APPLICATION OF REAL-TIME REVERSE TRANSCRIPTION PCR FOR THE DETECTION OF BLUETONGUE VIRUS

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Abstract

Real-time RT-PCR (rRT-PCR) for the detection of bluetongue virus (BTV) in EDTA treated blood samples taken from BTV infected animals was described. A combination of two primer sets (representing eastern and western BTV serotypes) and two Taqman probes specific for a highly conserved region in BTV RNA segment 1 were used. The assay detected the viral RNA in blood samples collected from seropositive cattle imported to Poland from Germany at the end of 2007. No BTV RNA was detectable in samples from uninfected sheep. The rRT-PCR can provide quantitative as well as qualitative information and is more sensitive and much faster to perform than the conventional RT-PCR. It can be used in large-scale screening, because of its ability to simultaneous analysis up to 96 samples per run. The applied rRT-PCR is an accurate and reliable technique for the detection of BTV in blood samples.

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

Bluetongue (BT) is an infectious but non-contagious, arthropod-borne viral disease. It affects camelids and other domestic and wild ruminant species (10). The most common symptoms of the disease include fever, catarrhal stomatitis, rhinitis, enteritis, and lameness. The mortality rate can vary from 0 to 30%, but may reach 75% (13). BT is caused by the BT virus (BTV), the species of the genus Orbivirus within the family Reoviridae. Twenty-four serotypes (BTV-1 to BTV-24) of the virus have been identified so far over the world (4). The BT viral genome comprises 10 double-stranded (ds) RNA segments varying in size from 0.5 to 2.7 kDa, which encode for seven structural proteins (VP1-VP7) and four non-structural proteins (NS1-NS3 and NS3A) (21). VP2 and VP5 are variable proteins located in the outer capsid of the virion determining the antigenic variability of the virus and serotype (3). VP7 is the major immunodominant serogroup antigen (21) and is used widely for the identification of the bluetongue serogroup by serological assays (5). VP1 is the viral RNA-dependent RNA polymerase (BTV Seg-1 encodes) that is found in the sub core of the virion (22). The pathogenesis of BTV infection is similar in sheep and cattle, and most probably in all ruminant species (10). The BTV is associated with the red blood cells. It may persist in these cells even after the development of a high humoral antibody response (2). It was shown that the BTV RNA could be detected in the whole blood of infected sheep for at least 30 d post infection (d p.i.) and in the blood of infected cattle even up to 90 d p.i. (1).

The distribution of BT is determined by the geographical distribution of the arthropod vector and includes Africa, southern Asia, Australia, the Middle East, and the Americas (26). Historically, Europe has experienced only sporadic incursions of BT, involving a single virus serotype on each occasion (14). However, since 1998, BT outbreaks have occurred annually, involving strains from six distinct BTV serotypes – 1, 2, 4, 8, 9 and 16 (15). Since August 2006, for the first time, the BTV passed the latitude 50°N and BT outbreaks occurred in the northern part of the EU: the Netherlands, Belgium, Germany, France, and Luxembourg (18, 25). In 2007, BTV-8 spread to the other regions of Europe, where the disease has never been noticed before: the United Kingdom, Denmark, Switzerland, and Czech Republic. In total, 31 588 outbreaks caused by BTV serotype 8 were reported by EU member States during 2007 in the North-Western Europe (7).

The occurrence of BT in the border countries suggest that Poland is now at high risk of BTV epizootic infection. The threat posed by BT highlights a need for reliable assay systems to detect BT infection. Since 2006, we introduced the c-ELISA and agar-gel immunodiffusion (AGID) assays to determine seroprevalence of BTV specific antibodies (18). The BT monitoring studies were continued in 2007, and up to the end of that year, about 10 000 serum samples collected from susceptible animals imported into Poland from European countries affected by BTV were tested. Subsequently, a conventional RT-PCR for the
detection of BTV in blood samples collected from seroreagents was applied in the laboratory (17).

The aim of this study was to determine diagnostic value of real-time RT-PCR (rRT-PCR) assay for the rapid detection of BTV in blood samples taken from BTV infected animals.

Material and Methods

Sample origin. A panel of EDTA treated blood samples collected in 2007 for the purposes of the ring trial for BTV (serotype 8) viral genome and antibody detection, originated from: sheep - uninfected, sheep - infected in the field in the Netherlands; dilutions 1/5, 1/10, 1/20, and 1/100. In addition, two samples (082 and 083) of blood taken from seropositive cattle imported from Germany were tested. The samples of blood collected from uninfected and experimentally infected sheep 5 d p.i. provided by CRL BTV, Pirbright, UK, were used as a negative (K-) and positive (K+) controls, respectively. Moreover, one sample of RNA extracted from foot-and-mouth disease virus (FMDV), serotype O, Kaufbeuren, kindly provided by Dr. O. Marquardt from the Federal Research Centre for Virus Diseases of Animals in Tübingen (Germany), was used.

RNA extraction and denaturation. RNA was extracted from the EDTA blood samples by use of the QIAamp Viral RNA Mini Kit (QIAGEN, cat. no. 52904), 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 cooling to 0°C (17).

Oligonucleotide primers. Combination of two primer sets (representing eastern and western BTV serotypes) and two probes labelled with 6-carboxyfluorescein (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. (23). The primers sequence was as follows: BTVrsaF (5'-GCGTTCGAAGTTTACATCAAT-3'), BTVrsaR (5'-CAGTCATCTCTAGACACTCTATAATTACG-3'), BTVuniF (5'-GCTTTTGAGGTGTACGTGAAC-3'), BTVuniR (5'-TCTCCCTTGAAACTCTATAATTACG-3'), BTV segment 1 probes sequence: RSA-BTV (5'-CGGATCAAGTTCACTCCACGGT-3'), and BTV (5'-TCCTCCGGATCAAGTTCACTCCAC-3'). Oligonucleotide primers and fluorogenic probes were prepared in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw.

Real-time RT-PCR conditions. The rRT-PCR was performed in MicroAmp optical 96-well reaction plate (Applied Biosystems) in one-step reaction using the Quantitect Probe PCR Kit (QIAGEN, cat no. 204443). 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 denaturated RNA was added to the reaction mix (total volume of 25 µl) and the reaction 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 (Cₜ) 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.

Results

The negative Cₜ value for any test and control sample that corresponded to Cₜ of ≥ 42.0 was selected as the positive/negative cut-off Cₜ values obtained as a consequence of examining the blood samples by three rRT-PCR assays. An example of rRT-PCR results are presented in Fig. 1. Both blood samples (082 and 085) collected from seropositive cattle imported to Poland from Germany were recognised as positive (CT < 42.0). Strong positive control (K+) had a Cₜ value of 20.86 and two blood samples 082 and 085 collected from seroreagents imported from Germany had higher CT values of 28.53 and 25.99, respectively. BTV RNA was undetectable in EDTA treated blood sample from uninfected sheep (K-) (Table 1). FMDV RNA sample also was negative in applied rRT-PCR (data not shown).

By rRT-PCR assay, it was possible to detect BTV RNA in all dilutions of blood sample collected from infected sheep (Fig. 2). All dilutions of blood sample were scored positive by fluorogenic RT-PCR. Undiluted sample of blood collected from sheep 5 d p.i. had a Cₜ value of 20.32. An increase in Cₜ value was observed for subsequent dilutions 1/5, 1/10, 1/20, and 1/100, Cₜ of 28.43, 29.22, 31.48, and 33.53 were found, respectively (Table 1).
Fig. 1. Logarithmic fluorescence plots versus cycle number resulting from the determination of BTV RNA in blood samples (082 and 085) collected from imported animals.

Fig. 2. Amplification plots of an undiluted and diluted blood samples from infected sheep for BTV by real-time RT-PCR.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>BTV serotype</th>
<th>$C_T$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K+ (sheep 5 d.p.i.)</td>
<td>BTV-8</td>
<td>20.86</td>
</tr>
<tr>
<td>K- (sheep uninfected)</td>
<td>BTV-8</td>
<td>No $C_T$</td>
</tr>
<tr>
<td>082 - cow (German origin)</td>
<td>BTV-8</td>
<td>28.53</td>
</tr>
<tr>
<td>085 - cow (German origin)</td>
<td>BTV-8</td>
<td>25.99</td>
</tr>
<tr>
<td>Sheep - blood undiluted</td>
<td>BTV-8</td>
<td>20.32</td>
</tr>
<tr>
<td>Sheep - blood dil. 1/5</td>
<td>BTV-8</td>
<td>28.43</td>
</tr>
<tr>
<td>Sheep - blood dil. 1/10</td>
<td>BTV-8</td>
<td>29.22</td>
</tr>
<tr>
<td>Sheep - blood dil. 1/20</td>
<td>BTV-8</td>
<td>31.48</td>
</tr>
<tr>
<td>Sheep - blood dil. 1/100</td>
<td>BTV-8</td>
<td>33.53</td>
</tr>
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</table>
Discussion

The rapid spread of BTV-8 outbreaks in the North-Western Europe during 2007 has highlighted that all diagnostic laboratories dealing with BTV must be well prepared for the rapid and reliable detection of this pathogen. The laboratory diagnosis and the identification of the BTV serotype involved, is today done using various antibody detection methods, including antigen capture, agar gel immunodiffusion (AGID), c-ELISA, and virus neutralisation (VN) assays (6, 18, 20, 24). Detecting of BTV in clinical samples is done by virus isolation in cell cultures (BHK-21, Vero) or in embryonated chicken eggs (ECE), ELISA, immunofluorescence, dot immunobinding assay (DIA), and immunoelectron microscopy (19). Virus isolation requires a long time before the results are available and is especially laborious (27). ELISA is very rapid and easy to perform but is unsuccessful for the detection of BTV in the blood samples (12). To avoid these problems, several RT-PCR assays based on nucleotide sequences of different genome segments were developed and evaluated for the detection of BTV serotypes (27). The conventional RT-PCR for BTV genome segment 7 was recently introduced to the routine BT diagnosis in our laboratory (17). However, this method requires agarose gel electrophoresis, which is time-consuming, insensitive, non-quantitative, subjective, and so limits the number of samples that can be tested during the day.

In recent years, various real-time PCR methods were implemented and applied for the diagnosis of human and animal diseases (8). The rRT-PCR offers certain advantages over conventional RT-PCR. It avoids the use of agarose gel electrophoresis, decreases the risk of contamination, and is suitable for large scale testing and automation. The target amplicon is usually smaller, reducing the potential for problems caused by target degradation. Detection of specific genes sequence by rRT-PCR involves monitoring fluorescence generated by cleavage of a target specific oligonucleotide probe during amplification. This format eliminates the need to open the reaction tube post-amplification, for either a nested step or final agarose PCR. It avoids the use of agarose gel electrophoresis in agarose gel. In addition, the used thermal cycler allows the use of 96-well plate formats, which further increases the capacity and speed of analysis. However, the results of rRT-PCR assay need to be interpreted with caution as BTV has been detected by RT-PCR from the blood of calves and sheep infected for at least 30 days, and sometimes longer (up to 90 days) (1, 11). The possibility of transmission of BTV by biting midges fed this blood, even though it is RT-PCR positive, is rather poor (1, 11). Thus the detection of BTV RNA by these methods indicates recent viral infection, but does not necessarily indicate the presence of contagious virus in the animal.

In conclusion, it may be assumed that the rRT-PCR recently introduced in our laboratory is a sensitive and reliable technique for the detection of BTV in EDTA treated blood samples. The method is more sensitive and much quicker to perform than the conventional RT-PCR because it does not require electrophoresis in agarose gel. In addition, the used thermal cycler allows the use of 96-well plate formats, which further increases the capacity and speed of analysis. However, the results of rRT-PCR assay need to be interpreted with caution as BTV has been detected by RT-PCR from the blood of calves and sheep infected for at least 30 days, and sometimes longer (up to 90 days) (1, 11). The possibility of transmission of BTV by biting midges fed this blood, even though it is RT-PCR positive, is rather poor (1, 11). Thus the detection of BTV RNA by these methods indicates recent viral infection, but does not necessarily indicate the presence of contagious virus in the animal.

References