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Diagnostic reliability of different RT-PCR protocols for the detection of bluetongue virus serotype 14 (BTV-14)

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Abstract

Introduction: The reverse transcription polymerase chain reaction (RT-PCR) is one of the most extensively used methods for identification of animals infected with bluetongue virus (BTV). There are several RT-PCR protocols published and several real-time RT-PCR (rtRT-PCR) commercial kits available on the market. Because Poland faced BTV-14 infection in 2012, different protocols were implemented in the country to confirm the RT-PCR results positive for this virus. The article presents a comparative study of several RT-PCR protocols and discusses their diagnostic reliability and applicability. **Material and Methods:** Six rtRT-PCR/RT-PCR protocols were compared for the laboratory diagnostic of fourteen BTV-14 isolates circulating in Poland in 2012–2014. **Results:** All 14 isolates were positive in the protocols of Shaw *et al.* (18), a commercial LSI NS3 kit, and Eschbaumer *et al.* (5). Four out of fourteen BTV-14 isolates gave positive results in Hoffmann's 2 and 6 protocols and none of the 14 isolates yielded positive results in Maan *et al.* (8) method. Phylogenetic study of a short fragment of 450 nt of BTV segment 2 (258–696 positions) revealed 100% identity within Polish variants and with Russian and Spanish isolates. **Conclusion:** The paper points to the possible false negative results in the diagnosis of BTV infections depending on the protocol used.

Keywords: bluetongue, typing, genetic variability, Poland.

Introduction

Bluetongue (BT) is an anthropod-borne viral disease caused by the bluetongue virus (BTV) belonging to the *Orbivirus* genus of the *Reoviridae* family. The disease is economically important, affecting domestic and wild ruminants especially sheep, goats, and cattle (3, 4). Primarily the disease has occurred in tropical regions of the world. Significant changes in the distribution of BTV have been recorded since 1998, and previously exotic serotypes (1, 2, 3, 4, 9, and 16) have spread throughout Mediterranean Europe causing BT outbreaks in ruminants (24). In 2006, BTV serotype 8 emerged in Northern Europe and spread rapidly through Belgium, the Netherlands, France, and Germany (3, 7, 22–24). BTV-14 was identified for the first time in Northeastern Europe in the Smolensk region of Russia in 2011. In 2012 national bluetongue monitoring programmes and pre-movement animal testing reported BTV-14 in Lithuanian cattle imported to Spain, Poland, Latvia, and Estonia (10, 12, 13).

The BTV genome, consisting of approximately 19,200 base pairs, is composed of ten linear segments of double stranded RNA (dsRNA) coding 11 distinct proteins (8). Seven are structural viral proteins (VP1 to VP7) and the remaining four (NS1 to NS4) are non-structural proteins (15). The majority of BTV proteins are highly conserved, exemplified by the more than 75.9% nt/81.2% aa identity shown for segment 10 of BTV coding NS3 protein involved in virus exit from infected cells. Therefore, this segment is the target most frequently used for BTV detection in animals.

Seg-2 is the highly variable region of the BTV genome coding VP2, the outer capsid protein involved in cell attachment and entry. VP2 is particularly relevant in diagnosis due to the epitope responsible for virus neutralisation as well as serotype determination (2). Serological characterisation of BTV is often complicated by cross-neutralisation among different

serotypes (4), therefore, conventional serological methods are replaced with molecular techniques. Phylogenetic analyses of Seg-2 of multiple BTV isolates has separated BTV isolates into 27 distinct clades formed in two major "topotypes" (eastern and western) and several additional groups (11).

Genetic variability of viruses is a natural evolutionary process comprising mainly genome recombination, gene reassortment, and nucleotide substitution. Viruses with segmented RNA, of which BTV is an example, can exchange genetic material generating new variants when co-infecting a single host, and these new serotypes emerging as a result of genome mutation can cause difficulty in molecular diagnosis. Nucleotide sequence variability within and between different BTV serotypes hinders the designing of one universal set of primers that could cover the plethora of variants emerging in the field. There are a few papers describing molecular methods for BTV detection based on segments 1, 2, or 7 of the BTV genome (1, 5, 8, 18). Besides these methods there are commercial kits on the market which can be used for BTV typing (*e.g.* the LSI VetMAX Bluetongue Virus NS3 kit and the Virotype BTV Plus real-time RT-PCR test kit). However, some molecular-based techniques can produce false or confusing results due to the high genetic variation of BTV isolates.

The paper presents the reliability of a few molecular protocols applied for detection and identification of BTV-14 isolates collected in Poland between September 2012 and October 2014. It also briefly discusses the phylogenetic variability of Polish BTV-14 isolates.

Material and Methods

Samples. The study involved 14 EDTA blood samples described in Table 1 originating from cattle, sent to the National Veterinary Research Institute in Puławy, Poland for BTV diagnosis. The presence of viral RNA was examined in the sample as a follow up to seropositive results in BT ELISA.

RNA extraction and denaturation. Total RNA was extracted with a commercial QIAmp Viral RNA Mini Kit (Qiagen, Germany), according to the manufacturer's instructions, from 140 µL non-coagulated blood samples. Extracted dsRNA was exposed directly to heat denaturation at 100ºC for 5 min followed by rapid cooling on ice and then used immediately for RT-PCR or stored frozen at −20ºC.

rtRT-PCR. The commercially available LSI VetMAX Bluetongue Virus NS3 kit (LifeTechnologies, Thermo Fisher Scientific, USA) was used as the first step. To verify the results of the LSI test, the rtRT-PCR assay described by Shaw *et al.* (18) was applied.

In order to serotype BTV positive samples, rtRT-PCR procedures designed and provided by Dr. Bernd Hoffmann from the Friedrich Loeffler Institute (FLI) in Germany were used. Dual procedures are performed at the FLI for detection of animals suspected of having BTV-14 infection. The protocol consists of two duplex rtRT-PCR methods (mix 2 and mix 6), both dedicated to BTV-14 detection with β actin/EGFP as internal controls. A mixture of 5 µL of RNA template and 2 µL of the mix of BTV primers and probe after heat denaturation at 95ºC for 3 min were added to reaction mixture containing 12.5 µL of master mix, 2 µL of mix of primers for β actin/EGFP, 1 µL of enzyme mix, and 2.5 µL of RNAse-free water. The reactions were carried out in a Stratagene Mx3005P thermocycler (Thermo Fisher Scientific, USA) with the following programme: 1 cycle of reverse transcription at 45ºC for 10 min, 1 cycle of 95ºC for 10 min followed by 42 cycles of 95ºC for 15 s, 56ºC for 20 s, and 72ºC for 30 s.

RT-PCR and sequencing. Two methods of RT-PCR for the detection of BTV-14 described previously by Eschbaumer *et al.* (5) and Maan *et al.* (8) were used. Since the procedure published by Maan *et al.* (8) had failed in detection of Polish BTV-14 isolates, the method was optimised by increasing the annealing temperature from 49 to 55°C. The remaining parameters, including reagent concentrations, temperature, and time of cycling, were as described previously (8).

After separation in 2% agarose gel, the amplicons obtained with the method of Eschbaumer *et al.* (8) were subjected to sequencing which was performed in both directions in an ABI PRISM 310 Genetic Analyzer automated sequencer (Applied Biosystems, Thermo Fisher Scientific, USA) using a BigDye Sequencing Kit (Applied Biosystems) with GeneScan analysis software (Applied Biosystems).

Phylogenetic analysis. Nucleotide sequences of 14 positive for BTV-14 samples were aligned with Clustal W multiple alignment software based on the 450 bp region of Seg-2. To determine phylogenetic relationships, Polish BTV-14 isolates were compared to reference sequences (available in the GenBank database and described briefly in Table 1). A phylogenetic tree was generated using the neighbour joining (NJ) method with the Kimura 2-parameter mode and bootstrapped on the set of 1,000 replicates with Mega software v. 4.1 (21).

Results

rtRT-PCR. All blood samples included in the study were positive in rtRT-PCR with the LSI NS3 kit (Ct 25.96 to 36.95) designed to detect BTV Seg-10 and confirmed by the method of Shaw *et al.* (18) (Ct values ranged from 25.5 to 35.68) targeting BTV Seg-1. Four out of fourteen BTV-14 positive samples were positive with Hoffmann's protocols, either with mix 2 or mix 6. Ct values were slightly higher when mix 2 was used (29.0 to 30.26) than when mix 6 was applied (30.35 to

35.48). The remaining 10 samples were negative with both protocols (Table 2).

RT-PCR and phylogenetic analysis. The application of the RT-PCR method described by Maan *et al.* (8) has revealed no products of expected size. Reoptimisation of the method, *i.e.* increasing the annealing temperature to 55ºC, resulted only in non-specific product generation, approximately 600 bp, in 6 out of 14 samples. Viral RNA in all blood samples was detected by the RT-PCR method described by Eschbaumer *et al.* (5). The results are presented in Table 1. RT-PCR products of BTV Seg-2 of the expected 450 bp size were subjected to sequencing. Multiple sequence alignment revealed 100% nucleotide sequence identity of the Seg-2 of all Polish BTV isolates and BTV-14 isolates collected in South Africa, Spain, and Russia. Fig. 1 demonstrates the phylogenetic relationships of the dataset (n = 9) of BTV Seg-2 sequences (KY075918–KY075926) using the NJ method.

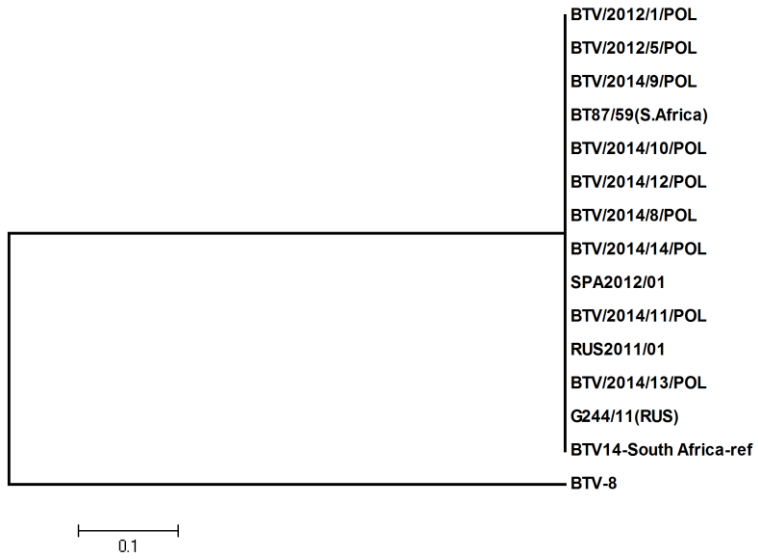

Fig. 1. The phylogenetic tree comparing the Polish BTV-14 isolates with the reference BTV-14 strains. The phylogenetic analysis was conducted with the NJ method. Bootstrap values were obtained for 1,000 replicates. The BTV-8 strain was used as the outgroup

Table 1. Description of BTV isolates used in the present study. POL means Poland

Isolate	Collection data	Origin	GenBank accession number
BTV/2012/1/POL	15.09.2012	Sokółka (POL)	KY075919
BTV/2012/2/POL	15.09.2012	Sokółka (POL)	-
BTV/2012/3/POL	15.09.2012	Sokółka (POL)	-
BTV/2012/4/POL	15.09.2012	Sokółka (POL)	-
BTV/2012/5/POL	17.09.2012	Suwałki (POL)	KY075924
BTV/2012/6/POL	17.09.2012	Augustów (POL)	-
BTV/2012/7/POL	17.09.2012	Augustów (POL)	-
BTV/2014/8/POL	27.01.2014	Białystok (POL)	KY075918
BTV/2014/9/POL	27.01.2014	Białystok (POL)	KY075922
BTV/2014/10/POL	25.10.2014	Grajewo (POL)	KY075923
BTV/2014/11/POL	29.10.2014	Olecko (POL)	KY075920
BTV/2014/12/POL	29.10.2014	Olecko (POL)	KY075925
BTV/2014/13/POL	29.10.2014	Mrągowo (POL)	KY075926
BTV/2014/14/POL	29.10.2014	Giżycko (POL)	KY075921
Rus2011/01	09.2011	Russia: Smolensk-Galloway	KP821096
BT87/59 (S. Africa)	-	South Africa	JX272480
SPA2012/01	08.2012	Spain (cow imported from Lithuania)	KP821097
G244/11(RUS)	2011	Russia	KR233814
BTV14-SouthAfrica-ref	-	-	AJ585135
BTV-8	02.2011	Brazil	KM110785

Table 2. Comparison of the results obtained with different RT-PCR based amplification techniques

Sample	rtRT-PCR (Ct result)				RT-PCR	
	LSI NS3 Kit	Shaw *et al.* (18)	Hoffmann mix 2	Hoffmann mix 6	Maan *et al.* (8)	Eschbaumer *et al.* (5)
BTV/2012/1/POL	31.66	33.3	negative	negative	non-specific product	positive
BTV/2012/2/POL	31.02	32.2	negative	negative	negative	positive
BTV/2012/3/POL	36.95	30.6	negative	negative	negative	positive
BTV/2012/4/POL	26.05	25.5	30.35	29.0	non-specific product	positive
BTV/2012/5/POL	25.96	31.2	32.75	29.38	negative	positive
BTV/2012/6/POL	27.87	27.87	33.09	29.32	non-specific product	positive
BTV/2012/7/POL	26.53	26.53	33.94	29.88	non-specific product	positive
BTV/2014/8/POL	29.18	33.1	negative	negative	non-specific product	positive
BTV/2014/9/POL	30.08	34.2	negative	negative	non-specific product	positive
BTV/2014/10/POL	30.08	33.46	negative	negative	negative	positive
BTV/2014/11/POL	29.69	34.24	negative	negative	negative	positive
BTV/2014/12/POL	30.54	33.99	negative	negative	negative	positive
BTV/2014/13/POL	33.4	35.41	negative	negative	negative	positive
BTV/2014/14/POL	32.62	35.87	negative	negative	negative	positive

Discussion

In field diagnosis of cases, monitoring of regional epidemiological status, and control of the disease, molecular detection of BTV is usually based on the conserved region of the viral genome, but the serotype can only be ascertained from a highly variable region like Seg-2 (8). A variety of BTV serotypes/variants can circulate in the same endemic region (13, 16) and co-infections by different types may significantly influence the laboratory diagnosis of BT and in consequence the control of the disease. Genomic sequence variation within BTV serotypes isolated from the particular topotype or different geographical regions makes it difficult to design universal primer sets for PCR-based amplification techniques (8, 14). Therefore, laboratories performing molecular detection of BTV have to be aware of the limitations resulting from the RT-PCR protocols used. In the case of the Polish samples, the application of the RT-PCR method described by Maan *et al.* (8) yielded no products of expected size. Despite the fact the method applies three pairs of primers covering almost the whole Seg-2 of BTV-14 (107 nt–2142 nt position), the Polish BTV-14 isolates were not detected. The primers in the method of Maan *et al.* were developed based on the nucleotide sequence of isolates collected from sheep in the USA (Marion County, Florida) in 2003 and were successfully tested for amplification of Seg-2 BTV-14 strains belonging to a "Western" topotype. Nevertheless, Polish BTV-14 isolates previously characterised as "Western" topotypes were not detected when this method was used.

Positive results for BTV-14 detection in all blood samples were obtained with the RT-PCR assay developed by Eschbaumer *et al.* (5). Shaw *et al.* (18) presented rt-RT-PCR protocol and the LSI NS3 commercial kit. Only 4 of 14 samples positive in the above protocols were positive in the Hoffmann mix 2 and 6 protocols. These comparative results of various RT-PCR protocols clearly indicate how important for monitoring and control of BTV infection the results from diagnostic laboratories can be. Probably, the discrepancy in the detection of Polish isolates by different PCR protocols listed in Table 1 signifies intra-type sequence variation within primer from annealing applied by the different protocols.

Our study shows that although BTV detection based on the conserved region of the genome gives reliable results, BTV typing can produce more confusing outcomes. There are many primers designed for BTV-14 typing but there is still a need to improve the detection methods, especially for the niche isolates circulating in restricted areas. Thus, the general conclusion of the study is that false negative typing results generated by one method or protocol should be verified by other protocols. It should be also remembered that new BTV serotypes are still appearing (6, 17, 20). Therefore, laboratory diagnosticians performing BTV diagnosis and veterinarians making administrative decisions, *e.g.* on consignments of animals entering the country, should be aware of the limitations of the RT-PCR protocols used by the laboratory for the detection of BTV-infected animals.

Multiple sequence alignment of the 450 bp nucleotide sequence fragment within Seg-2 of BTV-14 has revealed 100% nucleotide sequence identity of the Seg-2 of all 14 Polish isolates, which suggests the circulation of one BTV-14 serotype. The high homology between Polish BTV-14 isolates and the vaccine strain containing BTV-14 seems to confirm previous suggestions of the spread of the attenuated BTV-14 vaccine strain in the field (10, 13). BTV isolates closely related to this vaccine strain or one of the other two (BTV-6 and BTV-11) were detected in Northern Europe, although their route of entry is still unclear. These live attenuated vaccine strains are not excluded from the rapid and progressive reassortment taking place between co-circulating BTV strains, as phylogenetic analysis of strains of European and North

African origin has shown (9). Reassortment between two serologically unrelated BTV strains is flexible and can involve any genome segment (19).

A close relationship of Polish BTV-14 isolates was shown with BTV-14 strains diagnosed in Russia and Spain (in the case of a calf imported from Lithuania). Regarding the close relationship of Polish, Russian, and Spanish BTV-14 isolates, plotting the time of their appearance may suggest the direction of BTV-14 transmission in Europe. As we mentioned previously, BTV-14 emerged in Eastern Europe in 2011 (in Smolensk, Russia) and spread to the West causing BTV outbreaks in Lithuania and Poland in the subsequent years (10, 13). In Poland the virus was circulating from September 2012 to October 2014.

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