DETECTION OF BLUETONGUE VIRUS IN BLOOD SAMPLES OF INFECTED RUMINANTS BY RT-PCR FOR GENOME SEGMENT 7

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Abstract

RT-PCR for the detection of bluetongue virus (BTV) in blood samples, collected from infected animals, were described. Two primer sets targeting genome segment 7 of BTV were selected. The full-length S7 cDNA (1156 bp) was amplified in all samples of EDTA blood taken from BTV infected animals. No viral RNA was detected in samples from uninfected animals and seropositive cattle of Dutch origin, imported from Belgium on 7 August 2006. The method proved to be specific, as no positive reaction for foot and mouth disease virus, serotype O and A, was observed. The applied RT-PCR is an accurate and reliable technique for the detection of BTV in EDTA blood samples. This assay is easy and quick to perform and the results are available within 10 h.

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

Bluetongue (BT) is an economically important, infectious, non-contagious, insect-transmitted disease of domestic and wild ruminants (4, 12). It is caused by BT 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 (13). BTV is a small (90 nm in diameter)icosahedral virus with a ten-segmented, double stranded RNA (dsRNA) genome (20). Each of the ten segments codes for at least one of ten distinct viral proteins, seven of which are constructed components of the virus particle, and three of which are non-structural. The inner capsid of BTV is composed of five polypeptides: three minor proteins (VP1, VP4, and VP6) and two major proteins (VP3 and VP7) (18). The outer capsid is composed of two major viral proteins, VP2 and VP5, which determine the antigenic variability of the BTV (6).

BT was first described in South Africa (7), but later was recognised in most countries in the tropics and sub-tropics between latitudes 40°N and 35°S of the Americas, Africa, Asia, and Australia (22). In the Southern Europe, it only caused periodic epizootics involving a single virus strain on each occasion (16). From 1998, this range has extended gradually further north (as far as 44°N) into areas of Mediterranean Europe and the Balkans, involving five different BTV serotypes: 1, 2, 4, 9, and 16 (22). Since 2006, for the first time, the BTV passed latitude 50°N, and BT outbreaks occurred in the northern parts of the EU. On 18 August 2006, the Netherlands reported an outbreak of BT in sheep, in the province of Limberg (Southern Netherlands), close to the border with Germany and Belgium (8). Subsequently, BTV was introduced to the other countries. As of 15 December 2006, the Netherlands had reported 456 outbreaks, Belgium 704, Germany 868, France 5, and Luxembourg 3 outbreaks (9). A “non-European” BTV strain of serotype 8 (BTV-8) has been identified by RT-PCR, and a sequence analysis to determine the cause of the outbreaks (10). As a result of these events, the whole of Europe must now be considered as vulnerable to the incursion of the disease. BT has never been reported in Poland. However, during the routine checks of animals imported to Poland since June 2006, three seroreagents in a consignment of 47 bovines of Dutch origin imported from Belgium were detected in our laboratory.

The aim of the present study was to apply a RT-PCR assay for the rapid detection of BTV in blood samples taken from BTV-8 infected animals.

Material and Methods

Sample origin. A selection of EDTA blood samples collected in 2006, for the purposes of a Ring trial for BTV (serotype 8) viral genome and antibody detection, originated from: sheep – uninfected, sheep – 5 d post infection (d p.i.), sheep – 8 d p.i., a cow – 10 d p.i, sheep – field sample (The Netherlands 2006), and a cow – field sample (Germany 2006). In addition, 3 samples of EDTA blood taken from seropositive cattle imported from Belgium were tested. Moreover, two samples of RNA extracted from foot and mouth disease
virus (FMDV), serotypes O1 Kaufbeuren and A5 Westerwald, kindly provided by Dr. O. Marquardt from the Federal Research Centre for Virus Diseases of Animals in Tübingen (Germany), were used.

**Extraction of viral dsRNA.** RNA was extracted from the EDTA blood samples by use of the QIAamp Viral RNA Mini Kit (QIAGEN, cat. no. 52904), according to the protocol of the manufacturer.

**RNA denaturation.** Extracted RNA was denatured by incubation of the samples for 5 min at 100°C, and then cooling to 0°C.

**Oligonucleotide primers.** Two sets of primers according to Anthony et al. (3): BTV/S7/SA1 (5’–GTTAAAAAATCGTTCAAGATG–3’), BTV/S7/SA2 (5’–GTAAGTTTAAATCGCAAGACG–3’) and BTV/S7/SZ1 (5’–GTTAAAAAATCTATAGAGATG–3’), BTV/S7/SZ2 (5’–GTAAGTGTAATCTAAGAGA–3’) targeting genome segment 7 of BTV were used. The expected size of RT-PCR product was 1156 bp. Oligonucleotide primers were prepared in the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw.

**One-step RT-PCR.** The OneStep RT-PCR Kit (QIAGEN, cat no. 210210) was used for the amplification of BTV and FMDV RNA. The master mix contained as follows: 10 μl of template RNA (about 1 μg total DNA), 10 μl of 5 x Qiagen RT-PCR buffer, 2 μl of dNTPs mixture (0.2 mM each), 0.5 μl (0.6 μM) of each of four primers: BTV/S7/SA1, BTV/S7/SA2, BTV/S7/SZ1, and BTV/S7/SZ2, 2 μl of Qiagen Enzyme Mix, and 25 μl of RNase free water. The samples were transferred to a thermal cycler (Mastercycler personal, Eppendorf) and incubated at 45°C for 30 min. This was followed by an activation step at 94°C for 15 min, in order to activate the DNA polymerases and inactivate the reverse transcriptases. Then, 40 amplification cycles were performed: 94°C/1 min, 45°C/1 min, and 72°C/2 min. The PCR ended with a final elongation for 7 min at 72°C.

**Analysis of PCR products.** The PCR products were separated in 1.3% agarose gel in 1 x TBE buffer, and stained for 30 min in ethidium bromide (1 μg/mL). The gel was analysed and data stored using the Gel Documentation System Imagistore 5000.

**Results**

By using the conventional one-step RT-PCR, it was possible to detect BTV in all samples of EDTA blood taken from infected animals (Fig. 1). The product of expected size (1156 bp) was amplified from RNA extracted from: sheep 5 d p.i. (lane 4), 8 d p.i. (positive control – lane 2), and a cow 10 d p.i. (lane 5) as well as in the field samples from BT outbreaks in the Netherlands (lane 6) and Germany (lane 7). No viral RNA was detected in samples from an uninfected animal (negative control – lane 3) and seropositive cattle of Dutch origin imported to Poland from Belgium (lanes 8-10). Both samples of RNA from FMDV of O1 Kaufbeuren and A5 Westerwald serotypes were negative in the applied RT-PCR (lanes 11-12).

**Discussion**

The last incursion of BT virus into the Netherlands, Belgium, France, Germany, and Luxemburg, has highlighted that all national reference laboratories and others acting as a BT diagnostic laboratories, must establish a level of preparedness that shall enable them rapid and reliable detection methods of this pathogen. Currently, diagnosis of BTV infection is based primarily on serological methods, which detect virus specific antibodies in serum: competitive ELISA and agar gel immunodiffusion (AGID). A number of other procedures are also currently used to detect BTV from the blood or tissues of infected animals: inoculation of susceptible mammalian or insect cells or intravenous inoculation into 10–12-d-old embryonated chicken eggs (ECE).
ELISA, immunofluorescence, and dot immunobinding assay (DIA), and immunoelectron microscopy (17). However, some of diagnostic methods (virus isolation in cell culture and EEC) are laborious and time-consuming, and the others, e.g. ELISA, are unsuccessful for the detection of BTV in the blood samples (15). To avoid these problems, PCR-based assays were developed and evaluated for the detection of BTV serotypes based on nucleotide sequences of different genome segments (1, 2, 5, 11, 14, 19, 21, 23).

In this study, we have applied RT-PCR for the rapid detection of BTV in blood samples taken from BTV-8 infected animals. We introduced the rapid method for viral RNA extraction by the use of the QIAamp Viral RNA Mini Kit. Using this procedure, it was possible to isolate viral RNA within three to four hours. Due to the double stranded nature of extracted RNA, before application to the RT-PCR, the sample must be denatured in order to separate positive and negative copies. It can be achieved by the chemical or heat treatment. The chemical denaturation in 0.1 mM methyl mercury hydroxide (MMOH), described by Wade-Evans et al. (21), is regarded as a more efficient method. However, the use of MMOH can be hazardous. As the heating to 100°C is an equally suitable method of dsRNA denaturation (P. Mertens, personal information), we decided to apply it in our studies. The RT-PCR was performed using the Qiagen OneStep RT-PCR Kit with the primers targeting genome segment 7. This segment of viral RNA was chosen, because it encodes VP7, the main group-specific antigen of the BTV, and is highly conserved (2). Using the mixture of four primers, we were able to amplify full-length S7 cDNA (1156 bp) from blood samples of all BTV infected animals. As the clinical signs of BT are similar to those caused by the FMDV, each suspicion of the disease should be considered as FMD, and the precise differential diagnosis must be performed. In our study, it was shown that both pathogens can be differentiated in the RT-PCR assay with BTV specific primers. The specificity of the applied RT-PCR is evident.

In conclusion, it may be assumed that the RT-PCR is an accurate and reliable technique for the detection of BTV. The assay is easy and quick to perform and the results are available within 10 h. Therefore, this assay can be useful to detect the infection in a field situation.

References