Typing of European Bluetongue Virus Serotypes 1, 6, and 8 by Real-Time RT-PCR

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Abstract

A novel real-time RT-PCR (rtRT-PCR) for the detection and typing of bluetongue virus (BTV) in EDTA treated blood samples taken from BTV infected animals was described. This rtRT-PCR was based on BTV Seq-2 target gene encoding the highly variable outer shell protein VP2. The applied PCR was accurate, specific, and reliable technique for the detection of a specific sequence for a BTV type and endogenous internal positive control (IPC) in the same well. Using this technique, it was possible to identify the European BTV serotypes 1, 6, and 8 in archival blood samples supplied during 2008-2010 for the purpose of the ring trial for BTV genome and antibody detection. Moreover, it was shown that all archive BTV positive blood samples taken from seropositive cattle imported to Poland from Germany were positive for BTV8. This method was much faster (approximately 4 h) and more precise than conventional serological typing methods. In addition, the used thermal cycler allows the use of 96-well plate formats, which further increases the capacity and speed of the analysis. Therefore, it seems to be a valuable complementing tool for a routine diagnosis of BTV infection.

Key words: cattle, bluetongue virus, typing, real-time RT-PCR.

Bluetongue (BT) is a non-contagious viral disease of domestic and wild ruminants that induces variable clinical signs depending on the host species and breed (15). The disease is transmitted by arthropod vectors of the genus Culicoides (17). It is caused by bluetongue virus (BTV), the species of the genus Orbivirus within the family Reoviridae. Twenty-four immunologically distinct serotypes (BTV1 to BTV24) of the virus have been identified worldwide until 2008 (9). Recently, a novel bluetongue virus (BTV25?) termed Toggenburg orbivirus (TOV) was detected in goats from Switzerland by using real-time reverse transcription-PCR (rtRT-PCR) (5). BTV is a small (about 70 nm in diameter) icosahedral virus with a ten-segmented, double-stranded RNA (dsRNA) genome (27), which is packaged within an icosahedral nucleocapsid composed of seven structural proteins (23). The inner capsid, forming the core, is made up by two major (VP7 and VP3) and three minor (VP1, VP4 and VP6) viral proteins, which are relatively conserved among members of the BTV serogroup. The outer capsid is composed of two proteins, VP2 and VP5, which exhibit a high degree of sequence variation both between and within each serotype (23). Besides, the viral subcore consist of nonstructural proteins NS1, NS2, NS3, and NS3A, which participate in the control of BTV replication, maturation, and export from infected cell (23, 24). The BTV is associated with the red cells, it may persist in these cells even after the development of a high humoral antibody response (4).

The distribution of BT is determined by the geographical distribution of the arthropod vector (17). Until recently, BTV was confined mainly to tropical and temperate areas, including America, Australia, Africa, and some regions of Asia (28). Historically, Europe has experienced only sporadic incursions of BT, involving a single virus serotype on each occasion (16). However since 1998, BT outbreaks have occurred annually, involving strains from six distinct BTV serotypes – 1, 2, 4, 8, 9, and 16 (18). In August 2006, for the first time, the BTV passed the latitude 50° and BT outbreaks occurred in Central-Western Europe: the Netherlands, Belgium, Germany, France, and Luxembourg (29). In 2007-2008, the BT situation changed for the worse; BTV serotype 8 spread to the other regions of Europe, the number of outbreaks increased rapidly and new BTV serotypes (BTV1 and BTV6) were detected (30). However, the implementation of BT compulsory vaccination programs in Europe in spring 2008 resulted in reduction of BTV8 cases to 81 in the season 01 May 2009 – 16 November 2010 and in the same period, 670 cases caused by BTV serotype 1 were noticed (11).

As described above, especially BTV serotypes 1, 8, and 6 are the concern to veterinary authorities in Europe. Therefore, the fast, reliable, and sensitive assays are needed to correctly identify the circulating BTV
serotypes in the field. In Poland, the virological surveillance of all susceptible animals imported from EU member states was started in December 2007. So far, the BTV RNA was detected by rRT-PCR in 38 samples of blood collected from German cows, one sample from a Dutch fallow deer, and one from a 4-week-old calf born in Poland from a seropositive German dam (21). It was supposed, that all BTV positive samples were serotype 8 but no precise typing was performed.

The aim of this study was to apply a rRT-PCR assay for the typing of BTV serotypes currently circulating in Europe.

**Material and Methods**

**Sample origin.** Three panels (each of 10 samples) of EDTA treated blood samples collected in 2008-2010 for the purposes of the ring trial for BTV genome and antibody detection were tested. In addition, 38 archive BTV positive samples of blood taken from seropositive cattle imported from Germany were used. The blood samples collected from uninfected and experimentally infected sheep 5 d post infection provided by CRL BTV, Pirbright, UK, were used as negative (K-) and positive (K+) controls, respectively.

**RNA extraction and denaturation.** RNA was extracted from the EDTA blood samples using the QiAamp Viral RNA Mini Kit (QIAGEN), according to the method recommended by the manufacturer. Extracted RNA was denatured by incubation of the samples for 5 min at 100°C, and then cooled to 4°C (20).

**Oligonucleotide primers.** Combination of two primer sets (representing eastern and western BTV serotypes) and two probes labelled with 6-carboxyfluorescein (FAM) at the 5′ end and with 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end, targeting BTV segment 1 were used according to Shaw et al. (25). The primers sequences were as follows: BTVrsaF (5′-GGTCTTGAAGTTTACATCAA-3′), BTVrsaR (5′-TGTCATCTCTTCTAGACA-3′), BTVuniF (5′-GGTTTGTAGGTGTACGTGAA-3′), BTVuniR (5′-TCTTGGAACTCTATAATTACG-3′), BTV segment 1 probes sequences: RSA-BTV (5′-CGGATCAGTGACTCCACAGG-3′), and BTV (5′-TCCTCAGTCAAGTTCACTCCAC-3′). Oligonucleotide primers and fluorogenic probes were prepared in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences in Warsaw.

**Real-time RT-PCR conditions (all genotypes).** The rRT-PCR was performed in MicroAmp optical 96-well reaction plate (Applied Biosystems, USA) in one-step reaction using the QuantiTect Probe PCR Kit (QIAGEN). The reaction mixture at the volume of 19 µl contained as follows: 12.5 µl of 2x QuantiTect Probe RT-PCR Master Mix, 1 µl (20 pmol) of each of four primers: BTVrsaF, BTVrsaR, BTVuniF, and BTVuniR, 0.5 µl (5 pmol) of probes RSA-BTV and BTV, 1.25 µl of MgSO4 (25 mM), 0.1 µl of RNasin, 0.2 µl of QuantiTect RT Mix, and 1.15 µl of RNase free water. The volume of 6 µl of the extracted and denatured RNA was added to the reaction mix (total volume of 25 µl) and the reaction plate was capped using optical caps (Applied Biosystems). The plate was transferred to the thermal cycler (7300 Real Time PCR System, Applied Biosystems) and amplification was carried out using 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. 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 PCR reactions after the amplification.

**Real-time RT-PCR conditions (BTV typing).** BTV serotypes 1, 6, and 8 were specifically detected with the Taquet European BTV Typing (1-2-6-8-9-11-16) Kit (Laboratoire Service International, France). The reaction mixture contained 20 µl of the BTV-Type 1, 6, or 8 mix and 5 µl of the extracted and denatured RNA (total volume of 25 µl) and the reaction plate was capped using optical caps (Applied Biosystems). The plate was transferred to the thermal cycler (7300 Real Time PCR System, Applied Biosystems) and amplification was carried out using 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. 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 PCR reactions after the amplification. According to the manufacturer’s specification, a sample was considered to be positive if its C_T value was lower than 40. The C_T value of internal positive control (IPC) should be lower than 30.

**Results**

The 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 the blood samples by rRT-PCR assay for all BTV genotypes. Then, all BTV positive samples were retested by rRT-PCR for BTV typing. An exemplary image of this rRT-PCR result is presented in Fig. 1.
Fig. 1. Logarithmic fluorescence plots versus cycle number resulting from the determination of BTV RNA in blood samples by rRT-PCR for typing of BTV.

<table>
<thead>
<tr>
<th>Sample</th>
<th>BTV serotype</th>
<th>C&lt;sub&gt;T&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2815/08</td>
<td>BTV1</td>
<td>20.36</td>
</tr>
<tr>
<td>2820/08</td>
<td>BTV1</td>
<td>27.46</td>
</tr>
<tr>
<td>2310/09</td>
<td>BTV1</td>
<td>25.07</td>
</tr>
<tr>
<td>2301/09</td>
<td>BTV6</td>
<td>24.12</td>
</tr>
<tr>
<td>2305/09</td>
<td>BTV6</td>
<td>23.78</td>
</tr>
<tr>
<td>2811/08</td>
<td>BTV8</td>
<td>28.72</td>
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<tr>
<td>2819/08</td>
<td>BTV8</td>
<td>30.48</td>
</tr>
<tr>
<td>2303/09</td>
<td>BTV8</td>
<td>25.25</td>
</tr>
<tr>
<td>2306/09</td>
<td>BTV8</td>
<td>23.78</td>
</tr>
</tbody>
</table>

BTV blood samples from the 2010 bluetongue inter-laboratory proficiency test collected from BT seropositive cattle imported from Germany had C<sub>T</sub> values of 16.57 (IPC 21.34) for BTV1, 18.85 (IPC 23.48) for BTV6, and 28.24 (IPC 20.68) for BTV8, respectively (Fig. 1). Moreover, some of the archival BTV positive samples from the 2008 and 2009 ring trials for BTV genome and antibody detection were positive for BTV serotypes 1, 6, and 8 (Table 1). All archive BTV positive samples of blood taken from seropositive cattle imported from Germany were positive for BTV8 and had C<sub>T</sub> values from 21.16 to 35.74 (data not shown).

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 typing BTV involve virus isolation, adaptation to cell culture, and serological neutralisation assay that may take several weeks to complete. These serological assays can also give inconclusive results, particularly if the sample contains more than one BTV serotype (1, 7). BTV serotype can also be identified by the specificity of neutralising antibodies generated by the specific strain during infection. However, sequential infection with multiple BTV serotypes generates antibodies that neutralise serotypes not encountered previously (12), potentially making typing by this method unreliable, particularly in areas where multiple serotypes are co-circulating. Differentiation between coexisting BTV serotypes can also be achieved using sequence analysis (14), but this technique is not suitable for routine high-throughput diagnosis.

In recent years, several RT-PCR were 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 (2). BTV2 and BTV9 specific real-time RT-PCR assays were used to differentiate Italian BTV field strains from South African modified live vaccine strains (8). The others developed a rRT-PCR for the detection of BTV4 in the Mediterranean region (22) and BTV8 rRT-PCR in regional German laboratories (6). Mertens et al. (19) 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. Recently, the highly sensitive rRT-PCR assays directed to BTV genome Seg-2, for specific detection of BTV1, 6, and 8 in animal samples was developed and introduced to the routine diagnostic at the German National Reference Laboratory for BT (10). Besides, Vandenbussche et al. (26) described and validated four real-time RT-PCR assays for the serotyping of BTV serotypes 1, 6, 8, and 11, which usefulness was clearly demonstrated during the 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 diagnostic in our laboratory at the end of 2007. So far, by this assay we were able to detect the presence of viral RNA in 40 blood samples from animals imported from BTV-affected countries (21). However, using this technique we could detect all BTV genotypes but the precise identification of the virus serotype was not possible. Therefore, we decided to apply the fast, sensitive, and specific method, which allows the simultaneous detection of a specific sequence for a BTV type and endogenous IPC in the same well. The IPC allows verification of the efficiency of RNA isolation and the absence of inhibitors in samples (3). This rRT-PCR is based on BTV Seq-2 target gene encoding the highly variable outer shell protein VP2 (23). Sequencing studies (of all 24 serotypes) have confirmed that variations in the nucleotide sequence in Seq-2 correlate with differences in virus serotype (13), therefore this BTV genome segment was chosen as a target for the serotype-specific RT-PCR assay. Using this technique we were able to identify European BTV serotypes 1, 6, and 8 in archival blood samples supplied during 2008-2010 for the purpose of the ring trial for BTV viral genome and antibody detection (Fig. 1, Table 1). Moreover, we found, that all archive BTV positive samples of blood taken from seropositive cattle imported from Poland to Germany were positive for BTV8.

In conclusion, it may be assumed that the novel rRT-PCR recently introduced in our laboratory is a powerful technique for the identification and typing of BTV serotypes now present in Europe. This method is much faster (approximately 4 h) and more reliable than conventional serological typing method. In addition, the used thermal cycler allows the use of 96-well plate formats, which further increases the capacity and speed of the analysis. Therefore it is seen as a valuable tool to complement the routine diagnostic procedure for BTV diagnosis.

References
11. http://eubtnet.izs.it/btnet.reports/BTV8.html


