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Validation of universal and serotype-specific real-time RT-PCR assays for the detection of European bluetongue virus serotypes

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Summary

The aim of this study was to validate real-time RT-PCR (rRT-PCR) assays for the detection and typing of bluetongue virus (BTV) serotypes recently circulating in Europe. The universal rRT-PCR assay (for all BTV genotypes) was based on a highly conserved region in BTV RNA segment 1, and the serotype-specific (BTV typing) rRT-PCR was based on the BTV Seg-2 target gene encoding the highly variable outer shell protein VP2. The rRT-PCR techniques applied here are very fast (approximately 4 h), specific and sensitive for the detection and identification of BTV serotypes. Using the BTV-typing rRT-PCR, it was possible to identify European BTV serotypes 1, 2, 4, 6, 8, 11 and 16 in archival blood samples collected in 2008-2011 for the purpose of a ring trial for BTV genome and antibody detection. This assay may therefore be considered as a valuable tool complementing the routine diagnostic procedure for BTV diagnosis.

Keywords: bluetongue virus, detection, typing, real-time RT-PCR

Bluetongue (BT) is an infectious, non-contagious arboviral disease of domestic and wild ruminants that induces variable clinical signs depending on the host species and breed (14). BT has a heavy economic impact, mainly due to the effect of the disease on animals (morbidity, mortality, reproductive failure, reduction in milk yields and weight gain) and, most of all, to the disruption of international trade in animals and animal products (23, 24). The disease is transmitted by blood-feeding midges of the genus *Culicoides* (*Diptera Ceratopogonidae*) (15). The aetiological agent of BT, bluetongue virus (BTV), belongs to the family *Reoviridae* and the genus *Orbivirus* (17). Twenty-four immunologically distinct serotypes (BTV1 to BTV24) of the virus were identified worldwide by 2008 (24). In 2008 an additional putative BTV serotype 25 (Toggenburg virus) was isolated from goats in Switzerland (4) and recently a novel BTV serotype 26 was identified in Kuwait (10). The genome of BTV consists of ten linear double-stranded RNA genome segments encoding seven structural proteins, from VP1 to VP7, and three non-structural proteins, NS1, NS2 and NS3/NS3a (22).

Historically, BTV was confined mainly to tropical and temperate areas, including America, Australia, Africa, and some regions of Asia (28). In August 2006, for the first time, BTV passed the latitude 50°, and BT outbreaks caused by BT virus serotype 8 occurred in north-western Europe: the Netherlands, Belgium, Germany, France, and Luxembourg (29). In 2007 and 2008, an infection caused by BTV8 spread rapidly across Europe, and new BTV serotypes (6, 11 and 16) were detected (6, 30). Mass vaccination campaigns implemented in Europe in spring 2008 quickly limited the spread of BTV-8, and as a result only one case of BTV8 was noted from May 1, 2011, to February 12, 2012. In the same season, 8, 4 and 2 cases of BTV serotypes 1, 4 and 16 were noted, respectively (http://eubtnet.izs.it/btnet/reports/Outbreaks.html).

The laboratory diagnosis and the identification of the BTV serotype involved is currently done by various antibody-detection methods, including antigen capture, agar gel immunodiffusion (AGID), c-ELISA and virus neutralisation (VN) assays (5, 8, 20). BTV in clinical samples is detected by virus isolation in cell cultures (BHK-21, Vero) or in embryonated chicken eggs (ECE), ELISA, immunofluorescence, dot immunobinding assay (DIA) and immunoelectron microscopy (5). The detection of the viral RNA is usually done by various traditional gel-based reverse transcription

– polymerase chain reaction (RT-PCR) or real-time RT-PCR (rRT-PCR) assays (2, 25, 26, 31).

As mentioned above, especially BTV serotypes 1, 4, 6, 8, 11 and 16 have been a concern to veterinary authorities in Europe since 2008. Therefore, fast, reliable, and sensitive assays are needed to correctly detect members of specific BTV serotypes. The aim of this study was to validate rRT-PCR assays for the detection and typing of BTV serotypes currently circulating in Europe.

Material and methods

Sample origin. Four panels (of 10 samples each) of EDTA-treated blood samples collected in 2008-2011 for the purposes of a ring trial for BTV genome and antibody detection were tested. In addition, 38 archival BTV-positive samples of blood taken from seropositive cattle imported from Germany were used. A blood sample collected from uninfected sheep, provided by the Community Reference Laboratory for BT (CRL BTV), Pirbright, UK, was used as a negative control (K–).

RNA extraction and denaturation. RNA was extracted from the EDTA blood samples with a QIAamp Viral RNA Mini Kit (Qiagen), according to the method recommended by the manufacturer. Extracted RNA was denaturated by the incubation of the samples for 5 min at 100°C, and then cooled to 4°C (18).

Oligonucleotide primers. A combination of two primer sets (BTVrsaF and BTVrsaR, BTVuniF and BTVuniR) representing eastern and western BTV serotypes and two probes (RSA-BTV and BTV) labelled with 6-carboxyfluoroscein (FAM) at the end 5' and with 6-carboxytetramethylrhodamine (TAMRA) at the end 3' targeting BTV segment 1 were used according to Shaw et al. (25).

Real-time RT-PCR conditions (all BTV genotypes). The rRT-PCR was performed in a MicroAmp optical 96--well reaction plate (Applied Biosystems, USA) in one-step reaction, using a QuantiTect Probe PCR Kit (Qiagen). The reaction mixture at a volume of 20 µl contained as follows: 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 1 µl (20 pmol) of each of four primers, 0.5 µl (5 pmol) of both probes, 1.25 µl of $MgSO_4$ (25 mM), 0.1 µl of RNasin, 0.2 µl of QuantiTect RT Mix, and 0.95 µl of RNase-free water. A volume of 5 µl of the extracted and denatured RNA was added to the reaction mix (total volume of 25 µl), and the reaction was capped with optical caps (Applied Biosystems). The plate was transferred to a thermal cycler (7300 Real Time PCR System, Applied Biosystems), and amplification was carried out according to the following programme: 55°C for 30 min, 1 cycle (reverse transcription), 95°C for 15 min (1 cycle) to activate the DNA polymerases and inactivate the reverse transcriptases, and 45 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescence was measured at the end of the 60°C annealing/extension step. A cycle threshold (C_T) value (the point on the x-axis showing the number of cycles of replication where the fluorescence breached a threshold fluorescence line) was assigned to all PCRs after the amplification.

Real-time RT-PCR conditions (BTV typing). BTV serotypes 1, 2, 4, 6, 8, 11 and 16 were specifically detected with a TaqVet European BTV Typing (1-2-4-6-8-9-11-16) Kit (Laboratoire Service International, France). The reaction mixture contained 20 µl of the BTV-type 1, 2, 4, 6, 8, 11, or 16 mix and 5 µl of the extracted and denatured RNA (total volume of 25 µl), and the reaction was capped with optical caps (Applied Biosystems). The plate was transferred to the thermal cycler (7500 Real Time PCR System, Applied Biosystems), and amplification was carried out according to the following programme: 45°C for 10 min, 1 cycle (reverse transcription), 95°C for 10 min (1 cycle) to activate the DNA polymerases and inactivate the reverse transcriptases, and 40 cycles of 95°C for 15 s and 60°C for 45 s. The fluorescence was measured at the end of the 60°C annealing/extension step. A cycle threshold (C_T) value (the point on the x-axis showing the number of cycles of replication where the fluorescence breached a threshold fluorescence line) was assigned to all PCRs after the amplification. According to the manufacturer's specification, a sample was considered positive if its C_T value was lower than 40.

Results and discussion

The coexistence of several BTV serotypes in Europe requires the use of rapid, sensitive, and specific methods for the precise identification of currently circulating BTV serotype in the field. Conventional procedures for BTV typing involve virus isolation, adaptation to cell culture, and serological neutralisation assay that may take several weeks to complete. These serological assays may also give inconclusive results, particularly if the sample contains more than one BTV serotype (1). Differentiation between coexisting BTV serotypes can also be achieved using sequence analysis (13), but this technique is not suitable for routine high--throughput diagnosis.

In recent years, several RT-PCR have been developed to serotype circulating BTV strains. A multiplex RT-PCR-based assay was used for simultaneous detection and differentiation of five North American BTV serotypes 2, 10, 11, 13, and 17 in cell culture and clinical samples (3). Maan et al. (12) developed RT--PCR-based assays for the typing of European strains of BTV and the differential diagnosis of field and vaccine strains. Others developed a rRT-PCR for the detection of BTV4 in the Mediterranean region (21) and BTV8 rRT-PCR in regional German laboratories (7). Mertens et al. (16) described the design and evaluation of rapid, sensitive and specific RT-PCR-based assays (and primers) to detect members of European BTV serotypes and to distinguish eastern and western Seg-2 topotypes within each serotype. The highly sensitive rRT-PCR assays directed to BTV genome Seg-2, for a specific detection of BTV1, 6, and 8 in animal samples was developed and introduced to the routine diagnosis at the German National Reference Laboratory for BT (9). Besides, Vandenbussche et al.

Tab. 1. C_T value of archival BTV-positive blood samples from the 2008-2011 ring trials for BTV genome and antibody detection obtained by a rRT-PCR assay

Sample	BTV serotype (isolate)	C_T value
2815/08	BTV-1 (Spanish pool 1,2)	20.36
2820/08	BTV1(Spanish pool 3,4,5)	27.46
2310/09	BTV1 (GIB 2007/01)	25.07
27-3/10	BTV1 (OMN 2009/01)	24.08
27-9/10	BTV1 (MOR 2009/01)	19.43
29-14/11	BTV1(ALG 2006/04)	18.87
29-15/11	BTV1(ALG 2006/04)	19.23
2812/08	BTV2 (ITL 2002/07)	24.51
27-8/10	BTV2 (FRA 2001/06)	27.04
2814/08	BTV4 (SPA 2005/05)	25.71
27-7/10	BTV4 (MOR 2009/07)	29.42
29-13/11	BTV4 (MOR 2009/09)	22.18
2301/09	BTV6 (NET 2008/06)	24.12
2305/09	BTV6 (NET 2008/05)	23.78
27-4/10	BTV6 NET 2008/05)	23.75
2811/08	BTV8 (Denmark)	28.72
2817/08	BTV8 (Denmark)	22.64
2819/08	BTV8 (356/07)	30.48
2303/09	BTV8 (UKG 2008/01)	25.25
2305/09	BTV8 (NET 2008/05)	23.78
2306/09	BTV8 (GIB 2007/01)	23.78
27-10/10	BTV8 (UKG 2008/01)	29.46
29-11/11	BTV8 (FRA 2009/01)	20.72
29-18/11	BTV8 (FRA 2009/01)	17.48
2308/09	BTV11 (USA 2005/02)	23.95
2304/09	BTV16 (ISR 2008/03)	33.82
27-5/10	BTV16 (OMN 2009/02)	36.98
29-16/11	BTV16 (CYP 2010/03)	22.75

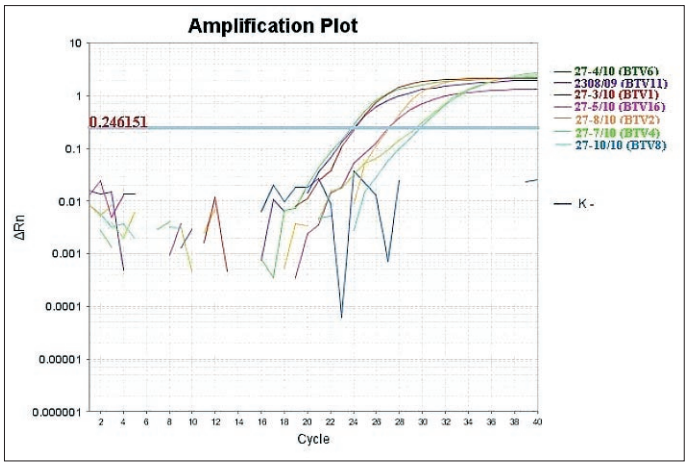

Fig. 1. Logarithmic fluorescence plots versus cycle number resulting from the determination of BTV RNA in blood samples by rRT-PCR for the typing of BTV

(27) described and validated four real-time RT-PCR assays for the serotyping of BTV serotypes 1, 6, 8, and 11, the usefulness of which was clearly demonstrated during a BT outbreak in Belgium in 2008.

An accurate and reliable rRT-PCR technique for the detection of BTV RNA in blood samples was introduced to the routine diagnosis in our laboratory at the end of 2007. Using this assay, we were able to detect the presence of viral RNA in 38 blood samples from animals imported from BTV-affected countries (19). However, no precise identification of virus serotype was possible with this technique. That is why we introduced a rRT-PCR assay for the typing of BTV serotypes currently circulating in Europe. This rRT--PCR is based on a BTV Seg-2 target gene encoding the highly variable outer shell protein VP2 (22). Since sequencing studies (of all 24 serotypes) have confirmed that variations in the nucleotide sequence in Seg-2 correlate with differences in the virus serotype (11), this BTV genome segment was chosen as a target for the serotype-specific RT-PCR assay.

A negative C_T value for any test and control sample that corresponded to C_T of = 40 was selected as the positive/negative cut-off C_T values obtained as a consequence of examining blood samples by the rRT-PCR assay for all BTV genotypes. Then, all BTV-positive samples were retested by rRT-PCR for BTV typing. Using this technique, we were able to identify European BTV serotypes 1, 2, 4, 6, 8, 11 and 16 in archival blood samples collected during 2008-2011 for the purpose of the ring trial for BTV viral genome and antibody detection (Fig. 1, Tab. 1). Moreover, we found that all archival BTV-positive samples of blood taken from seropositive cattle imported to Poland from Germany were positive for BTV8 and had C_T values from 21.16 to 35.74 (data not shown).

In conclusion, it may be assumed that the rRT-PCR recently introduced in our laboratory are a useful technique for the identification and typing of BTV serotypes recently circulating in Europe. The rRT-PCR applied in our research is very fast (approximately 4 h), specific and sensitive for the typing of BTV serotypes currently circulating in Europe. It may therefore be considered as a valuable tool complementing the routine diagnostic procedure for BTV diagnosis.

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