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Short communication

Critical parameters of real time reverse transcription polymerase chain reaction (RT-PCR) diagnostics: Sensitivity and specificity for bluetongue virus

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ABSTRACT

A new variant of bluetongue virus serotype 3, BTV3 ITL 2018 (here named: BTV3), was included in serial dilutions in the BT Proficiency Test 2020. Although the OIE-recommended panBTV real time RT-PCR test targeting genome segment 10 (Seg-10) detected this variant, we showed that reverse transcription (RT) at 61 °C instead of 50 °C completely abolished detection. Another Seg-10 panBTV real time RT-PCR test detected BTV3, irrespective of the temperature of RT. *In silico* validation showed that each of the OIE-recommended PCR primers using IVI-primers contain single mismatches at the -3 position for BTV3. In contrast, WBVR-primers of a second test completely match to the BTV3 variant. Our results suggest that single mismatches caused false negative PCR results for BTV3 at high RT temperature. Indeed, correction of both IVI-primers for BTV3 led to positive results for BTV3 but negative results for all other samples of the BT Proficiency Test 2020. Apparently, variability of the -3 position is sufficient for discriminative PCR detection, although the single mismatch in the IVI-reverse primer was the most important for this phenomenon. Extensive *in silico* validation showed that targets of both Seg-10 panBTV RT-PCR tests are not completely conserved, and the detailed effect of single mismatches are hard to predict. Therefore, we recommend at least two panBTV RT-PCR tests to minimize the risk of false negatives. Preferably, their PCR targets should be located at completely different and highly conserved regions of the BTV genome to guarantee adequate detection of future BTV infections.

Bluetongue is an arthropod-borne disease of a wide range of ruminants and causes significant losses of sheep and goats. Bluetongue is listed as a 'notifiable disease' by the World Organisation for Animal Health (OIE) (OIE, 2019b). Bluetongue virus (BTV) (family *Reoviridae*, genus *Orbivirus*) contains a ten-segmented dsRNA genome and includes 24 otifiable serotypes 1–24 (Maan et al., 2007), and new additional serotypes have been proposed (Bumbarov et al., 2020; Hofmann et al., 2008b; Maan et al., 2011; Marcacci et al., 2018; Ries et al., 2020; Sun et al., 2016). So far, BTV serotypes 1, 2, 3, 4, 6, 8, 9, 11, 14, 16 and 25 have been reported in Europe, of which BTV-3, BTV-3 SAR2018 or BTV3 ITL 2018 is the most recent incursion in the southern part of Italy (Cappai et al., 2019).

Molecular diagnostics, like polymerase chain reaction (PCR) tests, has become the frontline diagnostic tool to control diseases of animals and humans. Primers and probe as well as buffer conditions and annealing temperature determine the diagnostic sensitivity and specificity. The majority of viral pathogens contain an RNA genome; positive or negative single stranded RNA or double stranded RNA (dsRNA). Consequently, prior to PCR amplification, reverse transcription (RT) of viral RNA into copy DNA is required. Previously, hexamers were used for random copy DNA synthesis by reverse transcriptase, but current RT-PCR tests use PCR primers to initiate RT.

Highly sensitive and specific real time RT-PCR tests have been developed targeting different BTV genome segments, reviewed in (Hoffmann et al., 2009). These are supposed to detect all and future BTV variants (panBTV PCR tests). However, several tests fail to detect newly discovered serotypes and variants, such as BTV serotype 26 by the Seg-1 panBTV RT-PCR test (Maan et al., 2011; Toussaint et al., 2007). Several panBTV RT-PCR tests target highly conserved regions within genome segment 10 (Seg-10) (Akita et al., 1992; Hofmann et al., 2008a; Leblanc et al., 2010; Orru et al., 2006; van Rijn et al., 2012), for an overview (van Rijn et al., 2013). Because of the dsRNA genome, RT-PCR tests for BTV

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require denaturation of extracted viral dsRNA (external denaturation) before RT (predenaturation) and PCR are performed as described for many RT-PCR tests, i.e. the OIE-recommended panBTV RT-PCR test (OIE, 2019a). To reduce labour and the risk of contamination, we have developed the all-in-one method by which all steps, predenaturation (internal denaturation), RT, PCR, and real time detection are performed after the complete RT-PCR mix was added to isolated dsRNA (van Rijn et al., 2012). This all-in-one RT-PCR protocol uses a set of primers and probe (WBVR-primers), which is also applicable for many other sets of primers and probe, including the OIE-recommended set (IVI) using slightly modified primers and probe compared to the original set (Hofmann et al., 2008a; OIE, 2019a) (Table 1). *In silico* validation by the automated tool PCRv demonstrated that Seg-10 panBTV PCR tests target highly conserved, specific regions of Seg-10 (van Weezep et al., 2019). Diagnostic sensitivity and specificity are potentially high, since, with a very few exceptions, a maximum of one mismatch per primer or probe was found for BTV sequences, while for nonBTVsequences contain at least four mismatches.

Nevertheless, we found false negative PCR results with IVI-primers and probe for BTV3 ITL 2018 by use of a higher temperature for RT (Table 2, column 1). Three dilutions of BTV3 were included twice in the BT Proficiency Test 2020 organized by the European Reference Laboratory for Bluetongue, Madrid, Spain. RNAs were automatically isolated using MagNA Pure 96 system and the accompanying DNA and Viral NA Small Volume Kit (Roche, Almere, the Netherlands). Both serial dilutions of BTV3 were repeatedly PCR-negative, in contrast to results with WBVR-primers and probe. The main difference between the OIE-recommended RT-PCR protocol and the all-in-one RT-PCR protocol using Lightcycler RNA Master HybProbe® (Roche) is RT at 48 °C instead of 61 °C. However, modifications such as annealing at 50 °C for 20 s prior to initiate RT, elongation temperature at 61 °C instead of 72 °C, and use of the Lightcycler 480 RNA Master Hydrolysis Probes® (Roche) did not result in detection of BTV3 (Table 2, column 2). Apparently, these modifications were not sufficient to increase the sensitivity for this variant of BTV3. Both supplied kits of Roche use Thermus thermophilus DNA polymerase (Tth) for RT as well as for PCR. Tth is thermostable and has intrinsic reverse transcriptase activity at high temperature in the presence of manganese. Possibly, the Tth enzyme failed to transcribe RNA of BTV3 into copy DNA. Therefore, predenaturation (external denaturation) of isolated dsRNA samples was carried out before adding RT-PCR mix based on the OneStep RT-PCR Kit® (Qiagen) to allow RT at 50 °C, followed by PCR with annealing at 55 °C and elongation at 72 °C. PCR results of all samples were similarly considered positive or negative for both panBTV RT-PCR tests (Table 2, column 3). We suggest that the lower RT temperature at 50 °C is crucial for successful detection in the case of IVI-primers and probe. We proposed that mismatches in IVI-primers or probe likely cause the difference in detection of BTV3, although use of different enzymes, reverse transcriptase and DNA polymerase, cannot be excluded.

Table 1
List of primers and probe of Seg-10 panBTV PCR tests. Sequences of forward primers (F), probes (P) and reverse primers (R) (5′ -> 3′) indicated by IVI and WBVR have been described (Hofmann et al., 2008a; OIE, 2019a; van Rijn et al., 2012), but IVI-primers slightly modified compared to the original sequences (Hofmann et al., 2008a)(underlined). Sequences are indicated in the 5′ -> 3′ order. Mismatches and corrected nucleotides for BTV-3 SAR2018 are in bold and double underlined, respectively.

WBVR-F:	AGTGTCGCTGCCATGCTATC
WBVR-P:	CGAACCTTTGGATCAGCCCGGA
WBVR-R:	GCGTACGATGCGAATGCA
IVI-F:	TGGAYAAAGCRATGTCAAA
IVI-P:	ARGCTGCATTCGCATCGTACGC
IVI-R:	ACRTCATCACGAAACGCTTC
IVIc-F:	TGGAYAAAGCRATGTCTAA
IVIc-R:	ACRTCATCACGAAACGCCTC

Table 2
RT-PCR results for the BT Proficiency Test 2020 using different RT-PCR protocols. RNAs were automatically isolated using MagNA Pure 96 system and the accompanying DNA and Viral NA Small Volume Kit (Roche). The used RT-PCR kits are indicated: Hybrid; Lightcycler RNA Master HybProbe® (Roche), Hydrol; Lightcycler 480 RNA Master Hydrolysis Probes® (Roche), QS5; OneStep RT-PCR Kit® (Qiagen). Sets of primers and probe are indicated by WBVR and IVI according to Table 1. Predenaturation: int; all-in-one method (internal denaturation), ext; predenaturation prior adding RT-PCR mix (external denaturation). Time and temperature of different intervals, and total running time of 45 PCR cycles are indicated. No PCR signal after 45 cycles is indicated as -.

samples of BT Proficiency Test 2020		1 Hybrid.		2 Hydrol.		3 QS5	
		WBVR	IVI	WBVR	IVI	WBVR	IVI
BTV8 FRA 18.01 (4557) (a) -4	1	32.2	32.1	32.3	31.9	34.6	32.4
Non spiked ovine blood	2	–	–	–	–	–	–
BTV3 ITL 2018 (a) -2	3	26.1	–	26.9	–	27.0	30.5
BTV3 ITL 2018 (a) -3	4	29.3	–	29.9	–	30.6	33.8
BTV3 ITL 2018 (a) -4	5	32.3	–	32.2	–	34.4	38.9
BTV4 MOR 2009 (b) -4	6	31.9	34.2	32.0	33.3	34.3	31.9
Mix of #1 and #14	7	32.0	32.1	31.9	31.9	42.1	32.0
BTV3 ITL 2018 (a) -2	8	26.0	–	26.6	–	26.9	30.8
BTV3 ITL 2018 (a) -3	9	29.5	–	29.7	–	30.7	34.1
BTV3 ITL 2018 (a) -4	10	32.3	–	32.3	–	34.3	38.0
Non spiked bovine blood	11	–	–	–	–	–	–
Non spiked pool of ovine bloods	12	–	–	–	–	–	–
BTV16 ITL 2004/01 (b) -4	13	33.9	34.1	34.7	34.8	37.7	31.5
EHDV1 15.02 (5374) (a) -1	14	–	–	–	–	–	–
BTV4 MOR 2009 (b) -4	15	32.5	35.9	32.6	35.2	34.3	31.7
controls							
BTV weak	W	33.0	35.5	32.8	31.9	33.6	31.5
BTV middle	M	29.6	32.6	29.6	28.6	29.8	28.0
BTV strong	S	31.2	30.9	27.9	26.7	27.4	26.0
negative	N	–	–	–	–	–	–
RT-PCR protocol							
predenatur.		20″ 98 int		20″ 99 int		3′ 95 ext	
RT		20′ 61		20″ 50		30’ 50	
RT				20′ 61			
Activ. Denat.		30″ 95		–		15′ 95	
PCR denaturation		1″ 95		1″ 95		10″ 95	
PCR annealing		10″ 61		10″ 50		30″ 55	
PCR elongation		15″ 72		30″ 61		10″ 72	
total time (45x)		01:12		01:26		02:03	

In order to elucidate the discrepancy of prediction and PCR results, we rechecked the *in silico* validation results in more detail. Clearly, both panBTV RT-PCR tests fulfilled the maximum of one mismatch per primer or probe for BTV-3 SAR2018 (accession number: MK348546). However, in contrast to WBVR-primers, both IVI-primers contain one mismatch at position -3 for BTV3 (Fig. 1). Notably, all differences between BTV-6 NET2008 and BTV-3 SAR2018 as shown in Fig. 1 are synonymous mutations, including both mismatches. This observation could suggest that these regions can vary bymutations without changing translated NS3/NS3a protein, however, this genetic variability is limited (Boyce and McCrae, 2015; Feenstra et al., 2016). The observed single mismatches on the -3 positions of IVI-primers were corrected for BTV3, IVI-Fc and IVI-Rc (Table 1). Different primers sets were studied using the all-in-one method at the critical RT temperature of 61 °C. Serial dilutions of BTV3 were detected with corrected IVI-primers, but all other previously PCR-positive samples of the BT Proficiency Test 2020 were PCR negative

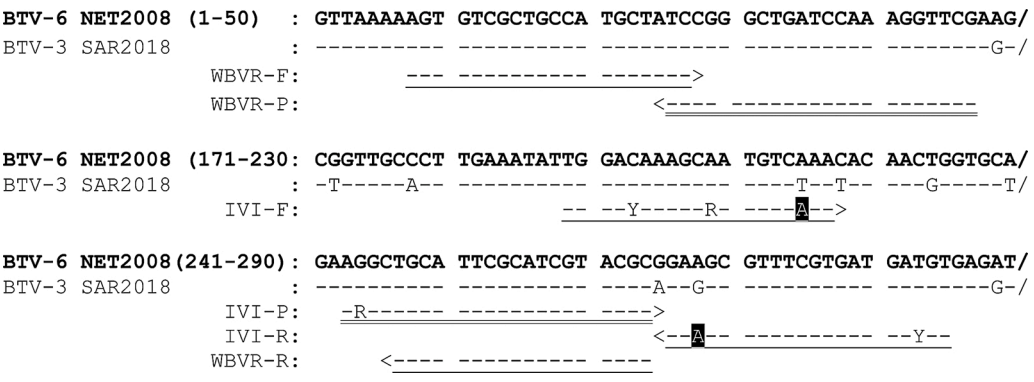

Fig. 1. Overview of the location of primers and probes in genome segment 10. Differences in BTV-3 SAR2018 (accession nr.: MK348546) compared to BTV-6 NET2008 (accession nr.: FJ183383) are indicated. Mismatches in primers and probes for BTV3 are shadowed. Forward (F) and reverse (R) primers (underlined), and probes (P) (double underlined) of panBTV RT-PCR tests are indicated by "IVI" and "WBVR" (OIE, 2019a; van Rijn et al., 2012). Their orientations are arrowed but sequences of the positive strand are shown.

Table 3

RT-PCR results for the BT Proficiency Test 2020 using different sets of primers and probe. RNAs were automatically isolated using MagNA Pure 96 system and the accompanying DNA and Viral NA Small Volume Kit (Roche). RT-PCR was performed by use of the Lightcycler 480 RNA Master Hydrolysis Probes® kit (Roche) (Hydrol.) according to the all-in-one method (van Rijn et al., 2012). Sets of primers and probe are indicated by WBVR and IVI according to Table 1. The sets named IVIc-F and IVIc-R consists of the set of primers and probe according to IVI, except for the indicated primer corrected for BTV-3 SAR2018 (Table 1). Time and temperature of different intervals, and total running time of 45 PCR cycles are indicated. A very weak signal without Ct value is interpreted as doubtful (d). No PCR signal after 45 cycles is indicated as -.

samples of BT Proficiency Test 2020		Roche Hydrolysis kit				
		WBVR	IVI	IVIc primers	IVIc-F	IVIc-R
BTV8 FRA 18.01 (4557) (a) -4	1	31.7	32.3	–	37.5	38.3
Non spiked ovine blood	2	–	–	–	–	–
BTV3 ITL 2018 (a) -2	3	26.9	–	28.1	–	32.2
BTV3 ITL 2018 (a) -3	4	29.3	–	32.3	–	36.8
BTV3 ITL 2018 (a) -4	5	31.3	–	35.0	–	37.6
BTV4 MOR 2009 (b) -4	6	31.7	35.3	–	40.0	–
Mix of #1 and #14	7	31.9	31.7	–	35.1	35.5
BTV3 ITL 2018 (a) -2	8	26.6	–	28.8	–	33.9
BTV3 ITL 2018 (a) -3	9	29.7	–	31.5	–	35.6
BTV3 ITL 2018 (a) -4	10	31.8	–	35.3	–	40.0
Non spiked bovine blood	11	–	–	–	–	–
Non spiked pool of ovine bloods	12	–	–	–	–	–
BTV16 ITL 2004/01 (b) -4	13	31.5	35.4	–	40.0	d
EHDV1 15.02 (5374) (a) -1	14	–	–	–	–	–
BTV4 MOR 2009 (b) -4	15	32.9	36.7	–	–	39.6
controls						
BTV weak	W	33.5	32.6	40.0	40.0	33.9
BTV middle	M	29.7	29.1	37.6	39.2	31.9
BTV strong	S	27.1	26.6	31.2	35.3	29.2
negative	N	–	–	–	–	–
RT-PCR protocol						
predenatur.		20″ 99 int				
RT		20″ 50				
RT		20′ 61				
PCR denaturation		1″ 95				
PCR annealing		10″ 50				
PCR elongation		30″ 61				
total time (45x)		01:26				

(Table 3). Apparently, the -3 position of PCR primers is critical if RT is performed at 61 °C, and caused discriminative detection. Remarkably, strong, middle and weak positive PCR controls, dilutions of cultured BTV, were still detected with BTV3 corrected IVI-primers, although showing much higher Ct values than with uncorrected IVI-primers (Table 3).

Single correction of the forward IVI-primer did not lead to detection of BTV3, while Ct values for other samples were higher indicating reduced sensitivity (Table 3). Thus, this single mismatch did not improve detection of BTV3. Further, single correction of the reverse primer did lead to detection of BTV3, but with higher Ct values than for both corrected IVI-primers. Remarkably, several other BTV samples were also detected with the single corrected reverse IVI-primer, although with very high Ct values. Taken together, diagnostic sensitivity is strongly affected by matching of primers and details of the RT-PCR protocol, in particular the temperature used for RT. Further, mismatches in primers can reduce the diagnostic sensitivity, although their effect is hard to predict.

Frequent updating of PCR tests in the light of upcoming variants is required to avoid unnoticed false negative results. However, access to all variants and performing PCR tests for all these variants is laborious and hardly possible. In addition, more sequences become available while most of these virus variants have not been isolated. The automated tool PCRv predicts sensitivity and specificity (*in silico*) based on the number of mismatches in primers and probe (van Weezep et al., 2019). Nonetheless, *in silico* validation has shown its limitations. Potential false negatives and positives should be checked manually on source, relevance and correctness of the submission and sequence. Further, the kind and position of mismatches as well as the total number of mismatches in primers and probe(s) are not taken into account by PCRv. In addition to a summarizing table, per primer or probe, potential false negatives by descending number of mismatches and potential false positives by ascending number of mismatches are listed by PCRv (supplemented data A and B). Only accession numbers in the upper part of both lists require special attention and significantly reduce labour. In an upgraded version of PCRv the total number of mismatches in primers and probe per accession number is used as second criteria to generate the list of potential false negatives. Thus, these accession numbers then move upwards in the list and will be more pronounced.

The effect on detection by the kind and position of mismatches is hard to be evaluated automatically. For example, diagnostic sensitivity is strongly affected by the temperature of RT (Table 2). Further, despite of the proposed allowance of one mismatch per primer or probe, the effect of a single mismatch can vary between negligible and complete loss of detection (Table 3). On the other hand, a critical temperature for annealing will potentially enhance the diagnostic specificity. Nevertheless, extensively validated panBTV RT-PCR tests are highly specific even with lower, less critical, RT temperature. Of course, the specificity is also defined by PCR primers and probe, and their relative positions on the target sequence.

Summarizing, a new variant of BTV serotype 3, BTV3 ITL 2018 or BTV-3 SAR 2018, was detected with the OIE-recommended panBTV RT-PCR test using slightly modified primers as previously published (Hofmann et al., 2008a; OIE, 2019a). We here showed that single mismatches can lead to false negative RT-PCR results by use of a critical (higher) RT temperature. An extensively validated panBTV PCR-test not

showing mismatches detected this BTV variant, irrespective of the used RT-PCR protocol (van Rijn et al., 2012). Since all studied PCR targets of panBTV PCR tests are not completely conserved among the BTV species together with the unpredictability of the effect of mismatches on diagnostic sensitivity, we recommend at least two panBTV RT-PCR tests, preferably targeting completely different regions, to guarantee adequate laboratory diagnosis for BTV infected animals.

Author statement

Both authors have seen and approved the final version of the manuscript being submitted. They warrant that the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Akita, G.Y., Chinsangaram, J., Osburn, B.I., Ianconescu, M., Kaufman, R., 1992. Detection of bluetongue virus serogroup by polymerase chain reaction. J. Vet. Diagn. Investig. 4, 400–405.

Boyce, M., McCrae, M.A., 2015. Rapid mapping of functional cis-acting RNA elements by recovery of virus from a degenerate RNA population: application to genome segment 10 of bluetongue virus. J. Gen. Virol. 96, 3072–3082.

Bumbarov, V., Golender, N., Jenckel, M., Wernike, K., Beer, M., Khinich, E., Zalesky, O., Erster, O., 2020. Characterization of bluetongue virus serotype 28. Transbound. Emerg. Dis. 67, 171–182.

Cappai, S., Rolesu, S., Loi, F., Liciardi, M., Leone, A., Marcacci, M., Teodori, L., Mangone, I., Sghaier, S., Portanti, O., Savini, G., Lorusso, A., 2019. Western Bluetongue virus serotype 3 in Sardinia, diagnosis and characterization. Transbound. Emerg. Dis. 66, 1426–1431.

Feenstra, F., van Gennip, R.G., Schreuder, M., van Rijn, P.A., 2016. Balance of RNA sequence requirement and NS3/NS3a expression of segment 10 of orbiviruses. J. Gen. Virol. 97, 411–421.

Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A., van Rijn, P.A., Slomka, M.J., Banks, J., Brown, I.H., Alexander, D.J., King, D.P., 2009. A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. Vet. Microbiol. 139, 1–23.

Hofmann, M., Griot, C., Chaignat, V., Perler, L., Thur, B., 2008a. [Bluetongue disease reaches Switzerland]. Schweizer Archiv fur Tierheilkunde 150, 49–56.

Hofmann, M.A., Renzullo, S., Mader, M., Chaignat, V., Worwa, G., Thuer, B., 2008b. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. Emerg. Infect. Dis. 14, 1855–1861.

Leblanc, N., Rasmussen, T.B., Fernandez, J., Sailleau, C., Rasmussen, L.D., Uttenthal, A., Zientara, S., Belak, S., Hakhverdyan, M., 2010. Development of a real-time RT-PCR assay based on primer-probe energy transfer for the detection of all serotypes of bluetongue virus. J. Virol. Methods 167, 165–171.

Maan, S., Maan, N.S., Samuel, A.R., Rao, S., Attoui, H., Mertens, P.P., 2007. Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. J. Gen. Virol. 88, 621–630.

Maan, S., Maan, N.S., Nomikou, K., Batten, C., Antony, F., Belaganahalli, M.N., Samy, A. M., Reda, A.A., Al-Rashid, S.A., El Batel, M., Oura, C.A., Mertens, P.P., 2011. Novel bluetongue virus serotype from Kuwait. Emerg. Infect. Dis. 17, 886–889.

Marcacci, M., Sant, S., Mangone, I., Goria, M., Dondo, A., Zoppi, S., van Gennip, R.G.P., Radaelli, M.C., Camma, C., van Rijn, P.A., Savini, G., Lorusso, A., 2018. One after the other: a novel Bluetongue virus strain related to Toggenburg virus detected in the Piedmont region (North-western Italy), extends the panel of novel atypical BTV strains. Transbound. Emerg. Dis. 65, 370–374.

OIE, 2019a. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Paris.

OIE, 2019b. Terrestrial Animal Health Code. Paris.

Orru, G., Ferrando, M.L., Meloni, M., Liciardi, M., Savini, G., De Santis, P., 2006. Rapid detection and quantitation of Bluetongue virus (BTV) using a Molecular Beacon fluorescent probe assay. J. Virol. Methods 137, 34–42.

Ries, C., Sharav, T., Tseren-Ochir, E.O., Beer, M., Hoffmann, B., 2020. Putative novel serotypes' 33' and' 35' in clinically healthy small ruminants in Mongolia Expand the group of atypical BTV. Viruses 13.

Sun, E.C., Huang, L.P., Xu, Q.Y., Wang, H.X., Xue, X.M., Lu, P., Li, W.J., Liu, W., Bu, Z.G., Wu, D.L., 2016. Emergence of a novel bluetongue virus serotype, China 2014. Transbound. Emerg. Dis. 63, 585–589.

Toussaint, J.F., Sailleau, C., Breard, E., Zientara, S., De Clercq, K., 2007. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. J. Virol. Methods 140, 115–123.

van Rijn, P.A., Heutink, R.G., Boonstra, J., Kramps, H.A., van Gennip, R.G., 2012. Sustained high-throughput polymerase chain reaction diagnostics during the European epidemic of Bluetongue virus serotype 8. J. Vet. Diagn. Invest. 24, 469–478.

van Rijn, P.A., van de Water, S.G., van Gennip, H.G., 2013. Bluetongue virus with mutated genome segment 10 to differentiate infected from vaccinated animals: a genetic DIVA approach. Vaccine 31, 5005–5008.

van Weezep, E., Kooi, E.A., van Rijn, P.A., 2019. PCR diagnostics: In silico validation by an automated tool using freely available software programs. J. Virol. Methods 270, 106–112.