Epidemiology of bluetongue virus with special reference to the Mnisi area, Mpumalanga Province, South Africa

By

Jumari Steyn

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Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

Pretoria

South Africa

Supervisor:

Prof EH Venter

Co-supervisors:

Dr P Coetzee and Dr G Venter

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Declaration

I hereby declare that this dissertation submitted to the University of Pretoria for the degree M. Sc (Veterinary Science) has not been previously submitted by me or anyone for the degree at this or any University, that it is my own work in design and in execution, and that all material contained therein has been duly cited.

Signature of candidate

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Date: / /

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List of Abbreviations

AHSV	African Horse sickness virus
ARC-OVI	Agricultural Research Council – Onderstepoort Veterinary Institute
BHK-21	Baby hamster kidney-21
BT	Bluetongue
BTV	Bluetongue virus
CMH	Cochran-Mantel-Haenszel statistical test
CPE	Cytopathic effect
C_q	Quantification cycle
DVTD	Department of Veterinary Tropical Diseases
EHDV	Epizootic hemorrhagic disease virus
ELISA	Enzyme-linked immunosorbent assay
cELISA	Competitive ELISA
FAM	Fluorescein amidite, used in the preparation of fluorescein-labelled oligonucleotide probes for PCR
FVS	Faculty of Veterinary Science
H’	Shannon-Weiner Index
J’	Species evenness
KNP	Kruger National Park
MagMax™	Express Magnetic Particle Processor
MEGA	Express Magnetic Particle Processor
MGB	Minor groove binder
MLV	Modified live virus vaccine
NJ	Neighbour-joining
NS	Non-structural
NS3/A	NS viral protein 3 alternative form
OD	Optical density

OIE	World Organisation for Animal Health
OVI	Onderstepoort Veterinary Institute
PBS (+)	Phosphate-buffered saline containing calcium and magnesium
PCR	Polymerase chain reaction
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
RT-PCR	Reverse transcription-polymerase chain reaction (legacy RT-PCR)
Vero	African green monkey cells
VI	Virus isolation
TaqMan®	PCR methods that uses a hydrolysis probe derived from a thermostable Taq polymerase derived from the thermophilic bacteria *Thermus aquaticus*.
$TCID_{50}$	50% tissue culture infective dose
VN	Virus neutralisation test
VP	Viral protein

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Summary

Bluetongue virus (BTV) is the prototype member of the genus *Orbivirus* in the family *Reoviridae*. This virus causes an economically important infectious, non-contagious disease, bluetongue, rendering it a notifiable disease at the OIE. Twenty six serotypes of the virus have been identified that are transmitted primarily by certain species of biting midges that belong to the genus *Culicoides* (Diptera: Ceratopogonidae). The distribution of BTV is determined by the occurrence of competent *Culicoides* midge species, climatic conditions and susceptible ruminant hosts. During the last decade BTV has become a major concern worldwide as well as the focus point of many epidemiological studies and surveillance programmes.

The first experimental study was based in Mnisi, a rural area located in Mpumalanga. This area is adjacent to the Kruger National Park and ideally represents the interphase in a wildlife-domestic animal interaction. Cattle farming is the major source of income of the local community.

Chapter 2 focused on determining the prevalent *Culicoides* spp. in the area as well as to determine whether BTV is circulating among cattle in the area. The epidemiology of bluetongue virus is very complex due to the involvement of several mammalian hosts and vector species. The role of cattle in the epidemiology of BT in SA is not well understood. Light traps were used to collect midges over 16 trap nights during autumn and winter. *Culicoides* midges were identified to species level and pooled (200 midges/ pool). Midge pools were subjected to real-time RT-qPCR to test for the presence of BTV RNA. Serum samples were randomly collected from 1 260 cattle and screened for antibodies specific to bluetongue virus using a BTV-specific cELISA. Blood samples were collected from seronegative cattle and screened for the presence of BTV RNA with a real-time RT-qPCR. Twenty-five different *Culicoides* spp. were identified of which *C. imicola* were found to be the most abundant. Of the 25 species collected, 19 species yielded parous females with 16 *Culicoides* species demonstrating a vector rating higher than 25%. Bluetongue virus RNA was detected in 51.2% and 75.9% of midge pools collected during autumn and winter, respectively resulting in an infection prevalence of 0.3% and 0.7%.

Antibodies specific to BTV were detected in 1 206 (95.7%) of the sera samples tested with significant differences ($p < 0.05$) in seroprevalence between age groups and between villages. No significant differences in seroprevalence were observed between different breeds or sex. A total of 16 out of 45 (35.5%) blood samples from seronegative cattle tested positive for BTV RNA. These results demonstrate that *C. imicola* is the most abundant midge species and that BTV is highly prevalent in autumn as well as throughout winter in Mnisi.

The second part of the study focused on the overwintering of BTV in *Culicoides* populations at the ARC-OVI. The exact method(s) of overwintering of BTV is unknown. Recent studies have suggested that the virus is overwintering within the midge vector. Climatic conditions in South Africa are suitable for adult *Culicoides* midges to remain active throughout much of the year with only certain areas where temperatures can become unsuitable. During such periods ambient temperature might be too low for midge emergence or viral replication within the vector. It is hypothesised that during summer, BTV infection becomes more prevalent in the *Culicoides* population and therefore disease only occurs in late summer to early autumn. Real-time RT-qPCR was used to detect BTV in *Culicoides* midges collected from July 2010 to August 2011. Bluetongue virus RNA was detected in 52 out of 57 (91.2%) midge pools tested. The results obtained strengthen the findings of chapter 2 demonstrating that BTV is present throughout winter in *Culicoides* populations at various temperatures. It is therefore clear *Culicoides* are present throughout the year and that BTV are capable of overwintering within the midge even though temperatures fall well below the normal activity range of these midges. This also forms part of a study focussing on overwintering of AHSV in the *Culicoides* vector.

In the last research chapter of the dissertation, segment 10 of BTV isolates previously used as reference strains in the Department of Veterinary Tropical Diseases, from 1972 - 2000 were compared to establish the topotypes of these strains. Bluetongue virus can be divided into five topotypes based on segment 10 i.e. western 1, 2 and 3 and eastern 1 and 2. The groupings are based on evolutionary distinct geographical variants and phylogenetic analysis. It is hypothesised that the segment 10 genes have co-evolved with respect to the specific *Culicoides* species

found in a geographical area. Comparing the highly conserved NS3 gene region to newly isolated strains in South Africa as well as to representative global strains could give us an indication of the degree of variability between strains and serotypes. Segment 10 from these samples were sequenced and compared to global NS3 sequences. A total of 11 sequences were obtained and submitted to GenBank. All sequences demonstrated the conserved cysteine regions as well as the tryptophan residue. Both hydrophobic regions and the proline-rich regions were also conserved throughout all 11 isolates as well as the two glycosylation sights. Both a neighbour-joining and a neighbour-net tree were compiled using MEGA 6 and SplitsTree 4 respectively. Three lineages groups were established with i.e. western group 1 and 2 and eastern group 1. The phylogenetic trees coincide with previous studies done on segment 10 of the BTV genome. These results also confirm that BTV-20, BTV-21 and BTV-23 are exotic to South Africa. BTV-2 demonstrated the ability of strains to cluster together, irrespective of their serotype providing evidence of genetic differences within serotypes.

The presence of both competent midges and amplifying host, e.g. cattle and wildlife, contribute in the epidemiology of the disease, especially in episystems where distinct grouping of viruses co-evolved with respect to specific *Culicoides* species. The epidemiology of BTV is therefore likely to be largely influenced by the level of contact between livestock (and wildlife) and BTV-infected *Culicoides* species that influences both the distribution and the genetics of the virus

Chapter 1: Literature review

1.1 General introduction

During the last decade bluetongue virus (BTV) has become a major concern worldwide as well as the focus point of many epidemiological studies and surveillance programmes. The virus is the prototype member of the genus *Orbivirus* in the family *Reoviridae* and causes an economically important infectious, non-contagious disease in domestic and wild ruminants called bluetongue (BT) (Spreull, 1905; Borden *et al.,* 1971). Twenty six serotypes (Howell, 1960; Maan *et al.,* 2011) of the virus have been identified, that are transmitted primarily by certain species of biting midges that belong to the genus *Culicoides* (Diptera: *Ceratopogonidae*) (Du Toit, 1944; Mellor *et al.,* 2000). Bluetongue is an International Office of Epizootics (OIE) notifiable disease and is described as a rapidly spreading disease that has a considerable impact on the health of livestock (MacLachlan, 2011). Non-tariff trade barriers have been implemented in many countries across the globe to prevent the spread of BTV to unaffected areas (MacLachlan and Osburn, 2006). The incidence and geographical distribution of BTV is partly determined by the distribution of vector-competent *Culicoides* species, which, in turn, is restricted by environmental parameters. Consequently, BTV has been considered to be endemic in certain regions of the world from latitudes of approximately 40 °N and 35 °S. However, since 1998, BTV has been documented at latitudes up to 50 °N and by 2006, the distribution of BTV expanded beyond 51 °N (Gibbs and Greiner, 1994; Mellor, 1994, 2004; Mellor and Wittmann, 2002; Purse *et al.,* 2005).

The epidemiology of BTV has changed substantially since 1998 (Mellor and Witmann, 2002). The recent northwards expansion of BTV is speculated to be coupled to unprecedented climate changes (Cook, 1992; Purse *et al.,* 2008). Various climatic drivers are involved in BTV transmission and emergence, including

temperature and moisture availability (Mellor *et al*., 2000; Wittmann and Baylis, 2000). These climatic factors influence the life cycle of the vector species, as well as virus transmission. An increase in range and abundance as well as the seasonal activity of the traditional vector, *Culicoides imicola*, was hypothesised to be due to a change in climate rather than biotic and/or abiotic factors (Purse *et al*., 2005). The ability of, historically, 'non-vector' species to transmit BTV is also attributed to an increase in temperature as population sizes and survival rates increase and compensates for their low vector competence levels (Purse *et al*., 2005).

1.1.1 History

Bluetongue (BT) was first recorded in the Cape Province of South Africa in the late eighteenth century (Spreul, 1905). It is believed that the disease had been endemic in certain wild ruminants in sub-Saharan Africa before the virus first spread to domestic ruminants more than 100 years ago (Barzilai and Tadmor, 1971; Stott *et al.,* 1987). The disease was first recognized following importation of susceptible Merino sheep from Europe. Initially the disease was called 'fever' or 'epizootic catarrh' but was later renamed as 'malarial catarrhal fever of sheep' as it was initially thought that it was caused by a parasite (Hutcheon, 1880). In 1905 the name was changed to 'bluetongue', referring to the development of a cyanotic tongue in some infected animals (Spreull, 1905). Sir Arnold Theiler demonstrated that the aetiological agent of BT could be passed through a filter thereby establishing that BTV was caused by a virus (Theiler, 1906). Theiler, the founder of the Onderstepoort Veterinary Institute (OVI), contributed to the establishment of OVI as a major southern African research centre focusing on BT. The first vaccine strain (BTV-4) was developed at the institute and was used by sheep farmers throughout South Africa for the first 40 years of the 19th century (Theiler, 1908; Dungu *et al.,* 2004). After numerous failures of the Theiler vaccine, Neitz (1948) started investigating the variation of BTV strains that are found in the field, by cross-protection studies in sheep. Not only was Neitz able to show that sheep infected with a particular field strain develop life-long protective immunity against re-infection, but also that this immunity is ineffective against other heterologous field strains (Neitz, 1948). Studies such as these made it possible to serotype BTV field isolates and to develop cross-

protective vaccines against various circulating BTV serotypes. Howell (1960) initially used 22 field isolates and a neutralization test to identify 12 serotypes. Later, a plaque inhibition assay identified four more serotypes from South Africa (Howell *et al.,* 1970). The development of *in vitro* and *in vivo* systems facilitated large scale propagation of BTV that guided physiochemical, antigenic and genetic studies of the virus. These studies allowed scientist to establish that BTV is a dsRNA virus, with a genome comprising ten linear genome segments, enclosed within a triple layered protein capsid (Verwoerd 1970; 1972). BTV was later classified into the *Orbivirus* genus (Borden, 1971).

1.1.2 Bluetongue

Bluetongue virus causes severe disease in certain ruminants and camelid species; however, the severity of the disease differs between species and within breeds (Neitz, 1948; Vosdingh *et al.,* 1968; MacLachlan *et al.,* 2009). European fine wool and mutton breeds and the North American white-tailed deer show severe disease with prominent clinical signs, while cattle and goats are usually sub-clinically affected (Spreull, 1905; Vosdingh, 1968). Clinical signs can also vary amongst susceptible sheep of the same breed, ranging from sub-clinically infected individuals to individuals showing acute disease that can lead to death. This type of variation can be explained by intrinsic differences in virulence between infecting field strains as well as age, nutritional status and differences in innate immunity of the host. The titre of viral inoculum and environmental factors such as UV radiation also contributes to this variation (Erasmus, 1975). The severity of the disease within a population of susceptible sheep varies, but morbidity can be as high as 70%, whereas mortality varies from 5% to 50% (Verwoerd and Erasmus, 2004; Venter *et al*., 2011).

Initial replication of the virus, after a bite from an infected competent vector, occurs in the regional lymph node of a susceptible animal (Pini, 1976; Barratt-Boyes and MacLachlan, 1994). From there the virus disperses throughout the body tissues where it replicates in endothelial cells, mononuclear phagocytes and lymphocytes (MacLachlan *et al*.,1990, 1994; Barratt-Boyes and MacLachlan, 1995). The virus replicates primarily in the lymph nodes, spleen and lungs (Barratt-Boyes and MacLachlan, 1994; Sanchez-Cordon *et al*., 2010). During viraemia, BTV is primarily

associated with the platelets and erythrocytes and entirely associated with the latter, during later stages of infection. Due to this association, the ruminant host show viraemia for a prolonged period, albeit not being necessarily infectious to the vector (Barratt-Boyes and MacLachlan, 1995; Bonneau *et al.,* 2002; Darpel *et al.,* 2007).

Clinical signs of BT in ruminants may be mistaken for diseases such as foot-and-mouth disease, acute photosensitisation, epizootic haemorrhagic disease, rinderpest and malignant catarrhal fever (Bekker *et al.,* 1934; Bexiga *et al.,* 2007; Williamson *et al.,* 2008). Infected hosts may show fever, depression, anorexia, nasal or serosal fluid discharge, coronitis that can manifest as lameness, and muscle degeneration. Other signs include congestion, oedema and haemorrhage in the mucus membranes of the digestive and respiratory system, cyanosis of the tongue and facial and pulmonary oedema (Erasmus, 1975; Pini, 1976; Mahrt and Osburn, 1986; Verwoerd and Erasmus, 2004; MacLachlan *et al.,* 2008). Reproductive failures in infected animals include abortions, still births and malformations in offspring (Desmecht *et al*., 2008; Vercauteren *et al*., 2008; Wouda *et al*., 2008).

1.1.3 Bluetongue virus vaccines

Attenuated modified live vaccines (MLVs), produced by Onderstepoort Biological Products, are administered annually from August to October to sheep and goats in South Africa and Namibia as prophylaxis (Gerdes, 2004). These multivalent vaccines contain five serotypes each; Bottle A consists of serotype -1, -4, -6, -12 and -14; bottle B serotypes -3, -8, -9, -10 and -11 and bottle C serotypes -2, -5, -7, -13 and -19 (Dungu *et al.,* 2004). In southern Europe and the Middle East cattle have been vaccinated with formulations containing BTV-2, -4, -9 and -16 with little or no adverse effects (Savini *et al.,* 2008). Regardless, inactivated vaccines against BTV-2, -4 and more recently BTV-1 and -8 are predominantly used in Europe (Savini *et al.,* 2008).

Modified live vaccines are capable of reassorting with wild type and/or other vaccine strains in the field to create novel viruses (Batten *et al.,* 2008). These viruses may display altered biological properties that could potentially include enhanced virulence or an enhanced capacity to infect *Culicoides* midges. Live vaccine virus have also been isolated from field caught *Culicoides* in Italy (Batten *et al.,* 2008; Maan *et al.,*

2010). Other disadvantages of MLV vaccines in the field include mild to severe clinical signs and/or a decrease in milk production in sheep (Savini *et al.,* 2008; Veronesi *et al.,* 2010), a reduction in semen quality in rams (Breard *et al.,* 2007) and a suppression of specific lymphocyte blastogenesis in goats (Lacetera and Ronchi, 2004). Transplacental infection of MLVs has also been demonstrated in South Africa, USA as well as Southern Europe (Osburn, 1994; MacLachlan *et al.,* 2000). Prolonged circulation of vaccines in the field could also potentially result in a reversion to virulence as a result of genetic drift (Veronesi *et al.,* 2005). The fact that MLV vaccines are not DIVA (Differentiating Infected from Vaccinated Animals) compliant also complicates disease surveillance (Savini *et al.,* 2008).

1.2 Virion structure

The BTV genome is composed of 10 segments of linear double stranded (ds) RNA which code for seven structural (VP1 to VP7) and four non-structural proteins (NS1 to NS4) (Van Dijk and Huismans, 1988; Belhouchet *et al*., 2011). Segments 9 and 10 encodes two alternative proteins VP6a and NS3a (Verwoerd *et al.,*1970; Van Dijk and Huismans 1988; Belhouchet *et al*., 2011; Ratinier *et al*., 2011).

1.2.1 Structural proteins

The seven structural proteins are organised into a diffuse outer protein layer and two inner shells (Fig. 1.1) (Verwoerd *et al.,* 1969). The outer diffuse protein layer consists of triskelion-shaped viral protein 2 (VP2) that is arranged as trimers and that protrudes from the surface of the virus. Viral protein 2 mediates virus-receptor binding to mammalian cells The VP2 trimers are interspersed with globular trimers of VP5 (segment 6), that facilitate viral fusion during receptor-mediated endocytosis (Hassan and Roy, 1999). Viral protein 2 (segment 2) is the most variable of the proteins and the interaction of the protein with the ruminant humoral immune system determines virus serotype (Huismans and Erasmus, 1981). A clear correlation between amino acid sequence diversity in VP2 and viral serotype has previously been demonstrated (Maan *et al*., 2007). The outer protein layer is bound to an inner

scaffold composed of VP7 (segment 7) that overlays the inner icosahedral shell composed of VP3 (segment 3). Infection of insect cells is mediated by VP7 that protrudes through the outside of the virion and which mediates the binding of the virus to insect cell receptors via an arginine-glycine- aspartate (RGD) tripeptide motif (Xu *et al.,* 1997; Tan *et al.,* 2001). Ten to twelve transcriptase complexes with "flower"-like arrangements facilitate viral transcription and replication. This complex is composed of VP1 (segment 1), VP4 (segment 4) and VP6 (segment 9) and is located on the inner side of the sub-core. The primary function of VP1 (viral RNA-dependant RNA polymerase) is viral transcription and replication (Boyce *et al.,* 2004), VP4 caps newly synthesized mRNA strands (Ramadevi and Roy, 1998) and VP6 unwinds and re-anneals the viral dsRNA, during genome transcription and replication. The genome is situated within the viral sub-core and is arranged as four layered spirals below pores that are present at the five-fold axes of the sub-core (Gouet *et al*., 1999).

1.2.2 Non-structural proteins

The four non-structural proteins are found in BTV infected cells (Van Dijk and Huismans, 1988; Ratinier *et al*., 2011). Non-structural protein 1 (segment 5), which is the most abundant protein in infected cells, forms tubular structures in the cytoplasm. The exact role of these structures in viral replication has yet to be determined although it is thought that viral tubules help with the translocation of progeny viral particles to the cell membrane (Huismans and Els, 1979; Owens *et al*., 2004). NS1 is also a positive regulator of BTV gene expression that increases protein synthesis from viral mRNAs (Boyce *et al.,* 2012). Viral assembly occurs in association with viral inclusion bodies that are formed by the phosphoprotein, NS2 (segment 8). The phosphoprotein rapidly accumulates as a granular matrix in the cytoplasm of infected cells. The NS2 protein is thought to assort the genome segments into progeny viral pre-cursor (Lymperopoulos *et al*., 2006).

Non-structural viral glycoproteins, NS3 and its alternative form NS3/A (segment 10), mediate viral release from infected cells. NS3/A also facilitates the transport of the virus to the host cell surface by acting as a bridge between VP2 and components of the host cell cytoskeleton (Wirblich *et al.*, 2006; Celma and Roy, 2009). At the cell

surface NS3/A mediates the release of viral particles either through cell lysis or budding. In mammalian cells, virus release is typically mediated by cell lysis, resulting in cytopathic effect (CPE) and cell death. However, in insect cells, virions bud from the cell membrane leaving the host cells intact (Owens *et al.,* 2004).

It has recently been demonstrated that the non-structural protein NS4 (segment 9) localizes in the nucleus of BTV infected cells (Belhouchet *et al*., 2011; Ratinier *et al*., 2011). NS4 is thought to be a nucleic acid binding protein that inhibits transcription of interferon genes, thereby allowing the virus to spread, and replicate unhindered. NS4 has been proposed to either repress or enhance transcription of genes linked to interferon response of the cell (Belhouchet *et al*., 2011; Ratinier *et al*., 2011).

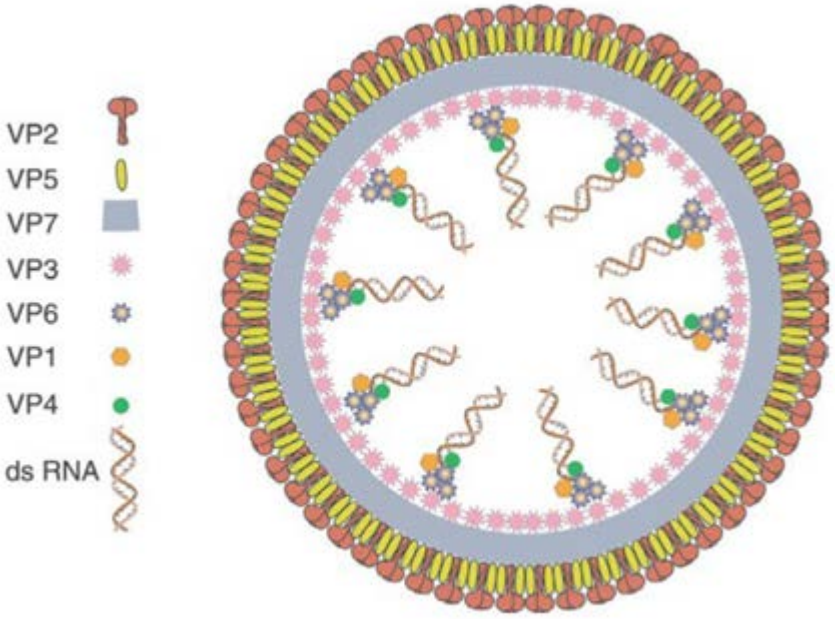

Figure 1.1: Schematic representation of the bluetongue virion. Structural proteins VP1 to VP7 are illustrated. VP2 triskelion-shaped trimers are interspersed with globular VP5 trimers on the surface of the core. The outer protein layer is bound to the inner core that is composed of VP7. This layer overlay the inner icosahedral shell composed of VP3. Ten to twelve transcriptase complexes with “flower”-like arrangements composed of VP1, VP4 and VP6, are found on the inner side of the sub-core. Source: Schwartz-Cornil *et al*., (2008).

1.3 *Culicoides* midges

In the early 1900 ‘s, little was known about the transmission of BTV. It later became evident that the disease demonstrated a seasonal infection pattern and that by stabling sheep at night the number of BTV-infected animals could be decreased. This led researchers to believe that a crepuscular insect vector might be responsible for the transmission of the virus (Hutcheon, 1902; Spreull, 1905; Du Toit, 1944). Adult female haematophagous biting midges that belong to the genus *Culicoides* (Diptera: *Ceratopogonidae*) were later identified as the primary vector for BTV transmission (Du Toit, 1944).

1.3.1 *Culicoides* species as vectors

The capacity of vector midges to act as disease agents was first established in 1927 when it was reported that *Culicoides* could transmit the filarial worm, *Mansonella perstans*, to humans in West Africa (Meiswinkel *et al.,* 2004). It was only in the 1940’s that *Culicoides* were demonstrated to be able to transmit BTV (Du Toit, 1944). The development of light traps in South Africa enabled researchers to collect insects throughout the night. Large numbers of trapped *Culicoides* made it possible for scientists to induce BT in sheep by inoculating animals with field-collected *Culicoides* (*Avaritia*) *imicola* (Du Toit, 1944). Du Toit (1944) was also able to demonstrate that *C. imicola* fed on viraemic sheep, were able to transmit the virus to apparently healthy sheep. These findings confirmed the status of *Culicoides* haematophagous biting midges as vectors of BTV. The transmission of BTV by *Culicoides* midges was universally accepted following further transmission experiments in the USA (Foster *et al.,* 1963).

Apart from BTV, more than 50 arboviruses have been isolated from *Culicoides* species (Meiswinkel *et al.,* 2004; Borkent, 2005). Of these arboviruses, the families *Reoviridae* (*e.g.* African horse sickness virus (AHSV)), *Bunyaviridae* (*e.g.* Akabane virus) (St George *et al.,* 1978; Theodoridis *et al.,* 1979) and *Rhabdoviridae* (*e.g.* Bovine ephemeral fever) (Davies and Walker, 1974; Muller and Standfast, 1986) were especially prominent. At least 30 *Culicoides* species have been associated with

the transmission of *Orbiviruses* worldwide, with 13 of these *Culicoides* species occurring in SA (Meiswinkel *et al.,* 2004).

1.3.2 *Culicoides* morphology

Culicoides midges are small in size, ranging from 1 - 3 mm in body length and typically have white and grey patterned wings. Worldwide, more than 1 400 species have been identified of which 96% are obligate haematophages (Mellor *et al*., 2000, 2009). *Culicoides* ontogeny is holometabolous and the life cycle comprises egg, four larval instars, a pupa and an adult stage. Under optimal conditions the life cycle takes between two to six weeks to complete (Mellor *et al.,* 2000). Larvae occupy various moist habitats such as fresh and salt water marshes, animal dung, rotting vegetation and water troughs, muddy grounds and areas covered with organic and/or faecal matter (Braverman *et al.,* 1974; Lubega and Khamala, 1976; Mellor and Pitzoltis 1979; Edwards 1982; Mellor *et al.,* 2000; Mullen, 2002). Adult midges have been shown to only fly short distances and no evidence suggesting long distance migration has been published. However, passive wind transport of over 700 km has been recorded (Chapman *et al*., 2004; Ducheyne *et al.,* 2007). Recent studies that investigated both active and passive dispersal of *Culicoides* midges, determined that disease outbreaks in previously unaffected areas such as northern Europe can be explained by passive vector dispersal (Sanders *et al.,* 2011).

Adult *Culicoides* midges, both male and females, feed on plant fluids and nectar. Female *Culicoides* midges can be divided into two groups; parous females, which have laid at least one batch of eggs or nulli-parous females which have never developed eggs nor taken a blood meal (Braverman and Mumcuoglu, 2009). Female *Culicoides* need a blood meal to develop eggs. Parous and gravid females are therefore the only proportion of a population that could potentially become infected and transmit BTV (Nelson and Scrivani, 1972; Allingham and Standfast, 1990, Venter and Meiswinkel, 1994). After feeding on a BTV-infected animal, the virus replicates inside the cells of the mid-gut of the midge from where progeny virus particles, may or may not be contained by a mid-gut barrier. In competent females the virus passes through the mid-gut barrier into the haemocoel to infect and replicate in the salivary gland. Following infection of the salivary gland the virus can

be transmitted, during subsequent blood feeding, to a susceptible host. An apparent ovarial barrier safeguards the ovarium from becoming infected. In non-vector competent females, infections are restricted to the infection of the cells of the mid-gut. *Culicoides* saliva facilitates viral transmission to the host by inhibiting the host's immunological response, by enhancing viral replication and by regulating blood flow at the bite site of the host (Wilson and Mellor, 2009). It takes 3 – 21 days, depending on the temperature, for the virus to replicate to transmissible levels, with infection being life-long in infected midges (Eaton *et al.,* 1990; Mellor, 1990; Mellor *et al*., 2000; Paweska *et al*., 2002; Mullens *et al*., 2004). Winter and prolonged droughts can inhibit the transmission of BTV, as vector midges are either inactive or occur at very low population levels in dry areas. Despite this, ruminants still show signs of BT shortly after winter in some areas, when adult vectors are either absent and/or present at low numbers (Taylor and Mellor, 1994). This observation raises questions as to the mechanism that is used by BTV to overwinter from one vector season to another (Nunamaker *et al*., 1990).

1.3.3 *Culicoides* species distribution

Traditionally, *C. imicola* (Fig. 1.2) was known to be the primary BTV vector of BTV in Africa, Asia and some parts of southern Europe (Mellor, 1990). However, recent studies demonstrate that additional *Culicoides* species have been associated with the transmission of BTV, particularly in northern Europe. In northern Europe, temperate species such as *Culicoides* (*Avaritia*) *obsoletus* and *Culicoides* (*Culicoides*) *pulicaris* groups are found in temperature ranges of 6°C to 16°C and have been involved in the transmission of BTV-8 (Mehlhorn *et al*., 2007, 2009). In the USA, *Culicoides* (*Monoculicoides*) *sonorensis* is regarded as the primary vector with *Culicoides* (*Hoffmania*) *insignis* being involved in south east USA to a lesser extent (Jones, 1985). *Culicoides insignis* is believed to be the primary vector in South America although BTV has also been isolated from *Culicoides* (*Avaritia*) *pusillus* in Central America and the Caribbean (Mo *et al.,* 1994) as well as in South America. The primary vectors in Australia are *Culicoides* (*Avaritia*) *fulvus, Culicoides* (*Avaritia*) *wadai and Culicoides* (*Avaritia*) *brevitarsis* and to a lesser extent *Culicoides actoni* and *Culicoides* (*Avaritia*) *dumdumi* (Standfast *et al.,* 1985, 1992). Most of

these species have also been reported in Southeast Asia and India (Wirth and Hubert, 1989) (Fig. 1.3). In South Africa, *Culicoides* (*Avaritia*) *bolitinos* has been found to be another important vector of BTV, especially in colder, high lying regions (Venter and Meiswinkel, 1994) (Fig. 1.3). *Culicoides bolitinos* show high activity in temperatures ranging from 13-35°C, whereas *C. imicola* prefers temperatures of ≥18°C to ≥38°C (Ortega *et al.,* 1999; Carpenter *et* al., 2011; Verhoef *et al*., 2014). Other species identified near livestock throughout South Africa are *Culicoides* (*Meijerehelea*) *leucostictus*, *Culicoides* (*Beltranmyia*) *pycnostictus*, *Culicoides* (*Remmia*) *subschultzei*, *Culicoides* (*Remmia*) *enderleini*, *Culicoides magnus*, *Culicoides zuluensis*, *Culicoides* (*Beltranmyia*) *nivosus* and *Culicoides* (*Remmia*) *schultzei* (Nevill *et al.,* 1992; Meiswinkel *et al*., 2004, Venter *et al.,* 2006).

Figure 1.2: *Culicoides* (Avaritia) *imicola* Kieffer, the major bluetongue virus vector in Africa and Asia. Photo: Bruno Mathieu, (EID).

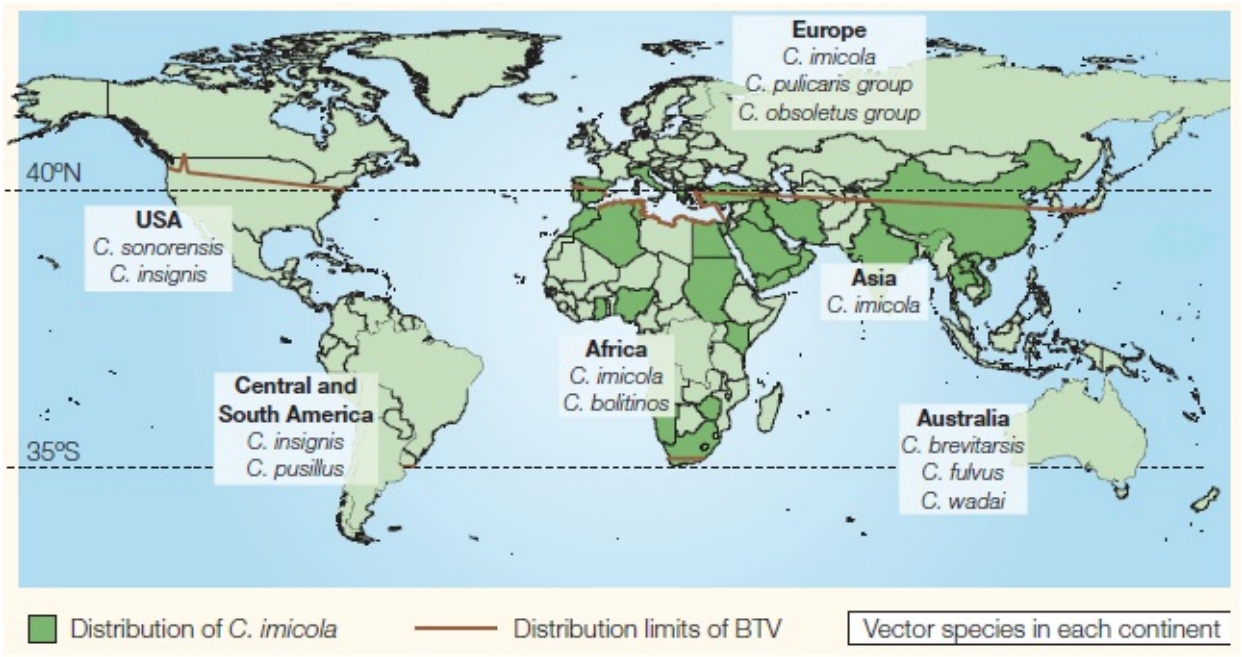

Figure 1.3: Geographical distribution of *Culicoides* vector species responsible for BTV transmission within different geographic regions. Dark green shading indicates the distribution of the "old world" vector species, *C. imicola* from 1998 to 2005. Source: Adopted from Purse *et al.,* (2005).

1.4 Bluetongue virus and *Culicoides* vectors

Factors that influence the infection and transmission of BTV by *Culicoides* midges include environmental, midge and virus-specific factors.

1.4.1 Vector capacity and vector competence

Vector capacity refers to the ability of a vector population to transmit a pathogen. It is defined as the average number of infective *Culicoides* midge bites while feeding on a host. Vector capacity is influenced by midge density, host preference, biting frequency, midge life-span, duration of viraemia and vector competence. Vector competence is measured by the number of midges that become infected after feeding on a viraemic host. Vector competence is dependent upon genetic make-up of the midge, which influences the ability of the vector to support viral infection, replication and dissemination. Vector competence is also greatly influenced by

environmental factors, particularly temperature (Mullens *et al*., 2004; Mellor *et al.,* 2009).

1.4.2 Oral susceptibility and transmission potential of *Culicoides* species

Oral susceptibility studies have demonstrated that significant differences in infection rates exist between different strains/populations of *Culicoides* midges, depending on the BTV strains used (Venter *et al*., 2006, 2007). For example, one study demonstrated that BTV-8 could not be isolated from 234 *C. imicola* females 10 days after the ingestion of a 6.8 $\log_{10}TCID_{50}$/ml blood meal. This indicates a comparatively low susceptibility of *C. imicola* for BTV-8. In contrast, nine of 142 *C. imicola* tested positive for the attenuated vaccine strain of BTV-8 after feeding on blood with a virus concentration of 4.6 $\log_{10}TCID_{50}$/ml (Venter *et al.,* 2006, 2007). Anecdotal evidence further suggest that certain strains of BTV which are more frequently isolated from *Culicoides* midges in the field in South Africa may have a higher transmission potential as compared to other strains which are only rarely isolated (Dungu *et al.,* 2004). Ambient temperature also plays a crucial role in the rate of virogenesis, the length of the EIP and the infection rate of the virus with regards to the vector (Wittmann and Baylis, 2000; Wittmann *et al.,* 2002).The duration of the EIP, which is the interval between virus ingestion and when the midge becomes capable of transmitting the virus, is shorter at higher temperatures. Replication of the virus in the midge will concurrently, also decrease until a threshold temperature of 11°C to 13°C is reached, after which replication will stop completely (Carpenter *et al*., 2011).

1.4.3 Bluetongue virus sequence variation and *Culicoides* species

The evolution of distinct variants of BTV is observed throughout the globe due to viral evolutionary processes coupled with the localised circulation of BTV in different ecological niches. Evolutionary processes such as intragenic recombination, gene mutation and segment reassortment are the main driving forces behind the emergence of novel BTV lineages (He *et al*., 2010; Shaw *et al.,* 2013). Phylogenetic analyses of the viral genome indicate that global variants of BTV cluster into two major lineages. Viruses circulating in America, the Caribbean and Africa make up a

western lineage, whereas viruses circulating in Australia, Asia and Indonesia form part of an eastern lineage (Gould and Hyatt, 1994; Carpi *et al.,* 2010).

Different genome segments of BTV demonstrate varying degrees of sequence conservation. Differences in the sequence conservation between segments reflect both the error prone replication processes and the selective pressure that acts on the encoded proteins (Bonneau *et al*., 2001; Bonneau and MacLachlan, 2004). Only a limited number of studies have been done to delineate the intrinsic BTV-specific genetic markers that influence oral susceptibility and/or transmission potential in *Culicoides* midges (Nevill *et al.,* 1992; Bonneau and MacLachlan, 2004; Venter *et al.,* 2007). Intrinsic virus-specific factors that may influence the infection and transmission dynamics in *Culicoides* midges may be reflected in variation in VP7 and NS3/A which, respectively, mediate infection and dissemination of the virus in the insect vector. Genetic variation in these proteins have been considered to influence the transmission of the virus by different midge species in different geographical areas (Bonneau *et al*., 2000; Wilson *et al*., 2000; Maan *et al*., 2008, 2010) (Fig. 1.3). Although relatively conserved, VP7 demonstrates diversity based on deduced amino acid sequences that cluster the virus into several clades (Wilson *et al.,* 2000). Virus isolates can be divided into several clades by phylogenetic analysis using segment 7 (VP7); however, only a weak correlation between these clades and particular geographic locations and/or *Culicoides* species/populations have been established (Wilson *et al.,* 2000). In fact it has been demonstrated that the RGD binding motif in VP7 remains conserved amongst a global panel of BTV isolates (Grimes *et al*., 1995; Tan *et al*., 2001). Similarly to VP7, the amino acid sequence of the alternative NS3 encoding gene of various BTV isolates failed to demonstrate adaptive selection. Recent findings in northern Europe indicated that *Culicoides* species not known for transmitting BTV can be competent vectors. The findings support the less specific nature of BTV and argue against more specific co-evolution (Gibbs and Greiner, 1994; Tabachnick, 2004; Balasuriya *et al*., 2008). Regardless of the lack of evidence for co-evolution of NS3/A with particular *Culicoides* species/populations, at least one study with the closely related AHSV has suggested that this protein may be involved in modulating transmission potential (Riegler, 2002).

The infectivity for *Culicoides* species among BTV field strains may also differ based on the amino acid sequence of the outer core protein, VP2. Previous studies have revealed that, after treatment with chymotrypsin, VP2 is cleaved resulting in the generation of infectious sub-viral particles with an enhanced infectivity for *Culicoides* cells (Mertens *et al*., 1987). The same could be observed after cleavage of VP2 by proteases found in the saliva of vector competent *Culicoides* midges (Mertens *et al.,* 1996; Darpel *et al.,* 2011). Importantly, the protease present in the saliva of *Culicoides* females differ in its ability to cleave the VP2 protein of viruses from either eastern or western origins, suggesting that the infectivity of particular serotypes may be influenced by variation in the amino acid sequence of the protein (Darpel *et al*., 2011).

1.5 Overwintering of bluetongue virus

The exact mechanism by which the virus is able to survive during cold periods when few or no midges are present is poorly understood. Overwintering mechanisms include overwintering either within the vector, host population or by alternative transmission cycles in novel vector/host species (Takamatsu *et al*., 2003; Wilson *et al*., 2007, 2008).

1.5.1 Overwintering within the vector

Culicoides midges usually inhabit temperature ranges of 13°C to 37°C, although it has been recorded that during the mild winter of 2006/2007 in Europe, adult *Culicoides* midges were able to survive up to three months at much lower temperatures (Losson *et al*., 2007; Lysyk and Danyk, 2007). *Culicoides imicola* adults can survive for >15 days and some species, e.g. *Culicoides pycnostictus,* up to 54 days at temperatures as low as -1.5°C (Nevill, 1971). Studies have also confirmed that both *C. imicola* and *C. bolitinos* are able to harbour BTV for more than 20 days at 10°C (Paweska *et al.,* 2002). The implication of these findings is that BTV-infected midges might survive for the duration of winter and are capable of re-infecting ruminant hosts when conditions are again more favourable (Takamatsu *et al*., 2003; Wilson *et al*., 2007, 2008).

A transovarial transmission study, using infected adult *Culicoides variipensis*, detected RNA fragments of the virus in the protein yolk bodies and the vitelline membrane using immunoelectron microscopy. Detection of BTV could, however, not be confirmed, following plaque assays, from any of the progeny midges refuting the possibility of transovarial transmission. This confirms that some virus particles and/or viral nucleic acid are capable of moving through the pores of the midge oocytes (Nunamaker *et al.,* 1990). The detection of BTV in the larval stage of the midge's live cycle has been demonstrated. Viral RNA was detected in the larvae of *C. sonorensis*, suggesting that BTV may overwinter in larvae stages of *Culicoides* (White *et al.,* 2005). It might be plausible that infected adults might emerge without ever feeding on an infected host (Comer *et al.,* 1990; Beaty and Calisher, 1991; White *et al.,* 2005).

1.5.2 Overwintering within the ruminant host

An alternative overwintering mechanism for BTV might involve persistent infection in the ruminant population during the winter (Taylor and Mellor, 1994; Takamatsu *et al*., 2003; Osmani *et al*., 2006; Wilson *et al*., 2007). The duration of viraemia in sheep and cattle is known to be approximately 50-and 60-days, respectively (Sellers and Taylor, 1980; MacLachlan *et al.,* 1990; Koumbati *et al.,* 1999). Studies have shown BTV viraemia in cattle can occasionally last up to 100 days without showing any clinical signs (Sellers and Taylor, 1980). Prolonged infectious viraemia in host species might allow the virus to survive vector-free periods (Taylor and Mellor, 1994; Takamatsu *et al.,* 2003; Purse *et al.,* 2005; Osmani *et al.,* 2006; Wilson *et al.,* 2007).

Infection with laboratory-adapted virus has demonstrated that BTV could persistently infect γδ T-cells of sheep *in vitro* and *in vivo* (Takamatsu *et al.,* 2003; Purse *et al*., 2005). These cells possibly become infected during the initial stages of virus replication and viraemia within the host (Takamatsu *et al.,* 2003). Animals will be seropositive and aviraemic but will still carry BTV in their γδ T-cells. Recovered virus is capable of replicating continuously within the skin cells without showing any obvious CPE. The same could not be demonstrated in bovine γδ T-cells where persistent BTV infection does not occur in the skin of naturally infected cattle (Melville *et al.,* 2004). The authors concluded that more evidence of BTV persistence

in sheep skin is essential before accepting these finding as an 'overwintering' mechanism.

1.5.3 Overwintering through transplacental transmission

Transplacental transmission of BTV was first documented in the 1970 's when ewes were sub-cutaneously inoculated with a cell culture isolate of BTV-4 during mid-gestation. None of the ewes developed clinical signs, but 80% of the offspring tested positive for BTV through virus isolation (Gibbs *et al.,* 1979). It was subsequently demonstrated that MLVs of BTV serotypes (BTV-10, -1, -13 and -17) are also able to cross the placenta (MacLachlan *et al.,* 2000). Since then, several studies have confirmed the capability of MLV strains to cross the placental barrier of sheep, cattle and goats, resulting in abortions, deformity and/or foetal death (Kirkland and Hawkes, 2004; MacLachlan and Osburn, 2008; Savini *et al.,* 2014).

Prior to 2008, transplacental transmission of BTV was considered solely a property of laboratory-adapted viruses (Roeder *et al.,* 1991; Parsonson *et al.,* 1994). However, studies conducted on the wild-type BTV-8 strain, which caused the recent epidemic in Europe between 2006 and 2008, has demonstrated that this virus also has the ability to cross the placental barrier of ruminants. This outbreak resulted in a significant quantity of calves being born with congenital abnormalities and hydranencephaly, as well as high numbers of bovine abortions. *In utero* infection rates of 33-44% have been reported in the Netherland, Belgium and the United Kingdom (De Clercq *et al.,* 2008; Desmecht *et al.,* 2008; Darpel *et al.,* 2009). The implication of transplacental transmission with regards to the epidemiology and overwintering of BTV is still unclear.

1.6 Laboratory diagnosis of bluetongue virus

The identification and differentiation of BTV serotypes, strains and topotypes are essential for the understanding the epidemiology of BTV (Mertens *et al.,* 2009). The identification of the exact serotypes of BTV circulating within a region are essential to ensure the use of appropriate vaccines, whereas the detection of the virus based on

group-conserved genes or antigens is the most reliable method to diagnose BTV (Hamblin, 2004). Bluetongue virus can also be isolated from blood, semen and various other tissue samples (Parsonson, 1990; Afshar, 1994; Tweedle and Mellor, 2002).

For diagnostic purposes, assays must be able to distinguish BTV from other orbiviruses. Tests also need to be sensitive enough to detect low levels, early or late infection (Mertens *et al.,* 2009). Specific serological and molecular assays have been designed to test for the presence of conserved BTV antigens and RNA. Tests that target conserved antigens and RNAs are referred to as serogroup-specific assays and typically target the non-structural and core proteins of the virus, especially VP7 and NS1. Serogroup-specific tests include enzyme-linked immunosorbent assays (ELISA) and reverse transcriptase-polymerase chain reactions (RT-PCR) and/or real-time RT-qPCR (Howell and Verwoerd, 1971; Erasmus, 1975; Gumm and Newman, 1982; Pearson *et al.,* 1991; Clavijo *et al,* 2000; Orru *et al.,* 2004, 2006).

Serotype-specific tests are able to distinguish between different virus serotypes, and include RT-PCR (including real-time RT-qPCR) and serum neutralisation tests (SNT). These assays target the outer core proteins (VP2 and VP5) of the BTV genome, the determinants of virus serotype, in order to differentiate between BTV serotypes (Maan *et al.,* 2007; Mertens *et al.,* 2007).

1.6.1 Virus isolation

Traditionally, BTV was isolated from field samples, using embryonated chicken eggs that had been inoculated either by yolk sac or via the intravenous route (Mason *et al*., 1940; Goldsmit and Barzilai, 1985). Other methods such as sheep inoculation and cerebral inoculation of suckling mice have also been used (Afshar, 1994). In some cases it may also be possible to do direct isolation in mammalian cells [Baby hamster kidney-21 cells (BHK-21) or African green monkey cells (Vero)] or insect cells [*Aedes albopictus* (C6/36) or *C. sonorensis* cells (KC cells)] (Wechsler and McHolland, 1988; Mecham, 2006).

Cell culture contain the advantage in that virus isolation (VI) from samples, e.g. blood and spleen, surpasses the need for experimental animals (Homan *et al*., 1990; Mo *et*

al., 1994; Prasad *et al*., 1994; Sharifah *et al*., 1995). The development of PCR assays has reduced the need for virus isolation although VI is still widely used to distinguish infectious virus from non-infectious virus. Virus isolation is still of use as it provides large amounts of viral RNA needed for phylogenetic studies and is still a crucial part in the determination of virus serotype using VN.

1.6.2 Antibody/antigen detection

The indirect ELISA is able to detect BTV serogroup-specific antibodies although this assay lacks specificity due to the use of semi-purified antigens (Hubschle *et al.,* 1981). To solve this problem, a blocking ELISA (b-ELISA) and competitive ELISA (c-ELISA) were designed (Anderson, 1984; Afshar *et al.,* 1987). The blocking ELISA tests for the presence of BTV antibodies in test serum based on the reaction of the test serum with an immobilized BTV antigen. This is followed by the addition of BTV group-specific murine monoclonal antibody (MAb). If anti-BTV antibodies are present in the test serum binding of BTV specific antibodies blocks the antigen, thus preventing a reaction with the MAbs (Anderson, 1984). These tests are highly sensitive and detect antibodies to 22 BTV serotypes that include all of the BTV serotypes circulating in Europe (Anderson *et al.,* 1984; Mertens *et al.,* 2009). The c-ELISA is based on the same principle except the test serum and MAbs react simultaneously to compete for immobilized BTV antigens (Afshar *et al.,* 1987). The c-ELISA detects antibodies against the major serogroup specific antigen VP7, and therefore minimize cross reactions between other orbiviruses (Afshar *et al.,* 1987). The test is therefore also the prescribed test for detection of BTV-specific antibodies by the OIE (OIE, 2008). Since the development of b-ELISAs and c-ELISAs, novel reagents, including a greater number of group-specific BTV MAbs as well as genetically engineered BTV antigens have been described (Lunt *et al.,* 1988; Eaton *et al.,* 1990; House *et al*., 1990). Serogroup-specific indirect ELISA 's that use recombinant non-structural NS3 protein capable of detecting antibodies to BTV-NS3 as well as the non-structural protein NS1, are used as a diagnostic test to differentiate between vaccinated and BTV infected animals (DIVA) (Anderson *et al*., 1993; Barros *et al.,* 2009).

The virus neutralisation test (VNTs) is used to detect antibodies against specific BTV serotypes. This assay is dependent on the antigenic specificity of segment 2 (VP2) and to a lesser extent segment 6 (VP5). This test can differentiate between BTV serotypes but is not suitable for routine high-throughput diagnosis as it is time consuming and infection with multiple serotypes would complicate serotype identification (Mertens *et al*., 2009).

1.6.3 Molecular assays

Molecular assays are based on the detection of viral RNA but are not able to distinguish between infectious and non-infectious virus. Molecular methods include methods such as polyacrylamide and/or agarose gel electrophoresis (PAGE and AGE) (Laemmli, 1970) as well as legacy reverse transcriptase-polymerase chain reactions (RT-PCR) (Wade-Evans *et al.,* 1990; Afshar *et al*., 1994, Tabachnick *et al*., 1996, Bonneau *et al*., 2000; Wilson *et al.,* 2000; Mertens *et al.,* 2007) and real-time quantitative RT-PCR (RT-qPCR) tests (Orru *et al*., 2004; Vandenbussche *et al.,* 2009).

Serogroup-specific RT-PCR assays target segment 1, 5 or 7 of the viral genome (Wade-Evans *et al.,* 1990; Pearson *et al*., 1992; Afshar *et al*., 1994, Tabachnick *et al*., 1996, Bonneau *et al*., 2000; Wilson *et al.,* 2000; Mertens *et al.,* 2007), whereas serotype-specific RT-PCRs target segment 2 and to a lesser extent segment 6. RT-PCRs allow for faster serotype identification compared to VNT although more serotype-specific primers are needed to identify serotypes faster and with greater accuracy and sensitivity (Mertens *et al*., 2009). Sequence analysis of segment 2 and 6 from different genome regions can also help to determine and compare BTV serotypes and to identify novel strains or BTV topotypes by means of phylogenetics (Maan *et al*., 2010, 2011). Phylogenetic analysis based on the nucleotide sequences of the BTV genome have been widely used to identify the geographical origins of different BTV strains (Van Niekerk *et al*., 2003; Balasuriya *et al*., 2008; Maan *et al*., 2008). Full as well as partial sequence data is available on GenBank for all 26 BTV serotypes (Maan *et al.*, 2012a; 2012b).

Numerous serogroup-specific real-time RT-qPCR assays have been designed for the detection of BTV. Such assays target BTV genome segment 1 (VP1) (Shaw *et al.*, 2007; Toussaint *et al.,* 2007), segment 5 (NS1) (Jimenez-Clavero *et al.,* 2006; Toussaint *et al.,* 2007) segment 9 (VP6) and/or segment 10 (NS3) (Orru *et al.,* 2006; Van Rijn *et al.,* 2012). Duplex assays targeting segment 1 from eastern and western BTV topotypes have also been developed and is used by reference laboratories to detect BTV RNA irrespective of serotype (Shaw *et al.*, 2007). Real-time RT-qPCR assays that can distinguish between wild-type and vaccine strains have been described. These assays target nucleotide polymorphisms in segments 5 and 10 (Orru *et al*., 2004; Elia *et al.,* 2008). A serotype-specific real-time RT-qPCR assay targeting genome segment 2 (VP2) of BTV-1, -6, 8 and -11 have been described. This assay, in combination with a serogroup-specific pan-BTV/seg5, is able to detect and differentiate BTV serotypes circulating in central and western Europe (Vandenbussche *et al*., 2009). Real-time RT-qPCRs are also used as a screening tool to detect orbivirus RNA in *Culicoides* vectors (Carpenter *et al*., 2008; Veronesi *et al*., 2008; Vanbinst *et al*., 2009; Scheffer *et al.,* 2011). With RT-qPCRs the amount of viral RNA in an insect can be determined, although RT-qPCR positive results only demonstrate previous BTV exposure, not infection. The better understanding of the relationships between quantification cycle (Cq) values of RT-qPCRs, virus titer and transmissibility of infection could replace virus isolation as means of diagnostic tool. Until then, diagnostic PCRs together with virus isolation are being used as screening techniques and in surveillance programmes in the detection of BTV.

1.7 Bluetongue epidemiology

Bluetongue virus was confined to Africa until the early 1940s. The first BTV outbreak outside of the African continent was recorded in 1943 in sheep in Cyprus (Gambles, 1949; MacLachlan, 2004). This was followed by outbreaks in Israel in 1949 (Komarov and Goldsmith, 1951). During 1956-1957 BTV was recorded in Portugal and Spain. Subsequently, BTV-10 spread to the Middle East, Asia and other southern European countries (Gibbs and Greiner, 1994; Mellor and Wittmann, 2002; MacLachlan, 2004). The spatial and temporal distribution of BTV is determined by

the availability of susceptible ruminants, vector-competent midge species, genetics of the virus and suitable climatic conditions (Mellor and Wittmann, 2002).

1.7.1 Host range

The majority of BTV-infected ruminants develop mild or no clinical signs, especially ruminant breeds that are native to BTV-endemic areas (Gibbs and Greiner, 1994; MacLachlan and Osburn, 2006). The virus shows similar pathogenicity in ruminants although marked differences in the severity of disease is evident between different ruminant species and breeds. Improved European sheep breeds are especially susceptible to the more severe clinical manifestations of BT disease (MacLachlan, 1994; Barratt-Boyes and MacLachlan, 1995; Verwoerd and Erasmus, 2004; Darpel *et al.,* 2009). The BTV-8 outbreak during 2006/2007 in Europe was atypical as cattle and goats, which are usually sub-clinically infected, demonstrated clinical signs (Dercksen *et al.,* 2007; Dal Pozzo *et al.,* 2009).

BTV infection in cattle was first diagnosed in 1933 and was named pseudo-foot-and-mouth disease, based on the similarities in clinical signs with foot-and-mouth disease (Bekker *et al.,* 1934). Cattle can play a major role in the epidemiology of BT. This is mainly due to the prolonged viraemia of 50 to 60 days, that is typically accompanied by a lack of clinical signs (Thiry *et al.,* 2006; Darpel *et al.,* 2007; Elbers *et al.,* 2008). Clinical signs that have occasionally been seen in cattle in endemic regions include anorexia, nasal discharges, decreased milk production, lethargy and oedema in the distal limbs (Thiry *et al.,* 2006; Guyot *et al.,* 2008). Lesions of the muzzle, characterised by ulcers and necrotic lesions, have also been recorded (Anderson, 1984). Less prominent symptoms are found in goats. Most goats present with a high fever of up to 42°C and a decrease in milk production. Goats may also show oedema of the lips and head area, as well as crusting on the lips and nose and nasal discharge (Dercksen *et al.*, 2007).

Bluetongue virus infection, under natural conditions, has also been confirmed with serological tests in American white-tailed deer (*Odocoileus virginianus*) (Parsonson, 1990), African antelope and elephants (Rivera *et al.,* 1987; Henrich *et al.,* 2007; Meyer *et al.,* 2009), and various other wild ruminant species such as mouflon (*Ovis*

orientalis musimon) (Fernández-Pacheco *et al.,* 2008) and red deer (*Cervus elaphus*) (Linden *et al.,* 2008). Even though BTV infection is mainly associated with ruminant and camelid species, infection of domestic dogs (*Canis lupus famliaris*) with the virus has been reported after inoculation with BTV-infected canine vaccines (Wilbur *et al.,* 1994; Evermann, 2008). Infectious virus has also been isolated from the lungs of Eurasian lynx (*Lynx lynx*) that had ingested BTV-8-infected foetuses in Europe (Jauniaux *et al.,* 2008). In Africa, BTV-specific antibodies have been detected in lions (*Panthera leo*), cheetahs (*Acinonyx jubatus)*, wild dogs (*Lycaon pictus*), hyenas (*Crocuta crocuta*), genets (*Genetta maculate*) and several jackal species (*Canis* spp.) (Alexander *et al.,* 1994).

1.7.2 Bluetongue virus in Europe

Before 1998, the distribution of BTV in southern Europe was sporadic, short-lived and was typically associated with only one serotype (Mellor and Boorman, 1995; Mellor *et al.*, 2000; Mellor and Wittmann, 2002). Climate change might have been a contributing factor to the expansion of BTV into Mediterranean countries never before affected (Hammami, 2004) by extending the distribution of *Culicoides* vectors. The virus first spread to the Greek islands in 1998 after which BTV was reported around the Mediterranean Basin, in countries including Bulgaria, Tunisia and Turkey (Zientara *et al.,* 2000). The route of entry of the different serotypes into southern Europe is believed to be of different origins (Fig. 1.4). Bluetongue virus serotype 2 was introduced from the south where it occurred in Tunisia in 1999 and subsequently spread to the Italian island of Sardinia in 2000 and from there to Balearic Islands of Spain and to Corsica (Mellor and Wittmann, 2002). Serotypes 4, -9 and -16 were introduced from the east, probably Greece, into Italy, France and Spain (Mellor and Wittmann, 2002; OIE, 2003). The expansion of BTV into naïve ruminant populations was the cause of huge economic losses in southern Europe. In 2006 to 2008, Europe experienced yet another BTV outbreak when a strain of BTV-8 was introduced through an unknown route into north-western Europe and spread by Palearctic midge species, *e.g. Culicoides obsoletus, Culicoides scoticus and Culicoides pulicaris* (Mellor and Pitzolis, 1979; Meiswinkel *et al*., 2008). The virus persisted throughout the 2006-2007 winter period and re-emerged in May and June

2007. This outbreak affected over 55 000 holdings in 2007, resulting in economic losses of hundreds of millions of Euros (Maan *et al*., 2008; Velthuis *et al*., 2010). The outbreak was unique since BTV-8 caused clinical disease, not only in sheep, but also in cattle and goats (Thiry *et al.,* 2006; Dercksen *et al*., 2007; Dal Pozzo *et al*., 2009) and was able to cross the placenta (Desmecht *et al*., 2008; Vercauteren *et al*., 2008; Wouda *et al.,* 2008).

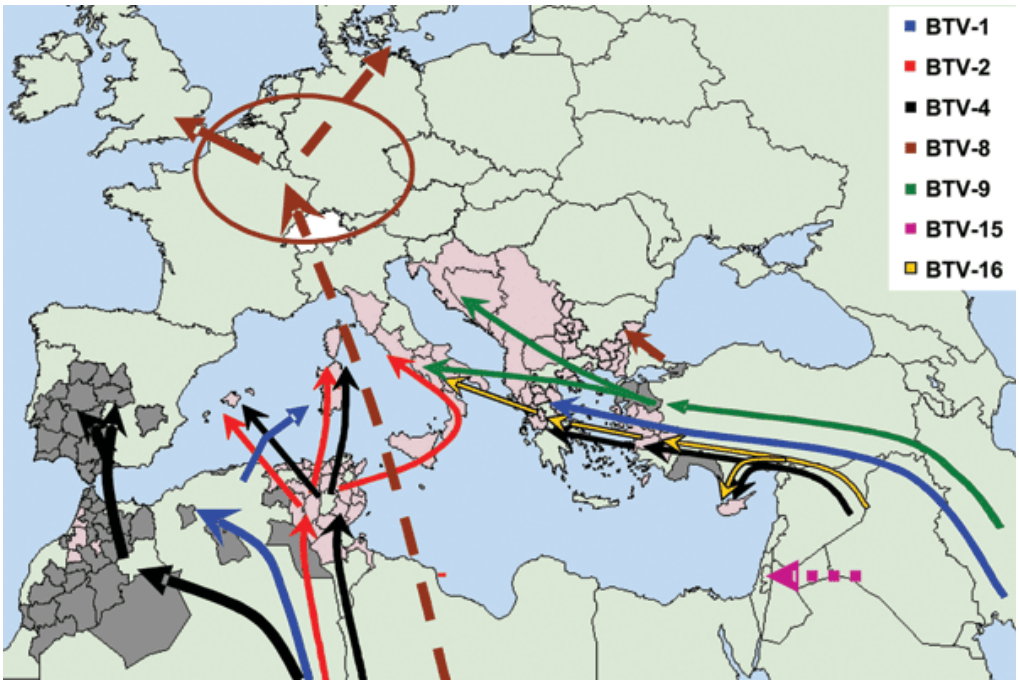

Figure 1.4: Route of introduction and spread of different BTV serotypes in Europe since 1998. Source: Saegerman *et al*., 2008.

1.7.3 Bluetongue virus in Australia

BTV was first recognised in Australia in 1977 (Gibbs and Greiner, 1994) and was identified as BTV-20. Virus neutralization tests indicated that this serotype was not distributed throughout the country, therefore, it was believed that there was more than one serotype circulating in Australia (Delle-Porta *et al*., 1983). Virus neutralization tests later confirmed the presence of BTV-1 and -21 (St George *et al.,* 1980). Over a period of four years another five serotypes were isolated (BTV-3, -9, -

15, -16 and -23) (Gard *et al.,* 1985, 1987, 1988). Genetic analysis of the isolates obtained since 1992 to 2001 showed incursions of the virus from South-East Asia (Daniels *et al*., 1995; Melville *et al*., 1997; Melville, 2004; Pritchard *et al*., 2004).

1.7.4 Bluetongue virus in Asia

In China, BTV has been recognised since 1979 when an outbreak occurred amongst cattle in the subtropical south-west region (Zhang and Kirkland, 1998). The serotypes associated with this outbreak were identified as BTV-3, -4 and -5. An additional five serotypes were later isolated from sentinel cattle (BTV-1, -2, -12, -15 and -16). Serotypes 1 and 16 have also previously been isolated from sheep with clinical disease (Zhang *et al.,* 2004). Phylogenetic analyses revealed Chinese strains form monophyletic groups distinct from USA strains and are more closely related to Australian strains (Zhang *et al.,* 2004). BTV serotypes isolated in South-East Asia are BTV- 1, -2, -3, -7, -9, -12, -16, -21 and -23. Molecular analysis of these isolates confirmed grouping with Australasian topotypes as well as isolates from India (Sendow *et al*., 1997; Pritchard *et al*., 2004).

1.7.5 Bluetongue virus in the New World

Bluetongue virus was first reported in Texas in the USA in 1948 and again in California in 1952. In both of these outbreaks, viruses were isolated from sheep (Hardy and Price, 1952). Since then, BTV-10 and -11 (1955), -13 (1967) -17 (1962) and -2 (1983) have been isolated from different regions in the USA (Johnson *et al*., 2007; Mertens *et al.,* 2007). The primary vector, *C. Sonorensis,* along with serotypes BTV-10, -11, -13 and -17, forms an episystem in North America that consists of a distinct grouping of viruses with a unique vector found on no other continent (Tabachnick, 2004). Bluetongue virus serotype 2 is restricted to the southern parts of the USA and is primarily associated with *C. insignis.* By 2005, six more BTV serotypes (BTV- 3, -5, -6, -14, -19 and -22) were identified (Johnson *et al.,* 2007; Mertens *et al.,* 2007), although these serotypes are considered to be exotic. BTV was also isolated from cattle, with mild clinical signs, in Canada (Dulac *et al*., 1992). It should, however, be emphasized that Canada, along with the north-eastern and New England states of the USA, are considered as being BT-free due to the poor

vector competence of *C. variipennis* populations found in these areas (Thompson *et al*., 1992).

The first isolation of BTV in South America was made in Brazil in 1978 from Zebu cattle with the virus being identified as BTV-4 (Groocock and Campbell, 1982). Today, seven BTV serotypes are present across South America namely, BTV-4, -6, -12, -14, -17, -19 and -20. The sparse serological and virological data of BTV and the lack of information on available vectors involved in virus transmission have hindered definition of the virus-vector situation in South America.

1.7.6 Bluetongue virus in South Africa

Bluetongue virus was first described in the late eighteenth century by Francois de Vaillant during his travels in the Cape of Good Hope (Gutsche, 1979). Currently 21 of the 26 known serotypes are known to be circulating in SA. These include BTV-1 to 15, -18, -19, -22 and -24, with BTV-17, -20, -21, 23, -25 and -26 regarded as exotic (Dungu *et al*., 2004; Verwoerd and Erasmus, 2004). Although considered exotic, serotype 17 has been isolated in 1985, 1986 and 2000 (Gerdes, 2004). Previous data suggested that BTV-23 is circulating in South Africa, however, no evidence of this serotype since 1983 has been recorded therefore altering it as exotic (Gerdes, 2004). BTV serotypes in South Africa are randomly distributed with no definite pattern with regards to livestock (Gerdes, 2004). During 1996, South Africa had exceptionally high rainfall which resulted in BTV outbreaks in both sheep and cattle in various parts of the country (Barnard *et al.,* 1997). The serotypes responsible for disease in cattle were identified as BTV- 2, -3, -6 and -8 with serotypes responsible for disease in sheep being BTV-1, -2, -3, -4, -6, -8, -9 and -12 (Barnard *et al.,* 1997). BTV outbreaks in SA vary from year to year with 21 reported outbreaks to almost 100 reported outbreaks from 1998 to 2000. BTV is sometimes isolated from asymptomatic animals, especially during routine testing as part of export programmes. Routine BTV screening of sheep and goat embryo donor females, all tested negative between 1997 and 2002 except in 1999 when 12 isolates from asymptomatic animals were identified (Gerdes, 2004). The epidemiology of BTV, especially with regards to cattle and wildlife, in South Africa is not clear. It was hypothesised that BTV was historically a disease of African antelope, and that the

role of African antelope in BT disease epidemiology was supplanted by domestic ruminants due to large-scale agricultural developments (Mellor *et al.,* 2009). Cattle later replaced wild ruminants as the primary epizootiological host in the BTV cycle in the country. Cattle which demonstrate prolonged viraemia are suitable amplifying hosts for BTV. The vast number of cattle and the wide variety of vector competent midge species could possibly help in determining seasonal prevalence, distribution and overwintering of BTV in South Africa.

1.8 References

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Chapter 2: The epidemiology of bluetongue in the Mnisi community, Mpumalanga Province South Africa: *Culicoides* vectors and cattle as hosts

Abstract

The role of cattle in the epidemiology of BT in South Africa, as well as the distribution of different vector species throughout the country is not well understood. Mnisi, a rural area located in the north-eastern corner of Bushbuckridge, South Africa, was selected for an epidemiological study. The area represents a wildlife-livestock interface. The prevalence of *Culicoides* species associated with this area, as well as whether BTV is circulating in the area has not been reported before and was determined. Light traps were placed at four different sites in the Mnisi area during the autumn and winter periods. Midges were identified to species level and pooled (200 midges/pool). A total of 82 and 79 midge pools from autumn and winter sampling periods, respectively, were tested for the presence of BTV RNA with real-time RT-qPCR. Additionally, sera were randomly collected from 1 260 cattle and screened with a BTV-specific cELISA. Blood samples from 45 seronegative cattle were also screened for BTV RNA. Twenty-five different *Culicoides* species were identified, of which *C. imicola* was found to be the most abundant. *Culicoides enderleini* and *C. bolitinos* were the second most abundant species during autumn and winter sampling, respectively. Of the 25 species collected, 19 species yielded parous females with 16 *Culicoides* species demonstrating a vector rating higher than 25%, indicating that the species are both abundant and wide spread in the area. Real-time RT-qPCR detected BTV RNA in 51.2% and 75.9% of midges sampled in autumn and winter, respectively, with an infection prevalence of 0.3% and 0.7%. Bluetongue virus-specific antibodies were detected in 1 206 (95.7%) of the serum samples tested demonstrating that infection is highly prevalent, with a statistical significant difference ($p < 0.05$) between cattle age groups and villages sampled. There were no significant differences in seroprevalence between breeds or sex. A total of 35.5% seronegative cattle tested, positive for the presence of BTV RNA after the blood was screened with real-time RT-qPCR. These results demonstrate that BTV, as well as different vectors of the virus are circulating in the Mnisi area. The circulation of the virus between

cattle and different midge species will assist in the understanding of the epidemiology of the disease especially at the wildlife-livestock interface.

Keywords: Bluetongue virus, *Culicoides,* Cattle*,* cELISA, Epidemiology, Real-time RT-qPCR

2 .1 Introduction

As discussed earlier the distribution of BTV is determined by the occurrence of vector-competent midge species, climatic conditions that support the large population of vectors, as well as susceptible hosts. In South Africa, BT is most commonly detected in late summer and autumn (February to April). The occurrence of multiple BTV serotypes circulating at the same time during vector seasons have been demonstrated, although the occurrence of serotypes are unpredictable and probably determined by herd immunity (Nevill *et al*., 1992a). Most outbreaks occur in areas with high rainfall, even though serosurveys provided evidence of BTV in all vegetational zones, with different rainfall parameters, throughout the country (Barnard*,* 1997). In cooler high lying areas such as the southern Free State, BT usually disappears with the first winter frost, while the disease is likely to occur year-round in warmer regions such as the KwaZulu-Natal and Limpopo Provinces (Erasmus, 1980; Verwoerd and Erasmus, 2004).

The geographical distribution and relative abundance of *Culicoides* midges in South Africa varies between regions. In South Africa, *C. imicola* is the major vector of BTV, followed by *C. bolitinos* (Nevill *et al.,* 1992a, b; Venter *et al.,* 1996; Meiswinkel *et al.,* 2004). *Culicoides bolitinos* has, however, been shown to be significantly more susceptible to oral infection with BTV than *C. imicola* (Venter *et al.,* 1998, 2006; Paweska *et al.,* 2002*).* Following feeding on a BTV-infected blood meal, virus has been isolated under laboratory conditions from *C. imicola, C. bolitinos, C. milnei, C. leucostictus, C. pycnostictus, C. expectator, C. gulbenkiani, C. zuluensis, C. magnus, C. enderleni, C. bedfordi and C. huambensis.* In addition, BTV has been isolated from six field-caught *Culicoides* species namely, *C. imicola, C. bolitinos, C.*

milnei, C. leucostictus C. pycnostictus and C. expectator (Paweska *et al.,* 2002; Venter *et al.,* 2010).

The susceptibility of cattle to BTV was already first demonstrated in 1905 (Spreull, 1905) when calves were experimentally infected with BTV. Since then, BTV has been isolated from single cases of cattle suffering from clinical signs similar to BT as well as from subclinically infected cattle throughout SA (B.J.H. Barnard, unpublished data, 1984, cited by Barnard *et al*., 1998). Viraemia in ruminants are highly cell-associated, especially to erythrocytes at late stage of infection. The prolonged viraemia in cattle could be due to the adsorption of BTV to blood cells and not because of prolonged replication of the virus in infected tissue (MacLachlan *et al.,* 1990). It is believed that this prolonged viraemia in cattle does not reflect a persistent viral infection, but rather that blood cells delay the elimination of the virus by the immune system (MacLachlan *et al.,* 1990). Prolonged viraemia within the cattle may allow for an extended transmission of the virus to the vector.

Mnisi is a rural area located in Mpumalanga Province of South Africa. With its subtropical climate and large numbers of livestock, this area makes an ideal environment for the maintenance of arboviruses. Subclinically infected cattle could potentially be important reservoirs of BTV and aid in the amplification, spread and maintenance of the virus in this region of the country (Barratt-Boyes and MacLachlan, 1995). Studies on BTV in cattle are not well reported and the role of cattle in the epidemiology of the disease in South Africa is not clear. The recent outbreaks in Europe (2006 – 2008) that affected mainly cattle initiated studies to specifically look at the role of cattle in the epidemiology of BT in South Africa.

Aim:

The objective of the study was to determine the prevalence of different *Culicoides* species associated with livestock in the Mnisi area, in the Mpumalanga Province of South Africa, and to establish the presence of BTV RNA in field-collected midges. In addition, serological tests, i.e. cELISA, were used to screen cattle serum samples that were collected from dip tanks in the area for BTV group-specific antibodies in order to evaluate the sero-prevalence of the virus. Blood samples from cattle with a serum percentage negativity of less than 50% were screened for the presence of BTV RNA.

Objectives:

- To determine *Culicoides* midge abundance and species prevalence in the Mnisi area of the Mpumalanga Province, South Africa.
- To use real-time RT-qPCR to detect BTV RNA in field-collected midges and to determine whether there are any seasonal differences in viral RNA detection.
- To use a BTV group-specific cELISA to determine whether there are BTV-specific antibodies in randomly selected cattle serum samples, collected from Mnisi, and
- To screen blood samples for BTV RNA with real-time RT-qPCR.

2.2 Materials and Methods

2.2.1 Study area

Mnisi is a rural area situated in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa (Fig. 2.1). The study area comprises a savannah ecosystem and a semi-arid region with regards to water and grazing availability. The region has a sub-tropical climate and a summer rainfall pattern. Crops and vegetables are produced mostly for home consumption with livestock as their main agricultural activity. Livestock in Mnisi consists of indigenous

breeds of cattle, goats and donkeys. Over two thirds of the study area's land is on the interface with provincial and private game reserves. Mnisi is surrounded by Andover- and Manyeleti provincial game reserves, and Sabi Sand, a private game reserve (Fig. 2.1).

To accurately predict the abundance of *Culicoides* midges in the Mnisi area, villages were chosen according to the following criteria (Table 2.1, Fig. 2.1):

- Village location
- Availability of 220 V power source in sampling proximity
- Consent from farmer/owner

Serum samples and blood in EDTA were collected from cattle at 11 sites in or near the following villages: Clare (A and B), Dixie, Gottenburg, Hlalakahle, Seville (A and B), Tlhavekisa, Utha A, Welverdiend (A and B). Hlalakahle and Welverdiend B correspond with the midge collection sites (Fig. 2.1). Collections were made from 8 April 2013 to 9 September 2013.

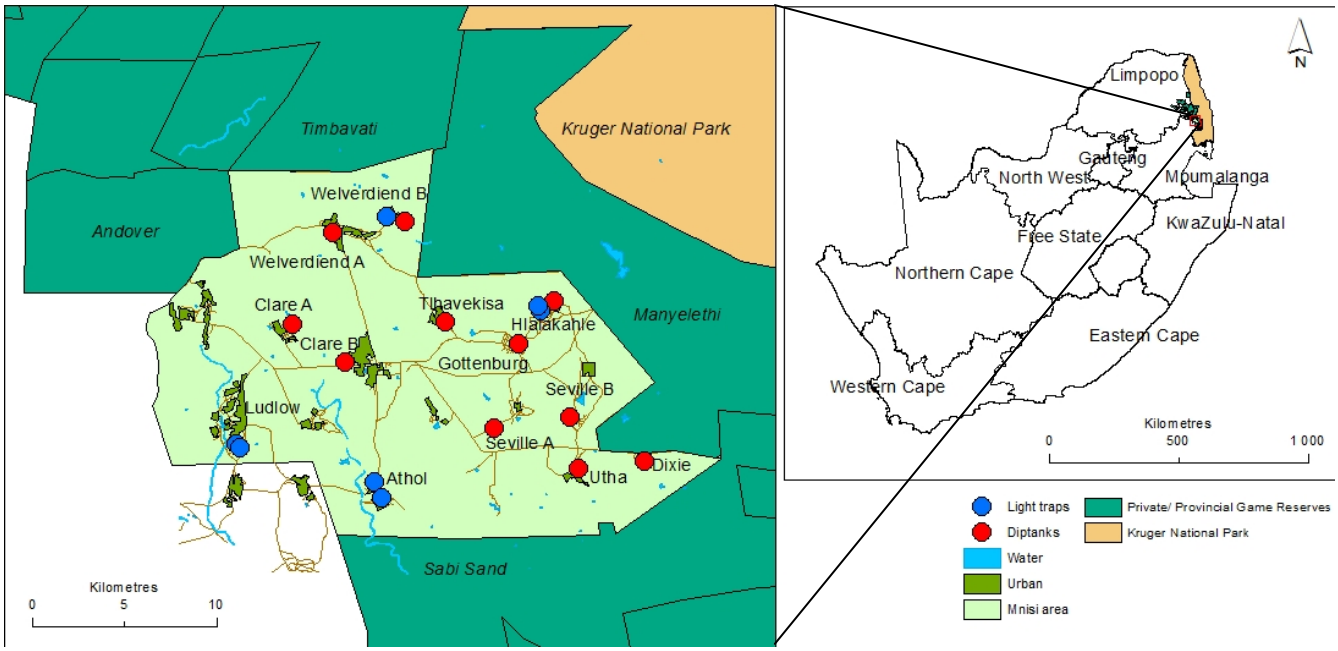

Figure 2.1: Enlarged map of Mnisi, in the Mpumalanga Province, indicating sampling sites as well as the position of Mnisi within South Africa surrounded by Kruger National Park (green) and private/provincial game reserves (light green). Red dots indicate villages/dip tanks were serum samples from cattle were collected, whereas blue dots indicate villages from which midge samples were collected. Map also indicates the nine Provinces of South Africa Source: Drawn by J. Steyn on ArcGIS 10.2.

Table 2.1: Grid references of the sites in the Minis area where 220V light traps were used to collect *Culicoides* midges. Village names and livestock associated with the site are also indicated.

Site	Grid reference	Height above sea level (m)	Village name	Animals in vicinity of light trap
1	S 24°70586', E 031°34587'	424	Athol	Cattle and chickens
2	S 24°71386', E 031°34947'	419	Athol	Cattle
3	S 24°57623', E 031°35163'	459	Welverdiend B	Cattle
4	S 24°57635', E 031°35109'	467	Welverdiend B	Cattle
5	S 24°68782', E 031°27770'	473	Ludlow	Cattle and goats
6	S 24°68932', E 031°27997'	481	Ludlow	Cattle
7	S 24°62205', E 031°42685'	431	Hlalakahle	Cattle and donkeys
8	S 24°62001', E 031°42599'	431	Hlalakahle	Cattle

2.2.2 *Culicoides* midges

2.2.2.1 Collection of *Culicoides* midges

Sampling of *Culicoides* midges were conducted once every four nights, starting on 23 April to 3 May 2013 (autumn) and again from 17 June to 15 July 2013 (winter). Two 220 V Onderstepoort light traps equipped with 8 W black light tubes (Fig. 2.2) were used to collect midges into 500 ml plastic beakers filled with 0.5% Savlon® (Johnson & Johnson) (Clorhexidinegluconate 0.3 g/100 ml and Cetrimide 3.0 g/100 ml) antiseptic. Since adult *Culicoides* are usually crepuscular species, i.e. flight activity peaks around dusk and/or dawn, the light traps were operated from one hour before sunset to one hour after sunrise. Traps were placed near 10 to 30 cattle, depending on the site. Mosquito netting placed around the trap excluded large insects from the collection beaker. The beakers were collected every morning after a trapping night. *Culicoides* midges were then separated from other insects. Large collections were sub-sampled (Van Ark and Meiswinkel, 1992). Sub-sampled

collections were subsequently identified to species level. This was accomplished by comparing insect morphology to slide reference collections, morphological keys and a wing picture atlas of Afrotropical *Culicoides* (R. Meiswinkel, OVI, unpublished data 1994.) Males and females were separated, and females age-graded into freshly blood-fed, parous, nulliparous or gravid, based on abdominal pigmentation and morphological differences (Dyce, 1969).

2.2.2.2 *Culicoides* processing

Midges were divided into pools of 200 individuals. Midge pools were washed in 500 µl PBS (+) solution containing 1 ml Gentamycin (50 mg/ml) (Virbac) and 0.125 ml Fungizone (10 mg/ml) (Bristol-Myers Squibb) per 1000 ml stock solution (v/v) by centrifuging the midge suspension for 30 seconds at 1 300 g. Midges were subsequently macerated in 1000 µl reconstituted Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) LTD) (without serum) containing a sterile glass bead. Following maceration, insect debris was collected by centrifuging at 1 300 g for 1 minute and the supernatant aliquoted into three 0.5 ml microcentrifuge tubes. One aliquot was used to conduct real-time RT-qPCR, the second aliquot was sent to the Equine Research Centre, Faculty of Veterinary Science (FVS), UP, and the third stored at -80°C as a back-up. Midge suspensions were tested for BTV in a DAFF-accredited Virology Laboratory of the Department of Veterinary Tropical Diseases (DVTD), FVS, UP, using real-time RT-qPCR (van Rijn *et al*, 2012). Meteorological data for the four months (April, May, June and July) were obtained from the Southern African Wildlife College (personal communication).

Figure 2.2: A 220V Onderstepoort light trap used for the collection of *Culicoides* species in Mnisi, Mpumalanga Province. © Jumari Steyn

2.2.2.3 Distribution and relative abundance of *Culicoides*

All the midges collected during April/May from Sites 1, 2, 3, 4, 7 and 8 were characterized (Table 2.1). Due to time constraints and the large number of midges collected at Sites 5 and 6, only subsamples were characterized (1.4% and 50%, respectively). All collections made during June/July were subsampled. The data was adjusted for the difference as follow:

Approximately 200 individuals were identified per site with only one replicate per site from samples collected during winter.

- Calculation: Subsample per species / (Subsample/Total) = Relative abundance across all sites

From the samples collected during autumn, Sites 1, 2, 3, 4, 7 and 8 were readjusted to one replicate per site, while subsamples from Site 5 and Site 6 were readjusted from 1.4% and 50%, respectively to 100%

- Calculation: (Abundance per species on Sites 1, 2, 3, 4, 7, 8) / 2 = Relative abundance across all sites
- Site 6: Subsample abundance per species * 2 = Relative abundance across all sites
- Site 5: Subsample per species / (1.4%) = Relative abundance across all sites

Species diversity and evenness of both autumn and winter collections were calculated by the Shannon-Weiner index using a diversity calculator (http://lbsite.zxq.net/programs/diversity.html). The Shannon-Weiner index describes the evenness in distribution of species abundances taking sample size into account.

To compensate for localised abundance, vector rating was calculated by averaging relative abundance and percentage of positive sites for all collections for a specific species (Venter *et al.,* 1996). A vector rating >25% was used to indicate that a species was both abundant and wide spread in the area (Venter *et al.,* 1996).

The percentages of parous individuals of specific species in the population were also determined by dividing the number of parous females of a specific species by the total number of individuals of that species collected. This was done separately for autumn and winter collections.

The Wilcoxon rank-sum test was used to compare the mean of midges collected between autumn and winter and a cut-off with a 5% significance level ($p < 0.05$) was used. The same test was also used to determine any significant difference in the mean of parous females collected by sites between sampling periods.

2.2.3 Bluetongue virus detection

2.2.3.1 Bluetongue virus RNA extraction

Two different commercial kit methods were used to extract RNA from *Culicoides* midges:

2.2.3.1.a TRIzol® Reagent (Ambion®) extraction from *Culicoides* midges (winter)

Total RNA was extracted from midge pools collected between 17 June and 15 July 2013 using TRIzol® Reagent (Ambion®). In short, 1000 µl of MEM containing homogenised midges were transferred to a 1.5 ml Eppendorf tube (Merck (Pty) LTD)) and centrifuged at 3 500 g for 5 minutes to sediment cell debris and to form a

pellet. The pellet was dissolved by adding 1 ml of TRIzol® Reagent and shaking vigorously. Chloroform was added to make up a 1.2 ml solution. Tubes were then incubated at room temperature for 2 minutes and centrifuged at 5 600 g for 15 minutes at 4°C. The aqueous phase was transferred to a new Eppendorf tube and 900 µl of isopropanol was added. The tubes were incubated at -20°C for 2 hours and centrifuged at 8 000 g for 10 minutes. The supernatant was removed and 1 ml 70% ethanol was added. Tubes were again centrifuged at 3 500 g for 5 minutes to re-pellet. The pellet was dried after removing the supernatant and re-suspended in 50 µl of TE buffer.

2.2.3.1.b MagMAX™ Express (Ambion®) from *Culicoides* midges (autumn)

Total RNA was extracted from samples collected between 23 April to 3 May 2013 using an Express Magnetic Particle Processor, the MagMAX™ Express (Ambion®), using the MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems part number AM1830). This kit is designed for rapid, high-throughput purification of RNA and combines an effective sample disruption method with magnetic bead-based nucleic acid purification technology. Extractions were performed according to the manufacturer's instructions with minor changes. In brief, 50 µl of sample homogenate was added to the Bead Mix in the first rows of the 96-well processing plate wells, along with 65 µl of lysis solution and 65 µl isopropanol. In the second and third rows, 150 µl of wash solution 1 was added while wash solution 2 was added to rows four and five. The final row was filled with 50 µl of Elution buffer. The extraction program was according to the manufacturer's instruction. Briefly, lysis and binding was conducted for 5 minutes, followed by washing with each wash solution for 30 seconds. The RNA was then dried and dissolved in Elution buffer.

2.2.3.1.c TRIzol® Reagent (Ambion®) extraction from blood samples

A total of 45 blood samples that presented a serum percentage negativity of less than 50% on the cELISA were collected from cattle at seven villages, i.e Hlalakahle, Welverdiend B, Clare B, Tlhavekisa, Gottenburg, Utha A and Seville A. Collections were made from May to September 2013. The TRIzol® Reagent extraction method was used to extract RNA from whole-blood samples. In short, 360 µl blood together

with 1440 µl TRIzol® Reagent was transferred to a 1.8 ml cryo tube and vortexed. From there, two 900 µl aliquots were made into 1.5 ml Eppendorf tubes. One aliquot was stored at -20°C for back-up, while the other aliquot was used for RNA extraction. Chloroform was added to the second aliquot to make up a total volume of 1.1 ml. Samples were incubated at room temperature for 2 minutes and centrifuged at 5 600 g for 15 minutes at 4°C. The aqueous phase was then transferred to a new Eppendorf tube and 900 µl of isopropanol was added. Samples were again incubated at -20°C for 2 hours and centrifuged at 8 000 g for 10 minutes. The supernatant was removed afterwards and 1 ml 70% ethanol was added. Samples were centrifuged for a last time at 8 000 g for 5 minutes to re-pellet. The pellet was dried and 50 µl of TE buffer was added to re-suspend the pellet.

2.2.3.2 Screening of *Culicoides* midges with Real-time RT- qPCR

Real-time RT-qPCR was performed on a StepOnePlus™ PCR thermocycler (Applied Biosystems) using a published assay targeting segment 10 (encoding protein NS3/A) of the BTV genome (Van Rijn *et al.,* 2012). In order to conduct the assay, TaqMan® Fast Virus Master Mix reagent (Applied Biosystems) was used in conjunction with 200 nM of the forward primer (5'- GTGTCGCTGCCATGCTATC-3') and reverse primer (5'- CGTACGATGCGAATGCA -3'), and 100 nM of hydrolysis probe (5'-FAM-CGAACCTTTGGATCAGCCCGGA-MGB-3'). The forward primer binds from nucleotides 8-27 of the NS3/A-encoding gene, while the reverse primer binds to nucleotides 247-264. The probe binds to nucleotides 461- 483 of the BTV segment 10 gene (EMBL-EBI 2014©).

Double-stranded RNA was denatured by heating for 3 minutes at 95°C and snap cooled by placing it at -20°C for 5 minutes. Five µl was added to the MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems), along with 5 µl of a 4x TaqMan® Fast Virus Master Mix reagent, the primers and probes. The total volume was adjusted to 20 µl with RNAse-free PCR-grade water and covered with MicroAmp® Optical Adhesive Film (Applied Biosystems). The samples were centrifuged briefly and the following cycling program was used: 50°C for 5 minutes, 95°C for 20 seconds and 40 cycles of 95°C for 2 seconds and 60°C for 30 seconds.

In total, 82 autumn midge pools and 79 winter midge pools from the different collection sites were tested. Random positive samples were viewed by gel electrophoresis to confirm primer binding specificity. The same samples were also sent to a commercial service provider (Inqaba Biotechnical Industries (Pty) Ltd) for sequencing to verify the presence of BTV amplicons in the samples.

2.2.3.2.a Field infection prevalence of bluetongue virus

Field infection prevalence in each sampling period was calculated using Chiang and Reeves's (1962) formula.

$$P = 1 - [(n-x)/n]^{1/m} \times 100$$

P = estimate of the infection rate

m = pool size

n = number of pools tested

x = number of positive pools

2.2.3.3 Competitive enzyme-linked immunosorbent assay

Veterinarians and/or animal health technicians were contracted to collect serum samples from cattle from April to September 2013. Microsoft Excel was used to randomly select serum samples routinely collected in the Mnisi area for use in other projects to be tested for BTV-specific antibodies using a competitive enzyme-linked immunosorbent assay (cELISA) (Veterinary Medical Research and Diagnostics, Bluetongue Virus Antibody Test Kit, Inc., Pullman, U.S.A). A total of 1 260 serum samples that included males and females, of four different cattle breeds and different age groups, were selected and tested using a cELISA. The assay is able to detect group-specific antibodies against BTV and was done according to the manufacturer's instructions. Plates were read using a microplate absorbance spectrophometer with the optical density (OD) set at 630 nm. Plates were valid if the mean of the negative control was > 0.3 and < 2.0, and if the mean of the positive control was < 50% of the mean of the negative control. Percentage negativity values were calculated by using

the following formula: [1 – (OD sample/OD negative reference)] x 100 and a cut-off value of > 50% was used to distinguish between positive and negative serum samples.

2.2.3.3.a Statistical analyses of seroprevalence

The Shapiro-Wilk test and a histogram were used to determine the normality of the data where p > 0.05 indicated normal distribution. Non-parametric Kruskal-Wallis one-way analysis of variance test was used to calculate significant difference in antibody percentages between different breeds, sex, age groups and collection site (villages). Significant differences were confirmed or excluded using the Cochran-Mantel-Haenszel (CMH) statistical test to exclude any influence of covariates.

2.2.3.4 Screening of blood samples with Real-time RT-qPCR

Blood in EDTA collected from 45 cattle were screened for BTV RNA with real-time RT-qPCR, as discussed in Section 2.2.3.2.

2.3 Results

2.3.1 Weather conditions

During *Culicoides* collection, the average outside temperatures varied between 17.4°C and 20.8°C with an average rainfall of 0.02 mm during the four months (Table 2.2). In April the average outside temperature was 20.5°C with 0.05 mm rain. In May the average outside temperature was 19.1°C with no rain, during the month. June had an average outside temperature of 18.4°C also with no rain and July had an average outside temperature of 17.7°C with 0.01 mm rain during the month. Throughout the sampling months, the relative humidity was above 50% but lower than 87% with an average wind speed of 2.9 km/h (Table 2.2).

Table 2.2: Weather conditions for Mnisi, Mpumalanga Province during the course of the study period i.e., April, May, June July 2013. Average high and low temperatures, rain fall, humidity and wind speed for all four months are listed. Source: Southern African Wildlife College weather station.

	High temp (°C)	Low temp (°C)	Rain (mm)	Relative humidity (%)	Wind speed (km/h)
April	20.8	20.2	0.05	70.0	3.3
May	19.5	18.8	0.00	65.7	2.3
June	18.8	18.0	0.00	86.3	2.7
July	18.0	17.4	0.01	55.0	3.4

2.3.2 *Culicoides* midges

Species analysis of *Culicoides* midges collected in the Mnisi area revealed 25 different *Culicoides* species (including a number of taxonomically yet undescribed species) (Table 2.3). A total number of 91 659 *Culicoides* midges were collected in 16 light trap collections made between 23 April to 3 May 2013 and 17 June to 15 July 2013 (Table 2.3). During April/May 2013 (autumn) 23 species were identified from 16 collections made at eight sites, while 20 species were identified during June/July (winter) from the same sites (Table 2.3). *Culicoides dekeyseri* and *Culicoides glabripennis* were only collected in winter, while *Culicoides engubandei, Culicoide kanagai, Culicoides milnei,* Culicoides. sp # 50 and Culicoides sp. # 54 p/f were absent (Table 2.3).

Species diversity, as represented by the Shannon-Weiner index, for autumn and winter were H' = 0.5 and H' = 1.2, respectively. Species evenness were calculated to be J' = 0.1 and J' = 0.4 for autumn and winter respectively. A total of 73 321 *Culicoides* midges were collected from 16 collections made at eight sites throughout April/May 2013. The same number of sampling at the same eight sites took place during June/July 2013 where 18 338 midges were collected. The largest number of midges collected in a single trap in one night was 68 928 *Culicoides* midges during April/May, whereas the second largest collection consisted of 12 024 *Culicoides*

individuals collected during June/July. Both of these collections were made at Site 5 (Table 2.3). In both collections the most abundant species was *C. Imicola,* representing 92% and 80% of total midge catches in autumn and winter, respectively. Site 6 yielded the third and fourth highest numbers of *Culicoides* collected, i.e. 1 683 and 2 768 for autumn and winter, respectively. *Culicoides imicola* proved to be the most abundant species, representing 84% (autumn) and 66% (winter) of the total number of *Culicoides* collected at this site. The numbers collected at the rest of the sites varied between autumn and winter (27.5 to 546.50 and 109 to 610, respectively). Further, during winter, Site 6 demonstrated the greatest species diversity (13/20 species), whereas Site 7 had the greatest species diversity in autumn (13/23 species) (Table 2.3). No statistical significant difference ($p = 0.96$) was found in the mean number of *Culicoides* midges collected in autumn and winter by site.

Culicoides imicola was the most abundant species present during both autumn (91%) and winter (75%) sampling (Table 2.3). The second and third most abundant species collected during autumn was *Culicoides nevilli* (4.0%) and *Culicoides enderleini* (1.3%), respectively. During winter collection, *C. bolitinos* (4.4%) was the second most abundant with *C. nevilli* (3.4%) being the third most abundant. *Culicoides imicola* was also most abundant at all sites during both sampling periods, except at Site 3 (winter), where *C. bolitinos* replaced *C. imicola* as the most abundant species (Table 2.3).

Of the 25 *Culicoides* species sampled, 13 species in autumn and 16 species in winter demonstrated a vector rating higher than 25% (Table 2.4). These species are considered as being both abundant and wide-spread. Species associated with both autumn and winter are *C. bolitinos, C. enderleini, Culicoides exspectator, C. imicola, Culicoides leucostictus, Culicoides neavei, C. nevilli, Culicoides pycnostictus, Culicoides simillis,* C. sp. # 54 d/f, *Culicoides subschultzei, Culicoides tropicali and Culicoides tutttifruitti. Culicoides bedfordi, Culicoides zuluensis and Culicoides nivosus* were present in winter only (Table 2.4).

Parous females were present throughout both sampling periods. A total of 19 out of the 25 species yielded parous females with 18 of these 19 species collected in

autumn and 16 species collected in winter (Table 2.5). The percentage of parous females in the population differed between autumn and winter, i.e. 3.4% and 1.8%, respectively. Culicoides. sp. # 50 d/f had the highest number of parous females in winter (29%) and the second highest during autumn (49%), while *Culicoides brucei* yielded the most parous females in autumn (50%). The mean number of parous females collected in autumn (101) was significantly higher ($p > 0.05$) than that collected in winter (13).

Table 2.3: Summary of different *Culicoides* species associated with 16 collections, at the eight collection sites, per season, in Mnisi. Collections per site were pooled. Midges collected in winter and autumn are presented separately. Species absence is indicated by * in June/July (winter) and ** in April/May (autumn).

	Winter sampling (June/July)									Autumn sampling (April/May)								
Site no.	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Total	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Total
Culicoides spp																		
C.bedfordi	1.9	0.0	0.0	1.0	456.6	84.6	2.0	1.0	547.1	0.0	0.5	0.0	0.0	142.9	0.0	1.0	0.0	144.4
C.bolitinos	27.2	17.0	243.4	5.0	456.6	25.4	14.0	11.0	799.5	3.0	6.5	0.5	0.0	285.7	0.0	44.5	31.0	371.2
C.brucei	0.0	0.0	0.0	0.0	76.1	42.3	0.0	0.0	118.4	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0	4.0
C.coarctutus	0.0	0.0	0.0	0.0	76.1	42.3	0.0	0.0	118.4	0.0	0.0	1.0	0.0	0.0	44.0	0.0	0.0	45.0
*C.dekeyseri***	0.0	0.0	0.0	0.0	0.0	8.5	0.0	0.0	8.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.enderleini	3.9	3.4	9.5	1.0	152.2	0.0	2.0	3.0	175.0	7.5	12.5	4.0	0.5	857.1	102.0	23.0	3.5	1010.1
*C.engubendei**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	2.0
C.exspectator	3.9	45.8	88.5	2.0	76.1	25.4	3.0	6.0	250.7	2.0	9.5	0.0	1.5	142.9	8.0	20.0	4.5	188.4
*C.glabripernis***	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.imicola	279.4	101.8	85.3	55.0	12024.1	1116.4	39.0	49.0	13750.0	268.5	231.0	31.0	18.5	63428.6	2318.0	307.0	360.0	66962.6
*C.kanagai**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
C.leucostictus	21.3	61.1	50.6	43.0	152.2	33.8	7.0	10.0	379.0	1.5	7.0	3.0	3.0	714.3	10.0	15.0	14.0	767.8
*C.milnei**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0
C.neavei	0.0	1.7	0.0	1.0	152.2	84.6	0.0	0.0	239.5	0.0	0.5	0.0	0.5	142.9	2.0	0.0	0.0	145.9
C.nevilli	1.9	0.0	31.6	0.0	532.7	50.7	0.0	0.0	617.0	0.5	3.5	0.0	0.0	2785.7	202.0	1.0	1.0	2993.7
C.nivosus	3.9	8.5	25.3	13.0	0.0	8.5	3.0	1.0	63.1	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	1.0
C.pycnostictus	17.5	5.1	25.3	5.0	0.0	16.9	4.0	3.0	76.8	0.0	0.5	0.0	0.5	0.0	0.0	3.5	4.0	8.5
C.simillis	7.8	22.1	22.1	3.0	152.2	33.8	2.0	6.0	249.0	1.0	0.5	1.5	0.0	0.0	0.0	3.0	1.5	7.5
*C.sp # 50**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
C.sp # 54 d/f	1.9	1.7	0.0	3.0	0.0	8.5	10.0	9.0	34.1	0.0	0.0	0.0	0.0	0.0	0.0	46.5	0.5	47.0
*C.sp # 54 p/f**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	32.5	0.0	32.5
C.subschltzei	7.8	11.9	12.6	6.0	152.2	33.8	0.0	0.0	224.3	1.5	5.0	4.0	0.5	357.1	58.0	5.5	1.5	433.1
C.tropicalis	7.8	42.4	9.5	5.0	228.3	25.4	17.0	17.0	352.3	0.5	7.0	0.5	2.5	0.0	6.0	27.0	1.0	44.5
C.tutttifruitti	1.9	11.9	6.3	33.0	152.2	33.8	4.0	4.0	247.2	0.0	1.5	7.0	0.0	0.0	10.0	16.0	1.0	35.5
C.zuluensis	0.0	1.7	0.0	0.0	76.1	8.5	1.0	0.0	87.3	0.5	0.0	0.0	0.0	71.4	2.0	0.0	0.0	73.9
Total:25	388.0	336.0	610.0	176.0	14916.0	1683.0	109.0	120.0	18338.0	286.5	285.5	53.5	27.5	68928.6	2768.0	546.5	425.0	73321.1

'df' (Dark Form) and 'pf' (Pale Form) refers to the colour of the wing of the unidentified species. (Numbering system is that of R. Meiswinkel and refers to yet undescribed *Culicoides* species).

Table 2.4: The number of individual *Culicoides* species collected across eight sites, during winter and autumn in Mnisi, their relative abundance and vector rating. Grey-shaded species indicate a vector rating >25% in both autumn (A) and winter (W), while * indicates *Culicoides* species with a vector rating >25% present in winter only. A vector rating >25% indicates that the species is both abundant and wide spread in the area. Known vectors of BTV and/or other orbiviruses are indicated in bold.

Culicoides spp	Winter (W) abundance	Positive sites in winter	Percentage positive sites (W)	Vector rating (W)	Autumn (A) abandunce	Positive sites autumn	Percentage positive sites (A)	Vector rating (A)
C. imicola	75.0	8	100.0	87.5	91.3	8	100	95.7
C. leucostictus	2.1	8	100.0	51.0	1.0	8	100	50.5
C. exspectator	1.4	8	100.0	50.7	0.3	7	87.5	43.9
C. tropicalis	1.9	8	100.0	51.0	0.1	7	87.5	43.8
C. bolitinos	4.4	8	100.0	52.2	0.5	6	75	37.8
C. simillis	1.4	8	100.0	50.7	0.0	5	62.5	31.3
C. tutttifruitti	1.3	8	100.0	50.7	0.0	5	62.5	31.3
C. enderleini	1.0	7	87.5	44.2	1.4	8	100	50.7
C. pycnostictus	0.4	7	87.5	44.0	0.0	4	50	25.0
*C. nivosus**	0.3	7	87.5	43.9	0.0	1	12.5	6.3
C. subschultzei	1.2	6	75.0	38.1	0.6	8	100	50.3
C. sp # 54 d/f	0.2	6	75.0	37.6	0.1	2	50	25.0
*C. bedfordi**	3.0	6	75.0	39.0	0.2	3	37.5	18.8
C. nevilli	3.4	4	50.0	26.7	4.1	6	75	39.5
C. neavei	1.3	4	50.0	25.7	0.2	4	50	25.1
*C. zuluensis**	0.5	4	50.0	25.2	0.1	3	37.5	18.8
C. coarctutus	0.6	2	25.0	12.8	0.1	2	25	12.5
C. brucei	0.6	2	25.0	12.8	0.0	1	12.5	6.3
C. dekeyseri	0.0	1	12.5	6.3	0.0	0	0	0.0
C. glabripernis	0.0	1	12.5	6.3	0.0	0	0	0.0
C. engubendei	0.0	0	0.0	0.0	0.0	1	12.5	6.3
C. kanagai	0.0	0	0.0	0.0	0.0	1	12.5	6.3
C. milnei	0.0	0	0.0	0.0	0.0	1	12.5	6.3
C. sp # 50	0.0	0	0.0	0.0	0.0	1	12.5	6.3
C. sp # 54 p/f	0.0	0	0.0	0.0	0.0	1	12.5	6.3
Total				16.00				13.00

Table 2.5: The total number and percentage of parous females in the population between autumn and winter cathes across all sites. Species sheded in light grey indicate a lack of parous females across all sites and species highlighted in dark grey indicates a lack of parous famales for a specified season.

Culicoides spp	No. of midge in winter	Portion of parous females in winter	Percentage parous females winter (%)	No. of midges in autumn	No. of parous feamles in autumn	Percentage parous females in autumn (%)
C. sp # 54 d/f	34.1	10.0	29.3	47.0	23.0	48.9
C. leucostictus	379.0	22.0	5.8	767.8	20.0	2.6
C. tutttifruitti	247.2	11.0	4.5	35.5	14.0	39.4
C. enderleini	175.0	6.0	3.4	1010.1	47.0	4.7
C. bolitinos	799.5	27.0	3.4	371.2	38.0	10.2
C. nivosus	63.1	2.0	3.2	1.0	0.0	0.0
C. pycnostictus	76.8	2.0	2.6	8.5	2.0	23.5
C. simillis	249.0	6.0	2.4	7.5	3.0	40.0
C. subschltzei	224.3	4.0	1.8	433.1	13.0	3.0
C. imicola	13750.0	211.0	1.5	66962.6	2246.0	3.4
C. tropicalis	352.3	5.0	1.4	44.5	19.0	42.7
C. neavei	239.5	3.0	1.3	145.9	1.0	0.7
C. nevilli	617.0	7.0	1.1	2993.7	65.0	2.2
C. brucei	118.4	1.0	0.8	4.0	2.0	50.0
C. coarctatus	118.4	1.0	0.8	45.0	6.0	13.3
C. bedfordi	547.1	4.0	0.7	144.4	2.0	1.4
C. sp # 54 p/f	0.0	0.0	0.0	32.5	9.0	27.7
C. exspectator	250.7	0.0	0.0	188.4	12.0	6.4
C. zuluensis	87.3	0.0	0.0	73.9	2.0	2.7
C. dekeyseri	8.5	0.0	0.0	0.0	0.0	0.0
C. engubendei	0.0	0.0	0.0	2.0	0.0	0.0
C.glabripernis	1.0	0.0	0.0	0.0	0.0	0.0
C. kanagai	0.0	0.0	0.0	1.0	0.0	0.0
C. milnei	0.0	0.0	0.0	1.0	0.0	0.0
C. sp # 50	0.0	0.0	0.0	0.5	0.0	0.0
Total	18338.0	322.0	1.8	2681.8	2524.0	3.4
No. of species with parous females		17.0			18.0	

2.3.3 Real-time RT-qPCR PCR analyses of *Culicoides* midges

The real-time RT-qPCR targeting segment 10 was tested before the start of the study using two BTV positive controls composed of extracted RNA from a BTV-5-infected cell culture (Vero) and from a *C. imicola* midge that fed on blood infected with BTV-8 (5.9 $\log_{10}$ $TCID_{50}$/ ml) (Fig. 2.3). PCR-grade water was used as negative control and was included in all steps from RNA extraction to real-time RT-qPCR. A total of 82 and 79 pools, each containing 200 *Culicoides* collected during autumn and winter, respectively, were tested for the presence of BTV RNA. Sites 3 and 4 of autumn collections were pooled due to the low numbers of midges associated with these sites.

The quantification cycle (C_q) reflected by the real-time PCR is an indication of viral load. In autumn, viral RNA loads ranged from the highest C_q-value of 27.4 to the lowest value of 37.8, with an average C_q-value of 35.0 (Fig. 2.4A). Viral RNA loads from midges collected during winter ranged from the highest C_q-values of 26.7 to the lowest value of 38.5, with an average C_q-value of 30.5 (Fig. 2.4B). A C_q-value of ≥ 40 indicated the cut-off point for this analysis. Bluetongue virus-positive results were obtained in 51.2% of the midge pools collected in autumn (42/82 positive) and 75.9% (60/79 positive) of the midge pools collected during winter. Bluetongue virus RNA was not detected in the rest of the midge pools. Midges caught during autumn demonstrated a field infection prevalence of 0.3%, while midges collected during winter showed an infection rate of 0.7%.

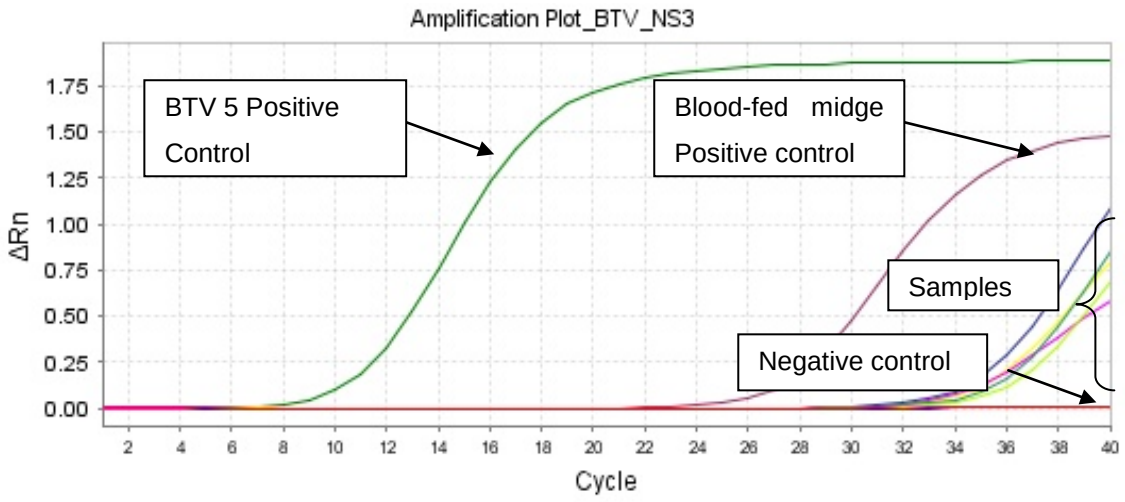

Figure 2.3: Amplification plot of real-time reverse transcriptase quantitative PCR targeting segment 10 of a BTV cell culture-adapted positive (BTV-5) and a BTV (BTV-8) positive blood-fed *C. imicola*. PCR-grade water was used as negative control.

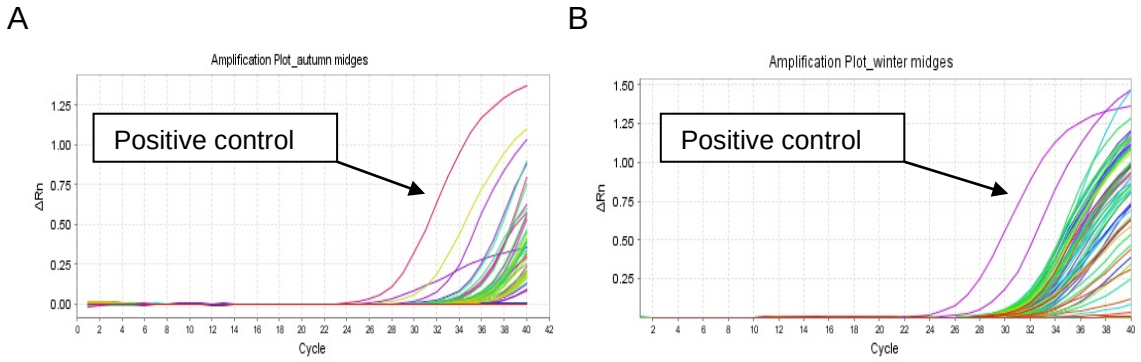

Figure 2.4: Amplification plot of real-time reverse transcriptase quantitative PCR. A) 42 out of 82 midge pools (200 midges/pool) collected during autumn tested positive for BTV RNA. C_q-values ranged from 27.4 to 37.7, with an average value of 35. B) 60 out of 79 midge pools (200 midges/pool) collected during winter tested positive for BTV RNA. Cq-values ranged from 26.7 to 38. 4, with an average value of 30.5.

2.3.4 Competitive enzyme-linked immunosorbent assay analyses

A total of 1 260 serum samples were obtained from cattle at 11 villages (Fig. 2.5). Animals were placed within an age group according to year of birth. Seven age groups were identified spanning from less than 1 year to more than 10 years (Fig. 2.6a). Antibodies specific to BTV were detected in 1 206 (95.7 %) of the serum samples tested. Non-parametric tests were used to establish any statistical significant differences between seroprevalence and breed, sex, age and specific villages (Table 2.6). There was a significant difference between the seroprevalence of BTV antibodies and the different villages sampled (Table 2.6, Fig. 2.5), as well as between age of the animals ($p < 0.05$) (Table 2.6, Fig. 2.6A). No significant difference for BTV antibody seroprevalence in either breed ($p = 0.4$) (Table 2.6) or sex ($p = 0.8$) (Table 2.6, Fig. 2.6B) were found. There was a significant difference ($p < 0.05$) when age groups and sex were compared with regards to seroprevalence. The Cochran-Mantel-Haenszel test/analysis confirmed that the significant difference found between age groups and sex with regards to antibody inhibition as well as the difference in seroprevalence between villages sampled is caused by covariates.

2.3.5 Real-time RT-qPCR PCR analyses of blood samples

Real-time RT-qPCR targeting segment 10 was used to screen blood samples for the presence of BTV from 54 cattle that tested negative for antibodies specific to BTV with a cELISA. A total of 16 blood samples tested positive for BTV RNA (35.5%) (Fig. 2.7). Viral RNA loads ranged from the highest C_q-values of 31.4 to the lowest value of 37.0, with an average C_q-value of 34.5 (Fig. 2.7). A C_q-value of ≥ 40 indicated the cut-off point.

Table 2.6: Significance of different factors that might play a role in the presence of antibodies specific to BTV in samples collected from cattle in Mnisi. Non-parametric Kruskal-Wallis test was used to determine any statistical significant differences within groups where p < 0.05 indicates a significant difference.

Factor	Group	No. of cattle tested	Number of positive animals	Number of negative animals	p-value
Breed	Brahman (cross)	694	659	35	0.4
	Brahman (typical)	43	42	1	
	Sanga (typical)	522	504	18	
	Other	1	0	1	
Sex	Male	339	324	15	0.8
	Female	921	882	39	
Age	>1	334	294	40	2.92E-15
	1-2	212	211	1	
	2-3	111	110	1	
	3-4	117	114	3	
	4-5	120	117	3	
	5-10	309	304	5	
	10-15	57	56	1	
Village	Clare A	121	120	1	1.15E-13
	Clare B	173	169	4	
	Dixie	20	20	0	
	Gottenburg	154	141	13	
	Hlalakahle	61	54	7	
	Seville A	126	120	6	
	Seville B	96	93	3	
	Tlhavekisa	85	78	7	
	Utha A	108	101	7	
	Welverdiend A	265	263	2	
	Welverdiend B	51	47	4	

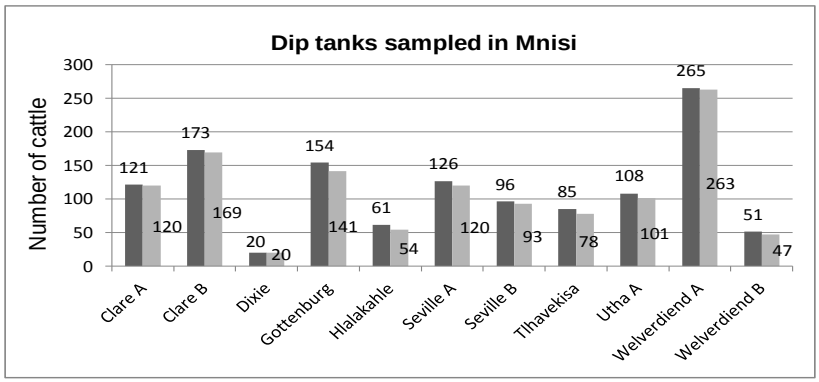

Figure 2.5: Total number of individual cattle sampled and tested for BTV-specific antibodies across 11 dip tanks in Mnisi, Mpumalanga Province. Non-parametric Kruskal-Wallis test revealed a significant difference ($p < 0.05$). Dark grey bars indicate the total number of cattle serum samples tested per dip tank (village), while light grey bars indicate the presence of BTV antibodies in individuals.

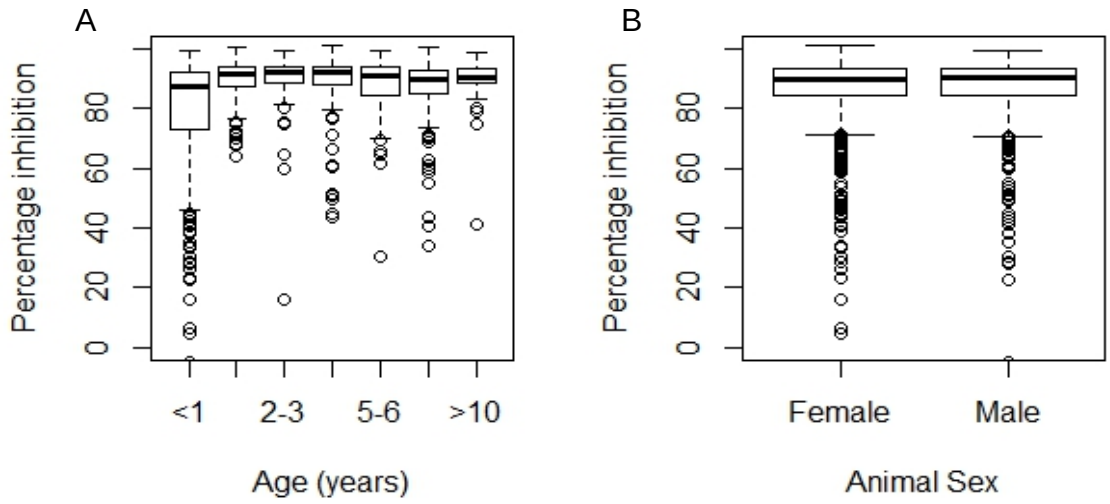

Figure 2.6: A) Box plot representing seroprevalence of BTV-specific antibodies across cattle age groups ($p < 0.05$) i.e., less than 1 year, 1-2 years; 2-3 years; 3-4 years; 4-5 years; 5-6 years 6-10 years and older than 10 years. B) Box plot representing the BTV antibody seroprevalence in female and male cattle. A total of 921 cows and 339 bulls were tested. No significant difference were found ($p = 0.8$).

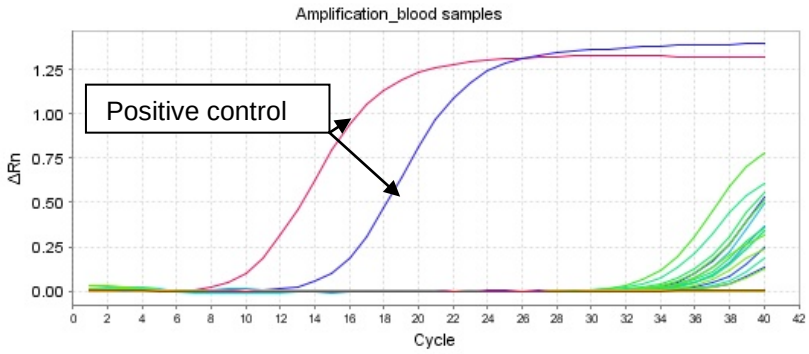

Figure 2.7: Amplification plot of real-time RT-qPCR on blood collected from 45 seronegative cattle in Mnisi. A total of 16 out of 45 samples tested positive for BTV RNA (35.5%). C_q–values ranged from 31.4 to 37.0, with an average C_q-value of 34.5.

2.4 Discussion

The first natural outbreak of BTV in bovine in South Africa was described in field-roaming cattle in 1933 (Bekker *et al.,* 1934). Bluetongue virus was also isolated from cattle, although no clinical signs were present (De Kock *et al.,* 1937; Mason and Neitz, 1940). The BTV outbreak in South Africa during 1995 to 1997 confirmed the presence of this disease in cattle after serological tests verified antibodies in 84% of the cattle tested. During the outbreak, BTV was also isolated from 12 cattle as well as from *C. imicola* and *C. bolitinos*. Unusually wet conditions due to an above-average rainfall were identified as the main trigger for the BTV outbreak (Barnard *et al.,* 1998). Clinical symptoms associated with the European BTV-8 field strain were for the first time observed in a previously BTV naïve cattle population in Europe from 2006 to 2007 (Thiry *et al.,* 2006; Darpel *et al.,* 2007, Elbers *et al.,* 2008). Bluetongue virus antibodies have also been detected in wild ruminants in different vegetational zones and rainfall areas in South Africa, with a lower incidence in semi-desert areas. This demonstrates that the disease occurs country-wide and is likely to affect domestic ruminants throughout South Africa (Barnard, 1997). The susceptibility of blesbuck (*Damaliscus albifrons)* to BTV, was also demonstrated in 1933 by Neitz (1933) when viraemia was detected between 8 and 17 days post-infection after experimental infection with BTV although no clinical signs were present. Clinical disease has been verified in USA where wild ruminants such as white-tailed deer

(*Odocoileus* virginianua) and pronghorn (*Antilocapra* Americana) developed severe clinical symptoms (Hoff and Hoff, 1976). Antibodies specific to BTV were also detected in roe deer (*Capreolus c. capreolus*), red deer (*Cervus e. elaphus*), Alpine chamois (*Rupicapra r. rupicara*) and Alpine ibex (*Capra i. ibex*) in Switzerland, although at low levels (Casaubon *et al.,* 2013). Low seroprevalence indicated that BTV is not circulating at a large scale within the wildlife-livestock interface and that wildlife in Switzerland probably does not act as a reservoir for BTV. The authors further suggested that susceptibility of wildlife to BTV differs between species and that wildlife should be included in BT surveillance programs (Casaubon *et al.,* 2013). During the 2008 outbreaks of BT in Europe the role of cattle was re-emphasised after cattle developed clinical disease (Thiry *et al*., 2006; Dercksen *et al*., 2007; Dal Pozzo *et al*., 2009). Clinical signs of BT in cattle are not generally found in South Africa and the role of cattle in the epidemiology of BT is not clear. This study focused on *Culicoides* surveillance, as well as the role of cattle in the epidemiology of the disease at the wildlife-livestock interface.

Livestock farming in the Mnisi area comprises primarily indigenous cattle and a few donkeys and goats. Mnisi is located in the subtropical Mpumalanga Province of South Africa where the summers are hot and rainy, and the winters are warm and dry seldom reaching sub-zero temperatures. Temperature fluctuations are therefore small and the area is not known for being windy. Meteorological factors influencing *Culicoides* abundance were therefore limited. Vector surveillance periods spanned from 23 April to 3 May 2013 (autumn) and again from 17 June to 15 July 2013 (winter). Throughout these periods the average outside temperature never rose above 25°C or fell below 15°C. Temperatures above 25°C or below 15°C have been shown to decrease the biting rate of *Culicoides* midges (Mellor *et al.,* 2000; Tweedle and Mellor, 2002). The wind speed never exceeded 3.4 km/h, with an average wind speed of 2.9 km/h, which is lower than the wind speed at which *Culicoides* are expected to stop flying (10.8 km/h) (Hendry, 1996). Except for June, the relative humidity was in an optimal range for *Culicoides* species, i.e. 50 - 70% (Venter *et al.,* 2004).

Numerous studies have been conducted on the affect of meteorological factors such as temperature, wind speed and humidity on the presence of *Culicoides* biting midges (Braverman, 1988; Blackwell, 1997; Meiswinkel *et al.,* 2000). These factors have been shown to influence the distribution and vectorial capacity of *Culicoides* midges and could influence the occurrence, as well as the number of midges collected (Meiswinkel *et al.,* 2000; Carpenter *et al.,* 2008). In previous studies, wind has been described as the most influential meteorological factor when collecting *Culicoides* midges (Braverman, 1988). In a study on *C. imicola* and *C. bolitinos,* Meiswinkel *et al.,* (2000) found that the number of midges collected correlate positively to temperature, and negatively to both humidity and wind speed. In contrast, a study on *C. impunctatus* demonstrated a positive correlation between the number of *Culicoides* collected and temperature, as well as a positive correlation between *Culicoides* numbers and humidity (Blackwell, 1997). In the United Kingdom, it was found that air temperature and wind direction significantly influences the number of blood-fed midges in a collection where high humidity decreases the activity of biting midges (Carpenter *et al.,* 2008).

2.4.1 Distribution and relative abundance of *Culicoides* species

A total of 25 *Culicoides* species were identified in Mnisi. This is less than 23% of the 112 *Culicoides* species found in South Africa (Meiswinkel, 1995). The Shannon-Weiner index indicated that collections made during autumn (H' = 0.5) is concentrated to one species with more rare species, while winter (H' = 1.2) have a greater species diversity. The Shannon-Weiner index calculates species diversity within an area; the higher the number, the higher the species diversity. Species evenness were also determined for autumn (J' = 0.1) and winter (J' = 0.4), respectively, indicating that there is variation in midge species collected between the seasons. Species richness refers to the proportion that each species comprises of the population and is affected by the number of individuals, as well as by the heterogeneity of the sample (Nolan and Callahan, 2005).

The dominant *Culicoides* species collected throughout the study was *C. imicola.* This species accounted for 91% and 75% of midges collected during autumn and winter, respectively. The abundance of *C. imicola* throughout the study area confirms that

this species is probably still the most prevalent *Culicoides* species throughout South Africa and highlights the importance of this species as a vector for BTV and other related orbiviruses such as AHSV (Nevill *et al.,* 1992b; Meiswinkel *et al.,* 2004). The super-abundance of *Culicoides imicola*, especially at Sites 5 and 6, may be attributed to a stream that is located near the sites. *Culicoides imicola*'s pupae require moist, nutrient-rich soil to breed. Midge numbers have been demonstrated to increase with ground moisture and increasing wetness (Jenkins and Young, 2010).

In winter, *C. bolitinos* was the second most abundant species accounting for 4.4% of total midges collected. The relatively low abundance, compared to *C. imicola,* could be due to the fact that this species occurs mostly in cooler, highland areas with higher rainfall such as the eastern Free State Province (Venter and Meiswinkel, 1994). *Culicoides bolitinos* species' numbers increased at the sites closer to the wildlife-livestock interface. Previous studies have found that *C. bolitinos* breed in the dung of large herbivores such as various cattle breeds (various *Bos* breeds) as well as African buffalo (*Syncerus caffer*) and blue wildebeest (*Connochaetes taurinus*) (Meiswinkel, 1989). The large number of cattle, together with the bordering wildlife reserves, could contribute to the abundance of this species outside its normal distribution range.

Culicoides nevilli was the second most abundant species during autumn (4%) and the third most abundant during winter (3.4%). This is in agreement with to reports by Venter *et al.,* (in press), where this species was found to be the third most abundant species in Pretoria, Gauteng. Although the vector competence of this midge species for BTV has not been proven yet, the closely related EHDV was previously isolated from *C. nevilli* (Barnard *et al.,* 1998).

The second most abundant species during autumn, *C. enderleini*, accounted for 1.3% of the total number of midges collected. This species is considered as a livestock-associated species and has demonstrated to be susceptible to BTV under laboratory conditions (Nevill *et al.,* 1992b; Meiswinkel *et al.,* 2004, Venter *et al.,* 2006). *Culicoides enderleini* is also known to be associated with livestock (Nevill *et al*., 1992a, b; Meiswinkel *et al.,* 2004).

The presence of C. sp. # 54 d/f may be due to their breeding preferences. This species breeds in the dung of African elephants (*Loxodonta Africana*), white rhinoceros (*Ceratotherium simum*), black rhinoceros (*Diceros bicornis*) and Burchell's zebras (*Equus burchelli*) (Dyce and Marshall,1989; Meiswinkel, 1992). The scarcity of C. sp. # 50 *and* C. sp. # 54 p/f is unexpected, considering the numerous reserves bordering Mnisi that provides excellent breeding sites for these species.

The chance of a midge to become infected with BTV, surviving the incubation period and then transmitting the virus to a receptive host is very low (Venter *et al.,* 1996). For this reason, rare species are unlikely to be important vectors (Standfast and Dyce, 1972), whereas the more wide-spread and abundant species would potentially make better vectors. Species that were both widespread and abundant, resulting in a vector rating of more than 25%, were present in both autumn and winter. During autumn, 13 species demonstrated a vector rating higher than 25%, while 16 species were identified during winter. *Culicoides imicola* demonstrated the highest vector rating, i.e. 95.7% and 87.5% in autumn and winter, respectively. This supports previous studies on BTV and related orbiviruses in SA where *C. imicola* was the most abundant species with the highest vector rating (Nevill *et al.,* 1992a; Venter *et al*., 1996). Other potentially important species that demonstrated a vector rating of more than 25% and that have been demonstrated to be susceptible to BTV infection under either laboratory conditions or in the field are *C. bolitinos, C. bedfordi, C. exspectator, C. leucostictus, C. pycnostictus* and *C. subschultzei (*Nevill *et al.,* 1992a; Venter *et al.,* 1998, 2006; Paweska *et al.,* 2002; Paweska and Venter, 2003).

Parous females were present in both autumn and winter sampling. The percentage of parous females could theoretically give an indication of the rate of transmission. Most species, including *C. Imicola*, presented parous females during autumn and winter sampling. Although significantly lower ($p > 0.05$), the presence of parous females in winter indicates that blood feeding and therefore the potential to transmit BTV continues throughout the colder months in the Mnisi region. Previous studies on AHSV in Pretoria, South Africa, also confirmed the circulation of both the vector and the virus throughout winter months (Venter *et al.,* in press). This could prove useful

in understanding the overwintering mechanism of BTV and other related orbiviruses. Further, the presence of males during the winter is an indication that mating continues throughout the year (Venter *et al.,* in press).

Light traps, although widely used, have the disadvantages that they become less efficient as wind speed increases and that they do not reflect midge biting rates (Taylor, 1962; Meiswinkel *et al.,* 2000; Carpenter *et al.,* 2008). The latter is important in determining the risk of pathogen transmission by insects (Gerry *et al.,* 2008). Light traps alone are not sufficient in determining vector populations and should be accompanied or replaced by mechanical aspiration (Scheffer *et al.,* 2012), which has the advantage of determining the numbers of midges on an individual animal, thereby providing a more accurate representation of midge biting rate (Carpenter *et al.,* 2008). The counterflow geometry trap is also widely used for *Culicoides* surveys (Logan and Birkett, 2007). This trap uses various baits such as pheromones or host odours to lure midges. Vehicle-mounted traps have also been demonstrated to be effective in monitoring *Culicoides* species, especially near wildlife (Dyce *et al.,* 1971). In future studies, it would be advisable to make use of more than one trapping method to accurately determine the presence and abundance of a specific vector species. This would improve vector surveillance and ultimately contribute to understanding the epidemiology of BTV.

Dyce's method (Dyce, 1969) was used to age-grade the midges. This method uses abdomen pigmentation to differentiate between nulliparous and parous females. In recent studies the effectiveness of this method was questioned and it was shown to be inaccurate by a factor of 23% (Braverman and Mumcuoglu, 2009). This method, however, does not affect the ability to sort midges between blood-feds, gravid and male midges as this is based on morphological differences. Dyce's method, although criticized, was still used in this study due to the fact that no other method has been described.

2.4.2 Bluetongue virus detection in *Culicoides*

In the current study, real-time RT-qPCR was used to detect the presence BTV RNA in field-collected *Culicoides* biting midges. The real-time RT-qPCR confirmed BTV

RNA in 51.2% of the midge pools collected during autumn and 75.9% in midge pools collected during winter. This resulted in a field infection prevalence of 0.3% in autumn and 0.7% in winter. The higher prevalence of BTV within the vectors during winter could be due to the fact that they are not transmitting the virus in colder temperatures. This then leads to a build-up of BTV within the midges that are only transmitted once favourable conditions arise (Van Dijk and Huismans, 1982; Mellor *et al*., 2000).

The results contradict previous findings where it was established that the field infection prevalence of BTV in *Culicoides* is a mere 0.06% (Nevill *et al.,* 1992b; Venter *et al.,* 2006). Variation in results could possibly be explained by the different diagnostic tools used. Nevill, Venter and co-workers used cell culture techniques to detect the presence of the virus. This relies on virus isolation and the detection of CPE that represents infectious virus. The use of real-time RT-qPCR is therefore much more sensitive and allows the detection of very low levels of viral RNA, even if the virus is non-infectious. Real-time RT-qPCR, targeting segment 5, has also been used to detect BTV RNA in European *Culicoides* species (Vanbinst *et al.,* 2009). With much smaller pools (5 to 10 midges), BTV RNA was detected in 20/103 pools, resulting in a field infection rate of 2.6% (Vanbinst *et al.,* 2009). A similar study on the closely related AHSV demonstrated an AHSV field infection rate of 1.35% in *Culicoides* (Scheffer *et al.,* 2012) compared to the previously determined field infection rate of less than 0.0001% to 0.03% (Nevill *et al.,* 1992b; Venter *et al.,* 2006; Venter and Paweska, 2007).

In this study, midges were pooled and screened for BTV. A disadvantage of pool testing is that it is not known how many midges within the pool are positive. Theoretically, just one midge or all of the midges can be positive. Quantitative real-time PCR should be validated and used in the future since C_q-values are not directly related to RNA or viral copy number within the midges.

Two different RNA extraction methods were used. This was to determine whether the methods were sensitive to extract enough RNA from *Culicoides* midges. Both the Trizol® and the MagMax™ extraction methods gave similar PCR results. Real-time RT-qPCR assays targeting different genes have also been evaluated. At the start of

the study primers and probes targeting segment 1 and segment 5 of the BTV genome, developed and validated by Toussaint *et al,* (2007), were used in the real-time RT-qPCR. These primer and probe sets unfortunately did not give the desired results as unspecific amplification was present. Primers and probes, designed by Van Rijn *et al.* (2012), targeting segment 10, was adapted by replacing the TAMRA probe with a minor groove binding (MGB) probe and used in the assay.

2.4.3 Seroprevalence of BTV in cattle

Results of the cELISA corroborate the PCR results for widespread occurrence of BTV in Mnisi. The seroprevalence for antibodies specific to BTV in sera collected from cattle was 95.7%. This study revealed a significant difference in antibodies prevalence to BTV among various age groups and different villages sampled ($p < 0.05$). The significant difference among age groups could be explained due to the number of young animals (< 1 year) that demonstrated a lower seroprevalence. Young animals are less likely to have become infected with BTV, but as animals mature the chance of becoming infected with BTV increases, as they are exposed to more BTV-infected vector periods.

The significant difference between age groups and sex with regards to antibody inhibition was explained due to fewer males being present in the community. In Mnisi, farmers sell the bulls after weaning, resulting in higher numbers of older females than older males. This could also possibly explain previous results indicating a higher seroprevalence in females than males (Elhassan *et al.,* 2014). Alternative possibilities e.g., olfactory response such as cattle-derived volatile compounds that might influence host seeking activities of *Culicoides*, should also be investigated (Bhasin *et al.,* 2000; Oyarzún *et al.,* 2009).

The significant difference in seroprevalence between villages is hard to explain, although this could be due to sample size bias. Alternate factors such as vegetation, interface areas e.g., Welverdiend B and Hlalakahle or the presence of water in the village e.g., Tlhavekisa and Seville B could also possibly affect the results (Fig. 2.1). No significant variations for prevalence of BTV antibodies were observed between cattle breeds ($p > 0.05$). A similar study conducted on dairy cattle breeds in Sudan

also found no significant difference in seroprevalence between local and cross breeds (Elhassan *et al*., 2014). Limited studies have been done on the effect of cattle breeds on BTV susceptibility. During the BTV outbreak in Europe (2006 - 2008), reports identified clinical disease associated with BTV-8 in Dutch dairy herds (Darpel *et al*., 2007; Schaik *et al*., 2008). These herds also demonstrated a higher seroprevalence than small-scale and suckler herds (Darpel *et al*., 2007; Schaik *et al*., 2008). Housing and grazing management of different cattle breeds might have played a role in this observation. Dairy herds are usually kept indoors during the night, whereas small-scale and suckler herds graze outside throughout the night making them more prone to *Culicoides* bites (Schaik *et al*., 2008). In general, cattle breeds appear to play a limited role in BTV susceptibility unlike sheep breeds where European breeds have been demonstrated to be more susceptible (Spreull, 1905; Neitz, 1984).

Antibody prevalence on its own is not sufficient to determine the role of a specific host in the epidemiology of a disease as seroprevalence might be under-or over-represented in a population (Barnard, 1997). This could be caused by overexposure due to low population density of susceptible animals or insufficient transmission as a result of small vector populations. The need to include several diagnostic tests such as PCR and VI, as well as vector surveillance is therefore essential in epidemiological studies of a specific disease (Barnard, 1997).

2.4.4 Bluetongue virus detection in blood samples

Blood samples from animals that were seronegative were taken on the same day from the same animal as the serum samples. A total of 45 blood samples were screened for the presence of BTV RNA. Bluetongue virus was detected in 16 out of 45 (35.5%) of the blood samples. Seronegative animals were expected to remain PCR negative, although results could indicate early infection. Similar results were obtained by Toussaint *et al*. (2007) where BTV was detected with RT-PCR, although no antibodies were present at the time. The PCR results do not indicate infectious virus in the animal as real-time RT-qPCR is able to detect viral nucleic acid long after viraemia. In rams, BTV-8 RNA was detected for up to 116 days post-infection with real-time RT-qPCR (Leemans *et al*., 2012). Another study demonstrated that a dam

infected with BTV-8 at eight months of gestation gave rise to a real-time RT-qPCR positive but seronegative calf (Backx *et al*., 2013).

Seronegative animals were used to increases the chance of detecting the virus with PCR. When the viral load is higher than the antibodies, it enhances the chances to acquire the virus. This would also allow for virus isolation and/or sequencing of the specific strain involved. The latter will be part of a future study based on the positive results found with the real-time RT-qPCR.

Real-time RT-qPCR can be used in rapid screening of field samples for BTV (Shaw *et al*., 2007). The real-time RT-qPCR described in this study includes first-strand cDNA synthesis, PCR and detection by real-time technology. The PCR was adapted from Van Rijn *et al.* (2012) with minor adjustments. Van Rijn *et al.* (2012) confirmed the presence of BTV in EDTA blood samples and demonstrate a diagnostic specificity close to 100%. Definite negative and positive results were observed in this study. Sequence analysis of three BTV-positive midge pools collected during winter using BTV segment 10 and BLAST (Basic Local Alignment Search Tool) confirmed the presence of BTV, thereby verifying the PCR results. The real-time RT-qPCR proved to be efficient in the detection of BTV RNA in both field-caught *Culicoides* midges and EDTA blood samples and could possibly aid in vector surveillance programmes (Vanbinst *et al.,* 2009), and as a screening technique to identify possible novel vectors.

The results indicate that BTV is circulating in Mnisi between vectors and hosts even though no clinical signs have been reported in cattle. The high seroprevalence, BTV RNA-positive blood and the lack of clinical disease in cattle support the hypothesis that cattle could potentially be important maintenance hosts of the BTV cycle in SA.

2.5 Conclusion

The epidemiology of BT is very complex due to the involvement of several mammalian hosts and vector species. Most domestic and wild ruminants are susceptible to BTV infection and at a wildlife-ruminant interface the primary cycle most probably involves one or more species of African antelope and biological *Culicoides* vectors. It has been hypothesised that cattle superseded the role of African antelope in areas where wildlife have been replaced by domestic ruminants (Du Toit, 1962).

The lack of a significant difference in the total midge numbers collected in this study during autumn and winter indicates that *Culicoides* are present in high numbers throughout the year in this region. The high seroprevalence as well as BTV RNA-positive midges indicate that BTV might possibly be circulating throughout the year.

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Chapter 3: Detection of bluetongue virus in *Culicoides* populations at the ARC-OVI, Pretoria using real-time RT-qPCR

Abstract

Several studies have demonstrated the ability of certain viruses to overwinter in arthropod vectors. The overwintering mechanism of BTV is unknown. One hypothesis is overwintering within adult *Culicoides* midges that survive mild winters where temperature seldom drops below 10°C. The reduced activity of midges and the absence of outbreaks during winter may create the impression that the virus has "disappeared" from an area. Onderstepoort light traps were used to collect *Culicoides* midges from July 2010 to September 2011 at the ARC-OVI. More than 500 000 *Culicoides* midges were collected from 88 traps and sorted to species level, revealing 27 different species. *Culicoides* midges were present throughout the 15-month study. Nine *Culicoides* species potentially capable of transmitting BTV were present during the winter months. Midges were screened for the presence of BTV RNA with real-time RT-qPCR. In total, 91.2% midge pools tested positive for BTV RNA. PCR results were compared to previous VI results that demonstrated viable virus in summer and autumn months. The results indicate that infected *Culicoides* vectors are present throughout the year at the ARC-OVI. Viral RNA-positive midges are also found throughout the year, with viable virus only in summer and early autumn. Midges that survive mild winter temperatures could therefore harbour BTV but a decreased vector capacity, as the population size and viral replication decreases, could halt BTV transmission. Overwintering of BTV at the ARC-OVI could therefore be a result of re-emergence rather than reintroduction.

Keywords: Bluetongue virus, *Culicoides*, overwintering, real-time RT-qPCR, virus isolation

3.1 Introduction

In South Africa, BT is common during late summer and autumn in areas with high rainfall (Verwoerd and Erasmus, 1963). Bluetongue usually disappears after the first frost, resulting in BT-free seasons (Verwoerd and Erasmus, 1963). During these apparent BT-free periods, no clinical cases are recorded. The virus is therefore believed to be absent from the area or dormant in either its vector or its vertebrate host. Seasonal recurrences of BTV raise the question whether the virus is re-introduced into an area or does it merely re-emerge as a result of an increase in vector capacity. Overwintering (or absence of cases during winter) of BTV has been documented (Nevill, 1971; Taylor and Mellor, 1994; Osmani *et al*., 2006), although the exact mechanism is still unknown (Taylor and Mellor, 1994; Singer *et al*., 2001; Osmani *et al*., 2006). As an example, apparent overwintering of BTV was demonstrated in Europe after BTV-8 persisted throughout the 2006-2007 winter period and re-emerged in the spring of 2007 (Maan *et al*., 2008; Velthuis *et al*., 2010).

Insect vectors, although relatively short-lived, are usually infectious for the rest of their life span after becoming infected (Wilson *et al*., 2008). Several arboviruses such as Ross River virus (Lindsay *et al*., 1993), West Nile virus (WNV) (Goddard *et al*., 2003; Reisen *et al*., 2006) and Orungo virus (Cordellier *et al*., 1982), have demonstrated the ability to be transmitted vertically by infected arthropod females. Apparent, vertical transmission of BTV in the genus *Culicoides* has been demonstrated in *C. sonorensis,* the primary North American vector, after BTV nucleic acid was detected in field-collected larvae (White *et al*., 2005). This study, however, did not demonstrate whether the BTV RNA-positive larvae would have emerged and been able to transmit infectious virus as adults.

In most parts of South Africa climatic conditions are suitable for adult *Culicoides* midges to remain active throughout the year and temperatures seldom reach sub-zero temperatures. It is therefore theoretically possible that small populations of virus-infected adult midges might survive long enough to bridge the gap between transmission seasons during mild winters. To ensure continuous transmission of BTV, vector-free periods must be of shorter duration than the maximum period of

viraemia in the vertebrate population (Mellor, 1994). Bluetongue virus viraemia can last up to 50 and 60 days in sheep and cattle, respectively (Sellers and Taylor, 1980; MacLachlan *et al.,* 1990; Koumbati *et al.,* 1999). Cattle as hosts have been implicated in certain parts of the country where more than 95% of cattle had antibodies specific to BTV, as discussed in Chapter 2. This indicates that there are therefore hosts in South Africa that can act as a source for BTV. Midge-free periods must therefore be longer than 60 days to break the cycle in transmission. Surveys have demonstrated the presence of large numbers of *Culicoides* midges throughout the year in areas where temperatures rarely drop below 0°C (Venter *et al.*, 1997; Meiswinkel *et al*., 2004) but have been recorded at -4.5°C (Becker *et al*., 2012; Venter *et al*., in press). Throughout such periods, ambient temperature might be too low for midge emergence or viral replication within the vector which only occur as soon as the temperature becomes favourable (Wellby *et al*., 1996; Mellor *et al*., 1998; Wittmann, 2000; Paweska *et al*., 2002). The mosquito *Culex pipiens*, an important vector for West Nile virus, is able to survive the winter as infected adults and can tolerate temperatures as low as -25°C (Nasci *et al*., 2001). *Culicoides* midges, unlike certain mosquito vectors, are less tolerant to sub-zero temperatures.

Adult *Culicoides* midges have a lifespan of 10 to 20 days (Mellor *et al*., 2000), although studies have demonstrated that their lifespan may be extended during mild winter months with some individuals surviving for up to three months at 10°C (Lysyk and Danyk, 2007). It is generally accepted that survival will increase, despite lower relative humidity, with a decrease in temperature (Hunt *et al*., 1989; Welby *et al*., 1996; Gerry and Mullens, 2000; Mullens *et al*., 2004). The rate of virus replication and the minimum temperature required for replication are generally consistent for different orbiviruses across different *Culicoides* vector species (Carpenter *et al*., 2011). Members of the Obsoletus complex can be active at temperatures as low as 3.5°C (Glukhova, 1989). In the laboratory, *C. bolitinos* can survive for >90 days and 10 days at 17°C and 4°C, respectively (Goffredo *et al*., 2004). *Culicoides imicola* adults can survive for >15 days and *C. pycnostictus* for up to 54 days at -1.5°C (Nevill, 1971). Previous studies have also demonstrated the capability of *C. imicola* and *C. bolitinos* to harbour BTV for more than 20 days at 10°C (Paweska *et al*., 2002). *Culicoides bolitinos* is also more adapted to cold temperatures than *C.*

imicola (Verhoef *et al*., 2014), suggesting that they are capable of surviving the mild winter temperatures in Gauteng.

It was recently shown that *Culicoides* midges can, despite relative low temperatures, occur throughout the colder winter months in the Onderstepoort area, Gauteng (Venter *et al.,* in press). It was also shown that the detection of viable African horse sickness virus, in particular, in these samples is linked to relative large insect collections (Venter *et al.,* in press).

Aim:

In this study, *Culicoides* collections previously used in a study to test for AHSV (Venter *et al*., in press), were screened for the presence of BTV RNA. Detection of BTV in midges collected throughout the winter months could possibly indicate overwintering of BTV in adult *Culicoides* populations at the ARC-OVI.

Objectives:

- To screen for the presence of BTV RNA in adult *Culicoides* populations collected throughout a 15-months survey at the ARC-OVI using real-time RT-qPCR.
- To compare real-time RT-qPCR with virus isolation results and to demonstrate viable BTV in the *Culicoides* population.

3.2 Materials and Methods

3.2.1 *Culicoides* collection and processing

The *Culicoides* midges used in this study were collected, as described by Venter *et al.* (in press). *Culicoides* midges were collected overnight at the ARC-OVI (25°39'S, 28°11'E; 1 219 m above sea level) using 220 V down-draught Onderstepoort black lights traps. Trapping was conducted one night a week from July 2010 to September 2011. Frequency of collection was increased (up to 5 nights/week) during winter months (July and August 2011). Traps were operated in the vicinity of 15 to 20 horses. Although not present in the vicinity of the light trap, cattle were abundant in a radius of 500 - 750 meters from the collection site. Midges were recovered from 88 black light trap collections. To preserve the *Culicoides* midges for virus isolation, collections were made into phosphate-buffered saline containing calcium and magnesium (PBS (+)) to which 0.5% Savlon® antiseptic had been added (Walker and Boreham, 1976). After retrieval in the morning, insects were first washed and then stored in PBS (+) at 4°C in the dark until analysed. Large collections were sub-sampled (Van Ark and Meiswinkel, 1992). Based on abdominal pigmentation (Dyce, 1969), the females of all species were age-graded into nulliparous, parous, gravid or freshly blood-fed. Males were also counted. Following gradation to species level and age, midges were removed from the PBS (+) and stored in 1.5 ml vials at -80°C until assayed for the presence of viruses.

Daily ambient temperatures were recorded using Tinytag Explorer (Gemini Data Loggers, UK, Ltd) (Venter *et al.,* in press). Midges (20 to > 3 000 per pool) were subsequently macerated in 1000 µl Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) LTD) (without serum) containing a sterile glass bead. Following maceration, insect debris was collected by centrifuging at 1 300 g for 1 minute after which the supernatant was added to confluent BHK-21. Samples were incubated at 37°C in a CO_2 incubator for 10 to 14 days or until CPE was observed. Samples were passaged three times on BHK-21 cells and stored at 4°C. Samples were numbered according to collection week (1 - 60).

3.2.2 RNA Extraction and Real-time RT-qPCR analyses

In brief, 2000 µl detached cell cultures was centrifuged for 5 minutes at 8 000 g to collect the pellet. Supernatants were discarded and 300 µl Millipore ultra-pure water (18.2 Ω) was added to break the cells by means of osmosis. RNA extractions were performed, as described in Section 2.2.3.1b. Real-time reverse transcriptase quantitative PCR, targeting segment 10, was performed as described in Section 2.2.3.2.

3.3 Results

3.3.1 Daily ambient temperature

Throughout the study period the maximum, minimum and average temperature at the trap site were recorded (Fig. 3.1). The maximum temperature measured was >40°C during December 2010, while the minimum temperature measured was -4.5°C during June 2011.

3.3.2 *Culicoides* midges

More than 500 000 *Culicoides* midges were collected from July 2010 to September 2011 (Fig. 3.2) (Venter *et al*., in press). Midges were present throughout the winter months (May, June, July and August). The mean number of *Culicoides* midges collected per trap per night ranged from 262 173 in March 2011 to 67 in July 2010 (Venter *et al*., in press). A total of 27 *Culicoides* species were present throughout the 15 month survey. Midge numbers decreased with a decrease in temperature, although nulliparous, parous, freshly blood fed, gravid and male midges were present throughout the study period (Venter *et al*., in press). A total of nine *Culicoides* species potentially capable of transmitting BTV under either laboratory or field conditions were present throughout the winter months, *C. imicola*, *C. enderleini*, *C. pycnosticus*, *C. bolitinos*, *C. leucostictus*, *C. zuluensis*, *C. magnus*, *C. gulbenkiani* and *C. expectator (*June, July and August) (Nevill *et al.,* 1992b; Venter *et al.,* 1998, 2006; Paweska *et al.,* 2002, 2003).

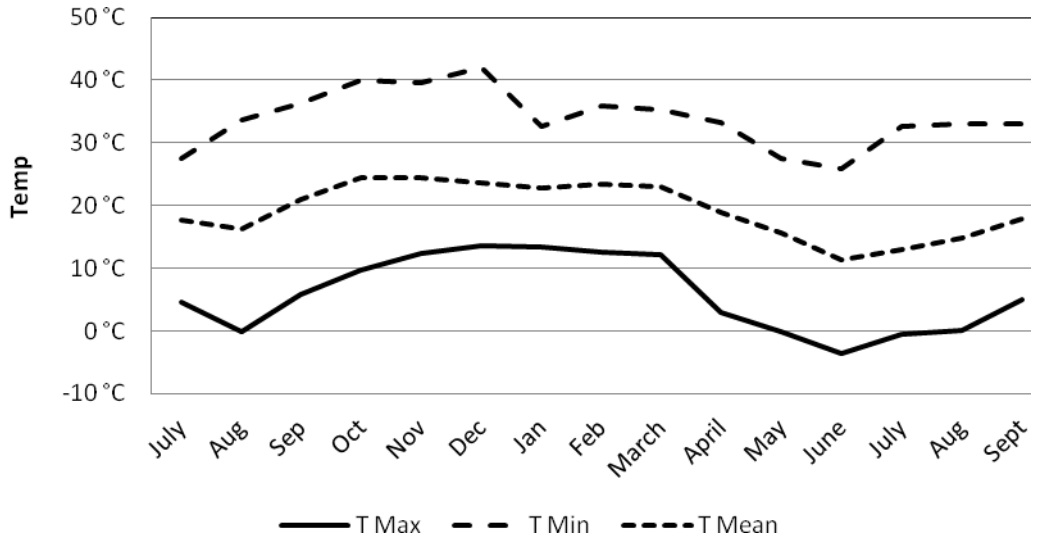

Figure 3.1: Graph representing the maximum, minimum and average temperature as measured during the study period i.e., July 2010 to September 2011 at the ARC-OVI. Source: Venter et al. (unpublished data).

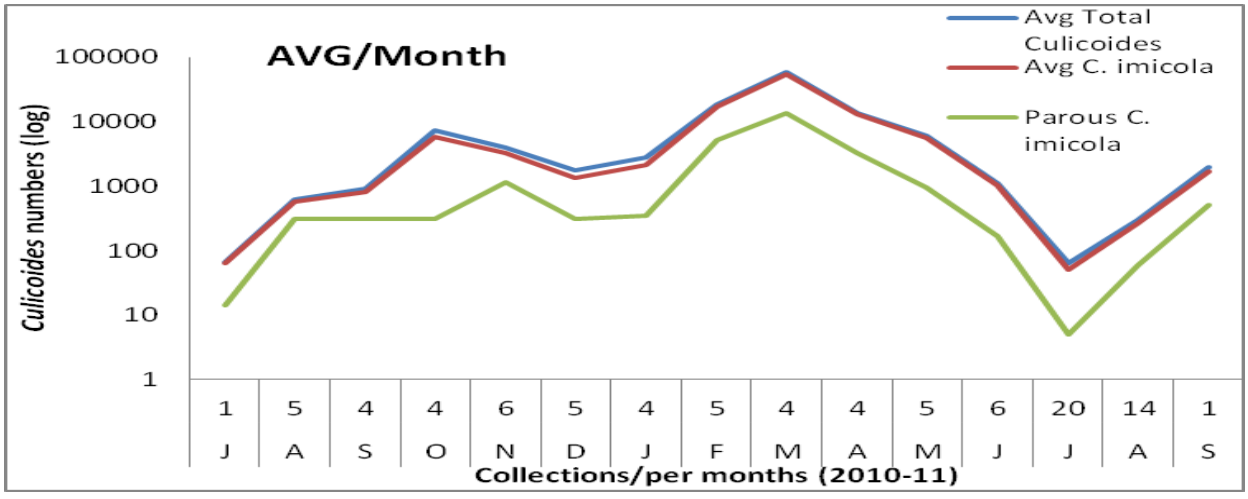

Figure 3.2: Mean number of *Culicoides* midges collected in weekly light trap collections made from July 2010 to September 2011 at the ARC-OVI. Source: Venter et al. (unpublished data).

3.3.3 Real-time RT-qPCR analyses of *Culicoides*

A total of 23 midge pools collected during 2010 (July to December) and 34 midge pools collected during 2011 (January to August) were subjected to real-time RT-qPCR (Table 3.1 Appendix). Pool sizes varied between 20 to > 3000 based on catch size. Negative results (undetectable at C_q > 40) were obtained from five samples, i.e. two in August 2010, one in October 2010, one in February 2011 and one in May 2011. All other samples, 52 out of 57 (91.2%), tested positive for BTV RNA with C_q-values ranging from 9.8 to 36.9 with an average C_q-value of 29.9.

3.3.4 Virus isolation of *Culicoides* pools

Virus isolation previously performed on all of the samples demonstrated CPE in only four samples [December (n = 1 770, C_q = 9.8), January (n = 1 514, C_q = 10.9), February (n = 10 728, C_q = 34.7) and March (n = 13 519, C_q = 11.2)] although the virus was not identified at the time (Venter *et al*., in press). These results were verified by PCR-positive results.

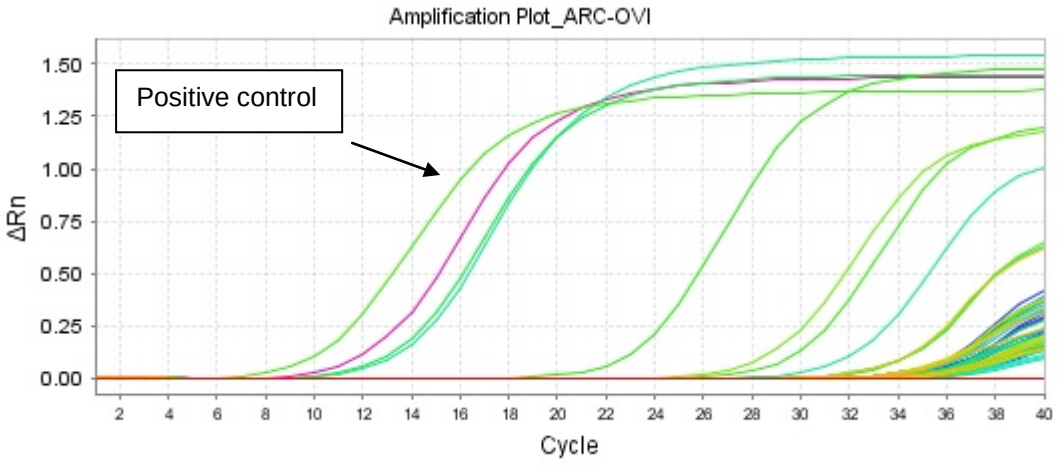

Figure 3.3: Amplification plot of real-time reverse transcriptase quantitative PCR. BTV RNA was detected in 52 of 57 (91.2%) of the midge pools tested. C_q-values ranged from 9.8 to 36.9 with an average C_q-value of 29.9.

3.4 Discussion

A previous study on the closely related AHSV demonstrated a relatively low virus isolation rate in the *Culicoides* population at the ARC-OVI (Venter *et al*., in press). No AHSV was detected during the winter, although continuous adult *Culicoides* activity indicates that transmission can potentially occur. The detection of AHSV is linked to high midge numbers collected, while the absence of AHSV in the midges during winter months was ascribed to the relatively low numbers of midges collected (Venter *et al.,* in press). Similar to AHSV, the overwintering mechanism for BTV is unknown. This study focused on the possibility that *Culicoides* that survived throughout winter in an area can harbour BTV and be an overwintering mechanism for the virus.

In total more than 500 000 midges were collected during the 15-month survey with speciation revealing 27 different species. The presence of both parous and freshly blood-fed females throughout the year indicates that feeding continues throughout winter (Venter *et al*., in press). This could indicate the potential transmission of BTV throughout the winter. The decrease in midge numbers during winter months could be ascribed due to a shift in activity. Midges will feed earlier (late afternoon rather than after dusk) to avoid the colder night temperatures, which would influence the catch size as the light traps will not work sufficiently (Nathan, 1981; Scheffer *et al*., 2012). This has been demonstrated in various studies as discussed in Section 2.4.1, and highlights the importance of using more than one trapping method to establish *Culicoides* abundance, especially in winter (Nathan, 1981; Scheffer *et al*., 2012).

3.4.1 Bluetongue virus detection in *Culicoides* population

Real-time RT-qPCR was used to screen the samples for the presence of BTV RNA in the *Culicoides* population. Bluetongue virus RNA was detected in 91.2% of the midge pools tested in comparison to 40% AHSV RNA in the same samples (Venter *et al.,* in press). The difference in positive results could either be due to BTV outcompeting AHSV within the midge or that BTV is predominant at the ARC-OVI due to a higher number of BTV host species such as sheep and cattle. All but two of the pools (20 to 3000 midges) that tested positive for AHSV RNA (12/35) also tested

positive for BTV RNA (53/58). This demonstrates co-circulation of both BTV and AHSV within the midge population and could indicate possible co-infection of *Culicoides* midges. Similar results were observed during a six-year survey where BTV and AHSV were detected with legacy PCR in 11.7% and 1.5% of the midge population, respectively (Nevill *et al*., 1992a). The difference in midge pool infection with BTV and AHSV is evident, although lower than the current study. This could be due to the difference in the sensitivity of legacy PCR versus real-time PCR where real-time PCR is much more sensitive.

Negative midge pools were present in all four seasons (August, October, February 2011 and May). No distinct pattern in BTV RNA-negative *Culicoides* with regards to season was therefore observed. At the time of VI virus(es) isolated in three of the four midge samples were not identified as PCR tested negative for AHSV in these samples (Venter *et al*., in press). The VI positive samples consequently tested positive for BTV RNA with real-time RT-qPCR, demonstrating viable virus in the isolates. Viable virus was only detected during summer (December 2010 to January 2011) and at the beginning of autumn (February 2011 to March 2011) and in only one midge pool per month. This could indicate that although BTV RNA is present throughout winter, virus capable of replicating to infectious levels is only present in summer and early autumn. The five midge pools that tested negative for BTV using PCR also tested negative with virus isolation, thus confirming the results.

Comparing real-time RT-qPCR and VI, it illustrates the importance of the diagnostic technique used when determining the presence/absence of viruses. Only 7.0% of the samples tested positive with cell culture isolation techniques in comparison with 91.2% that tested positive with real-time RT-qPCR. Virus isolation is of use to detect infectious virus if the viral titre is high enough and capable of showing CPE. In contrast, real-time RT-qPCR only detects viral RNA even if the virus is non-infectious. Real-time RT-qPCR is therefore important to establish whether the virus is present in a specific area/population, even if it is not infectious.

These results strengthen the findings in Chapter 2 where BTV RNA was found in midges collected during winter months. It is therefore evident that *Culicoides* are present throughout the year, even if temperatures fall well below the normal activity

range of these insects and that viral RNA is detectable in midges throughout most of the year. One hypothesis is that BTV overwinters in the midges and as soon as summer approaches and temperatures rise above 15°C, BTV starts replicating. Bluetongue virus then builds-up in the midge population to sufficient levels for transmission during summer and therefore disease only occurs late summer, early autumn (Van Dijk and Huismans, 1982; Mellor *et al*., 2000). The increase in virus replication within the midges, as a result of an increased temperature, also shortens the period between blood meals (increase feeding rate) and leads to larger population sizes. Re-emergence of BTV at the ARC-OVI is therefore due to overwintering rather than reintroduction (Wilson *et al*., 2008).

3.5 Conclusion

Culicoides midges were present throughout the 15-month survey period, demonstrating that the insects are able to survive mild winter temperatures recorded at the ARC-OVI, Gauteng. The presence of males, freshly blood-feds and parous females during the winter months also indicate that breeding and blood feeding continues throughout winter. BTV RNA was detected in most of the midge pools and viable virus was detected in summer and autumn collections. This study demonstrates that vector-free periods are not present at Onderstepoort and therefore possible overwintering of BTV within midges in this area.

3.6 References

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Chapter 4: Sequence analysis and evaluation of the NS3/A gene region of BTV isolates from South Africa

Abstract

Phylogenetic networks and sequence analyses allow for a more accurate understanding of the serotype, genetic relationships and the epidemiology of viruses. Bluetongue virus can be divided into five topotypes, based on conserved segment 10 (NS3) gene sequences. In this molecular epidemiology study, segment 10 sequence data of 11 isolates obtained from the Virology Section of the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, were analyzed and compared to sequence data of BTV strains available on GenBank from elsewhere in the world. The consensus nucleotide sequences of NS3/A showed intermediate levels of nucleotide variation with a nucleotide identity ranging from 79.72% to 100%. All 11 strains demonstrated conserved amino acid characteristics. Phylogenetic networks were used to identify BTV topotypes. The phylogeny obtained from the nucleotide sequence data of the NS3/A-encoding gene presented three major and two minor topotypes. The clustering of strains from different geographical areas into the same group indicated spatial spread of the segment 10 genes, either through gene reassortment or through the introduction of new strains from another geographical area via trade. The effect of reassortment and genetic drift on BTV and the importance of correct serotyping to identify viral strains are highlighted.

Keywords: Bluetongue virus, MEGA 6, phylogenetic networks, neighbour-joining, neighbour-net, NS3A

4.1 Introduction

Molecular phylogenetic analyses are imperative in epidemiological studies. Phylogenetics have been used widely to identify and categorize different viruses such as Newcastle disease virus (Miller *et al*., 2009), avian influenza A virus (He *et al*., 2009), foot-and-mouth disease virus (FMDV) (Knowles and Samuel, 2003) and Dengue virus (Holmes *et al*., 1999), and to determine the origin of viruses, source of outbreaks and spread that could assist in the control of a disease (Maan *et al.,* 2009; Mellor *et al.,* 2009; Vandenbussche *et al.,* 2009). Phylogenetic networks that include phylogenetic trees and split networks are important in the reconstruction of evolutionary history (Huson and Bryant, 2006). Split networks are also very useful to explore and visualize the different genetic differences such as reassortment in a data set (Huson and Bryant, 2006). Reassortment and genetic drift and shift have been considered key factors responsible for the evolution of various segmented genome viruses to create novel viruses (Holmes *et al*., 1999; He *et al*., 2009; Miller *et al*., 2009).

During a recent BTV outbreak in northern Europe, phylogenetic analyses were used to identify the origins of the responsible viruses (Maan *et al.,* 2008, 2009). Sequence analysis revealed that several of the viruses originated from sub-Sahara Africa, including a South African vaccine strain (Mellor *et al.,* 2009; Vandenbussche *et al.,* 2009). The release of vaccine strains into the field has increased the level of genetic diversity within the BTV population and emphasized the complexity of the epidemiology of BTV (Vandenbussche *et al.,* 2009).

The segmented nature of the BTV genome allows for reassortment within the host or vector when simultaneously infected with two or more strains (Bonneau *et* al., 2001; Batten *et al.,* 2008). This has complicated the genetic classification of BTV to some extent and it is desirable to conduct viral topotyping through sequence analyses of more than one gene segment (Samal *et al.,* 1987; He *et al.,* 2010). The relevant sequence data used in phylogenetic networks are therefore dependant on the application of the data or the information provided by the data, for example, determining geographical distribution, source of outbreak, speciation, etc.

Arthropod-borne RNA viruses that rely on both a vertebrate host and an insect vector have demonstrated slower rates of evolution compared to single-host RNA viruses. The reason that selective pressure within the two hosts is relatively low could be due to the need for the virus to compromise between two very different environments (Scott *et al*., 1994; Novella *et al.,* 1999; Weaver *et al.,* 1999). The non-structural protein NS3/A has been associated with the release of virus particles from insect cells (Wirblich *et al.,* 2006; Celma and Roy, 2009). It has been suggested that the variations observed in this protein may relate to transmission of the virus by distinct insect vector species in different geographical regions (Gibbs and Greiner, 1994). No evidence of co-evolution in the segment 10 gene has been demonstrated, only negative selection due to genetic drift and the founder effect (Bonneau *et al.,* 2000, 2001; Balasuriya *et al.,* 2008).

The BTV genome segment 10, coding for the protein NS3/A, is conserved intermediately with a nucleotide sequence identity of more than 80% across all isolates, independent of serotype (Maan *et al.,* 2008). The NS3/A protein has conserved characteristics that are shared by orbiviruses, e.g. BTV, AHSV and equine encephalosis virus (EEV) (Van Niekerk *et al*., 2003). These regions include a second in-phase start codon, a proline-rich domain and hydrophobic regions (Huismans *et al*., 2004). Based on evolutionary distinct geographical variants and phylogenetic analyses obtained from segment 10, BTV can be divided into three Eastern and two Western topotypes (Balasuriya *et al*., 2008; Maan *et al.,* 2008; Carpi *et al.,* 2010).

Aim:

The aim of this study was to compare sequence data from BTV isolates and to establish the group/topotypes of the newly sequenced strains using phylogenetic networks. The strains used in this study were isolated between 1972 and 2000 and labelled 'reference strains' by the Department of Veterinary Tropical Disease, University of Pretoria. The highly conserved NS3/A-encoding gene region was compared to NS3/A sequences of strains representative of different geographical regions globally. This could give us an indication of the degree of variability between BTV strains and add to the knowledge of the epidemiology of BTV in South Africa.

Objectives:

- To define the molecular epidemiology of BTV field strains by comparing previously sequenced serotypes isolated in South Africa and other countries.
- To investigate the level of nucleotide variation between the strains.
- To use molecular phylogenetic networks to establish different topotypes.
- To verify the presence of the conserved amino acid (aa) domains within each isolate.

4.2 Materials and Methods

4.2.1 Cell culture

Bluetongue virus field isolates were cultivated in African green monkey kidney (Vero) cells. Vero cells were grown in Dulbecco's minimal essential medium (MEM) (containing Earle's salts and $NaHCO_3$) (Highveld Biological (Pty) LTD) supplemented with 10% gamma-irradiated foetal calf serum (v/v) (Highveld Biological (Pty) LTD), 10% tryptose phosphate broth (v/v) (Sigma-Aldrich Co. LLC), 1 ml Gentamycin (500 µg/ml) (Virbac Animal Health) and 0.125 ml Fungizone (10mg/ml) (Bristol-Myers

Squibb) in tissue culture flasks (containing plug seal caps) at 37°C for seven days until confluent. Confluent flasks were infected with each of the 26 BTV field isolates and incubated for 7 days or until 100% CPE were observed. Cell cultures were frozen and thawed three times after which cell debris were collected by centrifugation for 10 minutes at 2000 g.

4.2.2 Viral RNA extraction

RNA extractions were done as described in Section 2.2.3b.

4.2.3 Legacy RT-PCR

Double stranded RNA was denatured by heating for 3 minutes at 95°C and placing it at -20°C for 5 minutes. Reverse transcription was carried out with the GeneAmp® Gold RNA PCR Reagent Kit (Applied Biosystems®) using segment 10-specific forward and reverse primers. Each 20 µl reaction contained 1 µl of denatured ds-RNA, 7.2 µl PCR-grade water (Millipore® ultra pure water) 4 µl of a 5x RT-PCR buffer, 2 µl magnesium chloride (2.5 mM), 2 µl dNTPs (1 mM), 0.5 µl RNase inhibitor, 2 µl DTT (1.25 mM), 0.5 µl forward (200 nM) (5'–GTTAAAAAGTGTCGTGCC–3') and reverse (5'-GTTAGTGTGTAGAGCCGCG-3') primers, respectively, and 0.3 µl of MultiScribe Reverse Transcriptase enzyme™. The mixture was incubated for 10 minutes at 25°C and then for 12 minutes at 42°C in a thermocycler (Applied Biosystems®).

4.2.4 Legacy PCR

Before the start of the experiment, BTV-positive cell culture-adapted isolates were used to optimize the PCR. Amplification of cDNA was performed using Takara® Taq and the ABI PCR system. Each 50 µl final reaction contained 0.25 µl Taq, 5 µl 10x buffer, 4 µl dNTPs, 1 µl (200 nM) of the forward (5' –GTTAAAAAGTGTCGTGCC– 3') and reverse (5' -GTTAGTGTGTAGAGCCGCG- 3') primers, respectively, and the volume was adjusted with 37.75 µl PCR-grade water (Millipore ultra pure water). One microlitres of denatured cDNA was then added to the master mix. Samples were vortexed and briefly centrifuged. Amplification was carried out by 30 cycles of

denaturation at 98°C for 10 seconds, annealing at 58°C for 30 seconds, and extension at 72 °C for 2 minutes. Positive results were defined on the basis that BTV segment 10 is 822 base pairs long. This was established with gel electrophoresis using a 2% agarose (Bio-Rad Laboratories, Inc) gel and visualized by a UV transilluminator after staining with ethidium bromide (stock: 10 mg/ml). PCR-grade water was used as negative control and was included in all steps from RNA extraction to PCR.

4.2.5 DNA sequencing and analysis

Amplified PCR products were sent to Inqaba Biotechnical Industries (Pty) Ltd for purification and Sanger sequencing. The purified DNA was sequenced in both directions using the same primers as in the PCR amplification step. Resulting sequence data were edited and analysed using the Staden Package (http://staden.sourceforge.net/), while multiple sequence alignments of the NS3/A-encoding gene and translated amino acid (aa) sequences were done using default parameters of CLUSTAL X and BioEdit. Bluetongue virus sequences were selected based on serotype and downloaded from GenBank as well as sequence data from another member of the *Orbivirus* genus, epizootic hemorrhagic disease virus (EHDV), to use as an out-group to root the trees (Table 4.1).The number of nucleotide differences between BTV strains was determined using pairwise distance with 1000 bootstrap replicates in MEGA 6 (Molecular Evolutionary Genetics Analysis software). Conserved regions were identified and aa positions were compared between strains. The best nucleotide substitutions model was determined using MEGA 6. The model with the lowest Bayesian Information Criterion (BIC) i.e., Tamura-3 using a gamma distribution (T92+G) (Tamura, 1992), was use to construct a neighbour-joining (NJ) tree with 1000 bootstrap replicates. A splits network inferring a neigbor-net analysis was used to extract phylogenetic signals that are missed by tree-based methods and to exclude any systematic error (Bryant and Moulton, 2004). This analysis was undertaken in SplitsTree 4 (Huson and Bryant, 2006).

Table 4.1: The accession number, origin, year of isolation and reference of sequence data of the segment 10 gene of different BTV isolates obtained from GenBank and used to compare newly sequenced isolates from SA.

Accesion number	Origin	Year of isolation/ submitted	Reference
AB473808	Japan	2008	Inumaru and Hosamani, 2008
AF044373	TX, USA	1962	Pierce *et al.* , 1998
AF044377	USA	1998	Pierce *et al.* , 1998
AF044383	CA/ Kern	1981	Pierce *et al.* , 1998
AF044386	NE/ Pershing	1979	Pierce *et al.* , 1998
AF044702	CA/ Kern	1980	Pierce *et al.* , 1998
AF044703	CA/Kern	1981	Pierce *et al.* , 1998
AF044704	CA/Kern	1981	Pierce *et al.* , 1998
AF481093	Corsican, France	2001	Sailleau *et al.* , unpublished
AF481094	SA	2002	Breard et al., 2001
AF512913	India	1999	van Niekerk *et al.*, 2003
AF512916	Ermelo, Mpumalanga, SA	1958	van Niekerk *et al.*, 2003
AF512919	SA	2002	van Niekerk *et al.*, 2003
AF512920.	Free State, SA	1999	van Niekerk *et al.*, 2003
AF512921	Gauteng, SA	1999	van Niekerk *et al.*, 2003
AF512922	Beaufort West, Western Cape, SA	1944	van Niekerk *et al.*, 2003
AF512923	Nelspoort, Western Cape, SA	1944	van Niekerk *et al.*, 2003
AF512924	Onderstepoort, SA	1937	van Niekerk *et al.*, 2003
AY120938	SA	2002	van Niekerk *et al.*, 2003
AY426604	Caribbean	1990	Balasuriya *et al* ., unpublished
AY449652	Greece	2003	Nikolakaki *et al.*, 2005
AY775164	SA	2004	Monaco *et al.*, 2006
AY857506	SA	2004	Breard *et al.*, 2007
EF434179	Portugal	2005	Barros et al.,2007
EU131022	India	2007	Desai et al., 2009
EU131027	India	2007	Desai et al., 2009
FJ183383	Netherlands	2008	Potgieter *et al.*, 2009
FJ713322	USA	2009	Wilson et al., 2009
GQ506460	Netherlands	2007	Maan et al., 2012
JN255871	Italy	2000	Caporale *et al* ., 2011
JN255931	SA (reference strain/wild type)	2001	Caporale *et al* ., 2011
JN255941	SA (Vacc)	2000	Caporale *et al* ., 2011
JN671915	China	1996	Yang *et al* ., 2011
JQ086240	Beatrice Hill/CPRS, NT, Australia	1986	Boyle *et al.*, 2012
JQ086250	Douglas Daly, NT, Australia	2008	Boyle *et al.*, 2012
JQ086260	Beatrice Hill/CPRS, NT, Australia	1977	Boyle *et al.*, 2012
JQ086270	VRRS, NT, Australia	1979	Boyle *et al.*, 2012
JQ086280	TFRF, NT	1982	Boyle *et al.*, 2012
JQ086300	Beatrice Hill/CPRS, NT, Australia	2007	Boyle *et al.*, 2012
JQ240330	Australia	2010	Boyle *et al.*, 2012
JQ713563	India	1982	Maan *et al* ., 2012
JQ904064	India	2008	Minakshi *et al.*, unpublished
JQ972840	Belgium	2010	Vandenbussche et al., in press
JQ972850	Belgium	2010	Vandenbussche *et al.* , in press
JQ972860	Germany	2011	Vandenbussche *et al.* , in press
JQ972870	Caribbean	2010	Vandenbussche et al., in press
JX129386	Pakistan	2012	Maan *et al* ., 2012
JX272398	SA (*Culicoides* pools)	2012	Koekemoer *et al.*, unpublished
JX272418	Australia	1979	Koekemoer *et al.*, unpublished
JX272428	Australia	1975	Koekemoer *et al.*, unpublished
JX272438	SA	1976	Koekemoer *et al.*, unpublished
JX272468	Hazara, West Pakistan	1960	Koekemoer *et al.*, unpublished
JX272518	Nelspoort, Cape Province, SA	1944	Koekemoer *et al.*, unpublished
JX272548	Onderstepoort, Transvaal, SA	1937	Koekemoer *et al.*, unpublished
JX272558	Utrecht, Natal, SA	1955	Koekemoer *et al.*, unpublished
JX272608	Ermelo, Transvaal, SA	1959	Koekemoer *et al.*, unpublished

4.3 Results

A total of 26 viral strains dated from 1972 to 2000 and stored by the Virology Section, DVTD, were used in this study. The NS3/A gene of 11 isolates could be amplified and sequenced successfully (Table 4.2), which included original isolates, vaccine strains and reference antigens. PCR products were viewed by agarose gel electrophoresis (Fig. 4.1) and sequence data were uploaded to NCBI GenBank to obtain accession numbers (Table 4.2). All of the BTV segment 10 sequences analysed were > 800 nucleotides, with the NS3/A start codon (ATG) at nucleotide position 40 to 42. The open reading frame of the NS3/A protein varied between 648 – 690 nucleotides and the NS3/A protein ranged from 215 – 229 aa. The differences are due to partial sequencing of some of the isolates.

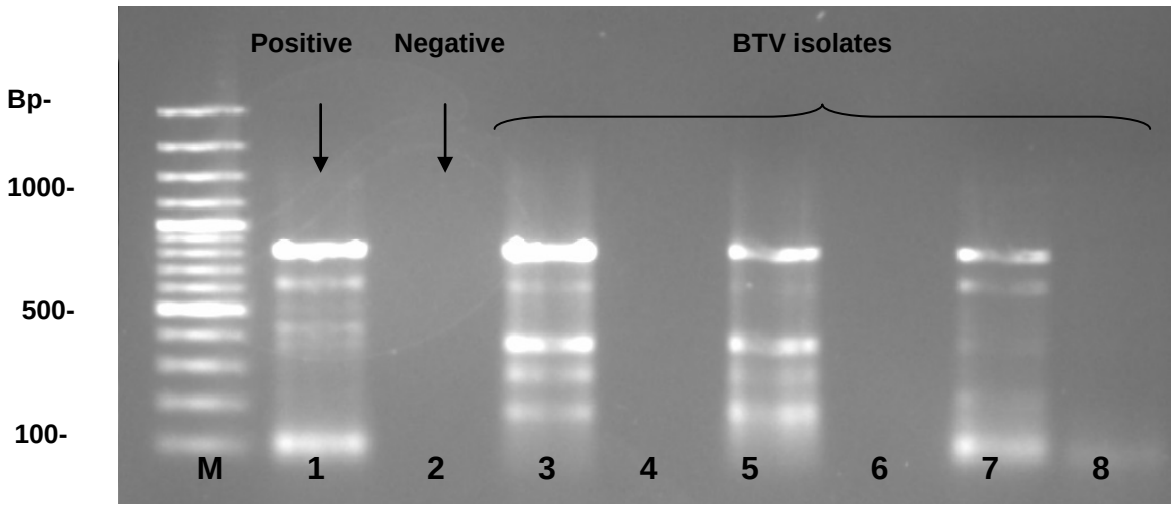


Figure 4.1: Electrophoretic analysis of PCR amplicons generated from segment 10 of BTV isolates. Bands with a size of 820 bp indicate amplification of the NS3/A-encoding gene region. Lane M indicates the 100 base pair marker (Fermentas Life Science). Lane 1 indicates a cell culture-adapted BTV isolate used as positive control. Lane 2 indicates the negative water control. Lanes 3 to 8 indicate amplified BTV isolates, while samples 4, 6 and 8 did not amplify.

Table 4.2: Bluetongue virus isolates obtained from 1972 to 2000 in South Africa, the isolation date and GenBank accession number, indicating sequence data of segment 10 for all isolates.

Strain	Year received at DVTD, OP	Accession number
BTV-2_NS3/A	1972	KJ506696
BTV-7_NS3/A	1972	KJ506695
BTV-8_NS3/A	1972	KJ506703
BTV-11_NS3/A	1972	KJ506694
BTV-16_NS3/A	1972	KJ506697
BTV-19_NS3/A	1995	KJ506699
BTV-20_NS3/A	1995	KJ506702
BTV-21_NS3/A	1995	KJ506701
BTV-22_NS3/A	1995	KJ506698
BTV-23_NS3/A	1995	KJ506700
BTV-S299_NS3/A	2000	KJ506704

4.3.1 Nucleotide variation

Nucleotide variation of newly sequenced strains relevant to this study (Table 4.2) has been described. Segment 10 or the NS3/A-encoding gene show intermediate levels of nucleotide variation between isolates with a nucleotide identity that ranged from 100% to 79.72% (Table 4.3). Segment 10 of BTV-S299 (**KJ506704**) was most closely related to BTV-2 from Portugal (EF434179) and three BTV-2 strains from South Africa (AF481094, JX272608 and JN255941) (96.86% identity), and had the greatest nucleotide variation to the newly sequenced BTV-2 (**KJ506696**) (80.35%). BTV-8 (**KJ506703**) showed the least variation to BTV-7 from South Africa (AY857506) (96.70%) and most nucleotide differences to BTV-2 (**KJ506696)** and BTV-19 (JX2724389) from South Africa and BTV-8 (AY426604) from the Caribbean (80.97%). Both BTV-23 (**KJ506700**) and BTV-20 (**KJ506702**) showed the greatest

percentage variation to BTV-11 (AF044386) from USA (81.76%). Segment 10 of BTV-23 (**KJ506700**) showed the least variation to BTV-2 (JQ713563) from South Africa (99.00%), while BTV-20 (**KJ506702**) was identical to two BTV-20 strains of Australian origin (JX272428 and JQ086260) (100%). The segment 10 gene sequence of BTV-21 (**KJ506701**) demonstrated the least variation to Australia's BTV-2 (JQ086250) (97.80%), and the most variation to BTV-19 (**KJ506699** and JX2724389) and BTV-11 (AF512921) from SA (81.45%). Segment 10 from BTV-19 (**KJ506699**) was identical to BTV-19 from SA (JX2724389) (100%) and had the most nucleotide differences to BTV-8 also from SA (AY120938) (80.19%). BTV-2 (**KJ506696**) and BTV-16 (**KJ506697**) showed the least difference in nucleotide composition to two strains of BTV-7 (**KJ506695** and JX272558) (97.80% and 96.70%, respectively). Five strains demonstrated the most nucleotide difference to BTV-11 (AF044702) from USA i.e., BTV-2 (**KJ506696**), BTV-16 (**KJ506697**), BTV-7 (**KJ506695**), BTV-11 (**KJ506694**) and BTV-22 (**KJ506698**) (79.72%, 81.29%, 80.03%, 80.97% and 80.66%, respectively). The following strains all demonstrated 100% identity to the relevant compared strains i.e., BTV-7 (**KJ506695**) to BTV-7 from SA (JX272558 1955), BTV-22 (**KJ506698**) to BTV-2 from SA (JX272398) and BTV-7 (**KJ506695**) and BTV-11 (**KJ506694**) to BTV-11 (JQ972840) from Belgium, BTV-11 (JX272518) from SA and BTV-11 (JQ972860) from Germany (100%) (Table 4.3).

Table 4.3: Comparison of nucleotide sequences between newly sequenced BTV segment 10 and reference strains obtained from GenBank. The number of differences between strains is presented as a percentage. Most nucleotide differences (upper percentile) and the least number of nucleotide differences (lower percentile) are given in the table.

BTV isolates from this study	Maximum nucleotide identity (%)	Compared strain/s	Minimum nucleotide identity (%)	Compared strain/s
s299 (2000)	96.86	BTV-2 (POR_EF434179) BTV-2 (SA_AF481094) BTV-2 (SA_JX272608) BTV-2 (SA_(V) JN255941)	80.35	BTV-2 (KJ506696)
8 (1972)	96.70	BTV-7 (SA_AY857506)	80.97	BTV-2 (KJ506696) BTV-19 (SA_JX2724389) BTV-8 (CAR_AY426604)
23 (1995)	99.00	BTV-2 (SA_JQ713563)	81.76	BTV11 (USA_AF044386)
21 (1995)	97.80	BTV-2 (AUS_JQ086250)	81.45	BTV-19 (KJ506699) BTV-19 (SA_JX2724389) BTV-11 (SA_AF512921)
20 (1995)	100.00	BTV-20 (AUS_JX272428) BTV-20 (AUS_JQ086260)	81.76	BTV-11 (USA_AF044386)
19 (1995)	100.00	BTV-19 (SA_JX2724389)	80.19	BTV-8 (SA_AY120938)
7 (1972)	100.00	BTV-7 (SA_JX272558)	80.03	BTV-11 (USA_AF044702)
2 (1972)	97.80	BTV-7 (KJ506695) BTV-7 (SA_JX272558)	79.72	BTV-11 (USA_AF044702)
22 (1995)	100.00	BTV-22 (SA_JX272398)	80.66	BTV-11 (USA_AF044702)
16 (1972)	96.70	BTV-7 (KJ506695) BTV-7 (SA_JX272558)	81.29	BTV-11 (USA_AF044702)
11 (1972)	100.00	BTV-11 BEL(R)_JQ972840) BTV-11 (SA_JX272518) BTV-11 (GER_JQ972860)	80.97	BTV-11 (USA_AF044702)

*AUS=Australia; GER=Germany; BEL=Belgium; SA=South Africa; POR=Portugal; USA=US of America; CAR=Caribbean

4.3.2 Phylogenetic network analyses

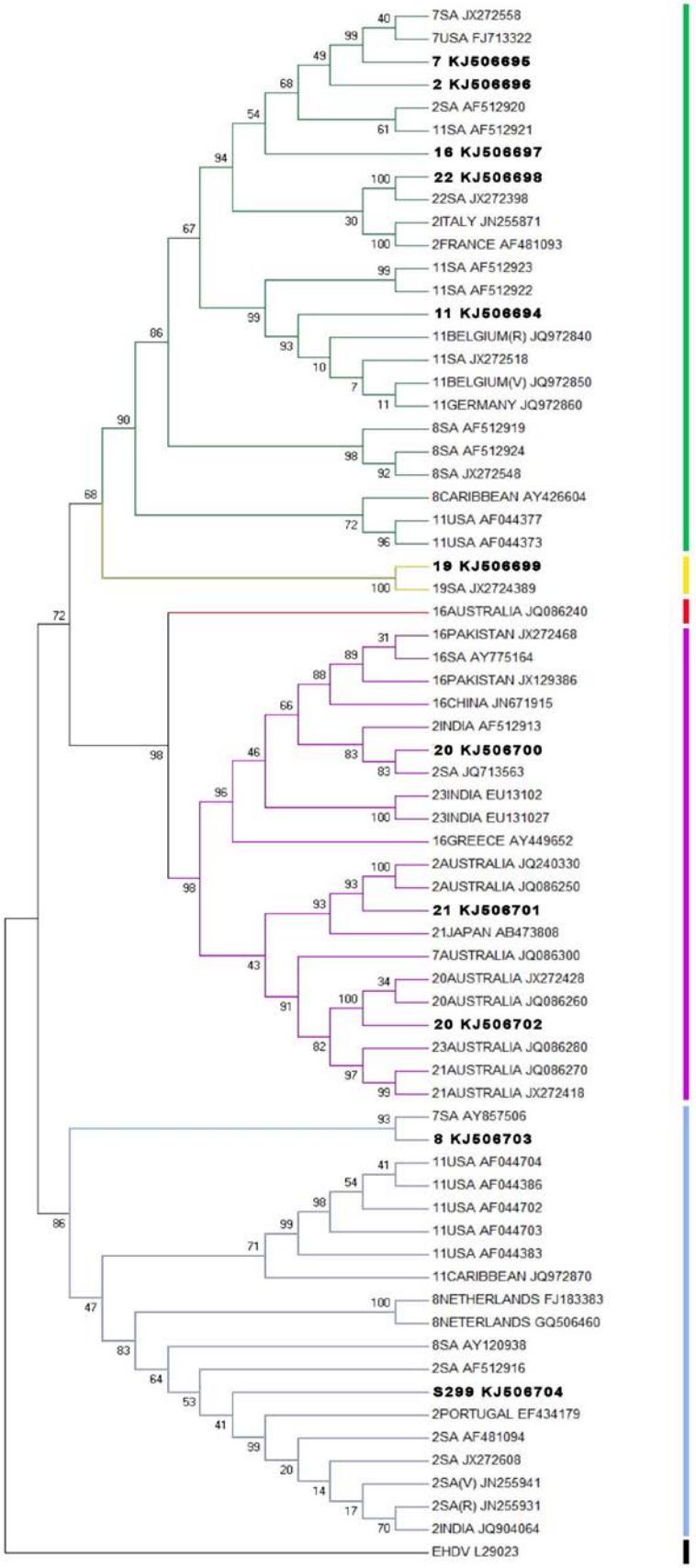
There was no difference in topotyping between the two phylogenetic network methods (NJ and neighbour-net analyses), suggesting that there was no error in the tree-based phylogenetic model. The NJ tree (Fig. 4.2) revealed two distinct monophyletic lineages with 72% (Fig. 4.2a) and 86% (Fig. 4.2c) confidence levels, and three minor lineages [Fig. 4.2 (a, b and c)] with confidence levels of 68%, 98% and 93% (Fig. 4.2b). The segregation of segment 10 corresponded to clusters determined previously i.e. Western Group 1, 2 and 3 and Eastern Group 1 and 2 (Balasuriya *et al.,* 2008; Maan *et al.,* 2008). The neighbour-net tree [Fig. 4.3 (a - e)] also provided strong evidence for networked evolution among BTV strains.

Segment 10 of BTV-2 from Italy (JN255871), France (AF481093) and SA (AF512920), BTV-7 from USA (FJ713322) and SA (JX272558), BTV-8 from SA (AF512919, AF512924 and JX272548) and the Caribbean (AY426604), BTV-11 from SA (AF512921, AF512923, AF512922 and JX272518), Germany (JQ972860), Belgium (JQ972850 and JQ972840) and USA (AF044377 and AF044373) and -22 from SA (JX272398), along with the sequences obtained from this study, BTV-2 (**KJ506696**), BTV-7 (**KJ506695**), BTV-11 (**KJ506694**), BTV-22 (**KJ506698**) and BTV-16 (**KJ506697**) clustered together in Western Group 2 (Fig. 4.2a, Fig. 4.3a). The minor lineages, Western group 3, consist of BTV-19 (JX2724389) and BTV-19 (**KJ506699**) from SA (Fig. 4.2a, Fig. 4.3d).

Segment 10 of BTV-2 from Australia (JQ240330 and JQ086250), India (AF512913) and SA (JQ713563), BTV-7 from Australia (JQ086300), BTV-16 from China (JN671915), Pakistan (JX129386 and JX272468), SA (AY775164) and Greece (AY449652), BTV-20 from Australia (JX272428 and JQ086260), BTV-21 from Australia (JQ086270 and JX272418) and Japan (AB473808), BTV-23 from Australia (JQ086280) and India (EU13102 and EU131027) along with newly sequenced strains BTV-20 (**KJ506702**), BTV-21 (**KJ506701**) and BTV-23 (**KJ506700**) clustered together in Eastern Group 1 (Fig. 4.2b, Fig. 4.3b), while segment-10 of BTV-16 from

Australia (JQ086240) formed the minor Eastern Group 2 lineage (Fig. 4.2b, Fig. 4.3e).

Segment 10 of BTV-2 from India (JQ904064), SA (AY120938, AF512916, JN255931, AF481094, JX272608 and JN255941) and Portugal (EF434179), BTV-8 from the Netherlands (FJ183383 and GQ506460), BTV-7 from SA (AY857506), and BTV-11 from USA (AF044704, AF044386, AF044703, AF044702 and AF044383) and Caribbean (JQ972870) as well as two of the sequences of interest, BTV-S299 (**KJ506704**) and BTV-8 (**KJ506703**) cluster together in Western Group 1 (Fig. 4.2c, Fig. 4.3c).



a) Western group 2 & 3:

Europe, USA, Caribbean and SA

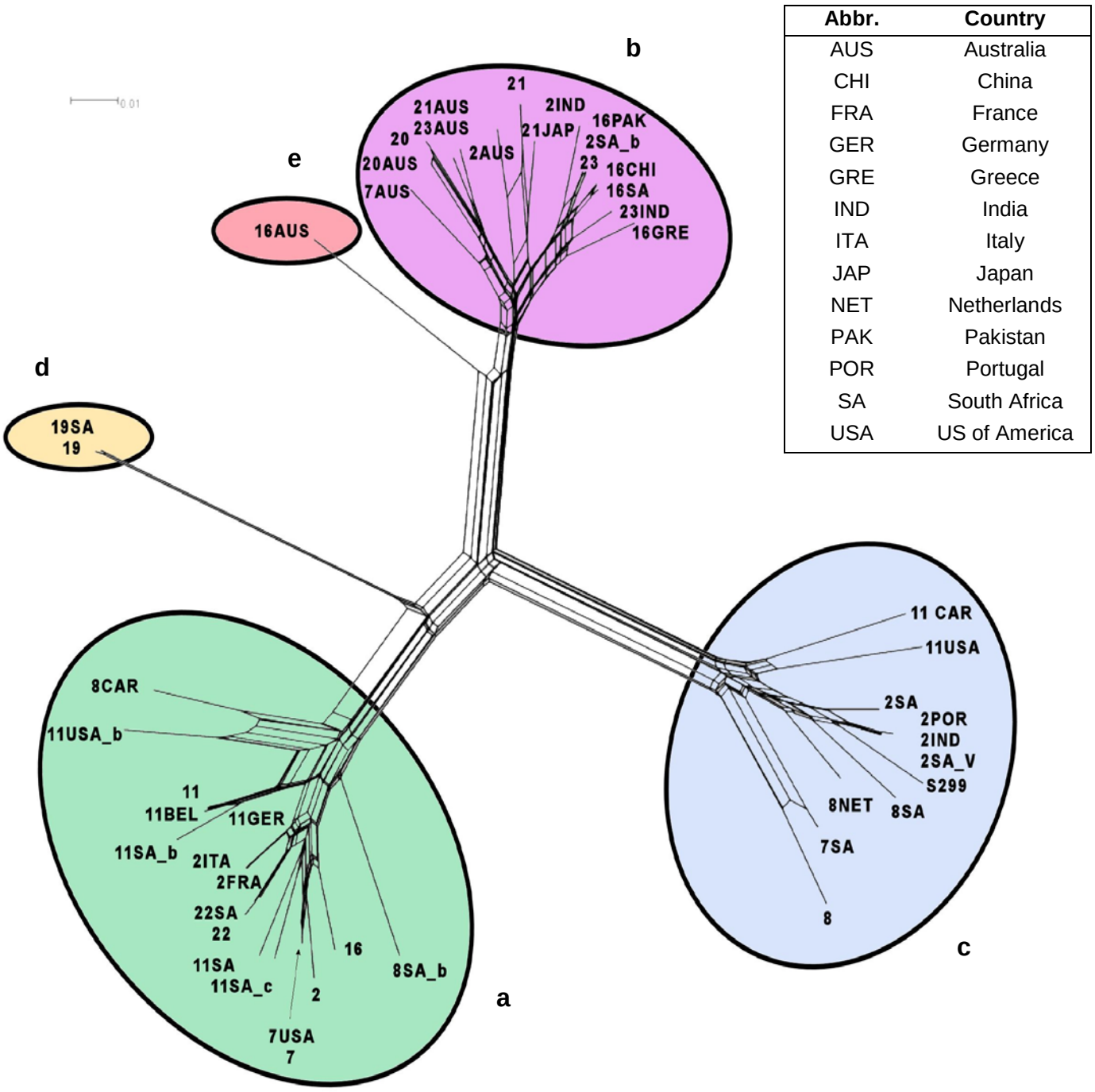
b) Eastern group 1 & 2:

Australia, Asia, Europe and SA

c) Western group 1:

SA, USA, Caribbean and Europe

Figure 4.2: Neighbour-joining tree (calculated by BI, T92+G substitution model and bootstraps estimates from 1000 replicates), showing the relationships of 67 BTV segment 10 nucleotypes using EHDV as an out-group. Strains relevant to this study are indicated in highlighted. The tree reveals 3 major groups, i.e. western 1(c) and 2 (a) and eastern group (b), and 2 minor groups, i.e. western 3(c) and eastern 2(b). Confidence scores are shown on the branches.



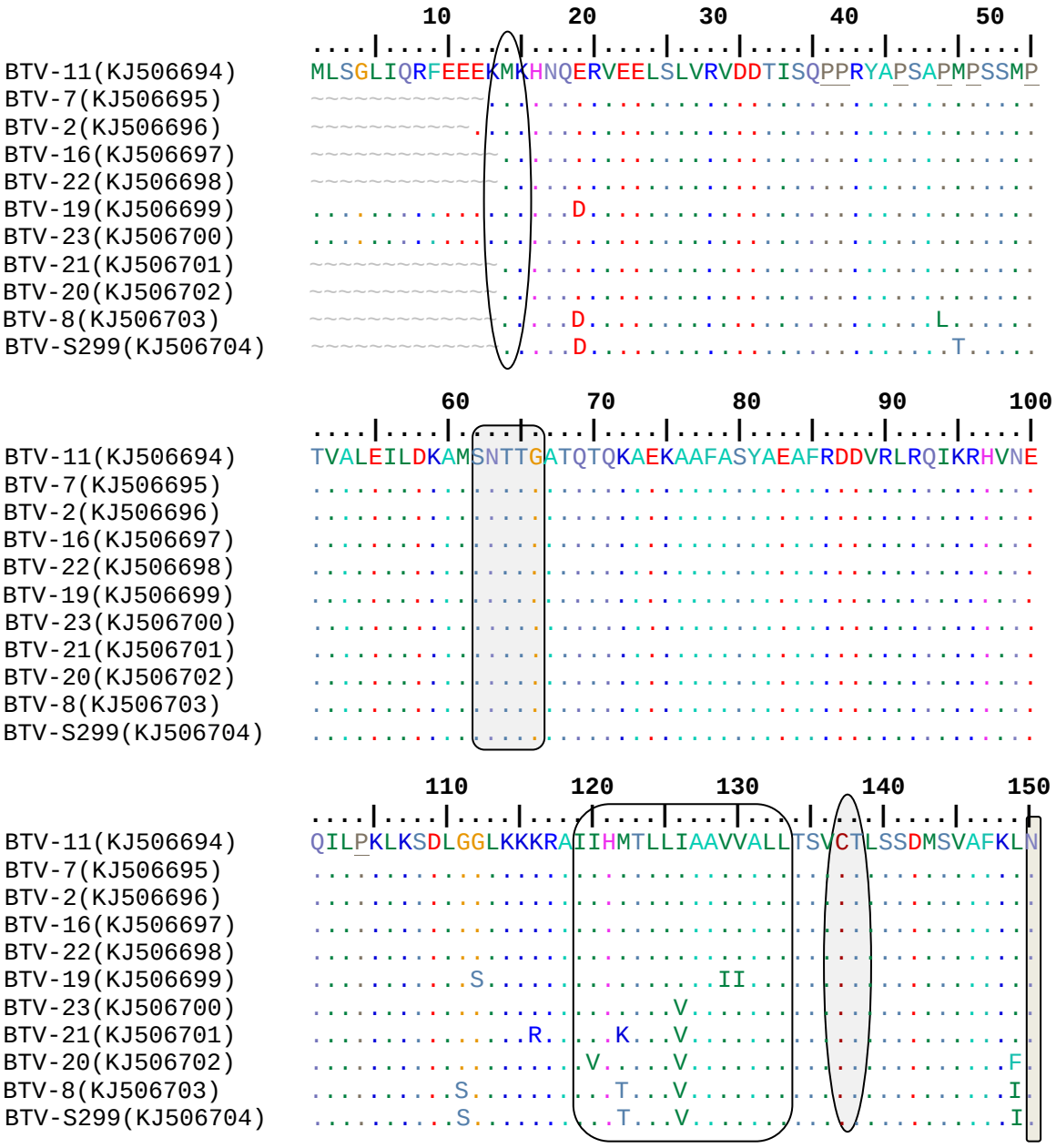
Abbr.	Country
AUS	Australia
CHI	China
FRA	France
GER	Germany
GRE	Greece
IND	India
ITA	Italy
JAP	Japan
NET	Netherlands
PAK	Pakistan
POR	Portugal
SA	South Africa
USA	US of America

Figure 4.3: Neigbour-net analyses of 46 different BTV segment 10 gene sequences. The tree reveals 3 major groups, i.e. Western 1(c) and 2 (a) and Eastern group (b), and 2 minor groups, i.e. Western 3(d) and Eastern 2(e). Accession numbers indicated in the Appendix.

4.3.3 Amino acid sequence analyses

The BTV segment 10 sequences were conserved between the isolates sequenced in this study. Some isolates demonstrated unique aa changes. Overall, the isolates used in this study were 76% conserved at the amino acid level. All 11 sequences demonstrated a conserved cysteine (C) at amino acid positions 137 and 181, and a conserved tryptophan (W) residue at amino acid position 159 (Fig. 4.4). Potential N-linked glycosylation sites are present at amino acid position 63 to 65 (NTT) for all isolates, while the N-linked glycosylation sites at position 150 to 152 (NGT) were conserved in 10 of the viruses, albeit that BTV-19 (KJ506699) contains a NNA sequence instead. Other regions present in all 11 isolates were the prolines (P) at amino acid positions 36, 37, 41, 44 (except BTV-8 (KJ506703)), 46, 50, 104, 157, 165, 220 and 227, confirming previous results suggesting conserved regions (Hwang *et al.,* 1992). Isolates BTV-19 (KJ506699) and BTV-20 (KJ506702) had an additional proline (P) at amino acid 158, whereas BTV-8 (KJ506703) and BTV-S299 (KJ506704) have a serine (S) residue. The rest of the isolates had a glutamine (Q) at amino acid position 158. Two hydrophobic regions are conserved between amino acid positions 119 to 133 and 167 to 183, respectively, with five viruses possessing a valine (V) instead of an isoleucine (I) residue at amino acid position 126 (Fig. 4.4). The amino acid region 110 to 130 had up to three amino acid changes in six of the strains, while region 149 to 200 had up to eight amino acid changes (e.g. BTV-19 (KJ506699)) (Fig. 4.4). BTV-8 (KJ506703) and BTV-S299 (KJ506704) both had the most amino acid changes when compared to BTV-11 (KJ506694) i.e. 13 changes each (Fig. 4.4). These two viruses demonstrated the same amino acid changes, except at position 44 where BTV-8 (KJ506703) posseses a leucine (L) and at position 45 where BTV-S299 (KJ506704) posseses a threonine (T). Amino acid position 154 differs in BTV-8 (KJ506703), BTV-S299 (KJ506704), BTV-23 (KJ506700), BTV-21 (KJ506701) and BTV-19 (KJ506699) replacing the alanine (A) residue with a threonine (T). Amino acid position 177 differs between the viruses. In BTV-20 (KJ506702) and BTV-21 (KJ506701) the leucine (L) residue is replaced with an isoleucine (I) and in BTV-23 (KJ506700) it is replaced with a valine (V) (Fig. 4.4).

Five viruses also demonstrated amino acid differences at position 187 where BTV-23 (KJ506700), BTV-21 (KJ506701) and BTV-20 (KJ506702) have a glycine (G) and BTV-8 (KJ506703), and BTV-S299 (KJ506704) an alanine (A) instead of a serine (S) residue (Fig. 4.4).



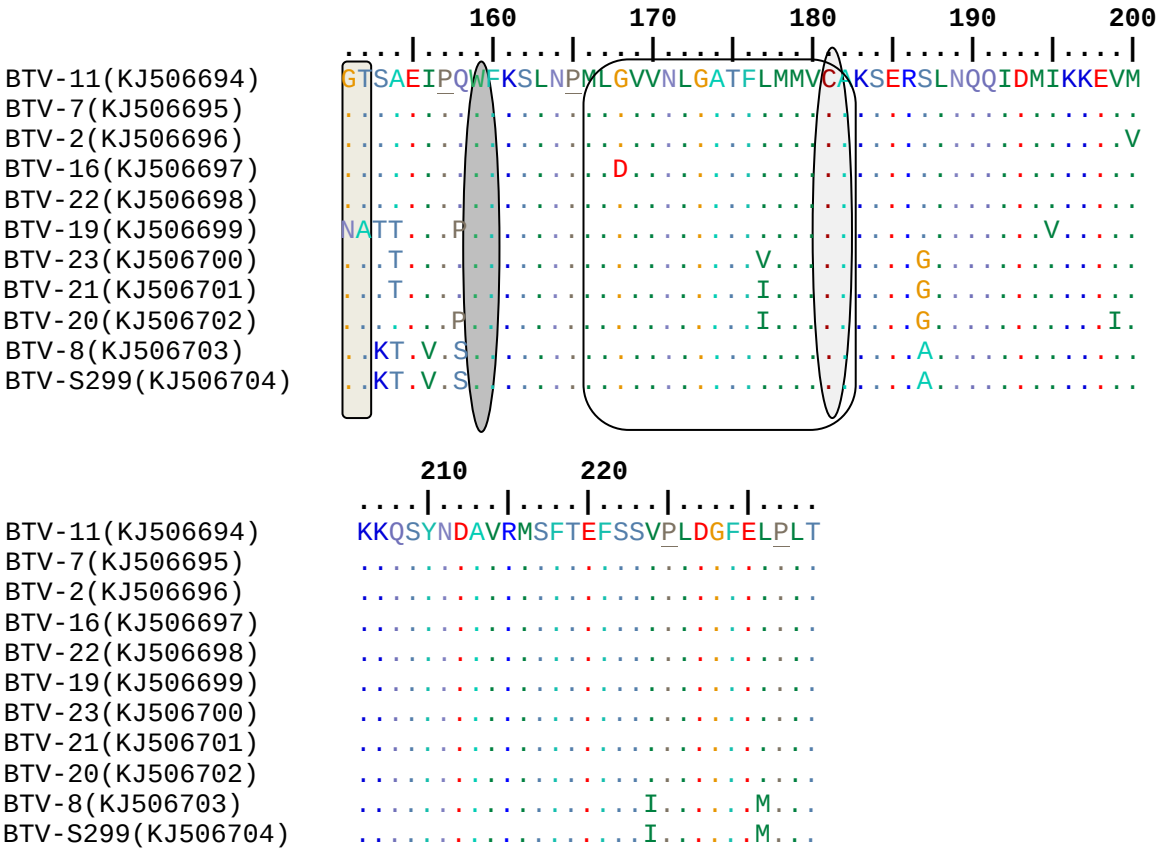


Figure 4.4: Amino acid sequence alignment, with conserved regions, of NS3/A from 11 BTV isolates. The NS3/A start codon (aa 14) is indicated by a sphere shape. The proline residues (aa 36, 37, 41, 46, 50, 104, 157, 165, 220 and 227) are underlined. The two N-linked glycosylation sites (aa 62-66 and 150-152) are indicated by light grey shaded rectangles. Hydrophobic sites (aa 119-133 and 167-183) are indicated by open rectangles. The conserved cysteines (C) at amino acid positions 137 and 181 are indicated by light grey-shaded sphere shapes, while the tryptophan (W) residue at amino acid position 157 is indicated by a dark grey-shaded sphere shape. Dots indicate the identity between the 11 isolates.

4.4 Discussion

Phylogenetic networks have an important role to play in determining the source of an outbreak. Numerous viruses such as Newcastle disease virus (Miller *et al*., 2009), avian influenza A virus (He *et al*., 2009), FMDV (Knowles and Samuel, 2003), Dengue virus (Holmes *et al*., 1999) and BTV (Maan *et al.,* 2008, 2009; Mellor *et al.,* 2009; Vandenbussche *et al.,* 2009) have been identified and categorized based on geographical distinct groups. In this molecular epidemiological study, segment 10 nucleotide sequence data from different BTV isolates obtained in South Africa from 1972 to 2000 were analyzed and compared to strains from the New World, Europe, Asia and Australia. Previous studies have shown considerable sequence identity and conservation of the NS3/A protein encoded by segment 10 of the BTV viral genome, especially at amino acid level (Hwang *et al.,* 1992; Pierce *et al.,* 1998; van Niekerk *et al*., 2003; Balasuriya *et al.,* 2008; Maan *et al.,* 2008). Balasuriya *et al.* (2008) and Maan *et al.* (2008) demonstrated that BTV strains can be divided into five groups/topotypes consisting of three major lineages, i.e. one Eastern group and two Western groups and two additional minor lineages of western and eastern origin using sequence data from segment 10 of the BTV genome.

Phylogenetic network analyses (Fig. 4.2, Fig. 4.3) revealed that the three major groups contain the majority of the segment 10 sequences with less sequences in the two minor groups (Balasuriya *et al*., 2008; Maan *et al*., 2008, 2009). From both the NJ tree and the neigbor-net tree it was possible to determine the origin of the strains sequenced in this study as discussed in the following sections. The BTV strains used in the study were isolated between 1937 and 2012. The isolation dates of the strains do not appear to influence the nucleotide identity between newly sequenced strains and global BTV strains (Table 4.1, Table 4.3).

4.4.1. Western groups

Bluetongue virus **8 (KJ506703)** and **BTV-S299 (KJ506704**) clustered into Western Group 1 (Fig. 4.2c, Fig. 4.3c). Bluetongue virus **S299** is closely related (96.86%) to BTV-2 strains from SA (including a vaccine strain) and a BTV-2 strain from Portugal. This could be the result of reassortment between the SA BTV-2 MLV strain and a BTV-2 strain from Portugal. Bluetongue virus serotype 2 vaccine strains were isolated in both Italy and Portugal (Ferrari *et al*., 2005; Barros *et al*., 2007).

Neighbour-net analysis does not support the split of **BTV-8 (KJ506703)** and **BTV-7 (AY857506)** into a separate cluster but rather clustered them in Western Group 1 (Fig. 4.3c). This was also evident in the nucleotide differences between **BTV-8 (KJ506703)** and strains from Western group 2 (80.97%) (Table 4.3). The high nucleotide identity between **BTV-8 (KJ506703)** and **BTV-7 (AY857506)** (96.70%) indicated the impact of genetic drift and/or reassortment between different serotypes and the importance of relying on segment 2 to identify serotypes rather than segment 10 (Huismans and Erasmus, 1981; Mertens, 1999; Maan *et al*., 2007). The segment 10 gene sequences of **BTV-2 (KJ506696)**, **BTV-7 (KJ506695)**, **BTV-11 (KJ506694)**, **BTV-16 KJ506697)** and **BTV-22 (KJ506698)** all clustered into Western group 2 (Fig. 4.2a, Fig. 4.3a). These serotypes, along with **BTV-8 (KJ506703)** and **BTV-7 (AY857506)** from Western group 1, are all endemic to South Africa although they formed two separate groups/topotypes.

The presence of **BTV-16 (KJ506697)** in Western Group 2 is unexpected (Fig. 4.2a). This serotype is endemic to the Mediterranean Basin and Asia, with a few isolates from Australia. The latter strain has a nucleotide identity of 96.70% to BTV-7 from South Africa (KJ506695 and JX272558) and a BTV-11 reference strain (JQ972840) from Belgium (Table 4.3). Gene reassortment between serotype 16 and/or BTV-7 and BTV-11 could explain the unexpected grouping. A double reassortant field isolate of BTV-16 was also isolated in 2002 in Italy, which contained a segment 2 derived from a SA BTV-16 MLV and a segment 5 derived from a SA BTV-2 MLV strain (Ferrari *et al*., 2005; Batten *et al*., 2008; Listes *et al*., 2009). The neighbour-net

analysis verifies the grouping of BTV-16 in the Western Group 2 but rather illustrates reassortment between BTV-2 (KJ506696) which, in turn, is closely related to BTV-7 (KJ506695 and JX272558) (Fig. 4.3a).

Bootstrap values and split network analysis supported the clustering of the segment 10 genes from two South African BTV-19 strains (KJ506699 and JX2724389) into Western Group 3 (Fig. 4.2a, Fig. 4.3d). Serotype 19 is endemic to both South Africa and the New World, and showed 100% nucleotide identity (Table 4.3). In both Western Groups 1 and 2, segment 10 sequences obtained from isolates from the New World and Europe clustered together with South African strains. The clustering of the New World strains and South African strains was first described by van Niekerk *et al*., (2003). This appears to demonstrate the movement of South African strains into the New World in the past and suggests a history of genetic continuity between the strains (van Niekerk *et al*., 2003; Balasuriya *et al*., 2008).

4.4.2. Eastern groups

In Eastern Group 1, strains isolated in the Mediterranean Basin (Greece) and Asia clustered together with Australian strains (Fig. 4.2b, Fig. 4.3b). The clustering of segment 10 from **BTV-20 (KJ506702)**, **BTV-21 (KJ506701)** and **BTV-23 (KJ506700)** into an Eastern group is expected. These serotypes are exotic to South Africa (Gerdes, 2004) and originated in Australia (Delle-Porta *et al*., 1983; St George *et al.,* 1980), Asia (Sendow *et al*., 1997; Pritchard *et al*., 2004) and/or South America (Cunha, 1990). Bluetongue virus serotype **20 (KJ506702)** demonstrated 100% nucleotide identity to two Australian strains of BTV-20 (Table 4.2). The segment 10 nucleotide sequence from **BTV-21 (KJ506701)** was 97.80% identical to that of a serotype 2 strain from Australia, while **BTV-23 (KJ506700)** was 99% identical to a serotype 2 strain from South Africa from Eastern Group 1 (Table 4.3, Fig. 4.2b, Fig. 4.3b). This suggests that BTV-23 circulated in South Africa in the past and that gene reassortment between the latter two strains occurred. Clustering of BTV-23 into Eastern Group 1 could clarify the exotic status of this serotype in South Africa, although phylogenetic analyses of segment 2 are needed to be used to confirm this.

No published data of BTV-23 having been isolated in South Africa in the last 20 years could be found. Conversely, previous studies demonstrated that geographical origin has little or no influence on the variation in the NS3/A gene (van Niekerk *et al*., 2003).

The neighbour-net analysis provides strong evidence for networked evolution in segment 10 gene sequences of BTV isolates (Fig. 4.3). The Neigbor-net analysis demonstrated reassortment of BTV segment 10 within the different topotypes. It highlighted the role that vaccine strains play in the epidemiology of BTV, e.g. BTV vaccine strain 2 from SA (JN255941), BTV-2 from India (JQ904064) and BTV-2 from Portugal (EF434179) all demonstrated similar splits probably due to reassortment of the three strains (Fig. 4.3c). Reassortment of genes could be due to co-circulation of strains and/or co-infection of either vector or host. Split networks such as the neighbour-net analysis are capable of representing incompatible and ambiguous signals in data sets by allowing additional splits with weights. This allows split networks to fit the data better than a single bifurcating tree, considering that weights are analogous to the length of a branch in a phylogenetic tree (Huson and Bryant, 2006).

4.4.3 Conserved amino acid regions

Five conserved regions of segment 10 identified previously were present in all 11 BTV isolates analysed (Hwang *et al.,* 1992; Pierce *et al.,* 1998; van Niekerk *et al*., 2003). These regions included the cysteine (C) at amino acid positions 137 and 181, and the tryptophan (W) residue at amino acid 159. Furthermore, two potential N-linked glycosylation sites at amino acid position 63 to 65 (NTT) and 150 to 152 (NGT) (Hwang *et al.,* 1992; Pierce *et al.,* 1998) were present. The hydrophobic regions, N-linked glycosylation as well as the proline-rich regions were also conserved in all 11 isolates. The amino acid variation of NS3/A between strains complimented the phylogenetic networks inferred from the nucleotide sequences. Amino acid changes appear to be topotype-associated, with similar changes in closely related strains. Segments 10 encoding NS3/A from BTV-8 (KJ506703) and

BTV-S299 (KJ506704) had the most amino acid differences of all 11 sequences. Differences in amino acid sequences could be a result of nucleotide substitution or non-synonymous single nucleotide polymorphism caused by genetic reassortment or mutation (Pierce *et al.,* 1998; Bonneau *et al.,* 2001; Balasuriya *et al.,* 2008).

The implication of the differences between non-conserved amino acids with regards to biological properties such as virulence and the ability to be transmitted between the vector and the host needs to be examined. Adaptive selection is not evident (Balasuriya *et al.,* 2008) but corresponding amino acid differences within topotypes could be due to genetic drift of co-circulating viruses in a host-specific fashion (Bonneau *et al*., 2004). The conserved nature of the segment 10 gene suggests that the encoded protein is critical to the biological function and the fitness of the virus in this ecosystem (Pierce *et al*., 1998; He *et al.,* 2010).

4.4 Conclusion

Due to negative selection pressure such as reassortment, genetic drift and/or the founder effect on the BTV NS3/A encoding gene, it was suggested to classify BTV into topotypes (Bonneau *et al.,* 2001; Balasuriya *et al.,* 2008). This allowed for viral strains to be assigned to a specific geographical region based on conserved gene regions such as NS3 (Gould and Pritchard, 1990). In this study, newly sequenced BTV field strains were compared to segment 10 sequences from global BTV isolates. Phylogenetic network analyses (neighbour-joining and neighbour-net) of 11 BTV segment 10 sequences revealed five topotypes (Balasuriya *et al.,* 2008; Maan *et al.,* 2008). The newly sequenced isolates also demonstrated the five previously identified conserved regions (van Niekerk *et al*., 2003; Maan *et al.,* 2008) with intermediate nucleotide identity (>79%) (Maan *et al.,* 2008). Non-structural protein NS3/A shows very little overall variation within the proteins of global BTV strains.

4.5 References

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Chapter 5: Discussion

Global climate changes are believed to be the major driving force for the expansion in the distribution of viral vectors like *Culicoides* and therefore the occurrence of BTV beyond the northern latitude. The recent "global warming" has also allowed for longer time/seasonal periods of activity of biting midges and thus longer periods for the transmission cycle of BTV between vector and host, as an increase in ambient temperature results in an exponential increase in vector capacity (Tweedle and Mellor, 2002; Mullens *et al*., 2004).

The 2006-2008 BTV outbreaks in Europe highlighted the importance of the disease, also in South Africa, yet again. The epidemiology of a disease and the awareness of potential vectors of the diseases are crucial in the implementation of integrated control measures, disease risk analysis and management of the disease. The epidemiology of BT in South Africa is far less studied than in countries such as Europe and the New World. In South Africa, nine *Culicoides* species have been identified that are capable, either under laboratory conditions or in the field, of transmitting BTV (Nevill *et al.,* 1992; Venter *et al.,* 1998, 2006; Paweska *et al.,* 2002, 2003). This along with various host species such as sheep, goats and cattle, with the latter two usually subclinically infected, and the interaction with wildlife complicates the epidemiology of BTV and therefore surveillance and control programs.

In this study, the primary vector associated with BTV within the Mnisi area was *C. imicola.* This result was expected as *C. imicola* thrives in warm subtropical areas (Chapter 2). The occurrence of *C. bolitinos*, also capable of transmitting BTV, was expected during the winter months as *C. bolitinos* prefers cooler temperatures and breeds in the dung of bovine species. The absence of *Culicoides* vector-free periods

indicates that BTV might be present throughout the year and that once vector capacity reaches critical levels, BTV outbreaks can occur. The high abundance of *Culicoides* in both autumn and winter months theoretically also indicates that transmission could continue throughout the winter. This was also demonstrated by the presence of BTV in midges screened with real-time RT-qPCR. The study on the *Culicoides* population at the ARC-OVI in Pretoria (Chapter 3) strengthened the findings after midges collected throughout the winter (June, July and August) tested positive for BTV RNA. Although real-time RT-qPCR cannot distinguish between viable and non-infectious virus the detection of antibodies specific to BTV in serum samples collected from cattle between April (autumn) 2013 and September (spring) 2013 indicated that infectious virus circulated at some point in the area. The occurrence of BTV-specific antibodies, BTV RNA-positive blood samples and the lack of reports of clinical disease demonstrate that BTV infection in cattle in Mnisi is subclinical. This is not surprising, as clinical signs in local breeds of ruminants in Africa, where the virus is known to be endemic, are seen to be less overt or even unapparent than in exotic European breeds (MacLachlan, 1994).

Phylogenetic networks have an important role to play in the reconstruction of evolutionary history (Huson and Bryant, 2006), especially where reassortment of viruses occur. Co-circulation of Western and/or Eastern topotypes of BTV, along with reassortants, could lead to novel BTV strains or serotypes which could be further spread by animal movement, global warming and/or passive vector movements. This could expand the occurrence of the virus and ultimately complicate the epidemiology of BTV.

This study (Chapter 4) demonstrated that BTV isolated in South Africa can be divided into five topotypes, based on gene sequences of segment 10. Newly sequenced BTV strains from South Africa also demonstrates intermediate conservation in the NS3/A protein and display five conserved regions possibly important in viral release from the infected cell and to facilitate transport of the virus to the host cell surface.

Overall, this study contributed in identifying indigenous cattle in South Africa as BTV carriers, albeit being subclinically infected, and supports the hypothesis that cattle can be reservoir maintenance hosts to BTV (Chapter 2). Strong evidence of overwintering of BTV within the *Culicoides* vector is also evident and although no positive VI was found during the winter months (Chapter 3) the high seroprevalence of cattle in the Mnisi area throughout winter indicates that infectious virus might be present (Chapter 2). Newly sequenced strains from South Africa were also added to GenBank and made available for future research (Chapter 4). This would expand the knowledge of BTV in South Africa where the first ever case of BT was recorder and could help in identifying reassortment of viruses when compared to strains elsewhere in the world.

Future studies should focus on oral susceptibility to identify the vector capacity of novel midges such as C*.* sp # 54 (d/f and p/f) and C*.* sp # 50, not just for BTV but also for other arboviruses. Oral susceptibility to different reassortant viruses should also be determined. Results obtained by pool-testing of *Culicoides* do not necessarily demonstrate infection of the whole populations. Individual midges rather than midge pools should be tested for a specific Orbiviruses as to identify the exact species that are able to become infected. This is especially important since *Culicoides* susceptibility to infection with a virus is a genetically heritable trait (Mellor, 2004). Different populations of the same midge species may therefore have varying oral susceptibilities to infection and transmission of a particular serotype or strain of BTV. The need to explain and demonstrate the exact overwintering mechanism of BTV is also vital for control strategies and livestock trade. Virus isolation and/or sequencing of BTV-positive blood samples should be included in future work. The results could demonstrate whether viable virus is present, including the serotype present, and define the occurrence of the virus and therefore the epidemiology of BTV in the north-eastern corner of Bushbuckridge, Mpumalanga Province.

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Appendix Figure 4.3.

Accession numbers of 46 different BTV segment 10 gene sequences from Genbank used in neigbor-net analyses: 8=KJ506706; 7SA=AY857506, 2SA=AF512916; 2SA_V=JN255941, 2IND=JQ904064, 2POR=EF434179, S299= KJ506704, 8SA=AY120938, 8NET=FJ183383, 11CAR=JQ972870, 11USA=AF044704, 8SA_b=AF512919, 16=KJ506697, 2=KJ506696, 7=KJ506695, 7USA=JX272558, 2SA_c=AF512920, 11SA_b=AF512921, 22=KJ506698, 22SA=JX272398, 2ITA=JN255871, 2FRA=AF481093, 11GER=JQ972860, 11=KJ506694, 11BEL=JQ972850, 11SA=AF512922, 11USA=AF044377, 8CAR=AY426604, 9SA=JX2724389, 19=KJ506699, 16AUS=JQ086240, 7AUS=JQ086300, 20AUS=JX272428, 20=KJ506702, 23AUS=JQ086280, 21AUS=JX272418, 2AUS=JQ240330, 21=KJ506701, 21JAP=AB473808, 2IND_b=AF512913, 16PAK=J129386, 16CHI=JN671915, 2SA_b=JQ713563, 23=KJ506700, 16SA=AY775164, 23IND=EU13102, 16GRE=AY449652