CHAPTER 3.1.3.

BLUETONGUE (INFECTION WITH BLUETONGUE VIRUS)

SUMMARY

Bluetongue (BT) is an infectious, primarily vector-borne viral disease that affects wild and domestic ruminants such as sheep, goats, cattle, buffaloes, deer and most species of African antelope, and camelids as vertebrate hosts. The virus is primarily transmitted between susceptible vertebrate hosts by competent species of Culicoides *midges, and the global distribution of the disease is largely determined by the distribution of competent vectors. Infection with bluetongue virus (BTV) is inapparent in the vast majority of animals but can cause fatal disease in a proportion of infected sheep, deer and wild ruminants. Infection of cattle is usually subclinical, with the exception of BTV-8 infection. Cattle are particularly significant in the epidemiology of the disease due to the prolonged viraemia that occurs following infection. When apparent, clinical signs of BT are mainly attributable to an increase in vascular permeability and include fever, hyperaemia and congestion, facial oedema and haemorrhages, erosion of the mucous membranes, coronitis and laminitis, and pleural and pericardial haemorrhages.*

Detection of the agent: *BTV is the type member of the* Orbivirus *genus of the family* Reoviridae*. There are 27 recognised BTV serotypes and several recently isolated but as yet unclassified unique 'atypical' strains. Virus serotype identification traditionally requires isolation and amplification of the virus in embryonated chicken eggs,* Culicoides *cells or other tissue culture and the subsequent application of serogroup- and serotype-specific tests including virus neutralisation assays. The reverse-transcription polymerase chain reaction (RT-PCR)* assay *has permitted rapid amplification of BTV cDNA in clinical samples and well validated real-time RT-PCR-based systems for virus detection are now routine, allowing for more rapid and sensitive diagnostic testing. The combination of RT-PCR amplification and capillary sequencing or whole genome sequencing now offer relatively rapid unequivocal agent identification at the genome level. Collectively, these procedures augment the classical virological techniques to provide information on virus serogroup, serotype and topotype.*

Serological tests: *Serological responses appear 7–14 days after BTV infection. Infected animals produce both neutralising and non-neutralising anti-BTV antibodies that are generally long-lasting. Enzyme-linked immunosorbent assay (ELISA) and virus neutralisation (VN) are the most frequently used serological tests. A monoclonal antibody-based competitive ELISA to specifically detect anti-BTV (serogroup-specific) antibodies is the test recommended to certify animals as free from infection prior to movement; the test is highly sensitive and specific (compared with the agar gel immunodiffusion), quick, inexpensive and reliable. However, the ELISA may have reduced sensitivity for some serotypes. Procedures to identify and quantify BTV serotype-specific antibodies are more complex, being typically based on cell culture- and live virus-dependent neutralisation tests.*

Requirements for vaccines: *Vaccination is the preferred method of BT control in endemic regions. Vaccination has been used successfully to limit direct losses, minimise the circulation of BTV, eradicate BTV from country/regions and allow safe movement of animals. However, use of live vaccines can be associated with adverse outcomes and live attenuated vaccine strains can be spread by vectors, with eventual reversion to virulence or reassortment of vaccine virus genes with those of wild-type virus strains. Inactivated vaccines are safer, but require multiple doses to become efficacious and incur higher costs. Such vaccines have been very effective in combatting the spread of BTV-8, BTV-1 and BTV-4 in Europe.*

A. INTRODUCTION

1. Description of the disease

Bluetongue (BT) is an infectious, primarily vector-borne viral disease that affects wild and domestic ruminants. Midges of an increasing number and geographical range of species (Carpenter *et al*., 2015) in the genus *Culicoides* (the insect host) typically transmit bluetongue virus (BTV) among susceptible ruminants, having become infected by feeding on viraemic animals (the vertebrate host). Other routes of transmission have been documented including direct vertical, oral and possibly venereal transmission and indirect transmission through reused needles; however the epidemiological significance of these routes remains uncertain (Belbis *et al*., 2017; Darpel *et al*., 2016; Kirkland *et al*., 2004). The more recently recognised serotypes, such as BTV-25, BTV-26 and BTV-27 appear to be transmitted exclusively by these vector-independent routes, and may result in persistent infection in goats (Belbis *et al*., 2017; MacLachlan *et al*., 2015; Vogtlin *et al.,* 2013).

The global distribution of BTV is determined by epidemiological systems (episystems) that are in turn primarily delimited by specific vector species, the presence of susceptible host species and their collective natural history. Observations in Europe, India and the USA indicate that strains of BTV can move between episystems through movement of animals, wind dispersion of infected vectors and by adaptations of both vector species and virus (Carpenter *et al*., 2015; Jacquot *et al*., 2019; Maan *et al*., 2015). The expanding global range of BTV infection has been notable, particularly at the traditional northern- and southern-most limits of BTV distribution with incursions of multiple serotypes into Europe, Australia, both North and South America, and Asia, in countries that had not previously reported BTV infections (MacLachlan *et al*., 2015; MacLachlan & Mayo, 2013). In addition to detecting and confirming an increasing number of serotypes, developing sequencing technologies, phenotyping software and molecular assays have revealed that two major ancestral lineages, a Western (Africa, Europe, the Americas) and an Eastern (Australia, and Asia) exist globally (Maan *et al*., 2015; Mertens *et al*., 2007).

In temperate parts of the world, infection has a seasonal occurrence (Verwoerd & Erasmus, 2004), whereas in tropical regions BTV infection can occur year-round (MacLachlan *et al*., 2015). Survival of BTV in the environment is associated with insect factors such as over-wintering (Mayo *et al*., 2016). Additionally, long-term persistence of BTV in a region only occurs when multiple virus serotypes circulate (MacLachlan *et al*., 2015). The ability of the more recently detected BTV serotypes 25, 26 and 27 to be transmitted in the absence of vector insects reduces the likelihood of seasonality in their occurrence or dependence on the geographical distribution of vector species for transmission.

The vertebrate hosts for BTV include both domestic and wild ruminants, such as sheep, goats, cattle, buffaloes, deer, most species of African antelope and other *Artiodactyla* such as camels. Although detection of antibodies to BTV antigen or viral nucleic acid or live virus has been demonstrated in some carnivores, black and white rhinoceroses and elephants, the role of non-ruminant species in BTV epidemiology is considered minimal. The outcome of infection ranges from subclinical in the vast majority of infected animals, especially wild African ruminants, cattle and goats, to serious or fatal in a proportion of infected sheep, goats, deer and some wild ruminants (Verwoerd & Erasmus, 2004). Infection in cattle and goats is typically subclinical and these species are considered to be amplifying reservoir hosts in endemic regions, making control measures for BTV in those animals important. However, a higher incidence and severity of clinical disease has been observed in naïve cattle infected with BTV-8. As breeds of sheep have varying levels of susceptibility to disease, BTV infections of livestock can occur unobserved and be detected only by active surveillance (Daniels *et al*., 2004).

Clinical signs of disease in sheep vary markedly in severity, influenced by the type or strain of the infecting virus, husbandry factors and animal breed (Verwoerd & Erasmus, 2004). In severe cases there is an acute febrile response characterised by hyperaemia and congestion, leading to oedema of the face, eyelids and ears, and haemorrhages and erosions of the mucous membranes. The tongue may show intense hyperaemia and become oedematous, protrude from the mouth and in severe cases become cyanotic. Hyperaemia may extend to other parts of the body particularly the coronary band of the hoof, the groin, axilla and perineum (MacLachlan & Mayo, 2013). Sheep that develop chronic disease often have severe muscle degeneration, and breaks in the wool may occur associated with pathology in the follicles. A reluctance to move is common and torticollis may occur in severe cases (Maclachlan *et al.,* 2009). In fatal cases, the lungs may show interalveolar hyperaemia, severe alveolar oedema and the bronchial tree may be filled with froth. The thoracic cavity and pericardial sac may contain varying quantities of plasma-like fluid. Most cases show a distinctive haemorrhage near the base of the pulmonary artery (Verwoerd & Erasmus, 2004). In cases of vertical transmission of BTV-8, the central nervous system may be severely affected resulting in a failure of the cerebral hemispheres to develop (dummy calf syndrome). With virulent strains, infection in naïve sheep can result in mortality rates as high as 70%.

Control of BTV in animals is covered in Chapter 8.3 of the WOAH *Terrestrial Animal Health Code*. Because of the high percentage of subclinical infections, persistent viraemia, and challenges with vector control, traditional methods of control and eradication of BTV, such as movement controls, stamping out and vector control have not always been successful in controlling the disease. Thus, a safe and efficacious vaccine that meets the objectives of control, eradication and prevention requirements is an important component of disease control in many settings (MacLachlan & Mayo, 2013).

2. Nature and classification of the pathogen

Taxonomically, BTV is classified as the type species in the *Orbivirus* genus in the family *Reoviridae*, one of 22 recognised species in the genus that also includes epizootic haemorrhagic disease virus (EHDV), equine encephalosis virus and African horse sickness virus. There is significant immunological cross-reactivity among members of the BTV serogroup. Within species, individual BTV are differentiated on the basis of genotype and neutralisation tests; currently 27 serotypes of BTV are recognised including Toggenburg Orbivirus (BTV-25), BTV-26 from Kuwait and BTV-27 from Corsica. These most recently detected serotypes and several other as yet serotypically unclassified unique BTV strains (Belbis *et al*., 2017) may exhibit quite distinct modes of transmission associated with their respective abilities to infect *Culicoides* spp. and specific ruminant hosts (Breard *et al*., 2018, Chaignat *et al*., 2009, Maan *et al*., 2015; Savini, 2015). Both genetic shift and genetic drift account for the diversity and heterogenicity of the BTV strains in the field (Pritchard *et al*., 2004).

BTV particles are composed of three protein layers. The outer capsid layer contains two proteins, VP2 and VP5. VP2 is the major neutralising antigen and determinant of serotype specificity. Removal of the outer VP2/VP5 layer leaves a bi-layered icosahedral core particle that comprises an outer layer composed entirely of capsomeres of VP7 and a complete inner capsid shell (the subcore layer) comprising VP3, which surrounds 10 dsRNA genome segments and minor structural proteins (VP1, VP4 and VP6). VP7 is a major determinant of serogroup specificity and the site of epitopes used in competitive enzyme-linked immunosorbent assays (C-ELISAs) to detect anti-BTV antibodies (Mertens *et al*., 2005). VP7 can also mediate attachment of BTV to insect cells.

Genetic sequencing of specific BTV genome segments and movement towards full genome analyses allows for additional differentiation and analysis of strains apart from serotyping, with potential to identify multiple different clades for each genome segment[1] (Gould, 1987; Jacquot *et al*., 2019). Even for strains within one serotype it is therefore possible to identify the likely geographical origin (topotype) for each genome segment (Gould, 1987). Identification of apparent associations between some genotypes of virus and some vector species has led to further development of the concept of viral-vector episystems (Daniels *et al*., 2004). Movements of several BTV serotypes between vector species and into new geographical regions, leading to multiple reassortment events and the emergence of novel strains containing new combinations of genome segments from different origins (Jacquot *et al.,* 2019; Nomikou *et al.,* 2015), indicate that a more complete identification of the genetic makeup of each strain would contribute to a better understanding of BTV epidemiology.

3. Zoonotic potential and biosafety and biosecurity requirements

There is no known risk of human infection with BTV. Biocontainment measures should be determined by risk analysis as described in Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*.

4. Differential diagnosis

Depending on the clinical presentation, affected species and epidemiological data, differential diagnoses for suspected cases of BTV infection may include other infectious diseases including but not limited to epizootic haemorrhagic disease, foot and mouth disease, peste des petits ruminants, sheep and goat pox, vesicular stomatitis and contagious ecthyma. In cases of dummy calf syndrome, bovine viral diarrhoea virus should be taken into consideration. Non-infectious causes, including photosensitisation, should also be considered.

1 See BTV-Glue database http://btv-glue.cvr.gla.ac.uk

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of bluetongue and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Detection of the agent[a]						
Real-time RT-PCR	–	+++	–	+++	++	–
RT-PCR	–	+++	–	+++	++	–
Classical virus isolation	–	+++	–	+++	–	–
Detection of immune response						
C-ELISA (serogroup specific)	+++	+++	++	–	+++	++
VN (serotype specific)	+	+	++	+	++	++
AGID	+	+	+	–	+	+

Key: +++ = recommended for this purpose; ++ recommended but has limitations;
+ = suitable in very limited circumstances; – = not appropriate for this purpose.
RT-PCR = reverse-transcription polymerase chain reaction; C-ELISA = competitive enzyme-linked immunosorbent assay;
VN = virus neutralisation; AGID = agar gel immunodiffusion
[a]A combination of agent identification methods applied on the same clinical sample is recommended.

1. Detection of the BTV agent

1.1. *In-vitro* and *in-vivo* cultures

Specimens for virus isolation can include unclotted blood (heparin or EDTA [ethylene diamine tetra-acetic acid]-treated whole blood) from suspected viraemic animals, blood clots after separation of serum, spleen or lymph nodes collected at necropsy of clinical cases, or midges. Several virus isolation systems for BTV are in common use, including inoculation of embryonated chicken eggs (ECE) and primary inoculation of cell cultures such as the KC cell line (a cell line derived from *C. sonorensis* midges). The KC cell line has been proven to be very sensitive (McHolland & Mecham, 2003) and from an animal ethics perspective reduces the use of embryonated eggs. Attempts to isolate virus *in vitro* in mammalian cell culture systems may be considered more convenient, however the success rate is frequently much lower than that achieved with embryonated chicken egg and KC cell systems. The same diagnostic procedures are used for domestic and wild ruminants. Inoculation of sheep continues to be used for *in-vivo* testing and amplification of virus but should be avoided wherever possible in accordance with chapter 7.8 of the *Terrestrial Animal Health Code*.

1.1.1. Isolation in embryonated chicken eggs

i) Blood is collected from suspected viraemic animals into an anticoagulant such as EDTA, heparin or sodium citrate, and the blood cells are washed three times with sterile phosphate-buffered saline (PBS). Washed cells are re-suspended in PBS or isotonic sodium chloride

and either stored at 4°C or used immediately for attempted virus isolation. Tissue and midge suspension can be also prepared and stored as described above or immediately used.

ii) For long-term storage at 4°C or where refrigeration is not possible for short periods of time, blood samples are collected in oxalate–phenol–glycerine. Washed red blood cells and tissue homogenates can be stored directly at –70°C. The virus is not stable at –20°C. BTV has remained viable for several months or even years in whole blood in anticoagulant stored at 4°C.

iii) In fatal cases, spleen and lymph nodes are the preferred organs for virus isolation attempts. Organs and tissues should be kept and transported at 4°C to a laboratory where they are homogenised in PBS or isotonic saline (1/10), centrifuged at 1500 rpm for 10 minutes, and filtered (0.2–0.4 μm). The tissue suspensions can be used as described below for blood cells.

iv) Washed blood cells are re-suspended 1/1 in distilled water (to disrupt whole red blood cells) then diluted to 1/10 in PBS to ensure isotonic balance. 0.1 ml amounts are inoculated intravascularly into 5–12 ECE that are 9–12 days old. This procedure requires skill and practice. Details are provided by Clavijo *et al*. (2000).

v) The eggs are incubated in a humid chamber at 32–33.5°C and candled daily. Any embryo deaths within the first 24 hours post-inoculation are regarded as nonspecific.

vi) Embryos that die between days 2 and 7 and embryos remaining alive at 7 days are retained at 4°C overnight before harvesting. Infected embryos may have a haemorrhagic appearance. Dead embryos and those that live to 7 days are homogenised as two separate pools. Whole embryos, after removal of their heads, or pooled organs such as the liver, heart, spleen, lungs and kidney, are homogenised and the debris removed by centrifugation at 1500 ***g*** for 10 minutes.

vii) Virus in the supernatant may be identified either directly as described in Section B.1.2 below or after further amplification in cell culture, as described in Section B.1.1.2.

1.1.2. Isolation in cell culture

Virus isolation is best achieved by primary isolation (or amplification) of virus in ECE, followed by a passage in the *Aedes albopictus* (AA) clone C6/36 insect cell culture or primary isolation in cells derived from *Culicoides sonorensis* (free of BT viruses) and designated as KC or CuVa cells (McHolland & Mecham, 2003). These two amplification steps are then followed by a passage in mammalian cell lines such as baby hamster kidney (BHK 21) or African green monkey kidney (Vero). Passages in BHK 21 and Vero will enable further replication of virus and visual confirmation of virus isolation by cytopathic effect (CPE).

There have been occasions where CPE is not seen in a mammalian cell line, but BTV antigen has been visualised when staining by direct immunofluorescence. Cell monolayers are incubated at 37°C in 5% CO_2 with humidity and monitored for the appearance of CPE after 5–7 days. If no CPE appears, a second passage is made in the mammalian cell culture.

Positive CPE or immunofluorescence detection or negative cell culture results for BTV must be confirmed after each ECE, KC or cell culture passage by antigen detection ELISA or polymerase chain reaction (PCR) techniques.

1.2. Virus detection and characterisation

The success of virus isolation techniques is assessed by testing for the presence of BTV in the cell culture supernatants or embryo tissues using a variety of detection systems. Virus detection systems include antigen-capture ELISA, direct immunofluorescence, reverse-transcription PCR (RT-PCR) or real-time RT-PCR, as described in Section B.1.3 below. Currently, testing of the isolation media by real-time RT-PCR is the preferred screening method.

Detection and characterisation are typically step-wise processes, with serogroup-specific tests used initially to detect the presence of a BTV. Subsequent genotype and serotype identification of BTV isolates provides valuable epidemiological information and is critical for the implementation of vaccines or for vaccine development. RT-PCR assays employing serotype-specific primers will provide the most rapid

and specific information regarding isolate serotype (Mertens *et al.,* 2007). Genotyping for molecular epidemiology can be based on RT-PCR tests and sequencing of the amplicon. Different laboratories have standardised several different gene sequences for this purpose. Where available, full genome sequencing may also be performed to provide serotype, as well as other unique sequence information of isolates.

Neutralisation procedures using individual serotype antisera may also be employed for serotyping, although some serotypes are cross-reactive and interpretation can be difficult. For laboratories without serotyping capabilities, BTV isolates may be submitted to any WOAH BT Reference Laboratory for serotyping of isolates.

1.2.2. Immunological serogrouping of viruses

Orbivirus isolates are typically serogrouped on the basis of their reactivity with specific standard antisera that detect proteins, such as VP7, that are conserved within each serogroup. The cross-reactivity between members of the BT and EHD serogroups raises the possibility that an isolate of EHDV could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. Polyclonal and monoclonal antibodies (MAbs) used for serogrouping BTV isolates must be characterised as appropriate for the purpose. There exists significant VP7 variation within BTV, as well as antigenic relatedness between other closely related orbiviruses, such as EHDV, that will influence antibodies binding in different assay formats (IFA, ELISA, AGID). For this reason, a BT serogroup-specific MAb can be used. A number of laboratories have generated such serogroup-specific reagents. Commonly used methods for the identification of viruses to serogroup level are as follows.

i) *Immunofluorescence/immunoperoxidase staining*

Monolayers of BHK or Vero cells in various tissue culture substrates (including chamber slides, glass cover-slips, 96 or 24 well plates or other suitable formats) are infected with either tissue culture-adapted virus or virus in ECE lysates. After 24–48 hours at 37°C, or after the appearance of a mild CPE, infected cells are fixed with agents such as paraformaldehyde, acetone or methanol, dried and viral antigen detected using anti-BTV antiserum or BTV-specific MAbs and standard immunofluorescent procedures. Standard immunoperoxidase staining procedures can also be used when fluorescent microscopes are not available for reading immunofluorescence, as stained antigen can be read by eye or using light microscope at 100× magnification.

ii) *Antigen capture enzyme-linked immunosorbent assay*

Viral antigen in ECE, culture medium harvests and infected insects may be detected directly using an antigen-capture ELISA (Ag ELISA). In this technique, virus derived proteins are captured by antibody adsorbed to an ELISA plate and bound materials detected using a second antibody. The capture antibody may be polyclonal or a serogroup-specific MAb. Serogroup-specific MAbs and polyclonal antibody raised to VP7 proteins or whole virus have been used successfully to detect captured virus. Antigen detection from whole blood is not always successful using the Ag ELISA.

1.2.2. Serotyping of isolates by virus neutralisation

Neutralisation tests are type specific for the currently recognised BTV serotypes that have been isolated in culture. Reference antibodies can be used to serotype a virus. In the case of an untyped isolate, the characteristic regional localisation of BTV serotypes can generally obviate the need to attempt neutralisation by antisera to all isolated serotypes, particularly when endemic serotypes are well known.

There is a variety of tissue culture-based methods available to aid in typing isolates. Cell lines commonly used are BHK, Vero and L929. Three methods to serotype BTV isolates are outlined briefly below. For antibody typing virus neutralisation methods, see Section B.2. below. There is also a fluorescence inhibition test, not described. BTV serotype-specific antisera generated in guinea-pigs or rabbits have been reported to have less serotype cross-reactivity than those made in cattle or sheep. It is important that antiserum controls be included.

i) *Plaque reduction*

The virus to be serotyped is diluted to contain approximately 100 plaque-forming units (PFU) and incubated with either no antiserum (virus control) or with serial dilutions of individual standard antisera to a panel of BTV serotypes. Virus/antiserum mixtures are added to monolayers of cells. After adsorption and removal of inoculum, monolayers are overlaid with agarose or carboxy-methyl cellulose (medium viscosity) for easy removal of semi-solid agar before staining. The neutralising antibody titres are determined as the reciprocal of the serum dilution that causes a fixed reduction (e.g. 80%) in the number of PFU. The unidentified virus is considered serologically identical to a standard serotype if the latter is run in parallel with the untyped virus in the test and is similarly neutralised.

ii) *Plaque inhibition*

Tests are performed in 90 mm diameter Petri dishes containing confluent cell monolayers that are infected with approximately 5×10^4 PFU standard or untyped virus. After adsorption and removal of inoculum, monolayers are overlaid with agarose. Standard anti-BTV antisera are added to individual filter paper discs and placed on the agarose surface. Dishes are incubated for at least 4 days. A zone of virus neutralisation, with concomitant survival of the cell monolayer, will surround the disc containing the homologous antiserum.

iii) *Microtitre neutralisation*

Approximately 100 $TCID_{50}$ (50% tissue culture infective dose) of the standard or untyped virus is added in 50 µl volumes to test wells of a flat-bottomed microtitre plate and mixed with an equal volume of standard antiserum serially diluted in tissue culture medium. Approximately 10^4 cells are added per well in a volume of 100 µl, and plates incubated for up to 7 days, depending on the level of CPE observed in negative serum wells of the test. When CPE has developed to 4+ (100%) in the negative control serum wells of each serotype tested, the test is read using an inverted microscope. Wells are scored for the degree of CPE observed. Those wells that contain cells only or cells and antiserum, should show no CPE. In contrast, wells containing cells and virus should show 75–100% CPE. The unidentified virus is considered serologically identical to a standard BTV serotype if both are neutralised in the test to a similar extent.

1.3. Molecular methods

1.3.1. Detection of nucleic acid

RT-PCR techniques provide rapid identification of BT viral nucleic acid in blood and other tissues of infected animals. Importantly, RT-PCR-based diagnostics should be interpreted with caution because the RT-PCR procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian hosts. Hence a positive RT-PCR result, does not necessarily indicate the presence of infectious virus. In addition, RNA originating from vaccine strains can be detected.

Multiple RT-PCR formats are available that can be used to detect BTV specifically to 'serogroup' Orbiviruses and to 'serotype' BTV. These molecular approaches are much more rapid than traditional virological and immunological approaches, which may require up to 4 weeks to generate information on serogroup and serotype. Developments in molecular assays, new sequencing technologies and phenotyping software have identified increasing numbers of new serotypes, methods of transmission, and host-specific susceptibility patterns.

The nucleic acid sequence of cognate BTV genes may differ depending on the geographical area of virus isolation (Gould, 1987; Maan *et al.,* 2015). This has provided a unique opportunity to complement studies of BTV epidemiology by providing information on the potential geographical origin of virus isolates, a process termed genotyping or topotyping. While sequencing of BTV isolates from different parts of the world may allow detection of various clades for each genome segment, which may permit finer discrimination of geographical origin, the relationship between sequence and geographical origin is not absolute in all cases. Thus, BTV sequencing information is vitally important and all data regarding BTV segment sequences should be made widely available by submitting appropriately validated data to reputable sequence databases such as

GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The BTV-Glue website (http://btv-glue.cvr.gla.ac.uk) can provide phylogenetic tree analyses of BTV isolates based on the sequence of RNA segments. These compiled data will provide a resource for epidemiological studies, the identification of new isolates, as well as supporting *in-silico* analyses for maintenance and validation of existing RT-PCR assays and the design of new primers probes for development of additional assays, e.g. for novel/variant BTV types/strains.

The capacity of RT-PCR assays to detect very small numbers of nucleic acid molecules means that such tests are exquisitely sensitive to contamination by extraneous nucleic acids leading to false positive results. False negatives, due for example to poor nucleic acid preparation or inappropriate primers, may also be encountered. This is covered in detail in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*.

There are many RT-PCR assays currently in use that use different extraction methods, reverse transcriptases, amplification enzymes, primers and conditions. Technology is changing rapidly, and the genetic diversity of the BTV genes makes the choice and validation of RT-PCR assays conditional on its application in a regional setting. Therefore, the procedures listed below are examples only.

Two BTV RT-PCR assays are presented here: a real-time assay (Hofmann *et al*., 2008), targeting the NS3 gene segment and a conventional nested assay targeting the NS1 gene segment, using primers designed by Katz *et al*. (1993). The nested assay has been successfully used for over 20 years and can detect serotypes 1–24 and 26 (there are no reports of testing of other serotypes from multiple species. The nested assay may be beneficial for laboratories without the capacity to perform real-time RT-PCR. The real-time RT-PCR assay presented below has been tested at several laboratories world-wide and has been found capable of detecting all 27 serotypes of BTV (as well as other recently detected novel BTV strains), and equals or surpasses the sensitivity of the nested assay while providing rapid, quantitative detection of BTV without the contamination risks associated with nested RT-PCR assays.

1.3.1.1. Real-time reverse-transcription polymerase chain reaction

Real-time RT-PCR methods provide sensitive and rapid detection of BTV in a one-step procedure. Advantages of real-time methods over traditional PCR methods include rapidity of testing, quantitation of the virus present, and the reduced opportunity for contamination to occur as no post-amplification handling such as gel electrophoresis is needed. The real-time RT-PCR assays are the tests of choice for diagnosis.

The method presented here is an adaption from Hofmann *et al.* (2008) and is capable of detecting all known BTV serotypes and strains currently circulating. The assay targets BTV segment 10 (NS3). The procedure given may require modification to accommodate individual laboratory or different RT-PCR kit requirements.

i) RNA extraction from blood, tissue samples, and midges

Commercial kits are widely available; the RNA extraction step can be performed according to the procedures specified in each kit.

ii) Real-time reverse-transcription polymerase chain reaction

Kits for the one-step real-time PCR are available commercially. Below are some basic steps as described by Hofmann *et al*. (2008), which can be modified depending upon local/case-specific requirements, kits used and equipment available.

Primer and probe sequences for the detection of BTV species viruses:

BTV_IVI_F 5'-TGG-AYA-AAG-CRA-TGT-CAA-A-3'

BTV_IVI_R 5'-ACR-TCA-TCA-CGA-AAC-GCT-TC-3'

BTV_IVI_P 5'FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG- C-3' BHQ1

a) Primer stock solutions are diluted to a working concentration of 20 pmol/µl, whereas probe is diluted to a working concentration of 5 pmol/µl.

b) A test plate layout should be designed and loaded into the real-time PCR machine software. Using the layout as a guide, 0.5 µl of each primer working stock (20 pmol/µl) is added to each well that will go on to contain RNA samples, positive or negative controls. The plate is held on ice.

Note: PCR plates can be replaced with tubes or strips as appropriate.

c) 2 µl of RNA samples, including test and positive and negative controls, are added to appropriate wells of the plate following the layout (note: these wells already contain primers from step b).

d) Heat denaturation: 95°C for 5 minutes, hold on ice for further 3 minutes.

e) An appropriate volume of real-time one-step RT-PCR master mix for the number of samples to be tested is prepared, following the manufacturer's instructions. Probe should be included in the master mix to give a final concentration of 0.2 pmol/µl per sample.

f) 22 µl of master mix is distributed in each well on the PCR plate containing the denatured primers and RNA.

g) The plate is placed in a real-time thermal cycler programmed for reverse transcription and cDNA amplification/fluorescence detection as suggested by the manufacturers. The following thermal profile is an example:

	48°C	30 minutes
	95°C	2 minutes
50 cycles:	95°C	15 seconds
	56°C	30 seconds
	72°C	×30 seconds

1.3.1.2. Reverse-transcription polymerase chain reaction – nested PCR

Select RT-PCR (reverse-transcription and first stage amplification) and PCR (nested amplification) kits are available to perform the nested assay. The assay presented below for illustration uses the parameters associated with a specific kit. The PCR parameters should be adjusted according to the manufacturer's recommendations for the specific kits to be used.

The nested assay employs the use of the following primers:

First stage amplification (outer) (dilute to 25 pmol/µl; final concentration in PCR is 0.6 µM each):

FW: GTT-CTC-TAG-TTG-GCA-ACC-ACC
RV: AGG-CCA-GAC-TGT-TTC-CCG-AT

Nested amplification (dilute to 25 pmol/µl; final concentration in PCR is 0.5 µM each):

nFW: GCA-GCA-TTT-TGA-GAG-AGC-GA
nRV: CCC-GAT-CAT-ACA-TTG-CTT-CCT

i) Prepare the first stage amplification mixture (one-step RT-PCR kit) of the following reagents (per sample):

Nuclease-free water	11.8 µl
5× one-step RT-PCR buffer	5.0 µl
dNTP mix	1.0 µl
Enzyme	1.0 µl
FW primer (25 pmol/µl	0.6 µl
RV primer (25 pmol/µl)	0.6 µl

ii) Dispense 20 µl of the mixture into each PCR tube included in the assay. Add 5 µl of sample or control denatured RNA (described above) to the appropriate tube. Place tubes in a thermal cycler and run the following programme:

Reverse transcription	50°C	30 minutes
Taq activation	95°C	15 minutes
Followed by 35 cycles of:		
Denature:	94°C	45 seconds
Anneal:	58°C	45 seconds
Extension	72°C	60 seconds (final extension 10 minutes)

iii) Prepare a nested PCR mixture (HotStarTaq DNA Polymerase kit) of the following reagents (per sample):

Nuclease-free water	40.75 µl
10×HotStar buffer	5.0 µl
dNTP mix	1.0 µl
HotStarTaq	0.25 µl
nFW primer (25 pmol/µl)	1.0 µl
nRV primer (25 pmol/µl)	1.0 µl

iv) Dispense 49 µl of the mixture into each PCR tube. Transfer 1.0 µl of the amplified DNA from the first stage reaction (step 2) to the appropriate nested tube. Change gloves between samples and use caution when transferring the DNA to avoid cross contamination of samples. Place tubes in a thermal cycler and run the following programme:

Taq activation	95°C	15 minutes
Followed by 28 cycles of:		
Denature:	94°C	45 seconds
Anneal:	62°C	45 seconds
Extension:	72°C	1 minute (final extension 10 minutes)

Perform gel electrophoresis followed by modern gel visualisation methods on the nested PCR product. The positive control(s) and any positive samples will have a 101 base pair band. Negative controls and negative samples should not have a visible band. Positive samples may be sequenced for verification.

1.3.2. Nucleic acid sequencing

Although PCR-based methods can provide a rapid prospective determination of the serotype and genotype of a BTV isolate, the nucleic sequence of specific BTV genome segments is required for ultimate unequivocal identification. Through the exploitation of information provided by real-time RT-PCR or serology, and the now extensive availability of BTV sequence databases that better inform primer design, the combination of RT-PCR amplification and high throughput capillary sequencing can offer a relatively rapid, confirmative diagnostic approach. The now more economically viable option of whole genome sequencing (WGS) is also being increasingly routinely applied to BTV diagnosis (Belbis *et al*., 2017). However, the concentration of BTV in field isolations can provide limitations to the effectiveness of these sequencing approaches and prior establishment of growth in cell culture may be required for some isolates. Care should be taken concerning interpretation of results obtained with tissue culture grown virus samples, as the process of adaptation to cell culture inevitably involves selection of those viruses that can infect and replicate in the culture system used. The sequences generated may not therefore fully represent the virus population present in the original diagnostic sample.

2. Serological tests

Serological responses appear 7–14 days after BTV infection. Infected animals produce both neutralising and non-neutralising anti-BTV antibodies that are generally long-lasting. Anti-BTV antibodies generated in infected animals can be detected in a variety of ways that vary in sensitivity and specificity. Both serogroup-specific and serotype-specific antibodies are elicited and if the animal was not previously exposed to BTV, the neutralising antibodies generated are specific for the serotype of the infecting virus. Multiple infections with different BTV serotypes lead to the production of antibodies capable of neutralising serotypes to which the animal has not been exposed.

2.1. Competitive enzyme-linked immunosorbent assay

The BT competitive or blocking ELISA (C-ELISA) was developed to measure BTV-specific antibody without detecting cross-reacting antibody to other Orbiviruses (Afshar *et al*., 1989; Lunt *et al*., 1988). The specificity is the result of using BT serogroup-reactive MAbs. These antibodies were derived in separate laboratories, and although possessing different properties or epitope specificities, all appear to bind to the amino-terminal region of the major core protein VP7. In the C-ELISA, antibodies in test sera compete with the MAbs for binding to antigen. The following procedure for the C-ELISA has been standardised after comparative studies in a number of international laboratories. Importantly, monoclonal antibody-based competitive ELISA formats have been known to not detect all serotypes of BTV (particularly some BTV-15). Use of such ELISAs therefore requires an understanding of the test's fitness for purpose, and the assay must be validated accordingly to avoid missing detection of some BTV serotypes, including the novel serotypes recently identified.

2.1.1. Test procedure

There are several test procedures described; this is an example of one BT ELISA procedure.

i) First, 96-well microtitre plates are coated at 4°C overnight or at 37°C for 1 hour with 50–100 µl of either tissue culture-derived sonicated cell culture antigen or the major core antigen VP7 expressed in either *Baculovirus* (Oldfield *et al*., 1990) or yeast (Martyn *et al*., 1990) and diluted in 0.05 M carbonate buffer, pH 9.6.

ii) The plates are washed five times with PBST (0.01 M PBS containing 0.05% or 0.1% Tween 20, pH 7.2).

iii) Next, 50 µl of test sera is added in duplicate at a single dilution, either 1/5 (Afshar *et al.,* 1989) or 1/10 (Lunt *et al*., 1988) in PBST containing 3% bovine serum albumin (BSA).

iv) Immediately, 50 µl of a predetermined dilution of MAb diluted in PBST containing 3% BSA is added to each well. MAb control wells contain diluent buffer in place of test serum.

v) Plates are incubated for 1 hour at 37°C or 3 hours at 25°C, with continuous shaking.

vi) After washing as described above, wells are filled with 100 µl of an appropriate dilution of horseradish peroxidase-labelled rabbit anti-mouse IgG (H+L) in PBST containing 2% normal bovine serum.

vii) Following incubation for 1 hour at 37°C, the conjugate solution is discarded and plates are washed five times using PBS or PBST. Wells are filled with 100 µl substrate solution containing 1.0 mM ABTS (2,2'-azino-bis-[3-ethylbenzothiazoline-6-sulphonic acid]), 4 mM H_2O_2 in 50 mM sodium citrate, pH 4.0, and the plates are shaken at 25°C for 30 minutes. (Other substrates may be used and the reaction continued with shaking for an appropriate length of time to permit colour development.)

viii) The reaction is stopped by addition of a stopping reagent, such as sodium azide.

ix) After blanking the ELISA reader on wells containing substrate and stopping reagent, the absorbance values are measured at 414 nm. Results are expressed as per cent inhibition and are derived from the mean absorbance values for each sample by the following formula:

% inhibition = 100 – [(mean absorbance test sample)/(mean absorbance MAb control) × 100].

NB: Some laboratories prefer to use a negative control serum that has previously been shown to have a percentage inhibition of zero as an alternative to the MAb control.

x) Percentage inhibition values >50% are deemed positive. Inhibition between 40% and 50% is considered to be suspicious. The results of the test sera duplicates can vary as long as the results do not lie either side of the positive cut -off.

xi) Strong and weak positive sera and a negative serum should be included on each plate. The weak positive should give 60–80% inhibition and the negative should give less than 40% inhibition.

A number of commercially produced C-ELISAs based on recombinant VP7 and anti-VP7 MAb are now available. These commercial assays are routinely used in many laboratories across the world and have been proved to be fit for purpose in ring-trials (Batten *et al*., 2008). Formal acceptance for trade purposes should depend on adoption of individual kits to the WOAH Register.

Genetic divergence of certain BTV strains (e.g. different regional groups or topotypes) may affect the nature of serogroup-reactive antibodies. It is therefore possible that diagnostic characteristics for antibody detection are not uniform for all viruses encompassed by the serogroup. Diagnostic reagents and kits produced in one region may not have the same performance characteristics when used in another region. This should be addressed in considerations of fitness for purpose.

2.2. Indirect ELISA

An indirect ELISA for bulk milk samples has been shown to be reliable and useful for surveillance purposes (Kramps *et al*., 2008). It should be validated for relevant serotypes before use. While an indirect ELISA may have the disadvantage of cross reactivity with related viruses such as EHDV, it does have high analytical sensitivity and could be a useful screening assay in some situations.

2.3. Virus neutralisation serology

VN serology can identify serotype-specific neutralising antibodies as well as determine their titre. It is an important additional test in endemic areas where multiple serotypes are likely to be present. Its capability to identify the serotype involved in an outbreak is essential for putting in place appropriate control measures such as vaccination or animal movement restrictions. It is also useful for epidemiological surveillance, transmission studies and for determining useful antibody response to vaccination. Cross-neutralising antibodies can develop in animals that have experienced BTV infection. Importantly, infection with a second or third serotype can broaden the neutralising antibody response to include antibodies to serotypes to which the animal has not been exposed. The application of VN serology is frequently most useful in conjunction with other virological investigations that, in combination, can provide a more definitive basis for resolving serotype distribution. The use of the plaque reduction test (modified from Section B.1.2.2) can improve resolution of serotype involvement, where this is unclear on a standard VN test due to its quantitative nature.

2.3.1. Test procedure

Several methods to determine titre and serotype of BTV have been described; here the procedure that has been standardised after comparative studies in various international laboratories is briefly outlined. Indicator cell lines commonly used are BHK and Vero. It is important that well characterised, reference positive and negative control antiserum be included in each test.

i) 50 µl of serial sera dilutions, from 1/10 to 1/1280, are added to each test well of flat-bottomed microtitre plates and each well is mixed with an equal volume of medium containing approximately 100 $TCID_{50}$ of suitable, well characterised, BTV reference viruses. Note the selection of reference strains used will be dependent on the circulating (or possibly circulating) serotypes in the testing population.

ii) The plates are incubated in a humid chamber at 37°C in 5% CO_2.

iii) After 1 hour of incubation, approximately 10^4 Vero or BHK-21 cells are added per well in a volume of 100 µl of suitable media (containing) able to sustain growth of the chosen cells.

iv) Incubate at 37°C for up to 7 days, depending on the level of CPE obtained in normal serum wells of the test. When CPE has developed to 100% in the normal serum wells of each serotype tested, the test is read using a light microscope at 100× magnification to determine presence of CPE.

v) Wells are scored for the degree of CPE observed. A sample is considered positive when it nears full neutralisation, allowing for the acceptance of trace CPE at the lowest dilution (1/10). The presence of trailing trace CPE may indicate evidence of partial neutralisation. In these situations, the reference virus used may not be representative of contemporary circulating viruses and further investigation may be required (for example, use of the plaque reduction

neutralisation test, which is superior in resolving cross reactivity between related serotypes). The serum titre represents the highest serum dilution capable of near complete neutralisation of 50% of replicate test wells. A fourfold difference in endpoint titre obtained from different serotypes tested can indicate the serotype involved in an infection. Cross reactions are known to occur between serotypes in the VN test even when only one serotype is involved.

2.4. Agar gel immunodiffusion

It must be recognised that a major disadvantage of the AGID used for BT is its lack of specificity in that it does not exclusively differentiate between antibodies to the BT and EHD serogroups. Hence, it cannot be used definitively to detect antibodies to BTV as a positive reaction may have been the result of an infection to another *Orbivirus* species. Notably, the AGID test is simple to perform and the antigen used in the assay relatively easy to produce. Since 1982, the test has been one of the standard testing procedures for international movement of ruminants, however, it is no longer considered sufficiently accurate for use in the support of international trade. The assay does have a role in the investigation of samples that give inconsistent results in other assays. AGID positive sera should be retested using a BT serogroup-specific assay if BTV specificity is required. The preferred test, a C-ELISA, is described in Section B.2.1.

C. REQUIREMENTS FOR VACCINES

1. Background

Vaccination with effective vaccines is the preferred method of BTV control in endemic countries. Vaccination has been used successfully to limit direct losses, minimise the circulation of BTV, eradicate BTV from a region, and allow safe movement of animals. Both live attenuated and inactivated BTV vaccines are currently available for use in sheep and sheep and cattle, respectively. Recombinant BT vaccines based on various approaches are under development (van Rijn, 2019), but none has been licensed and these vaccines will not be addressed here.

Live attenuated vaccines are inexpensive to produce in large quantities; they generate protective immunity after a single inoculation and have proven effective in preventing clinical BT disease in the areas where they are used. In South Africa, live attenuated vaccines have been used for over 50 years and are known to induce an effective and lasting immunity. Live attenuated vaccines are produced by adapting BTV field isolates to growth *in vitro* through serial passages in tissue culture or in ECEs. Stimulation of a strong antibody response by these vaccines is directly correlated to their ability to replicate in the vaccinated host. However, live attenuated BTV vaccines suffer from a variety of documented or potential adverse outcomes, including depressed milk production, abortion/embryonic death, teratogenesis and congenital defects, and have been documented to be spread by vectors with considerable potential for reversion to virulence and reassortment of vaccine virus genes with those of wild-type virus strains (Ferrari *et al*., 2005; Ranjin *et al*., 2019; Savini *et al.,* 2014). Under-attenuation, the impact of which may vary with different breeds of sheep (Veronesi *et al*., 2010), and reassortment with other vaccine and field strains (Nomikou *et al*., 2015) may also occur. The frequency and significance of these events remain poorly defined but transmission of vaccine strains by vector midges has already been documented in the USA, South Africa and Europe (Ferrari *et al*., 2005).

Inactivated vaccines containing tissue-culture grown and chemically inactivated vaccine strains are far safer but require multiple doses to become efficacious and incur higher costs. Such vaccines have been very effective in combatting the spread of BTV-8 in Europe. Whilst inactivated vaccines (as with live vaccines) are not compatible with serological assays for detection of infection in vaccinated animals (DIVA strategies), surveillance can be maintained after their use by RNA based (RT-PCR) assays.

Additionally, non-BTV vaccines for both target and non-target species have been found to be contaminated with BTV leading to abortion, heart failure, respiratory distress and death in non-ruminant species. These drawbacks have resulted in a great deal of research and development of BTV vaccines that are safe, efficacious against multiple serotypes, inexpensive, are DIVA capable, only require a single dose with rapid onset of immunity, and block viraemia. Recent efforts with reverse genetics and molecular technology has led to next generation vaccines that balance these often competing requirements while meeting specific field situation needs.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 *Principles of veterinary vaccine production*. The guidelines given below and in chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

2. Outline of production and minimum requirements for vaccines

2.1. Characteristics of the seed

See chapter 1.1.8 for general requirements for master seeds and allowable passages for vaccine production.

2.1.1. Biological characteristics of the master seed

For live attenuated vaccines, the master or primary virus seed is prepared from a single plaque of serially passaged, attenuated BTV. Vaccine viruses have been attenuated by either passage in ECE, tissue culture cells or a combination of both. Each primary seed virus lot should also be tested for transmissibility and reversion to virulence prior to vaccine manufacture. Samples of vaccine prepared from secondary seed virus at the maximum permitted passage level should be tested in sheep for avirulence, safety and immunogenicity.

For inactivated vaccines, the issues of attenuation do not apply, and the approach adopted has been to use field strains of low passage level with the intent of achieving high antigenicity.

2.1.2. Quality criteria (sterility, purity, freedom from extraneous agents)

Primary seed virus must be free of contaminating bacteria, viruses, prions, fungi and mycoplasmas, particularly pestivirus contamination. For the latter, attention should be paid to the fetal bovine serum used in cell cultures, as it may be contaminated. Seed viruses must be shown to have the desired serotype specificity. BTV seed lot viruses should be sequenced and the data made available to relevant databases. Secondary seed lots, which are used as inocula for vaccine production, are usually not more than three passages beyond the primary seed lot.

2.1.3. Validation as a vaccine strain

Live attenuated BT vaccines must be safe and efficacious, and a brief description of appropriate tests for these parameters is given below. In addition, attenuated viruses should not revert to virulence during replication in vaccinated animals or be able to be transmitted from such animals by insect vectors. The latter criterion is very important because insect-mediated transmission of attenuated virus from vaccinated to non-immune animals, with the subsequent replicative steps in each host species, increases the possibility of reversion to virulence. Although tests for reversion to virulence and transmissibility are rarely, if ever performed, a brief description of what may be necessary is outlined.

There is a variation in BT susceptibility between breeds of sheep; it is important that sheep that have been proven to be susceptible to infection with BTV be used for vaccine validation.

2.1.4. Emergency procedure for provisional acceptance of new master seed virus (MSV) in the case of an epizootic

In a number of different circumstances (see Chapter 1.1.10 *Vaccine banks*) and in the event that a new or different serotype or variant of BTV results in an emergency epizootic situation that cannot be controlled by currently available vaccines, and where there is not enough time to fully test a new MSV for all extraneous agents, provisional acceptance of the new strain could be based on a risk analysis of the possibility of contamination of the antigen produced from the new MSV with extraneous agents. This risk assessment should take into account the characteristics of the process, including the nature and concentration of the inactivant for inactivated vaccines, before allowing or not the early release of the new product.

2.2. Method of manufacture

2.2.1. Procedure

Attenuation of field isolates of BTV was first achieved by serial passage in ECE. Because of the concern about transmission of the egg propagated attenuated virus, it has been recommended that animals receiving vaccines produced in ECE should not be moved internationally. More recently, it is accepted that passage in cultured cells will also result in attenuation of virulence. No studies have been done to precisely relate passage number and extent of attenuation for individual virus isolates or serotypes. To prepare attenuated virus, field isolates are adapted to cell culture and passaged *in vitro* up to 40 times or more. Ideally, a number of plaque-purified viruses are picked at this stage and each is examined to determine the level of viraemia they generate and their ability to elicit a protective immune response in vaccinated sheep. The most suitable virus is one that replicates to low titre but generates a protective immune response, and this may represent the source of vaccine primary seed stock virus.

BTV for inactivated vaccines is produced in large-scale suspension cell systems under aseptic and controlled conditions. Cell lines adapted for large scale industrial cultures are used and these are proven to be free from contaminating microorganisms. When the viral suspension virus reaches its maximum titre, followed by cell disruption, the culture is clarified and filtered. Subsequently inactivation is performed according to processes adopted by the manufacturer, such as by addition of binary ethyleneiminee (BEI) or other inactivating agents. The process must comply with legislation relevant for the intended market, be validated to ensure complete inactivation and supported by appropriate documentation. The inactivation process should not significantly alter the immunogenic properties of the viral antigens. Purification is carried out by chromatography. The inactivated virus is then concentrated by ultrafiltration and stored. The inactivated, chromatography-purified and concentrated BTV antigens are made into vaccine by dilution in a buffer solution and addition of adjuvants.

2.2.2. Requirements for ingredients

All ingredients of animal origin, including serum and cells must be checked for the presence of viable bacteria, viruses, fungi or mycoplasma. For further details, see chapter 1.1.8 for general guidance on ingredients of animal origin. Ingredients of animal origin should be sourced from a country with negligible risk for transmissible spongiform encephalopathies.

2.2.3. In-process controls

Virus concentration of live attenuated vaccines is assessed by infectivity and ELISAs.

For inactivated vaccines, during inactivation of the virus, timed samples are taken at regular intervals for the purpose of monitoring the rate and linearity of the inactivation process. Virus titres in the samples are determined by inoculation of BHK-21 or other appropriate cell cultures. At the end of the inactivation process, the vaccine is checked to ensure that there is no live virus.

2.2.4. Final product batch tests

i) Sterility

Every batch of vaccine should be tested for the presence of contaminant viruses, viable bacterial, fungi or mycoplasma. For example, in South Africa a pool of ten randomly selected ampoules are inoculated into soya broth and thioglycolate broth, and incubated at room temperature and 37°C for 14 days, respectively. If contaminated, the batch is disqualified.

ii) Safety

Every batch of attenuated vaccine is safety tested in newborn and adult mice or guinea-pigs. If any adverse reactions or significant signs are noted, the test is repeated. Any increase in the body temperature of the test animal that is above the level expected for the strain of attenuated virus under test should be regarded as symptomatic. If the results are unsatisfactory after a second attempt, the batch is disqualified.

Safety testing of inactivated vaccines is conducted to ensure side effects are not observed.

iii) Potency

Each batch is tested by inoculation of susceptible sheep. Pre-vaccination, 21- and 28-day post-vaccination sera are tested by VN assay to determine neutralising antibody levels. To be passed, the antibody titre must be equal to or higher than a set standard based on international vaccine standards.

iv) Duration of immunity

Studies with live attenuated BTV vaccine have shown that antibodies in sheep may appear before day 10 post-vaccination, reach a maximum approximately 4 weeks later and persist for well over a year. There is a temporal relationship between the increase in neutralising antibody titre and clearance of virus from the peripheral circulation. Live attenuated BTV vaccines have been in use for over 50 years and are known to induce an effective and lasting immunity in sheep (Verwoerd & Erasmus, 2004). Many serotypes of BTV may be present in endemic areas of South Africa, and polyvalent vaccines are used. The inclusion of 15 serotypes in three polyvalent vaccines that are administered sequentially sometimes means that an effective immune response is not generated to all serotypes, presumably because of the antigenic mass of individual serotype-specific antigens is small. In an attempt to broaden the response, vaccination is repeated annually.

Initial studies with inactivated vaccines show that antibody against BTV can be detected by day 7 post-vaccination and increase in titre to days 14–21. A second dose of vaccine boosts the titre. Data to demonstrate the expected duration of immunity are being acquired.

v) Stability

Procedures have been developed for attenuated vaccines. Stability should be tested over a period of 2 years. Vaccines in liquid and lyophilised forms are deemed to have shelf lives of 1 and 2 years, respectively. Each batch of vaccine is subjected to an accelerated shelf-life test by storing it at 37°C for 7 days. It is then titrated and evaluated according to a set standard, as determined in the initial testing of the vaccine. Shelf life of stock aliquots stored at +4°C should be tested periodically.

2.3. Requirements for regulatory approval

2.3.1. Manufacturing process

For registration of vaccine, all relevant details concerning manufacture of the vaccine and quality control testing (see Section C.2.1 and C.2.2) should be submitted to the authorities. This information shall be provided from three consecutive vaccine batches with a volume not less than 1/3 of the typical industrial batch volume.

2.3.2. Safety requirements

All vaccines must be safety tested. Safety tests for attenuated vaccines do not address the issue of their teratogenicity. Attenuated virus vaccines are teratogenic and should not be administered to pregnant sheep during the first half of pregnancy as this may cause fetal abnormalities and embryo death.

i) Target and non-target animal safety

Demonstration of avirulence is necessary for live, attenuated vaccines. A number of sheep seronegative by an appropriate, sensitive serological test (that will reliably detect antibodies even in vaccinated animals), are inoculated with the primary seed stock. Temperatures are noted twice daily. The animals are monitored at regular intervals over a period of 28 days for clinical signs and any local or systemic reactions to ensure avirulence and innocuity. Blood samples removed at regular intervals can be used to measure level of viraemia and antibody responses. The test shall be valid if all of the vaccinated sheep show evidence of virus replication and do not display signs of disease other than mild transient illness. In South Africa, a clinical reaction index is calculated for each animal between days 4 and 14 and must be below a specific standard value.

ii) Reversion-to-virulence for attenuated/live vaccines and environmental considerations

Transmissibility is an issue with live attenuated vaccines but not with killed vaccines. Procedures to determine if attenuated virus can be transmitted by insects that feed on vaccinated, viraemic sheep are difficult to perform and analyse statistically, and consequently, this criterion of vaccine validation is rarely sought. Laboratory data indicate that laboratory-adapted viruses can be transmitted by insect vectors (Ferrari *et al.,* 2005). A suitable procedure to determine attenuated virus transmissibility requires that sheep be vaccinated and, during viraemia, that they be exposed to competent, uninfected *Culicoides*, which are then permitted to feed on uninfected animals that are monitored for the presence of BTV and anti-BTV antibody. As the titre of attenuated virus in the blood of vaccinated sheep is usually low, very large numbers of *Culicoides* may be needed and only a small proportion of these would become infected and live long enough to feed on and potentially transmit the virus to other uninfected sheep. It is difficult to design a laboratory experiment that takes account of the large numbers of vaccinated sheep and insects that would be present in field situations. Although virus titres in blood less than 10^3 $TCID_{50}$/ml have traditionally been considered a "safe" threshold, authentic instances of insects acquiring BTV from animals with viraemic titres much less than 10^3 $TCID_{50}$/ml have been reported. Given the complex interaction of BTV, *Culicoides* vectors and animal hosts in the life cycle of infection, virus titres induced by live attenuated vaccine should be kept to an absolute minimum especially if field transmission of vaccine strains is a concern.

Current data indicate that during viraemia and in contrast to wild-type virus, laboratory-adapted strains of BTV may be found in the semen of bulls and rams (Kirkland *et al.,* 2004). The implications of these observations for virus transmissibility are unclear. A recent study of semen from rams vaccinated with BTV-2 live attenuated vaccine showed that even if BTV was not detected in the semen, the vaccine caused a decrease in the quality of the semen (Breard *et al.,* 2007).

Validation studies confirm that attenuated viruses do not revert to virulence in vaccinated sheep. However, if attenuated viruses can be transmitted from vaccinated animals, reversion to virulence following several sheep-insect replication cycles becomes a distinct prospect. The only appropriate way to monitor for reversion to virulence under these circumstances is to compare the virulence of the vaccine virus with that which had been subject to several sheep–insect replication cycles as described above. As indicated, this is difficult to achieve. Consequently, the effect of sheep/insect passages on the virulence of attenuated viruses has not been determined. In South Africa, it is accepted that if blood from vaccinated animals during the viraemic stages is serially passaged three times in sheep without reversion to virulence, the chances of reversion in the field will be infinitely small. In Europe, five passages are required.

iii) Precautions (hazards)

Attenuated vaccines should be used in the cooler months when adult *Culicoides* vector populations are at a minimum. They should not be used in ewes during the first half of pregnancy and in rams 2 months before the breeding season

2.3.3. Efficacy requirements

Vaccinated and unvaccinated sheep known to be susceptible to BT disease should be challenged with virulent homologous serotype. It is recommended that the challenge model preferably use virus passaged only in ruminant animals and with no or limited ECE or cell culture passages. Passage in such an isolation system results in viral cultures that might induce clinical BT disease that is milder than the natural disease. Animals are monitored for clinical signs of BT disease, rectal temperatures are taken twice daily and blood samples removed at regular intervals to measure viraemia and antibody responses. Unvaccinated control sheep should show clinical signs of BT disease and viraemia. However, it is difficult to be certain of the appearance of clinical disease following inoculation of sheep with certain BTV serotypes and isolates, and consequently, evidence of infection of unvaccinated control sheep may rest on the appearance of a temperature rise above 40°C and a viraemia. As a further evidence of infection pre- and post-vaccination sera are checked for the presence of neutralising antibody.

2.3.4. Vaccines permitting a DIVA strategy (detection of infection in vaccinated animals)

The live attenuated and inactivated products now commercially available do not allow any DIVA strategy via serological testing.

RT-PCR may be used as DIVA assays, allowing surveillance to be maintained during inactivated vaccination campaigns. DIVA strategies may be possible using PCR-based test systems in populations in which inactivated vaccines have been applied. The inactivated vaccines may generate a very weak RT-PCR signal although this disappears a few days post-vaccination. In contrast, infected animals usually maintain a high RT-PCR signal that can last for several weeks to months, even in the presence of neutralising antibodies, due to the haemagglutinin activity associated with outer capsid protein VP2. A similar approach is not possible with currently available live attenuated vaccines. The generation of weak signal due to very early infection should also be considered if using this strategy.

2.3.5. Duration of immunity

Studies to determine a minimum duration of immunity should be conducted before the vaccine receives final approval. Duration of immunity should be demonstrated in a manner similar to the original efficacy study, challenging animals at the end of the claimed period of protection. At a minimum, the duration should be for the length of the mosquito season in areas with seasonal infections. It may be desirable to demonstrate longer immunity for animals at higher risk and in infected areas with year-round mosquito activity.

2.3.6. Stability

Live and inactivated vaccines are typically assigned an initial dating of 18 or 24 months, respectively, before expiry. Real-time stability studies should be conducted to confirm the appropriateness of all expiration dating. Product labelling should specify proper storage conditions.

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NB: There are WOAH Reference Laboratories for bluetongue
(please consult the WOAH Web site:
https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3).
Please contact the WOAH Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for bluetongue

NB: First adopted in 1991. Most recent updates adopted in 2021.