Real-time polymerase chain reaction to detect bluetongue virus in blood samples

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Summary

The authors describe a real-time reverse transcriptase polymerase chain reaction (RT-PCR) to detect bluetongue viruses (BTV) in blood samples. The primers and Taqman probes used were specific for a conserved region of BTV RNA segment 5, which encodes non-structural protein NS1. The method was able to detect strains of BTV serotypes 2, 4, 9 and 16 isolated in Italy, and their respective vaccine strains. The limit of detection was 5.0×10^{-3} $TCID_{50}$ per ml of sample. The assay did not amplify RNA from other *Orbiviruses*, including epizootic haemorrhagic disease virus (EHDV) or African horse sickness virus (AHSV), or from viruses in the *Reoviridae* family or those that cause a similar clinical picture to that of BTV. Its accuracy was evaluated on 104 blood samples in ethylene-diamine tetra-acetic acid (EDTA) and the results were compared with those obtained with the conventional RT-PCR used in routine diagnosis. Both tests gave negative results on 40 blood samples from bluetongue-free farms (confidence interval: 95%, 92.5-100%). Real-time PCR detected BTV RNA in 64 sentinel cows that had recently seroconverted to serotypes 2 and 16 (confidence interval: 95%, 95.5-100%), whereas conventional RT-PCR detected only 47 of these (confidence interval: 95%, 61.5-82.7%) ($P<0.05$). The method is rapid, thereby reducing execution times, and does not require any post-amplification manipulation, thus avoiding the inherent risk of contamination of amplified products.

Keywords

Bluetongue virus, Diagnosis, Polymerase chain reaction, Real-time reverse transcriptase polymerase chain reaction.

Introduction

Bluetongue (BT) is a non-contagious infectious disease with a viral aetiology which affects sheep and other domestic and wild ruminants. It is transmitted by insects of the *Culicoides* genus (29) and is characterised by fever, weight loss, mucosal ulceration and cyanosis, oedema and lameness. Mortality can be high in sheep (5), while other species usually present an asymptomatic form of the disease (3).

The BT virus (BTV) belongs to the *Reoviridae* family and is the prototype of the *Orbivirus* genus (6, 20). It is a naked double-stranded RNA virus with a genome consisting of 10 gene segments that encode 7 structural proteins (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3 and NS3a). Twenty-four serotypes of BTV have currently been identified that are variably related, as revealed by *in vivo* cross-protection and *in vitro* cross-reaction (8).

Bluetongue is one of the 16 former list A diseases of the World Organisation for Animal Health (OIE: Office International des Épizooties) (34) and the cause of significant financial losses in the livestock sector. These losses reflect not only the direct and indirect costs resulting from the infection itself, but also those incurred by restrictions on the movement of livestock and their products in

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countries where the disease is present (20, 24, 30). BTV infections have been recorded in temperate and tropical areas worldwide, but the spread of the disease is limited to regions inhabited by insect vectors capable of transmitting the virus (8, 16). These vectors become infected after consuming the blood of infected animals and are able to transmit the virus to a new host after 10 to 14 days (31).

The virus reached the Mediterranean basin in 1998 and between 1999 and 2006 affected numerous countries, resulting in the most widespread and severe BT epidemic ever witnessed in Europe. In Italy, six successive epidemics occurred between summer 2000 and April 2006, with approximately 15 000 clinical foci in eight regions (Abruzzo, Calabria, Campania, Molise, Puglia, Sardinia, Sicily and Tuscany). During these epidemics which saw an intense and constant spread of the virus northward and the simultaneous appearance of widely distributed foci, virus serotypes 2, 4, 9 and 16 were isolated. To tackle the constant spread of the virus and protect animals from infection, the health authorities decided to vaccinate all domestic ruminants in and around the areas where the virus was known to be in circulation. In the various 2002 vaccine campaigns, animals were vaccinated with preparations containing one or more virus serotypes depending on their geographic origin. The product used was a live vaccine supplied by Onderstepoort Biological Products (OBP) in Onderstepoort, South Africa. One of the drawbacks of this type of vaccination is the possible spread of the vaccine strain to the environment, an event that already has occurred with attenuated BT vaccine serotypes 2 and 16 (7, 18). It is obvious that the situation of virus serotypes circulating in Italy is just as complex and does not exclude the possibility of serotypes 2, 4, 9 and 16 originating from vaccines being present alongside the homologous field serotypes. The situation is further complicated by the fact that BTV, the biological cycle of which includes replication phases in both insect and ruminant cells, undergoes continuous genetic evolution leading to variation in each gene segment, both between serotypes and within a single serotype (25. For this reason, although at the serological level there are four circulating viral serotypes, the possible existence of at least eight BTV variants cannot be excluded. This scenario considerably complicates the accuracy and rapidity of diagnostic methods, especially those that detect the virus directly. If it is true that the availability of diagnostic tests that are capable of rapidly revealing the presence of a virus is important for all diseases, it is even more so for those diseases previously on OIE list A, where speed becomes of the essence in containing the dramatic consequences of the spread of these pathogens.

In this context, molecular biology has made a valuable contribution by greatly reducing diagnosis times. The use of nucleic acid amplification technology (NAT) is now widespread in a number of microbiological and virological diagnostic sectors (21, 27). Qualitative assays are now complimented more often by quantitative tests, essential in monitoring infections and treatments (12, 14, 17). Numerous commercial and 'home made' NAT-based diagnostic methods have been introduced recently, such as reverse transcriptase polymerase chain reaction (RT-PCR), nested-PCR, branched-DNA, real-time PCR and competitive-PCR. Molecular methods based on RT-PCR have been developed for the BTV (2, 13, 15, 28). As well as offering greater speed than traditional methods, these methods also enable further virus characterisation through sequencing of the amplified nucleic acid. Specific real-time RT-PCR protocols have recently been designed for some BTV serotypes that are circulating in given geographic areas (11, 33), or to differentiate between vaccine and field strains of the virus (1, 4, 19), or able to recognise all 24 BTV reference strains (32).

This study describes a real-time RT-PCR method able to detect BTV serotypes 2, 4, 9 and 16 isolated in Italy as well as their vaccine variants in animal blood.

Materials and methods

Viral strains

The method was developed using strains of BTV serotypes 2, 4, 9 and 16 that were originally isolated by the Virology Department of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise 'G. Caporale', the OIE reference centre for BT, during the bluetongue epidemic that occurred in Italy between 2000 and 2004, and the homologous vaccine strains produced by OBP and used in the 2002-2005 vaccine campaigns. Before use, viruses were propagated on Vero cells (African green monkey kidney) cultivated in minimum essential medium (MEM) (Eurobio, France) containing L-glutamine, antibiotics (penicillin 100 IU/ml (Sigma, Munich), streptomycin 100 µg/ml (Sigma), gentamicin 5 µg/ml (Sigma) and nystatin 50 IU/ml (Sigma) and 10% foetal calf serum (FCS) (Sigma). The viral load of each strain was determined by micro-titration using the Reed and Muench formula (23).

Method development

Viral RNA extraction

Total RNA was extracted from the supernatant of cell cultures infected with BTV and from vaccine strains using the high pure nucleic acid extraction kit (Roche, Nutley, New Jersey), starting from a volume of 200 µl and a viral load of at least 10^4 $TCID_{50}$/ml, in accordance with the instructions of the manufacturer and eluting the RNA in 50 µl of the buffer provided. RNA was extracted from the blood samples in ethylenediamine tetra-acetic acid (EDTA) as described for the BTV strains, except that 500 µl was taken instead of 200 µl and, before the RNA extraction, the blood was lysated by adding 1 ml cold water and keeping the sample on ice for 10 min; the sample was then centrifuged at 4°C for 10 min at 16 000 g, eliminating the supernatant and resuspending the pellet in 200 µl of binding buffer.

Sequencing

Segment 5 of each strain was sequenced from the extracted viral RNA; this gene codes for the protein NS1. Briefly, individual genes were retrotranscribed and amplified using the forward primer 5-AGTTCTCTAGTTGGCAAC CACC-3′ and the reverse primer 5′-CTAATAC TCCATCCACATCTGAGAC-3′. The amplified product was purified using the QIAquick gel purification kit (Qiagen, Cologne). The sequence reaction was conducted using the big dye terminator kit 3.1 (Applied Biosystems, Foster City, California) and purified with Montage™ PCR (Millipore, Billerica, Massachusetts). Electropherograms were obtained using the DNA sequencer ABI PRISM 3100-Avant (Applied Biosystems); they were then analysed and the sequences aligned using EditSeq and MegAllign software (DNA Star Inc., Madison, Wisconsin).

Real-time 'one-step' RT-PCR

Primers and probes were designed using the Primer Express software (version 1.0) (Applied Biosystems). The segment 5 sequence (accession no. DQ017960) of the field strain BTV-16 (IT8054/02) was chosen as a model. The total amplicon length is 81 bases and the forward (5′-TGGCAACCACCAAACATGG-3′) and reverse (5′-CCAAAAAAGTCCTCGTGG CA-3′) primers identify an area of segment 5 between nucleotides 12 and 93. The TaqMan probe (6FAM-5′-CGCTTTTTGAGAAAATACA ACATCAGTGGGGAT-3′-TAMRA) was labelled at the 5′ end with 6-carboxy-fluorescein and at the 3′ end with 6-carboxy-tetramethylrhodamine and hybridised within the primer-amplified area between nucleotides 33 and 65.

The RT-PCR was conducted in a single step using the SuperScript™ III RT/Platinum® one-step quantitative RT-PCR system (Invitrogen, Carlsbad, California). In the initial denaturation phase, 5 µl viral RNA, 2.25 µM of each primer and RNase-free water to 10 µl volume were heated to 95°C for 5 min and then submerged in ice. The following were then added to each sample: 1× Reaction Mix, 1× ROX reference dye, 0.50 µl SuperScript™III RT/Platinum® Taq Mix, 250 nM TaqMan probe and RNase-free water to a final volume of 25 µl. The retrotranscription was conducted at 50°C for 15 min, followed by a Taq polymerase activation cycle at 95°C for 2 min and 45 PCR cycles of 15 sec at 95°C and 30 sec at 60°C. Samples were analysed in 96-well optical

plates for real-time PCR using the ABI Prism 7700 instrument and sequence detection system software (SDS version 1.7°) (Applied Biosystems).

All samples that demonstrated a threshold cycle (TC) value of less than 40 cycles were considered positive, after the detection threshold had been manually set at 0.1 in line with the instructions of SDS version 1.7a.

Limit of detection

To evaluate the limit of detection (LOD) of the method, serial log dilutions (10^{7}-10^{-3}TCID$_{50}$/ml) of vaccine and field BTV strains of serotypes 2, 4, 9 and 16 were prepared. Each dilution was then subjected to RNA extraction and RT-PCR in quadruplicate, using both the real-time 'one step' and conventional 'one step' RT-PCR methods (13). The LOD was taken as the highest dilution capable of revealing the presence of BTV in all four replicas. Serial log dilutions of RNA extracted from vaccinal BTV-2 with a load of 10^{5} TCID$_{50}$/ml were examined to assess the effect of the extraction phase on the LOD. Results were compared against those obtained when the method was applied to the RNA extracted from each of the log dilutions of the same viral strain.

To assess whether the sample matrix had any effect on the test, a blood sample with EDTA taken from a goat known to be BTV-negative was divided into three aliquots which were then spiked in the laboratory with 6×10^{-1}, 6×10^{-2} and 6×10^{-3} TCID$_{50}$/ml of BTV-2 IT8341/00. Each aliquot was then examined 30 times and the results compared with those obtained when the method was applied to the same dilutions from the virus strain.

Specificity

Specificity was determined through the analysis of viruses genetically related to BTV including African horse sickness (AHS) and epizootic haemorrhagic disease (EHD) viruses of the genus *Orbivirus*, and the avian Orthoreovirus which belongs to the *Reoviridae* family. Further tests were conducted on viruses able to induce clinical pictures similar to that of BTV, specifically contagious ecthyma (orf) virus, bovine viral diarrhoea (BVD) virus and border disease virus (BDV).

Comparison with other virological methods

The accuracy of the diagnostic system developed was evaluated on 104 EDTA blood samples ithat were collected between 2004 and 2005 from sheep and cattle in various regions of Italy (Abruzzo, Emilia, Marche, Sicily and Umbria). Of these, 64 originated from sentinel cattle that had seroconverted to serotypes 2 (n=62) and 16 (n=2), and 40 from sheep and goat farms in officially bluetongue-free areas. Results were compared with those obtained by the RT-PCR assay that is routinely used in our laboratory (18) and virus isolation (26).

Statistical analysis

The TC values obtained for each viral concentration logarithm are reported in orthogonal Cartesian axes. The lines that provided the best fit of points and limiting lines, i.e. those with the maximum and minimum slopes of all those fitting the graph points, were then drawn. The coefficient of linear regression (R), the intercept and the angular coefficients (slope) were calculated for each line. The 95% confidence intervals were then compared.

The efficiency of the real-time PCR was also determined using the formula $[10^{(-1/slope)} -1]$, while the significance of any differences between the various diagnostic tests used and the confidence intervals were calculated using the beta distribution (e+1, n–e+1) where e, the number of events, is the total number of positives and n, the number of tests, is the total number of animals examined. The peak of distribution curve represents the most probable percentage of positive animals, while its breadth gives information on the uncertainty of the estimate due to the sample size. For each statistical analysis, differences with $P<0.05$ were considered as significant.

Results

The sequences obtained were published in Genbank. Their alignment led to identification of a region of ~200 bp, the sequence of which was conserved for all amplified serotypes at the 5′ terminal end, on which a pair of primers and a TaqMan probe were designed (Table I).

Table I
Alignment of region 5′ of segment 5 of the bluetongue virus and localisation of the primers and probe

BTV strain	GenBank access code	Real-time RT-PCR results 5′-3′ sequence	5′ primer position 12-30 TGGCAACCAC CAAACATGG	TaqMan probe position 33-65 CGCTTTTTGAGAAAATAC AACATCAGTGGGGAT	3′ primer position 74-93 TGCCACGAGGACT TTTTTGG
BTV2IT8341/00	DQ017956	+	----------	----------	---------A----------
BTV4IT3036/03	DQ017958	+	----------	----------	---------A----------
BTV4vaccineOBP	DQ017957	+	----------	----------	C--------A----------
BTV9IT12217/00	DQ017959	+	----------	----------	---------A----------
BTV9vaccineOPB	DQ017962	+	----------	----------	---------A----------
BTV16IT8054/02	DQ017960	+	----------	----------	----------
BTV16vaccOBP	DQ017961	+	----------	----------	---------A----------
BTV2vaccineOBP	AY138895	+	----------	----------	----------

BTV bluetongue virus
RT-PCR reverse transcriptase-polymerase chain reaction

The real-time RT-PCR assay detected all BTV serotypes evaluated, whether they had been isolated from the field or originated from vaccine (Table II). A strong linear negative correlation (R^2=0.99) was seen between the virus concentration and the signal emitted by the probe at each amplification cycle (Fig. 1). When compared, the confidence intervals of the intercepts and the angular coefficients of the field BTV-4 did not overlap with those of BTV-2 of both field and vaccine origin indicating that for these strains the real-time system did not have the same performances (Table III). However, the fact that all strains analysed in this study had the confidence intervals of the angular coefficients ranging from –3.1 to –3.6, indicates that the real-time system efficiency is between 90% and 100%. Comparable regression lines were obtained, even for interpolation of the TC values obtained from the analysis of the serial dilutions of the BTV-2 strain and from the analysis of dilutions of RNA extracted from vaccinal BTV-2 (Fig. 2).

The LOD of the real-time RT-PCR method fluctuated between 0.005 and 0.05 $TCID_{50}$/ml, depending on the virus strain. In contrast, the conventional RT-PCR generally yielded higher LODs with a far more variable interval, fluctuating from 0.025 $TCID_{50}$/ml for BTV-4 of vaccine origin to $10^{3.7}$ $TCID_{50}$/ml for field BTV-9 (Table IV).

Table II
Limits of detection of the real-time reverse transcriptase-polymerase chain reaction method and parameters of the correlation line obtained from serial dilutions of the supernatant of tissue cultures infected with the bluetongue virus
Correlation lines were calculated reporting the threshold cycle value on the y-axis and the corresponding log virus concentration on the x-axis
The RT-PCR efficiency was calculated using the formula $E=10^{(-1/m)}-1$

BTV strain	Limit of detection $TCID_{50}$/ml	Correlation coefficient R^2	RT-PCR efficiency
BTV-2 vaccine	0.05	0.999	88%
BTV-4 vaccine	0.02	0.991	101%
BTV-9 vaccine	0.05	0.998	96%
BTV-16 vaccine	0.01	0.996	86%
BTV-2 IT8341/00	0.05	0.995	86%
BTV-4 IT9034/03	0.01	0.997	99%
BTV-9 IT12217/00	0.05	0.998	91%
BTV-16 IT8054/02	0.005	0.990	95%

BTV bluetongue virus
TCID tissue culture infective dose
RT-PCR reverse transcriptase-polymerase chain reaction

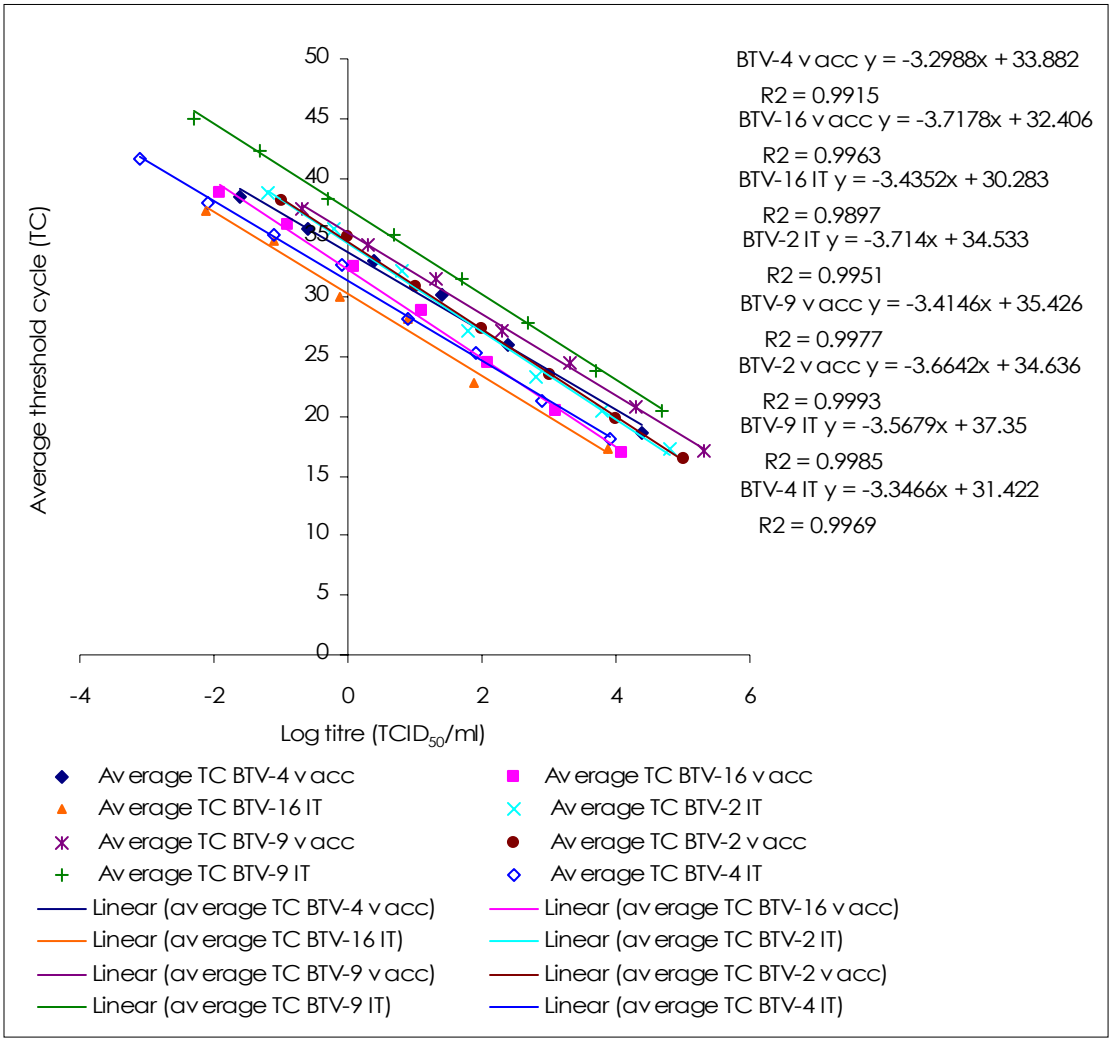

Figure 1
Correlation lines obtained by interpolating the threshold cycle values obtained from the analysis of RNA extracted from successive dilutions of the bluetongue virus serotypes 2, 4, 9 and 16 field strains and their respective vaccine strains

Table III
Estimation of 95% confidence limits calculated for the intercept and angular coefficient of the regression lines of the bluetongue virus strains examined

Strain	Intercept	Lower limit (95%)	Upper limit (95%)	m	Lower limit (95%)	Upper limit (95%)
BTV-16 field	30.661	30.314	31.008	–3.435	–3.602	–3.269
BTV-4 field	31.756	31.518	31.995	–3.347	–3.448	–3.245
BTV-16 vaccine	31.964	31.622	32.306	–3.693	–3.846	–3.540
BTV-4 vaccine	32.722	31.977	33.468	–3.374	–3.732	–3.016
BTV-9 vaccine	34.402	34.114	34.690	–3.415	–3.516	–3.313
BTV-2 vaccine	34.636	34.344	34.928	–3.664	–3.768	–3.561
BTV-2 field	35.276	34.778	35.773	–3.714	–3.890	–3.538
BTV-9 field	38.685	37.969	39.400	–3.654	–3.902	–3.405

BTV bluetongue virus

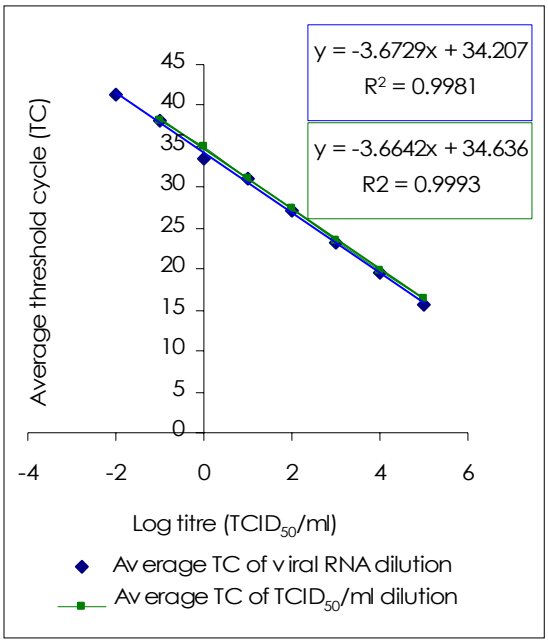

Figure 2
Correlation lines obtained by interpolating threshold cycle values generated by the analysis of RNA extracted from successive dilutions of the vaccine viral strain BTV-2 and those obtained by dilution of RNA extracted from a single aliquot of a known concentration of the same viral strain

Tests of the three groups of blood samples spiked with the field strain of BTV-2 gave 100% positivity (confidence interval: 95%, 90.8-100%) at a virus concentration of 0.63 $TCID_{50}$/ml, 60% positivity (confidence interval: 95%, 42.2-75.5%) at 0.06 $TCID_{50}$/ml and only 10% positivity (confidence interval: 95%, 3.6-25.7%) at 0.006 $TCID_{50}$/ml. The method did not amplify the nucleic acids of the viruses genetically similar to BTV or of those that cause a clinical picture similar to that caused by BTV.

The 40 blood samples that originated from BT-free farms tested negative for BTV with all three tests used in the study (confidence interval: 95%, 92.5-100%). Real-time PCR revealed the presence of genetic BTV material in all sentinel cattle (confidence interval: 95%, 95.5-100%), compared with only 47 detected by RT-PCR (confidence interval: 95%, 61.5-82.7%) ($p<0.05$). BTV was isolated from four samples; three were typed as BTV-2 and one as BTV-16.

Table IV
Comparison of detection thresholds obtained with the conventional 'one-step' reverse transcriptase-polymerase chain reaction I and real-time reverse transcriptase-polymerase chain reaction

Serotype	Detection threshold ($TCID_{50}$/ml)	
	Real-time RT-PCR	Conventional 'one-step' RT-PCR
BTV-2 field	0.05	$10^{1.4}$
BTV-2 vaccine	0.05	0.1
BTV-4 field	0.01	2.5
BTV-4 vaccine	0.02	0.025
BTV-9 field	0.05	$10^{3.7}$
BTV-9 vaccine	0.05	0.2
BTV-16 field	0.005	$10^{2.4}$
BTV-16 vaccine	0.01	$10^{2.1}$

BTV bluetongue virus
TCID tissue culture infective dose
RT-PCR reverse transcriptase-polymerase chain reaction

Discussion

Recent BT epidemics in the Mediterranean Basin, and Italy in particular, have graphically shown the need for an automated diagnostic test that can rapidly process large numbers of samples and also identify all variants of the virus that have circulated since 2000, with sensitivity and specificity comparable or superior to the currently used laboratory tests. NATs in molecular diagnostics are now considered the 'gold standard' for the highly sensitive and specific identification of genome targets in biological samples, and have become an essential tool for all test laboratories (14). Real-time PCR (9, 10) is an innovative quantitative PCR model with enormous potential compared with the 'old style' PCR, thanks to its diagnostic flexibility and rapid results. These features make it an extremely useful tool not only for routine diagnosis but also for identification of new pathogens. As there is no detection phase after the amplification process, the test procedure is considerably shorter than the conventional RT-PCR and, as the amplification product is

detected during the PCR cycles, the process can be easily automated with ease.

The use of this method in the virus diagnostic sector is constantly increasing. In the diagnosis of BT, the real-time processes reported to date have given encouraging results but their use has been limited by the application to only some serotypes, making them suitable for a reduced number of epidemiological situations. There is no real-time RT-PCR method which has been formally validated to detect all 24 BTV serotypes in field conditions. Consequently, the application of each protocol must be performed in every different epidemic situation in a given geographic area since a single nucleotide mismatch in the probe hybridisation area can drastically reduce the sensitivity of the method (11).

In the recent BT epidemic, the geographic position of Italy facilitated the introduction of BTV serotypes 2, 4, 9 and 16. Vaccination campaigns and the possible spread of homologous vaccine strains into the environment have complicated the epidemiological picture, to the extent that it is possible that Italy – in contrast with all other countries in the Mediterranean Basin – may have as many as eight distinct BTV strains in circulation, and further variants of these likely will arise over time. For this reason, it was necessary to design an ad hoc real-time protocol capable of satisfying these specific diagnostic requirements, given that the real-time RT-PCR recently refined by Jiménez-Clavero *et al.* (11) was unable to identify the 16 BTV vaccine strains or the field BTV-9 strains isolated in Italy (data not shown). It was decided to use segment 5 as the amplification target, due to its high level of conservation in the various serotypes (2, 33).

To reduce the test time, the RT-PCR was executed in a single step. In the extraction phase, the high pure viral nucleic acid kit was preferred over Trizol, which is already used in the one-step RT-PCR but which does not guarantee an adequate level of RNA purity and does not enable automation of the extraction process. The real-time system thus developed was able to rapidly identify (in no more than 4 h) all the serotypes examined, with comparable LODs, reaching thresholds far superior to those obtained with the RT-PCR used in routine diagnosis. Following the preliminary tests, the method was also shown to identify serotypes 1, 8, 13, 14 and 19 (data not shown).

The improved sensitivity was also confirmed by the greater number of correctly identified field positive samples although they all belong to serotypes 2 and 16, despite differences in one or two nucleotides between the reverse primer and the complementary viral RNA sequence (Table I). Results confirm the above observations; i.e. while differences in even one nucleotide in the probe hybridisation zone can cause a drastic loss of sensitivity, differences of one or two nucleotides on the primer sequences seem to have a lesser impact (11). The high RT-PCR efficiency (>86%) further confirms the accuracy and reproducibility of the test. The closer the value to 100%, the more accurate and reproducible the results. This value is affected by the length of the amplicon, its G/C content and its secondary structure. It also depends on the reaction dynamics and variations may be due to the enzymes used or non-optimal reagent concentrations (22).

The decision to conduct the RT-PCR in a single step did not affect its sensitivity or efficiency (Table II), while the similarity of the regression lines deriving from the interpolation of the TC values, obtained from the serial viral RNA dilutions and those from the serial viral strain dilutions (Fig. 2), confirms that the extraction process had no effect on the sensitivity of the method. In addition to its high sensitivity, the method was also found to be highly specific, as it did not amplify any nucleotide sequence of genetically similar viral strains (AHS, EHD and avian reovirus) or those causing a similar clinical picture to that of BT. It is therefore extremely valuable in the differential diagnosis of diseases such as orf, BDV and BVD.

The real-time diagnostic efficacy was further corroborated when the method was tested on spiked blood samples, where the viral RNA, similar to the field situation, is found in a medium that is relatively rich in nucleic acids of cellular origin. Here too, the real-time PCR was able to detect virus concentrations

(6×10^{-3} $TCID_{50}$/ml) similar to those it detected for the viral strains. At the concentration of 0.06 $TCID_{50}$/ml, 60% of samples tested positive for BTV with a mean TC value of 37.8 ± 0.6 cycles. No differences were observed when cattle, goats or sheep blood was used (G. Savini, personal communication). This result indicates the importance of considering the advantage offered by the real-time method over conventional RT-PCR when the quantity of target viral RNA is close to the LOD of the method. With conventional PCR, the nucleic acid band amplified in the gel must be read visually to interpret the final qualitative result, thus giving a subjective evaluation of positivity, with real-time PCR, the result is expressed quantitatively and objectively. Furthermore, as the nucleic acid is measured during the amplification cycles, without necessitating further product manipulation, this test method considerably reduces the risk of contamination due to the amplified products, which are often a further source of doubt in interpreting results in conventional PCR systems, especially in nested-PCR methods.

Conclusion

The real-time RT-PCR method described detected all BTV serotypes present in Italy, including those of vaccinal origin and serotype 1 that had recently been recorded in Greece. It has the requisites necessary to monitor the spread of the virus through the foci or can be used as a screening test before proceeding with more complex and costly methods. Given that the presence of viral nucleic acid is not indicative of ongoing viremia (31), confirmation of the result with viral isolation is recommended. As well, in view of the continued evolution and possible emergence of new BTV strains in Italy, the test, as it was designed, requires a constant monitoring. Its constant use in parallel with other virological tests would give additional information on its diagnostic sensitivity and specificity related to the viral strains circulating in Italy.

Not least of the advantages of the method over conventional RT-PCR is its ability to quantify nucleic acids and detect multiple targets with the same amplification reaction (multiplexing). The ability to use multiplexing enables constant control of the entire test process, by including a viral target of known concentration among the test samples (internal positive control or IPC). The identification and optimisation of a suitable IPC to be amplified alongside the target viral nucleic acid enables detection of any false reactions attributable to the poor quality of the extracted RNA or to PCR inhibition phenomena.

The selection and optimisation of a suitable IPC and verification of RT-PCR ability to recognise all 24 BTV serotypes is therefore the priority for further improvement of the method.

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