Review

Bluetongue: Virus proteins and recent diagnostic approaches

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Bluetongue (BT) is an infectious and non-contagious arthropod borne viral disease of domestic and wild ruminants namely sheep, goat, cattle, camels, llamas, deer and antelopes. It is characterized by high fever, catarrhal inflammation of the buccal and nasal mucous membranes, and inflammation of the tongue, intestine and sensitive laminae of the foot. It is caused by Blue tongue virus. Bluetongue virus is a member of the genus Orbivirus in the family Reoviridae. Its genome consists of ten double-stranded (ds) RNA segments coding for seven structural proteins (VP1-VP7) and four non-structural proteins (NS1-NS3 or NS3A, and NS4). At present, 26 serotypes have been reported throughout the world. Bluetongue can be diagnosed from several kinds of samples. For virus isolation, embryonated eggs, 9 to 12 days old, at intravenous inoculation is best however, cell lines like KC line, the insect cell line C6-36 derived from Aedes spp., Baby hamster kidney (BHK-21) and African green monkey kidney (Vero) lines can be used. For antigen identification sandwich enzyme linked immunosorbent assay (ELISA), serotype specific reverse transcriptase Polymerase chain reaction (RT-PCR), Real time quantitative PCR, Sequencing and phylogenetic analysis (targeting conserved genome segments), Restriction enzyme profile analysis (REPA), Molecular probes, RNA polyacrylamide gel electrophoresis (RNA-PAGE) are now available in an increasing number of laboratories for the identification of Bluetongue virus (BTV). For antibody identification, it is possible to use blocking ELISA, competition ELISA, indirect ELISA, agar-gel immunodiffusion test (AGID), Complement-fixation test (CFT) and Haemagglutination inhibition test (HI), serum or virus neutralisation test (VNT/SNT), Immunofluorescence, Immunoperoxidase, and Dot immunobinding assays (DIA).

Key words: Antibody, antigen, bluetongue, bluetongue virus, diagnosis, structure, molecular technique.

INTRODUCTION

Bluetongue (BT) is an infectious and non-contagious arthropod borne viral disease of domestic and wild ruminants namely sheep, goat, cattle, camels, llamas, deer and antelopes. BT primarily affects sheep and deer with frank clinical symptoms, but subclinical disease occurs in cattle and goat. In 2006, serotype 8 has been reported to be implicated in causing clinical signs in cattle in UK (Maclachlan, 2011). It is characterized by high fever, catarrhal inflammation of the buccal and nasal mucous membranes, and inflammation of the tongue, intestine and sensitive laminae of the foot. Bluetongue has been known in South Africa for over a 100 years and endemic in wild ruminants since antiquity (Maclachlan et al., 2009, Maclachlan, 2011; Sperlova and Zendulkova, 2011; Maan et al., 2012a; Bitew et al., 2013).
includes tropical, subtropical, and temperate regions of the world between latitudes of approximately 40° North and 35° South that is, America, Africa, Australia and Asia where vectors (Culicoides sp.) are present (Maclachlan, 2011; Bitew et al., 2013). BT is a disease of ruminants in temperate zones. However, clinical disease is reported in tropical and subtropical areas of the world when non-native breeds of ruminants are introduced in virus endemic area (Sperlova and Zendulkova, 2011). The European outbreaks of BT due to BTV-8 in 2006 have dramatically changed the geographic and ecological epysystems around the world (Maclachlan, 2011; Maan et al., 2008). Due to segmented nature of virus, it often undergoes mutation by the process of drift and shift (reassortment of BTV gene segments).

The economic losses due to bluetongue is around 3 billion US$ per year in the world (Sperlova and Zendulkova, 2011). The direct losses are death, abortions, weight loss and reduced milk and meat productions and indirect losses are export restrictions of live animals, semen and foetal calf serum (Bitew et al., 2013).

At present 26 serotypes have been reported throughout the world (Maan et al., 2012 a, b, c) with recent additions of the 25th serotype (“Toggenburg orbivirus”) from Switzerland in goat and 26th serotype from Kuwait in sheep and goat (Hofmann et al., 2008; Maan et al., 2011; Maan et al., 2012c; Bitew et al., 2013). There is only low level of cross-protection among the BT virus serotypes and making vaccination strategies and control programs a daunting task (Hofmann et al., 2008; Eschbaumer et al., 2009; Bitew et al., 2013).

BT is a multiple species disease to the OIE, World Organisation for Animal Health (OIE, 2009; Maclachlan, 2010; 2011.) and to veterinary authorities in many countries (Eschbaumer et al., 2009). BTV is almost exclusively spread by Culicoides spp. biting midges (Diptera) and occurs worldwide. All the serotypes can cause bluetongue disease (BT), a non-contagious hemorrhagic disease of domestic and wild ruminants and camels with no known zoonotic potential (Eschbaumer et al., 2009).

Although a galaxy of serological and molecular diagnostic tools are available for the prompt, reliable and precise detection and characterization of BTV strains/serotypes and large number immunoprophylactic agents have been developed for the control of the disease however, it is still endemic in many countries with substantial economic losses. Further, X-ray crystallography and cryo-electro-microscopy studies showed the minute details of the molecular structure of the bluetongue virus as well as different proteins coded by the different RNA segments of BTV (Schwartz-Cornil et al., 2008).

This review would provide the structural details of the BTV and the diagnostic tools developed over the times for the sensitive detection and molecular characterization of BTV for the benefit of the scientific fraternity, researchers, scientists and academicians involved in the bluetongue virus research.

**THE BLUETONGUE VIRUS**

BTV is the etiological agent of BT, an insect transmitted disease of ruminants. Bluetongue virus with closely related species African Horse Sickness virus (AHSV) and Epizootic Hemorrhagic Disease virus (EHDV) belongs to the genus Orbivirus (comprising at least 20 species overall) in the family Reoviridae (Eschbaumer et al., 2009; Maan et al., 2012a). The virions have a diameter of 90 nm. Bluetongue virus is having density 1.337 g/cm³ and relative molar mass of about 10.8 × 10⁴, 12% of which is genomic RNA (Eschbaumer et al., 2009; Schwartz-Cornil et al., 2008). The virus is a non enveloped with a genome of approximately 19.2 kbp and composed of ten linear double-stranded RNA (dsRNA), containing 57% AU and 43% GC, with conserved 5' and 3' terminal sequences (GUUAAA at 5', and ACUAC at 3' ends of the positive strand) (Schwartz-Cornil et al., 2008). The genome segments are numbered 1-10 (Large: L1-3; medium: M4-6; small: S7-10) in the order of decreasing size on agarose gels (Bhanuprakash et al., 2009).

Ten (10) dsRNA segments are packaged within a triple layered icosahedral protein capsid (90 nm in diameter) (Maan et al., 2012 a, b, c, d, e). The genome encodes seven structural and four non-structural proteins. Each segment contains one open reading frame flanked by non-coding regions. The open reading frame on segment 10 encodes two proteins by alternate translation initiation (Eschbaumer et al., 2009) (Figure 1). The outer layer of BTV particle is composed of two structural proteins (60 trimers of VP2) and 120 trimers of VP5). The intermediate layer consists of the major immunodominant VP7 structural protein organized in 260 trimers. VP7 forms the outer layer of the transcriptionally active virus ‘core’ (Eschbaumer et al., 2009) (Figure 1). The subcore consists of the 12 decamers of the VP3 protein, one centered on each of the five fold axes of the icosahedral particle structure. The 120 molecules of VP3 houses the viral genome segments and three minor proteins involved in transcription and replication, namely the RNA-dependent RNA polymerase (VP1), the RNA capping enzyme (VP4) and the dsRNA helicase (VP6) (Eschbaumer et al., 2009; Maan et al., 2012 a, b, c, d, e) (Figure 1). Non-structural proteins (NS1, NS2, NS3, NS3A and NS4) probably participate in the control of BTV replication, maturation and export from the infected cell. Unlike most single stranded RNA (ssRNA) viruses, the orbiviruses are genetically and antigenically stable throughout infection; point mutations do not appear to arise *in vivo*, at least at the high frequency noted with
many non-segmented ssRNA viruses (Ratinier et al., 2011) (Table 1).

There are 26 serotypes of BTV (Maan et al., 2012c) which are distinguished by epitopes on the outer capsid protein VP2 although VP5 also can influence neutralization through its conformational influence on VP2. The L2 gene which encodes VP2 is the only serotype specific BTV gene with a considerable variation amongst the different serotypes. The two outer capsid proteins VP2 and VP5 are responsible for virus entry and establishment of virus infection within the host cells, the core proteins as well as three non-structural proteins are less variable and responsible for replication of the viral genome. This genetic diversity of BTV is consequence of both drift (point mutation) and shift (reassortment of BTV genome). This genetic diversity of BTV is consequence of both drift (point mutation) and shift (reassortment of BTV genome) (Maan et al., 2012a, b, d, e).

Structural proteins

Outer shell protein

Outer shell composed of two structural proteins VP2 and VP5. The trimers of the VP2 form ‘triskelion’ motifs (three interlocked spirals) on the outer layer (Schwartz-Cornil et al., 2008). VP2 is responsible for receptor binding, hemagglutination and eliciting serotype-specific neutralizing antibodies (Dahiya et al., 2004; Schwartz-Cornil et al., 2008). Recombinant VP2 has a strong affinity for glycolphorin A, a sialoglycoprotein component of erythrocytes, an interaction that could be involved in BTV binding to erythrocytes. Furthermore, VP2 and glycophorin can inhibit BTV attachment to susceptible cells, suggesting that the BTV receptor involves VP2 interaction with a cell surface glycoprotein (Dahiya et al., 2004; Schwartz-Cornil et al., 2008). Inside the cell, VP2 associates to vimentin, which allows the proper sub-cellular localization of the protein and the interaction of mature BTV particles to intermediate filaments (Bhattacharya et al., 2007). Disruption of the VP2/vimentin interaction by pharmacological inhibitors leads to blockade of the virus egress (Bhattacharya et al., 2007).

VP2 is the major determinant of BTV serotype, with a minor role for VP5. Phylogenetic comparisons of VP2 from the 24 reference strains (Dahiya et al., 2004; Maan et al., 2007; Maan et al., 2012f) show a perfect correlation between sequence variation in genome segment 2 (Seg-2), coding for VP2, and determining BTV serotype. Sequences of seg-2 from the 24 BTV types cluster as ten distinct evolutionary lineages, identified as nucleotypes A-J. The inter-serotype VP2 nucleotide sequences varied from 29% (BTV-8 and BTV-18) to 59% (BTV-16 and BTV-22). Sequencing and phylogenetic comparisons of VP2 gene also revealed significant variations between strains of the same serotype that were derived from different geographical areas, with a maximum of 30% nucleotide sequence variation within the same serotype (Maan et al., 2007). These geographical variations define eastern and western VP2 topotypes within individual serotypes (Figure 2).

Oligonucleotide primers can be designed targeting Seg-2 that can be used in RT-PCR assays to facilitate typing of BTV field isolates and vaccine virus of each serotype and topotype (Mertens et al., 2007). Despite the overall sequence variability, some features of VP2 appeared to be conserved across serotypes, including the hydrophobicity profile, charge distribution and the position of certain cysteine residues (Maan et al., 2007).

In contrast to VP2, VP5 is significantly more conserved but shows some degree of variations that reflects the geographic origin (Singh, 2005). Trimers of VP5 form the globular motifs of the outer layer of the BTV virus particle (Nason et al., 2004). VP5 has recently been shown to be a membrane penetration protein that mediates release of viral particles from endosomal compartments into the cytoplasm. Analysis of the VP5 sequence using secondary structure prediction algorithms indicates that this protein is predominantly α-helical, with an amphipathic helical domain at the N terminus followed by a coiled domain, thus sharing structural features with class I fusion proteins of enveloped viruses (Nason et al., 2004). Furthermore, VP5 undergoes pH-dependent conformational changes that allow membrane fusion and syncytium formation (Forzan et al., 2004). The syncytium formation by VP5 is inhibited in the presence of VP2 when expressed in a membrane-anchored form.

The major core proteins

VP3 and to a lesser extent VP7 are conserved proteins, hydrophobic in nature and are forming major core protein
Table 1. Bluetongue virus genome segments and proteins.

<table>
<thead>
<tr>
<th>Segment</th>
<th>Size (nt)</th>
<th>Encoded protein</th>
<th>Location (number of copies per virion), proposed function</th>
<th>Protein size* (weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3954</td>
<td>VP1</td>
<td>Within the core (12), RNA-dependent RNA polymerase</td>
<td>1302 aa (150 kDa)</td>
</tr>
<tr>
<td>2</td>
<td>2926</td>
<td>VP2</td>
<td>Outer capsid (180), type-specific structural protein</td>
<td>961 aa (111 kDa)</td>
</tr>
<tr>
<td>3</td>
<td>2772</td>
<td>VP3</td>
<td>Inner (sub-core) capsid (120), scaffold for VP7 layer</td>
<td>901 aa (103 kDa)</td>
</tr>
<tr>
<td>4</td>
<td>2011</td>
<td>VP4</td>
<td>Within the core (24), RNA capping enzyme</td>
<td>644 aa (75 kDa)</td>
</tr>
<tr>
<td>5</td>
<td>1770</td>
<td>NS1</td>
<td>Non-structural protein (0), forms tubules of unknown function in host cells</td>
<td>552 aa (64 kDa)</td>
</tr>
<tr>
<td>6</td>
<td>1639</td>
<td>VP5</td>
<td>Outer capsid (360), structural protein, co-determinant of virus serotype</td>
<td>526 aa (59 kDa)</td>
</tr>
<tr>
<td>7</td>
<td>1156</td>
<td>VP7</td>
<td>Core capsid (780), group-specific structural protein</td>
<td>349 aa (39 kDa)</td>
</tr>
<tr>
<td>8</td>
<td>1123</td>
<td>NS2</td>
<td>Non-structural phosphoprotein (0), forms viral inclusion bodies in host cells</td>
<td>354 aa (41 kDa)</td>
</tr>
<tr>
<td>9</td>
<td>1046</td>
<td>VP8</td>
<td>Within the core (72), RNA helicase</td>
<td>329 aa (36 kDa)</td>
</tr>
<tr>
<td>10</td>
<td>822</td>
<td>NS3</td>
<td>Non-structural glycoprotein (0), membrane protein, aids virus release from host cells</td>
<td>229 aa (26 kDa)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NS3A</td>
<td>Expressed by alternate translation initiation</td>
<td>216 aa (24 kDa)</td>
</tr>
</tbody>
</table>

*Size (amino acids, aa) and weight (Dalton, Da) data are for the European reference isolate of BTV-8. (Kindly taken from Eschbaumer et al., 2009).

Figure 2. Unrooted neighbour-joining tree showing relationships between nucleotide sequences of Seg-2 from the 24 BTV types (Maan et al., 2007).

(Schwartz-Cornil et al., 2008). They play an important role in the structural integrity of the virus core. They express group-specific antigenic determinants defining several distinct phylogenetic groups (Anthony et al., 2007). Importantly, cores are poorly infectious or even non-infectious in different mammalian cells but they are at least 100 fold more infectious for adult Culicoides midges or a Culicoides cell line (KC cells) (Schwartz-Cornil et al., 2008). VP7 can mediate attachment and penetration of insect cells in the absence of either VP2 or
VP5, a process that may involve an arginine-glycine-aspartate (RGD) tripeptide motif present at amino acid residues 168 to 170 on the outermost surface of the VP7 trimers on the BTV core. VP7 can bind to glycosaminoglycans, although it appears likely that other specific receptors are also involved in cell attachment and penetration. The VP3/VP7 complex protects the viral dsRNA genome from intracellular surveillance, thus preventing activation of type I interferon (IFN) production (Sutton et al., 2004). VP1 can extend IFN synthesis from the genomic dsRNA template. It unwinds duplexes of dsRNA and could assist mRNA synthesis from oligo(A) primers and acts as the BTV replicase that synthesizes dsRNA from a viral plus-strand RNA template (Boyce, 2004). VP1 has an optimal activity at 27 to 37°C, allowing efficient replication in both insect and mammalian cells. The early BTV mRNAs are capped. The cap (methylguanosine connected to the first nucleoside) stabilizes the mRNA and allows efficient translation. In cells, capping requires the action of four distinct enzymes. In BTV, all four reactions are catalyzed by the single VP4 protein, whose crystal structure shows an elongated modular architecture that provides a scaffold for an assemblage of active sites (Sutton et al., 2007). The VP6 protein has ATP binding activity and displays RNA-dependent ATPase and helicase functions. It unwinds duplexes of dsRNA and could assist mRNA synthesis from the genomic dsRNA template.

Minor core proteins

Minor core proteins also called transcription complex comprises VP1, VP4 and VP6. VP1 is present in a low molar ratio (approximately 12 copies per particle) within the virion (Schwartz-Cornil et al., 2008). VP1 can extend RNA synthesis from oligo(A) primers and acts as the BTV replicase that synthesizes dsRNA from a viral plus-strand RNA template (Cornil et al., 2008). VP1 has an optimal activity at 27 to 37°C, allowing efficient replication in both insect and mammalian cells. The early BTV mRNAs are capped. The cap (methylguanosine connected to the first nucleoside) stabilizes the mRNA and allows efficient translation. In cells, capping requires the action of four distinct enzymes. In BTV, all four reactions are catalyzed by the single VP4 protein, whose crystal structure shows an elongated modular architecture that provides a scaffold for an assemblage of active sites (Sutton et al., 2007). The VP6 protein has ATP binding activity and displays RNA-dependent ATPase and helicase functions. It unwinds duplexes of dsRNA and could assist mRNA synthesis from the genomic dsRNA template.

Non structural proteins (NSP)

The two larger BTV non structural proteins, NS1 and NS2, are the first and second most highly expressed proteins in infected cells whereas the two closely related minor proteins NS3 and NS3A are barely detectable in mammalian cells. However, NS3 and NS3A are synthesized in much larger amounts in insect cells, suggesting that their role may be primarily related to BTV replication and dissemination within the insect vector (Schwartz-Cornil et al., 2008). NS4 has been recently identified as additional to non structural proteins (Ratinier et al., 2011).

NS1

Electron microscopic analysis of thin sections of BTV-infected cells have revealed a large number of virus-specific tubules (52.3 nm diameter and 1000 nm long) composed of multimers of the NS1 protein, a striking intracellular morphological feature of BTV infection. NS1 has a role in BTV cytopathogenesis (Owens et al., 2004).

NS2

NS2 is the major constituent of the viral inclusion bodies (VIB) seen in infected cells mainly in the vicinity of the nucleus. NS2 binds to viral ssRNA and hydrolyses nucleotidetriphosphates to nucleotide monophosphates (Schwartz-Cornil et al., 2008). These two properties imply that NS2 might be involved in some way in selection and condensation of the BTV ssRNA segments prior to genome encapsidation. NS2 expression in cells is sufficient for formation of inclusion bodies and it recruits VP3, suggesting that NS2 is a key player in virus replication and core assembly (Kar et al., 2007).

NS3

NS3 and its shorter form, NS3A, which lacks the N-terminal 13 amino acids of NS3, are the only membrane proteins encoded by orbiviruses. Interestingly, NS3 and NS3A appear to be associated with smooth intracellular membranes, although they are also present at the plasma membrane. NS3 functions as a viroporin, facilitating virus release by inducing membrane permeabilization (Han and Harty, 2004). NS3 allow BTV particles to leave host cells by a budding mechanism similarly to retroviruses. This budding mechanism might be involved in BTV egress from insect cells in which BTV does not induce significant cytopathic effect, whereas the viroporin mechanism would be more prominent in mammalian cells (Schwartz-Cornil et al., 2008).

NS4

It has been recently identified that BTV expresses a fourth non-structural protein (NS4) encoded by an open reading frame in segment 9 overlapping the open reading frame encoding VP6. NS4 is 77-79 amino acid residues in length and highly conserved among several BTV serotypes/strains (Ratinier et al., 2011).

RECENT DIAGNOSTIC APPROACHES

A preliminary diagnosis based on clinical signs, post-mortem findings and epidemiological assessment should be confirmed by laboratory examination (Sperlova and Zendulkova, 2011). Samples to be examined in the labo-
ratory should include non-coagulated blood (use of EDTA or heparin is preferred), serum, post-mortem tissue samples such as spleen, lymph nodes, lungs, liver, bone marrow heart and skeletal muscles. In addition, brain of the aborted foetus can be collected (Sperlova and Zendulkova, 2011). For transport, serum samples should be frozen at -20°C and the other samples should be kept on ice. The blood samples can be stored at +4 °C for a long time; isolated blood cells in 10% dimethyl sulphoxide require storage at a temperature of -70°C (Sperlova and Zendulkova, 2011).

Bluetongue virus isolation

BTV can be isolated from blood, semen and various other tissue samples including liver, spleen, brain, lymph nodes and mucosal epithelium. Bluetongue virus can be propagated in embryonated chicken eggs (ECE), cell cultures or in sheep. Embryonated eggs, 9 to 12 days old are inoculated with the materials by intravenous route for BTV isolation. This method is 100-1000 fold more sensitive than yolk sac inoculation (Dadhich, 2004; Sperlova and Zendulkova, 2011; Biswas et al., 2010), but needs technical skills and experience. The material obtained from ECE can either be further propagated in cell culture or directly examined using molecular methods (PCR or in situ hybridisation) (Dadhich, 2004; Sperlova and Zendulkova, 2011).

Bluetongue virus can also be isolated in cell lines of different animal origin. Cell lines of insect origin include the KC line derived from Culicoides sonorensis cells or the C6/36 line from Aedes albopictus (AA) cells. The mammalian cell lines for BTV isolation like BHK-21, calf pulmonary artery endothelium (CPAE) or Vero cell lines can also be used (Mecham, 2006). The cytopathic effect produced by BTV is observed only on cell lines of mammalian origin at 3 to 5 days after inoculation and appears as foci of rounded and refractile cells. If Cytopathic effect (CPE) doesn’t appear, a second passage is made in cell culture. The isolation of virus in cell culture is usually preceded by its passage in ECE which are more susceptible to BTV than cell lines (Sperlova and Zendulkova, 2011; Biswas et al., 2010). Sheep can provide a sensitive and reliable system for BTV isolation; however, today they are used only occasionally, for example, in cases when a sample contains a very low virus titre (Sperlova and Zendulkova, 2011). The identity of BTV in the culture medium of cells manifesting a CPE may be confirmed by antigen-capture ELISA, immunofluorescence, immunoperoxidase, SNT or VNTs.

Antigen identification

Sandwich ELISAs have been described for the detection of BTV antigens in infected cell cultures or adult Culicoides midges. Although antigen ELISAs are specific, they are insensitive requiring relatively large amounts of antigen (equivalent to ≥ 2.5 – 3.0 log10 infectious units of virus) to give a positive result and consequently are rarely used as a front line test for the detection of BTV (Batten et al., 2008). In addition to ELISA, molecular assay can be used to detect and identify the viral RNA of BTV or related viruses. A direct identification of BTV in blood or tissue samples is possible with use of the reverse transcription-polymerase chain reaction (RT-PCR) method that allows for serotyping and can detect BTV RNA in samples as late as 6 months after infection (Sperlova and Zendulkova, 2011). A quantitative assessment of RNA in a sample is possible by real time-RT-PCR (Shaw et al., 2007; Toussaint et al., 2007; Vanbinst et al., 2010; De Leeuw et al., 2013). RNA polyacrylamide gel electrophoresis (PAGE) has been used as a diagnostic tool for the identification BTV 10 segments. RNA PAGE has also been used to identify different genotypes of the same serotype, as well as to indicate different serotypes of BTV. Group specific non-radio-labelled probes, based on the NS1 and VP3 genes, have been developed in India for detection of BTV in clinical specimens or infected cell cultures. Serogroup-specific RT-PCR, sequencing, restriction enzyme profile analysis (REPA) and phylogenetic analyses (targeting conserved genome segments) are now available in an increasing number of laboratories for the identification of BTV. Serotype-specific RT-PCR assays (targeting genome segments 2 or 6) have also been used to identify different BTV serotypes (Maan et al., 2007, 2008; Mertens et al., 2007).

Reverse transcription PCR (RT-PCR)

Primer-directed amplification of viral nucleic acid has revolutionised BT diagnosis. Results to date indicate that RT-PCR (particularly nested or real-time assays) techniques may be used, not only to detect the presence of viral nucleic acid (starting from 1-3 days of pi), but also to ‘serogroup’ orbiviruses and provide information on the serotype and possible geographic source (topotype or genotype) of BTV isolates within a few days of receipt of a clinical sample, such as infected sheep blood. Traditional approaches, which rely on virus isolation followed by virus identification, may require at least 3 to 4 weeks to generate information on serogroup and serotype and yield no data on the possible geographic origin of the isolated virus (Dadhich, 2004). Oligonucleotide primers used to date have been derived from RNA 7 (VP7 gene), RNA 6 (NS1 gene), RNA 3 (VP3 gene) and RNA 2 (VP2 gene). The size of the amplified transcripts is usually small (in the order of several hundred nucleotides) but can also be a full-length gene. Primers derived from the highly conserved genes, such
as VP3, VP6, VP7, NS1 and NS3, may be used for serogrouping (i.e. they will react with all members of the BT serogroup) and topotyping (that is, they will react with BTV isolates from the same geographic area). NS1 is currently recommended as an RT-PCR target by the OIE (OIE, 2009). Primers whose sequence was determined from VP2 gene sequences provide information on virus serotype (Dadhich, 2004). Two major geographic groups of BTVs have been identified and have been designated as ‘eastern’ and ‘western’ topotypes. The eastern includes viruses from Australia and the Middle/Far East, and the western includes Africa and the Americas, respectively (Maan et al., 2008; Maan et al., 2012a, b, c, d, e).

The RT-PCR requires agar gel electrophoresis to show the amplification of the target sequence, which severely limits the speed of testing. The RT-PCR assay involves three separate procedures (Maan et al., 2008). In the first, BTV RNA is extracted from blood using a chaotropic agent such as guanidine thiocyanates (GuSCN) to denature protein and release viral RNA. A number of commercial kits are available. The reagents provided with the kit are numbered and their use is indicated in the protocol. Again, Trizol is useful for the extraction of viral nucleic acid from spleen or blood clots. Operators should follow the procedures specified in each kit and use reagent solutions either provided or recommended for the kit of their choice. The second procedure is the denaturation of viral double-stranded RNA and reverse transcription (RT) to generate cDNA, which is amplified by PCR. Equivalent kits and reagents are available from other sources. The final step of the process is the analysis of the PCR product by electrophoresis (Dadhich, 2004; Maan et al., 2008; Maan et al., 2012).

**Real time RT-PCR (qPCR)**

Real-time RT-PCR is a sensitive method that can be used for the laboratory detection of viral RNA. Several types of real-time RT-PCR exist, most being based on either SYBR (where an intercalating molecule fluoresces upon binding to double stranded DNA) or on fluorescence resonance energy transfer (FRET) (for example, TaqMan and molecular beacon assays) (Yin et al., 2010). In the FRET-based assays, a fluorescent ‘probe-oligonucleotide’ binds specifically to the region between the two primers. A positive signal is generated when the probe is degraded by the polymerase as it synthesizes new complimentary DNA strands. The results of real-time RT-PCR assays are expressed as a cycle-threshold (Ct) value. This represents the number of amplification cycles that are required under standard test conditions to cross a certain threshold level of fluorescence and higher Ct values therefore indicate that smaller amounts of the target gene are present in the test sample than do lower Ct values. Blood samples taken from an infected animal at the peak of viraemia may give Ct values of <20, whereas a low level viraemia could still be detectable with a Ct value >35. Negative control samples should not achieve a positive Ct value. Standardization of the assay, described by Shaw et al. (2007) and using a dilution series of viral RNA indicated that a change of 3 Ct units was approximately equivalent to a 10-fold dilution of the sample.

There are two published real-time assays that have been shown to detect all 26 serotypes (Shaw et al., 2007; Toussaint et al., 2007). Of the two, the assay of Shaw et al. (2007) has been tested against more serotypes and topotypes to date. So far the real-time RT-PCR assays have not been validated to the level required by the OIE, although ring trials have been conducted (Batten et al., 2008). Vanbins et al. (2010) developed a duplex real-time RT-PCR for the detection of bluetongue virus in bovine semen. De Leeuw et al. (2013) also reported Bluetongue virus RNA detection by real-time RT-PCR in post-vaccination samples from cattle.

**Antibody identification**

Serogroup-specific antibodies against BTV can be detected by a blocking ELISAs, competitive ELISAs and dot immunobinding assays (DIA) test targeted to the VP7 protein. This is a rapid method permitting determination of serum or plasma antibody as early as the 6th day of post-infection (PI) (Mars et al., 2010; Kramps et al., 2008; Batten et al., 2008). Again an indirect ELISA based on VP 7 protein has been developed at Indian veterinary research institute (IVRI), Mukteswar (Chand et al., 2009). In addition, serogroup-specific antibodies can be identified by an agar-gel immunodiffusion test (AGID), a complement-fixation test and a haemagglutination-inhibition test (Sperlova and Zendulkova, 2011). Agar gel immuno-diffusion (AGID) tests, historically, have been widely used for the detection of group-specific antibodies against BTV. The AGID test relies on the availability of purified soluble antigens, derived from BTV-infected cell cultures and positive control serum from hyper-immunised animals. However, AGID may produce cross-reactions with other orbiviruses like African Horse Sickness virus (AHSV) and Epizootic Hemorrhagic Disease virus (EHDV) (Sperlova and Zendulkova, 2011).

Complement fixation tests (CFT) have been used to identify BTV or to detect a rise in BTV-specific antibody titre following infection. These assays that primarily detect early antibodies, IgM, depend on inhibition of the complement-mediated lysis of activated erythrocytes by BTV antigen/antibody complexes that can also fix the available complement. However, they may only be effective for a relatively short period of time following infection and have largely been superseded by the use of
the ELISA. There are several ELISA techniques recommended for the detection of humoral antibody response to BTV but the blocking and competition are the best methods. The current edition of the OIE Manual of Standards for Diagnostic Tests and Vaccines (2009) cites the competition ELISA as a prescribed test for the detection of BTV-specific antibodies (OIE, 2009). A new indirect ELISA for the detection of BTV-specific antibodies in bulk milk (Kramps et al., 2008) and other samples (Chand et al., 2009; Gandhale et al., 2010) is reported to be robust, specific and sensitive. The SNT or VNT has the highest specificity and sensitivity of all the tests, but is also most expensive and time-consuming. Most of the antibodies that neutralize intact BTV particles are specific for VP2 although VP5 can also influence the specificity of the reaction, probably through its interactions with VP2 (Hamblin, 2004; Batten et al., 2008; Batten et al., 2013). Immunofluorescence, immunoperoxidase, and dot immunobinding assays (DIA) have been widely used for BTV antibody detection.

Serogrouping of BTV

*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 BT and epizootic haemorrhagic disease (EHD) viruses raises the possibility that an isolate of EHD virus could be mistaken for BTV on the basis of a weak immunofluorescence reaction with a polyclonal anti-BTV antiserum. For this reason, a BT serogroup-specific monoclonal antibody (MAb) can be used (Gandhale et al., 2010; Sperlova and Zendulkova, 2011). A number of laboratories have generated such serogroup-specific reagents. Commonly used methods for the identification of virus to serogroup level are immunofluorescence, antigen-capture enzyme-linked immunoassay (ELISA), immunospot test and indirect peroxidase/antiperoxidase identification (Sperlova and Zendulkova, 2011), but they are rarely used (Dadhich, 2004).

Serotyping of BTV

The serotype of each BTV strain is determined by the specificity of reactions between components of the outer capsid (proteins VP2 and VP5) of the virus particle and neutralizing antibodies that are generated during infection of the mammalian host (Maan et al., 2007). These reactions can be analysed and measured using a variety of micro-titre, plaque reduction or other neutralisation assays (SNT, VNT). SNT can be used to detect neutralizing antibodies that are specific for each BTV serotype in diagnostic serum samples. SNT is highly sensitive and is usually specific for each BTV serotype although circulation of more than one serotype in a region, leading to sequential infections with different serotypes, is likely to cause cross-reactions with multiple additional serotypes. VNT can be used to identify the serotype of BTV isolates. Alternative serotyping methods have used small filter-paper discs soaked in serotype-specific neutralizing antisera which are placed on an agar overlay to create a zone of protection in lawns of tissue culture cells that are challenged with the test virus isolate (OIE, 2009). More recently serotyping can be made by RT-PCR using serotype specific oligonucleotide primers. Primers have been developed by Maan et al. (2012).

Detection of BTV in Culicoides midges

The detection of BTV in field collected populations of adult Culicoides biting midges is most commonly attempted in areas where outbreaks are occurring or from endemic regions during periods of intense transmission. The techniques used are, with a few exceptions, the same as those used for detection of the virus in ruminants. The detection of BTV in Culicoides is most often carried out using ECE, followed by passage (often blind) in cell culture, to isolate the virus from pools of parous midges. As with samples from ruminants, the isolation of BTV is not always successful and virus strains which fail to grow under these conditions will remain undetectable. In these cases, other techniques (particularly RT-PCR) can be used to identify the presence of viral RNA in the pooled insects providing evidence that the virus itself is present (Veronesi et al., 2009).

Differential diagnosis

The clinical signs of bluetongue can easily be mistaken for those of other ruminant diseases such as orf (contagious pustular dermatitis), foot and mouth disease, acute photo sensitization, acute haemonchosis (with depression and submandibular oedema), facial eczema, *Oestrus ovis* infestation, pneumonia, plant poisoning, salmonellosis, sheep pox. Peste des Petits Ruminants (PPR) (Williamson et al., 2008), malignant catarrhal fever, pododermatitis, rinderpest, infectious bovine rhinotracheitis, bovine viral diarrhoea, bovine popular stomatitis, bovine herpes mamilitis and epizootic haemorrhagic disease of deer (Mehlhorn et al., 2008; Williamson et al., 2008; Savini et al., 2011).

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