

Serological and genetic characterisation of putative new serotypes of bluetongue virus and epizootic haemorrhagic disease virus isolated from an Alpaca

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Dissertation submitted in partial fulfilment of the requirements
for the degree *Magister Scientiae* in *Biochemistry* at the
Potchefstroom Campus of the North-West University

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May 2014

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TITLE AND ABSTRACT OF POSTER PRESENTED AT THE ANNUAL CONGRESS OF THE SOUTH AFRICAN ASSOCIATION OF VETERINARY TECHNOLOGISTS HOSTED AT BERG-EN-DAL, KRUGER NATIONAL PARK ON THE 30 TH AND 31 ST OCTOBER 2012	102
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ABSTRACT

Alpacas were first introduced into South Africa during the year 2000. They are valuable because of the fine quality wool they produce which has much better insulation properties than that of merino wool fibres. Alpacas are also used to act as guards of sheep herds against predators.

During 2008, blood samples from an alpaca that died acutely with severe lung oedema, respiratory distress and froth exuding from the nose were received at Elsenburg Veterinary Laboratory. The alpaca was from a herd of 23 alpacas of a British veterinarian in the Montagu district in the western Cape. Virus isolation attempts on the blood produced infrequent embryo mortalities. Embryonated chicken egg (ECE) material was sent to the Virology Department at the Onderstepoort Veterinary Institute (OVI). A bluetongue virus (BTV) PCR performed at the diagnostic PCR laboratory at OVI on the ECE material was positive. Further intra-venous (IV) inoculations in ECE produced embryo mortalities on two consecutive days, the 8th and 9th November. The dead embryos were harvested separately and named and treated as two separate virus samples, Alp8 and Alp9 which were further passaged on baby hamster kidney (BHK) cells.

The BTV virus neutralisation tests (VNT) performed at the Office International des Epizooties (OIE) Laboratory on both Alp8 and Alp9 were negative. Because of the close serological relationship between BTV and epizootic haemorrhagic disease virus (EHDV), an EHDV VNT was also performed and was also negative.

In the light of the negative VNT and the positive BTV PCR results, more in-depth molecular analyses were performed. RNA was purified from tissue culture material and agarose gel electrophoresis (AGE) performed. Both Alp8 and Alp9 had a typical orbiviral electrophoretic profile, but their respective profiles were different.

A sequence-independent reverse transcriptase PCR amplification method generated ample complementary DNA (cDNA) of both samples for sequencing. Sanger sequencing was used to partially sequence genome segments 5 (NS1) and 2 (VP2). BLAST analysis of the partial information of the genome segments 5 (NS1) of Alp8 confirmed it as being a BTV and Alp9 as being an EHDV. BLAST analysis of the deduced amino acid sequence generated of VP2 of both Alp8 and Alp9 established that these samples were possibly new serotypes of BTV and EHDV respectively. The complete genome of both Alp8 and Alp9 was sequenced with next generation 454 Pyrosequencing. This confirmed the partial sequencing results. BLAST

analysis of the complete sequence of S2 (VP2) of Alp8 showed that it has 73 % nucleotide and 77 % deduced amino acid identity to BTV15. In contrast the nucleic acid sequence of genome segment S2 (VP2) of Alp9 had no nucleotide sequence identity to any virus, but its deduced amino acid sequence had 71 % amino acid identity to EHDV2.

Hyper immune guinea pig (GP) serum prepared against the putative new BT (Alp8) and EHD (Alp9) virus serotypes were tested for serological cross-reactivity against the 24 OIE reference antigen strains of BTV and the 8 OIE reference antigen strains of EHDV. Alp8 had a neutralising antibody (NAb) titre of ≥ 32 against BTV15. Alp9 did not cross react with any of the OIE BTV and EHDV strains.

Six out of the remaining 22 alpacas on the farm had NAb to a greater or lesser extend against Alp8 (BTV) and Alp9 (EHDV) viruses, which confirmed that the viruses were also present in other alpacas in the herd. Very few cases of EHDV in alpacas have ever been reported in literature.

A small scale pilot vector susceptibility study showed that vector competence of *C. imicola* for both Alp8 and Alp9 was low, below 2 %. The fact that neutralising antibodies to Alp8 and Alp9 were detected in other alpacas in the herd raises the question as to whether there are other *Culicoides* species circulating in the area that could vector the viruses.

In conclusion, the results from the serological and virological analyses as well as the nucleic acid sequence data of the genomes of two virus samples, Alp8 and Alp9, from an alpaca that died in the Montagu district in the western Cape identified Alp9 as a definite new serotype of EHDV and Alp8 as a possible new serotype of BTV most closely related to BTV15.

Keywords:

Bluetongue virus, Epizootic haemorrhagic disease virus, Genetic characterisation, Serological characterisation, New serotypes

OPSOMMING

Alpakkas is die eerste keer gedurende die jaar 2000 in Suid-Afrika ingebring. Hulle is waardevol vir die besondere kwaliteit van wol wat hulle produseer wat baie beter isolerende eienskappe as die van merino wol vesels het. Alpakkas word ook gebruik as beskermers van skaap troppe teen roofdiere.

Gedurende 2008 is bloed monsters van 'n alpaka wat akuit gevrek het met erge long edeem, respiratoriese asemnood en 'n skuim uitloopsel uit die neus by Elsenburg Veterinêre Laboratorium ontvang. Die alpaka was een van 'n trop van 23 alpakkas van 'n Britse veearts in die Montagu distrik in die wes Kaap. Isolasië pogings uit die bloed het ongereelde vrektes veroorsaak in bebroeide hoender eiers. Bebroeide eier materiaal is na die Virologie Departement by die Onderstepoort Veeartsenykunde Instituut (OVI) gestuur. 'n Bloutong virus polimerase ketting reaksie (BTV PKR) toets wat uitgevoer is op die embryo materiaal was positief. Verdere intraveneuse inokulasie in kuiken embryos het vrektes op twee opeenvolgende dae, die 8^{ste} en 9^{de} November opgelewer. Die dooie embryos is apart geoes en benoem en verder behandel as twee aparte virus monsters, Alp8 en Alp9 wat verder passeer is op baba hamster nier (BHK) selkulture.

'n BTV virus neutralisasie toets (VNT) wat uitgevoer is by die Office International des Epizooties (OIE) laboratorium was negatief. As gevolg van die noue serologiese verwantskap tussen BTV en episootiese haemorrhagiese siekte virus (EHDV) is 'n EHDV VNT ook uitgevoer, en was ook negatief.

In die lig van die negatiewe VNT en die positiewe BTV PKR resultaat, is meer in-diepte molekulêre analise vervolgens gebruik. Ribonukleïensuur (RNS) is gesuiwer uit die selkultuur materiaal en agarose gel elektroforese (AGE) is uitgevoer. Beide Alp8 en Alp9 het 'n tipiese orbivirale elektroforese profiel gehad, hoewel die onderskeie profiele verskillend was.

'n Volgorde-onafhanklike omgekeerde transkripsie genoom amplifiseringsmetode het genoegsame komplementêre DNS van beide monsters gegenereer vir volgordebepaling. Sanger volgordebepaling is gebruik vir die gedeeltelike volgordebepaling van genoom segmente 5 (NS1) en 2 (VP2) van Alp8 en-9. 'BLAST' ontleding van die gedeeltelike inligting van genoom segment 5 (NS1) van Alp8 het bevestig dat dit 'n BTV is en Alp9 'n EHDV is. 'BLAST' ontleding van die afgeleide aminosuur volgorde wat verkry is van VP2 van beide Alp8 en Alp9 dui aan dat die monsters moontlik nuwe serotipes van BTV en EHDV onderskeidelik is. Die volledige nukleïensuurvolgorde van die genoom van beide Alp8 en

Alp9 is bepaal met volgende generasie 454 Pyro-volgordebepaling. Dit het die gedeeltelike volgordebepaling resultate bevestig. 'BLAST' analise van die volledige volgorde van S2 (VP2) van Alp8 wys dat dit 73 % nukleotied en 77 % afgeleide aminosuur identiteit toon met BTV15. Die nukleïensuur volgorde van genoom segment 2 (VP2) van Alp9 het geen nukleotied volgorde identiteit met enige virus getoon nie, maar die afgeleide aminosuur volgorde het 71 % aminosuur identiteit met EHDV2 getoon.

Hiper-immuun marmot serum wat berei is teen die vermeende nuwe BT- en EHD serotipes is getoets vir serologiese kruis reaktiwiteit teen die 24 OIE verwysings antigeen serotipes van BTV en die 8 OIE verwysing antigeen serotipes van EHDV. Alp8 het 'n neutraliserende teenliggaam titer van ≥ 32 gehad teen BTV15. Alp9 het geen kruisreaktiwiteit getoon met enige van die 24 BTV of 8 EHDV virus serotipes nie.

Ses uit die oorblywende 22 alpakkas op die plaas het neutraliserende teenliggame tot 'n meerdere of mindere mate gehad teen Alp8 (BTV) en Alp9 (EHDV) virusse, wat bevestig dat die virusse teenwoordig was in ander alpakkas in die trop. Baie min gevalle van EHDV in alpakkas is al aangemeld in literatuur.

'n Klein skaal vektor-vatbaarheids studie het aangedui dat vektor bevoegdheid van *C. imicola* vir beide Alp8 en Alp9 laag was, minder as 2 %. Die feit dat neutraliserende teenliggame teen Alp8 en Alp9 gevind is in die res van die trop laat die vraag ontstaan of daar ander *Culicoides* spesies sirkuleer wat die virus kan vektor.

Ter afsluiting, die resultate van die serologiese en virologiese analise sowel as die nukleïensuur volgorde data van die genome van twee virus monsters, Alp8 en Alp9, van 'n alpakka wat gevrek het in die Montagu distrik in die wes Kaap identifiseer Alp9 as 'n definitiewe nuwe serotipe van EHDV en Alp8 as 'n moontlike nuwe serotipe van BTV wat die naaste verwant is aan BTV15.

Sleutelwoorde:

Bloutong virus, Episootiese haemorrhagiese siekte virus, Genetiese karakterisering, Serologiese karakterisering, Nuwe serotipes

ACKNOWLEDGEMENTS

My sincere appreciation towards the following persons:

- Prof. A.A. van Dijk for the valuable support you gave me in completing this project.
- Prof. A.C. Potgieter for your guidance, understanding and patience in leading me through the molecular aspects of this thesis. I admire you for your knowledge.
- Dr. G.J. Venter and Entomology personnel for the help and support with the vector susceptibility study.
- Dr. G.H. Gerdes for sharing your enthusiasm in Virology.
- All personnel at the Virology Department and other Departments at OVI for your help in different aspects of this study.
- My mother and late father. You always believed in me, and taught me that anything is possible with hard work and dedication and the faith in our Lord to lead us through all we pursue.
- My dear husband. Your understanding, love and support through all the difficult times made this task easier.
- My children, I want to be an inspiration for you.
- Our HEAVENLY FATHER, for giving me all I have and for what I am, I praise YOU.

Romeine 11:33-36 Lofsang

O Diepte van die rykdom en wysheid en kennis van God! Hoe ondeurgrondelik is sy oordele en onnaspeurlik sy weë! Want wie het die gedagte van die Here geken, of wie was sy raadsman gewees? Of wie het eers iets aan Hom gegee, dat dit hom vergeld moet word? Want uit Hom en deur Hom en tot Hom is alle dinge. Syne is die heerlijkheid tot in ewigheid.

Amen

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND TO THIS STUDY

Original observation

In November 2008, chicken embryo material from a virus isolation attempt from heparin blood from an alpaca (*Vicugna pacos*) (Figure 1) was submitted to Onderstepoort Veterinary Institute (OVI) from Deltamune for serotyping. The alpaca was from a herd kept for the production of wool in the Montagu district in the Western Cape, and was imported from Chile the previous year.

Alpacas were first introduced into South Africa in 2000. Alpacas are farmed for their fine quality wool fibre and to work on commercial sheep farms as herd guards against small predators. The herd guard alpacas learn to adopt the sheep as their family and protect them instinctively <http://www.alpacasociety.co.za>.



Figure 1: Alpacas. They are farmed for the fine quality wool they produce, and are kept also to guard flocks of sheep against predators.

(Photo with permission from Manor House Alpacas)

The owner of the alpaca (a veterinarian) had previous concerns of bluetongue (BT) infection in the herd when subclinical signs were observed. Antibody responses were however negative.

The alpaca suffered from an acute disease onset and died within 3 hours from the time clinical signs were first observed. Clinical signs included respiratory distress, severe lung oedema, froth exuding from the nose and subcutaneous bleedings of the buccal cavity (Figure 2A). Post mortem findings included point bleedings on the lungs and severe hydrothorax (Figure 2B).

An initial observation by the owner based on the clinical signs raised the suspicion of bluetongue virus (BTV) involvement. The owner's suspicion was confirmed with a BTV PCR performed by the PCR diagnostic laboratory at OVI.

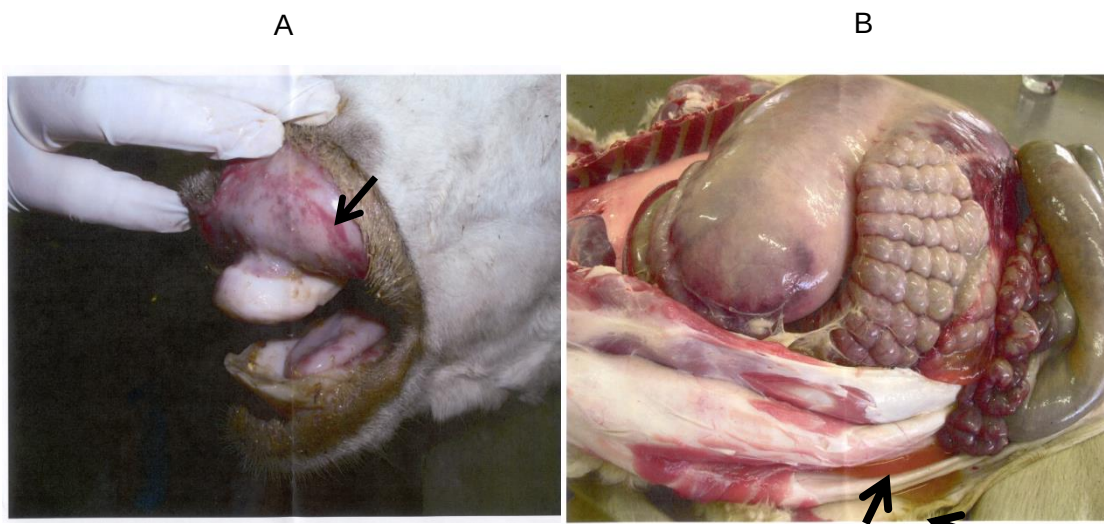


Figure 2: Clinical signs of BT in the alpaca. A) Subcutaneous bleedings of the buccal cavity. B) Post mortem of the alpaca shows severe hydrothorax (accumulation of serous fluid in the pleural cavities around the heart/lung area) and point bleedings on the lungs.

Subsequent passages of the chicken embryo material caused mortalities on the third and fourth day post intra-venous (i.v.) inoculation in embryonated chicken eggs (ECE). The embryos were harvested separately and kept separate in subsequent passages on baby hamster kidney clone 21 (BHK-21) cells. The isolates were labelled Alp8 and Alp9, according to the different dates the embryos died.

All positive BT and epizootic haemorrhagic disease (EHD) as well as African horsesickness (AHS) and equine encephalosis (EE) samples, isolated in the Virology Division of OVI are submitted to the Office International des Epizooties (OIE) reference laboratory. These samples are serotyped as part of the OIE laboratories mandate. Annual reports are made to

the OIE head office. This data is used in surveillance studies of the epidemiology of specific diseases.

1.2 LITERATURE REVIEW

1.2.1 General Virology

The earliest description of, what later would be classified as viruses (Latin *virus*, poison, slime) – was a “filterable, living liquid”, too small to be observed through a light microscope, but being capable to cause disease (Beijerinck, 1898). The first virus to be described under Beijerinck’s classification was the tobacco mosaic virus, a plant virus. However, this study entails viral infections of ruminants.

Foot-and-mouth disease was the first animal virus to be described under the term for a ‘filterable agent’ in 1898 by Loeffler and Frosch (1898). This outbreak of foot-and-mouth disease followed the rinderpest pandemic that swept through Europe during the 18th century in cattle.

Rinderpest is a devastating infectious disease of cattle, and has a mortality rate higher than 70 %. The virus killed an estimated 200 million cattle in Europe alone during the 18th century (Yamanouchi, 2012). Arnold Theiler faced the same picture in South Africa when he was commissioned to prevent or stop the rinderpest pandemic in the late 1800s. Prevention of rinderpest was reported by Theiler in 1896 with the use of immune serum (Taylor *et al.*, 2006). The rinderpest ‘pandemic’ in the Cape of Good Hope during the first years of the 20th century left only around 5 % surviving cattle and wild ungulates, leading to a standstill in animal transport and famine amongst people (Roeder, 2011).

During the same period as the rinderpest outbreak, Theiler faced another challenge in a disease infecting mostly merino sheep, namely BT (Theiler, 1906).

More recently, BT outbreaks between 2006 and 2008 in Belgium caused 42 % mortalities in sheep and 18 % in cattle (Webb, 2008).

1.2.2 *Identification of unknown viruses*

“To distinguish between viruses belonging to different species of a genus, one obviously cannot rely on properties that are shared by all the members of the genus like virion morphology, genome organisation or method of replication. Properties that are useful for differentiating members of different species are the natural host range, cell and tissue tropism, pathogenicity, mode of transmission, certain physiochemical and antigenic properties of virions and genome sequence similarities” Quoted from Van Regenmortel (2007).

The isolation and identification of any microorganism causing disease rely heavily of the postulates of Robert Koch which are as follows (Koch, 1876):

- The microorganism must be found in abundance in all organisms suffering from the disease, but should not be found in healthy organisms.
- The microorganism must be isolated from a diseased organism and grown in pure culture.
- The cultured microorganism should cause disease when introduced into a healthy organism.
- The microorganism must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

With the dawn of veterinary research in South Africa in the late 1800s and early 1900s these basic guidelines were applied in efforts to conquer disease in animals. Isolation attempts are usually directed by the expected outcome.

Methods used for the identification of viruses are described below.

Using the principles of Koch's postulates, the blood of an infected animal or infected organs from animals that died of disease, would be used in the inoculation of the same animal species as the original animal species. Work done on BTV by Spreull (1905) and Theiler (1906) used blood from infected sheep to re-infect healthy sheep in studies that challenged the immune status of other sheep. Theiler (1909) described a fever in horses in 1907 that resembles AHS, but with no protective immunity against challenge with blood from infected mules. In his studies to determine the connection between this ephemeral fever he used blood from horses that recovered from AHS to inoculate mules. The mules were monitored for fever and the duration of fever. In the ensuing experiments, Theiler could clearly distinguish between more virulent virus strains and less virulent virus strains as well as animals more and less protected against the virus strains used.

Other systems for virus isolation was the use of ECE (Mason *et al.*, 1940). The ECE is the most sensitive and versatile system for isolation of viruses. Mason *et al.* (1940) was the first to isolate BTV using ECE and manage to attenuate virus through serial passages in ECE. Goldsmit and Barzilai (1965) found that with i.v. inoculation rather than inoculation in the yolk sac of ECE as described by Alexander (1947), two- to three-fold better sensitivity is achieved in isolation of BTV. Small laboratory animals such as suckling mice (Kipps, 1958) or adult mice may also be used. Suckling mice were found to be also suitable for the isolation of BTV. The susceptibility of suckling mice for BTV was later used by Kipps for the production of complement fixation testing (CFT) antigen (Kipps, 1956).

The first reports of work on TC were from Wilhelm Roux in 1885. The medullary plate of an embryonic chicken could be maintained in warm saline for several days, thereby establishing the basic principle of TC. Sophistication of the technique led to *in vitro* cultivation of numerous TC systems from frog nerve cells by Ross Granville Harrison in 1907, the growth of small pox virus (variola) on fragments of guinea pig (GP) corneal tissue (Steinhardt and Grund, 1915) to insect TC systems, and the widely used BHK-21 clone 13 system as described by Macpherson and Stoker (1962). The advantage of TC systems is its use in numerous applications, from virus isolation to large scale vaccine production (Howell, 1963). Research in virology without the use of TC systems is nowadays unthinkable. Moreover so, as the use of experimental animals become more and more problematic in terms of ethical issues.

Haig *et al.* (1956) were the first to use TC for antibody detection studies in BTV work. The first report of *in vitro* cross neutralisation testing of BTV was by Kipps (1958). The virus neutralisation test (VNT) system evolved from using serum-virus mixtures inoculated in suckling mice to applying the same principle using TC systems. VNT assays as applied currently, were developed by Parker *et al.* (1975) where a known mono-specific GP hyper-immune serum is titrated against an unknown virus.

Plaque assays were proven to be very helpful in a study of the immunological differences between BTV strains (Howell *et al.*, 1967). Howell *et al.* (1970) described the use of fish-spine beads filled with hyper immune GP serum against the 16 different serotypes of BTV to identify newly isolated strains of BTV.

The development of molecular techniques greatly enhanced the capabilities for identification of different viruses involved in causing disease. Verwoerd *et al.* (1970) determined the double-stranded nature of RNA of the BTV genome. Molecular studies on the structure of BTV (Verwoerd *et al.*, 1972) showed the segmented nature of BTV that led to its

classification as an *Orbivirus*. Electron micrographs of the same virus supported the results by Verwoerd *et al.* (1972). The finding that the outer layer of the BT virion contains virus specific proteins by Verwoerd *et al.* (1972) led to the first attempts to use VP2 (S2) as a subunit vaccine for sheep (Huisman *et al.*, 1987a).

Following the sequencing of the 10 genome segments of BTV by Roy (1989) it was possible to perform hybridisation studies and reverse transcriptase PCR techniques (RT-PCR) (Dangler *et al.*, 1990). RT-PCR techniques are used as a diagnostic tool in numerous diagnostic laboratories for the detection of BTV nucleic acids. Advances made on molecular studies of Orbiviruses were published by Potgieter *et al.* (2009), with reports of sequence-independent complementary DNA (cDNA) synthesis and PCR amplification of dsRNA genomes. Sequence data derived from the sequencing of individual genome segments, are more frequently used for the identification of new virus serotypes (Maan *et al.*, 2007).

1.2.3 *History of BTV and EHDV*

1.2.3.1 *History of BTV*

Twenty six serotypes of BTV have to date been analysed and described (Maan *et al.*, 2011b) (Chaignat *et al.*, 2009). The collection and identification of BTV serotypes 1 to 24 were done over an 87 year period, from 1905 to 1992 by Spreull (1905) and Theiler (1906), Neitz *et al.* (1948), Howell *et al.* (1970), and Erasmus (unpublished reports). Twenty one of these serotypes were first identified in South Africa from samples collected within South Africa.

BT as an infectious disease in sheep first became apparent with the introduction of merino and European sheep breeds into the Cape Colony of South Africa in 1652 and again later in 1870. Merino and European sheep breeds are much more susceptible to BT than indigenous sheep breeds (Hutcheon, 1881).

The existence of various BTV serotypes was suspected when it was observed that reinfection of sheep that have recovered from BT occurred (Spreull, 1905) (Theiler, 1906). During the early 1900s, Theiler developed a crude vaccine against BT. Theiler used blood from recovered sheep, and performed serial passages through sheep to attenuate the virus. Theiler's BTV vaccine was used to immunise sheep flocks between 1907 and 1943 (Henning, 1956). The serotype used then as the vaccine, was later serotyped as BTV4, and the production was stopped as it became evident that it did not protect sheep against the

multiple circulating serotypes (Henning, 1956). Neitz (1948) demonstrated the first evidence of the plurality of antigenically different strains of BTV by cross protection studies in sheep.

In an effort to solve the problem on concurrent reinfection, Howell *et al.* (1970) undertook a series of experiments to identify the various circulating serotypes of BTV. Data of all reported serotypes, as discovered by Howell, Erasmus and others are recorded in Table 1.

1.2.3.1.1 The identification of BTV serotypes 1 – 24

In his studies, Howell used infective blood, of which 2 samples were submitted from outside South Africa and stored in oxalate-carbolic-glycerine (OCG) that were submitted over a 58 year period, as antigen. Serum from inoculated sheep was collected 30-45 days post inoculation. This serum was used in the first cross neutralisation studies on lamb-kidney cells. Neutralisation testing was performed using TC roller tubes (Howell, 1960).

From this initial data, 12 serotypes were identified. Serotype 3 was from Cyprus, and serotype 10 from Portugal (Howell *et al.*, 1970). Following the first experiment and grouping of 12 serotypes of BTV, an attempt to isolate as many serotypes as possible was made during the BT season of 1959 in South Africa. One hundred and fourteen further isolates were made. Of the 114 isolates, 8 isolates from the Ermelo district and from Natal were not neutralised by the 12 sheep antisera. Cross-neutralisation was done on antigen and serum derived from the untyped isolates, and 2 further serotypes were identified. Experimental data clearly indicated that serotype 2 was the most prevalent serotype circulating in the Transvaal region, and serotype 1 in the Cape Province. Following work done by Du Toit (1962) on the role cattle play in the epizootiology of BT, Howell *et al.* (1970) reported the isolation of serotype 15 from viraemic sentinel cattle. During this study, neutralising antibodies (NAb) against multiple serotypes, namely serotypes 1, 2, 5, 7, 8, 12 and 15 were detected in their blood samples.

Serotype 16 was isolated from sheep during an outbreak of BT during 1959 in Pakistan (Sapre, 1964). BTV17 was isolated in the United States of America by Barber (1979), and serotyped by Erasmus (Unpublished). Serotype 18 and 19 were isolated from clinically ill sheep during 1976 (Erasmus, Unpublished), BTV20 was isolated in Australia and BTV21 was isolated from cattle in Australia by St George (1978, 1980) and serotyped by Erasmus (unpublished). BTV22 (Nevill *et al.*, 1992) and BTV23 were isolated by Nevill from wild caught *Culicoides*. Erasmus (Unpublished data) isolated BTV24 from sheep clinically ill with BT.

1.2.3.1.2 The identification of BTV serotypes 25 and 26

During November and December 2007, BTV was detected in clinically healthy goats from the Toggenburg region in Switzerland by BTV-specific antibody enzyme linked immune absorbent assay (ELISA) and real time reverse transcriptase PCR (rtRT-PCR), based on BTV genome segment 10, (Chaignat *et al.*, 2009). Further studies, using an rtRT-PCR were able to detect the current 24 circulating serotypes (Toussaint *et al.*, 2007) yielded negative or weak positive results. This virus was initially named Toggenburg *Orbivirus*, and has since been designated BTV25 after sequencing results of genome segments 2, 5, 6, 7, 8, 9, and 10 of the virus (Hofmann *et al.*, 2008) indicated a high sequence similarity to BTV.

BTV26 (Kuwait) was isolated and identified shortly after BTV25 (Maan *et al.*, 2011b). Blood samples of 2 animals from a goat and sheep flock in Kuwait showing clinical signs consistent with BT infection, tested positive on an RT-PCR assay selective for genome segment 10 (Orru *et al.*, 2006). Blood from one of the animals with a RT-PCR cycle threshold value (Ct) of 28 was used for isolation attempts. RNA from cell culture material showing atypical cytopathic effects (CPE) generated a typical BTV–dsRNA pattern on agarose gel electrophoresis (AGE). Comparative studies of sequences of genome segments 2, 3, and 7 with other BTV serotypes suggested a new serotype of BTV. Further confirmation was gained when reference GP serum against the BTV known serotypes (1-24) and goat serum from BTV25 did not neutralise virus in VNT (Maan *et al.*, 2011a)

Table 1: Summary of isolation information of the serotypes of BTV

*Serotypes	Year	Origin	Species isolated from	References
1	1958	Biggarsberg – South Africa	Sheep	(Howell <i>et al.</i> , 1970)
2	1958	Vryheid - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
3	1944	Cyprus	Sheep	(Gambles, 1949)
4	1900	Cape Province - South Africa	Sheep	(Theiler, 1906)
5	1953	Machadodorp - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
6	1958	Vryheid - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
7	1955	Utrecht - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
8	1937	Onderstepoort - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
9	1942	Pretoria - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
10	1956	Portugal	Sheep	(Howell <i>et al.</i> , 1970)
11	1944	Beaufort -West - South Africa	Sheep	(Howell <i>et al.</i> , 1970)
12	1941	Pretoria - South Africa	Cattle	(Howell <i>et al.</i> , 1970)
13	1959	Transvaal/Natal - South Africa	Unknown	(Howell <i>et al.</i> , 1970)
14	1959	Transvaal/Natal - South Africa	Unknown	(Howell <i>et al.</i> , 1970)
15	1960	Ermelo district - South Africa	Cattle	(Howell <i>et al.</i> , 1970)
16	1959	West Pakistan	Sheep	(Sapre, 1964)
17	1979	United States	Unknown	(Barber, 1979)
18	1976	South Africa	Sheep	(Erasmus, Unpublished)
19	1976	South Africa	Sheep	(Erasmus, Unpublished)
20	1978	Australia	<i>Culicoides</i>	(St. George <i>et al.</i> , 1978)
21	1980	Australia	Cattle	(St George <i>et al.</i> , 1980)
22	1992	South Africa	<i>Culicoides</i>	(Nevill <i>et al.</i> , 1992)
23	1987	Australia	Cattle	(Gard <i>et al.</i> , 1987)
24	1992	South Africa	Sheep	(Erasmus, Unpublished)
25	2007	Toggenburg – Switzerland	Goats	(Chaignat <i>et al.</i> , 2009, Hofmann <i>et al.</i> , 2008)
26	2010	Kuwait	Sheep	(Maan <i>et al.</i> , 2011b)

*Data on BTV1 - BTV16 was partly compiled from Howell, P.G., DVSc. thesis (1969)

1.2.3.2 History of EHDV

Recordings of EHD in deer since 1890 throughout the south-eastern USA were made by woodsmen and hunters. EHD was known as “black tongue” (Shope *et al.*, 1955). EHDV was formally identified by Shope *et al.* (1955) who isolated EHDV from white-tailed deer (WTD) (*Odocoileus virginianus*) from New Jersey. EHD is regarded as the most important infectious disease of WTD in the USA (Nettles and Stallknecht, 1992). However, acute disease can also be caused by EHDV in various domestic and wild ruminants (Shope *et al.*, 1960), especially when introduced to new areas (Allison *et al.*, 2012). The different EHDV isolates were initially serotyped using VNTs, but the lack of well characterised reference strains caused mistyping of serotypes. For example the initial untyped EHDV strain “318” isolated from Bahrain during 1983 was identified as serotype 6 by means of serological and phylogenetic comparison of the full genome (Anthony *et al.*, 2009b). During the same study it was demonstrated that serotypes 1 and 3 are analogous, based on genetic and phylogenetic comparison (Anthony *et al.*, 2009b, Anthony *et al.*, 2009a). Phylogenetic and serological data presented by Anthony *et al.* (2009b) suggest that the EHDV group consists of seven as opposed to the 8 serotypes generally recognised in literature (Mertens *et al.*, 2005).

1.2.4 Classification of the *Orbivirus* genus in the family *Reoviridae* with specific reference to BTV and EHDV

Bluetongue virus and EHDV both belong to the *Orbivirus* genus (Matthews, 1982, Borden *et al.*, 1971) of the family *Reoviridae* (respiratory enteric orphan – viruses considered to be not associated with disease, hence called ‘orphan’ viruses). Twenty two virus species are listed under the genus, as well as a further 10 tentative unassigned virus species (Mertens *et al.*, 2005) (Table 2). BTV is regarded as the type-species in the family *Reoviridae* (Borden *et al.*, 1971, Verwoerd, 1970). Both BTV and EHDV have a double-stranded RNA genome (Verwoerd, 1969, Livingston and Moore, 1962). The distinctive characteristic features of the genus *Orbivirus*, is that the genomes of all the orbiviruses are composed of 10 linear dsRNA segments (Verwoerd, 1969). Seven of the ten genome segments encode for structural virus proteins (VP) and the other 3 for non-structural viral proteins (NS) (Mecham and Dean, 1988).

The large doughnut shaped capsomeres seen on the surface of virus particles on electron microscopic (EM) observation (Borden *et al.*, 1971) led to the name *Orbivirus* (Latin: ring) – defining the structure of the particle.

All Orbiviruses (Table 3) are transmitted by specific arthropod vectors, namely different species of ticks, mosquitoes or midges (Du Toit, 1944, Mertens *et al.*, 2005).

BTV and EHDV are serologically the most closely related viruses in the *Reoviridae* family (Inaba, 1975).

Of all the Orbiviruses, BTV has had the biggest impact on the global economy (Attoui *et al.*, 2009). Losses associated with BTV for 2007 was estimated at \$130 million in the United States. The BTV8 outbreak in France resulted in losses estimated at \$1.4 billion (Tabachnick *et al.*, 2008). These losses were calculated comprehensively, and include direct losses to animals, losses due to vaccination, and losses due to trade restrictions. The BTV8 outbreak in 2007 did cost the Netherlands an estimated \$85 million (Tabachnick *et al.*, 2008). BT also causes breakage in wool. Often, the entire fleece will be casted (Erasmus, 1975), causing serious losses in the wool industry. Trade restrictions are also affecting the export of embryos. To determine whether ewes were exposed to a recent BTV infection, ewes are tested twice to determine whether any change in the BTV antibody titre occurred. Besides the testing of ewes to determine serostability before export, embryos for export must undergo a comprehensive washing process to wash away any potential BTV particles adhering to the embryo (Venter *et al.*, 2011). These measures contribute greatly to the cost of BTV control.

BTV has been studied quite extensively. It will therefore, for the scope of this study, be dealt with in greater depth than EHDV. The difference between BTV and EHDV in terms of hosts and vectors will, however, be discussed specifically.

Table 2: The genera of the family *Reoviridae* With permission P.P.C. Mertens

Genera	Number of genome segments(genome size)	Hosts (vectors)
<i>Aquareovirus</i>	11 (~23.7 kbp*)	<i>Molluscs</i> , finfish, <i>Crustacea</i>
<i>Cardoreovirus</i>	12 (~20.15 kbp)	Crabs
<i>Coltivirus</i>	12 (~29 kbp)	Mammals (including humans) [ticks]
<i>Cypovirus</i>	10 (~24.8 to 33.3 kbp)	Insects (<i>Lepidoptera</i> , <i>Diptera</i> and <i>Hymenoptera</i>), a single isolate was reported from a freshwater daphnid
<i>Fijivirus</i>	10 (~28.7 kbp)	Plants (<i>Graminae</i> , <i>Liliaceae</i>) (delphacid plant hoppers)
<i>Dinovernavirus</i>	9 (~23.35 kbp)	Mosquitoes
<i>Idnoreovirus</i>	10 + 1 (~25.1)	Insects (<i>Hymenoptera</i>)
<i>Mimoreovirus</i>	11 (~25.56 kbp)	Phytoplankton
<i>Mycoreovirus</i>	11 or 12 (~24.4 kbp)	Fungi
<i>Orbivirus</i>	10 (~19.2 kbp)	Mammals (including humans), birds [<i>Culicoides</i> ,mosquitoes, <i>phlebotomines</i> , ticks]
<i>Orthoreovirus</i>	10 (~23.5 kbp)	Birds, reptiles, mammals
<i>Oryzavirus</i>	10 (~26.1 kbp)	Plants (<i>Graminae</i>) [<i>delphacid</i> plant hoppers]
<i>Phytoreovirus</i>	12 (~25.1 kbp)	Plants (<i>cicidellid</i> leafhoppers)
<i>Rotavirus</i>	11 (~18.5 kbp)	Birds, mammals
<i>Seadornavirus</i>	12 (~21 kbp)	Mammals (including humans) [mosquitoes]
Unassigned <i>Reoviruses</i> (potentially representing new genera)	10, 11 or 12	

*kbp – Kilo-basepairs

Table 3: Summary of members of the genus *Orbivirus* (With permission, P.P.C. Mertens)

Virus species	Number of serotypes/strains	Vector species	Host species
African horse sickness virus (AHSV)	9 numbered serotypes (AHSV-1 to AHSV-9)	<i>Culicoides</i> spp.	Equids, dogs, elephants, camels, cattle, sheep, goats, humans (in special circumstances), predatory carnivores (by eating infected meat)
Bluetongue virus (BTV, <i>Orbivirus</i> type-species)	24 numbered serotypes (BTV1 to BTV24)	<i>Culicoides</i> spp.	All ruminants, camelids and predatory carnivores (by eating infected meat)
Changuinola virus (CGLV)	12 named serotypes	Phlebotomine sand flies, culicine mosquitoes	Humans, rodents, sloths
Chenuda virus (CNUV)	7 named serotypes	Ticks	Seabirds
Chobar Gorge virus (CGV)	2 named serotypes	Ticks	Bats
Corripata virus (CORV)	6 named serotypes/strains	Culicine mosquitoes	Humans, rodents
Epizootic haemorrhagic disease virus (EHDV)	7 numbered or named serotypes/strains	<i>Culicoides</i> spp.	Cattle, sheep, deer, camels, llamas, wild ruminants, marsupials
Equine encephalosis virus (EEV)	7 numbered serotypes (EEV-1 to EEV-7)	<i>Culicoides</i> spp.	Equid
Eubenangee virus (EUBV)	4 named serotypes	<i>Culicoides</i> spp. anopheline and culicine mosquitoes	Unknown host
Ieri virus (IERIV)	3 named serotypes	Mosquitoes	Bird
Great Island virus (GIV)	36 named serotypes/strains	Argas, Ornithodoros, Ixodes ticks	Seabirds, rodents and human
Lebombo virus (LEBV)	1 numbered serotype (LEBV-1)	Culicine mosquitoes	Humans, rodent
Orungo virus (ORV)	4 numbered serotypes (ORUV-1 to ORUV-4)	Culicine mosquitoes	Humans, camels, cattle, goats, sheep, monkey
Palyam virus (PALV)	13 named serotypes/strains	<i>Culicoides</i> spp., culicine mosquitoes	Cattle, sheep
Peruvian horse sickness virus (PHSV)	1 numbered serotype (PHSV-1)	Mosquitoes	Horse
St. Croix River virus (SCRV)	1 numbered serotype: (SCRV-1)	Ticks	Hosts unknown
Umatilla virus (UMAV)	4 named serotypes	Culicine mosquitoes	Bird
Wad Medani virus (WMV)	2 named serotypes	Boophilus, Rhipicephalus, Hyalomma, Argas ticks	Domesticated animal
Wallal virus (WALV)	3 serotypes/strains	<i>Culicoides</i> spp.	Marsupial
Warrego virus (WARV)	3 serotypes/strains	<i>Culicoides</i> spp., anopheline and culicine mosquitoes	
Wongorr virus (WGRV)	8 serotypes/strains	<i>Culicoides</i> spp., mosquitoes	Cattle, macropod
Yunnan orbivirus			Yunnan orbivirus

Tentative species			
Andasibe virus (ANDV)		Mosquitoes	Unknown host
Codajas virus (COV)		Mosquitoes	Rodents
Ife virus (IFEV)		Mosquitoes	Rodents, birds, ruminants
Itupiranga virus (ITUV)		Mosquitoes	Unknown hosts
Japanaut virus (JAPV)		Mosquitoes	Unknown hosts
Kammavanpettai virus (KMPV)		Unknown vectors	Birds
Lake Clarendon virus (LCV)		Ticks	Birds
Matucare virus (MATV)		Ticks	Unknown hosts
Tembe virus (TMEV)		Mosquitoes	Unknown hosts
Tracambe virus (TRV)		Mosquitoes	Unknown hosts

1.2.5 Taxonomy criteria

The classification of viruses was traditionally based on the clinical and pathogenic properties of the virus as well as ecological and transmission characteristics (Buschen-Osmond, 2002). The morphological properties of the virus are an important aspect in classification of virus. However Van Regenmortel *et al.* (1997) suggested that the morphological properties that were used earlier to classify virus species should not be the sole criteria for virus classification, but rather that the natural host range, tropism, pathogenicity, means of transmission, physiochemical and antigenic properties, and most importantly, genome sequence similarities should also be taken into account. The characteristics that are used to differentiate virus orders, families and genera are the type (RNA/DNA) and arrangement (single-stranded/double-stranded) of the viral genome, the process of viral replication and the virion structure (Buschen-Osmond, 2002).

In the family *Reoviridae* the suffix “Reo” refers to respiratory enteric orphan”. The common characteristics of the family are as follows (Attoui *et al.*, 2009):

- The genome contains linear dsRNA
- The genome consists of multiple segments
- Several viruses within the family is transmitted by an arthropod vector
- Similarities exist in the structure and replication strategies of the different reoviruses
- The genome of *Reoviridae* particles is surrounded by up to three concentric shells of viral structural proteins.

The taxonomic classification of BTV is as follows:

- Family - *Reoviridae*
- Genus - *Orbivirus*
- Virus group - Bluetongue virus
- Serotypes - 26 serotypes

Reassortment between different serotypes of a virus group is possible due to the segmented genome of the virus, (Allison *et al.*, 2012), but is not possible between different virus groups in the genera of the *Reoviridae* family.

1.2.6 Molecular characteristics of BTV

Studies that classified BTV under the family *Reoviridae* was conducted by Verwoerd *et al.* (1972) and Studdert *et al.*, (1966).

The BT virion is 68-70 nm in diameter in size (Verwoerd *et al.*, 1972) with the core particle containing 32 capsomeres (Verwoerd, 1969, Verwoerd *et al.*, 1972). (Figure 3) The virion is stable at a narrow pH range of between pH 8-9 (Huisman and Van Dijk, 1990). BTV has a dsRNA genome (Verwoerd *et al.*, 1972). The dsRNA property of BTV led to the classification under the genus *Diplorna viridae*, meaning two strands of RNA (Verwoerd *et al.*, 1972), but was later classified under the genus *Orbivirus* (Verwoerd *et al.*, 1979).

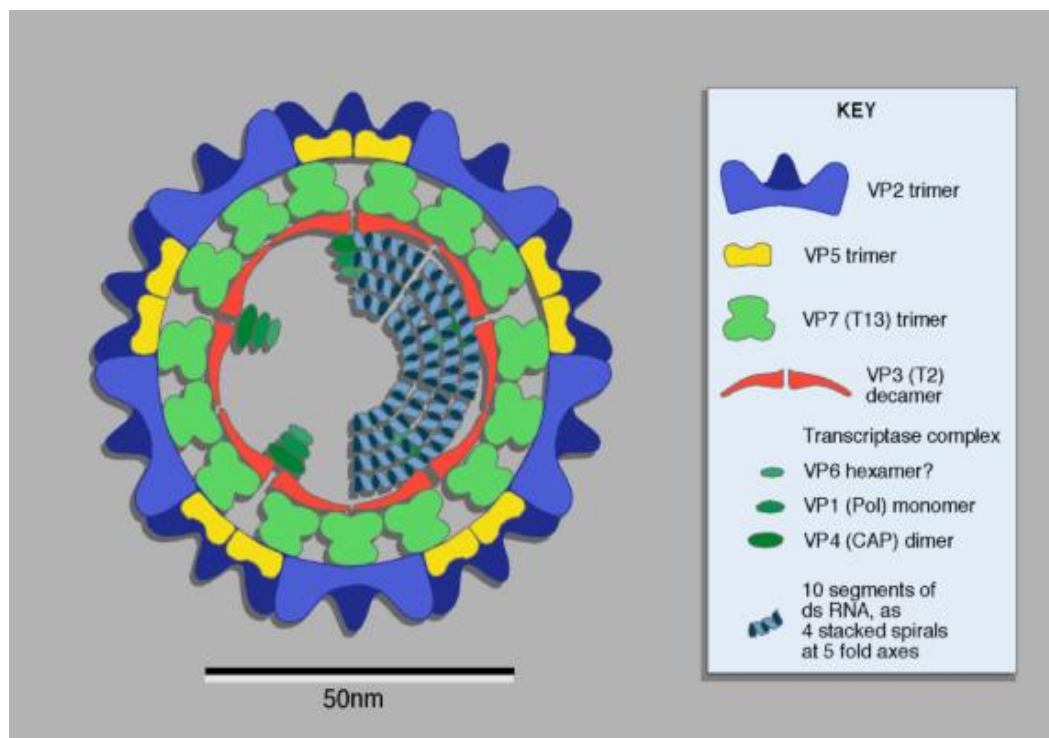


Figure 3: Schematic diagram of the structure of the bluetongue virus (BTV) particle based on structural data from several sources (Mertens *et al.*, 2005). With permission, P.P.C. Mertens

The BTV virion contains ten genome segments (Verwoerd *et al.*, 1972). The genome segments are numbered from segment one to ten based on their size on polyacrylamide gels (Table 4). The ten genome segments encode 7 structural viral proteins (VP1 to VP7) that are found in the BTV virion (Van Dijk and Huismans, 1988) and 5 non-structural viral proteins that are only found in infected cells (NS1 to NS4) (Van Dijk and Huismans, 1988, Grubman *et al.*, 1983). The genome segments 1-8 encode for a specific protein, while segments 9 encodes VP6 and NS4 (Firth, 2008), and segment 10 encodes NS3 and NS3a (Van Dijk and Huismans, 1980).

Information on the genome segments and proteins are summarised in Table 4.

Table 4. Summary of the dsRNA genome segments and proteins of bluetongue virus serotype 10 (BTV10) (genus *Orbivirus*: family *Reoviridae*). Table adapted from Mertens (2004)

Genome segment (Size: bp)	Coding assignment of genome segments	Number of Amino acids	Properties and Functions	References
1 (3954)	VP1	1302	RNA-dependent RNA polymerase; Forms the transcriptase complex with VP4 and VP6	(Roy <i>et al.</i> , 1988, Huismans, 1979, Verwoerd <i>et al.</i> , 1970) (Boyce <i>et al.</i> , 2004)
2 (2926)	VP2	956	Determines virus serotype; Cell attachment protein; Involved in determination of virulence; Most variable protein; Contains neutralising epitopes; Cleaved by proteases	(Huismans and Erasmus, 1981) (Verwoerd, 1970) (Huismans and Van Dijk, 1990)
3 (2770)	VP3	901	T=2 symmetry retains icosahedral symmetry by itself; VP3 controls the size and organisation of the capsid structure; RNA binding interacts with internal minor proteins; Highly conserved protein; Physical organisation of genome; Innermost protein	(Loudon and Roy, 1991, Verwoerd <i>et al.</i> , 1970, Huismans <i>et al.</i> , 1987b) (Stuart <i>et al.</i> , 1998) (Sharifah <i>et al.</i> , 1999)
4 (1981)	VP4	644	With VP1 and VP6, forms transcriptase complex; Capping enzyme; Guanylyltransferase transmethylese 1 and 2	(Verwoerd, 1970) (Mertens <i>et al.</i> , 1992)
5 (1769)	NS1	552	Forms tubules of unknown function; Role in morphogenesis and probably pathogenesis of BTV in the host.	(Huismans and Els, 1979)
6 (1638)	VP5	526	Inner layer of the outer capsid; Minor role to determine virus serotype variable protein; Mediate cell fusion during receptor-mediated endocytosis.	(Verwoerd <i>et al.</i> , 1970)(Forzan <i>et al.</i> , 2004, Mertens <i>et al.</i> , 1989, Roy, 1989)
7 (1156)	VP7	349	Trimer; One of the major polypeptides forming the outer core surface; Mediates attachment and infection in insect vector cells; Group specific antibodies are induced by VP7.	(Grimes <i>et al.</i> , 1998) (Huismans and Van Dijk, 1990) (Xu <i>et al.</i> , 1997) (Huismans and Erasmus, 1981)
8 (1124)	NS2	357	Phosphorylated protein continuously synthesised throughout the replication of BTV; Facilitate virus assembly by its ssRNA binding ability; Appears as viral inclusion bodies in the infected cell	(Huismans and Van Dijk, 1990)
9 (1046)	VP6 / NS4	329 / ?	VP6 Unwinds and re-anneals the dsRNA genome during transcription; NS4 is found in the nucleus of the infected cell	(Stauber, 1997) (Firth, 2008)
10 (822)	NS3 / NS3a	229 216	Glycoproteins; Membrane proteins, may be involved in determination of virulence and vector competence; Found in the infected cell membrane and may be involved in cell exit; Cytotoxic, can disrupt cell membranes	(Bansal <i>et al.</i> , 1998) (Hyatt <i>et al.</i> , 1991) (O'Hara <i>et al.</i> , 1998)

VP2 (S2) forms the outer capsid of the virion (Verwoerd *et al.*, 1972). VP2 (S2) determines the virus serotype, and is the most variable protein. VP2 (S2) is involved in the determination of virulence, and contains neutralising epitopes (Huismans *et al.*, 1987a). When VP2 (S2) is removed from the outer capsid of the virus particle, not only is there a decrease in infectivity (Verwoerd and Huismans, 1972), but attachment to BHK cells was adversely affected as well (Huismans *et al.*, 1983). In studies by Huismans *et al.* (1987a), the potential use of VP2 (S2) as a subunit vaccine, was first emphasised.

VP5 (S6) is also found in the outer capsid of the BTV virion (Verwoerd *et al.*, 1972), and also plays a role in the determination of virus serotype (Mertens *et al.*, 1989), although a higher degree of conservation within the different serotypes exist than of VP2 (S2) (Mecham *et al.*, 1986).

VP3 (S3) is found in the sub-core and its structural function has been described as to forming the support on which the capsomeres (VP7, VP5 and VP2) are arranged (Huismans *et al.*, 1987c).

VP7 (S7) mediates attachment and infection in insect vector cells (Tan, 2001) (Xu *et al.*, 1997). It is one of the major polypeptides that form the outer core. Group specific antibodies are induced by VP7 (S7) (Huismans and Erasmus, 1981).

VP1 (S1), VP4 (S4) and VP6 (S9) are all minor polypeptides found within the sub-core (Verwoerd *et al.*, 1972) and have enzymatic functions in the replication process of the virus (Roy *et al.*, 1988, Van Dijk and Huismans, 1980, Huismans *et al.*, 1987b).

NS1 (S5) forms large amounts of tubules very early on in infected cell cytoplasm (Huismans and Els, 1979). Eaton *et al.* (1988) suggests that NS1 (S5) has some role in the morphogenesis of BTV and that it may influence the pathogenesis in the host (Eaton *et al.*, 1988). These tubules are a characteristic of *Orbivirus* replication.

NS2 (S8) is a phosphorylated protein and is continuously synthesized throughout the replication of BTV (Huismans *et al.*, 1987a). NS2 (S8) has ssRNA binding abilities, and therefore facilitate virus assembly in the viral replication process (Brookes *et al.*, 1993).

NS3 (S10) is expressed more in insect cells than in mammalian cells and plays a role in vector competence (Hyatt, 1991) and in the determination of virulence (O'Hara *et al.*, 1998). Both proteins encoded by S10 (NS3 and NS3a) are required in the release of virions from the mammalian and insect vector cell (Mertens *et al.*, 1984).

NS4, also encoded by S9, was only recently discovered by Firth *et al.* (2008). It is found in the nucleus of the infected cell, the role it plays in the viral replication cycle is not yet clear at this stage (Ratinier *et al.*, 2011).

1.2.7 *Epidemiology of BTV and EHDV*

1.2.7.1 *Epidemiology of BTV*

BT is caused by BTV. BT disease causes morbidity and mortality in sheep. The disease was first described from outbreaks in South Africa (Theiler, 1906). For many years the disease was thought to be confined to the African continent. However, outbreaks in the Middle-East were the first to dismiss this assumption. Until the 1990s it was accepted that BT occurs throughout most of the world's tropical and subtropical regions as well as in southern Europe (Gibbs and Greiner, 1994). BT was reported from countries in the eastern Mediterranean (Gambles, 1949, Komarov and Goldsmit, 1951) north America (MaGowan, 1953, Bowne *et al.*, 1964) and south east Asia (Sapre, 1964). Its occurrence in Cyprus was described by Gambles (Gambles, 1949). Since then it has spread to Israel, the USA, Portugal, Spain, Pakistan, India, and Australia and has also recently spread to northern Europe (Mellor and Wittmann, 2002).

Since the late 1990s there have been widespread outbreaks of BT in Greece, Italy, Corsica and the Balearic Islands (Spain). It appears that the virus spread from Turkey and north Africa to these countries (Calistri and Caporale, 2003). Cases have also occurred in Bulgaria, Croatia, Macedonia, Kosovo and Yugoslavia. These cases were well north of its normal distribution (Purse *et al.*, 2005, Mellor *et al.*, 2008). The outbreaks after 1990 have expanded to latitudes of 58° N (Meiswinkel, R. personal communication). BT outbreaks have been reported from Poland, Russia and Belize implicating serotype 14 of the South African vaccine strain (European Comission, 2012).

In Europe, over 1.5 million sheep succumbed to the disease between 1998 and 2005 (Purse *et al.*, 2008, Calistri and Caporale, 2003, Mellor and Wittmann, 2002).

1.2.7.2 Epidemiology of EHDV

EHDV1 (New Jersey strain) and EHDV2 (Alberta strain) were first isolated in the United States, and were regarded as the only endemic circulating serotypes then (Shope *et al.*, 1955) (Chalmers *et al.*, 1964). However, during 2006, EHDV-6 was isolated from WTD in Indiana and Illinois (Allison *et al.*, 2010). After the initial detection, it was isolated during consecutive seasons in a couple of states in the USA, and has since been accepted as being endemic to the USA (Allison *et al.*, 2012). EHDV is endemic in South Africa, but rarely causes disease (Barnard *et al.*, 1998).

During an outbreak of EHD in west-Virginia in America, 70 deer died, whereas several cattle presented with NAb against EHDV and BTV, while no sheep were involved (Gaydos *et al.*, 2004). Gaydos *et al.* (2002) reported oral and faecal shedding of virus in WTD. The relevance of this data in epidemiology has not been determined. Virus has been isolated from aborted fetuses (Ohashi *et al.*, 1999) indicating trans placental transmission of the virus.

Clinical disease in cattle has been reported from USA (House *et al.*, 1998), South Africa (Barnard *et al.*, 1998), Japan (Omori *et al.*, 1969) and Reunion islands (Breard *et al.*, 2004).

1.2.8 Vector competence

1.2.8.1 Vectors of BTV

Vector competence and vector capacity is the ability of a vector to transmit virus to the ruminant host. Criteria that designate an arthropod as a confirmed arboviral vector have been established by the WHO (1967):

- Recovery of virus from the wild caught arthropod vector host
- Demonstration of the ability of the vector to become infected during feeding on an infected host or artificially on a blood meal spiked with virus
- Demonstration of the ability to transmit virus during feeding on the natural host
- Evidence of the association of the vector with the mammalian host
- The number of blood meals an infected vector can take during its lifetime

BTV and AHSV infection rates have been shown to be temperature dependent. In summer when infection rates are higher, rates of virogenesis are faster and transmission occurs earlier. (Mellor *et al.*, 2000, Mullens *et al.*, 1995, Wellby *et al.*, 1996, Meiswinkel *et al.*, 1994). During the

colder months of the year, vector activity is much lower, and so are infection rates in the susceptible mammal hosts. Overwintering periods ranging from three to eight months have been recorded (Nevill, 1971, Taylor and Mellor, 1994). The normal transmission cycle during summer and probable overwintering mechanism is shown in Figure 4 (Wilson *et al.*, 2008). Virus- infected biting midges transmit the virus during feeding on the susceptible hosts. Possible overwintering mechanisms have been proposed by several authors (Nevill, 1971, Meiswinkel *et al.*, 1994). Cattle can stay viraemic for up to 100 days (MacLachlan *et al.*, 1990). This period is long enough to enable BT virus to overwinter in cattle in South Africa. Other possible mechanisms of overwintering that have been mentioned by Wilson *et al.* (2008) include trans-ovarial transmission, survival of adult midges and persistent latent infection in the host.

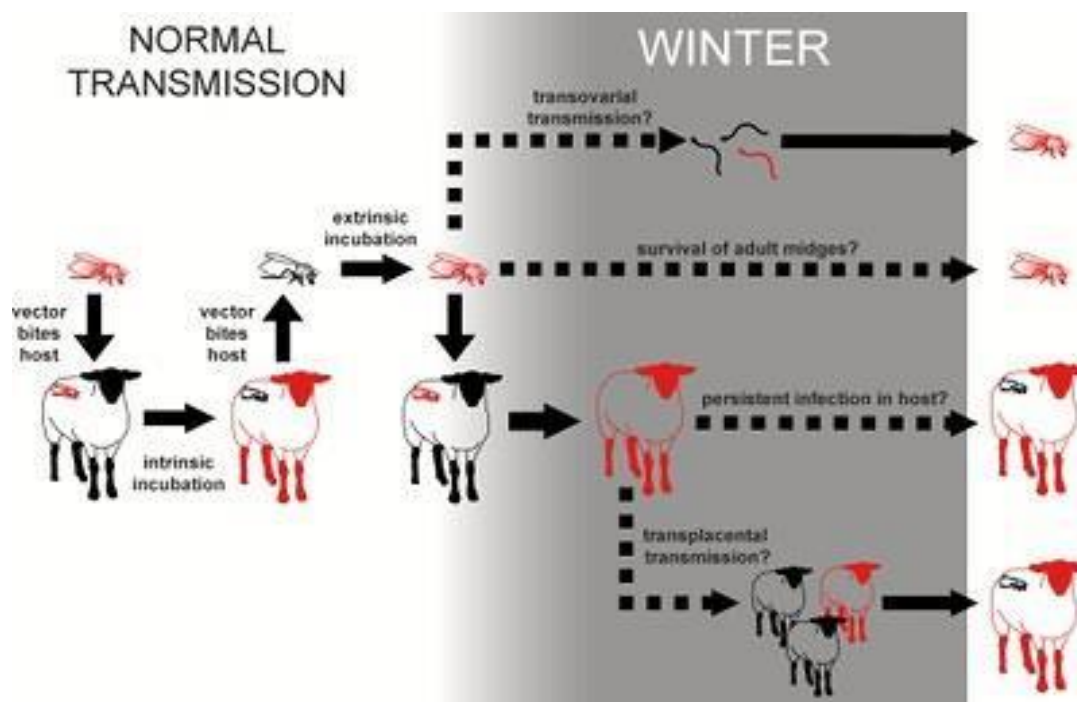


Figure 4: The transmission of BTV in summer (left), and winter (right). [Figure reproduced from Wilson, Darpel & Mellor (2008), PLoS Biology 6(8):e210; <http://dx.doi.org/10.1371/journal.pbio.0060210>. Published under a Creative Commons Attribution License (CCAL2.5).]

In South Africa BTV is transmitted between its ruminant hosts by certain species of the *Culicoides* biting midges, of which *C. imicola* and *C. bolitinos* are the major vectors of BTV. *C. imicola* and *C. bolitinos* are the most abundant BTV vectors throughout Africa (Du Toit, 1944, Meiswinkel *et al.*, 1994, Foster *et al.*, 1963, Walker and Davies, 1971). *C. bolitinos*

predominates in cooler regions in South Africa, and is up to 20 times more susceptible to infection under laboratory conditions than *C. imicola* (Venter *et al.*, 1998). Paweska *et al.* (2002) reported laboratory infection with BTV of *C. magnus*, *C. bedfordi*, *C. leucostictus*, *C. pycnostictus*, *C. gulbenkiani* and *C. milnei* in South Africa, albeit in very small numbers. Venter *et al.* (2007) showed that the potential exist of live-attenuated vaccine strains to be transmitted by *Culicoides* from vaccinated to unvaccinated susceptible hosts. This emphasises the problem of uncontrolled use of attenuated vaccines in non-endemic countries.

C. imicola is the most widespread species throughout the world (Howarth, 1985, Mellor, 1990, Tatem *et al.*, 2003, Purse *et al.*, 2005) and the mere presence of infected *C. imicola* indicates a risk of BTV exposure to susceptible animals.

In Europe *C. imicola* was also found to be distributed across the southern parts of Europe (Mellor *et al.*, 1985, Boorman, 1986) and this distribution of *C. imicola* was almost identical to BT outbreaks at that stage. *C. imicola* has not entered into northern Europe yet (Meiswinkel *et al.*, 2008). A few other species were, however, also identified to be competent vectors, like *C. obsoletus* and *C. pulicaris* (Caracappa *et al.*, 2003, Savini *et al.*, 2003, Purse *et al.*, 2005, Carpenter *et al.*, 2009). *C. obsoletus*, *C. pulicaris* as well as *C. dewulfi* are more predominant in the northern parts of Europe, thus leading to an increased risk of BT beyond the distribution of *C. imicola* (Mellor and Wittmann, 2002, Purse *et al.*, 2005).

1.2.8.2 Vectors of EHDV

EHDV is also transmitted by haematophagous midges of the genus *Culicoides* (Du Toit, 1962). *C. bolitinos* and *imicola* are the most abundant vectors in South Africa (Du Toit, 1962). In the USA *C. variipennis* is the primary natural vector of both BTV and EHDV (Jones *et al.*, 1977).

The distribution of EHDV in the USA, like BTV, reflects the distribution of *C. sonorensis*. The seasonality of the abundance of *C. sonorensis* coincides with the reporting of BT and EHD cases (Stallknecht and Howerth, 2004).

EHDV outbreaks have been reported in northern Europe far from its normal distribution, (Mellor *et al.*, 2008), and outside the normal distribution of the common vector species (Stallknecht and Howerth, 2004, Shapiro *et al.*, 1991). A possible indication is that the vectors could be another *Culicoides* species occurring in the colder regions such as New Jersey in the USA and western Canada (Mecham and Nunamaker, 1994). EHDV have been isolated from the following field collected *Culicoides* species; *C. cornutus* and *C. nevillii* from South Africa (Barnard *et al.*, 1998),

EHDV4 from *C. imicola* and *C. schultzei* from Sudan (Aradaib *et al.*, 1999, Mohammed and Mellor, 1990), Ibaraki (EHDV2) from *C. schultzei* in Nigeria (Lee, 1979), EHDV1 and EHDV2 from *C. sonorensis* from the USA (Jones *et al.*, 1977, Foster *et al.*, 1977), EHDV2 from *C. brevitarsis* from Australia (St George *et al.*, 1983), and from *C. oxystoma*, *punctatus* and *peregrinus* from Japan (Yanase *et al.*, 2005).

1.2.9 Host range and clinical disease signs caused by BTV and EHDV

1.2.9.1 Host range and clinical disease signs caused by BTV in sheep

BTV can infect most ruminants. Sheep, goats, cattle (Spreull, 1905, Bekker *et al.*, 1934, De Kock *et al.*, 1937), deer, bighorn sheep, most species of African antelope as well as various split-hoofed ruminants are susceptible to BTV (Hourrigan and Klingsporn, 1975, Hoff and Hoff, 1976). Different levels of severity of clinical signs, not only amongst the different species it affects, but also amongst different breeds of animals, their immunological condition, virulence of the BTV serotype and exposure to ultraviolet radiation have been reported (Osburn, 1994, Verwoerd and Erasmus, 1994). Disease in the fine-wool and mutton breeds is more severe than in indigenous sheep and in goat breeds (Koumbati *et al.*, 1999).

BTV can infect yaks, and has also been found in Eurasian lynx, (Mauroy *et al.*, 2008, Jauniaux *et al.*, 2008) and carnivores (Akita *et al.*, 1994, Alexander *et al.*, 1994). Infection in carnivores is rare, and is usually due to ingestion of BTV infected ruminant foetuses and meat (Jauniaux *et al.*, 2008, Mauroy *et al.*, 2008).

In South-Africa the mortality rate of sheep is between 2 and 30 percent (Verwoerd and Erasmus, 1994). Clinical signs usually start with a febrile reaction. The duration and extent of the febrile reaction often give an indication of the severity of disease signs to follow (Erasmus, 1990). The first signs occur in the facial area, with hyperaemia and petechia of the lips, muzzle and mucosa of the mouth. Oedema of the face develops and may extend down the neck. Erosions can develop inside the mouth, the tongue can become oedematous, and in very severe cases, may become cyanotic – from which the name bluetongue was derived (Spreull, 1905). Hyperaemia and cyanosis of the feet also appear, and some sheep tend to try and walk/stand on their knees because the feet become warm and painful (MacLachlan *et al.*, 2008, Henning, 1956). Breaks in the wool fibres occur, due to dermatitis that develops (Erasmus, 1990).

In studies done in Dorset Poll sheep (Veronesi *et al.*, 2010) commercial, freeze-dried attenuated, BTV4 and BTV16 modified live virus (MLV) vaccines were used to determine the viraemia of attenuated vaccines in naïve ruminant populations and the potential of virus transmission by the insect vector hosts, *Culicoides*. In this study clinical disease was observed similar to infection with wild type virus strains, with viraemic periods significant enough to enable vector transmission of the virus (Veronesi *et al.*, 2010).

Another risk of transmission is if semen is derived from infected bulls during the viraemic phase, which can infect inseminated cows (Bowen *et al.*, 1985). Embryos from infected donor ewes, collected during the viraemic phase, can transmit virus to recipient ewes (Venter *et al.*, 2011). Therefore, the International Embryo Transfer Society (IETS) has adopted a protocol to wash embryos between transfer from donor ewes to recipient ewes in the export trade to 'free' it from potential virus.

1.2.9.2 BT in alpaca

Very few reports of BT in alpaca have been published. Henrich and Reinacher (2007) reported BT infection in an alpaca during the 2007 BT outbreak in Germany, with high mortality in affected sheep from the area. Post mortem examination of the alpaca showed severe, acute, diffuse oedema of the lungs. Single small erosions and ulcerations were observed on the tongue, palate and buccal mucosa. Acute congestion of the liver, spleen and kidneys was also observed even though South American camelids (SAC), of which alpaca is a member, were considered to be resistant to BTV infection before 2007 (Afshar *et al.*, 1995). Ortega *et al.* (2010) reported the death of a 15-year-old alpaca in northern California during October 2008. Post mortem examination of this alpaca showed severe hydrothorax and hydro pericardium, but no lesions or ulcerations of the tongue or buccal cavity that could suggest BTV as the cause of illness. Histological examination revealed oedema and congestion of the lungs. This reported case has been regarded as the first report of lethal BT in a camelid in the USA (Ortega *et al.*, 2010).

In a large-scale serological and virological survey done in Germany amidst a BTV outbreak during 2008-2009, natural infection was recorded in three SAC. A llama and 2 alpaca died due to infection with BTV8, and the presence of BTV8 RNA in organ material was confirmed by PCR (Schulz *et al.*, 2012b). Serological data showed that only 14.3 % unvaccinated SAC were seropositive from a total of 1742 SAC present at that time in the region, amidst an vaccination campaign with inactivated BTV8 vaccine (Schulz *et al.*, 2012b).

Experimental infection with BTV8 with a virus titre of 10^5 TCID₅₀ (tissue culture infective dose), (Schulz *et al.*, 2012a) resulted in no clinical disease signs typical of BTV infection in llamas and alpacas. These results led to concerns that SACs may play a role as carriers of BTV, but findings indicated that transmission of BTV by SACs are negligible (Schulz *et al.*, 2012a) as BTV8 adsorbs less efficiently to SACs erythrocytes than to bovine erythrocytes.

1.2.9.3 Host range and clinical disease signs caused by EHDV

WTD is most susceptible for EHDV infection (Hoff and Trainer, 1981). However acute disease has been reported in cattle (Breard *et al.*, 2004), black tailed deer (Hoff and Trainer, 1981) and Rocky Mountain bighorn sheep (Noon *et al.*, 2002). A rare case of EHDV in an alpaca have been reported from Pennsylvania in the USA (Phillips, 2013).

The clinical pathology, epizootiology and morphology caused by EHD closely resemble that of BT (Gibbs and Lawman, 1977, Vosdingh *et al.*, 1968, Karstad and Trainer, 1967). Ulcerations of the buccal cavity and vascular alterations leading to extensive systemic haemorrhaging and oedema are typical clinical signs of haemorrhagic disease (Brannian *et al.*, 1983, Hoff and Trainer, 1981). The sloughing hooves, ascribed to BTV infection, were also recorded from WTD in the USA. Gross and microscopic lesions that have been recorded include haemorrhagic enteritis and the presence of serosanguineous fluid in the stomach, pleural and peritoneal cavities and pericardial sac (Brannian *et al.*, 1983). Reynolds *et al.* (1985) described a symptom in calves from infected cows, born at full term gestation, where striking lesions occurred in the eyes, called “white eyed syndrome”, animals were weak, did not have the ability to stand or suckle, which he ascribes to EHDV.

1.2.10 BTV Vaccines

In a country where BTV is endemic, the vaccination of animals is the only means to protect them against disease. The earliest attempt to immunise sheep against BTV was done by Spreull. He used hyperimmune serum together with infected blood to inoculate sheep (Spreull, 1905). Theiler anticipated that BTV could be attenuated by limited passages in sheep, and produced a vaccine that consisted of infected sheep blood (Theiler, 1906). Theiler’s vaccine however contained only serotype 4. This vaccine was used for almost 40 years with variable success.

The success of Theiler's monovalent vaccine was later explained by Erasmus (1990). Serotype 4 of BTV is serologically related to BTV9,-10,-11,-12,-17 and 20 to variable degrees, and provided low levels of cross-protection *in vitro* against the serologically related serotypes (Erasmus, 1990). The serological interrelationship between the 24 serotypes of BTV was determined by Erasmus (1990) by means of plaque-reduction testing (Figure 5) and later confirmed by Potgieter (2004) using phylogenetic sequence analysis of S2 (VP2) of the 24 strains of BTV.

The serotype combinations included in the commercial attenuated vaccine produced in South Africa (Erasmus and Potgieter, 2009) are indicated in Figure 5. The serotypes in the rectangle shapes (BTV20 and -21) indicate the serotypes that do not occur in South Africa (Erasmus, 1990). BTV17 has recently been isolated from samples submitted from South Africa (I.M. Wright, unpublished data). Serotypes 15, 16, 18, 22, 23 and 24 are not included in the vaccine due to their low pathogenicity in sheep, and their relative recent discovery since the development of the current vaccine (Verwoerd and Erasmus, 1994).

The serotype combinations included in the South African produced vaccine have been chosen on the following grounds; to prevent interference between the vaccine strains; on basis of the cross neutralisation provided between serotypes and differences in replication tempo between the serotypes. Vaccination in epidemic situations involving single serotypes induces lifelong protection against infection with the same serotype (Howell and Verwoerd, 1971). Erasmus (1980) determined that regardless of the number of live attenuated serotypes included in a BTV vaccine, sheep will develop NAb to three serotypes only, therefore the 3 week intervals indicated between usage of the BTV vaccine doses. Teratogenesis (hydranencephaly) of foetuses can, however, be caused if the vaccine is administered to ewes during the first half of gestation (Erasmus, 1990).

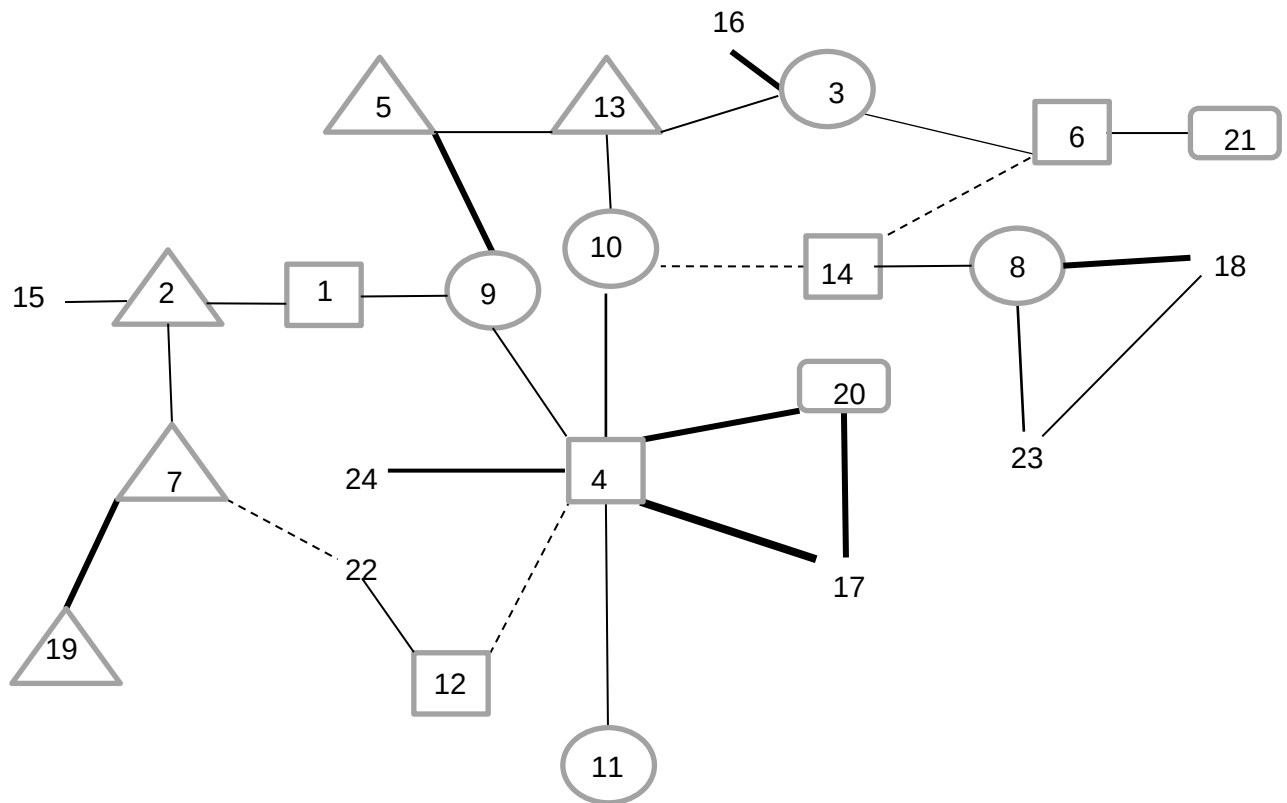


Figure 5: A schematic representation of the interrelationship between the 24 serotypes of BTV as determined by plaque reduction testing. The thickness of the lines indicate the degree of interrelationship between the different serotypes. The thicker lines indicate a stronger interrelationship, while the thin and broken lines indicate a lesser degree of interrelationship. Adapted from Erasmus (1990).

- Vaccine dose A:  The first dose of the polyvalent vaccine to be administered, and contains BTV serotypes 1, 4, 6, 12 and 14
- Vaccine dose B:  The second dose contains BTV serotypes 3, 8, 9, 10 and 11
- Vaccine dose C:  The third dose contains BTV serotypes 2, 5, 7, 13 and 19
-  The serotypes in the rectangle shapes does not occur in South Africa

When attenuated vaccines are used outside an endemic area, the risk of disease due to inadequate attenuation increases in a susceptible host species. Natural transmission due to inadequate attenuation was confirmed from a cow vaccinated with a modified live virus (MLV) produced by Onderstepoort Biological Products (OBP) in a study in Italy (Savini *et al.*, 2008). Monaco *et al.* (2006) reported viraemia in sheep from Sardinia resulting from vaccination with a pentavalent BTV vaccine, containing BTV16. The virus used for production in the pentavalent vaccine, was passaged 37 times in ECE, followed by 3 plaque selections and passages in BHK and African green monkey kidney (VERO) cells. Attenuated virus from the use of MLV, can be

transmitted post vaccination, which in turn can cause disease when transmitted to a naïve host population which is more susceptible than animals in South Africa (Savini *et al.*, 2008).

The use of modified live vaccines in countries where BT emerged after the 1990s caused concerns regarding the safety in a BT naïve animal population. Reports of attenuated BTV16 and BTV2 (identical to OBPs BTV2 vaccine strain) isolated from midge pools in France and Portugal were recorded during the BTV outbreak in Europe 1998-2005 (Barros *et al.*, 2007, Mellor and Wittmann, 2002, Sailleau *et al.*, 2004). Attenuated vaccine strains of BTV1, -4, -9 and -16 were recovered from blood-fed *C. imicola* after a 10 day incubation period, emphasising the risks involved in the use of attenuated vaccines in non-endemic countries (Venter *et al.*, 2007). The susceptibility of European sheep breeds to BTV disease is much higher than that of the ruminant populations in BTV endemic countries (Vercauteren *et al.*, 2008). Phylogenetic analysis of BTV isolated during autumn of 2012 from bovine samples from Poland and Russia showed 100 % nucleotide identity with the South African BTV14 vaccine strain (Nomikou *et al.*, 2012). Concern about incomplete attenuation of South African vaccines in European sheep breeds, encouraged the use of inactivated vaccines (Saegerman *et al.*, 2008). The use of beta-propiolactone and gamma irradiation to inactivate virus for use in monovalent and bivalent vaccines, delivered protective immunity (Campbell, 1985, Parker *et al.*, 1975). Inactivated polyvalent vaccines, however, are poorly immunogenic, and large vaccine doses are necessary to induce lasting neutralising antibody protection (Noad, 2009).

VP2 is the outer capsid protein that determines serotype and induces protective immunity (Huismans *et al.*, 1985). Sub-unit recombinant VLP vaccines including all different combinations of outer-capsid viral proteins did elicit immune responses in animal trials, and were proven to be safe to use (Roy *et al.*, 1990, Roy *et al.*, 1992). Unfortunately, to date, the production of a feasible recombinant BTV vaccine has not been realised.

1.3 PROBLEM FORMULATION

The alpaca society of South Africa currently has 45 breeders on their list (<http://www.alpacasociety.co.za>, 2012). Since the first introduction of alpacas to South Africa in 2000, the numbers have grown to more than 2000 animals. The introduction of exotic animal species, naïve to the circulating virus populations, creates the risk of losing animals to disease. Concerns of a putative new virus circulating in the Montagu region raised fear that more animals may become infected. Recent reports by Chaignat *et al.* (2009) and Maan *et al.* (2011b)

reported the isolation and molecular identification of the 25th and 26th serotypes of BTV from Switzerland and Kuwait respectively.

Homogenates from ECE from a virus sample isolated from heparin blood from an alpaca could not be serotyped using BTV VNT, using hyper-immune GP serum against the 24 BTV reference strains of the OIE laboratory, regardless of the initial positive BTV PCR results obtained from Dr. Marco Romito from the PCR diagnostic laboratory at OVI. We then performed VNT testing using EHDV GP hyper immune serum. The isolate could still not be serotyped. In the light of negative results with BTV and EHDV serotyping, additional testing had to be performed to determine the nature of the virus involved in causing a positive BTV PCR result and ECE mortalities.

1.4 AIMS OF THE STUDY

The purpose of my MSc project was to identify and characterise the infectious agent involved in the death of this alpaca.

Following negative VNT results and the positive result with BTV-PCR, further studies were undertaken to identify the two virus isolates from this dead alpaca. The specific aims of this MSc study were:

1. To perform agar gel electrophoresis in order to distinguish the electrophoretic profile from purified RNA of the two samples;
2. To perform partial nucleic acid sequencing and compare the data to published sequences on GenBank;
3. To perform complete nucleotide sequencing of the genome at Inqaba Biotec, analyse the sequence data and compare it to virus sequences published on GenBank;
4. To produce hyper immune GP serum to perform serum neutralisation testing (SNT) against the BTV and EHDV reference antigens kept in the virus bank of the OIE Reference Centre at OVI;
5. To test animals from the same region in the western Cape for the presence of antibodies against the isolated viruses to determine whether more animals on the farm were infected with the same viruses;
6. To perform midge susceptibility studies to determine the vector potential of the viruses.

CHAPTER 2

GENOME AMPLIFICATION, SEQUENCING AND SEQUENCE ANALYSIS OF THE TWO VIRUS ISOLATES OF AN ALPACA – ALP8 AND ALP9

2.1 INTRODUCTION

With the initial diagnostic PCR result in mind it was anticipated that the Alp8 and Alp9 viruses will have a dsRNA genome. The genomes of all viruses described in the *Orbivirus* genus consist of 10 linear dsRNA genome segments (Verwoerd *et al.*, 1972). The segments range in size – S1 (VP1) of BTV being the largest segment with 3945 nucleotide base pairs (bp) to S10 (NS3) being the smallest segment with 822 nucleotide bp. Each of the different *Orbivirus* species have a relatively uniform electrophoretic profile on SDS-PAGE (Verwoerd *et al.*, 1972) and as such can give an indication of virus species for the family *Reoviridae* (Verwoerd, 1970).

Potgieter *et al.* (2009) described a method for sequence-independent amplification and sequencing of viral double-stranded RNA genomes. This protocol can be used for the amplification of large (>2000 bp) dsRNA genome segments, making it possible to amplify genomes without the prior knowledge of sequence data to design primers. This method has advantages over previous methods, which used individual cloned genome segments for sequencing (Potgieter *et al.*, 2002, Attoui *et al.*, 2000, Vreede, 1998) or purified PCR products of separate genome segments (Maan *et al.* 2007). Disadvantages of the methods as described by Potgieter *et al.* (2002) and Vreede *et al.* (1998) are that the methods require the separation of genome segments in agarose gels, purification and primer walking of clones, where primers are designed from known sequences or conserved terminal ends (Maan *et al.*, 2007a, Potgieter *et al.*, 2009).

In this chapter the virus propagation, extraction and purification of dsRNA from viruses isolated from an alpaca will firstly be discussed. The purified dsRNA material will be used for genome amplification of Alp8 and Alp9. This will be followed by sequencing of the genomes of Alp8 and Alp9. Sequencing analysis of both genomes will be discussed. To sequence the genomes of the Alp8 and Alp9, we need to propagate the viruses in TC to increase the viral yield to have enough RNA to amplify. Total RNA will be extracted.

S2 encodes outer capsid viral protein 2 (VP2) which determines serotype. S5 encodes non-structural protein 1 (NS1). NS1 is one of the most conserved proteins amongst different serotypes of the orbiviruses. Genome segments S2 (VP2) and S5 (NS1) will be sequenced

partially at the sequencing unit at OVI by Ms. Junita Liebenberg using Sanger sequencing. Sequence data of S5 and S2 will indicate which virus species and serotype are involved.

2.2 MATERIALS AND METHODS

2.2.1 *Virus propagation*

One milliliter TC material of each sample (Alp8 and Alp9) was passaged in a 75 cm² flask (NUNC, catalogue no.: 156472) with a confluent BHK-21 cell monolayer. Flasks were placed on a Stuart Scientific platform shaker STR 6 set at its slowest speed in a 37° C incubator to facilitate adsorption of the virus to the cells. Following incubation, cells were washed twice with 5 ml of Eagles – minimum essential medium (E-MEM catalogue no.: 12-169F Lonza). Twenty milliliters of E-MEM containing 1 % penicillin/streptomycin/fungizone (PSF-catalogue no.; Lonza 17-603E) mixture was added as antibiotic and antimycotic. Flasks were placed in a 37° C New Brunswick incubator, containing 5 % CO₂. TC were observed daily for CPE. Supernatant and cells were harvested at a CPE score of 4 + (100 % cells infected).

2.2.2 *RNA extraction and purification*

Total RNA was extracted from infected BHK-21 cultures using the following commercially available kit and accompanying protocol:

TRI-Reagent®LS - RNA/DNA/Protein isolation reagent for liquid samples (Molecular Research Center, INC. Catalogue number TS-120)

TRI-Reagent®LS contains phenol and guanidine isothiocyanate. TC material was lysed and viral cells disrupted resulting in inactivation of viruses and inhibiting of RNase activity.

Approximately 14 ml of supernatant containing cells from one 75 cm² flask was centrifuged in an Eppendorf 5810R centrifuge with Rotor A-4-81(Merck) at 2500 x g for 7 minutes. The cell pellet was re-suspended in 750 µl TRI-Reagent®LS and homogenised. To separate the RNA from the TC DNA and protein, 200 µl chloroform was added and shaken vigorously. This was followed by an incubation period of 2 minutes. A centrifugation step using the Eppendorf 5415R centrifuge with rotor F45-24-11 (Merck) at 16 000 x g for 15 minutes resulted in an aqueous upper phase that contains total RNA. The aqueous phase was transferred to a clean microcentrifuge tube. Precipitation of RNA was accomplished by adding an equal volume of isopropanol to the

supernatant, followed by incubation for 2 minutes at room temperature, and a centrifugation step at 13200 x g for 15 minutes. The supernatant was removed, and followed by an additional centrifugation step to completely remove isopropanol.

Viral RNA was purified using the following commercially available kit: QIAamp MinElute® Virus spin kit (50) (QIAgen, Catalogue number 57704). This kit uses the selective binding properties of a silica based membrane and buffering conditions to provide optimal binding of the RNA to the QIAamp membrane.

The RNA pellet was solubilised using 100 µl of the Solubilisation Buffer (Qiagen).

90 µl Elution buffer was added to the spin columns (Qiagen) and 30 µl of 8 M LiCl to ensure a final concentration of 2 M (Sigma) added to precipitate the ssRNA. Following overnight incubation at 4°C the tube was centrifuged at 16 000 x g for 30 minutes. The supernatant was transferred to the Qiagen MinElute columns and centrifuged according to the manufacturer's specifications. A washing step was performed using Buffer PE (Qiagen) to remove any residual contaminants, with subsequent centrifugation to dry the column. Thereafter the dsRNA, if any present, was eluted from the column with 30 µl Buffer EB (Qiagen).

2.2.3 *Agarose gel electrophoresis*

AGE is a molecular technique used to separate the different molecules, in this case genome segments on the basis of their sizes. AGE and all accompanying buffers and agarose gels were performed and prepared as set out in the literature (Sambrook, 2001) unless specified otherwise. Tris-Borate-EDTA (TBE) buffer and 1% TBE agarose gels were used in the study, unless otherwise indicated. An electric field is applied to the TBE gel that contains ethidium bromide submerged in TBE buffer. The separation of the separate segments can be observed with an ultraviolet (UV) light, as the ethidium bromide fluoresces under the UV light. Loading buffer contains bromophenol blue and glycerol. It is mixed with the RNA so that the loaded material can be visualised and to keep dsRNA submerged in the wells in the agarose gel. Two µl purified dsRNA was loaded on a 1 % TBE gel. The genome segment sizes were evaluated by running O'GeneRuler ladder mix (Fermentas SM 1130) as size marker with the sample material. Gel electrophoresis was performed in a Cleaver system using a Bio-Rad PowerPac system at 90 V for an hour. dsRNA was visualised using a UVP UV trans illuminator (Scientific group). The agarose gel was visualised and documented using a gel documentation system Autochemi System UVP Bio-imaging systems (Scientific group).

2.2.4 Sequence-independent genome amplification

With the aim of identifying the two dsRNA viruses involved, it was decided to amplify the genomes of the viruses in a sequence-independent manner and to sequence amplicons.

Genome amplification was performed essentially as described by Potgieter *et al.* (2009) and involved the following steps:

Oligo-ligation

Primer PC3-T7 loop (5'-p-GGATCCCGGGAATTCGGTAATACGACTCACTATATTTTTATAGTG AGTCGTATTA-OH-3') synthesised by Tib Molbiol was ligated to sample dsRNA as described by Potgieter *et al.* (2009).

An oligo ligation reaction was assembled using 12 µl of purified dsRNA, 3 µl ligation buffer (50 mM HEPES pH 8.0 (Sigma), 18 mM MgCl₂ (Separations), 10 µg/ml % Bovine serum albumin (BSA) (TaKaRa), 1 mM ATP (Roche), 3 mM DTT (Roche)), 200 ng PC3-T7 loop primer, 20% polyethylene glycol (PEG₆₀₀₀ from BDH), 30 U T4 RNA ligase (TaKaRa) in a final volume of 30 µl.

The ligation reaction was incubated overnight at 37° C. The ligated dsRNA was purified using Qiagen's MinElute Gel extraction columns as follows: 70 µl HPLC water (Merck) was added to the ligated RNA. Three hundred µl QG buffer (Qiagen) and 100 µl Isopropanol was mixed with the ligated dsRNA and centrifuged over Qiagen MinElute columns. The columns were washed using 750 µl buffer PE (Qiagen), and dsRNA eluted in 10 µl elution buffer (Qiagen).

dsRNA denaturation

Denaturation of the purified ligated dsRNA was performed using a final concentration of 30 mM methyl mercury hydroxide (MMOH, Alfa Aesar).

c-DNA synthesis

Reverse transcription

cDNA was transcribed in a 30 µl reaction containing 50 mM Tris/HCl pH 8.3 (Sigma), 10 mM MgCl₂ (Separations), 70 mM KCl (Sigma), 30 mM Beta-mercaptoethanol (Sigma), 1 mM dNTPs (TaKaRa) and 15 U cloned AMV reverse transcriptase (Invitrogen).

Reactions were incubated at 42 ° C for 45 minutes and then for 15 minutes at 55 ° C.

RNA degradation and cDNA annealing

NaOH (Sigma) was added to a final concentration of 1M and the reaction incubated at 65 °C for 30 minutes to remove excess RNA. Tris HCl pH 7.5 (Sigma) and 0.1 M HCl (Sigma) was added to the cDNA before annealing to reduce the NaOH and create a NaCl concentration of 100mM in the cDNA reaction sufficient for annealing of cDNA.

The cDNA was annealed at 65 °C for 1 hour.

Note: All incubation steps were performed in an Eppendorf Master PCR cycler.

Genome amplification

Complete genomes were amplified in 50 µl PCR reactions. 100 picomoles PC2 primer (5'-CCGAATTCCCGGGATCC-3') was used for amplification in a 50 µl PCR reaction containing 35 µl DNase free water, 1 x Ex Taq Buffer (TaKara), 0.2 mM dNTPs (TaKaRa), 2.5 U Ex Taq Enzyme (TaKaRa) and 5 µl cDNA.

PCR cycling steps were performed as follows:

- 72 °C for 1 minute to fill incomplete cDNA ends to produce intact DNA
- 94 °C for 2 minutes - initial denaturation step
- 25 cycles of 94 °C for 30 seconds, 67 °C for 30 seconds and 72 °C for 4 minutes
- A final extension step of 72 °C for 5 minutes

The PCR products were separated on a 1 % agarose gel (TBE) containing 0.01 % Ethidium bromide.

2.2.5 Gel Extraction of S2 (VP2) and S5 (NS1) from amplified genomes

Genome segments 2 and 5 of the cDNA were purified from the agarose gel using a QIAgen MinElute gel extraction kit and protocol as per instructions of the manufacturer (QIAGEN - Catalogue number 28604) in preparation for sequencing.

Genome segments 2 and 5 were excised from the agarose gel using a clean, sharp scalpel blade, ensuring that as much as possible of the surrounding agarose was removed. The cDNA segments were placed in weighed 2 ml DNase, RNase free microcentrifuge tubes, weighed again and 3 volumes Buffer QG (QIAgen) added. These were incubated at 50° C for 10 minutes in a heating block (Monitoring and control supplies) until the gel slices have completely

dissolved. One gel volume of isopropanol (Sigma) was added to each gel slice and mixed properly. This was transferred to separate MinElute columns (QIAgen) to bind the cDNA. The MinElute columns were placed in collection tubes, and centrifuged at 17200 x g for 1 minute. The flow-through was discarded and 500 µl of buffer QG (QIAgen) added. After another quick spin the membranes were washed with 750 µl of Buffer PE (QIAgen) and centrifuged to remove any contaminants. cDNA was eluted in 12 µl Buffer EB (QIAgen).

Spectrophotometric analysis of the cDNA segments was performed using a Nanodrop Spectrophotometer ND 1000. Fifty ng of the amplicons were used in the sequencing reaction.

2.2.6 *Partial sequencing of genome segments 2 (VP2) and 5 (NS1) with Sanger sequencing*

Genome segments 2 (VP2) and 5 (NS1) were initially sequenced using Sanger cycle sequencing at OVI.

The Sanger method uses fluorescently marked ddNTPs as chain terminators. Each of the ddNTPs is marked or labelled with a different fluorescent dye, each of which emit light at a different wavelength, giving different peaks in the capillaries of which the readout is by a chromatogram (Sanger, 1977).

Partial sequencing of VP2 (S2) and NS1 (S5) was performed at OVI with the ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems) using the Sanger sequencing method. Analysing of sequencing reactions was done using the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems). The aim was to identify genetic relationship with regard to other orbiviruses by comparing results to published sequence data on Genbank. Amplification using the following Orbi-R primer sequence (CCGAATTCCCGGGATCC GTA) was performed. Analyses of sequencing reactions were performed using the ABI PRISM® 3100 Genetic Analyser (Applied Biosystems).

The partial S2 (VP2) and S5 (NS1) sequences were subjected to BLASTanalysis using the National Centre for Biotechnology Information (NCBI) website on <http://blast.ncbi.nlm.nih.gov/Blast.cgi> using BLASTN for nucleotide sequences and BLASTX for protein sequences.

2.2.7 454 Pyrosequencing

Pyrosequencing using GS20 FLX technology (Margulies *et al.*, 2005) can be used to sequence genomes of any size from genomic DNA, PCR products, and cDNA. Samples such as genomic DNA or RNA are fractionated into small, 300- to 800-basepair fragments (Margulies *et al.*, 2005).

A single-stranded DNA library is constructed from genomic material and added to DNA capture beads. These are emulsified with amplification reagents resulting in micro reactors containing just one bead with one unique sample-library fragment. Each unique sample library fragment is amplified within its own micro reactor. Amplification of the entire fragment collection is done in parallel; for each fragment, this results in a copy number of several million per bead. Subsequently, the emulsion PCR is broken while the amplified fragments remain bound to their specific beads. The fragments are then loaded onto a PicoTiterPlate device for sequencing. The diameter of the PicoTiterPlate wells allows for only one bead per well. After addition of sequencing enzymes, the fluidics subsystem of the Genome Sequencer FLX Instrument flows individual nucleotides (nt) in a fixed order across the hundreds of thousands of wells containing one bead each. Addition of one (or more) nucleotide(s) complementary to the template strand results in a chemiluminescent signal recorded by the CCD camera of the Genome Sequencer FLX Instrument (Margulies *et al.*, 2005)

To perform complete genome sequencing preparation of the cDNA only involved removing contaminants using the QIAquick PCR purification kit (QIAGEN – cat. nr. 28104) as per the instructions of the manufacturers. Two hundred and fifty µl of Buffer PB (QIAGEN) was added to the PCR product. The PCR product was transferred to a QIAquick column to bind the cDNA, and placed in a clean DNase, RNase free microcentrifuge tube, followed by a centrifugation step at 17200 x g for 1 minute. The flow through was discarded and the column placed back in the centrifuge tube. The column was washed with 750 µl of Buffer PE (QIAGEN) and centrifuged. The flow through was discarded and the column was placed back in the microcentrifuge tube followed by another quick spin to remove any residual wash buffer. The QIAquick column was transferred to a clean DNase, RNase free tube. To elute the cDNA 50 µl Buffer EB (QIAGEN) was carefully applied to the centre of the QIAquick (QIAGEN) column, ensuring that the silica membrane is not compromised, followed by an incubation step of 1 minute, and centrifugation at 17200 x g for 1 minute. There was no need for the separation of genome segments using AGE as the 454 GS FLX has the advantage over the Sanger method that the full genome can be sequenced without prior separation of segments.

Sequencing was done on the Roche GS 454 sequencer by Inqaba Biotec™ (pyrosequencing was developed by 454 Life Sciences).

2.2.8 *Genome sequence assembly and analysis*

The Lasergene 8 package from DNASTar was used for assembly of the complete genome from the 454 Pyrosequencing, essentially as described by Potgieter *et al.* (2009). Genome sequences were assembled de novo. The parameters used for assembly were set as prescribed by DNASTar:

- End trimming – Based on quality (set to medium)
- Match spacing – 15
- Minimum sequence length – 30
- Maximum mismatch end bases – 0

After assembly primer sequences (PC2) at the beginning and ends of contigs were deleted. All Bluetongue and EHDV genome segment sequences start with 5' GUU (GTT) and end with UAC 3' (TAC) and therefore the junction with the PCR primers could easily be identified. Assembled sequences were again subjected to BLASTanalysis using the NCBI website on <http://blast.ncbi.nlm.nih.gov/Blast.cgi> using BLASTN for nucleotide sequences and BLASTX for protein sequences. The search algorithm was set at megaBLAST (highly similar sequence) for nucleotide searches of all ten segments for both viruses.

2.3 RESULTS AND DISCUSSION

2.3.1 *RNA extraction and analysis*

BHK-21 cultures in a 75 cm² TC flask were seeded with Alp8 and Alp9 respectively and were observed daily. The TC flask with infected BHK-21 cells showed progressive viral activity rated as CPE after approximately 48 hours post infection and 4 + CPE after approximately 72 hours incubation (results not shown).

Total RNA was extracted from these BHK-21 cultures using a commercially available kit and accompanying protocol as described under Materials and Methods (2.2.2). It was anticipated that BTV dsRNA would be isolated. BTV has a dsRNA genome (Verwoerd *et al.*, 1972). To determine if infection as seen by CPE in TC cells was due to a virus with a dsRNA genome,

ssRNA was removed by precipitation with 2 M LiCl. ssRNA was observed as tiny white opaque pellets at the bottom of the microcentrifuge tubes (Results not shown).

The BTV genome is composed of 10 dsRNA genome segments (Verwoerd, 1969). An electrophoretic analysis was done to analyse the dsRNA of Alp8 and Alp9. AGE was performed using a TBE agarose gel and TBE buffer as set out under Materials and Methods (3.2.2). O'GeneRuler ladder mix (Fermentas SM 1130) was loaded with the sample material as size marker. This size marker, in the 3500 – 800 bp range was chosen on grounds of the size of the genome segments of the *Orbivirus* group (Verwoerd *et al.*, 1972). Figure 6 shows the electrophoretic profile of the purified dsRNA of Alp8 and Alp9. Segments from Alp8 and from Alp9 were visible. In both Alp8 and Alp9 the size of the largest genome segment was in the range of 4000 bp and the smallest genome segment in the range of 800 bp. Genome segments 7, 8 and 9 of Alp9 did not separate in clear distinguishable bands (Figure 6 lane 2). Very poor resolution was achieved for genome segments 2 and 3 of Alp8 (Figure 6 lane 3) as well. In both cases, it could be due to overloading of dsRNA of the gel, and due to the fact that the molecular sizes of the specific segments of both viruses are so close that they did not separate in distinguishable bands with the use of AGE (Verwoerd *et al.*, 1972). The results obtained by AGE for Alp8 and Alp9 were however consistent of an orbiviral dsRNA electrophoretic profile as determined by Potgieter *et al.* (2002) and for an BTV electrophoretic profile with the use of polyacrylamide gel electrophoresis by Verwoerd *et al.* (1972). The lack of distinct additional bands for either Alp8 or Alp9 confirmed that pure virus cultures were obtained from ECE that died on consecutive days. Regardless of both virus samples grown in 75cm² BHK-21 cultures (see 2.2.1), much more dsRNA product was present from Alp8 than of Alp9 (Figure 6).

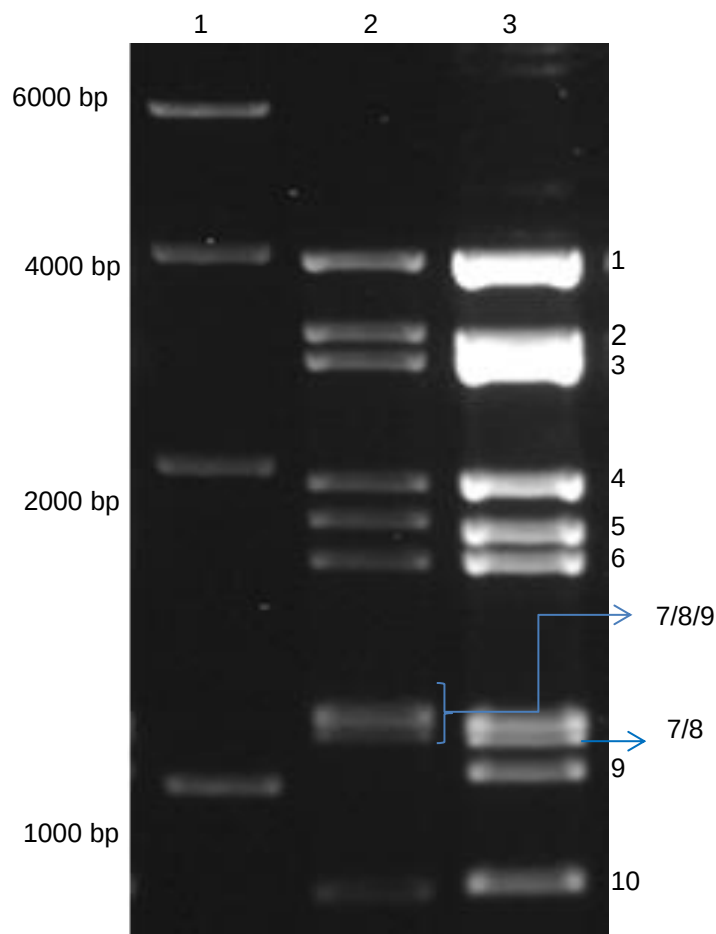


Figure 6: Agarose gel electrophoretic analysis of the purified dsRNA genomes of Alp8 and Alp9. Lanes: 1) O'GeneRuler ladder mix (Fermentas SM 1130); 2) 2 µl of 30 µl of purified dsRNA of Alp9. 3) 2 µl of 30 µl of purified dsRNA of Alp8.

2.3.2 Genome amplification

It was indicated that Alp8 and Alp9 contained dsRNA genomes, which both separated in 8 distinguishable bands respectively on AGE. As there was no prior knowledge of the sequence of Alp8 and Alp9 genomes, we used a sequence independent amplification protocol to amplify the two alpaca virus genomes. The genomes of both Alp8 and Alp9 were amplified successfully using the sequence independent amplification method for dsRNA genomes as described by Potgieter *et al.* (2009). The major advantage of using the amplification method as described by Potgieter *et al.* (2009) is that genome amplification could be performed even though we had no prior knowledge of which virus or viruses were present. Amplification products of the cDNA genome segments can be seen in Figure 7 which shows the agarose gel electrophoretic profile of the amplified cDNA of Alp8 and Alp9. All the genome segments of Alp8 were amplified

including the largest segment of approximately 4 kb. The electrophoretic profiles from cDNA of both Alp8 and Alp9 were similar to the agarose gel electrophoretic profiles obtained from the dsRNA of Alp8 and Alp9. The AGE result is supporting evidence of the successful amplification of the genomes of Alp8 and Alp9 by the method as described by Potgieter *et al.* (2009). Eight cDNA genome segments of Alp8 were visible in Figure 7. This was consistent with an AGE profile of BTV. The cDNA AGE profile of Alp9 is also similar to that obtained from the dsRNA, showing 8 clear bands. As with the AGE profile on the dsRNA of Alp9, genome segments 7, 8 and 9 did not separate into clear distinguishable bands (Figure 7), due to overloading of cDNA on the gel, and due to the fact that the molecular sizes of the specific segments of both viruses are so close that they did not separate in distinguishable bands with the use of AGE as also shown by Potgieter *et al.* (2002).

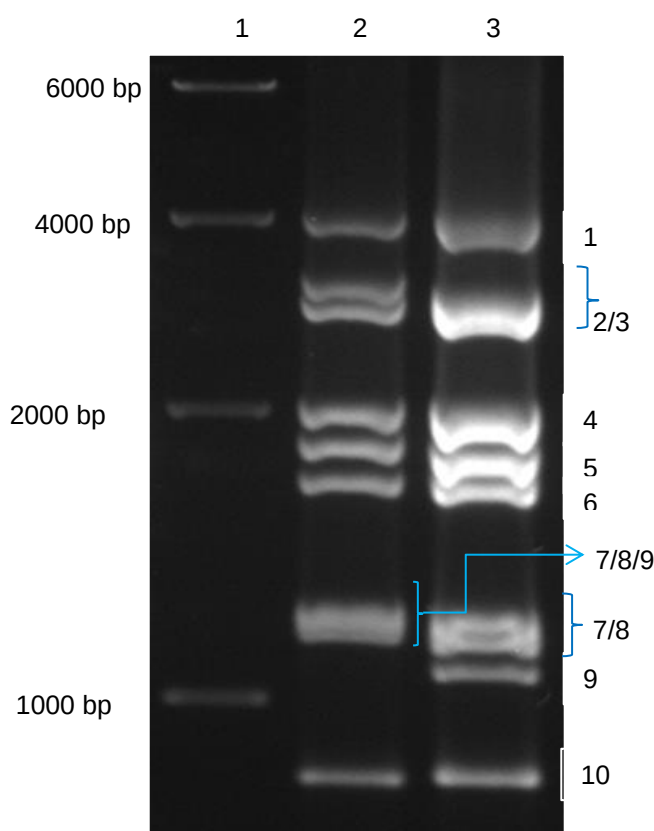


Figure 7: Agarose gel electrophoretic analysis of the cDNA genomes of Alp8 and Alp9.

Lanes: 1) O'GeneRuler DNA ladder mix (Fermentas SM 1123);
2) 2 µl of 30 µl of purified cDNA of Alp9; 3) 2 µl of 30 µl of purified cDNA of Alp8

2.3.3 *Partial sequencing of genome segments 2 (VP2) and 5 (NS1)*

The 3' ends of S2 (VP2) and S5 (NS1) of both virus isolates were sequenced successfully using Sanger sequencing of amplicons excised from an agarose gel. Approximately 500 bp of high quality sequence was obtained for each genome segment. The NS1 (S5) sequences of the viruses confirmed that the one virus was BTV and other virus was EHDV. NS1 (S5) of Alp8 shared 99 % deduced amino acid identity with BTV2. NS1 (S5) of Alp9 shared 99 % amino acid identity with EHDV6. Analysis of the VP2 (S2) sequences (which is an indication of serotype and genotype) showed that neither of the viruses belonged to one of the known serotypes of BTV or EHDV. BLASTP analysis of translated S2 (VP2) of Alp8 showed that this isolate was related to BTV15, but that it only shared 73 % deduced amino acid identity with BTV15. Sequence analysis of VP2 (S2) of Alp9 showed that it shared 76 % deduced amino acid identity with EHDV serotype 2 (Table 5).

Table 5: BLAST results from partial sequences of VP2 (S2) and NS1 (S5) of Alp8 and Alp9 indicating the closest related deduced amino acid sequence.

Virus Genome segment	¹ Closest related deduced amino acid sequence
Alp8 S2 (VP2)	73 % BTV15
Alp8 S5 (NS1)	99 % BTV2
Alp9 S2 (VP2)	76 % EHDV2
Alp9 S5 (NS1)	99 % EHDV6

1 Only approximately 500 bp were sequenced of each segment

2.3.4 *Complete genome sequencing*

The Roche 454 Pyrosequencing of Alp8 and Alp9 yielded 1564188 bp and 2035724 bp of sequence respectively. The average read length was 223 (Alp8) and 227 (Alp9) respectively. The complete genomes of both viruses could be assembled *de novo* using Lasergene software. Alp8 and Alp9 sequences each assembled into 10 individual contigs. Each contig contained the complete sequence of one of the ten genome segments starting with the conserved 5'-GTT and ending with the conserved TAC-3'.

The results of BLAST analysis of each genome segment of Alp8 is shown in Table 6 and that of Alp9 in Table 7. Analysis of the complete sequences of Alp8 confirmed that all genome segments were typical BTV genome segments. S7 (VP7) of Alp8 showed 97 % nucleotide

identity with the BTV15 Reference strain. VP7 (S7) is the virus species specific protein. The high similarities of 97 % nucleotide and 100 % of the deduced amino acid sequence of S7 (VP7) indicate that Alp8 falls in the BTV group. The overall nucleotide and amino acid comparison of genome segments S1, 3, 5, 8 and 10 of Alp8 showed no specific consistent correlation with any specific BTV serotype sequences in GenBank. The nucleotide sequence of S1 (VP1) showed 96 % nucleotide identity with BTV1, while the deduced amino acid sequence showed 99 % identity with BTV11. The nucleotide sequence of S3 (VP3) showed 90 % identity with BTV4 vaccine strain while the deduced amino acid sequence shared 99 % identity with BTV8. The nucleotide sequence of S5 (NS1) showed 97 % identity with BTV12 while the deduced amino acid sequence showed 99 % identity with BTV2. The nucleotide sequence of S8 (NS2) showed 97 % identity with BTV2 while the deduced amino acid sequence showed 98 % identity with BTV8. The nucleotide sequence of S10 (NS3) showed 96 % identity with BTV3 while the deduced amino acid sequence showed 99 % identity with BTV17.

The complete sequences of S7 (VP7) of Alp9 showed 95 % nucleotide and 100 % deduced amino acid identity with EHDV7 (Israel 2006 isolate). VP7 is the group-specific protein and induces group-specific antibodies (Huismans and Erasmus, 1981). VP2 (S2) is the serotype specific protein in the *Orbivirus* group and induces serotype specific antibodies (Huismans and Erasmus, 1981). No identity was however found with S2 (VP2) for Alp9 nucleotide sequence. The overall nucleotide and amino acid comparison of genome segments 1, 3, 6 and 10 showed no correlation between nucleotide and amino acid relationship. The amino acid and nucleotide sequences of genome segments 1, 3, 6 and 10 have the closest relationships respectively of different serotypes of EHDV in these segments. The nucleotide sequence of S1 (VP1) showed 99 % identity with EHDV6 isolate M44/96 from SA, while the deduced amino acid sequence showed 99 % identity with EHDV7 isolate Israel 2006. The nucleotide sequence of S3 (VP3) showed 99 % identity with EHDV6 isolate M44/96 while the deduced amino acid sequence showed 99 % identity with EHDV4 strain Ibaraki 3385. The nucleotide sequence of S6 (VP5) showed 81 % identity with EHDV2 strain KSB-14/E/97 while the deduced amino acid sequence showed 96 % identity with EHDV7 strain CSIRO from Australia. VP5 also plays a minor role in the determination of virus serotype, and the nucleotide sequencing result of S6 (VP5) endorsed the sequencing results obtained for S2 (VP2) for Alp9. The nucleotide sequence of S10 (NS3) showed 99 % identity with EHDV6 strain M44/96, while the deduced amino acid sequence showed 99 % identity with the EHDV7 strain Israel 2006. Potgieter *et al.* (2003) showed that the percentage identity between the deduced amino acid sequences of VP2 of the nucleic acid sequence of genome segment S2 (VP2) of the nine different AHSV serotypes correlate with serological cross reactions. The highest percentage identity indicated between VP2 (S2) of the different serotypes of AHSV was 71.4 % and the lowest identity 47.6%.

Table 6: Summary of the comparison of the nucleotide (nt) and deduced amino acid (aa) sequences of Alp8 with the closest related viruses found in GenBank. Accession numbers of the closest relatives as found on the National Centre for Biotechnology Information website on <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

Nucleic acid comparison					Amino acid comparison			
Genome Segment (protein)	No. nt identities/ total	Closest related BTV serotype	% Identity	Accession no. of closest related BTV segment	No. aa identities/ total	Closest relation to BTV serotype	% Identity	Accession no. of closest related BTV protein
1 (VP1)	3386/3944	BTV1	96	JN848759.1	1295/1302	BTV11	99	AFN71120.1
2 (VP2)	1982/2730	BTV15	73	JQ086222.1	731/952	BTV15	77	AFK91805.1
3 (VP3)	2500/2773	BTV4 vaccine strain	90	JN255944.1	896/901	BTV8	99	CAM57244.1
4 (VP4)	1829/1981	BTV11 vaccine strain	92	JQ972844.1	636/644	BTV11	99	AFN71113.1
5 (NS1)	1714/1773	BTV12	97	FJ713345.1	483/484	BTV2	99	AAN28946.1
6 (VP5)	1485/1639	BTV15 RSA Reference strain	91	AJ586716.1	519/527	BTV15	98	CAE53034.1
7 (VP7)	1123/1155	BTV15 RSA Reference strain	97	DQ465027.1	349/349	BTV15 RSA Reference strain	100	ABG35248.1
8 (NS2)	1097/1127	BTV2 vaccine strain	97	JN255939.1	292/299	BTV8	98	CAM57249.1
9 (VP6/ NS4)	972/990	BTV1	98	FJ437560.1	324/329	BTV1	98	ACJ65035.1
10 (NS3/ NS3a)	785/ 819	BTV3	96	FJ713318.1	226/229	BTV17	99	AAC42314.1

Table 7: Summary of the comparison of the nucleotide (nt) and deduced amino acid (aa) sequences of Alp9 with the closest related viruses found in GenBank. Accession numbers of the closest relatives as found on the National Centre for Biotechnology Information website on <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

Nucleic acid comparison					Amino acid comparison			
Genome Segment (protein)	No. nt identities/ total	Closest related EHDV serotype	% Identity	Accession no. of closest related EHDV segment	No. aa identities/ total	Closest relation to EHDV serotype	% Identity	Accession no. of closest related EHDV protein
1 (VP1)	3894/3942	M44/96	99	HM636907.1	1296/1302	EHDV7 Israel 2006	99	AEK21265.1
2 (VP2)	3002	No hits found	-	-	695/982	EHDV2 strain 439 Austr.	71	CAN89087.1
3 (VP3)	2746/2769	M44/96	99	HM636909.1	776/779	EHDV4 strain Ibaraki 33853	99	CAN89115.1
4 (VP4)	1955/1983	M44/96	99	HM636910.1	637/644	EHDV6 M44/96	99	AEK21268.1
5 (NS1)	1788/1802	M44/96	99	HM636911.1	550/551	EHDV6 M44/96	99	AEK21269.1
6 (VP5)	1291/1585	EHDV2 strain KSB-14/E/97 IBARAKI	81	AB078630.1	414/433	EHDV7 strain CSIRO 775	96	CAN89144.1
7 (VP7)	1089/1147	EHDV7 Israel 2006	95	JQ070183.1	349/349	EHDV7	100	AEY69034.1
8 (NS2)	1171/1186	M44/96	99	HM636914.1	368/373	EHDV6 M44/96	99	AEK21272.1
9 (VP6)	1096/1116	EHDV7 Israel 2006	98	JQ070185.1	349/359	EHDV7 Strain Israel 2006	97	AEY69036.1
10 (NS3)	805/810	M44/96	99	HM636916.1	227/228	EHDV7 Israel 2006	99	AEY69037.1

2.4 SUMMARY

BTV has a dsRNA genome that consists of 10 linear genome segments (Verwoerd *et al.*, 1972). The recent discovery of two new serotypes of BTV from Toggenburg in Switzerland (Chagnat *et al.*, 2009) and from Kuwait (Maan *et al.*, 2011b) once again highlighted the importance of molecular biology-based techniques ranging from AGE to genome sequencing to be able to rapidly identify virus isolates during disease outbreaks.

It was not possible to serotype the viruses Alp8 and Alp9 by means of BTV and EHDV VNTs at the OIE Reference Laboratory at Onderstepoort Veterinary Institute, despite a positive BTV PCR result on the original blood sample these two isolates were made of. In an effort to identify and characterise the viruses on a genetic level, dsRNA extraction and purification, followed by genome amplification and sequencing were performed.

The agarose gel electrophoretic profile of the dsRNA of Alp8 and Alp9 had 8 distinguishable dsRNA genome segments, ranging from approximately 800 bp to 4 000 bp (Figure 6). Such an electrophoretic profile is typical of orbiviruses. Since only eight clearly distinguishable bands were observed, it was assumed genome segments 7, 8 and 9 of Alp9 and 7 and 8 of Alp8 co-migrated.

Advances made in sequence-independent dsRNA genome amplification and sequencing by Pogieter *et al.* made it possible to amplify and sequence the genomes of both viruses isolated from the dead alpaca.

The cDNA AGE profile of Alp8 was similar to that of BTV with 8 identifiable segments visible; however genome segments 2 and 3 did not separate. Only 8 distinguishable segments were also visible for Alp9 (Figure 7).

Both the partial Sanger sequencing of VP2 (S2) and NS1 (S5) and complete 454 Pyrosequencing using the 454 GS FLX system (Roche) delivered similar results. Sequences of Alp8 and Alp9 were submitted to GenBank. BLAST analysis revealed that Alp8 is a BTV with BTV15 being the closest serotype with the highest percentage nucleic acid identity (73 %). The lowest percentage nucleotide variation in S2 (VP2) between the first 24 serotypes of over 300 BTV isolates (including reference strains) is 29 % (Maan *et al.*, 2009). The percentage nucleotide variation between S2 (27 %) of Alp8 to S2 of BTV15 strongly suggest that Alp8 may be a potential new serotype of BTV.

Whole genome sequencing data revealed that Alp9 is an EHDV, with no nucleotide identity to any known EHDV serotype. The deduced amino acid sequence of VP2 (S2) of Alp9 had 76 % identity to EHDV2 (Table 5) with the partial Sangar sequencing (500 bp) while the complete sequence of VP2 (S2) only had 71 % amino acid identity to EHDV2 (Table 7). The rest of the genome segments of Alp9 (S1, 3, 4, 5, 6, 7, 8, 9 and 10) had nucleotide identities ranging from 81 to 99 % and 96 to 100 % deduced amino acid sequence identity to different EHDV serotypes.

This sequencing result and subsequently BLAST analysis on Alp9 indicate that a new serotype of EHDV have been isolated. Very few cases of EHDV in alpacas have been reported in literature (Phillips, 2013).

In the next chapter results of serological investigations, especially VNT will be presented which were used to verify the sequencing results from a virological and serological perspective.

CHAPTER 3

SEROLOGICAL ANALYSIS

3.1 INTRODUCTION

In case of an outbreak and surveillance of disease it is of primary importance to determine which virus is involved, and secondly, to determine which serotype(s) are involved. The assays used mostly in laboratories for the detection of viruses and their components can be separated in 3 categories, namely a) those techniques that measure virus infectivity (plaque assays) (Howell *et al.*, 1967), b) those that measure the host's antibody response to BTV infection (ELISA, CFT), (Kipps, 1956, Anderson, 1984) (AGID, VNT) (Parker *et al.*, 1975, Klontz *et al.*, 1962), c) and those that rely on molecular techniques (RT-PCR) (Flint *et al.*, 1999).

VP7-based ELISA and CFT are both group-specific assays and can detect all the serotypes within a virus group. RT-PCR assays using primers from S7 (VP7) sequence that are conserved within virus species are used for group-specific PCR assays. A VP7-based competitive ELISA (cELISA) and CFT assay were used in this study to determine the *Orbivirus* group involved. Before the introduction of PCR assays, the diagnosis of virus infections often involved the isolation of the organism involved (Mertens *et al.*, 2009). This said, laboratories nowadays still need to perform virus isolation first in cases where too little viral genomic material is available to perform molecular techniques. The use of ECE and TC proved successful in the isolation of the two alpaca viruses involved in this study.

VNT is still regarded as the gold standard for the determination of virus serotype (Hamblin, 2004). VNT is used as a prescribed test in the OIE Reference Laboratory at OVI to determine virus serotype in all potential *Orbivirus* isolates. In the light of the initial positive BTV RT-PCR result as performed by Dr. Marco Romito, BTV VNT was performed on the alpaca samples. The determination of virus serotype is important in epidemiology studies and to determine the spread of virus in disease outbreaks (Mertens *et al.*, 2009). Molecular serotyping using RT-PCR steadily started to replace traditional serological methods in many laboratories (Mertens *et al.*, 2009), given the availability of serotype-specific primers of S2

(VP2), which is the primary genome segment determining serotype. The biggest disadvantage of VN testing is the duration of the test. It can take up to five days to serotype a virus from isolated material.

With a view to verify sequence results from the two alpaca virus isolates, Alp8 and Alp9, we performed a series of virological and serological assays. In this chapter the results of group-specific VP7-based tests, a competitive ELISA (cELISA) and CFT will be discussed. Thereafter, the results of neutralisation testing, the production of monovalent GP serum against Alp8 and Alp9 and SNT will be presented.

The owner of the alpaca reported that she had 22 more alpacas in the herd. No viraemia or clinical signs were reported in any of the alpacas in the rest of the herd (personal communication, Dr. J Tyrer). To determine whether any of the other alpacas had been infected and subsequently developed a serological response to the Alp8 and/or Alp9 viruses we also tested their serum for antibodies against Alp8 and Alp9 viruses.

To be able to test any other field samples that may contain the same serotypes of BTV or EHDV as in this case, we produced hyper immune GP sera to be used in serotyping.

The specific aims that were addressed in this chapter were:

- To verify the results obtained in the previous chapter from a virological and serological perspective;
- To determine whether the viruses were transmitted and have infected other animals in the herd by using SNT;
- Whether the testing methods currently applied in the laboratory detect the antibodies in the hyper immune GP serum produced against the alpaca viruses.

3.2 MATERIALS AND METHODS

3.2.1 *Virus neutralisation test*

All sterile procedures were executed in an Airvolution Biohazard class 11 cabinet. VNT testing was performed on virus suspensions isolated firstly on ECE, with subsequent passaging on chicken embryo rabies (CER) and BHK-21 cells. Virus suspensions were diluted 1:100 for use in the VNT.

All serum dilutions were diluted two-fold, with an initial dilution of 1:4 in the first wells in a 96 well NUNC TC plate. VNT testing was performed essentially as described in the OIE Reference Manual of 2009 (OIE, 2009) with minor modifications.

Twenty five µl of Eagles minimum essential medium (EMEM Cat. nr. 12-1169F, Bio Whittaker, Cambrex) containing 1 % of penicillin/streptomycin/fungizone mixture (Bio-Whittaker, cat nr. 17-745E), was added across the 96 well TC plates (NUNC, cat. nr. 167008). Aliquots of 25 µl of each of the 24 BTV OIE reference serums (produced in GP from OIE reference antigens) and the 8 EHDV (see Chapter 1.2.3.2) reference serums were made in duplicate in the top wells of a 96 well TC plate. Two-fold dilutions of the reference serums were made across the plate. A 25 µl volume of a 1/100 dilution in EMEM (Bio-Whittaker) of the virus isolate material (Alp8 and Alp9) was added to the serum dilutions on the plate, followed by an incubation step at 37°C for 30 minutes in an TC incubator (New-Brunswick) containing 5 % CO₂ to allow attachment of virus to specific antibody. Finally, 100 µl of VERO cells (diluted to be 75-80 % confluent in 24 hours) were added to the wells, before transferring the plates to a TC incubator containing 5 % CO₂. A back titration was performed to evaluate the progressing of CPE in the cell cultures. Field isolates are usually diluted 100 fold in VNT testing. This dilution of virus is then used as a working dilution on the 96 well TC plate. The back titration is a series of dilutions ranging from 10⁻¹ to 10⁻⁴ that is added to the back titration wells on the TC plate. Plates were incubated and evaluated daily for the progression of CPE until the back titration wells at the 10⁻² dilution (working dilution) showed 100 % CPE and the consequent wells at -3 and -4 showing CPE before reading.

VNT titres are expressed at the reciprocal of the dilution of serum that caused a 50 % inhibition of the virus to cause CPE of the VERO cells.

All TC medium used for passaging of cells contained 1 % of a 100 x penicillin, streptomycin and fungizone mixture (Bio Whittaker, Cambrex), 1 % non-essential amino acid mixture (Bio Whittaker, Cambrex, Cat. nr. 13-114E), a final concentration of 2 mM L-glutamine (Bio-Whittaker, Cambrex, Cat. nr 17-605), and 10 % foetal bovine serum (Bio-Whittaker, Cambrex, cat nr. 14-491E).

3.2.2 *Production of monovalent GP serum*

Monovalent GP serum was produced as described in the OIE manual for terrestrial animals, under the ethical clearance granted to the Virology Department's on-going project nr. 05/18

P001. Virus titres of Alp8 and Alp9 TC material were performed and determined according to the method published by Reed and Muench (1938). Serial dilutions of the antigen were prepared and 50 µl per dilution were aliquoted in 4 wells on a 96 well plate. Thereafter 100 µl VERO cell suspension was added. The Reed and Muench endpoint calculation method was performed as follows: (% of wells infected at dilution above 50% - 50%)/ (% of wells infected at dilution above 50% - % of wells infected at dilution below 50% endpoint). Titer = $10^{\log \text{total dilution above 50\%} - (I \times \log h)}$

Twenty millilitres of TC material was centrifuged at 2400 x g for 10 minutes to pellet TC cells. The supernatant containing virus was absorbed to 1 ml aluminium hydroxide gel (Al(OH)₃). This suspension was incubated on a platform shaker at room temperature for 1 hour, followed by a centrifugation step at 2400 x g for 10 minutes to pellet the Al(OH)₃. The supernatant tissue culture medium was discarded. Two ml preheated phosphate buffered saline (PBS) was then added to the Al(OH)₃ and gently mixed to resuspend the alhydrogel in the PBS. Female GP were inoculated via the intra-peritoneal route with 2 ml alhydrogel/PBS mixture. This process was repeated every week for 5 consecutive weeks with 1 week intervals, altering the side for intra-peritoneal inoculation of the GPs each week. The GPs were sedated and bled by cardiac puncture at 6 weeks from the first inoculation. The collected blood was left to clot, before it was centrifuged to separate the blood clot from the serum. The GP monovalent serum was then heat inactivated at 56°C for 30 minutes in a water bath, before it was sterile filtrated through a 0.22 micron syringe filter (Sartorius).

3.2.3 Serum neutralisation test

SNT was performed using OIE reference antigens and;

- 1) The GP produced monovalent serum as test serum and
- 2) Serum from additional alpacas from the same herd as the test samples.

All reference antigens were used at a dilution of 100 TCID₅₀ units from a pre-determined virus titre. SNT was essentially performed as described in the OIE manual for the VNT testing (OIE, 2009), with the only difference that the GP sera and alpaca test serums were used as the test serum to determine any cross reactivity between the OIE reference antigens and the GP produced serums and alpaca serums. The alpaca sera were heat inactivated at 56°C for 30 minutes prior to testing. The sera were diluted two-fold starting with a 1:4 dilution

on the 96 well TC plate. SNT titres are expressed as the reciprocal of the dilution that caused a 50 % inhibition of the virus to cause CPE of the VERO cells.

3.2.4 cELISA

A BTV cELISA was performed by Mr. H. Harris as per the instructions of the manufacturer (VMRD Catalogue nr. 287-2). Unfortunately, no ELISA methods were available for the detection of EHDV antibodies at the Serological Unit of the Virology Division at OVI.

All samples, including all controls, were tested in duplicate. All reagents were used at room temperature. The wash solution (prepared in-house), PBS containing 0.1 % v/v Tween-20 was pre-diluted to 1:50 with distilled water. Serum was tested undiluted.

Aliquots of 25 µl of the GP test serum were added to the wells of the antigen coated cELISA plate, to ensure that the bottoms of the wells were completely coated. The plates were incubated at room temperature for 15 minutes. Twenty five µl of antibody peroxidase conjugate was added to each well, followed by another incubation period of 15 minutes at room temperature. The plates were washed 3 times in an automatic ELISA washer. Fifty µl of the substrate solution was added to each well and the plate incubated for 10 minutes at room temperature. Fifty µl of the stop solution, at a final concentration of 1N H₂SO₄ (Associated Chemical Enterprises, South Africa) was added next where after reading was done on an ELISA plate reader (Biotek-ELx405, Analytical & diagnostic products). The optical density (OD) reading wavelength was set at 650 nm. The results were interpreted as follows: Test sera were positive if they produced an OD less than 50 % of the mean of the negative controls. Serums that produced an OD greater than or equal to 50 % of the mean of the negative controls were negative.

Results obtained from the cELISA are expressed at percentage inhibition, as determined by the following formula:

$$\% \text{ inhibition} = 1 - (\text{OD test serum} / \text{OD negative control}) \times 100$$

3.2.5 *Complement fixation test*

Test sera were heat inactivated at 56°C for 30 minutes prior to testing. This step is necessary to prevent anti-complementary reactions of the complement with the test sera. The antigens used in the CFT test are sucrose-acetone extractions of BTV- and EHDV-infected mouse brains respectively. The BTV and EHDV CFT antigens used were pre-titrated to contain four antigen units. The control wells contained uninfected mouse brain, and were treated in the same way as the positive antigens. Normal GP serum contains complement. This serum is not heat-inactivated as the heat inactivation step destroys complement found in mammalian serum. The haemolysin (amboceptor) used in the CFT test, is hyper-immune rabbit serum against sheep red blood cells. Amboceptor is used at two haemolytic units, mixed and diluted in veronal buffer with a 3 % concentration of sheep red blood cells.

Duplicate wells of a 96 well round bottom plate were used per test sample. Twenty five µl of veronal buffer was used as diluent on the plate. Twenty five µl of each test serum was aliquoted. Two-fold dilutions of the sera were made across the plate, followed by the addition of 25 µl of the antigen dilutions in every second well of the plate (a positive antigen row of wells followed by a negative antigen row). Twenty five µl complement was added to all the wells on the plate, except in the complement back titration wells. The plates were incubated overnight at 4°C. Twenty five µl sensitised sheep red blood cells were added to the wells on the following day, and the plate sealed with tape. The plates were shaken to re-suspend the sheep red blood cells. An incubation step of 15 minutes followed, the plate was shaken again and a further incubation of 15 minutes at 37°C followed. The wells were scored for the presence of haemolysis. The highest dilution of serum specifically fixing complement with the CF antigen is expressed as the titre in complement fixing units (CFU). A titre of 1/10 or more is positive, under 1/10 is regarded as negative.

3.3 RESULTS AND DISCUSSION

The specific aims that were addressed in this chapter were:

- To verify the results obtained in the previous chapter from a virological and serological perspective;
- To determine whether the viruses were transmitted and have infected other animals in the herd by using SNT;

- Whether the testing methods currently applied in the laboratory detect the antibodies in the hyper immune GP serum produced against the alpaca viruses. ELISA and CFT tests are both group-specific tests that detect the group-specific protein, VP7.

3.3.1 *Virus neutralisation test*

To determine the serotype of a specific typical BTV field sample isolated on either ECE or TC systems, VNT for BTV is firstly performed following the prescribed method in the OIE Reference Manual. The OIE reference serums produced in GPs against the 24 BTV serotypes did not neutralise either the Alp8 or Alp9 viruses (results not shown). Due to the very close serological relationship that exist between BTV and EHDV (Inaba, 1975) we decided to perform VNT for EHDV as well. None of the 8 EHDV (see Chapter 1.2.3.2) GP serums did neutralise any of the two alpaca viruses. The virus concentration in the back titration wells in both BTV and EHDV VNTs caused 4⁺ CPE in the wells containing the working dilutions of the viruses used in the VNT tests, and also in all the wells across the plates containing the serum dilutions. The cell control wells showed a confluent uninfected monolayer of cells (results not shown).

The VNT is regarded as the gold standard in the determination of *Orbivirus* serotypes (Hamblin, 2004) and are very specific. To be able to serotype a field sample, complete panels of verified diagnostic reference serum and antigen should be available.

Chaignat (2009) and Maan (2011b) described the existence of the 25th and 26th BTV serotypes. With the abovementioned results in mind and the initial PCR result (see Chapter 1.3) it was anticipated that Alp8 could be a new serotype of BTV.

3.3.2 *Serum neutralisation test*

To test for any cross reactivity between monovalent serum against Alp8 and Alp9 and OIE reference antigens for BTV and EHDV, and to have reference material for subsequent possible samples, we produced monovalent serum against Alp8 and Alp9 in GPs. The virus titre of Alp8 was 6.8 log₁₀TCID₅₀ and of Alp9 5.8 log₁₀TCID₅₀. For BTV SNT results see Table 8. Results of BTV SNT showed intermediate levels of cross reactivity (NAb titre of > 32), between serum produced against Alp8 and

BTV15, while the NAb titre of the positive control serum was high (NAb titre of 640). Much lower levels of cross reactivity were recorded between Alp8 and BTV serotypes 9 and 4, with NAb titres of 8 and 4 respectively. A titre of 4 was recorded for Alp9 against BTV15. NAb titres of 4 and 8 are regarded as negative. All reference antigens were used at a dilution of 100 TCID₅₀ units/ml. For EHDV SNT results see Table 9. Results of EHDV SNT showed low levels of cross reactivity between serums produced against Alp8 and EHDV serotype 1, 5 and 6, and are regarded as negative.

Table 8: Serum neutralisation (SNT) test results of serum produced against Alp8 and Alp9 viruses in guinea pigs (GP) against reference antigen strains of the 24 BTV serotypes.

BTV Serotype	Antigen dilution(TCID ₅₀)	NAb titre of Alp8 GP serum	NAb titre of Alp9 GP serum	Positive control OIE reference serum
1	4.16	-	-	640
2	4.16	-	-	160
3	2	-	-	320
4	4.5	4	-	640
5	4.16	-	-	320
6	3.8	-	-	320
7	3.8	-	-	320
8	3.5	-	-	40
9	3.5	8	-	160
10	3.5	-	-	160
11	4.16	-	-	320
12	2.16	-	-	160
13	2.5			160
14	4.16	-	-	160
15	4.16	≥32	4	640
16	3.5	-	-	640
17	2.83	-	-	1280
18	3.16	-	-	160
19	2.5	-	-	40
20	1.8	-	-	160
21	1.5	-	-	40
22	1.8	-	-	20
23	1.5	-	-	320
24	1.16	-	-	160

SNT titres are expressed at the reciprocal of the dilution that caused a 50 % reduction in CPE on VERO cells.

Table 9: Serum neutralisation test (SNT) results of serum produced against Alp8 and Alp9 viruses in guinea pigs (GP) against 8 reference antigen strains of EHDV (see Chapter 1.2.3.2).

EHDV Serotype	Antigen dilution (TCID ₅₀)	NAb titre of Alp8 GP serum	NAb titre of Alp9 GP serum	Positive control OIE reference serum
1	3.5	4	-	640
2	3	-	-	640
3 ¹	2.5	-	-	1280
4	2.5	-	-	640
5	1.5	4	-	1280
6	2.5	4	-	320
7	1.75	-	-	160
8	2	-	-	320

SNT titres are expressed at the reciprocal of the dilution that caused a 50 % reduction in CPE on VERO cells.

¹ The number of EHDV serotypes have been reduced from 8 to 7 serotypes (see Chapter 1.2.3.2)

As the alpaca from which the samples used in this study was from a herd of 22 alpacas, the possibility that the Alp8 and Alp9 viruses were transmitted to the rest of the herd was also investigated. As no reports of clinical responses were recorded in any of the rest of the alpaca herd (personal communication with owner), we decided to perform SNT. Both BTV and EHDV are transmitted between ruminant hosts by *Culicoides* vectors (Du Toit, 1962), and not through direct contact. SNT results (Table 10) indicated exposure with subsequent antibody responses to both viruses. Three alpacas out of 22 (Alpaca nr. 8, 11 and 18) had intermediate levels (16, ≥ 32) of NAb titres against both viruses, while 2 (Alpaca nr. 3 and 12) had an antibody response to Alp8 (≥ 32) and Alp9 (≥ 32) virus respectively. A further two alpacas (1 and 4) had low antibody titres (4, 8 and 12) against both Alp8 and Alp9. In all but one of the alpacas that were positive against both Alp8 and Alp9 viruses, the titres of Alp8 were higher than that of Alp9. Only alpaca 12 had a NAb titre (≥ 32) against Alp9 alone.

Table 10: Serum neutralisation test (SNT) results of antibody titres against Alp8 and Alp9 in the rest of the alpaca herd from which the samples used in this study were from.

Alpaca nr	Alp8	Alp9	Alpaca nr	Alp8	Alp9
1	12	4	12	-	≥ 32
2	-	-	13	-	-
3	≥ 32	4	14	-	-
4	8	4	15	4	-
5	-	-	16	-	-
6	-	-	17	-	-
7	-	-	18	≥ 32	≥ 32
8	≥ 32	16	19	-	-
9	-	-	20	-	-
10	-	-	21	-	-
11	≥ 32	≥ 32	22	-	-

SNT titres are expressed at the reciprocal of the dilution that caused a 50 % reduction in CPE on the VERO cells. Differing levels of antibody responses to the Alp8 and Alp9 viruses indicated dual infection in 6 out of the total of 22 animals.

3.3.3 cELISA and CFT

To measure the host's response to infection, ELISA and CFT are usually performed. For the purpose of this project, we performed a cELISA and an in-house OVI CFT to measure the antibody levels in the serum produced in GP against Alp8 and Alp9 viruses. cELISA and CFT assays have been developed to measure BTV and EHDV group-specific antibodies. The commercial BTV cELISA (VMRD Catalogue nr. 287-2) used in this work, is specific for the detection of BTV antibodies, and does not detect antibodies to EHDV, according to the manufacturer. The CFT is in essence a group-specific test. The antigen used in CFT testing is a sucrose-acetone mouse brain extract. The BTV CFT has the disadvantage that it detects antibodies of both the EHDV and Palyam virus groups as well (see Chapter 1.2.4), and is therefore not specific to detect antibodies to BTV (Della-Porta *et al.*, 1985).

For the cELISA test sera responses were interpreted as follows: test sera were positive if they produced an OD less than 50 % of the mean of the negative controls. Sera that

produced an OD at 650 nm greater than or equal to 50 % of the mean of the negative controls were negative.

Both alpaca samples, Alp8 and Alp9 tested positive on the BTV cELISA (Table 11).

In CFT testing (Table 11) Alp8 produced a higher antibody titre against BTV (1:16) than EHDV (1:4) and was regarded as positive. However Alp8 was negative for EHDV, as a titre of lower than 1:10 is regarded as negative. For Alp9 a two-fold higher antibody CFU titre was measured against EHDV than that of BTV. Alp9 produced high antibody titres against both EHDV (1:64) and BTV (1:32) (Table 11).

Table 11: cELISA and CFT results of Alp8 and Alp9 serum produced in GP against BTV and EHDV

Sample information	Tests		
	BTV cELISA ¹	BTV CFT(CFU) ²	EHDV CFT(CFU) ²
Alp8	0.2100	1:16	1:4
Alp9	0.2500	1:32	1:64
Pos. control	0.200	1:32	1:16
Neg. control	1.035	Neg.	Neg.

¹ cELISA results are reported as the percentage inhibition of binding as compared with the control serum.

² The CFT test result is expressed as complement fixing units (CFU)

From the cELISA and CFT results it was concluded that the mono-specific antibodies produced against Alp8 and Alp9 in guinea pigs showed levels of cross-reactivity between Alp8 and Alp9 on the BTV cELISA and the BTV and EHDV CFT. The BTV cELISA uses a VP7 based antigen. VP7 is the virus group-specific determinant (Huisman and Erasmus, 1981). Because of the close serological relationship between members of the BTV and EHDV group (Inaba, 1975), serological cross reactions between the two viruses is possible.

3.4 SUMMARY

Neither Alp8 nor Alp9 was neutralised by any of the 24 BTV or 8 EHDV OIE reference GP sera. To enable the identification of possible further samples from the same region in the western Cape near Montagu, GP serum was produced against both isolates. SNT of Alp8 showed a NAb titre of ≥ 32 against BTV15. Alp9 GP serum did not neutralise any of the 24 BTV serotypes, nor the 8 (see Chapter 1.2.3.2) EHDV serotypes. The SNT results showed that of the other 22 animals of the alpaca herd 7 also had NAbS against Alp8 and/or Alp9 (See Table 10). In three alpacas, namely numbers 8, 11, and 18 intermediate levels of NAb against both Alp8 and Alp9 were detected. Low levels of an antibody response against Alp8 and Alp9 were present in alpaca no's 1 and 4. Alpaca number 3 produced a NAb response (≥ 32) to the virus of Alp8, while alpaca number 12 produced a NAb response (≥ 32) to Alp9 virus alone. A NAb titre of 4 is regarded as negative. The fact that SNT showed that there were NAbS in the rest of the alpaca herd constitutes convincing evidence that laboratory contamination was not the cause of the presence of both Alp8 and Alp9 viruses in the original sample from which the viruses were isolated.

The BTV cELISA and CFT tests are group-specific and do not distinguish between virus serotypes. A cELISA detecting antibodies against BTV was performed. Serum produced against Alp8 and Alp9 was positive for BTV antibodies in the BTV cELISA. In the BTV CFT test, an antibody response was detected against Alp8 and against Alp9. In the EHD CFT test, an antibody response was detected against Alp9 (See Table 11).

These results support the genome sequencing evidence presented in Chapter 2 that Alp9 is a new serotype of EHDV causing disease in alpacas. To confirm whether Alp8 is indeed a new BTV serotype, the SNT with BTV15 antigen needs to be repeated. Alp8 GP serum needs to be titrated for an endpoint result to be able to compare Alp8 against BTV15 GP reference serum.

Orbiviruses are not transmitted via direct contact, but only through transmission by competent vectors. The fact that some of the animals in the rest of the alpaca herd also had Alp8 and Alp9 NAbS, indicate the involvement of a vector species. The competence of *Culicoides* vectors for Alp8 and Alp9 known to transmit BTV and EHDV will be presented in the following chapter.

CHAPTER 4

VECTOR SUSCEPTIBILITY OF *C. IMICOLA* FOR ALP8 AND ALP9

4.1 VECTOR TRANSMISSION OF BTV

The vector competency of *Culicoides* biting midges to transmit BTV and EHDV has been investigated extensively (Foster *et al.*, 1963, Du Toit, 1944, Jones *et al.*, 1977). The abundance of the particular potential vector of BTV and EHDV in a specific region increases the risk of transmission of the viruses to their mammalian hosts. *C. imicola* and *C. bolitinos* are the major vectors of BTV, and the most abundant vector of BTV throughout Africa (Du Toit, 1944, Meiswinkel *et al.*, 1994). Variations in the competence of different vectors of BTV have been recorded for the different serotypes of the virus and between different *Culicoides* species for the same virus serotype (Venter *et al.*, 2007).

S2 of Alp8 has a 73 % nucleotide and 77 % amino acid sequence identity to BTV15. In SNT a cross reaction with BTV15 was observed. BTV15 is regarded as having low epidemic potential, has low pathogenicity to sheep, and is mostly associated with viraemia in cattle (Du Toit, 1962, Erasmus *et al.*, 1984). In this chapter, I report on work done in collaboration with Dr. Gert Venter to investigate the potential of *C. imicola* to transmit the putative new serotype of EHDV (Alp9) and possible new serotype of BTV (Alp8).

4.2 MATERIALS AND METHODS

C. imicola were collected at Onderstepoort in South Africa using down-draught, 220 V light-traps equipped with 8 W UV light tubes during October 2009. Midges were sorted according to their wing pattern. Handling of field-collected *Culicoides* before feeding was carried out as described by Venter *et al.* (1991).

Field collected midges were fed on defibrinated sheep blood spiked with Alp8 and Alp9 TC material through a one-day-old chicken skin membrane as described by Jones *et al.* (1983). Virus dilutions were freshly prepared immediately before feeding as described by Paweska

et al. (2003). Virus titres used for feeding were 3.75- and 6.5 log₁₀ TCID₅₀/ml for Alp8 and 5.5 log₁₀ TCID₅₀/ml for Alp9 respectively.

Maintaining and processing of midges were performed according to methods described by Venter *et al.* (1991). Post-fed midges were chilled and replete females separated and retained in 250 ml un-waxed paper cups for 10 days at 23.5°C and 50-70 % relative humidity. During incubation, a 10 % (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 µg streptomycin and 1.25 µg of fungizone per 1 ml sucrose solution) was made available via cotton wool pads. *Culicoides* surviving incubation were chilled and sorted into species before being stored individually in 1.5 ml microcentrifuge tubes at -70°C prior to virus isolation. Individual midges were homogenised in 100 µl EMEM with the use of a battery operated micro-tissue grinder. Grinder pestles were pre-sterilised and changed between samples. Midge homogenates were centrifuged at 14 000 x g for 10 minutes. The supernatants were transferred to clean, sterile microcentrifuge tubes. After the addition of 100 µl EMEM containing 10 % foetal bovine serum (FBS), 25 µl aliquots of individual homogenates were made in triplicate in 96 well TC plates (NUNC). One hundred µl of a BHK-21 cell suspension in EMEM containing 5 % FBS was added to the TC plate. The TC plates were incubated in a 37°C TC incubator containing 5 % CO₂. After a 6 day incubation period the TC plates were evaluated for CPE. All wells showing CPE were marked, and the cells and TC medium harvested in separate microcentrifuge tubes for each individual sample showing CPE. The individual TC homogenates were used in a subsequent VNT test as described by House *et al.* (1990) using hyper immune GP sera prepared from Alp8 and Alp9 to confirm the identity of the virus causing CPE.

4.3 RESULTS AND DISCUSSION

Wild caught midges were fed on blood meals spiked with Alp8 and Alp9. Both Alp8 and Alp9 were isolated from midge homogenates. Isolation of virus from midges fed on a blood-spiked virusmeal complies with one of the WHO's criteria for determination of vector competence ((WHO), 1967). *C. imicola* is the most prevalent vector of BTV. Other species like *C. bolitinos*, which is most abundant in the eastern Free State, and then to a lesser extend *C. enderleini*, *zuluensis*, *milnei* and *pycnostictus* are also proven vectors of BTV in Africa (Meiswinkel *et al.*, 1994).

The individual midges that survived the 10 day incubation period were assayed for the presence of virus. The results are presented in Table 12. Besides the few other species

caught in the light trap, (results not shown) the most abundant *Culicoides* species from Onderstepoort was *C. imicola*. The susceptibility of midges fed with blood spiked with Alp8 virus with a titre of 3.75 log₁₀TCID₅₀ interestingly showed a higher percentage positive midges -1.7 % - than those fed with a titre of 10^{6.5} with 0.4 % positive. Alp9 virus however, delivered only 0.1 % positive midges fed with blood spiked with a virus titre of 5.5 log₁₀TCID₅₀.

Table 12: Virus isolation of individual *C.imicola* homogenates performed in BHK-21 cells

Virus in bloodmeal	Alp8		Alp9
Virus titre in blood meal log ₁₀ TCID ₅₀ /ml	3.75	6.5	5.5
Virus infection rates in <i>C. imicola</i>	¹ 12/686 (1.7 %)	¹ 1/237 (0.4 %)	¹ 1/673 (0.1 %)

¹Total midges positive out of the total that survived the 10 post-fed incubation period, and expressed as a percentage total positive

These infection rates of females fed on blood meals spiked with the different concentrations of Alp8 virus and Alp9 virus are relatively low. In BTV susceptibility studies of South African *C. imicola* by Venter *et al.* (2006) virus recovery rates for 5/11 different BTV serotypes were below 1 %, whilst virus recovery rates for *C. bolitinos* ranged from 1.6 % to as high as 50 %. However that does not necessarily implicate that *C. imicola* would not be an efficient vector of Alp8 and Alp9 viruses. Low virus recovery rates for certain *Culicoides* species may be compensated for by the high abundance and high biting rates (Venter *et al.*, 2006) of that specific midge species. The number of midges that were fed was also relatively low, and a more comprehensive study should be performed to include other potential vector species from the Montagu area where this alpaca was from. The SNT results (Chapter 3.3.2, Table 10) from 22 other alpacas from the same herd as where the alpaca isolates are from, suggested that there was transmission of both Alp8 and Alp9 viruses to 7 alpacas in the herd. Neither BTV nor EHDV are transmitted horizontally between ruminant species. The seropositive alpacas in the rest of the herd implicate that both Alp8 and Alp9 viruses were already established in the local midge population, and that another vector than *C. imicola* could possibly be involved in the transmission of Alp8 and Alp9 viruses. Pools of wild-caught midges from the Montagu area should also be subjected to virus isolation attempts to determine if and to what extend Alp8 and Alp9 viruses are present in the local midge population.

The competency of *C. imicola* for transmitting EHDV is much less compared to the competency of BTV.

The eradication of *Culicoides* is impossible, as adults occur in vast numbers, and their larval habitat is widespread and cannot be controlled nor treated due to the vastness thereof (Meiswinkel *et al.*, 1994).

4.4 SUMMARY

Both BTV and EHDV are not transmitted horizontally between ruminant species, but are transmitted between ruminant hosts via competent vector *Culicoides* midge species (Du Toit, 1944). *C. imicola* and *C. bolitinos* are the major vectors of BTV in South Africa (Du Toit, 1944, Meiswinkel *et al.*, 1994). Serological evidence that both Alp8 and Alp9 did spread to other alpacas in the same herd was presented in Table 10. Although the virus recovery rates from individual midge homogenates of laboratory infected wild caught *C. imicola* were rather low (1.7 % for Alp8 and 0.1 % for Alp9 respectively), *C. imicola* seemed to be a competent vector of both Alp8 and Alp9 viruses (Table 12).

CHAPTER 5

CONCLUDING SUMMARY

Historically, a febrile disease in sheep namely BT disease was first recognised 350 – 400 years ago with the introduction of merino and European sheep breeds into the Cape Colony of South Africa in 1652 and again in 1870. Merino and European sheep breeds are much more susceptible to BT than indigenous sheep breeds (Hutcheon, 1881).

More recently, the biggest incursion of BTV into southern Europe was most probably due to aerial movement of infected *Culicoides* (Wilson and Mellor, 2008) from Morocco, Tunisia and Turkey. BTV8, isolated from the 2006 outbreak, was very similar to strains isolated from sub-Saharan Africa previously (Wilson and Mellor, 2008), implicating aerial transmission of BTV8.

From this incursion, BTV8 infections spread through 2 122 holdings up to February 2007 and through 5 countries in Europe, expanded during 2007 and into 2008 to affect 57 545 holdings in 11 European countries and 27 139 holdings in 12 countries up to December 2008 (Wilson and Mellor, 2009). During the BTV8 outbreak in northern Europe in the summer and autumn of 2006 the mean morbidity rates were 20 % in sheep and 6.8 % in cattle, while the mortality rates were 5 % in sheep and 0.3 % in cattle (Guyot *et al.*, 2008). The total losses due to BTV8 infection in Europe during 2006/2007 were astronomical. In France losses were \$1.4 billion and \$85 million in the Netherlands (Tabachnick *et al.*, 2008).

Twenty six serotypes of BTV have been identified to date. The first 16 serotypes were isolated from 1900 to 1960, and serotyped by Howell (1970) in an attempt to solve the problem of continuous reinfection of sheep with BTV. The last serotypes of BTV to be identified was BTV25 from Switzerland during 2007 (Chaignat *et al.*, 2009, Hofmann *et al.*, 2008) and BTV26 from Kuwait during 2010 (Maan *et al.*, 2011a).

The introduction of exotic ruminant species to South Africa, mean that they will potentially be serologically naïve to circulating BTV serotypes. Alpacas were introduced into South Africa since 2000, and are mainly farmed with for their fine quality wool, and are also used as herd guards to protect sheep flocks from small predators. The current population of alpacas in South Africa is more than 2 000 (<http://www.alpacasociety.co.za>, 2012). The Virology

Division at OVI often received diagnostic samples from clinical cases of alpacas. BTV1 was isolated from alpacas in the western Cape shortly before the sample used in this study was received (Dr. Truuske Gerdes, personal communication). Clinical signs and lesions in the alpaca included respiratory distress, severe lung oedema, froth exuding from the nose and subcutaneous bleeding of the buccal cavity, signs consistent of BT infection. SAC, of which alpaca is a member, are susceptible to BTV as described by Schulz *et al.* (2012b), but studies indicate that transmission of BTV by SACs are negligible (Schulz *et al.*, 2012a), due to the fact that BTV binds less efficiently to the RBC of SAC than to that of sheep or cattle. The clinical signs and lesions caused by BTV and EHDV are indistinguishable (Inaba, 1975, Metcalf *et al.*, 1992).

Inconsistent chicken embryo mortalities complicated initial virus isolation attempts from heparin blood from the dead alpaca. Therefore, virus isolation was continued on BHK-21 cells from 3rd and 4th day mortalities in ECE. The 3rd and 4th day mortalities in ECE were harvested separately and passaged individually on 25 cm² flask with BHK-21 cells. The heparin blood from the alpaca tested positive for BTV on a RT-PCR. Neither of the isolated viruses (Alp 8 and Alp9) were neutralised by any of the 24 OIE BTV reference serums. Due to the close serological relationship between BTV and EHDV (Inaba, 1975), there is significant immunological cross-reactivity between members of the BT and EHD viruses (Monaco *et al.*, 2006). Following negative BT VNT testing, VNT was performed using EHDV hyper immune serum. No neutralisation of either the Alp8 or Alp9 virus was obtained with serum of any of the EHDV serotypes (see Chapter 3.3.1).

The dsRNA genome of BTV consists of 10 linear genome segments (Verwoerd *et al.*, 1972). The importance of the use of molecular techniques to rapidly identify virus isolates amidst disease outbreaks was once again highlighted with the recent identification of two new serotypes of BTV from Toggenburg in Switzerland (Chaignat *et al.*, 2009) and from Kuwait (Maan *et al.*, 2011b).

The electrophoretic profiles of dsRNA from Alp8 and Alp9 were typical of orbiviral genome profiles. Both virus genomes had agarose gel electrophoretic profiles with the genome segments ranging from approximately 800 bp to 4 000 bp (Figure 6). The absence of additional distinct genome segments was a first indication that pure cultures of BTV and EHDV were obtained after passaging in ECE and TC.

A sequence-independent reverse transcriptase cDNA synthesis and amplification method Pogieter *et al.* (2009) was used to generate, amplify and sequence cDNA of the genomes of

both viruses isolated from the dead alpaca. This method has the advantage over other amplification methods that primer walking and cloning bias are avoided.

In this MSc, genome segment S2 - which encodes VP2 that determines virus serotype, and genome segment S5 which encodes NS1 which is quite conserved within the virus groups was first analysed.

The PCR products were separated on a 1 % agarose gel and genome segments 2 and 5 were excised. The cDNA segments were submitted for partial sequencing at OVI using the Sanger sequencing method. BLAST analysis of the partial sequences (500 bp) of genome segments 2 and 5 showed that the deduced amino acid sequences of VP2 (S2) of Alp8 (Table 5) had 73 % amino acid identity to BTV15 and that NS1 (S5) had 99 % identity to BTV2. The deduced amino acid sequence of VP2 (S2) of Alp9 had 76 % amino acid identity to the EHDV6.

Complete genome sequencing using the next generation sequencing 454 GS FLX system from Roche was performed. The 454 GS FLX system has the advantage over Sanger sequencing that there is no need to separate genome segments beforehand and much more sequencing information is obtained. The sequences generated were submitted for BLAST analysis. The results from BLAST analysis, using MegaBLAST (highly similar sequence) of S2 (VP2) of the genome of Alp8 showed that there was only 73 % nucleotide sequence identity to BTV15 and 77 % deduced amino acid sequence identity to BTV15. BLAST analysis of S5 (NS1) showed 97 % nucleotide identity with BTV12 and 99 % deduced amino acid identity with BTV2 respectively (Table 6). The rest of the genome segments had nucleotide sequence identities ranging from 90 % to 98 % with different BTV serotypes, while the deduced amino acid sequences of the rest of the genome segments ranged from 98 % to 100 % amino acid identity with different serotypes of BTV.

The results from the BLAST analysis of S2 (VP2) of Alp9 showed that there was no nucleotide identity to any of the subjected sequences in the NCBI library using the MegaBLAST software, and that VP2 (S2) had 71 % deduced amino acid identity to EHDV2. S5 (NS1) showed 99 % nucleotide and deduced amino acid identity with EHDV6 strain M44/96 (Table 7). The other 8 genome segments of Alp9 showed nucleotide identity to other EHDV serotypes ranging from 95 % to 99 % identity. The deduced amino acid sequences of the rest of the genome of Alp9 showed sequence identity to other EHDV serotypes ranging from 96 % to 100 %. Potgieter *et al.* (2003) showed that there is 71.2 % identity between VP2 (S2) of two different serotypes (AHSV1 and 2) which cross react in serological analysis also. The highest percentage deduced amino acid sequence identity indicated between VP2

(S2) of the different serotypes of AHSV was 71.4 % and the lowest identity 47.6 %. The lowest percentage nucleotide variation in S2 (VP2) between the first 24 serotypes of over 300 BTV isolates (including reference strains) is 29 % (Maan *et al.*, 2009). A very low percentage identity exists between S2 (VP2) of the different species in the *Orbivirus* genus. In a study by Anthony *et al.* (2009b) it was found that even though there was a low percentage (71 % nt and 73.6 % aa) identity of nucleotide and deduced amino acid sequences of S2 between certain isolates of EHDV2, the virus was still neutralised by homologous sera in SNT. Because no nucleotide identity of S2 of Alp9 to any other serotype of EHDV and only 71 % amino acid identity of VP2 of Alp9 to EHDV2 could be found on NCBI library, the results of BLAST analysis strongly suggest that Alp9 is possibly a new serotype of EHDV.

No evidence of reassortment between the two viruses, Alp8 and Alp9, was found, which makes sense since they are different virus species. Very few cases of EHDV in alpacas have been reported in literature (Phillips, 2013).

VNT and SNT are regarded as the gold standards in determining virus serotype. In the light of the sequencing results it was decided to raise hyper immune GP serum against Alp8 and Alp9 that can be used in SNT and to test the rest of the alpaca herd on the farm where the alpaca died. The hyper immune sera against Alp8 and Alp9 were then subjected to SNT against the 24 OIE reference antigens against BTV and the 8 serotypes against EHDV. Alp8 GP serum had a NAb titre of ≥ 32 against BTV15 and did not cross-react with EHDV (see Table 8). In the preparation of hyper immune GP serum against a virus, the GPs were administered 5 consecutive intra-peritoneal inoculations over a period of five weeks. The question can be asked whether the 73 % nucleotide and 77 % amino acid identity between Alp8 and BTV15 could induce an antibody titre of ≥ 32 in GPs. Alp9 showed no cross reaction with any of the 24 BTV serotypes, neither with the 8 EHDV serotypes (Chapter 1.2.3.2, Table 9). To confirm whether Alp8 is indeed a new BTV serotype, the SNT with BTV15 antigen must be repeated. Alp8 GP serum needs to be titrated for an endpoint result to be able to compare Alp8 against BTV15 GP reference serum. A fourfold difference in the end titre between the test serum and the reference positive control serum will indicate involvement of another serotype of BTV (personal communication Dr. B.J. Erasmus).

SNTs were performed on serum samples from the rest (22 alpacas) of the alpacas in the herd. Three out of the 22 alpacas showed considerable antibody responses to both Alp8 and Alp9. Two more alpacas had antibody titres (12 and ≥ 32) against Alp8 (BTV) antigen, while one alpaca had an antibody titre of ≥ 32 against Alp9 (EHDV) antigen (Table 10).

A BTV cELISA was performed by the serological unit of the Virology Division at OVI on Alp8 and Alp9 hyper immune GP serum. Both samples tested positive for BTV, which reflects the known immunological cross-reactivity between BTV and EHDV (Inaba, 1975). Unfortunately, no EHDV ELISA assay was available for the detection of EHDV antibodies. BTV- and EHDV CFT were also performed on the hyper immune GP serum of Alp8 and Alp9. Alp8 hyper immune GP serum had a BTV antibody titre of 16, while hyper immune GP serum against Alp9 had an antibody titre of 64 against EHDV and showed a high level of cross reactivity with BTV with a CFT titre of 32 (Table 11).

Evidence that both Alp8 and Alp9 did spread to other alpacas in the same herd was presented in Table 10. The SNT results on the rest of the alpaca herd clearly showed that laboratory contamination was not the cause of the dual infection in the original isolated samples. Both BTV and EHDV are transmitted between its ruminant hosts via the competent vector *Culicoides* midge species and are not transmitted horizontally between ruminant species (Du Toit, 1944). *C. imicola* and *C. bolitinos* are the major vectors of BTV in South Africa (Du Toit, 1944, Meiswinkel *et al.*, 1994). The NAb titres in 6 of the animals in the rest of the herd are an indication that both the BT (Alp8) and EHD (Alp9) viruses were transmitted via a vector, and that these viruses may potentially spread to other susceptible hosts in the same region. *C. imicola* midges were fed on a blood meal spiked with Alp8 and Alp9 virus. Vector susceptibility was detected, albeit very low. The vector susceptibility of *C. imicola* for Alp8 was 1.7 %, while the susceptibility for Alp9 was 0.1 % (Table 12).

The importance of both serological results and nucleic acid sequencing were demonstrated to get an idea of the serotype of the two new viruses. The initial results of the molecular study and the subsequent serological study supported each other. The results obtained from the nucleic acid sequencing and serological analysis of two viruses isolated from the dead alpaca identified Alp9 as a new serotype of EHDV and Alp8 as a possible new serotype of BTV which is most closely related to BTV15.

This work was presented as a poster: (Appendix 4)

IM Wright, LH Maartens, S Gers, GH Gerdes, AA van Dijk and AC Potgieter

Serological and genetic characterisation of new serotypes of BTV and EHDV isolated from a single alpaca in South Africa.

BIG 5 Congress of the South African Association for Veterinary Technologists, Berg-en-Dal, Kruger National park, South Africa, October 2012.

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APPENDIX 3

LIST OF ABBREVIATIONS

ATP	Adenosine tri-phosphate
VERO	African green monkey kidney cells
AHS	African horsesickness
AHSV	African horsesickness virus
AGID	Agar gel immune diffusion
AGE	Agarose gel electrophoresis
AMV	Avian myoblastosis virus
BHK	Baby hamster kidney
BT	Bluetongue
BTV	Bluetongue virus
BSA	Bovine serum albumin
CO ₂	Carbon dioxide
cELISA	Competitive ELISA
CFT	Complement fixation test
CFU	Complement fixing units
cDNA	Complimentary DNA
Ct	Cycle threshold value
CPE	Cytopathogenic effect
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
ds	Double-stranded
dsRNA	Double-stranded ribonucleic acid
EMEM	Eagles – minimum essential medium
EM	Electron microscopic

ECE	Embryonated chicken eggs
ELISA	Enzyme linked immunoabsorbent assay
EHD	Epizootic haemorrhagic disease
EHDV	Epizootic haemorrhagic disease virus
EE	Equine encephalosis
EtBr	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
FBS	Foetal bovine serum
GS	Genome segment
GP	Guinea Pig
HCl	Hydrochloric acid
IETS	International Embryo Transfer Society
i.v.	Intra-venous
LiCl	Lithium chloride
MgCl ₂	Magnesium chloride
MMOH	Methyl mercury hydroxide
μl	Micro liter
ml	Milliliter
mM	Millimol
MLV	modified live virus
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
ng	Nanogram
nm	Nanometer
NCBI	National Centre for Biotechnology Information
NAb	Neutralising antibodies
NS	Non-structural viral proteins
OIE	Office International des Epizooties

OBP	Onderstepoort Biological Products
OVI	Onderstepoort Veterinary Institute
OD	Optical density
OCG	oxalate-carbolic-glycerine
PSF	Penicillin, streptomycin, fungizone
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
KCl	Potassium chloride
rtRT-PCR	Real time reverse transcriptase PCR
RT-PCR	Reverse transcriptase PCR
rpm	Revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
S	Segment (genome)
SNT	Serum neutralisation testing
ss	Single-stranded
NaCl	Sodium chloride
NaOH	Sodium hydroxide
SAC	South American camelid
TCID	Tissue culture infective dose
TBE	Tris-borate EDTA
US	United States
USA	United States of America
VP	Viral protein
VNT	Virus neutralisation test
WTD	White-tailed deer

APPENDIX 4

TITLE AND ABSTRACT OF POSTER PRESENTED AT THE ANNUAL CONGRESS OF THE SOUTH AFRICAN ASSOCIATION OF VETERINARY TECHNOLOGISTS HOSTED AT BERG-EN-DAL, KRUGER NATIONAL PARK ON THE 30TH AND 31ST OCTOBER 2012

Serological and genetic characterization of new serotypes of BTV and EHDV isolated from a single Alpaca in South Africa

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The introduction of exotic animal species into South Africa holds the risk of introducing new microbes too. Alternatively virus strains that normally circulate locally without causing disease may cause disease in exotic animals. During the summer of 2008 an Alpaca that had been imported from South America, was presented for necropsy to the Stellenbosch Regional Laboratory. It had suffered from an acute onset of respiratory distress and died

within hours with froth and fluids exuding from the nostrils. Two viruses were isolated from this one animal in embryonated chicken eggs. These viruses were both identified as orbiviruses based on the electrophoretic profile of their genetic material and partial sequencing of genome segments S2 and S5 which encode outer capsid protein VP2 and non-structural protein NS1 respectively. The NS1 sequences identified one of the viruses as bluetongue virus (BTV) and other as epizootic hemorrhagic disease virus (EHDV). Analysis of the S2 sequences (which indicate the serotype and genotype) suggested that neither virus is one of the known serotypes. BLASTP analysis showed that the putative BTV strain was related to BTV serotype 15 but that it only shared 73% amino acid identity with VP2 of BTV15. Sequence analysis of the EHDV strain showed that it shared 76% amino acid identity with EHDV serotype 2. Serological typing of these strains with BTV and EHDV guinea pig antiserum yielded negative results. We have thus confirmed that these isolates represented novel serotypes of BTV and EHDV.