

Diagnosing bluetongue virus in domestic ruminants: current perspectives

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Abstract: This review provides an overview of current and potential new diagnostic techniques against bluetongue virus (BTV), an *Orbivirus* transmitted by arthropods that affects ruminants. Bluetongue is a disease currently notifiable to the World Organization for Animal Health (OIE), causing great economic losses due to decreased trade associated with bluetongue outbreaks and high mortality and morbidity. BTV cross-reacts with many antigenically related viruses including viruses that causes African Horse sickness and epizootic haemorrhagic disease of deer. Therefore, reliable diagnostic approaches to detect BTV among these other antigenically related viruses are used or being developed. The antigenic determinant for differentiation of virus species/serogroups among orbiviruses is the VP7 protein, meanwhile VP2 is serotype specific. Serologically, assays are established in many laboratories, based mainly on competitive ELISA or serum neutralization assay (virus neutralization assay [VNT]) although new techniques are being developed. Virus isolation from blood or semen is, additionally, another means of BTV diagnosis. Nevertheless, most of these techniques for viral isolation are time-consuming and expensive. Currently, reverse-transcription polymerase chain reaction (RT-PCR) panels or real-time RT-PCR are widely used methods although next-generation sequencing remains of interest for future virus diagnosis.

Keywords: bluetongue virus, diagnostic tests, virus isolation, serological tools

Introduction

Bluetongue (BT) is an infectious disease transmitted by *Culicoides* biting midges, affecting mainly domestic and wild ruminants. The causative agent is bluetongue virus (BTV), one of the 22 species or serogroups in the genus *Orbivirus* in the *Reoviridae* family.¹

BTV causes severe morbidity and mortality in sheep, while the infection is subclinical in some domestic and wild ruminants. The BTV genomes are composed of ten fragments of double-stranded RNA encoding seven structural (VP1–7) and four nonstructural (NS1–4) proteins.^{2,3} It is classified into 27 serotypes based on the genetic and antigenic features of the neutralizing protein VP2.⁴ VP7 protein is a major determinant of serogroup specificity, and most of the serological assays to detect BTV are based on detecting anti-VP7 antibodies.⁵ The distribution of BTV has changed drastically in the last decades. It was primarily distributed in tropical regions of the world but since 1998 outbreaks through the Mediterranean to the Northern Europe have been reported.⁶

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BTV is notifiable to the World Organization for Animal Health (OIE), mainly because new outbreaks implicate movement and trade restrictions, causing severe economic losses. Vaccination with live-attenuated vaccines currently contributes to control BTV outbreaks.⁷ Nevertheless, active surveillance to detect BTV infection by either virus isolation or other detection test or serology is implemented. In this review, we provide an overview of current and new diagnostic procedures for BTV (summarized in Table 1).

BTV transmission and clinical signs

Transmission

BTV is an arbovirus, and until recently, its transmission was thought to be only mediated in cattle and ruminant through the bite of infected midges. This sole transmission route has been challenged recently with the emergence of reports of direct contact transmission with some serotypes and vertical transmission from mother to fetus.⁸

BTV is typically transmitted by the bite of *Culicoides*. Competent vectors were thought to be restricted to tropical and subtropical regions. However, outbreaks in North West Europe in 2006 have challenged this view, and it is now apparent that the virus can be transmitted by *Culicoides* species present in those latitudes.⁹ It is therefore critical to understand which *Culicoides* species are competent for BTV transmission. This is doubly important as this knowledge will allow for a better control of vector populations around livestock and for a better understanding of disease epidemiology.

Culicoides imicola in Africa and Asia, *Culicoides brevitarsis* in Australia, and *Culicoides sonorensis* in America are thought to be the main vectors in disease spread in those regions.^{10,11} *C. imicola* can now be found in the European Mediterranean basin and probably established itself from populations blown by winds from northern Africa.¹² *Culicoides obsoletus*, *Culicoides scoticus*, and *Culicoides pulicaris* are nonetheless the most likely competent vectors that spread

Table 1 Virus detection techniques

Test	Status	Advantage	Usage
Virus detection techniques			
Immunofluorescence	In use	Complement the full pathogen characterization	Used mainly in experimental pathogenesis
Competitive ELISA (c-ELISA)	Used routinely in many laboratories	Highly sensitive technique	Detection of the presence of BTV antigens
Dot immunoblotting	Not widely used	Sensitive for serotypes 1–18 and 20	Detection of virus
Electron microscopy	Not widely used for diagnosis	Identification of virus as well as cellular pathology caused by the virus	Diagnosis of unknown etiology virus
Standard RT-PCR	In use but replaced by real time RT-PCR	Sensitivity	Detection of BTV RNA
Real-time RT-PCR	In use	Analytically sensitive and reduced contamination	Detection of BTV RNA
Multiplex PCR	In use	Identified multiple serotypes simultaneously	Detection of BTV RNA
Sequencing	In use	Classification into serogroup, serotype, and toptotype	Obtain full genomic sequence of BTV
Antibody detection techniques			
Agar gel immunodiffusion	Into abeyance	Easy and quick to perform	Detection of BTV antigens
i-ELISA and c-ELISA	Most commonly used	Reliable and robust, commercially available	Detection of BTV antigens
VNT	Diminished use due to cost and labor	Very useful in epidemiology studies	Detection of serotype-specific antibodies
IgM-capture ELISA	Complementary to current tests	New technologies	Detection of BTV antibodies in recent infected cattle
Immunochromatographic strips	Not widely used	Rapid and high specificity	Detection of antibodies to VP7
Protein chips	Not widely used	New technique under development	Detection of antibodies to VP7
Latex agglutination test	Not widely used	New technique under development. Good for large number of samples	Detection of antibodies to VP7
Double-antigen MIA	Not widely used	High specificity and efficiency compared with c-ELISA, while VP2-MIA was as specific as a VNT	Detection of antibodies to VP2 and VP7 simultaneously

Abbreviations: BTV, bluetongue virus; i-ELISA, indirect ELISA; MIA, microsphere immunoassay; RT-PCR, reverse-transcription polymerase chain reaction; VNT, virus neutralization assay.

BTV in European northern latitudes.^{13–15} The distribution of these species in time and place matched BT outbreaks where *C. imicola* was absent.^{14,15} Moreover, BTV could be detected from wild-caught midge pools that did not contain *C. imicola*.¹⁵ Specific identification of the vector species responsible for outbreaks is however problematic as it often relies on morphological traits usually defined in male *Culicoides*, which limits the efficacy of trapping efforts as male do not contribute to BT epidemiology nor do they possess the same affinity for traps as female counterparts.¹⁶ Typically, midges are caught using light/suction traps, but different *Culicoides* species have preference for different light wavelengths.^{17,18} For instance, *C. brevitarsis* was found to prefer green light-emitting diode (LED) to incandescent light or ultraviolet (UV) LEDs,¹⁹ whereas *C. obsoletus* was caught in higher numbers with UV light traps than with incandescent light.²⁰ It should be noted that odor baiting can greatly enhance *Culicoides* trapping.¹⁷ Carbon dioxide is a major attractant of biting midges to their blood host. Sampling around livestock and analysis of preferred host-feeding pattern are also important factors to consider when identifying the transmitting vector.

BTV diagnostic could be greatly helped by improving methodology for molecular identification of vectors in affected areas along with parallel detection of virus in captured *Culicoides* populations. This has helped better define the *Culicoides* competence in transmission of the recently discovered *Schmallenberg virus* (SBV). *Culicoides* pools for SBV testing were prepared from midge heads, while the rest of the carcasses were stored so that the number of infected individuals and species competence could be evaluated.^{21–23} The application of molecular strategies to define *Culicoides* vector competence in BTV similar to those applied to SBV could greatly help to identify the vectors competent for BTV spread in Europe and entomological surveillance. Therefore, this will have major implications for risk assessment of BTV incursion and spread.

BTV can be spread through the transport of infected livestock. To control this, restriction in animal movement and massive vaccination programs are usually set up when outbreaks occur. These measures are however not always effective as observed in Italy where infection has been spreading. Infection of wild ruminants can be damaging to these programs as they can act as a reservoir for the virus.¹⁰ The main risk of breaking contention restrictions however remains the dissemination of infected vectors. *Culicoides* are bad fliers, but their small size allows them to be carried by wind for long distances. Indeed, *Culicoides* movements due to wind have been reported over hundreds of kilometers.^{12,24} As

a consequence, the regional spread of an outbreak is difficult to predict, which limits the efficacy of contention measures.

While in warmer African climates, BT outbreaks can occur throughout the year, in cooler climates like in the southern parts of South Africa the clinical disease usually becomes apparent in late summer and autumn and disappears with the first winter frosts.¹⁰ It has been suggested¹⁰ that this late emergence could be due to a BTV buildup in the vector during spring and early summer. Cattle rarely shows clinical signs but have prolonged viremia,²⁵ which could contribute to this buildup effect. In Europe, the warm and wetter summers that facilitate vector propagation and the warmer winters that permit survival of some adults^{14,26} have allowed the disease to disappear overwinter and reemerge in spring. It is still unclear how this occurs, but a combination of these favorable climatological factors for the vector, the long BTV viremia detected in some animals, vertical transmission from mother to fetus, and infection of wild ruminants are likely to explain why BTV has become endemic in Southern European latitudes.

Although the bite of infected *Culicoides* remains the main source of BTV infection, it is critical to be aware of other possible routes of transmission. Vertical transmission from mother to fetus has been described.^{8,27,28} Transplacental infection is of great economic importance as it can result in abortions, still births, or nonviable offsprings. Oral transmission through ingestion of contaminated placenta is also possible.²⁹ BTV can be detected in the semen of some viremic animals, which suggested that venereal transmission could be possible although this has not been documented.³⁰ The use of shared needles for subcutaneous inoculations can also be a source of BTV transmission.³¹ Horizontal transmission has also been reported for some serotypes through close direct contact between animals in vector-free environments.^{27,32–34} The route of infection in those cases remains unclear although direct contact between animals appears essential. BTV is unlikely to spread horizontally over large distances; however, these findings should be considered in the context of closely housed farmed animals. Hygiene and veterinary practices in outbreaks should therefore be adapted accordingly.

BTV clinical signs

BTV can infect ruminants and camelids,³⁵ but clinical disease is often more pronounced in sheep^{36–38} and in white tail deer.^{39,40} BTV seroconversion has been reported in numerous carnivores in Africa, although the epidemiologic significance of these findings remains unclear.⁴¹ Clinical signs however vary greatly depending on species and individual susceptibility. Cattle and goat are typically subclinically infected and are

often considered as amplifying hosts in endemic regions,¹⁰ Although some serotypes like BTV-8 produce clinical disease in cattle, particularly in naïve region in Europe.^{36,37,42} In all livestock cases, BTV can nonetheless produce economic losses even in subclinical cases, as transplacental transmission can result in fetus malformation.⁸ Since subclinical infections are prominent in cattle and goats, it is important to set up sanitary control for BTV status in those animals to limit disease spread.

BT clinical signs are triggered by its preferred replication in endothelial cells.⁴³ As a result of vascular damage, edema and hemorrhages take place. This damage in endothelial cells also produces a coagulopathy that participates in the hemorrhagic predisposition observed in BTV infections. Early signs of BT are however rather unspecific and resemble those of many other infectious diseases. Early clinical disease presents with pyrexia, lack of appetite, depression, or milk drop in lactating animals.⁴⁴

In sheep, where clinical disease is more pronounced, disease usually manifest with fever, conjunctivitis, lacrimation, congestion of nasal and oral mucosa, and edema of the face and lips. In some cases, the clinical signs do not progress further, but in most cases these signs develop to more severe clinical features like salivation, mucopurulent nasal discharge, severe facial edema, and erosions, ulcerations, and hemorrhages in the nose, lips, and tongue.⁴⁴ BT lesions are typically edematous, erosive, and hemorrhagic. These signs sometimes progress to cyanosis of the tongue, which gives its name to the disease. In most severe cases, pharyngeal and esophageal paresis and respiratory distress are observed. In most sheep, coronitis occurs 8–14 days after infection. This is characterized by lameness in more than one limb of variable intensity and diffused redness in the hoof coronary band. In most severe cases, serum exudate around the coronary band can be observed and animals appear hunched or kneeled and unwilling to stand or move.

In cattle, signs are usually milder than in sheep and only one or two signs manifest besides the pyrexia, lack of appetite, and depression.⁴⁴ Cocirculation of several BTV serotypes in one region could possibly increase clinical signs in cattle in some cases.⁴⁵

Considerations for BT differential diagnosis

Differential diagnosis for BT relies on the basic presentation of edema, ulcerations, and epithelial lesions. The type of lesions as well as their distribution in the infected animal can guide the veterinary practitioner toward a BT diagnosis.

OIE notifiable diseases with similar presentation are one of the main instances in which a clinical differential diagnosis could help set up early control measures. Differential presentation traits will thus be broadly discussed for some of these diseases. Differential diagnosis should nonetheless also include endemic diseases.

BT initial signs are very similar to foot and mouth disease (FMD); however, FMD lesions are vesicular and erosive, whereas BT lesions are hemorrhagic, edematous, and erosive.⁴⁴ Lesion distribution on the tongue also differs between FMD and BT. BT lesions are typically situated at the back and lateral borders of the tongue, whereas FMD lesions are situated at the tip and dorsum of the tongue. FMD lesions also rarely occur in eyes, whereas this is frequent in BTV infections.⁴⁶ Disease transmission is also an indicator for differential diagnosis. BT presentation can be sporadic due to its transmission by biting midges, whereas FMD is highly contagious and as such morbidity will be high in the herd. Vesicular stomatitis is another disease that could be mistaken for BT. Lesion presentation is very similar to FMD, so the same criteria can be applied for differential diagnosis.

Peste des petits ruminants (PPRs) is another infectious disease with similar presentation as BT. In the case of PPRs, nasal discharge can also be mucopurulent, with lesions appearing in the oral and nasal tract, the nares, and the eyes. PPR lesions are hemorrhagic, necrotic, and erosive. Lesion distribution also differs from BT. They appear in the dorsum of the tongue, and respiratory infections are more characteristic of PPR. PPR also produces severe alimentary tract disease with diarrhea, but no coronitis. Moreover, like FMD, PPR is highly contagious, and thus, morbidity will be high in flocks.

Capripoxvirus-induced disease should also be included in the differential diagnosis. These viruses produce lumpy skin disease in cattle or sheep and goat pox. The lumpy skin disease nodular lesions⁴⁷ and the sheep and goat pox pustular lesions that turn crusty⁴⁸ are characteristically proliferative in these diseases. Their wide distribution in the skin of affected animal makes them distinguishable from BT lesions.

Ultimately, although these differential characteristics of BT presentation can guide a field diagnosis, only serological and molecular methods can provide a definitive BT diagnosis.

Overview of current tests for virus detection

Despite advances in molecular biology techniques, traditional diagnostic methods for the detection and identification of BTV remain in force. Different assays have been developed for the detection of BTV infection, based on either antibody

detection or virus detection.⁴⁹ An overview of current tests for antibody detection included in the serological techniques for diagnosis will be provided later in this review. This paragraph aims at summarizing the virologic techniques for BTV detection.

One of the biggest challenges in the diagnosis of BTV has been not only the detection but also differentiation of the different BTV serotypes. One important method for the identification of BTV is viral purification, adaptation, and amplification in embryonated chicken eggs, tissue culture, *Culicoides*-derived cells, or inoculation of susceptible ruminants. At the beginning of 1990s, some diagnostic tests were implemented based on reverse-transcription polymerase chain reaction (RT-PCR) that allowed the detection of a limited number of serotypes. Parts of the viral genome were specifically amplified in a faster and cheaper way than the virus isolation, providing precise information on serogroup, serotype, and topotype, but these assays required to run the samples on agarose gels, which made them difficult to implement in the field or in developing countries. These procedures have been improved along the years, and many RT-PCR protocols are now available, validated, and published. The next step has been the use of micorarrays⁵⁰ and second- and third-generation sequencing⁵¹ for virus diagnostic, but these techniques are expensive and difficult to implement and maintain routinely for diagnosis and surveillance.

Virus isolation

BTV can be isolated from different types of samples:⁵²

1. Blood samples: BTV hosts present a transient viremia with a peak before the seroconversion, usually concomitant with fever. BTV has been found associated with red blood cells, and the isolation is possible from heparinized or sodium citrated whole blood.
2. Semen samples: BTV transmission through semen in sheep and cattle has been suggested,⁵³ and raw or extended semen samples can be used for virus isolation.⁵⁴
3. Tissue samples: Homogenized samples of different infected organs from the host collected in aseptic conditions and refrigerated also allows BTV isolation.⁵⁵ Dead animals or aborted fetuses are optimal to get lung, spleen, lymph nodes, brain, bone marrow, and liver samples,^{55–57} while live animals can also be a source of BTV, from red bone marrow biopsy of adult sheep or liver and spleen from lambs. BTV can be obtained from swabs from the different lesions produced during the disease, through the digestive, vascular, and respiratory tracts.

The most sensitive method for BTV isolation is the intravenous inoculation of 10- to 11-day-old embryonated chicken eggs⁵⁸ with sonicated blood or tissue suspension samples obtained from infected animals. In spite of the reduced isolation efficiency after virus inoculation of tissue culture cells with positively diagnosed samples, many cell lines are also used for this purpose such as the one derived from *Culicoides variipennis* or *sonorensis* midges (KC)^{59,60} or mouse L, baby hamster kidney (BHK-21), African green monkey kidney (Vero), or *Aedes albopictus* clone C6/36 (AA). However, as an alternative, that is, not routinely used, sheep can be inoculated intravenously or subcutaneously with washed blood cells or tissue homogenates from infected animals to amplify and isolate the virus.

Virus characterization

Once the virus is isolated, it can be serogrouped and serotyped.

Serogroup techniques are based on the reactivity of the antisera against conserved proteins, such as VP7, among *Orbivirus* isolates. Another serogroup, epizootic hemorrhagic disease virus, could be mistaken with BTV due to the cross-reactivity between them. For this reason, the use of monoclonal antibodies (MAbs) instead of polyclonal antibodies is recommended.

1. Immunofluorescence (IFA): Different research groups have developed MAbs that bind specifically to different *Orbivirus* isolates, usually based on the VP7 protein, that is conserved within serogroup, allowing its recognition. These MAbs or anti-BTV sera are employed as primary antibodies in IFA procedures to recognize specifically BTV being detected with secondary antibodies coupled to different fluorophores.^{61–63} An alternative technique is the immunoperoxidase method used to detect BTV antigen in fixed cells or tissues,⁶⁴ improving the sensitivity with the peroxidase–antiperoxidase detection, using BTV antiserum raised in sheep as primary antibody,⁶⁵ or the avidin–biotin complex detection.⁶⁶
2. Competitive antigen capture ELISA (competitive ELISA [c-ELISA]): The virus is detected with a double antibody sandwich ELISA. A guinea pig and a murine hyperimmune serum are used for BTV capture, and a third conjugated goat anti-murine antibody is used to develop the assay.⁶⁷ Despite being considered a highly sensitive technique, it has been questioned as a diagnostic tool for BTV detection from blood samples due to false negative registered as well as cross-reactivity between serotypes.⁶⁸

3. Dot immunoperoxidase assay: In this technique, the immune reaction takes place on nitrocellulose membranes where the samples are absorbed as dots and incubated with MAbs directed to the major group-specific antigen of BTV, detected with horseradish peroxidase-conjugated immunoglobulin showing a color reaction in the presence of H_2O_2 and a precipitating chromogen.⁵⁸ Samples from BHK-21 cells infected with available BTV serotypes 1–18 and 20 but no other *Orbivirus* isolates are identified with this assay. However, as with the ELISA, BTV detection directly in infected sheep blood cells or tissue suspensions does not work.⁶⁹
4. Electron microscopy: BTV serogroup particles can be detected by transmission electron microscopy,^{70,71} but immunomicroscopy techniques are needed to identify BTV, for example, with a specific MAb to BTV and gold-labeled protein-A.⁷²
5. Viral genome detection: The Northern and Southern blot techniques or dot blots and in situ hybridizations are used to detect BTV genome through hybridization with cDNA or RNA probes from different segments of the BTV genome and serotypes. Although these assays are sensitive and specific, hybridization techniques are difficult to perform requiring special treatment of the samples, such as electrophoresis (most of them) and optimization of the test conditions. They do not present the optimal properties to be included as routine diagnostic tests. These techniques have been displaced by the PCR or RT-PCR development. Specific oligonucleotide pairs have been developed for serogrouping and serotyping⁷³ BTV genome. The amplified product can be identified by hybridization,^{74–80} enzyme restriction, or sequencing techniques. A range of RT-PCR assays targeting different BTV genome segments have been developed since the early 1990s, but they were only able to detect some of the BTV serotypes.^{76,77,81,82} The rapid evolution of RT-PCR tests has resulted in many assays currently in use with different extraction and amplification methods (reverse transcriptase and polymerase enzymes, primers, or conditions), including the real-time PCR, the main diagnostic tool used in Europe for the detection of BTV in infected ruminants since 2006,⁸³ which is able to differentiate simultaneously all BTV serotypes. For example, Hofmann et al developed a real-time PCR based on the nonstructural protein 3 (NS3) fragment of the BTV genome⁸⁴ or the nested PCR⁸⁵ targeting the *NSI* gene, both able to differentiate 25 of 27 BTV serotypes. In the same way, duplex assays using two primer sets, one targeting the VP7 genome segment and the other the segment 1 (encoding the viral polymerase, VP1), were published.⁸⁶ Many real-time RT-PCR kits have been developed and validated during the last years, which are able to detect the genome of the 27 BTV serotypes. Multiplex PCR in which different primer pairs, based on VP2-specific genes,^{87,88} were developed allowing the identification of multiple serotypes simultaneously. These techniques are the most commonly used because they provide the easiest, cheapest, and fastest as well as specific information on the presence of BTV nucleic acid in blood and tissues from suspected infected samples.^{82,87,89} It may be taken into account that RT-PCR detects virus-specific nucleic acid even if it is no longer viable or infectious.⁹⁰
6. Sequencing techniques: Classification into serogroup, serotype, and even topotype (geographical origin) could be attempted by BTV genome sequencing.^{73,91–93} As the full genomic sequence of BTV isolates becomes available, the design of new primers for further RT-PCR and possibly serotype-specific assays for BTV is greatly facilitated. A wide range of molecular tests based on these sequences are available, requiring however modification or scaling-up in response to the emergence of new variants or serotypes. In spite of advances in sequencing, the reduction of prices, and the increase in companies that routinely sequence, it is still an expensive technique to use as a routine diagnostic test.

Overview of current tests for antibody detection

Serological techniques for the detection of BTV serum antibodies are widely used in Animal Health Laboratories across the world for virus infection confirmation. BTV serological response develops 7–14 days after infection, generating both neutralizing and nonneutralizing anti-BTV antibodies, and they are usually considered long-lasting.⁹⁴ Diagnostic tests for BTV infection can therefore be based around the detection of these anti-BTV antibodies.

Serological techniques for BTV detection can be divided into two main categories according to whether they aim at serogroup (detection of VP7 that is highly conserved within each serogroup) or serotype (neutralization techniques or detection of VP2 protein) specificity during detection. Agar gel immunodiffusion (AGID) and ELISA are the most used techniques for serogroup determination, although a wide range of different assays have been developed along the years for the same purpose. Serum neutralization is the most frequently used assay for BTV serotype identification.

Agar gel immunodiffusion

AGID⁹⁵ is a variant of a classical serological reaction in which a soluble antigen in a clear medium is precipitated by the addition of the specific antibody. Three positive sera are placed in alternate wells with three test sera, while central well is filled with a crude preparation of BTV-infected BHK or Vero cells. Soluble antigens and antibodies diffuse passively through the gel toward each other, forming an insoluble immune complex seen as a precipitin line. The test is easy and quick to perform, and the antigen required is relatively easy to produce. AGID is however only considered by the OIE as an alternative test due to its disadvantages.⁹⁶ Some of the disadvantages are its low sensitivity and specificity comparing with c-ELISA, the fact that is not a quantitative method and the result reading subjectivity factor.^{97,98} Since AGID can detect antibodies to other orbiviruses, especially those against the epizootic hemorrhagic disease, it is no longer considered a sufficiently accurate assay for international trade purposes, and AGID-positive sera results should be retested with a different serogroup-specific assay.⁹⁶

Indirect ELISA (i-ELISA)

i-ELISAs are commercially available for the detection of BTV antibodies in bulk milk. VP7 is used as a substrate for sample BTV antibodies and detected by an anti-ruminant-IgG-conjugate and substrate. It has been shown^{99,100} to be a reliable and robust assay, with a 96.5% specificity and a 98.9% sensitivity, was useful for surveillance purposes.⁹⁶ i-ELISA can also be used as a Differentiation of Infected from Vaccinated Animals (DIVA) system based on the detection of BTV NS3 antibodies.¹⁰¹ Recombinant NS3 was used as antigen, and unvaccinated cattle serum was compared with sera obtained after BTV infection or BTV vaccination with a bivalent inactivated BTV 2–4 vaccine. The greater levels of NS3 antibodies detected in infected animals than in vaccinated ones allow distinguishing between these two groups. This approach might be useful considering that competitive ELISA cannot differentiate infected animals from animals that received an inactivated vaccine.

Competitive ELISA

c-ELISA is a serogroup-specific assay able to measure BTV antibodies without the cross-reactivity problems of AGID.^{102,103} To do so, c-ELISA uses a known amount of one BT serogroup-reactive MAbs, which competes with the sample serum antibodies for the same conserved BTV VP7 antigen. This technique is a prescribed test for international trade by the OIE terrestrial manual⁹⁶ and is extensively used

in clinical laboratories for the detection of BTV antibodies. This test is a sensitive, specific, and reliable test compared with other aforementioned serological techniques like AGID or indirect ELISA.^{102,104} It is also quick and inexpensive and is considered an ideal technique to study BTV distribution, monitor vaccination campaigns, and eradicate campaign policies planning.^{68,96}

Serum neutralization test (SNT)

This test is used for both identification and titration of BTV serotype-specific neutralizing antibodies. Due to the great variety of serotypes cocirculating around the world, being able to distinguish them with a serological test is key to surveillance. There are different SNT protocols that makes it difficult to interpret and compare results.¹⁰⁵ Even though the OIE terrestrial manual⁹⁶ published a standardized procedure after comparing different studies, conditional changes have been proposed to improve sensitivity while retaining specificity by some authors.¹⁰⁵ SNT is based on serial dilutions of the test serum, which is incubated with a fixed amount of each BTV serotype. Cells (usually BHK or Vero) are then added and incubated for 3–5 days, after which cytopathic effect (CPE) is evaluated. Antibody titration is established by the maximum dilution capable of neutralizing >75% of CPE.⁹⁶ The main advantage of SNT probably is its ability to not cross-react with other *Orbivirus* serogroups while distinguishing between BTV serotypes. SNT is considered a highly sensitive and specific assay¹⁰⁶ that not only identifies the BTV serotype responsible for infection but can also be used to check the immune status of individual animals or populations after vaccination. SNT can also determine whether animals are free from infection before movement and provides essential information for eradication policy planning.⁹⁶ SNT present some disadvantages too: it can be time-consuming, laborious, and requires specific reference viruses stocks for each serotype.¹⁰⁶

Other serogroup-specific techniques

Numerous serogroup-specific assays have been developed along the years as alternative or, at least, complementary to the methods already in use.

1. IgM-capture ELISA: This technique was developed for the detection of BTV antibodies in recently infected cattle.¹⁰⁷ It is based on biotinylated capture of anti-bovine IgM antibodies bound to a streptavidin-coated ELISA plate, which can capture IgM antibodies present in test sera. These are detected by the addition of BTV VP7 antigen and an MAb specific for it.

2. Immunochromatographic strips (ICS): This test uses a recombinant BTV VP7 protein immobilized on nitrocellulose membranes to test for the presence of serogroup-specific antibodies. Colloidal gold particles bound to streptococcal protein G are then used to develop the chromatographic strip.¹⁰⁸ Its specificity and sensitivity correlated well with c-ELISA results. ICS thus appears as a rapid test that is suitable for the routine serological surveillance of BTV infection in the field, with a high specificity (97.6%) and sensitivity (100%) compared with c-ELISA.
3. Protein chip detection method: In this technique,¹⁰⁹ BTV VP7 is printed on a new solid supporting material known as polymer-coated initiator-integrated poly (dimethyl siloxane) (iPDMS). After test sera incubation, HRP-conjugated BTV-4H7 MAb is added to detect BTV anti-VP7 antibodies. Like other protein chip techniques, it allows performance of thousands of sample detections in parallel, plus a nearly zero nonspecific protein adsorption background due to iPDMS. Its sensitivity and specificity were reported as 98.6% and 94.8%, respectively.
4. Latex agglutination test: This assay uses latex beads coupled with recombinant VP7 protein to detect anti-BTV antibodies in ruminant serum. Specificity and sensitivity correlated well with c-ELISA (99.0% and 93.0%, respectively). This could be ideal for the screening of large numbers of serum samples due to its quick and easy use.¹¹⁰
5. Double-antigen microsphere immunoassay (MIA): This novel assay is both serogroup- and serotype-specific, as it was developed to detect BTV anti-VP2 and anti-VP7 serum antibodies simultaneously.¹¹¹ MIA is based on color-coded fluorescent beads, each one coated with a different viral antigen. First, test serum and antigen-coated color beads are mixed. Then, biotinylated antibodies specific to the analyte are added to form an antibody–antigen complex which, finally, will be detected, thanks to the addition of a phycoerythrin-conjugated streptavidin and a set of lasers to detect color beads. Authors argue that VP7-MIA showed high specificity and efficiency compared with c-ELISA, while VP2-MIA was as specific as a VNT.

Despite advances in diagnostic techniques over the years, the tests used for the diagnosis that allows serogroup and serotyping are still ELISAs and VNT, respectively. These are only replaced, in the reference laboratories, by the techniques based on RT-PCRs for BTV identification as routine protocols. In France, the annual ring trials based on RT-PCR

have been shown to be a good BTV surveillance method.⁸³ The development of easy, rapid, and cheap BTV serotype identification methods is important for BTV surveillance, vaccination programs, and epidemiology studies.

Current perspectives

In spite of the efficacy of all the techniques developed for BTV diagnosis, there is still room for improvement. In fact, most current methods are either slow or present a high cost. The advantage of a quicker diagnosis to producers is that authorities will be able to rapidly introduce control measures if an outbreak emerges. Molecular techniques such as real-time RT-PCR, although fast enough to identify BTV, need isolated genetic materials and sophisticated instruments, which make these methods unsuitable for in-field analysis. Consequently, development and improvement of diagnosis assays for rapid BTV detection and identification with high sensitivity and selectivity are challenges in the field. Biosensors are attractive solutions for fast and efficient infectious disease diagnostics due to their utilization simplicity and their potential as real-time analysis tools.¹¹² Biosensors recognize a target biomarker specific for the pathogen via an immobilized sensing element called bioreceptor that could be RNA, MAb, aptamers, or others. This bioreceptor is a crucial component that assures high sensitivity and selectivity, avoiding interferences from other pathogens. The interaction between the biomarker and the bioreceptor is converted into a measurable signal that is recorded, allowing qualitative and quantitative pathogen identification. Additional techniques to improve BTV diagnosis should also focus on the development of pen-side tests that allow for the rapid detection of target antigen or antibody, require little or no scientific equipment, and is sensitive and specific for the pathogen.

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Disclosure

The authors report no conflicts of interest in this work.

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