °C (Fig. 1). Thus, the annealing temperature of 47 °C, selected using an empirical approach, gave the highest specificity and sensitivity in the real time RT-PCR. At this hybridisation temperature the LF1 MB hybridised with the targets (LF2 and LF3) while no significant fluorescence signal was detected when the reaction was performed without targets. This indicated the absence of unwanted non-specific LF1 MB hybrids.

5.2. LF1 MB conventional RT-PCR and real time RT-PCR

Using the LF1 MB conventional RT-PCR, as evaluated here, identical results were obtained when compared with those of the newly developed real time RT-PCR (Table 2). The clinical samples collected from BTV-infected animals were correctly identified as positive and were easily separated from negative samples collected from healthy animals. All ten BTV serotypes, processed using the LF1 MB real time RT-PCR were tested positive (Fig. 2), such as the LF1 MB conventional RT-PCR. The quantification range test was performed using serial dilutions of the synthetic LF4 and LF5 targets (10^1 to 10^{11} molecules/PCR). The LF1 MB conventional RT-PCR had a limit of detection (LOD) of about 5×10^2 molecules per PCR reaction, while the LF1 MB real time RT-PCR showed a satisfactory LOD (10^3 molecules/PCR reaction) with correlation factor of $R^2 = 0.98$. These results, in accordance with melting curve analyses, showed that the real time RT-PCR is reliable for both the qualitative and quantitative detection of the two BTV target alleles.

All 10 serotypes examined using the LF1 MB conventional RT-PCR with agarose gel electrophoresis, gave a positive reac-

Table 2

Geographic origin, serotype and samples analysed using the LF1 MB fluorescent probe in real time RT-PCR and the conventional RT-PCR with the addition of the LF1 MB in the reaction mixture for the visualisation of the positive reaction under ultraviolet light (λ 365 nm)

Origin	RNA source	Serotype	No. of samples	LF1 MB conventional RT-PCR positive/negative	LF1 MB real time RT-PCR positive/negative
OVI ^a	Virus isolate	BTV-1	1	+	+
Latium	Virus isolate	BTV-2	1	+	+
Sardinia	Ovine blood	BTV-2	1	+	+
Sardinia	Ovine organ	BTV-2	2	+	+
Sardinia	Deer blood	BTV-2	1	+	+
Apulia	Ovine organs	BTV-2	6	+	+
Puglia	Caprine organs	BTV-2	3	+	+
Puglia	Bovine organs	BTV-2	1	+	+
Sicily	Bovine organs	BTV-2	1	+	+
Tuscany	Ovine Blood	BTV-2	1	+	+
Molise	Ovine organs	BTV-2	1	+	+
OVI ^a	Virus isolate	BTV-3	1	+	+
Greece	Virus isolate	BTV-4	5	+	+
OVI ^a	Virus isolate	BTV-5	1	+	+
OVI ^a	Virus isolate	BTV-6	1	+	+
OVI ^a	Virus isolate	BTV-7	1	+	+
OVI ^a	Virus isolate	BTV-8	1	+	+
Greece	Virus isolate	BTV-9	1	+	+
Molise	Bovine organs	BTV-9	17	+	+
Puglia	Ovine organs	BTV-9	10	+	+
Calabria	Ovine organs	BTV-9	2	+	+
Puglia	Ovine blood	BTV-16	1	+	+
Israel	Virus isolate	BTV-16	1	+	+
Sardinia	Ovine blood	Negative ^b	20	–	–
Sardinia	Ovine organs	Negative ^b	10	–	–

^a Onderstepoort Veterinary Institute.

^b Disease-free animals.

tion as indicated by the presence of a 91 bp band. By adding the LF1 MB to the reaction mixture, a clear green fluorescent light appears in the positive tubes as revealed under ultraviolet light (λ 365 nm). This demonstrates that the usual agarose gel electrophoresis detection of the RT-PCR reaction can now be bypassed making our test quicker and easier than a traditional RT-PCR (Fig. 3).

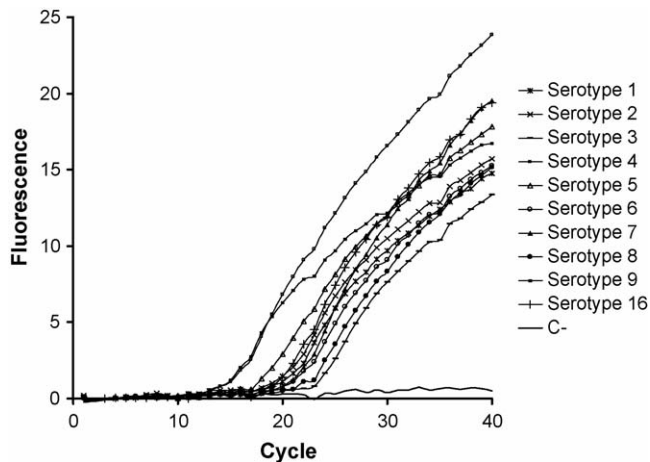


Fig. 2. Real time RT-PCR fluorescence curves obtained using ten RNA extracts from BTV-infected cell cultures (employed in non-homogenous concentrations) and the LF1 MB fluorescent probe.

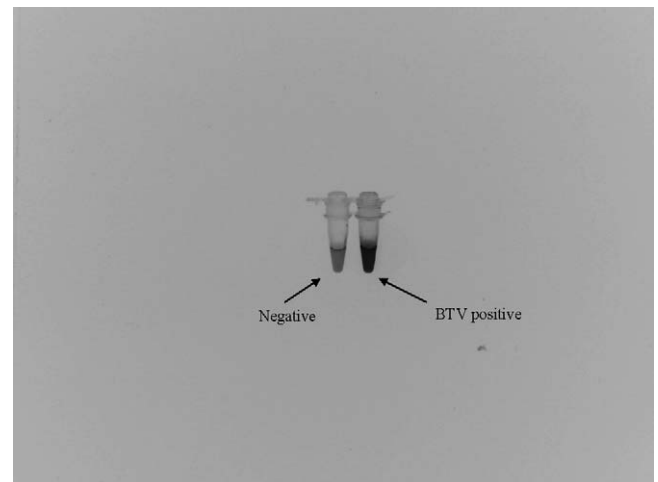


Fig. 3. Conventional RT-PCR performed with the addition of LF1 MB in the reaction mixture: only the positive samples showed an intense green fluorescence (left) under ultraviolet light (λ 365 nm), thereby eliminating subsequent gel electrophoresis detection of the amplified viral nucleic acid.

6. Discussion

BT is a serious disease of ruminant livestock and its eradication includes rigidly enforced animal movement controls. The Italian sheep and cattle industry has been severely affected by BT since the year 2000 (Caporale et al., 2004; Conte et al., 2005)

and, at present, is still engaged in a nationwide surveillance programme. Since BTV-2 first appeared on the island of Sardinia in 2000, a further three serotypes (BTV-4, BTV-9 and BTV-16) have entered Italy including the mainland. Simultaneously, BT spread to 14 other countries in the Mediterranean basin and as far as 44°N including some Balkan states (Mellor and Wittmann, 2002). The almost random and unpredictable circulation of at least eight serotypes of BTV within southern Europe, underlines the need for a reliable and quick diagnostic method and one that enables the identification of all serotypes of BTV.

The use of serological methods for BTV detection in infected ruminant blood has been unsuccessful (Mecham, 1993); in fact, they are either only able to detect antigens in animals with a heightened *viraemia* (Stanislawek et al., 1996) or have never been consistent enough to allow for the reliable diagnosis of BTV (Hawkes et al., 2000). Because of the epidemiological importance of serotype identification, a lot of effort has over the years been put into developing several serotyping procedures; these include the VN test and the plaque neutralisation, plaque inhibition and fluorescence inhibition tests (Howell et al., 1970; Afshar, 1994; Blacksell and Lunt, 1996). All these methods can be cumbersome and time consuming. A precise and simple method for detecting the level of *viraemia* in BTV-infected ruminants is needed also for veterinary authorities to implement logical trade policies (Bonneau et al., 2002). To provide more efficient serotyping systems a series of RT-PCR and real time RT-PCR methods have been developed over the last decade or more to detect and/or differentiate BTV serotypes and to differentiate between field and vaccine strains (McColl and Gould, 1991; Pearson et al., 1991; Akita et al., 1992; Katz et al., 1993; Stanislawek et al., 1996; Aradaib et al., 1998; Koumbati et al., 1999; Clavijo et al., 2000; Orrù et al., 2004). However, only a few molecular methods are available for the simultaneous detection of all BTV serotypes. This is because genetic variability within BTV, which is common to many RNA viruses, creates problems in regard to the use of molecular diagnostic tools which are highly specific and sensitive (Billinis et al., 2001). All current molecular tools, though useful, are either unable to simultaneously detect and quantify all BTV serotypes, or are not sensitive and simple enough to be applied in large scale testing. Furthermore, high throughput methods need to also consider the risk of contamination which are common to PCR-based methods. Some of the available BTV serogroup RT-PCRs are based on multiplex PCR for multiple serotype detection (Johnson et al., 2000; Aradaib et al., 2003) or on nested RT-PCR systems for increased sensitivity (Aradaib et al., 1998). Most of these methods use traditional agarose gel electrophoresis for the detection of the amplified viral nucleic acids, but this is not suited to large-scale routine diagnostics. Other authors (Katz et al., 1993) have developed an enzyme-linked oligonucleotide sorbent assay (PCR-ELOSA) as a large scale amplified viral nucleic acids detecting system, but still has the limitation that samples be handled carefully to avoid PCR product carry-over.

In recent years various real time PCR methods have been implemented and applied to the diagnosis of human and animal diseases (Lanciotti et al., 2000; Lanciotti and Kerst, 2001) and to

foodborne disease control (some of which are already available as commercial kits). The real time PCR technique is particularly attractive because it avoids the use of agarose gel electrophoresis, so decreasing the risks of contamination, and because it is suitable for large scale testing and automation. Despite the general interest only very few high throughput methods have been developed for the detection of BTV. Recently, a real time RT-PCR assay was developed (Jiménez-Clavero et al., 2006) to detect BTV during Mediterranean outbreaks of Bluetongue. It was designed using a conserved region in the BTV RNA segment 5 of BTV-2 and BTV-4; in this case the oligonucleotides and a Taqman-MGB fluorogenic probe were positioned in a part of the viral RNA segment 5 which had various mismatches. This reduced the sensitivity of the assay it being unable to detect 8 out of the 24 serotypes tested. Moreover, in regard to BTV-4 and BTV-16 the test was only able to detect the field isolates and not the reference strains. This may have been due to a drop in T_m because of an excessive number of mismatches in the probe and primer sequences. Only one other real time RT-PCR, using fluorescence resonance energy transfer (FRET) probe technology, has been described previously (Orrù et al., 2004). It was developed, during the vaccination campaign in Italy, to differentiate wild-type BTV-2 from the vaccine strain and to distinguish between vaccinated and naturally infected animals.

The molecular method described here is based on the use of an MB, with the aim of providing a versatile system that can be employed in both conventional RT-PCR or in real time RT-PCR assays. The MBs are hairpin-shaped oligonucleotide probes, and have the property of fluorescing upon hybridization with a specific target sequence. Their hairpin shape increases target specificity providing a lower background signal compared to that found in other linear probes (Tyagi et al., 1998). The LF1 MB, used here, is a “mismatch-tolerant” probe, as opposed to a “mismatch-sensitive” probe, which is mostly employed in allele discrimination assays (Kostrikis et al., 1998). Our use of this type of MB made it possible to overcome the interference which might have occurred because of the A/T mismatch located at the sixth position of its target sequence. This was confirmed by using two synthetic oligonucleotides (LF2 and LF3) in the melting curve analysis giving a fluorescence difference of only 5% between the two allelic forms. The use of the two 91 bp cDNA synthetic fragments (LF4 and LF5), representative of the two allelic variations in the S10 gene sequence of the various BTV serotypes, made it convenient to perform the quantitative real time RT-PCR under controlled conditions. The real time RT-PCR detected 10^3 molecules/PCR reaction and recognised all 10 serotypes studied (BTV-1–BTV-9 and BTV-16) and correctly differentiated between infected and non-infected animals irrespective of the type of sample used (blood, organs and cell cultures). These results were in agreement with the results obtained using the LF1 MB conventional RT-PCR. Even though the design of our LF1 MB is based only on 17 BTV serotype sequences, its ability to tolerate a single mismatch means that it will likely be able to detect all 24 serotypes of BTV known.

In summary, the versatility of the MB-based quantitative real time RT-PCR and conventional RT-PCR developed in this study allows one to use MB technology especially when limited finan-

cial resources impede access to real time PCR equipment. In fact most diagnostic veterinary laboratories do not have relatively expensive real time PCR apparatuses; in these instances the addition of the LF1 MB to the conventional RT-PCR eliminates the post-PCR detection steps that are required normally. The ability of the LF1 MB to develop a colour-forming fluorescent reaction in positive samples renders detection by gel electrophoresis unnecessary. Besides ease of detection there is also a reduction in the risk of contamination making the LF1 MB usable also under near field conditions.

References

- Afshar, A., 1994. Bluetongue: laboratory diagnosis. *Comp. Immunol. Microbiol. Infect. Dis.* 17, 221–242.
- Akita, G.Y., Chinsangaram, J., Osburn, B.I., Ianconescu, M., Kaufman, R., 1992. Detection of Bluetongue virus serogroup by polymerase chain reaction. *J. Vet. Diagn. Invest.* 4, 400–405.
- Aradaib, I.E., Schore, C.E., Cullor, J.S., Osburn, B.I., 1998. A nested PCR for detection of North American isolates of Bluetongue virus based on NS1 genome sequence analysis of BTV-17. *Vet. Microbiol.* 59, 99–108.
- Aradaib, I.E., Smith, W.L., Osburn, B.I., Cullor, J.S., 2003. A multiplex PCR for simultaneous detection and differentiation of North American serotypes of Bluetongue and epizootic hemorrhagic disease viruses. *Comp. Immunol. Microbiol. Infect. Dis.* 26, 77–87.
- Billinis, C., Koumbati, M., Spyrou, V., Nomikou, K., Mangana, O., Panagiotidis, A., Papadopoulos, O., 2001. Bluetongue virus diagnosis of clinical cases by a duplex reverse transcription-PCR: a comparison with conventional methods. *J. Virol. Methods* 98, 77–89.
- Blacksell, S.D., Lunt, R.A., 1996. A simplified fluorescence inhibition test for the serotype determination of Australian Bluetongue viruses. *Aust. Vet. J.* 73, 33–34.
- Bonneau, K.R., DeMaula, C.D., Mullens, B.A., MacLachlan, N.J., 2002. Duration of viraemia infectious to *Culicoides sonorensis* in Bluetongue virus-infected cattle and sheep. *Vet. Microbiol.* 88, 115–125.
- Bonneau, K.R., Mullens, B.A., MacLachlan, N.J., 2001. Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of Bluetongue virus upon passage between sheep, cattle, and *Culicoides sonorensis*. *J. Virol.* 75, 8298–8305.
- Bonneau, K.R., Zhang, N., Zhu, J., Zhang, K., Xiao, L., Xiang, W., MacLachlan, N.J., 1999. Sequence comparison of the L2 and S10 genes of Bluetongue viruses from the United States and the People's Republic of China. *Virus Res.* 61, 153–160.
- Brown, C.C., Myer, R.F., Grubman, M.J., 1993. Use of a digoxigenin-labeled RNA probe to detect all 24 serotypes of Bluetongue virus in cell culture. *J. Vet. Diagn. Invest.* 5, 159–162.
- Bowne, J.G., Jones, R.H., 1966. Observations on Bluetongue virus in the salivary glands of an insect vector, *Culicoides variipennis*. *Virology* 30, 127–133.
- Caporale, V., Giovannini, A., Patta, C., Calistri, P., Nannini, D., Santucci, U., 2004. Vaccination in the control strategy of Bluetongue in Italy. *Dev. Biol. (Basel)* 119, 113–127.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162 (1), 156–159.
- Clavijo, A., Heckertm, R.A., Dulac, G.C., Afshar, A., 2000. Isolation and identification of Bluetongue virus. *J. Virol. Methods* 87, 13–23.
- Conte, A., Colangeli, P., Ippoliti, C., Paladini, C., Ambrosini, M., Savini, L., Dall'Acqua, F., Calistri, P., 2005. The use of a web-based interactive geographical information system for the surveillance of Bluetongue in Italy. *Rev. Sci. Tech.* 24 (3), 857–868.
- Davies, F.G., Mungai, J.N., Pini, A., 1992. A new Bluetongue virus serotype isolated in Kenya. *Vet. Microbiol.* 31, 25–32.
- Du Toit, R.M., 1944. The transmission of Bluetongue and horse-sickness by *Culicoides*. *Onderstepoort J. Vet. Sci. Anim. Ind.* 19, 7–16.
- Erasmus, B.J., 1975. Bluetongue in sheep and goats. *Aust. Vet. J.* 52, 196–198.
- Gorman, B.M., 1990. The Bluetongue viruses. *Curr. Top. Microbiol. Immunol.* 162, 1–19.
- Han, Z., Harty, R.N., 2004. The NS3 protein of Bluetongue virus exhibits viroporin-like properties. *J. Biol. Chem.* 279, 43092–43097.
- Hawkes, R.A., Kirkland, P.D., Sanders, D.A., Zhang, F., Li, Z., Davis, R.J., Zhang, N., 2000. Laboratory and field studies of an antigen capture ELISA for Bluetongue virus. *J. Virol. Methods* 85, 137–149.
- Howell, P.G., Kumm, N.A., Botha, M.J., 1970. The application of improved techniques to the identification of strains of Bluetongue virus. *Onderstepoort J. Vet. Res.* 37, 59–66.
- Howell, P.G., Verwoerd, D.W., 1971. Bluetongue virus. In: Gard, S., Hallaver, C., Meyer, K.F. (Eds.), *Virology Monographs*, vol. 9. Springer Verlag, New York, pp. 35–74.
- Hwang, G.Y., Yang, Y.Y., Chiou, J.F., Li, J.K., 1992. Sequence conservation among the cognate non-structural NS3:3A protein genes of six Bluetongue viruses. *Virus Res.* 23, 151–161.
- Jiménez-Clavero, M.A., Agüero, M., San Miguel, E., Mayoral, T., Cruz López, M., Ruano, M.J., Romero, E., Monaco, F., Polci, A., Savini, G., Gómez-Tejedor, C., 2006. High throughput detection of Bluetongue virus by a new real-time fluorogenic reverse transcription-polymerase chain reaction: application on clinical samples from current Mediterranean outbreaks. *J. Vet. Diagn. Invest.* 18, 7–17.
- Johnson, D.J., Wilson, W.C., Paul, P.S., 2000. Validation of a reverse transcriptase multiplex PCR test for the serotype determination of U.S. isolates of Bluetongue virus. *Vet. Microbiol.* 76, 105–115.
- Katz, J.B., Alstad, A.D., Gustafson, G.A., Moser, K.M., 1993. Sensitive identification of Bluetongue virus serogroup by a colorimetric dual oligonucleotide sorbent assay of amplified viral nucleic acid. *J. Clin. Microbiol.* 31, 3028–3030.
- Kostrikis, L.G., Tyagi, S., Mhlanga, M.M., Ho, D.D., Russell, F.K., 1998. Molecular beacons: spectral genotyping of human alleles. *Science* 279, 1228–1229.
- Koumbati, M., Mangana, O., Nomikou, K., Mellor, P.S., Papadopoulos, O., 1999. Duration of Bluetongue viraemia and serological responses in experimentally infected European breeds of sheep and goats. *Vet. Microbiol.* 12, 277–285.
- Lanciotti, R.S., Kerst, A.J., Nasci, R.S., et al., 2000. Rapid detection of West Nile from human clinical specimens, field collection mosquitoes, and avian samples by a TaqMan reverse-transcriptase-PCR assay. *J. Clin. Microbiol.* 38, 4066–4071.
- Lanciotti, R.S., Kerst, A.J., 2001. Nucleic acid sequence-based amplification assays for rapid detection of West Nile and St. Louis encephalitis viruses. *J. Clin. Microbiol.* 39, 4506–4513.
- Landry, M.L., Garner, R., Ferguson, D., 2005. Real-time nucleic acid sequence-based amplification using Molecular Beacons for detection of enterovirus RNA in clinical specimens. *J. Clin. Microbiol.* 43, 3136–3139.
- MacLachlan, N.J., Nunamaker, R.A., Katz, J.B., Sawyer, M.M., Akita, G.Y., Osburn, B.I., Tabachnick, W.J., 1994. Detection of Bluetongue virus in the blood of inoculated calves: comparison of virus isolation, PCR assay, and in vitro feeding of *Culicoides variipennis*. *Arch. Virol.* 136, 1–8.
- Marras, S.A., Kramer, F.R., Tyagi, S., 1999. Multiplex detection of single-nucleotide variations using Molecular Beacons. *Genet. Anal.* 14, 151–156.
- McCull, K.A., Gould, A.R., 1991. Detection and characterisation of Bluetongue virus using the polymerase chain reaction. *Virus Res.* 21, 19–34.
- Mecham, J.O., 1993. Detection of *Bluetongue virus* from blood of infected sheep by use of an antigen-capture enzyme-linked immunosorbent assay after amplification of the virus in cell culture. *Am. J. Vet. Res.* 54, 370–372.
- Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998–2001. *Vet. J.* 164, 20–37.
- Mertens, P.P., Diprose, J., 2004. The Bluetongue virus core: a nano-scale transcription machine. *Virus Res.* 101, 29–43.
- Orrù, G., De Santis, P., Solinas, F., Savini, G., Piras, V., Caporale, V., 2004. Differentiation of Italian field and South African vaccine strains of Bluetongue virus serotype 2 using real-time PCR. *J. Virol. Methods* 122, 37–43.

- Pearson, J.E., Gustafson, A.L., Shafer, A.L., Alstad, A.D., 1991. Diagnosis of Bluetongue and epizootic hemorrhagic disease. In: Walton, T.E., Osburn, B.I. (Eds.), *Bluetongue, African Horse Sickness, and Related Orbiviruses*. Proceedings of the Second International Symposium. Boca Raton. CRC Press, pp. 533–546.
- Pierce, C.M., Balasuriya, U.B., MacLachlan, N.J., 1998. Phylogenetic analysis of the S10 gene of field and laboratory strains of Bluetongue virus from the United States. *Virus Res.* 55, 15–27.
- Price, D.A., Hardy, W.T., 1954. Isolation of the Bluetongue virus from Texas sheep-*Culicoides* shown to be a vector. *J. Am. Vet. Med. Assoc.* 124, 255–258.
- Poddar, S.K., 1989. Detection of adenovirus using PCR and Molecular Beacon. *J. Virol. Methods* 82, 19–26.
- Roy, P., 1989. Bluetongue virus genetics and genome structure. *Virus Res.* 13, 179–206.
- Roy, P., Marshall, J.J.A., French, T.J., 1990. Structure of Bluetongue virus genome and its encoded proteins. In: Roy, P., Gorman, B.M. (Eds.), *Current Topics in Microbiology and Immunology*. Springer, Berlin.
- Roy, P., 1992. Bluetongue virus proteins. *J. Gen. Virol.* 73, 3051–3064.
- Singer, R.S., Boyce, W.M., Gardner, I.A., Johnson, W.O., Fisher, A.S., 1998. Evaluation of Bluetongue virus diagnostic test in free-ranging bighorn sheep. *Prev. Vet. Med.* 35, 265–282.
- Stanislawek, W.L., Lunt, R.A., Blacksell, S.D., Newberry, K.M., Hooper, P.T., White, J.R., 1996. Detection by ELISA of Bluetongue antigen directly in the blood of experimentally infected sheep. *Vet. Microbiol.* 52, 1–12.
- Tan, W., Fang, X., Li, J., Liu, X., 2000. Molecular Beacons: a novel DNA probe for nucleic acid and protein studies. *Chemistry* 6, 1107–1111.
- Tyagi, S., Bratu, D.P., Kramer, F.R., 1998. Multicolor Molecular Beacons for allele discrimination. *Nat. Biotechnol.* 16, 49–53.
- Wade-Evans, A.M., 1990. Complete sequence of genome segment 10, encoding the NS3 protein, of Bluetongue virus, serotype 1 from South Africa. *Nucleic Acids Res.* 18, 4920.
- Zhou, E.M., Ridd, D., Riva, J., Fernando, L., Clavijo, A., 2001. Development and evaluation of an IgM-capture ELISA for detection of recent infection with Bluetongue viruses in cattle. *J. Virol. Methods* 91, 175–182.