Artykuł przeglądowy *Review*

Molecular technologies for detection and typing of bluetongue virus

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Summary

Rapid and accurate diagnosis plays an important role in the implementation of effective measures to control the spread of disease. Historically, the laboratory diagnosis and typing of BTV were carried out by various serological and virological methods, including virus neutralization (VN) assay, ELISA, as well as virus isolation (VI) in cell cultures or in embryonated chicken eggs. At present, various molecular techniques to detect BTV genome are increasingly used as primary diagnostic tools for the serotyping and epidemiological investigations of BTV. Initially, the viral RNA was detected by simple nucleic acid hybridization technologies. Then, conventional RT-PCR assays were developed and evaluated for the detection of BTV serotypes based on nucleotide sequences of different genome segments. Although RT-PCR, with its increased sensitivity, has advantages over hybridization, it is almost impossible to quantify accurately by regular and multiplex PCR procedures, and regular PCR may produce false positive results. Over the recent years, a number of real-time RT-PCR (rRT-PCR) methods have been described. The rRT-PCR offers certain advantages over conventional RT-PCR assay, as it is more rapid, sensitive, and can provide quantitative as well as qualitative genetic information. It does not use agarose gel electrophoresis, decreases the risk of contamination because it is run within an enclosed tube, and is suitable for large-scale testing and automation. The target amplicon is usually smaller, reducing the potential for problems caused by target degradation. Loop-mediated isothermal amplification (LAMP), a novel rapid, accurate and cost effective gene amplification method, is an autocycling and strand displacement DNA synthesis method. LAMP assays have been applied as a method of detecting a variety of animal pathogens, including BTV. RT- LAMP assay can be a valuable tool complementing the routine laboratory diagnosis of BTV.

Keywords: BTV, detection, typing, molecular techniques

Bluetongue (BT) is an infectious but non-contagious viral disease of domestic and wild ruminants that induces variable clinical signs, depending on the host species and breed (18). BT is classified as a notifiable disease by the Office International des Epizooties (OIE), according to Council Directive 82/894/EEC of 21 December 1982, due to its potential rapid spread and serious economic consequences in affected countries. It is endemic in most countries in the tropics and sub-tropics between latitudes 40°S and 53°N of the Americas, Australia, Africa and some regions of Asia (46). In the summer of 2006, for the first time, BTV crossed latitude 50°N, and BT outbreaks caused by BTV serotype 8 occurred unexpectedly in north-western Europe (39). In 2007-2008, BTV-8 spread rapidly and widely throughout much of Europe (41). The implementation of BT compulsory vaccination programmes in Europe in the spring of 2008 led to the elimination of the BTV-8 disease from Europe by the end of 2011 (52). However, after a four-year break, on 11 September 2015, an outbreak of BTV-8 was confirmed again in central France (Allier region), and by 8 June 2016 a total of 285 outbreaks of the disease had been reported throughout France. The latest cases of the BTV-8 disease were detected on 18 July 2016 in the Lozere department in southern France (http://www.oie.int).

BT is caused by bluetongue virus (BTV), a RNA virus belonging to the *Orbivirus* genus in the *Reoviridae* family (22) and transmitted by biting midges of the genus *Culicoides* (19). BTV can be transmitted vertically or horizontally, via the oral route or by direct contact (3, 5). To date, twenty-seven immunologically distinct serotypes of BTV have been identified world-

wide (53). BTV is a small icosahedral virus (about 90 nm in diameter) with a genome of approximately 19200 base pairs, composed of 10 linear segments of double-stranded RNA (dsRNA) (20) which encode ten distinct virus proteins (45) (Fig. 1). Seven of these (VP1 to VP7) are structural components of the icosahedral virus capsid, and three are non-structural proteins (NS1-NS3), which play different key roles during the viral replication cycle (38). VP2 and VP5 constitute the outer capsid of BTV particles. The inner capsid is composed of VP3 and VP7, and encloses the viral genome and three minor proteins, VP1, VP4 and VP6 (6). The VP2 protein, encoded by genome fragment 2 (Seq-2), is the most variable of BTV proteins and the main determinant of the virus serotype (16).

Historically, the laboratory diagnosis and typing of BTV serotypes were carried out by various antibody detection methods, including agar gel immunodiffusion (AGID), enzyme-linked immunosorbent assay (ELISA) and virus neutralization (VN) (36). BTV in clinical samples can often be grown in embryonated chicken eggs (ECE), isolated in insect or mammalian cell cultures (BHK-21, Vero cells), and detected by ELISA, immunofluorescence, dot immunobinding assay (DIA) and immune electron microscopy (32). At present, various molecular techniques to detect BTV genome are increasingly used as primary diagnostic tools for the serotyping and epidemiological investigations of BTV. Initially, the viral RNA was detected by simple nucleic acid hybridization technologies (8). Although these methods are relatively easy to perform, they lack analytical sensitivity. Hybridization assays have recently been enhanced by new reporter systems, e.g. PCR-based methods, but these systems are not yet readily available (50). Then, the conventional RT-PCR assays were developed and evaluated for the detection of BTV serotypes based on nucleotide sequences of different genome segments e.g. segment 7 (1) and segment 2 (13, 51) (Fig. 1). Moreover, a multiplex RT-PCR-based techniques have been developed for the simultaneous detection and differentiation of five North American BTV serotypes 2, 10, 11, 13 and 17 in cell culture and clinical samples (2). Furthermore, RT-PCR-based assays for typing the European strains of BTV and for differential diagnosis of field and vaccine strains have been described (17). Other rapid, sensitive and specific RT-PCR methods have also been developed to detect the members of European BTV serotypes and to distinguish eastern and western topotypes within each virus serotype (21). An RT-PCR assay for the detection of BTV genome in blood samples of susceptible animals imported into Poland from BTV-affected countries was introduced in our laboratory in late 2006 (27). It was performed using a Qiagen OneStep RT-PCR Kit with primers targeting BTV genome segment 7, a highly conserved genome fragment that encodes VP7 capsid protein (1). By using this method we were able to amplify full-length S7 cDNA (1156 bp) from blood samples of BTV-8-infected animals imported from Germany and the Netherlands. The assay proved to be specific, as no positive reaction for foot-and-mouth disease virus (FMDV) serotypes O and A was observed. We found that this RT-PCR is an accurate and reliable technique for the detection of BTV in EDTA blood samples of infected animals. However, although the conventional RT-PCR, with its increased sensitivity, has advantages over hybridization, it is almost impossible to quantify accurately by regular and multiplex PCR procedures, and regular PCR may produce false positive results. Besides, this method requires agarose gel electrophoresis, which is time-consuming and limits the number of samples that can be tested during one day. The analytical sensitivity of RT-PCR can be increased by the use of nested RT-PCR, which also increases the risk of laboratory contamination with amplified PCR products. The resulting risk of false positive results is mitigated in well-managed and well-constructed laboratories with rigidly enforced work practices based on the separation of steps in the assay procedure. However, not every laboratory has been designed to enforce such work practices effectively, and work practices are still susceptible to human error. The sequencing of the cDNA amplicons generated in RT-PCR assays and phylogenetic comparisons to other strains that had previously been analysed also made it possible to identify individual virus lineages in a manner that is not currently possible by serological techniques. The phylogenetic comparisons of BTV isolates are very important during epidemiological investigation to detect the presumable source of BTV infection.

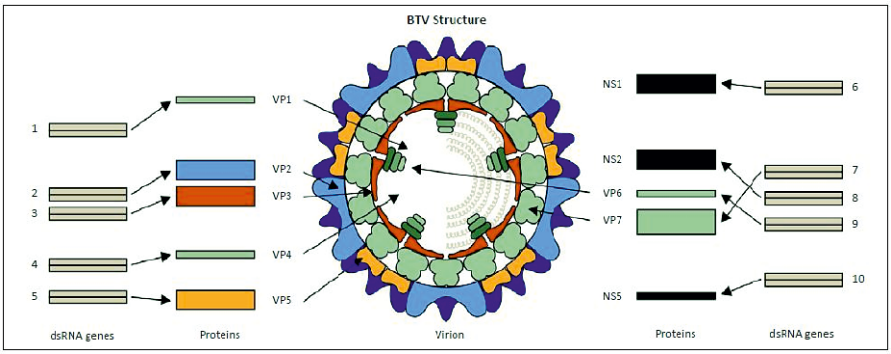

Fig. 1. Coding for viral proteins VP1-VP7 and non-structural proteins NS1--NS3 by the 10 dsRNA genes of the bluetongue virus. Modified, according to Vorwoerd (45)

Over the recent years, many real-time RT-PCR (rRT-PCR) techniques have been described for the diagnosis of human and animal diseases. The rRT-PCR offers certain advantages over the conventional RT-PCR assay. It avoids the use of agarose gel electro-

phoresis, decreases the risk of contamination because it is run within an enclosed tube, and is suitable for large scale testing and automation. The target amplicon is usually smaller, reducing the potential for problems caused by target degradation. Prior to the BTV-8 outbreaks in north-western Europe in 2006, several rRT-PCR methods had been described for the detection and identification of BTV strains. Primers targeting the fragment of NS1 gene (Seg-5) were used in an rRT-PCR assay to detect 11 out of 19 BTV serotypes tested (serotypes 20-24 were not tested) (48). Another rRT-PCR assay using primers from RNA segment 2 (VP2) was used to differentiate wild-type Italian field and South African vaccine strains of BTV-2 (35). In 2006, an RT-PCR method for the detection of viral RNA in blood samples was developed using a combination of primers for a highly conserved region in BTV RNA segment 5 (11). This assay detected all 17 serotypes of Mediterranean BTV isolates (including BTV-2, 4 and 16), vaccine strains for serotypes 2 and 4, and 15 out of the 24 reference strains available. However, BTV serotype 4 and 16 reference strains were not detected by this rRT-PCR, and this method was found to have a reduced sensitivity for the detection of field BTV-8 isolates compared to rRT-PCR assays used in a BTV inter-laboratory proficiency test (4). In the same year, another very sensitive rRT-PCR was developed using a molecular beacon fluorescent probe designed within the NS3 conserved region of BTV segment 10 (34). This assay recognized ten serotypes of BTV and 10^3 molecules/PCR reaction. Since the start of BTV-8 epizootic in Europe in August 2006, many rRT-PCR assays have been described. A one-step rRT-PCR assay has been developed that detects strains of BTV serotypes 2, 4, 9 and 16 isolated in Italy in epidemics between 2000 and 2004, as well as their respective vaccine strains (37). The limit of detection for the assay has been estimated at 0.005 to 0.05 $TCID_{50}$/ml, depending on the virus strain. Other rRT-PCR techniques were capable of identifying representative strains from 24 serotypes of BTV (40, 42). Two of them detected strains of different BTV serotypes from the Mediterranean region and prototype strains of 24 serotypes (42). Both assays have similar detection limits of less than 0.01 ECE_{50} and detect 100 RNA copies. In a combined duplex rRT-PCR assay, two sets of primers and probes were designed to target segment 1 of eastern and western group BTV viruses (40). It was evaluated with a wide variety of test samples, including tissue culture-derived viruses, infected tissue and blood samples from cattle, sheep and infected *Culicoides* midges. This rRT-PCR showed no cross-reactions with a closely related orbiviruses and gave positive Ct results with all of the viruses tested, including a panel of 129 BTV isolates derived from different geographical locations, reference strains of 24 BTV serotypes, and multiple field strains of BTV serotypes 1, 2, 4, 8, 9 and 16 from European outbreaks that had occurred since 1998. This assay detected less than 10 template copies per reaction. The same combination of two primer sets and two probes targeting BTV segment 1 of western and eastern BTV strains was used in our laboratory to develop a duplex rRT-PCR for the detection of viral RNA in blood samples from BTV-infected animals (25). We found that this method is more sensitive and much quicker to use than the conventional RT-PCR used earlier in our laboratory. Since December 2007, this procedure has been applied to detect BTV genome in blood samples from seropositive animals imported into Poland from BT-affected countries after 15 June 2006. On 5 December 2007, viral RNA was detected for the first time in samples from seropositive cattle from Germany (25). Forty (0.25%) out of 15676 blood samples tested by the end of 2009 were found to be positive for BTV RNA (28, 29). Among them, 38 positive blood samples were collected from cattle imported into Poland from Germany and one sample was taken from a Dutch fallow deer (Tab. 1). All of the BTV-positive animals were slaughtered. Moreover, using a novel rRT-PCR assay for the typing of BTV based on BTV Seq-2 target gene, we found that all positive samples taken from seropositive cattle imported into Poland from Germany were BTV serotype 8 (30). Most of these positive cattle were identified at the beginning of 2008, when the BTV-8 epidemiological situation in Europe was very serious. Since August 2008, only one BTV-8-positive cow of German origin has been detected by rRT-PCR (28). Mandatory vaccination against BT implemented in the European Union (EU) countries drastically reduced the number of infected animals, and, as a consequence, no more positive animals imported into Poland have been detected. The presence of BTV genome was also found in blood taken from a 4-week-old calf born from a positive dam imported from Germany. This result confirms the vertical transmission of BTV, described earlier by other authors (3). The long persistence of BTV in the

Tab. 1. Occurence of BTV in the population of animals imported into Poland from the EU (2007-2009). According to Niedbalski (28, 29)

Voivodeship	Animal species	Country of origin	Number (%) of BTV-positive samples
Lubelskie	cattle	Germany	5 (0.03%)
Mazowieckie	cattle	Germany, Poland*	5 (0.03%)
Podlaskie	cattle	Germany	14 (0.09%)
Wielkopolskie	cattle, fallow deer	Germany, the Netherlands	10 (0.06%)
Kujawsko-pomorskie	cattle	Germany	6 (0.04%)
			40 (0.25%)

Explanation: *a calf born in Poland from a BT-positive dam of German origin

blood of infected animals was demonstrated – viral RNA was detectable as late as one month after the first collection, although the C_T value of the samples was considerably reduced (29). As the virus has not been isolated from any of the PCR-positive samples, it can be concluded that the BTV-positive animals were probably in the late stages of infection, when animals remain PCR-positive, but are unlikely to be infective to midges. Recently, the first cases of BTV detected in native cattle from Poland have been described (33). In the National Reference Laboratory for Bluetongue, BTV serotype 14 was detected by type-specific rRT--PCR in animals reared near the Polish-Belarusian and Polish-Lithuanian borders. It was suggested that the most probable route of BTV introduction into Poland was transmission through *Culicoides* midges (33).

In 2006-2009, in addition to BTV-8, virus serotypes 1, 6 and 11 were introduced into Europe. In order to precisely identify BTV in circulation, four highly sensitive serotype-specific rRT-PCR techniques have been used (43). All assays exhibited a linear range of at least 0.05-3.80 $\log_{10}$ $TCID_{50}$/ml and PCR efficiency approaching the ideal amplification factor of two per PCR cycle. Additionally, highly sensitive rRT-PCR assays directed at BTV genome segment 2 for the specific detection of BTV serotypes 1, 6 and 8 have also been described (10). Each assay specifically detects one serotype with no cross-reactivity. Moreover, another rRT-PCR assay based on a primer-probe energy transfer for the detection of all BTV serotypes has been developed (12). Its sensitivity was in the range of 10-100 target copies, and specificity tests showed no positive results for heterologous pathogens. It was concluded that this technique provides an important tool for an early and rapid detection of a wide range of BTV strains, including emerging strains. A duplex rRT-PCR for the simultaneous detection of BTV in bovine extended semen for artificial insemination has also been developed and validated (44). The total assay was highly repeatable, and a preliminary analysis of its specificity was 100% in BTV-8-infected semen. This method has the potential to be used for the control of semen for international trade. Recently, a new multiplex rRT-PCR system for the detection of several viral genes in a single reaction (47) has been compared with the two previously described PCR methods (10, 42). For BTV-1 to BTV-24 and BTV-26, all assays yielded comparable results. However, BTV-25-positive samples were detected only by the newly designed multiplex technique. All 26 BTV serotypes were detected by the new rRT-PCR assay targeting the highly conserved genome segment 9 (encoding the viral helicase VP6 and NS4) (14). This assay can be used for a rapid and precise detection of BTV strains in cell culture, blood/tissues or insect samples. The efficiency of this method, evaluated with RNA derived from the reference strain of BTV-1, was 99.6%, detecting down to 4 copies per reaction. In common with other rRT-PCR assays described above, the capability of this method to detect BTV in blood, semen and other clinical samples for a significant period after viraemia, make this Seg-9-specific method a potentially valuable tool for studying the recent distribution and movement of BTV infections in the field. It should be noted, however, that some caution is needed in interpreting conventional RT-PCR and rRT-PCR results, because BTV RNA can be detected in blood from infected animals for many months, when viable virus is no longer present in the specimens (18). In experimentally infected animals, BTV was detected by RT-PCR at 16-20 weeks after infection, as compared to only 2-8 weeks by virus isolation (VI). This suggests an extended persistence of BTV RNA without the presence of infectious virus or active virus replication. A positive result indicates that the animal has been infected or vaccinated at some time prior to the collection of the specimen, but the animal is not necessary infectious and may no longer be capable of sustaining transmission of the virus (18).

The detection of BTV currently relies on various rRT-PCR assays, and although this technique is highly specific and sensitive, it has the disadvantages of requiring high-cost instruments and highly trained technicians. Loop-mediated isothermal amplification (LAMP), a novel rapid, accurate and cost-effective gene amplification method, is an autocycling and strand displacement DNA synthesis method (31). The main advantage of LAMP is the capacity to amplify target-specific sequences under isothermal conditions (63-65°C). Two or three inner and outer primer pairs that recognise six different target sequence regions are used to amplify the template. The results of LAMP can be observed directly with the naked eye after adding an intercalating dye and visualised by gel-electrophoresis or real-time fluorogenic analysis with a thermal cycler. LAMP assays have been used in detecting a variety of pathogens, including animal viruses (7, 9). A set of four primers targeting conserved segment 1 of BTV RNA was used in an RT-LAMP reaction developed in our laboratory for the detection of BTV genome (30). RT-LAMP and rRT-PCR assays were estimated to be equally sensitive, and no cross-reactivity of the primers with the genes of symptomatic look-alike diseases, such as foot-and-mouth disease (FMD) and peste des petits ruminants (PPR), was found. The RT-LAMP technique applied was very fast. Including the time required for the extraction of viral RNA, its presence in EDTA-treated blood samples could be detected within 2 hours. On the basis of the results obtained, we found that the RT-LAMP assay can be a valuable diagnostic tool complementing the routine methods of BTV detection (26). An accelerated RT-LAMP method using primers based on genome segment 2 (VP2 gene) of BTV-8 has been described by other authors (24). The assay was assessed using a full panel of BTV reference

strains and clinical samples. This RT-LAMP was highly specific for the detection of BTV-8 and showed no cross-reactivity with any of the other 24 BTV serotypes or with four strains of epizootic hemorrhagic disease virus (EHDV). It has considerable potential to be used in a pen-side setting, in the field or by smaller, less well-equipped laboratories in developing countries. Moreover, a single-step RT-LAMP method targeting NS1, a highly conserved gene among BTV serotypes 1, 2, 9 10, 16, 21 and 23, has been optimized and validated in India (23). The relative sensitivity of the assay was 0.3 $TCID_{50}$, and no cross-reactivity could be observed with FMDV, PPRV, goat pox (GP), sheep pox (SP) and orf viruses. The established RT-LAMP assay has also been assessed by screening clinical samples, and the results were comparable with the conventional RT-PCR. However, this assay cannot identify BTV topotypes, and it has not been tested against all circulating BTV serotypes. Recently, the development and evaluation of two separate accelerated RT-PCR assays, the eastern (e) RT-LAMP and western (w) RT-LAMP for a rapid and accurate detection and differentiation of RNA from eastern or western BTV strains circulating in India has been described (16). Each assay used four primers recognizing six distinct sequences of segment 1 BTV genome. The eRT-LAMP and wRT-LAMP assays detected BTV RNA in all positive isolates of Indian BTV strains 1, 2, 3, 5, 9, 10, 16, 21, 23 and 24 with high specificity and efficiency. The analytical sensitivity of these RT-LAMP techniques was comparable to rRT-PCR, but higher than that of conventional RT-PCR. The accelerated eRT-LAMP and wRT-LAMP assays generated detectable levels of amplified DNA, down to 0.216 fg and 108 fg of BTV RNA template, respectively. The two RT-LAMP techniques did not show any cross-reaction with each other. These assays can be adopted as a pen-side test, which makes them suitable for front-line diagnosis, helping to identify and contain field outbreaks of BTV (15). Another efficient and sensitive RT-LAMP method using four primers targeting the NS1 gene of BTV was applied to blood samples collected from 15 clinically healthy dairy cattle in Taiwan (49). It detected viral RNA in three animals, one of which was seronegative by ELISA. The authors suggest that this assay can be suitable for the screening of field samples with the potential to detect subclinical infection.

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