Reassortment of bluetongue virus vaccine serotypes in cattle

By

Carien van den Bergh

A dissertation submitted to the Faculty of Veterinary Science of the University of Pretoria in partial fulfillment of the requirements for the degree

Magister Scientiae (Veterinary Science)

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

University of Pretoria

South Africa

Supervisor:

Prof EH Venter

Co-Supervisor:

Dr P Coetzee

Date:

Table of Contents

Chapter 1: Literature review

Chapter 2: Materials and methods

Chapter 3: Results

Index of Tables

Index of Figures

Declaration

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

Signature of candidate

_____

Date: / /

Acknowledgements

I offer my sincere gratitude to my supervisor Prof Estelle Venter and co-supervisor Dr Peter Coetzee. I would not have been able to accomplish this research without your guidance and patience. Thanks you for all you taught me and making me a better researcher.

This project would not have been possible without the much appreciated funding provided by the University of Pretoria, the National Research foundation (NRF), the Meat Industry Trust (MIT) and Technology innovation Agency (TIA).

A special thanks to all the staff of the Department of Veterinary Tropical Diseases for all the assistance and expertise as well as the facilities you made available for me.

Thank you to van Zyl Boerdery for making their cattle available for my trial.

A special thanks to all my colleagues Ayesha Hassim, Anne-Mari Bosman, Karen Ebersohn, Carina Lourens and Rina Serfortein. **A special thanks** to Prof Alan Guthrie and Mischa le Grange for your help with the sequence analysis. **A Special thanks** to all the staff of the UPBRC for your friendly assistance during my animal trial.

Thank you to my family and to Desmond van Loggerenberg and Mike Diedericks for your continuous support and advice.

List of Abbreviations

AHSV	African horse sickness virus
BHK-21	Baby hamster kidney - 21
BT	Bluetongue
BTV	Bluetongue virus
bELISA	Blocking ELISA
cELISA	Competitive ELISA
CPE	Cytopathic effect
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DVTD	Department of Veterinary Tropical Diseases
ds	Double stranded
ECE	Embryonated chicken eggs
EDTA	Ethylenediaminetetraacetic acid
FLAC	Full length amplification of cDNA
ISP	Infectious sub-viral particles
IV	Intravenous route
MAb	Monoclonal antibodies
MEM	Minimal essential medium
MLV	Modified live vaccine
NGS	Next generation sequencing
NS	Non-structural
NS3/A	NS viral protein 3 alternative form
OD	Optical density
OBP	Onderstepoort Biological Products
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS (+)	Phosphate buffered saline containing calcium and magnesium
PCR	Polymerase chain reaction
RFLP	Restricted fragment length polymorphism
rpm	Revolutions per minute

RNA	Ribonucleic acid
RT – qPCR	Reverse transcriptase - quantitive polymerase chain reaction
RT - PCR	Reverse transcription – polymerase chain reaction
SA	South Africa
SNT	Serum neutralization assay
$TCID_{50}$	50% tissue culture infective dose
UCC	Untreated cell control
USA	United States of America
UPBRC	University of Pretoria's Biological Research Center
UPGMA	Unweighted Pair group Method with Arithmetic Mean
Vero	African green monkey kidney cells
VMRD	Veterinary Medical Research and Diagnostics
VP	Viral protein

Summary

Reassortment of bluetongue virus vaccine serotypes in cattle

By

Carien van den Bergh

Supervisor: EH Venter

Co-Supervisor: Peter Coetzee

Department of Veterinary Diseases

Faculty of Veterinary Science

Bluetongue (BT) is a non-contagious viral disease of domestic and wild ruminants. Bluetongue virus (BTV), an *Orbivirus* that belongs to the family *Reoviridae*, is the causing agent of the disease. The virus consists of a ten segmented double stranded (ds) RNA genome and currently 27 serotypes have been identified worldwide. The virus is transmitted by *Culicoide* biting midges (Diptera: Ceratoponidae) and the occurrence of the disease depends on the presence and abundance of competent vectors. In South Africa most European ovine breeds are more susceptible to the disease than indigenous sheep, while cattle and goats in general prove to be sub-clinically infected. During recent outbreaks in Europe (2008 – 2011) cattle showed severe clinical symptoms and mortality. The role of cattle in the epidemiology of the disease in South Africa is however poorly understood.

Bluetongue virus has the ability to reassort its genome segments in vertebrate hosts or vectors which have been infected with more than one strain at the same time. This phenomenon has been reported previously. In 1987, reassortment was investigated in cattle between BTV serotypes 11 and 17 where six reassortants with unique genetic profiles were described. In 2008, in Europe, segment 5 of BTV serotype 16 was identical to the South African vaccine strain of BTV serotype 2. In India in 2013 studies showed that in some isolates obtained from an outbreak segment 6 of BTV serotype 21 were 97.6% identical to segment 6 of BTV serotype 16.

Bluetongue disease is controlled by annual vaccination. In South Africa the freeze dried polyvalent BTV vaccine is mainly used to vaccinate sheep, and the vaccine consists of three bottles, each bottle includes five serotypes and each bottle is vaccinated at a three week interval, between August to October of each year. The vaccine proves to be effective in producing immunity against the disease but there are multiple side effects. The main concern is that vaccine virus can be detected during the viraemic period in inoculated sheep. The titre levels are also sufficient to be transferred to non-vaccinated animal hosts via *Culicoides* midges. The possibility of reassortment between genome segments of vaccine and wild type strain viruses when simultaneously infected therefore exists. This might result in the emergence of new virus serotypes with different phenotypic characteristics i.e. reversion of the live attenuated vaccine strain to a virulent strain.

The aim of the project was to investigate the potential generation of genetic reassortant viruses between field and vaccine serotypes of BTV within cattle. Six cattle between the ages of six and twelve months were used. Before the onset of the project cattle were tested for antibodies specific to BTV using a commercial available cELISA and for viral nucleic acid with RT-PCR. Only animals showing negative results by both the tests were used in the trial. The animals were housed in vector-free stables for the duration of the trial. The cattle were divided into two groups; the first group was infected with BTV serotypes from Bottle B of the Onderstepoort Biological Products (OBP) vaccine (BTV serotype 3, 8, 9, 10 and 11), while the second group was infected with the same vaccine serotypes and simultaneously infected with a wild type BTV serotype 4.

Blood samples were collected daily from the animals from Day 1 to Day 39 post inoculation. Viraemia was detected between day 2 to day 39 and in one of the animals viraemia could be detected until 39 days post inoculation using virus isolation. Buffy coats as well as first cell culture passages of buffy coats were used to isolate the virus using the plaque forming unit method. The vaccine parental strains were obtained from Bottle two of the vaccine using the plaque forming unit assay and the isolated viruses were serotyped using a serum neutralization assay. Plaques were isolated and amplified on Vero cells. BTV serotype 4 was isolated in the Department of Veterinary Tropical Diseases from a field sample. RNA was extracted from the isolated plaques as well as the six parental strains and compared using polyacrylamide gel electrophoresis (PAGE).

Clear genome segment shifts could be observed between the viruses isolated from cattle and the parental strains using PAGE. Gels were then analyzed using BioNumerics software and the 11 suspected reassortant viruses and the parental strains were sequenced using MiSeq sequencing. Sequences were analyzed using a sequence analysis program Geneious and neighbour-joining trees were created for each of the ten viral segments of all the possible reassortants. All isolated viruses were either BTV serotype 4 or BTV serotype 9. In two isolated viruses BTV serotype 9 exchanged segments 8 with BTV serotype 8.

The rate of replication was compared between the suspected reassortant viruses and the parental strains using a cell viability assay. Variation in cytotoxicity could clearly be demonstrated between isolates and parental strains using a CellTox Green Cytoxicity assay (Promega). The ability of the cells to maintain metabolic function when infected with each of the isolated samples as well as the parental strains was also compared with a CellTiter-Blue Cell Viability assay (Promega). Results of both tests indicated that the BTV serotype 9 isolate (2b/NGS 01) that had exchanged its segment 8 with the segment 8 of BTV serotype 8, was more cytotoxic when grown on Vero cells and inhibited normal cell metabolic function the most when compare to the parental or any of the other isolated viral serotype.

In this study the generation of reassortant viruses could only be demonstrated between the live attenuated vaccine serotype of BTV in cattle. It was however previously reported that reassortment could occurred between field and vaccines serotype. Segment 8 that exchanged between BTV serotype 9 and BTV serotype 8 codes for a non-structural protein, NS2 that assist in core assembly and virus replication. The use of the life attenuated BTV multivalent vaccine in South Africa causes a concentrated circulation of the different vaccine serotype in the environment and may create multiple opportunities for reassortment to occur. BTV may also overcome evolutionary constraints during replication in hosts such as cattle through a combination of reassortment of gene segments and this can contribute to genotypic and potentially phenotypic variability. However, the frequency and biological consequences of reassortment specifically remain poorly understood, especially in cattle in South Africa.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Bluetongue (BT) is a non-contagious viral disease of domestic and wild ruminants with serious socio-economic impact. The first record of BT was made in 1900; the disease was recorded in the Cape Province in South Africa (SA) (Spreull, 1905). The disease was commonly known as Malarial Catarrhal Fever. It was only later that the disease was called BT a direct translation of the Dutch word "Blaauwtong". The name referred to the characteristic cyanotic tongue caused by the disease in sheep (Spreull, 1905).

Bluetongue virus (BTV) is the causative agent of BT. Bluetongue virus is an *Orbivirus* that belongs to the family *Reoviridae*. There are 27 known BTV serotypes worldwide of which 21 are endemic in SA (Jenken *et al*., 2015). They have a 10- segmented double stranded (ds) RNA genome (Maan *et al*., 2012; Jenckel *et al*., 2015) and are transmitted by *Culicoides* biting midges (Diptera: Ceratopogonidae) (Mellor *et al.*, 2000; Verwoerd., 2004). In SA *Culicoides imicola* is the major vector of BTV. This is however not the only vector because *C. imicola* are not abundant in cooler regions where BTV is still prevalent. The other suspected vector, *C. bolitinos* is able to support replication of BTV to high titres after ingestion and is highly likely to be a competent vector of the BTV in cooler regions of the country (Venter *et al.*, 1998). Over 1000 species of biting midges have been identified worldwide, but only 30 have been identified as potential vectors of BTV (Meiswinkel *et al*., 2004, 2008).

Reassortment is a process of genomic exchange of segmented RNA viruses such as BTV. This mechanism plays an important role in the emergence of novel viruses that is still poorly understood. The frequency and the biological consequences of reassortment are still unknown (Nomikou *et al.,* 2015). An important prerequisite for reassortment is the entry of more than one strain of a virus such as BTV into a single host cell (Bodewes & Worobey, 2011). Reassortment in viruses can cause fundamental shifts in the phenotypic traits (Shaw *et al*., 2013). Reassortment could potentially lead to a change in transmissibility, pathogenicity or can cause the virus to jump the species barrier e.g. Group A Rotavirus (Shaw *et al*., 2013; Shelton *et al*., 2012; Watanabe *et al*., 2001)

1.2 Epidemiology

1.2.1 Hosts and clinical signs

Bluetongue virus will cause disease in areas where a susceptible mammalian reservoir, the virus and competent vectors have the opportunity to coexist. For the disease to occur the climatic conditions must be favorable for BTV replication and transmission of the virus from vector to susceptible animal (Carporale *et al.,* 2011).

Infection of BTV in ruminants is characterized by damage to endothelial cells lining small capillary blood vessels resulting in vascular thrombosis, thus increasing vascular permeability resulting in hypereamia, congestion, vascular permeability, ischaemic necrosis, haemorrhage and oedema (Melhorne *et al.,* 2007: Spreull, 1905; MacLachlan *et al.,* 2008; MacLachlan *et al.,* 2009; Erasmus, 1975). Bluetongue can cause disease in all of the sheep breeds but fine wool European breeds prove to be the most susceptible. Clinical signs of BT in sheep may include any combination of fever, depression, anorexia, nasal discharge, facial and pulmonary oedema, coronitis, and ulcerations of the mucosa of the buccal cavity, occasional cyanosis of the tongue, muscle degeneration and abortions (Spreull, 1905; Maclachlan *et al*., 2009). The clinical signs observed for the disease is extremely variable. Some infected animals may show mild signs of clinical infection while other can present mortality ranging from 2-30% during outbreaks (Erasmus, 1990), but can occasionally be as high as 70% (Gambles, 1949).

Certain species of deer can also be affected (Vosdingh *et al*., 1968), whereas goats, especially in SA, are usually sub-clinically affected (Luedke & Anakwenze, 1972; Anderson *et al*., 1984). This variation in severity has been ascribed to intrinsic differences in virulence between infecting field strains as well as host, vector (e.g. serotype) and environmental factors (e.g. breed, age, nutritional status, differences in innate immunity of each host and inoculum titre, high temperature, ultraviolet radiation in the environment) (Neitz & Reimerschmid, 1944; Erasmus, 1975). During outbreaks in Europe in 2008, cattle were severely affected and showed overt clinical signs (Wouda *et al*., 2009). This is not generally the case in SA. Cattle in Africa show some degree of resistance, but the period of viraemia is much longer than that of other domestic ruminants. This enables cattle to be a maintenance or amplifying host of infection (Darpel *et al.,* 2007; MacLachlan *et al.,* 2009).

Fatal BTV infections have been reported in canines. The symptoms were confirmed by reproducing the disease by experimentally infecting dogs (Akita *et al.*, 1994). The bitches aborted and died from severe pulmonary oedema. It has been documented that Eurasian Lynx have died from pulmonary oedema after consuming a BTV 8 contaminated carcass (Jauniaux *et al*., 2008). Other carnivores in SA including lions, African wild dogs (*Lycaon pictus*), spotted hyenas (*Crocuta crocuta*), jackals (*Canis mesomelas*) and large spotted genets (*Genetta tigrina*) were tested seropositive in BTV sero-prevalence study by Alexander *et al*., (1994). The role of canines in the epidemiology of the disease is however not clear.

1.2.2 Cattle

Cattle are considered to be maintenance hosts of the BTV. The infection is mostly asymptomatic with a prolonged viraemic period (Anderson *et al*., 1987; Hourrigan & Klingsporn, 1975; Du Toit, 1962; MacLachlan & Fuller, 1986; MacLachlan *et al*,. 1987; Nevill, 1971; Parsonson *et* al,. 1987a; Parsonson *et al*,. 1987b). This period is persistent despite the humeral immune response from the host (MacLachlan *et al*., 1987; Richards *et al.*, 1988). Viraemia provides more opportunities for the virus to reassort and to be transmitted to other hosts. Viraemic periods in cattle can last from 35 to 49 days (MacLachlan *et al*., 1990) but in some trials the virus has been isolated for up to 63 days in cell culture and the virus nucleic acid could be detected for up to 180 days using the polymerase chain reaction (PCR). The prolonged viraemic period is normally related to the lifespan of bovine erythrocytes (Singer *et al.*, 2001).

Cattle semen are exported and imported globally. This poses a potential problem since BTV isolation from semen using chicken embryos were possible 7, 14 and 21 days after inoculation of eggs. BTV isolation from sheep was possible after the sheep were injected with the semen of an experimentally infected bull; the semen of the bull was able to shed the virus for 3 years after initial experimental infection with BTV (Hourrigan & Klingsporn, 1975).

It is still not clear why BTV-infected cattle do not produce the same disease as it does in other ruminants (Spreull, 1905; Russell *et al*., 1996). The infection of both cattle and sheep shows identical pathogenesis, but the fundamental susceptibility of the bovine endothelial cells is different to that of sheep and is responsible for the different manifestation of the disease (De Maula *et al*., 2001, 2002a,b). Bovine endothelial cells are activated by BTV infection, thus resulting in increased transcription of genes

encoding the vasoactive and inflammatory mediators, as well as increased expression of cell surface adhesion molecules. When compared to endothelial cells of ovine, infection results in rapid cytolysis with minimal activation (De Maula *et al*., 2002a).

Although cattle generally show no clinical signs in SA it has been reported by field veterinarians in the United States that cattle infected with BTV present clinical signs such as laminitis and stiffness in all four legs. Some of the cattle showed erosions or even ulceration on the dental pads and ulcerative lesions on the tongue nose and muzzle. Most of the animals salivated excessively and had a nasal discharge with no appetite and only a few cows showed ulcerated udders (Bowne *et al*., 1966). Bowne and coworkers (1968) experimentally infected cows with BTV in vector free stables they showed minimal response. In the 2006 outbreak of BTV serotype 8 in Europe however cattle manifested with severe clinical signs and mortalities. The mortality rate in infected cattle was as high as 10% (Darpel *et al.,* 2007*).*

In a previous study it has been shown that ruminants can be infected with more than one serotype by various mechanisms. The host is infected by various vectors infected with different serotypes of the virus or the host is infected by one vector that carries more than one serotype as a result of previously feeding on more than one host (Stott *et al*., 1982).

1.2.3 Bluetongue virus

The BTV is an icosahedral particle of 85 nm in diameter and consists of 80% protein and 20% dsRNA (Xu *et al*., 1997). The BTV genome is composed of 10 segments of linear double stranded dsRNA which code for seven structural (VP1-VP7) and five non-structural proteins (NS 1, NS 2, NS 3/3a and NS 4) (Verwoerd *et al*., 1970; Ratinier *et al*., 2011; Legisa *et al*., 2015). Each genome segment codes for a more than one protein. The mature virion is non-enveloped and the 7 structural proteins are arranged into a triple layered icosahedral protein capsid as illustrated in Figure 1. The three concentric layers consists of a sub-core, a shell is constructed of twelve decamers of VP 3, encoded by segment 3. This layer provides a “scaffold” for VP 7 that is encoded by segment 7. Viral protein 7 is organized into trimers that form the core surface layer. The proteins of the BTV core particle have been studied by x-ray crystallography and cryo - electron microscopy and are illustrated by Figure 1.1. The outer layer of the virion consists of VP 2 encoded by segment 2. Viral protein 5 is encoded by segment 6 and is arranged in 120 globular

structures that are interspersed with the VP 2 to facilitate in viral fusion during receptor mediated endocytosis (Roy, 1989; Hassan & Roy, 1999).

The sub-core encloses the ten genome segments as well as multiple copies of three enzymatic proteins (Gouet *et al*., 1999; Grimes *et al*., 1998; Hewat *et al*., 1992). The first is VP 1 (RNA-dependent RNA polymerase, encoded by Seg-1).The primary function of VP 1 is transcription and replication (Boyce *et al*., 2004). VP 4 caps the newly synthesized mRNA (capping and trans-methylase enzyme, encoded by Segment 4) (Ramadevi *et al*., 1998) and VP 6 (encoded by segment 9) unwinds and re-anneal dsRNA during transcription and replication (RNA-dependent ATPase and helicase, encoded by segment 9) (Stauber *et al*., 1997). Viral protein 7 is a ligand for the insect cell receptor and appears to be able to mediate the attachment between the BTV and the membrane proteins of the cells (Basak *et al*., 1997; Xu *et al*., 1997). The outer capsid proteins are involved in the attachment and consist mainly of attach (Hassan & Roy, 1999) and release (VP 5) of the virus into the cytoplasm of mammalian cells (Forzan *et al*., 2004; Hassan *et al*., 2001). The NS 1 proteins play a key role in regulating viral protein expression.

As regards to the non-structural proteins, NS 1 (encoded by segment 5) is the most abundant in the infected cells and forms tubular structures in the cell cytoplasm; the NS 1 is also a positive regulator of the virus's expression that increases protein synthesis (Boyce *et al*., 2012). Non-structural protein 2 assorts the genome segments (encoded by Segment 8) (Kar *et al*., 2007). Non-structural protein 3 and the alternative nonstructural protein (encoded by Segment 10) facilitate the release of the virus from the infected cell. The proteins also functions as a bridging mechanism of VP 2 and the components of the cytoskeleton. The viral particles are released by means of lysis of infected cells (Celma & Roy, 2009; Hyatt *et al*., 1993). Non-structural protein 3 acts as an antagonist for Type I Interferon, an antiviral cellular response that is produced *in vitro* and *in vivo* when the host is infected with BTV (Chauveau *et al*., 2013). Non-structural protein 4 (encoded by segment 9) modulates the hosts' immunity by inhibiting the transcription of interferon genes. This protects the virus and gives it enough time to replicate and spread throughout the host (Ratinier *et al*., 2011).

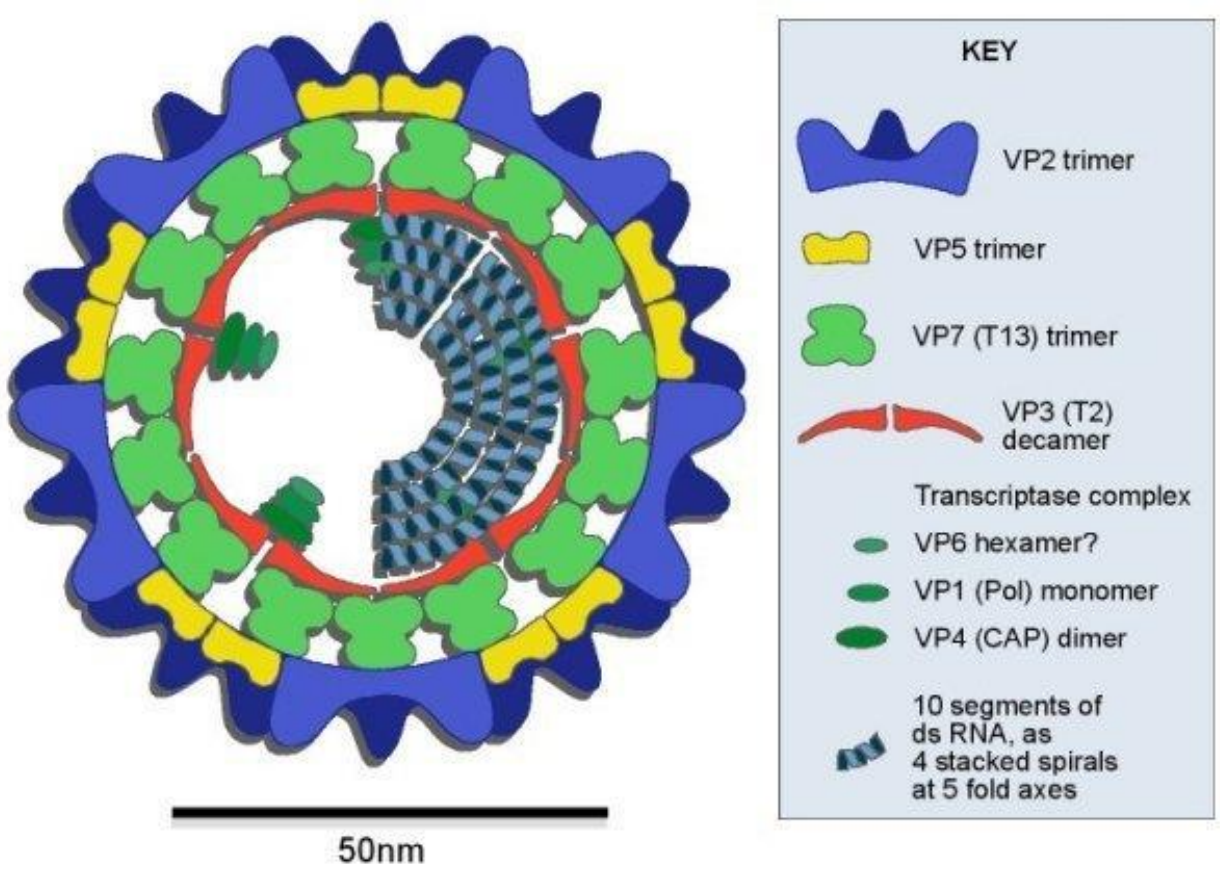


Figure 1.1: Schematic representation of BTV illustrating the interactions of the different proteins. (Mertens, 1999).

1.2.4 Occurrence of bluetongue virus

The biting *Culicoides* midges are sensitive to cold climates and extreme winters, but climate changes and the importation of animals have been blamed for the spread of the disease. The first recorded incident of the disease was in the Cape Province (now Western Cape Province), South Africa in the late eighteenth century (Spreull, 1905). Blue tongue virus has then been confined to the African continent up to 1943 when the first recorded outbreak in Cyprus occured (Gambles, 1949; MacLachlan, 2003). Although it is suspected that the disease might have been present in Cyprus since 1924 there are no documented records (Rodriguez-Sanchez *et al*., 2008). The disease soon made it its appearance in Isreal in 1949 (Komarov & Goldsmith, 1951), and between 1956 and 1960 the disease caused a major outbreak in Europe and 180 000 sheep died Portugal and Spain (Manso-Ribeiro *et al*., 1957). The disease was recorded in Texas in 1948 and again in California in 1952. Bluetongue was diagnosed and isolated in

1977 on the Australian continent (Gibbs & Greiner, 1994) and in 1979 BT was diagnosed amongst cattle in the subtropical South West region of China, (Zhu *et al.,* 2013). Sporadic outbreaks of BTV 10 occurred in Europe until 1998 (Mellor & Boorman,1995; Mellor *et al*., 2000) but BT has then spread to the Greek islands and was shortly reported in the Mediterranean Basin and other countries including Bulgaria, Tunisia and Turkey (Zientara *et al*., 2000). Bluetongue virus serotype 8 has caused major outbreaks in Northern Europe e.g. Belguim, France, Germany, Luxembourg and Netherlands around 2006 to 2008 involving disease in sheep, goats and cattle (Meiswinkel *et al*., 2008). Bluetongue virus has now been identified on all continents except Antarctica (Maclachlan *et al*., 2011; Gibbs & Greiner, 1994; Tabachnick, 2003; MacLachlan & Osburn, 2006; Mellor *et al*., 2008; MacLachlan, 2010).

1.2.5 Vectors

The serotypes prevalent in each region around the world are different and are mainly due to the presence of different competent vector species found in each of the regions (Balasuriya *et al*., 2008). Topotypes have also been divided into lineages, where Western lineages includes all serotypes that are endemic to Africa and the Americas while the Eastern lineages includes all the serotypes endemic to India, Malaysia and Australia (Maan *et al*., 2008; Mertens *et al*.,2007 and 2008). The main vector of BTV is adult female haematophagous biting midges that belong to the *Culicoides* genus (Du Toit, 1944). The midges are small flying insect about 1-3 mm in size and are present all over the world except in Anartica, Patagonia, Hawaiian Islands and New Zealand (Mellor *et al.,* 2000). There are 125 different genera and 5500 identified species. Species identification is mostly made by looking at different characteristics of their wings (Boorman, 1993) and molecular typing (Lassen *et al*., 2012). The specific vector species and the BTV serotypes that occur globally are outlined in Figure 1.2. The most abundant vector in Africa is *C. imicola,* but species such as *C. bolitinos* have been identified as another competent vector (Meiswinkel & Paweska, 2003; Venter *et al*., 2002). In Zimbabwe isolates of the BTV have also been made from *C. magnus* and in Kenya species of the Schultzei group are considered potential vectors of BT (Davies & Walker, 1974; Walker & Boreham, 1976; Davies *et al*., 1979). These vector species can transmit serotypes 1-15, 18, 19, 22, 24 and 25 between different vertebrate hosts (Walton, 2004).

In Europe the most common vectors have been proven to be *C. obsoletus, C. pulicaris, C. dewulfi* and *C. imicola*, but *C. scoticus* has also been implicated as a vector (Meiswinkel *et al*,. 2007). This is however

not illustrated by Figure 2 (Mellor & Pitzolis, 1979; Meiswinkel *et al*., 2008). Serotypes 1, 2, 4, 8, 9, 16 and 25 are prevalent in Europe but recently BTV serotype 27 has been isolated and sequenced in France after detection of the virus in goats (Jenckel *et al.,* 2015*).*

The primary vector in North America is *C. sonorensis* that is mainly responsible for transmitting BTV serotypes 10, 11, 13 and 17 (Tabachnick, 2003). The southern parts of the United States of America (USA) are mainly associated with BTV serotype 2, with *C. insignis* and *C. pussilus* as the transmitting vectors (Greiner *et al*., 1985; Mo *et al*., 1994). Since 2005, another 6 BT serotypes have been identified in the USA including BTV serotype 3, 5, 6, 14, 19, and 22 (Johnson *et al.*, 2007; Mertens *et al*., 2007). In east central Alabama a monthly 87% sero-conversion rate has been recorded in white tailed deer and cattle and the responsible vectors are *C. variipennis* and *C. pussillus* (Mullen & Anderson, 1998).

The prevalent BTV serotypes in Asia include 1-4, 12, 14-21. Bluetongue virus is mainly isolated in the topical, sub-tropical and temperate regions of Asia (St George *et al*., 1996). *Culicoides imicola* is the main vector in countries of the near and Middle East (Boorman, 1989; Braverman *et al.,* 1976; Jenning *et al*., 1983). In other regions like Tunisia, BTV have been isolated from several midge species, including *C. schultzei, C. gemellus, C. peregrinus, C. arakawae* and *C. circumscriptus* (Yunlong *et al*., 1996).

In Australia the most effective vector is considered to be *C. fulvus* but the species are restricted to areas with high summer rainfall. This limits the dominance of the vector in the sheep rearing areas which are in drier areas of the country and therefore influence the spreading of the disease. Species such as C. *actoni, C. wadai and C. brevitarsis* are considered important in spreading the 8 serotypes present in Australia as illustrated by Figure 1.2 (Standfast *et al*., 1985).

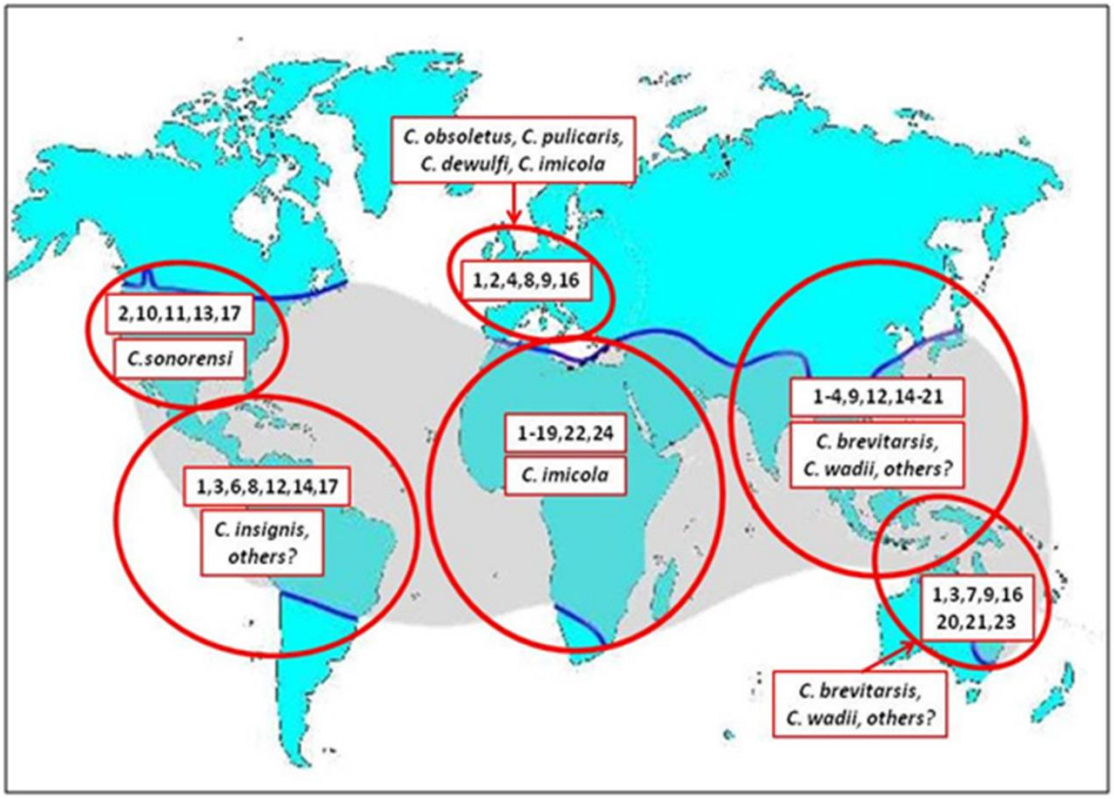


Figure 1.2: Global distribution of serotypes of BTV and *Culicoides* spp. in different geographic areas (Tabachnick, 2010).

1.2.6 Overwintering of bluetongue virus

Bluetongue virus needs a mechanism to survive cold winters when there are no or few midges. This mechanism includes overwintering in the vector and host population (Wilson *et al*., 2007, 2008). Different overwintering mechanisms have been proposed. The first mechanism is transovarial transmission where the infected female midge carries the BTV over to her offspring. A study in California investigated the hypotheses of vertical transmission of the BTV between the vectors and used quantitative RT-PCR to evaluate the parent, eggs and the progeny for viral nucleic acid after the midges were fed infected blood under laboratory conditions. Bluetongue virus was not detected in larvae pupae or in the F1 progeny (Osborne *et al*., 2015). The second mechanism which has been proposed a complicated overwintering cycle that involves unidentified intermediate hosts. This mechanism have not been studied in SA but a study in California showed that it is unlikely since most of the BTV transmitting midges only feeds on ruminants (Mayo *et al.,* 2014). In South Africa the presence and susceptibility of

wildlife to BTV can be seen as intermediate or amplifying hosts. Chronic infection in the ruminant population during winter is a third proposed overwintering mechanism (Takamatsu *et al*., 2003, Wilson *et al.*, 2007). The viraemic period of BTV is 50 days in ovine species (MacLachlan *et al*., 1990). Records have shown that the viraemic period in bovines can last up to 60-100 days without showing any clinical symptoms of the disease. The prolonged infection within bovine species might create a perfect mechanism for the virus to survive severely cold conditions during winter times when there is also no vectors available (Sellor & Taylor, 1980; Takamatsu *et al.,* 2003; Wilson *et al.*, 2007).

The fourth mechanism is which has been proposed is connected to the prolonged survival of adult *Culicoides* midges followed by a period of active BTV transmission. This mechanism is possible for South Africa since the preferred temperatures for the survival of midges are between 13°C and 37°C and winters in SA have mostly moderate temperatures. Records have also shown that an adult midge can survive for 90 days at much lower temperatures like during the winter of 2006/7 in Europe. *Culicoides imicola* survived for more than 15 days at temperatures as low as -1.5°C (Nevill, 1971). *Culicoides imicola* and *C. bolitinos* are able to harbor BTV successfully for 20 days at moderately low temperatures of e.g. 10°C (Paweska *et al*., 2002). This mechanism enables BTV infected midges to survive the winters and infect susceptible ruminants as soon as conditions become more favourable (Takamatsu *et al*., 2003; Wilson *et al*., 2007, 2008).

Another mechanism that was proposed was that the virus overwinters with ongoing or low-level cycle of infection and that transmission between cattle or other ruminants and the biting midges continuous during the winter period. The level of infection is not high enough to cause viral outbreaks but is enough to keep the virus circulating. When the climate changes to favour the reproduction of midges the level of infection increases and outbreaks occur (Neville, 1971).

The immune system of a calf fetus only becomes competent at 150 days of gestation leaving a fetus immunotolerant to BTV if the dam were infected before the calf's immune system becomes fully competent (Brown *et al*., 1979). Strong evidence was presented by Menzies and co-workers in 2008 that transplacental infection was occurring with BTV-8 circulating in Northern Europe. This mechanism

provides an opportunity for the virus to survive the vector free periods (Menzies *et al*., 2008; Backx *et al*., 2009).

1.3 Genetic reassortment

Evolutionary changes in segmented viruses like BTV occurs in a process called genome segment reassortment. This is a process in which different viruses can exchange or recombine their segments when they infected the same host simultaneously (Seagerman *et al*., 2007; Nomikou *et al*., 2015). Reassortment of BTV between field and vaccine strains as well as vaccine strains with vaccine strains have been studied extensively (Seagerman *et al*., 2007; Nomikou *et al*., 2015). Reassorted viruses show no bias towards specific segments, but a bias can be observed in the frequency of specific segments when associated with other segments.

In 1998, Europe was invaded by eight BTV strains from six BTV serotypes. This included Western lineage serotypes 1, 2, 4, and 8 and serotypes 1, 9, and 16 from the Eastern lineage (Darpel *et al*., 2007; Maan *et al*., 2007a; Mertens *et al*., 2007, 2006). The introduction of BTV into Europe has led to the use of the live attenuated vaccines produce in South Africa by Onderstepoort Biological Products (OBP). Live attenuated vaccine strains 2, 4, 9 and 16 were also used in Iberian, eastern Mediterranean islands and Italy for annual vaccinations in the Mediterranean outbreak in 2002. Bottle B including serotypes 3, 8, 9, 10 and 11 were briefly used in Bulgaria in 2000. BTV serotype 15 was identified in Israel in 2006 for the first time and an annual vaccination campaign was then launched for a few years using BTV serotypes 2, 4, 6, 10 and 16 (Shimshony, 2004). BTV serotype 2 and serotype 16 circulated in Europe between 1999 and 2005, as well as the vaccine strains mentioned above.

A study was reported where genome segment 2 and segment 5 of BTV 2 and BTV 16 from 15 samples isolated in Europe were compared. Sequence data of the isolates from the same serotype showed similarity in more than 92% of the nucleotides and less than 84% similarity between serotypes. But segment 5 of BTV serotype 16 (from Italy 2002) showed less than 83% similarity with other BTV serotype 16 isolates, but was identical to the BTV serotype 2 vaccine strain. This vaccine was used in Italy as an annual vaccine from 1995. Thus illustrating the generation of reassortment between a field strain and a vaccine strain (Batten *et al*., 2008).

Another study in India in 2013 isolates of BTV serotype 21, which at that time caused a severe outbreak in Andhra Pradesh, were compared to isolates of BTV 16 that was circulating at the same time. Full genome sequences of segment 2 and segment 6 were compared using phylogenetic analysis. The study revealed that segment 6 of BTV serotype 21 was 97.6% identical to segment 6 of BTV 16 and reassortment was therefore proven (Shafiq *et al*., 2013).

In 1987 Jeffrey Stott evaluated reassortment between 2 serotypes; BTV 11 and BTV 17, simultaneously infected in a Holstein bull. The viruses were isolated and dsRNA were subjected to soduim dodecyl-sulfate polyacrylamide gel electrophoresis. Six new reassortants with unique gel profiles of genome segments were showed.

1.4 Genetic and phenotypic diversity

Genetic diversity is the total number of genetic characteristics in the genetic make of a species while phenotypic diversity describes how the expression of the different traits can be influence by the environment and can change the expression of the gene reference. Phenotypic traits of the virus include viral replication, viral attachment and penetration and mechanism of viral release to name a few (Huismans *et al*., 2004). The differences in photogenic effect and the ability of the disease to be transmitted to vectors and hosts are related to variables of the genomes segments. *In vivo* virus replication can influence the transmissibility of the virus i.e. the efficiency of virus uptake by midges due to potential differences in the level of viraemia (Veronesi *et al*., 2010). The VP7 and NS3/A proteins mediates infection and dissemination in the *Culicoides* midge, and can suggest that the genotypic variation in these proteins can relate to the transmission of the virus by different midges in different geographic regions (Balasuriya *et al*., 2008; Maan *et al*., 2010).

The nucleotide sequences of the genome segments of BTV illustrate different degrees of conservation. This variation reflects on both the mutations and the selective pressures that act on the encoded proteins (Bonneau *et al*., 2001; Balasuriya *et al*., 2008). In general the genes encoding the proteins of the sub-core (segments 1, 3, 4 and 9) and non-structural proteins (segments 5 and 8) are conserved by

either structural or functional conservation, while the outer capsid proteins (segments 2 and 6) are exposed to external selective pressures are therefore more variable. Weak correlations between these clades and particular geographic locations and/or *Culicoides* species/populations have been established (Maan *et al*., 2007; Maan *et al*., 2008). Viral protein 7 is relatively conserved within the BTV sero-group, however the encoding gene also demonstrates sequence diversity that clusters the virus into several different clades (Bonneau *et al*., 2000; Wilson *et al*., 2000). Very limited variation in NS3/A was observed in a previous study, it was also illustrated that NS 3 is not evolved by positive selection and that is not influenced by different environmental pressures (Balasuriya *et al*., 2008).

There are differences in the ability of the protease to cleave the VP2 protein of BTV virus. The proteases from viruses belonging to the Western lineages are different from the proteases from the viruses belong to the Eastern lineages (Darpel *et al.,* 2011).

1.5 Bluetongue virus vaccine

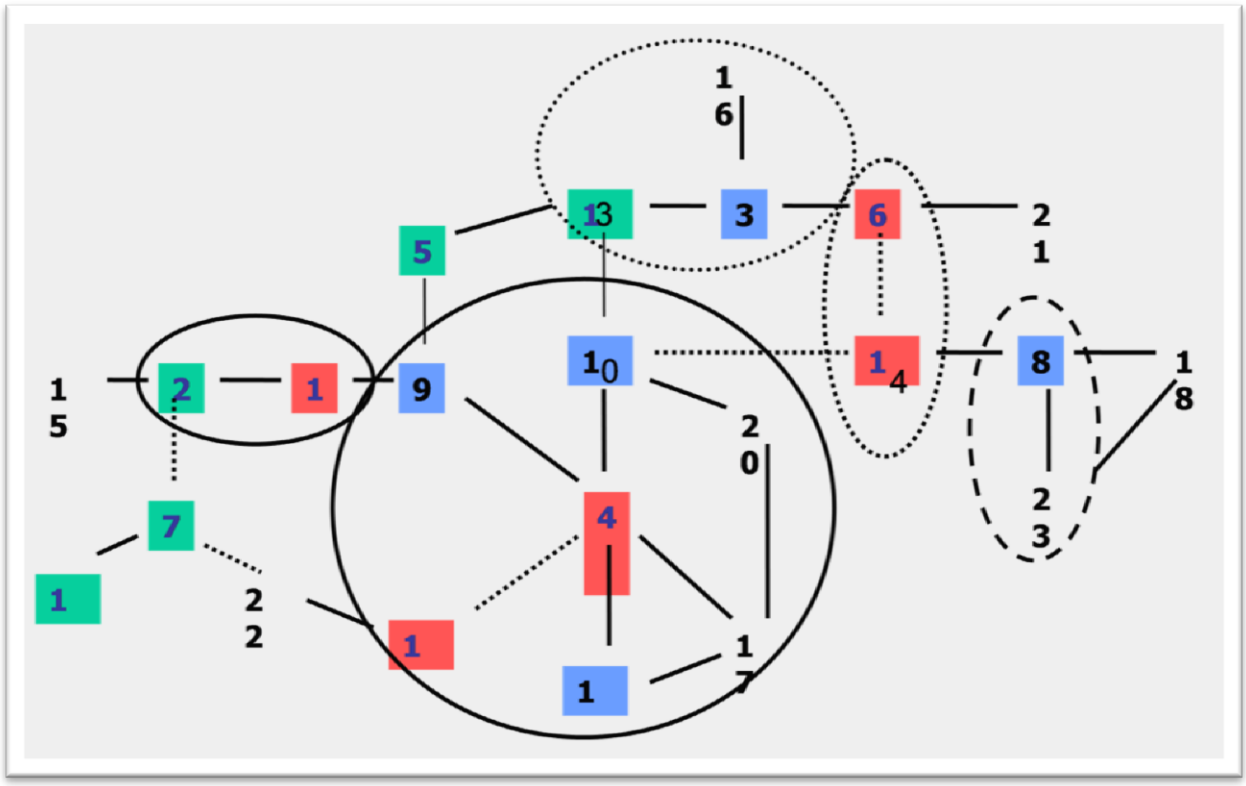
1.5.1 History

The disease is controlled by means of annual vaccination. The first vaccine was manufactured by Sir Arnold Theiler in 1906. The blood attenuated vaccine was made from BTV-infected blood samples collected from sheep and the virus was passaged until it was no longer virulent (Dungu *et al*., 2004; Theiler, 1908). The second vaccine was an egg passaged quadrivalent lyophilized BT vaccine. The animals responded better to this vaccine since the temperature reaction in animals was less severe and the incidence of vaccination induced disease were decreased (Alexander *et al*., 1951). After an outbreak in the Orange Free State in 1977/78 mainly caused by serotypes 18 and 19; serotype 19 was then added to the single vaccine containing only serotype 14 (Anon, 1985; Erasmus, 1975; Erasmus, 1980).

1.5.2 Live attenuated vaccine

Currently the OBP is producing a freeze dried polyvalent vaccine (Reg.No. G 358 Act No 36/1947). Selected BTV field strains are passaged in cell culture or embryonated eggs. In early studies the virus were passaged for as much as 80 times on cell cultures, but more recent studies showed that 20-50 passages are adequate (Murray & Eaton, 1996; Johnson *et al*., 1992a). This process involves careful

selection of the virus that is adapted to grow *in vitro*, but will be limited to its ability to replicate within the host and cause disease (Murray & Eaton, 1996). The serotypes were selected and placed in categories; the categories are structured in such a manner that more immunogenic and more dominant viruses provide cross protection to less immunogenic viruses as illustrated in Figure 1.3 (Erasmus, 1980). The vaccine consists of three bottles (A, B, C) consisting of five strains each; Bottle A includes serotypes -1, -4, -6, -12 and -14; Bottle B includes serotypes 3, -8, -9, -10 and -11 and Bottle C includes serotypes 2, -5, -7, 13- and -19 (Dungu *et al*., 2004).



Vaccine bottle A

Vaccine bottle B

Vaccine bottle C

Figure 1.3: An illustration of the cross-protection of different BTV serotypes using the SNT and the grouping of serotype combinations used in the vaccine (Erasmus 1990; Dungu *et al*., 2004).

The vaccine should be administered to sheep in three week intervals. The OBP vaccine should be administered nine to twelve weeks before mating season commences because it can cause abortions if ewes are vaccinated in the first halve of pregnancy. The rams should be vaccinated after the mating seasons and lambs at the age of six months. In most instances cattle and goats in SA are usually only sub clinically infected (Maan *et al*., 2010) and for this reason cattle and goats are not vaccinated in SA.

The vaccine has proven to be very affective in producing immunity in the vaccinated animals but there are some major side effects or possible risks at hand. The attenuated strains of South African, Australian and North American serotypes prove to be teratogenic (production of physical defects in offspring in utero) and cause foetal losses in sheep (Shultz & Delay 1955; Erasmus, 1990; Johnson *et al*., 1992a, b). The effect of the vaccine depends on the time of gestation of the vaccination of the animals. Ewes vaccinated between thirty and eighty days of gestation produce lambs with lesions on the brain, the severity differing from glial nodules to porencephaly and hydranencephaly (Flanagan & Johnson, 1995). Ewes that are vaccinated in the 6th week of gestation may suffer up to 40% fetal losses (Young & Cordy, 1964). Bluetongue virus induced central nervous system malformation in both calves and lambs range from thin walled cerebral hemispheres to the cerebellum only being represented by a fluid-filled sac if the dam was vaccinated in the early stages of gestation (MacLachlan *et al*., 2000).

Following vaccination as well as viraemic periods, attenuated viruses appear in the semen of inoculated bulls and rams (Johnson *et al*., 1992). A reduction in semen quality has also been recorded (Breard *et al*., 2007).

The vaccine can be detected in the inoculated animals. The titres reach levels that are sufficient to be transferred to non-vaccinated animal hosts via *Culicoides* midges (Elia *et al*., 2008). The vaccine strain and the wild-type strain can also exchange genome segments when simultaneously infection of animals occur, resulting in the emergence of new virus strains (Ferrari *et al*., 2005; Savini *et al*., 2008). Genetic drift is another mechanism of reversion of the live attenuated vaccine strain to a virulent strain if the vaccine is circulated in the field for prolonged periods (Veronesi *et al*., 2005).

1.6 Laboratory techniques and diagnosis

1.6.1 Virus isolation

This is a common technique traditionally used to isolate BTV field samples by inoculation of the yolk sac or using the intravenous route of embryonated chicken eggs (ECE) (Mason *et al*., 1940; Goldsmit & Barzilai, 1984). This method was also used by Alexander to passage the virus until it lost its virulent characteristic in the production of BTV vaccines. Inoculating the ECE intravenously is 100-1000 fold more sensitive in isolating the virus than inoculations via the yolk sac (Goldsmit & Barzilai, 1984). Virus isolation methods may also include cerebral inoculation of suckling mice (Afshar, 1994), although these days not commonly practice. The more frequently used technique is *in vitro* cell culture isolation. There are multiple cell cultures that can be used to culture viruses including African green monkey kidney cells (Vero), baby hamster kidney-21 cells (BHK-21) or insect cells (e.g. mosquito cell line, C6/36 or the *Culicoides* cell line, KC cells) (Wechler & McHolland, 1988). Cell culture enables researchers to isolate viruses from blood and organ samples and eliminates the use of animal experiments (Homan *et al.,* 1990; Mo *et al*., 1994; Sharifah *et al*., 1995).

1.6.2 Serology

1.6.2.1 Serum neutralization assay

Immunological assays have been used traditionally to confirm and classify BTV isolates. Serotyping is mainly conducted with fluorescent antibody staining and neutralization. There are 2 neutralization tests that are commonly used to distinguish between BTV serotypes. The first method is the virus neutralization test; this method utilizes standard concentrations of a known serum to be tested with different dilutions of an unknown virus (OIE, 2008). If the antibodies in the serum are adequate the virus will be neutralized, to measure the neutralization effect susceptible cells used to see if the virus will have a cytopathic effect or whether they were neutralized.

The other method is the plaque neutralization assay that is used to determine the presence and the titer of neutralizing antibodies to the virus or can be used to determine the virus serotype (Sundin *et al*., 1989). Reference serum from animals that were previously infected with known serotypes is used as antiserum against an unknown virus that needs to be serotyped i.e. if an unknown virus isolate is

neutralized by a particular reference antiserum, the virus serotype is identified. For antibody titers, different dilutions of an antiserum are tested against a fixed amount of virus. The maximum dilutions of serum where you still get neutralization represent the antibody titre (Hiag *et al*., 1956; Howell, 1960).

1.6.2.2 cELISA

The detection of BTV-specific antibodies can be done using a blocking ELISA (b-ELISA) or a competitive ELISA (c-ELISA) (Anderson, 1984; Afshar *et al*., 1987). The b-ELISA reacts on the presence of BTV antibodies in the test serum with an immobilized BTV antigen. This is possible with the addition of a BTV group-specific murine monoclonal antibody (MAb). If the BTV antibodies are present in the serum, the specific antibodies block the antigen and prevent it from reacting with the MAb (Anderson, 1984). The b-ELISA kits are extremely sensitive and can detect antibodies to 22 BTV serotypes (Mertens *et al*., 2009).

With the cELISA the test serum and the MAb reacts simultaneously to compete to react with the immobilized BTV antigens (Afshar *et al*., 1987). The cELISA uses the VP 7 epitope as antigen to minimize the cross reaction of other orbiviruses and is therefore the prescribed test for the detection of BTV-specific antibodies (OIE, 2008). Another ELISA called virus capture or a double sandwich ELISA has been used for detection of BTV in *Culicoides* biting midges as well as infected BHK-21 (El Hussain *et al*., 1989).

1.6.3 Molecular assays

Molecular assays that have been developed includes reverse transcriptase-polymerase chain reactions (RT-PCR) (Bonneau *et al*., 2000; Wilson *et al*., 2000; Mertens *et al*., 2007) and real-time quantitative RT-PCR (Orru *et al*., 2004) and are used to visualize amplified products. These assays can recognize viral RNA but are not specific to distinguish between infectious and non-infectious virus.

There are a number of serogroup-specific real-time RT-qPCR assays that have been designed for the detection of BTV. These assays targets specific genome segments of the BTV including segment 1 (VP 1) (Shaw *et al*., 2007), segment 5 (NS 1) (Toussaint *et al*., 2007) segment 9 (VP 6) and segment 10 (NS 3)

(Van Rijn *et al*., 2012). Currently Segment 5 is the most conserved genome that is recommended by the OIE to identify all 27 serotypes (OIE, 2008; Hofmann *et al.,* 2009).

Serotype-specific RT-PCRs are set up to target segment 2. PCRs are used for fast serotype identification but the accuracy and sensitivity is limited to the amount of serotype-specific primers that is available (Mertens *et al*., 2007).

1.6.4 Next generation sequencing

The principals of Sanger sequencing using nucleotide chain termination was developed more than 20 years ago, and are mainly used to sequence short DNA fragments (800-1000 nucleotides) (Sanger *et al.,* 1977). Sanger sequencing uses DNA polymerase catalysis for the incorporation of fluorescently identified deoxyribonucleotide triphosphate (dNTP's) into a DNA template strand during the sequential cycles of DNA synthesis (Lo and Chiu, 2009). Sanger sequencing is a useful technique when sequencing bacteria, archeal and eukaryotic genomes, but because of limitations Next Generation Sequencing was devolved. Next Generation Sequencing includes advantages such as a massive increase in throughput, increase speed, de novo sequencing and makes it possible for the researcher to analyze mutations in a population (Ronaghi, 2001). Next Generation Sequencing is a usefull to identify and characterize reassortment viruses of BTV (Maan *et* al., 2010). The technology provides and inexpensive, genomic-wide sequence end product that makes applications such as chromatin immune-precipitation, mutation mapping and polymorphism discovery to noncoding RNA discovery (Ram *et al*., 2011).

1.6.5 Analyzing software

BioNumerics consists of 10 software modules, used for the analysis of all major applications in bioinformatics: 1D electrophoresis gels, chromatographic and spectrometric profiles, phenotype characterizations, microarrays and sequences (Vauterin & Vauterin, 2006). BioNumerics is able to combine information from various genomic and phenotypic sources into one global database and conduct conclusive analyses (Schouls et al., 2009).

Genious is bioinformatic software that assists researchers in DNA alignment, assemblies and sequence alignment.

1.6.6 Cell viability assays

Cell viability is measured using two parameters; one that indicates whether the cell is capable to maintain its metabolic processes and another that detects changes in the cell membrane integrity (Riss & Morovec, 2003). The CellTiter-blue assay (Promega) provides a homogenous, fluorometric method for monitoring cell viability. This assay is based on the cells' ability to convert resazurin, a redox dye, into resofurin, a fluorescent end product as illustrated in Figure 4 (Riss and Moravec, 2003). Another available assay includes the CellTiter96, AQ_{ueous} One Solution Cell Proliferation Assay (Promega). These assays works on the same principal but uses the reduction of Methanethiosulfonate tetrazoluim to a coloured formazan end product. The CellTiter-Glo Luminescent Cell Viability Assay measures the ATP content of the cell in order to determine viability (CellTiter-Blue Cell Viability Assay Technical Bulletin).

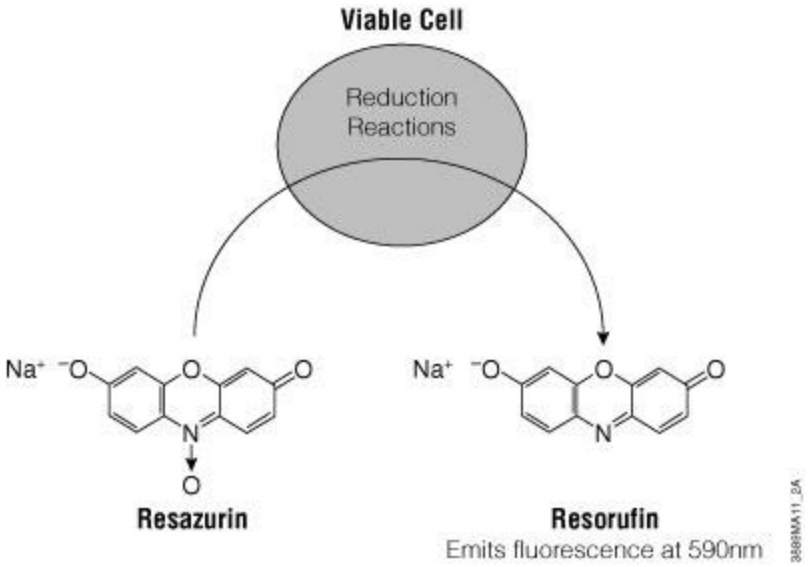


Figure 1.4: Conversion of Resofurin based on the cell's ability to reduce the product into a fluorescent end product (Riss and Moravec, 2003).

The CellTox Green Cytotoxicity Assay (Promega) assesses cytotoxicity in cell culture by measuring changes in the membrane integrity that occurs as a result of cell death. The cyanine dye cannot enter

the viable cell membrane, but as soon as the cell membrane is compromised the cyanine can bind to the DNA and the fluorescent properties of the dye are enhanced (McDougall & Dwight, 2010). As a result the viable cells produce no appreciable increase in fluorescence. The fluorescent signal that is produced is proportional to the cytotoxicity (Zhang *et al*., 1999).

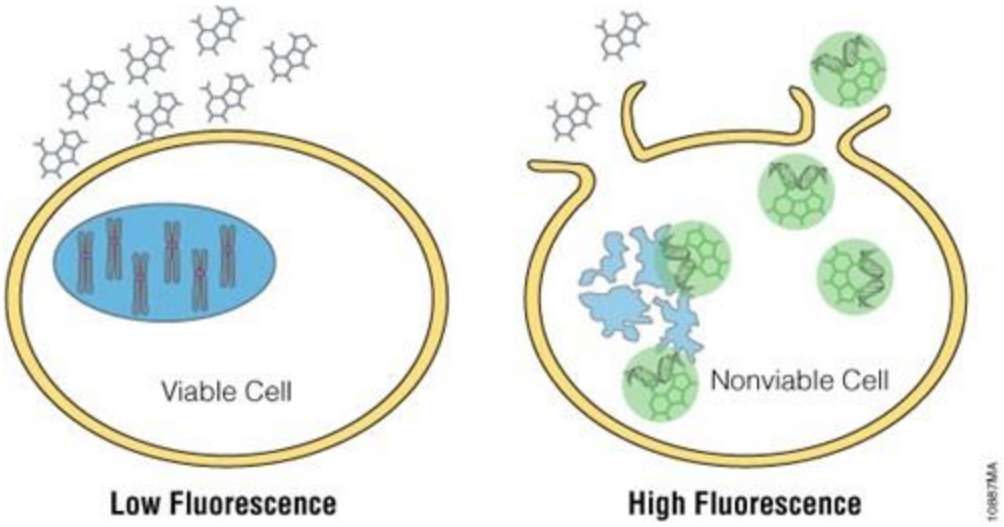


Figure 1.5: Illustration of Cyanine dye binding to the cytoplasmic content after the cell membrane integrity has been compromised (McDougall & Dwight, 2010).

1.7 Problem statement

The concern that reassortment may occur between genetically and phenotypically divergent BTV strains may lead to the emergence of new virus strains has been reported as previously discussed. The new viruses may demonstrate new undesired biological properties. That may include altered neutralization characteristics, increased virulence and possibly more severe pathogenic consequences for the mammalian host. Another concern may be that the reassortant virus may be optimally adapted to different environmental conditions which may lead to the spreading of the disease. The reassortant viruses may also have an increase ability to infect *Culicoides* species and may be more effectively transmitted than that of the original parental strains from which they were derived (Bansel *et* al., 1998; Xu *et al*., 1997).

The vaccination of sheep against BTV using a live attenuated vaccine is commonly practiced in sheep in SA and since the vaccine strains can replicate and cause viraemia in the vaccinated hosts, it increases the potential spread of vaccine strains. Although cattle are not normally vaccinated against the virus but can be naturally infected, the aim of this study is to investigate whether reasortment between vaccine strains and a wild type strain could occur in cattle. In previous kinetic studies reassortment frequencies in cattle was illustrated to be higher (89%) than reassortment in sheep (5%). This might assist in the understanding of not only the epidemiology of the BTV in SA but also add to a better understanding of the phenotypic characteristics of reassortant viruses.

1.8 Aims/Objectives

The aim of the project is to investigate the potential generation of genetic reassortant strains between field and vaccine strains within cattle.

The objectives include

- To infect six BTV negative bovines between the age of six months and one year with modified live virus (MLV) of BTV from Bottle B of the vaccine. The animals will then be divided into two groups of three cattle. The three randomly selected cattle will then be infected with a wild type BTV serotype 4 that was isolated in Rustenburg from a clinical infected ewe.
- Blood samples will be collected from all the bovines daily during the period of viraemia and the blood will be used to isolate BTV from the cattle.
- To identify the characteristics of the isolated viruses by conventional virology, polyacrylamide gel electrophoresis (PAGE) and sequencing in comparison to the parental vaccine strains. The parental strains will be isolated from the vaccine Bottle Band full genome sequencing will be done.
- To characterize cell growth/cell cytotoxicity of a subset of reassortant viruses compared to the parental strains using cell viability assays.

1.9 REFERENCES

1. Afshar, A.H.M.A.D, Thomas, F.C., Wright, P.F. Shapiro, J.L., Shettigara, P.T., and Anderson, J. (1987). Comparison of competitive and indirect enzyme-linked immunosorbent assays for detection of bluetongue virus antibodies is serum and whole-blood. *Journal of Clinical Microbiology*, 25(9), 1705-1710.
2. Afshar, A. (1994). Bluetongue: Laboratory diagnosis. *Comparative Immunology Microbiology and Infectious diseases,* 17(3), 221-242.
3. Akita, G.Y., Lanconescu, M., Maclachlan, N.J., Osburn, B.I. and Greene, R. (1994). Bluetongue disease in dogs associated with contaminated vaccine. *Veterinary record*, 135(11), 283 -284.
4. Alexander, R.A. and Haig, D.A. (1951). The use of egg attenuated bluetongue virus in the production of a polyvalent vaccine for sheep: a propagation of the virus in sheep. *Journal of Veterinary Research*, 25, 3-5.
5. Alexander, K.A., MacLachlan, N.J., Kat, P.W., House, C., O'Brien, S.J., Lerche, N.W., Sawyer, M., Frank, L.G., Holekamp, K. and Smale, L. (1994). Evidence of natural bluetongue virus infection among African carnivores. *American journal of Tropical Medicine and Hygiene*, 51(5), 568-576.
6. Anderson, J. (1984). Use of monoclonal antibody in a blocking ELISA to detect group specific antibodies to bluetongue virus. *Journal of Immunological Methods*, 74(1), 139-149.
7. Anderson, G.A., Stott, J.L., Gershwin, L.J. and Osburn, B.I. (1984). Subclinical and clinical bluetongue disease in cattle: clinical, pathological and pathogenic considerations. *Progress in Clinical Biological Research*, 178, 103-107.
8. Anderson, G.A., Stott, J.L., Gershwin, L.J., Osburn, B.I., (1987). Identification of bluetongue virus-specific immunoglobulin E in cattle. *Journal of General Virology*, 68, 2509-2514.
9. Anon (1985). Biennial report of the VRIO: Vaccine improvement 1984 – 1985. *VRIO*, 54.
10. Backx, A., Heutink, R., Van Rooij, E., & Van Rijn, P. (2009). Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in cattle after experimental infection. *Veterinary microbiology*, *138*(3), 235-243.
11. Bansal, O.B., Stokes, A., Bansal, A., Bishop, D. and Roy, P. (1998). Membrane organization of bluetongue virus nonstructural glycoprotein NS3. *Journal of Virology*, 72(4), 3362-3369.
12. Balasuriya, U.B.R, Nadler, S.A., Wilson, W.C., Pritchard, L.I., Smythe, A.B., Savini, G., Monaco, F., De Santis, P., Zhang, N., Tabachnick, W.J. and MacLachlan, N.J. (2008). The NS3 proteins of global

strain of bluetongue virus evolve into regional topotypes through negative (purifying) selection. *Veterinary Microbiology*, 126(1), 91-100.

13. Basak, A.K., Grimes, J.M., Gouet, P., Roy, P. and Stuart, D.I. (1997). Structures of Orbivirus VP 7: implications for the role of this protein in the viral life cycle. *Structure,* 5(7), 871-883.
14. Batten, C.A., Maan, S., Shaw, A.E., Maan, N.S., Mertens, P.P. (2008). A European field strain of bluetongue virus derived from two parental vaccine strains by genome segments reassortment. *Virus Research*, 137(1), 56-63.
15. Bodowes, R. and Worobey, M. (2011). Homologous recombination in negative sense RNA viruses. *Viruses*, 3, 1358-1373.
16. Bonneau, K.R., Zhang, N.Z., Wilson, W.C., Zhu, J.B., Zhang, F.Q., Li, Z.H., Zhang, K.L., Xiao, L, Xiang W.B. and MacLachlan, N.J. (2000). Phylogenetic analysis of the S7 gene does not segregate Chinese strains of bluetongue virus into a single topotype. *Archives of Virology*, 145(6), 1163-1171.
17. Boorman, J. (1989). *Culicoides* (Diptera; Ceratopoginidae) of the Arabian Peninsula with notes on their medical and veterinary importance. *Fauna of Saudi Arabia*, 10(1), 160-224.
18. Boorman, J. (1993). Biting midges (Ceratopogonidae). In *Medical insects and arachnids* (pp. 288-309) Springer Netherlands.
19. Bowne, J.G. and Jones, R.H. (1966). Observation on bluetongue virus in salivary glands of an insect vectors, *Culicoides variipennis. Virology*, 30(1), 127-133.
20. Bowne J.G., Luedke, A.J., Jochim, M.M. and Metcalf, H.E. (1968). Bluetongue disease in cattle. *Journal of the American Veterinary Medical Association,* 153(6), 662-668.
21. Boyce, M., Celma, C.C. and Roy, P. (2012). Bluetongue virus non-structural protein 1 is a positive regulator of viral protein synthesis. *Virology Journal*, 9, 178. doi: 10.1186/1743-422X-9-178.
22. Boyce, M., Wehrfritz, J., Noad, R. and Roy, P. (2004). Purified Recombinant bluetongue virus VP 1 exhibits RNA replicase activity. *Journal of Virology*, 78(8), 3994–4002.
23. Braverman, Y., Boorman, J. and Kremer, M. (1976). *Faunistic* list of *Culicoides* (Diptera: Ceratopogonidae) from Isreal. *Cah, ORSTOM Ser. Enthomology and Medical Parasitology*, 14: 179-85.
24. Breard, E., Pozzi, N., Sailleau, C., Durand, B., Catinot, V., Sellem, E., Dumont, P., Guerin, B. and Zientara, S. (2007). Transient adverse effect of an attenuated bluetongue virus vaccine on the quality of ram semen. *Veterinary Records*, 160(13): 431-435.

25. Brown, T.T., Schultz, R.D., Duncan, J.R. and Bistner, S.I. (1979). Serological response of the bovine viral diarrhea virus. *Infection and Immunity*, 25, 93-97.
26. Caporale, M., Wash, R., Pini, A., Savini, G., Franchi, P., Golder, M., Patterson-Kane, J., Mertens, P, Di, G.L., Armillotta, G., Lelli, R., Kellam, P. and Palmarini M. (2011). Determinants of bluetongue virus virulence in murine models of disease. *Journal of Virology* 85(21), 11479-11489.
27. CellTiter-Blue Cell Viability Assay Technical Bulletin #TH317, Promega Corporation.
28. Celma, C.C. & Roy, P. (2009). A viral nonstructural protein regulates bluetongue virus trafficking and release. *Journal of Virology* 83(13), 6806-6816.
29. Chauveau, E., Doceul, V., Lara, E., Breard, E., Sailleau, C., Vidalain, P.O., Meurs, E.F., Dabo, S., Schwartz-Cornil, I., Zientara, S. and Vitour, D. (2013). NS3 of Bluetongue virus interferes with the induction of type I interferon. *Journal of virology*, 87(14), 8241-8246.
30. Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A.E., Anthony, S., Bachanek-Bankawska, K., Kgosana, L., Bin-Tarif, A., Carpenter, S., Muller-doblies, U.U., Takamatsu, H.H., Mellor, P.S., Mertens, P.P.C. and Oura, C.A. (2007). Clinical signs and pathology shown by British sheep and cattle infected with Bluetongue virus serotype 8 from the 2006 outbreak in Europe. *Veterinary Record*, 161(8), 253-261.
31. Darpel, K., Monaghen, P., Anthony, S., Takamatsu, H and Mertens, P.C. (2009). BTV in the mammalian host and the induced immune response. In Mellor, P.S., Baylis, M., Mertens, P.C. (Eds.). Bluetongue. Elsevier Academic press, London, pp. 265-284.
32. Darpel, K.E., Langner, K.F., Nimtz, M., Anthony, S.J., Brownlie, J., Takamatsu, H.H., Mellor, P.S. and Mertens, P.P., 2011. Saliva proteins of vector *Culicoides* modify structure and infectivity of bluetongue virus particles. *PloS one*, *6*(3), p.e17545.
33. Davies, F.G., Walker, A.R., Ochieng, P. and Shaw, T. (1979). Arboviruses isolated from *Culicoides* midges in Kenya. *Journal of Comparative Pathology*, 89(4), 587-595.
34. Davies, F.G. and Walker, A.R. (1974). The isolation of ephemeral fever virus from cattle and *Culicoides* midges in Kenya. *Veterinary Records*, 95(3), 63-64.
35. De Clercq, K., De Leeuw, I., Verheyden, B., Vandemeulebroucke, E., Vanbinst, T., Herr, C., Méroc, E., Bertels, G., Steurbaut, N., Miry, C., De Bleecker, K., Maquet, G., Bughin, J, Saulmont, M., Lebrun, M., Sustronck, B., De Deken, R., Hooyberghs, J.,Houdart, P.,Raemaekers, M., Mintiens, K., Kerkhofs, P., Goris, N. and Vandenbussche, F. (2008). Transplacental infection and immunotolerance induced by a wild-type bluetongue virus serotype 8 natural infections. *Transboundary and Emerging Diseases,* 55(8), 352-359.

36. DeMaula, C.D., Julita, M.A., Wilson, D.W. and Maclachlan, N.J. (2001). Infection kinetics, prostacyclin release, and cytokine-mediated modulation of the mechanism of cell death during bluetongue virus infection of cultured ovine and bovine pulmonary artery and lung microvascular endothelial cells. *Journal of General Virology*, 82(4), 787-794.
37. DeMaula, C.D., Leutennegger, C.N., Bonneau, K.R. and Maclachlan, N.J. (2002a). The role of endothelial cell-derived inflammatory and vasoactive mediators in the pathogenesis of bluetongue. *Virology*, 296(2), 330-337.
38. DeMaula, C.D., Leutennegger, C.m., Jutila, M.A. and Maclachlan, N.J., (2002b). Bluetongue virus-induced activation of primary bovine lung microvascular endothelial cells. *Veterinary Immunology and Immunopathology*, 86(3), 147-157.
39. Du Toit, R.M. (1962). The role played by bovines in transmission of bluetongue in sheep. *Journal of the South African Veterinary Medicine Association*, 33, 483-490.
40. Du Toit, R.M. (1944). The transmission of bluetongue and horse sickness by *Culicoides*. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 19, 7-16.
41. Dungu, B., Gerdes, T. and Smit, T. (2004). The use of vaccination in the control of bluetongue in Southern Africa. *Veterinaria Italiana*, 40 (4), 616-622.
42. Elia, G., Savini, G., Decaro, N., Martella, V., Teodori, L., Casaccia, C., Di Gialleonardo, L., Lorusso, E., Caporale, V. and Buonavoglia, C. (2008). Use of real-time RT-PCR as a rapid molecular approach for differentiation of field and vaccine strains of bluetongue virus serotypes 2 and 9. *Molecular and Cellular Probes*, 22(1), 38-46.
43. El Hussain, A., Calisher, C.H, Holbrook, F.R., Schoepp, R.J. and Beaty, B.J. (1989). Detection of bluetongue virus antigens in *Culicoides variipennis* by enzyme immunoassay. *Journal of Clinical Microbiology*, 27(6), 1320-1323.
44. Erasmus, B.J. (1975). Bluetongue in sheep and goats. *Australian Veterinary Journal*, 51(4), 165-170.
45. Erasmus, B.J., (1980). The epidemiology and the control of bluetongue in South Africa. *Office International des Epizootics*, 92, 461-467.
46. Erasmus, B.J. (1990). Bluetongue virus. In *Virus Infections of Ruminants*. (Z. Dinter and B. Morein eds.) Elsevier Science Publishers, Amsterdam. The Netherlands, pp. 227–237.
47. Ferrari, G., De Liberato, C., Scavia, G., Lorenzetti, R., Zini, M., Farina, F., Magliano, A., Cardeti, G., Scholl, F., Guidoni, M., Scicluna, M.T., Amaddeo, D., Scaramozino, P. and Autorino, G.L. (2005).

Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Preventative Veterinary Medicine*, 68(2), 103-113.

48. Flanagan, M. and Johnson, S.J. (1995). The effects of vaccination of Merino ewes with an attenuated Australian bluetongue virus serotype 23 at different stages of gestation. *Australian Veterinary Journal*, 72(12), 455-457.
49. Forzan, M., Wirblich, C. and Roy, P., (2004). A capsid protein of non-enveloped Bluetongue virus exhibits membrane fusion activity. *Proceedings of the National Academy of Sciences of the United States of America,* 101(7), 2100-2105.
50. Gambles, R.M. (1949). Bluetongue of sheep in Cyprus. *Journal of Comparative Pathology*, 59, 176-190.
51. Gibbs, E.P. and Greiner, E.C. (1994). The epidemiology of bluetongue. *Comparative Immunology Microbiology and Infectious Diseases*, 17(3), 207 -220.
52. Goldsmit, L. and Barzilai, E. (1984). Isolation and propagation of bluetongue virus in embryonating chicken eggs*. Progress* in *Clinical and Biological Research*, 178, 307-318.
53. Gouet, P., Diprose, J.M., Grimes, J.M., Malby, R., Burroughs, J.N., Zientara, S., Stuart, D.I. and Mertens, P.P. (1999). The highly ordered double-stranded RNA genome of bluetongue virus revealed by crystallography. *Cell*, 97(4), 481-490.
54. Greiner, E.C., Barber, T.C., Pearson, J.E., Kramer, W.L. and Gibbs, E.P. (1985). Orbiviruses from *Culicoides* in Florida. *Progress in Clinical and Biological Research*, 178, 195-200.
55. Grimes, J.M., Burroughs, J.N., Gouet, P., Diprose, J.M., Malby, R., Zientara, S., Mertens, P. P. and Stuart, D. I. (1998). The atomic structure of the bluetongue virus core. *Nature,* 395(6701), 470-478.
56. Haig, D.A., McKercher. D.G. and Alexander, R.A. (1956). The cytopathogenic action of bluetongue virus on tissue cultures and its application to the detection of antibodies in the serum of sheep. *Onderstepoort Journal of Veterinary Research*, 27, 171-177.
57. Hassan, S.S. and Roy, P. (1999). Expression and functional characterization of bluetongue virus VP 2 protein: role in cell entry. *Journal of Virology,* 73(12), 9832-9842.
58. Hassan, S.H., Wirblich, C., Forzan, M. and Roy, P. (2001). Expression and functional characterization of bluetongue virus VP5 protein: role in cellular permeabilization. *Journal of Virology* 75(18), 8356-8367.
59. Hewat, E.A., Booth, T.F. and Roy, P. (1992). Structure of bluetongue virus particles by cryoelectron microscopy. *Journal Structural Biology,* 109(1), 61-69.

60. Hoffmann, B., Beer, M., Reid, S.M., Mertens, P., Oura, C.A., Van Rijn, P.A., Slomka, M.J., Banks, J., Brown, I.H., Alexander, D.J. and King, D.P. (2009). A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Veterinary microbiology*, *139*(1), 1-23.
61. Homan, E., Mo, C., Thompson, L., Barreto, C.H., Oviedo, M.T., Gibbs, E.P. and Greiner, E.C. (1990). Epidemiology study of bluetongue viruses in Central America and the Caribbean: 1986-1988. Regional bluetongue team. *American Journal of Veterinary Research*, 51(7), 1089-1094.
62. Howell, P.G.A. (1960). A preliminary antigenic classification of strains of bluetongue virus. *Onderstepoort Journal of Veterinary Research,* 28, 357-364.
63. Hourrigan, J.L. and Kingsporn, A.L. (1975). Bluetongue: the disease in cattle. Australian *Veterinary Journal,* 51 (4), 170-174.
64. Huismans, H., Van Staden, V., Fick, W. C., Van Niekerk, M., & Meiring, T. L. (2004). A comparison of different orbivirus proteins that could affect virulence and pathogenesis. *Veterinaria Italiana*, *40*(4), 417-425.
65. Hyatt, A.D., Zhao, Y. and Roy, P. (1993). Release of bluetongue virus-like particles from insect cells is mediated by BTV non-structural protein NS3/NS3A. *Virology* 193(2), 592-603.
66. Illumina. (2014). HiseqX ten preliminary systems specification sheet. (www.illlumina.com/documents/products/datasheet-hiseq-x-ten.pdf)
67. Jauniaux, T.P., De Clercq, K.E., Cassart, D.E., Kennedy, S., Vandenbussche, F.E., Vandemeulenbroucke, E.L., Vanbinst, T.M., Verbheyden, B.I., Goris, N.E. and Coignoul, F.L. (2008). Bluetongue in Eurasian Lynx. *Emerging infectious diseases,* 14, 1496-1408.
68. Jenckel. M., Breard, E., Schulz, C., Sailleau, C., Viarouge, C., Hoffmann, B., Hoper, D., Beer, M. and Zientara, S. (2015). Complete coding genome sequence of putative novel bluetongue virus serotype 27. *Genome announcements*, 3(2), e00016-15.
69. Jennings, D.M., Boorman, J.P.T. and Ergun, H. (1983). *Culicoides* from western Turkey in relation to bluetongue disease of sheep and cattle. *Revue D'elevage et de Medecine Veterinaire des Pays Tropicaux*, 36(1), 67-70.
70. Johnson, S.J., Hoffman, D., Flanagan, M., Polkinghorne, I.G. and Bellis, G.A. (1992a). Clinicopathology of Australian bluetongue virus serotypes for sheep. In *Proc. Of 2nd International symposium on Bluetongue, African horsesickness and Related Orbiviruses.* Edited by Walton, T.E., & Osburn, B.I., CRC Press, Boca Raton, (pp. 737-743).

71. Johnson, D.J., Mertens, P.P.C., Maan, S. and Oslund, E.N. (2007). Exotic bluetongue viruses identified from ruminants in the south-eastern U.S from 1999-2006. In: *Proceedings of the Annual AAVLD Conference. Reno, NV.* p.118.

72. Johnson, S.J., Polkinghorne, I.G., Flanagan, M. and Townsend, W.L. (1992b). In *Bluetongue, African horsesickness and related Orbiviruses*. Edited by Walton, T.E., & Osburn, B.I., CRC Press, Boca Raton, p 868.

73. Kar, A.K., Bhattacharya, B. and Roy, P. (2007). Bluetongue virus RNA binding protein NS2 is a modulator of viral replication and assembly. *BMC Molecular Biology* 8(1), 4.

74. Komarov, A. and Goldsmit, L.E.A.H. (1951). A disease, similar to bluetongue in cattle and sheep in Israel. *Refuah Veterinarith*, 8, 96-100.

75. Lassen, S.B., Nielsen, S.A., Skovgard, H. and Kirstensen, M. (2012). Molecular differentiation of *Culicoides* biting midges (Diptera: Ceratopogonidae) from subgenus *Culicoides latreille* in Denmark. *Parasitology Research*, 110(5), 1765 – 1771.

76. Ligisa, D.M., Perez Aguirreburualde, M.S., Gonzalez, F.N., Marin-Lopez, A., Ruiz, V., Wigdorovitz, A., Martinez-Escribano, J.A., Ortego, J. and Du Santos, M.J. (2015). An experimental subunit vaccine based on bluetongue virus 4 VP 2 protein fused to an antigen-presenting cells single antibody elicit cellular and humoral immune response in cattle, guinea pigs and IFNAR(-/-) mice. *Journal of Vaccines,* 33(22), 2614-2619.

77. Lo, Y.D. and Chiu, R.W. (2009). Next generation sequencing of plasma/serum DNA: an emerging analysis and molecular diagnostic tool. *Clinical Chemistry*, 55(4), 607-608.

78. Luedke, A.J. and Anakwenze, E.I. (1972). Bluetongue virus in goats. *American Journal of Veterinary Reseach Records*, 33, 1739-1745.

79. Maan, S., Maan, N.S., Samuel, A.R., Rao, S., Attoui, H. and Mertens, P.P.C. (2007). Analysis and phylogenetic comparisons of full-length VP2 genes of the 24 bluetongue virus serotypes. *Journal of General Virology*, 88(2), 621–630.

80. Maan. S., Maan N.S., Ross-smith, N., Batten, C.A., Shaw, A.E., Anthony, S.J. Sameul, A.R., Darpel., K.E., Veronesi, E., Oura, C.A., Singh, K.P., Nomikou, K., Potgieter, A.C., Attoui, H., Van Rooij, E., Van Rijn, P., de Clercq, K., Vandenbussche, F., Zientara, S., Breard, E., Sailleau, C., Beer, M., Hoffman, B., Mellor, B. and Mertens, P.P.C. (2008). Sequence analysis 28 of bluetongue virus serotype 8 from Netherlands 2006 and comparisons to other European strains. *Virology*, 377(2), 308-318.

81. Maan, S., Maan, N.S., Van Rijn P.A., Van Gennip, R.G., Sanders, A., Wright, I.M., Batten, C., Hoffmann, B., Eschbaumer, M., Oura, C.A., Potgieter, A.C., Nomikou, K. and Mertens, P. P. (2010). Full genome characterization of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other field and vaccine strains. *PLoS One*. 5(4):e10323.
82. Maan, N. S., Maan, S., Nomikou, K., Guimera, M., Pullinger, G., Singh, K.P., Belaganahalli, M.N. and Mertens, P.P. (2012). The Genome Sequence of Bluetongue Virus Type 2 from India: Evidence for reassortment between Eastern and Western topotype field strains. *Journal of Virology* 86(10), 5967-5968. doi:86/10/5967 [pii];10.1128/JVI.00536-12 [doi].
83. MacLachlan, N.J. (2003). Bluetongue: Pathogenesis and duration of viraemia. *Veterinaria Italiana* 40(4), 462-467.
84. MacLachlan, N.J. (2011). Bluetongue: history, global epidemiology and pathogenesis. *Preventive Veterinary Medicine,* 102(2), 107-111.
85. MacLachlan, N.J., Conley, A.J. and Kennedy, P.C. (2000). Bluetongue and equine viral arteritis as models of virus induced fetal injury and abortion. *Animal reproduction Science,* 60, 643 -651.
86. MacLachlan, N.J., Drew, C.P., Darpel, K.E. and Worwa, G. (2009). The pathology and pathogenesis of bluetongue. *Journal Comparative Pathology,* 141, 1-16.
87. MacLachlan, N.J. and Fuller, F.J. (1986). Genetic stability in calves of a single strain of bluetongue virus. *American Journal of Veterinary Research*, 47(4), 762-764.
88. MacLachlan, N.J., Heidner, H.W. and Fuller, F.J. (1987). Humeral immune response of calves to bluetongue virus infection. *American Journal of Veterinary Research*, 48, 1031-1035.
89. MacLachlan, N.J., Jagels, G., Rossitto, P.V., Moore, P.F. and Heidner, H.W. (1990). The pathogenesis of experimental bluetongue virus infection of calves. *Veterinary pathology*, 27(4)223-229.
90. MacLachlan, N.J. and Osburn, B.I. (2006). Impact of bluetongue virus infection on the international movements and trade of ruminants. *Journal of the American Veterinary Medical Association*, 228, 1346 – 1349.
91. MacLachlan, N.J., Crafford, J.E., Vernau, W., Gardner, U.A., Goddard, A., Guthrie, A.J. and Venter, E.H. (2008). Experimental reproduction of severe bluetongue in sheep. *Veterinary Pathology Online,* 45(3), 310-315.
92. MacLachlan, N.J. and Guthrie, A.J. (2010). Re-emergence of bluetongue, African horse sickness, and other orbivirus diseases. *Veterinary Research,* 41(6), 5.

93. Mason, J.H. and Neits, W.O. (1940). The susceptibility of cattle to the virus of bluetongue. *Onderstepoort, Journl of Veterinary Animal Industry*, 15, pp. 149-157.
94. Manso-Ribeiro, J., Rosa-Azevedo, J., Noronha, F., Braco-Forte-Junior, M., Grave-Periera, C. and Vasco-Fernandes, M. (1957). Fievre catarrhale du mouton blue-tongue).*Bulletin de l'Office International des Epizooties* 48, 350 – 367.
95. Mayo, C.E., Mullens, B.A., Reisen, W.K., Osborne, C.J., Gibbs, E.P.J., Gardner, I.A. and MacLachlan, N.J. (2014). Seasonal and interseasonal dynamics of Bluetongue Virus infections of dairy cattle and *Culicoides sonorensis* Midges in Northern California-implications for virus overwintering in temperate zones. E106975.
96. McDougall, M. and Dwight, S. (2010). Nucleic acid binding dyes and uses therefor US Patent Application 2010/0233710A.
97. McEllistern, M.C. (2009). Genetic diversity of pneumococcal capsule: implications for molecular based serotyping. *Future Microbiology*, 4, 857-865.
98. Meiswinkel, R., Baldet, T., de Deken, R., Takken, W., Delecolle, J.C. and Mellor, P.S. (2008). The 2006 outbreak of bluetongue in northern Europe – The entomological perspective. *Preventative Veterinary Medicine* 87(1), 55-63.
99. Meiswinkel, R., Gomulski, L.M., Delecolle, J.C., Goffredo, M. and Gasperi, G. (2004). The taxonomy of *Culicoides* vector complexes – unfinished business. *Veterinaria Italiana* 40(3), 151-159.
100. Meiswinkel, R. and Paweska, J.T. (2003). Evidence for a new field *Cullicoides* of African horse sickness in South Africa. *Preventative Veterinary Medicine*, 60(3), 243-253.
101. Meiswinkel, R., Van Rijn, P., Leijs, P. and Goffredo, M., 2007. Potential new Culicoides vector of bluetongue virus in northern Europe. *Veterinary Record*, 161(16), 564.
102. Melhorn, H., Walldorf, V., Klimpel, S., Jahn, B. and Jaeger, F. Eschweiler, J., Hoffman, B. and beer, M. (2007). First occurrence of *Culicoides obsoletus*-transmitted Bluetongue virus epidemic in Central Europe. *Parasitology research*, 101(1), 219-228.
103. Mellor, P.S and Boorman, J. (1995). The transmission and geographical spread of African horse sickness and bluetongue virus. *Annuals of Tropical Medicine and Parasitology*, 89(1), 1-15.
104. Mellor, P. S., Boorman, J. and Baylis, M. (2000). *Cullicoides* biting midges: Their role as arbovirus vectors. *Annual Review of Entomology*, 45(1), 307-340.

105. Mellor, P.S., Carpenter, S., Harrup, L., Baylis, M. and Mertens, P.P. (2008). Bluetongue in Europe and the Mediterranean Basin of occurrence prior to 2006. *Preventative Veterinary Medicine*, 87(1), 4-20.

106. Mellor, P.S. and Pitzolis, G. (1979). Observation on breeding sites and light trap collection of *Culicoides* during an outbreak of bluetongue in Cyprus. *Bulletin of Entomological Research*, 69(2), 229-234.

107. Mellor, P.S. and Wittmann, E.J. (2002). Bluetongue virus in the Mediterranean Basin 1998-2001. *Veterinary Journal*, 164(1), 20-37.

108. Menzies, F.D., McCullough, S.J., McKeown, I.M., Forster, J.L., Jess, S., Batten, C., Murchie, A.K., Gloster, J., Fallows, J.G., Pelgrim, W., Mellor, P.S. and Oura, A.L. (2008). Evidence for transplacental and contact transmission of bluetongue virus in cattle. *Veterinary Records*, 163(7), 203-209.

109. Mertens, P. P. C. (1999). Bluetongue Viruses in the Encyclopedia of Life Sciences.

110. Mertens, P.P.C., Maan, N.S., Prasad, G., Samuel, A.R., Shaw, A.E., Potgieter, A.C., Anthony, S.J. and Maan, S. (2007). The design of primers and use of RT-PCR assays for typing European BTV isolates: differentiation of field and vaccine strains. *Journal of General Virology*, 88(10), 2811–2823.

111. Mertens, P. (2008). Bluetongue – Europe (69): Netherlands, BTV-6 Vaccine Strain update. ProMED-mail; 31 Oct:20081031.3431.

112. Mo, C.L., Thompson, H.L., Homan, E.J., Oviedo, M.T., Greiner, E.C., Gonzales, J. and Seanz, M.R. (1994). Bluetongue virus isolations from vectors and ruminants in Central America and the Caribbean. *Interamerican Bluetongue Team. American Journal of Veterinary Research*, 55, 211-215.

113. Mullen, G.R. and Anderson, R.R. (1998). Transmission of Orbiviruses by *Culicoides Latreille* species (Ceratopogpnidae) among cattle and white-tailed deer in the southeastern United States. *Abstract Volume. Internasional Congress of Dipterology*, 4th, pp. 155-156. Oxford, UK.

114. Murray,P.K and Eaton, B.T. (1996). Vaccine of bluetongue. *Australian Veterinary Journal*, 73(6), 207-210.

115. Neitz, W.O. and Reimerschmid, G. (1944). The influence of sunlight on the course of bluetongue. *Ondertstepoort, Journal of Veterinary Science, Animal Industries,* 19, 69-70.

116. Nevill, E.M. (1971). Cattle and *Culicoides* biting midges as possible overwintering hosts of bluetongue virus. *Onderstepoort Journal of Veterinary Research*, 38, 65-72.

117. Niedbalski, W. (2013). The evolution of bluetongue virus: genetic and phenotypic diversity of field strains. *Polish Journal of Veterinary Science*, 16(3), 611-616.

118. Nomikou, K., Hughes, J., Wash, R, Kellam, P., Breard, E., Zientara, S., Palmirini, M., Biek, R. and Mertens, P. (2015). Widespred reassortment shapes the evolution and epidemiology of Bluetongue Virus following European Invasion. *PLOS Pathogens*, 11(8), DOI:10.1371/ journal.ppat.1005056.

119. OIE. (2008). Bluetongue. In: *Manual of standards for Diagnostic Test and Vaccine for Terrestrial Animals*, 6th ed., Chapter 2.1.3, Paris: OIE, pp. 158-174.

120. Orru, G., Santis, P.D., Solinas, F., Savini, G., Piras, V. and Carporale, V. (2004). Differentiation of Italian field and South Africa vaccine strains of bluetongue virus serotype 2 using real-time PCR. *Journal of Virological Methods*, 122(1), 37-43.

121. Osborne, C. J., Mayo, C. E., Mullens, B. A., McDermott, E. G., Gerry, A. C., Reisen, W. K., & MacLachlan, N. J. (2015). Lack of evidence for laboratory and natural vertical transmission of Bluetongue Virus in *Culicoides sonorensis* (Diptera: Ceratopogonidae). *Journal of Medical Entomology,* 52(2), 274-277.

122. Parsonson, I.M., Della-Porta, A.J., McPhee, D.A., Cybinski, D.H., Squire, K.R.E. and Uren, M.F. (1987a). Experimental infection of bulls and cows with bluetongue virus serotype 20. *Australian Veterinary Journal,* 64(1), 10-13.

123. Parsonson, I.M., Della-Porta, A.J., McPhee, D.A., Cybinski, D.H., Squire, K.R.E. and Uren, M.F. (1987b). Bluetongue virus serotype 20; Experimental infection of pregnant heifers. *Australian Veterinary Journal*, 64(1), 14-17.

124. Paweska, J.T., Venter, G.J. and Mellor, P.S. (2002). Vector competence of South African *Culicoides* species for bluetongue virus serotype 1 (BTV-1) with special reference to effect of temperature on the rate of virus replication in *C. imicola* and *C. bolitinos*. *Medical and Veterinary Entomology*, 16(1), 10-21.

125. Ram, J.L., Karim, A.S., Sendler, E.D. and Kato, I. (2011). Strategy for microbiome analysis using 16S rRNA sequence analysis on the Illumina sequence platform. *Systems Biology in Reproductive Medicine*, 57(3), 117-118.

126. Ramadevi, N., Burroughs, N.J., Mertens, P.P., Jones, I.M. and Roy, P. (1998). Capping and methylation of mRNA by purified recombinant VP 4 protein of bluetongue virus. *Proceedings of the National Academy of Sciences* USA 95(23), 13537-13542.

127. Ratinier, M., Caporale, M., Golder, M., Franzoni, G. Allan, K. and Nunes, S.F., Armezzani, A., Bayourny, Rixon, F., Shaw, A. and Palmarini, M. (2011). Identification and characterization of a novel non-structural protein of bluetongue virus. *PLoS Pathogenesis* 7(12):e1002477.

128. Richards, R.G., MacLachlan, N.J., Heidner, H.W. and Fuller, F.J. (1988). Comparison of virological and serological responses of lambs and calves infected with bluetongue virus serotype 10. *Veterinary Microbiology*, 18(3), 233-242.

129. Riss, T. and Moravec, R. (2003). Simplifying cytotoxicity screening; Introducing the CellTiter-Blue Cell Viability Assay. *Promega Corporation*, 83, 10-13.

130. Ronaghi, M. (2001). Pyrosequencing sheds light on DNA sequencing. *Genome Research,* 11,3-11.

131. Roy, P. (1989). Bluetongue virus genetics and genome structure. *Virus Research*, 13(3), 179-206.

132. Russell, H., O'Tool, D.T., Bardsley, K., Davis, W.C. and Ellis, J.A. (1996). Comparative effects of bluetongue virus infection of ovine and bovine endothelial cells. *Veterinary Pathology Online*, 33(3), 319-331.

133. Rodriguez-Sanchez, B., Iglesias-Martin, I., Martinez-Aviles, M. and Sanchez-Vizcaino, J.M. (2008). Orbiviruses in the Mediterranean basin: updated epidemiological situation of Bluetongue and new methods for the detection of BTV serotype 4. *Transboundary and Emerging diseases*, 55(5-6), 205-214.

134. Saegerman, C., Hubaux, M., Urbain, B., Lengele, L. and Berkvens, D. (2007). Regulatory issues surrounding the temporary authorization of animal vaccination in emerging situations: the example of bluetongue in Europe. *Revue scientifique et technique-Office International des Epizooties*, 26(2), 395.

135. Sanger, F., Nicklen, S., & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*, 74(12), 5463-5467.

136. Savini, G., MacLachlan, N.J., Sanchez-Viscaino, J.M. and Zientara, S. (2008). Vaccines against bluetongue in Europe. *Comparative Immunology, Microbiology Infectious Diseases*, 31(2), 101-120.

137. Schouls, L.M., Spalburg, E.C., van Luit, M., Huijsdens, X.W., Pluister, G.N., van Santen-Verheuvel, M.G., Van der Heide, H.G.J., Gundmann, H., Heck, M.E.O.C. and de Neeling, A.J. (2009). Multiple-locus variable number tandem repeat analysis of Staphylococcus aureus: Comparison with Pulsed-Field Gel Electrophoresis and spa-Typing. *PLoS ONE*, 4(4), e5082.

138. Schultz, G. & DeLay, P.D. (1955). Losses in lambs associated with Bluetongue vaccination of pregnant ewes. *Journal of the American Veterinary Medical Association,* 127(942), 224-226.

139. Sellers, R.F. and Taylor, W.P. (1980). Epidemiology of bluetongue and the import and export of livestock, semen and embryos. *Bulletin de l'Office International des Epizooties*, 92, 587-592.

140. Shafiq, M., Minakshi, P., Bhateja, A., Ranjan, K. and Prasad, G. (2013). Evidence of genetic reassortment between Indian isolate of bluetongue virus serotype 21 (BTV-21) and bluetongue virus serotype 16 (BTV -16). *Virus Research*, 173, 336 -343.

141. Shaw, A.E., Monaghan, P., Alpar, H.O., Anthony, S., Darpel, K.E., Batten, C.A., Guercio, A., Alimena, G., Vitale, M., Bankowska, K., Carpenter, S., Jones, H., Oura, C.A., King, D.P., Elliott, H., Mellor, P.S and Mertens, P.P.C. (2007). Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *Journal of Virological Methods*, 145(2), 115-126.

142. Shaw, A.E., Ratinier, M., Nunes. S.F., Nomikou, K., Carporale., M., Goldner, M., Allan, K., Hamers, C., Hudelet, P., Zientara, S., Breard, E., Mertens. P. and Palmarini, M. (2013). Reassortment between two serologically unrelated bluetongue virus strains is flexible and can involve any genome segments. *Journal of Virology*, 87(1), 543-557.

143. Sharifah, S.H., Ali, M.A., Gard, G.P and Polkinghorne, I.G. (1995). Isolation of multiple serotypes of bluetongue virus from sentinel livestock in Malaysia. Tropical Animal Health and production, 27(1), 37-42.

144. Shelton, H., Smith, M., Hartgroves, L., Stilwell, P, Roberts, K., Johnson, B. and Barclay, W. (2012). An influenza reassortant wit polymerase of pH1N1 and NS gene of H3N2 influenza A virus attenuated *in vivo. Journal of General Virology*, 93(Pt 5), 998-1006.

145. Shimshony, A. (2004). Bluetongue in Isreal-a brief historical overview. *Veterinia Italiana*, 40, 116-118.

146. Singer, R.S., MacLachlan, N.J. and Carpenter, T.E. (2001). Maximal predicted duration of viraemia in bluetongue virus – infected cattle. *Journal of Veterinary Diagnostic Investigation,* 13(1), 43-49.

147. Spreull, J. (1905). Malarial catarrhal fever (bluetongue) of sheep in South Africa. *Journal of Comparative Pathology and Therapeutics*, 18, 321-337.

148. Stauber, N., Martinez-Costas, J., Sutton, G., Monastyrskaya, K. and Roy, P. (1997). Bluetongue virus VP6 protein binds ATP and exhibits an RNA-dependent ATPase function and a helicase activity that catalyze the unwinding of double-stranded RNA substrates. *Journal of Virology*, 71(10), 7220-7226.

149. Standfast, H.A., Dyce, A.L. and Muller, M.J., (1985). Vectors of bluetongue virus in Australia. *Clinical and Biological Research*, 178, 177-186.

150. St George, T.D., Peng, K. Eds. (1996). Bluetongue disease in Southeast Asia and the Pacific. Canberra: ACIAR, pp 264.

151. Stott, J.L., Osburn, B.I. and Barber, T.L. (1982). Recovery of dual serotypes of bluetongue virus from infected sheep and cattle. *Veterinary Microbiology*, 7(3), 197-207.

152. Stott, J.L., Oberst, R.D., Channell, M.B. and Osburn, B.I. (1987). Genome segment reassortment between two serotype of bluetongue virus in a natural host. *Journal of Virology*, 61(9), 2670-2674.

153. Sundin, D. R., Dean, V. C., DuBard, K. M., & Mecham, J. O. (1989). In vitro neutralization of antigenic variants of bluetongue virus is related to in vivo protection. *Viral immunology*, 2(3), 195-203.

154. Tabachnick, W.J. (2003). *Culicoides* and the global epidemiology of the bluetongue virus infection. *Veterinia Italiana*, 40(3), 145 – 150.

155. Tabachnick, W.J. (2010). Challenges in predicting climate and environmental effects on vector-borne disease episystems in a changing world. *The Journal of Experimental Biology*, 213(6), 946-954.

156. Takamatsu, H., Mellor, P.S., Mertens, P.P.C., Kirkham, P.A., Burroughs, J.H. and Parkhouse, R.M.E. (2003). A Possible overwintering mechanism for bluetongue virus in the absence of the vector. *Journal of General Virology*, 84(1), 227-235.

157. Theiler, A. (1908). The inoculation of sheep against bluetongue and the result in practice. *Veterinary Journal*, 64, 600-607.

158. Toussaint, J. F., Sailleau, C., Breard, E., Zientara, S. and De Clerq, K. (2007). Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *Journal of Virological Methods*, 140(1), 115-123.

159. Van Rijn, P.A., Heutink, R.G., Boonstra, J., Kramps, H.A. and Van Gennip, R.G.P. (2012). Sustained high-throughput polymerase chain reaction diagnostics during the European epidemic of Bluetongue virus serotype 8. *Journal of Veterinary Diagnostic Investigations*, 24(3), 469-478.

160. Vauterin, L. and Vauterin, P. (2006). Integrated databasing and analysis. In*: Molecular Identification, Systematics, and Population Structure of Prokaryotes* (pp. 141-217) Springer Berlin Heidelberg, ISBN 978-3-540-23155-4

161. Venter, G.J., Groenewald, D., Venter, E., Hermanides, K.G. and Howell, P.G. (2002). A comparison of the vector competence of the biting midges, *Culicoides (Avarita) bolitinos* and *C. (A.) imicola*, for the Bryanstom serotype of equine encephalosis virus. *Medical and Veterinary Entomology*, 16(4), 372- 377.

162. Venter, G. J., Paweska, J.T., van Dijk, A.A., Mellor, P.S. and Tabachnick W.J. (1998). Vector competence of *C. bolitinos* and *C. imicola* for South African bluetongue virus serotypes 1, 3 and 4. *Medical and Veterinary Entomology*, 12:378-385.

163. Venter. G.J., Groenewald, D,. Venter, E., Hermanides, K.G. and Howell, P.G. (2002). A comparison of the vector competence of the biting midges, *Culicoides (Avaritia) bolitinos* and *C. (A) imicola* for the Bryanston serotype of equine encephalosis virus. *Medical and Veterinary Entomology,* 16(4), 372-377)

164. Veronesi, E., Darpel, K. E., Hamblin, C., Carpenter, S., Takamatsu, H. H., Anthony, S. J., Elliott. H., Mertens, P.P.S. and Mellor, P. S. (2010). Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4. *Vaccine*, 28(5), 1397-1403.

165. Veronesi, E., Hamblin, C., & Mellor, P. S. (2005). Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). *Vaccine*, *23*(48), 5509-5516.

166. Verwoerd, D.W., Louw, H. and Oellermann, R.A. (1970). Characterization of Bluetongue virus ribonucleic acid. *Journal of Virology*, 5(1), 1-7.

167. Verwoerd, D. W. and Erasmus, B.J. (2004). Bluetongue, In: *Coetzer J.A.W.* and *Tustin*, *R.C.* (*Eds*.), *Infectious Diseases of Livestock. 2nd edition*. Cape Town, Southern Africa: Oxford University Press; pp. 1201-1220.

168. Vosdingh, R.A., Trainer, D.O. and Easterday, B.C. (1968). Experimental bluetongue disease in white-tailed deer. *Canadian Journal Comparative Medical Veterinary Science* 32(1), 382-387.

169. Walker, A.R. and Boreham P.F.L. (1976). Blood feeding of *Cullicoides* (Diptera, Ceratopoginidae) in Kenya in relation to the epidemiology of bluetongue and ephemeral fever. *Bulletin of Entomology Research*, 66(1), 181-188.

170. Walton, T.E. (2004). The history of bluetongue and a current global overview. *Veterinaria Italiana*, 40(3), 31-38.

171. Watanabe, M., Nakagomi, T., Koshimura, Y. and Nakagmi, O. (2001). Direct evidence for genomic reassortment between concurrent circulating human rotavirus strains. *Archive of Virlology*, 146(3), 557-570.

172. Wechler, S.J, and McHolland, L.E. (1988). Susceptibility of 14 cell lines to bluetongue virus infection. *Journal of Clinical Microbiology*, 26(11), 2324-2327.

173. Wilson, W.C., Ma, H.C., Venter, E.H., Van Dijk, A.A. Seal, B.S. and Mecham, J.O. (2000). Phylogenetic relationships of bluetongue viruses based on. *Virus Research*, 67(2), 242-251.

174. Wilson, A.J., Carpenter, S., Gloster, J. and Mellor, P.S. (2007). Re-emergence of bluetongue in northern Europe in 2007. *Veterinary Record*, 161, 287-489.

175. Wilson, A.J., Darpel, K. and Mellor, P.S. (2008). Where does bluetongue virus sleep in the winter? *PLoS Biology, 6, 6-11.*

176. Wouda, W., Peperkamp, N.H., Roumen, M.P., Muskens, J., Van Rijn, A and Vellema, P. (2009). Epizootic congenital hydranencephaly and abortion in cattle due to bluetongue virus serotype 8 in the Netherlands. *Tijdschr Diergeneeskd,* 134(10), 422-427.

177. Xu G., Wilson W., Mecham J., Murphy K., Zhou E.M. and Tabachnick W. (1997). VP7: an attachment protein of bluetongue virus for cellular receptors in *Culicoides variipennis*. *Journal of General Virology, 78(7), 1617–1623*.

178. Young, S. and Cordy, D.R. (1964). An ovine fetal encephalopathy caused by bluetongue vaccine virus. *Journal of Neuropathology & Experimental Neurology*, 23(4), 635-659.

179. Yunlong, B., ChunDi, L., ShiYin, L., Bo, Q., Nan, Z., JinFeng, H S., Yang RuiLan. T.D., St George, and KeGao, P. (1996). An epidemiological survey of bluetongue disease in Yunnan Province, China. In *Bluetongue disease in Southeast Asia and the Pacific: Proceedings of the First Southeast Asia and Pacific Regional Bluetongue Symposium, Greenlake Hotel, Kunming, PR China, 22-24 August 1995* (pp. 51-56) Autralian Center for International Agricultural Reasearch (ACIAR, pp.264).

180. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999). A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *Journal of Biomolecular Screening*, 4(2), 67-73.

181. Zhu, J., Yang, H., Li, H., Xiao, L., Wang, J., Li, N., and Zhang, N. (2013). Full-genome sequence of bluetongue virus serotypes 1 (BTV-1) strain Y863, the first BTV-1 isolate of Eastern origin found in China. *Genome announcements*, 1(4), e00403-13.

182. Zientara, S., de La Rocque, S., Gourreau, J.M., Gregory, M., Diallo, A., Hendrikx, P., Libeau, G., Sailleau, C. and Delecolle, J.C. (2000). La fievre catarhale ovine and Corse 2000. *Epidemiologie et Sante Animale*, 38, 133-144.

CHAPTER 2

Materials and Methods

2.1 Experimental design

Six cattle were divided into two groups; one group was inoculated with the OBP BTV vaccine Bottle B while the other group was inoculated with the vaccine and a wild type BTV-4 serotype. Blood samples were collected for 39 days post inoculation and individual viruses were isolated from infected buffy coat using plaque purification. Isolated plaques were passaged once in cell culture, dsRNA extracted and electrophoretic profiles generated using PAGE. In order to identify possible reassortant strains, electrophoretic profiles of isolated plaques and parental viruses were compared as regards to mobility shifts by analyzing the PAGE gels with BioNumeric software. Full genome sequencing was done on selected viruses using MiSeq sequencing (Illumina) and reassortant and parental viruses phenotypically characterized using cell viability and cytotoxicity assays.

2.2 Animals

Six cattle free from antibodies specific to BTV were used in the trial and were purchased from a local buyer in Randfontein (GPS coordinates S26’14.757 E027’34.197). The cattle were first tested using a commercial available cELISA (Veterinary Medical Research and diagnostics) and RT-qPCR (Thermo Scientific). The cattle were transported to the Faculty of Veterinary Science and were housed in insect-free stables at the University of Pretoria’s Biologicall Research Centre (UPBRC) for the period of the trial

The animals were housed for a period of two weeks to adjust to new food, climate and surroundings before the trial started. The animal’s diet consisted of Eragrostis tef, Alfalfa (Lucerne hay) and a maintenance meal provided by the UPBRC. Animals were fed twice daily according to the animal’s daily maintenance requirements.

2.3 BTV vaccine serotypes

Viruses from Bottle B of the modified-live (attenuated) BTV vaccine currently used in SA were used to inoculate the cattle. Bottle B vaccine is produced by OBP and includes serotypes 3, 8, 9, 10 and 11. The vaccine was bought at a local supplier, Obaro in Pretoria.

2.3.1 Isolation of parental virus from OBP Bottle B vaccine

The BTV vaccine was resuspended in 1 ml reconstituted Dulbecco's minimal essential medium (MEM) containing 10 v/v tryptose phosphate broth, 5% v/v foetal bovine serum and 1 ml Gentamycing (50 mg/ml)) and then further diluted with 10 ml MEM (1:10 dilution). The vaccine was further diluted to approximately 100 dosages according to the manufacturer's instructions. In order to prepare viral plaques, a volume of 100 µl of diluted vaccine was added to 2.9 ml of reconstituted MEM and the diluted solution inoculated onto Vero cells in 6-well plates (Fisher Scientific). The plates were incubated for an hour for the virus to adsorb and penetrate the cells and excess vaccine dilution then aspirated. To allow plaque formation (as described in Section 2.7.2) a 1% agar (Highveld biological) layer stained with 1% neutral red (Sigma) was placed onto the cells. The plates were incubated at 37°C in an incubator containing 5% CO_2 and monitored daily for 7 days to allow the formation of plaques. Individual plagues were picked and amplified in 25 cm^2 flasks as described in Section 2.7.3.

2.3.2. Plaque neutralization assay and RT-PCR to identify viral serotype

In order to determine the specific BTV serotype of the isolated plaques, amplified viruses from plaques were diluted 1:100 and 1:1000 in MEM and 100 µl diluted virus per well was inoculated onto confluent Vero cells in 6-well plates. The plates were incubated for 1 hour at 37°C before the inoculated virus was removed and an agar overlay placed on top of the cells (as described in Section 2.7.2). Neutralization beads dipped in the antiserum of the parental viruses of interest i.e. 3, 8, 9, 10 and 11, were placed on top of the agar and the plate incubated for 7 days. The antiserum neutralizes the corresponding virus serotype, preventing plaque formation and facilitating the appearance of inhibition zones. It was however not possible to identify serotype 8 with the plaque neutralization assay method, and serotype 8 had to be identified by PCR using a *One Taq* RT PCR kit

(BioLabs) (Section 2.6.2). Bluetongue virus 8 segment 2 serotype-specific primers were designed to identify the BTV parental virus. The forward primer (5'GTAGGACGAAGCCAGGATTGACGCG3') with an annealing temperature of 69°C and the reverse primer (5'GATAGAATRCGTTCCCGCCT3') with an annealing temperature of 62°C were designed by Mertens *et al*, (2007). The PCR products were visualized by electrophoresis using a 1% agar gel (Sigma). The gel was run at 120V for 30 minutes and was then visualized with Chemdox XRS (Bio-Rad).

Plaques from the parental vaccine serotypes were then FLAC amplified (Section 2.7.3) and submitted to a commercial company (Inqaba Biotec) for sequencing (Section 2.8) and sequences obtained were submitted to GenBank.

2.4 BTV-4 field isolate

The BTV serotype 4 was isolated in 2011 from EDTA blood that was taken from a clinically affected Merino Dohne ewe on a farm in Rustenburg. The serotype was identified using a plaque neutralization assay as described in Section 2.3.2, and was further confirmed using FLAC amplification and Miseq sequencing (Section 2.7.3). In order to titrate the virus for animal inoculation, a series of 8 ten-fold dilutions of the virus was made. A 100 ul of each viral dilution was added to 96-wells in each row**.** A negative control was added to the last well in each row. The plate was incubated at 37°C in an incubator containing 5% CO_2 and observed daily for seven days for the development of cytopathic effect (CPE). The method of Kärber (Kärber, 1931) was used to determine the titre.

Calculation of $TCID_{50}$ according to Kärber formula:

$$= d + \frac{Wd+Wa}{N} - 0.5$$

$$=4 + \frac{Wd+Wa}{N} - 0.5$$

$$= 4 + \frac{6+2}{6} - 0.5$$

$$= 4 + 1.33 - 0.5$$

$$= 4.8/100ul$$

$$= 10^{5.8} / Ml$$

d = highest dilution in which all replicate wells shows CPE

Wd = Number of wells that shows CPE in d dilution

Wa = Number of wells at a dilutions higher than d

N = Number of wells inoculated used per dilutions

2.5. Animal trial

The animals were separated at random into two groups as illustrated in Table 2.1.Three animals were vaccinated with only the OBP BTV Bottle B vaccine (Group 1). The vaccine was diluted with sterile water as described by the manufacturer. The vaccine can be used to vaccinate up to 100 animals, however each of the bovines was only given one dose. The other three animals were simultaneously vaccinated with the OBP vaccine Bottle B as well as the wild-type BTV-4 isolate. A volume of 1 ml of the BTV-4 with a $TCID_{50}$ of $10^{5.8}$/ml was administered intravenously (IV) in the jugular vein, to each of the selected animals

Table 2.1: The selected groups of cattle and the inoculation regime

Group 1	Group 2
Bovine injected with vaccine	Bovines injected with vaccine and BTV-4
Animal 2 (Brahman)	Animal 1 (Bonsmara)
Animal 3 (Bonsmara)	Animal 4 (Bonsmara)
Animal 6 (Charolais)	Animal 5 (Bonsmara)

2.6 Sample collection

2.6.1. Before infection

Serum samples (n= 908 samples across SA) collected from animals from different farms were tested for the presence of group-specific antibodies to BTV using a competitive enzyme-linked immuno-absorbent assay (cELISA) (Veterinary Medical Research and Diagnostics (VMRD), Bluetongue Virus Antibody Test Kit, Inc., Pullman, U.S.A). Negative and positive controls were included in the kit and used according to manufacturer's instructions. A volume of 25 µl of the positive control (3 replicates), 25 µl of the negative control (3 replicates) and the serum samples to be tested was pipetted into an antigen coated plate. After 15 minutes incubation, 25 µl of the Antibody–Peroxidase conjugate was added to each well. After 15 minutes incubation the plate was washed 3 times using an automatic washer and a 1x wash solution provided in the kit. Directly after washing the plate, 50 µl Substrate Solution was added to each well and the plate then incubated for ten minutes. The reaction was stopped by adding 50 µl of stop solution (VMRD) to each well. The plate was read on a microplate absorbance spectrophotometer with the optical density (OD) reading set to a wavelength of 630 nm. Percentage negativity values were calculated by using the following formula: [1-(OD samples/OD negative reference)] x 100 and all the animals with a value of >50% were excluded from the trial.

2.6.2. RT-PCR

In order to confirm that animals were not recently/currently infected with BTV, a real-time RT-PCR was conducted on RNA that was extracted from EDTA blood samples by using a two-step procedure (Toussaint *et al*., 2007) targeting genome segment 5 of the BTV genome (encodes non-structural protein 1, NS1).

RNA extraction:

In order to conduct RNA extraction, 100 µl whole blood samples and a 1000 µl Trizol reagent (Qiagen) with 200 µl chloroform added simultaneously. The tubes were centrifuged at 14000 rpm in a MiniSpin plus centrifuge (Merck Millipore) for 10 minutes. The aqueous phase was transferred to a new Eppendorf tube and the RNA precipitated with 500 µl isopropanol. The tubes were stored at 4°C for 24 hours to allow RNA precipitation to occur, and then centrifuged at 14000 rpm in an Eppendorf centrifuge 5417R (Merck Millipore) for 40 minutes at 4°C.

The supernatant was removed and 90 µl of Elution buffer (Qiagen) added to tubes together with 30 µl 8 M lithium chloride (Sigma). The tubes were stored at 4°C for 24 hours to precipitate single stranded RNA. The tubes were then centrifuged at 14000 rpm in an Eppendorf centrifuge 5417R (Merck Millipore) for 40 minutes at 4°C and the aqueous phase was then transferred into a new Eppendorf tube with binding buffer (Qiagen). The mixture was transferred into MiniElute Spin columns (Qiagen) and centrifuged at 14000 rpm for 60 seconds in a Minispin centrifuge (Merck Millipore). The columns were washed with a PE buffer with 70% ethanol (Qiagen) and centrifuged again for 60 seconds. The columns were then transferred into new Eppendorf tubes and 22 µl Elution buffer were added to the columns. The columns with the new Eppendorf tubes were centrifuged for 1 minute and viral dsRNA eluted and stored at -20°C until used.

RT-PCR

A real-time RT-PCR targeting genome segment 5 (NS1 encoding gene) was used to test for the presence of BTV-specific RNA in the blood samples of the animals (Toussaint *et al*., 2007). The RNA was denatured with 10% DMSO (v/v) at 95°C for 3 minutes and snap cooled on ice. Complementary DNA synthesis was conducted using 2 µl high capacity RNA to cDNA master mix (Applied Biosystems) and 1.25 µM random hexamers (25°C for 5 minutes, 42°C for 30 minutes and 95°C for 5 minutes). The real-time PCR was done using a Taqman Universal Master Mix II (Applied Biosystems) with 10 pmol of each of the primers BTVS F1-19 and BTV S5 R76-57, 2.5 pmol TaqMan probe (BTV S5 P49-27) and 5 µl cDNA (20 µl in total). Cycling condition were 95°C for 10 minutes followed by 50 cycles at 95°C for 15 seconds and 58°C for 1 minute. Ribonucleic acid extracted from BTV serotype 4 was used as positive control and nuclease free water was included as a negative control. Samples were considered BTV positive if the cycle threshold value was <40.

2.7. Post Infection

2.7.1. Serological analysis

Serum samples were collected from the cattle from Day 1 to Day 39 post infection. The serum tubes were centrifuged at 3500 rpm for 5 minutes in a Rotafix 32 centrifuge (Hettich) to separate serum and blood cells. The serum was stored in cryo tubes (Lasec (Pty)) at -20°C until used. Group-specific antibodies to BTV were measured at days 13, 15, 17, 19 and 21 post inoculations using a cELISA (VMRD) according to the manufactures instruction as described in Section 2.6.1

2.7.2. Plaque purification of virus from infected blood

Heparin samples were collected from the cattle from Day 1 to Day 39 post infection. Immediately after collection the samples were centrifuged for 5 minutes at 3500 rpm in a Rotafix 32 centrifuge. The buffy coats were collected and transferred into Dulbecco phosphate-buffered saline (PBS+) solution and stored at -20°C until used.

Buffy coats (300 µl) from each sample was inoculated onto Vero cell monolayers in 25 cm^2 flasks (Corning), containing reconstituted MEM supplemented with 5% foetal calf serum (Highveld Biological). The flasks were incubated for 7 days at 37°C. The flasks were then frozen at -20°C for one hour and thawed to release virus particles. A volume of 300 µl of the cell cultured sample was then inoculated onto Vero cell monolayers in 6-well plates (Fisher Scientific). The plates were incubated for 1 hour at 37°C in an incubator in an atmosphere containing 5% CO_2, to allow for viral-cell adsorption and penetration. The monolayer was then washed twice with PBS+ and subsequently overlaid with agar (Sigma) stained with 1% neutral red (Sigma) and monitored for the formation of plaques for 7 days while incubated at 37°C in an atmosphere containing 5% CO_2. Visible plaques were picked and resuspended in 1000 µl PBS. A volume of 500 µl of the suspension was inoculated onto Vero cell monolayers in 25 cm^2 flasks in 10 ml MEM containing 2% foetal calf serum (Highveld Biological) and Gentamycin (50 mg/ml). When 100% CPE was evident at 5 days post infection the cellular debris was pelleted.

Double stranded RNA (dsRNA) was extracted from cell cultures and analyzed using PAGE analysis. The cell culture pellet and 100 µl were added to 1000 µl Trizol reagent (Qiagen) and 200 µl chloroform. The tubes were centrifuged at 14000 rpm in a MiniSpin plus centrifuge (Merck Millipore) for 10 minutes. The aqueous phase was transferred to a new Eppendorf tube and 500 µl isopropanol added. The tubes were then stored at 4°C for 24 hours to allow RNA precipitation, and then RNA was concentrated by centrifugation at 14000 rpm in an Eppendorf centrifuge 5417R (Merck Millipore) for 40 minutes at 4°C.

The supernatant was removed and 90 µl of Elution buffer (Qiagen) added to the total extracted RNA together with 30 µl 8 M lithium chloride (Sigma). The tubes were stored at 4°C for 24 hours to precipitate single stranded RNA. The tubes were then centrifuged at 14000 rpm in an Eppendorf centrifuge 5417R (Merck Millipore) for 40 minutes at 4°C and the aqueous phase was then transferred into a new Eppendorf tube with binding buffer (Qiagen). The mixture was transferred into MiniElute Spin columns (Qiagen) and centrifuged at 14000 rpm for 60 seconds in a Minispin centrifuge (Merck Millipore). The columns were washed with a PE buffer with 70% ethanol (Qiagen) and centrifuged again for 60 seconds. The columns were then transferred into new Eppendorf tubes

and 22 µl Elution buffer were added to the columns. The columns with the new Eppendorf tubes were centrifuged for 1 minute and viral dsRNA eluted and stored at -20°C until used.

2.7.3. Full length amplification of cDNAs

Full length amplification (FLAC) is a technique used to obtain the full length sequence of an RNA transcript found within a cell. This results in the production of a cDNA copy of the RNA sequence of interest, produced through reverse transcription, followed by PCR amplification of the cDNA copies. The amplified cDNA copies are then sequenced and used to map to a unique genomic region. s. FLAC amplification of the BTV genome is a prerequisite for NGS sequencing and was previously described by Potgieter, (2009). Next generation sequencing was done on the parental viruses and the viruses isolated from infected cattle so that one could compare the genomic sequences of the individual genome segments to confirm reassortment.

RNA extraction

Double stranded RNA was extracted from samples with the extraction method as explained in Section 2.6.2.

Ligation method

In order to ligate the double stranded RNA,v12 µl was mixed with 3 µl of ligation buffer (Potgieter, 2009), 3 µl DMSO (Sigma), 1 µl primer PC3-T7 loop, 10 µl 60% Poly-ethylene glycol 6000 (Sigma) and 1 µl T4 RNA Ligase (TAKARA). The samples were incubated overnight at 37°C. The ligation product was cleaned by adding 70 µl RNA free water, 300 µl QG buffer (Qiagen) and 100 µl isopropanol. The solution was transferred to a MiniELute (Qiagen) spin column and centrifuged for one minute. The samples were washed with 750 µl Wash Buffer (Qiagen) and centrifuged for one minute to collect the flow through. After an additional centrifugation step to remove residual ethanol, the ligated double stranded RNA was eluded into 12µl EB buffer (Qiagen).

cDNA synthesis

Purified ligated dsRNA (5 µl) was denatured through the addition of 1 µl 300 mM methyl mercury hydroxide (MMOH, Alfa Aeser) at 37°C for 30 minutes, then 1 µl β-mercapto-ethanol (Sigma) was added to reduce the MMOH. The cDNA was reverse transcribed in a cDNA reaction containing 50 mM Tris/HCL, pH 8.3 (Sigma), 10mM $MgCl_2$, (Seperations), 70 mM KCl (Sigma), 30 mM β-mercapto-ethanol (Sigma), 1 mM dNTPs (TaKaRa) and 15 U cloned AMV reverse transcriptase (Invitrogen). The reverse transcription reactions were incubated in a thermal cycler at 42°C for 45 minutes followed by 55°C for 15 minutes. After cDNA synthesis RNA was degraded by adding NaOH (Sigma) to a final concentration of 0.1 mM followed by incubation in a thermal cycler at 65°C for 30 minutes. The reaction was finally buffered with Tris, pH 7.5 (Sigma), to a final concentration of 0.1 M. Sodium hydroxide were buffered by adding 3 µl 1 N Hydrochloric acid. Individual cDNA strands were annealed into a duplex by incubating the mixture at 65°C overnight.

PCR Amplification

Amplification of cDNA was performed using a previously published primer (PC2) (Potgieter *et al*., 2009). The primer PCR 3-T7 loop folds back on itself (Figure 2.1) and is phosphorylated on the free end, thereby allowing the primer to self-anneal and prime first strand synthesis. The PCR mixture contained 1 x Ex Taq buffer, 2,5 mM dNTP's (TaKaRa), 5 µl cDNA and 2.5 mM TaKaRa Ex Taq and of 1 µl PC2 (25 pMol/µl). The reactions were incubated at 72°C for 4 minute to fill incomplete cDNA ends. This was followed by an initial denaturation step of 94°C for 2 minutes followed by 35 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 was included. The PCR products (all 10 genome segments) were analyzed by electrophoresis using a 1% agarose gel (Cleaver Scientific Pty) and visualized under UV light after staining the gel with 5 µl ethidium bromide (stock 10mg/mL) (Invitrogen).

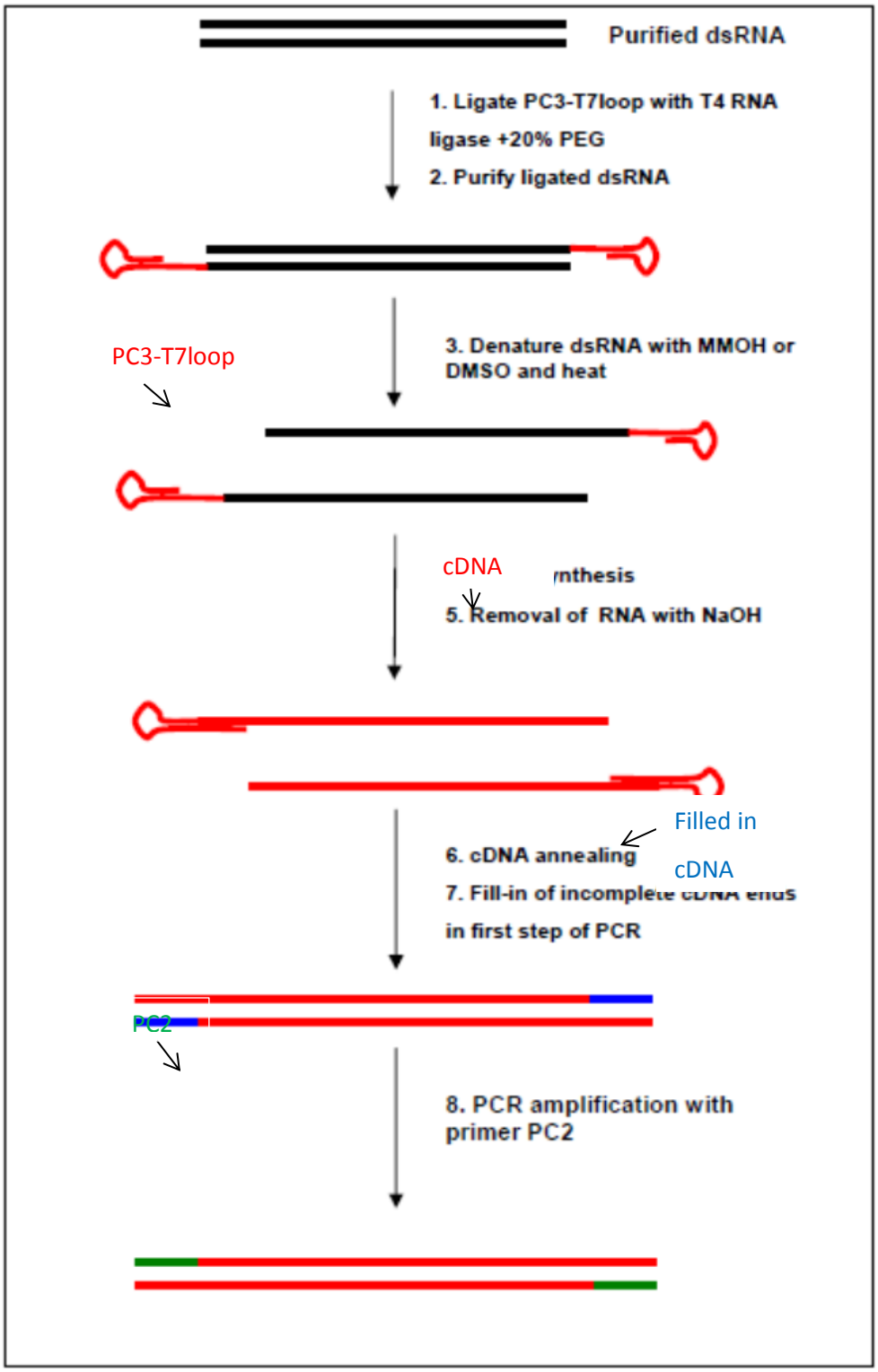


Figure 2.1: Schematic representation of the protocol for FLAC of dsRNA genome (Potgieter *et al.*, 2009).

2.8 Genome sequencing

Prior to sequencing, the amplified genomes (Section 2.7.3) were purified from the PCR reactions using a QIAquick PCR purification kit (Qiagen).Whole genome sequencing of suspected reassortant viral strains and the parental viruses was done using MiSec sequencing (Illumina). For virus samples purified PCR products of the 10 genomic segments of each virus were tagged and pooled (fragmented and indexed) using Illumina's Next era XT library preparation kit, followed by size selection using Ampure XP magnetic beads. Equa-molar pooled libraries were then sequenced using an Illumina MiSeq v3 kit (600 cycles) on an Illumina MiSeq platform at Inqaba Biotec (commercial service provider, Pretoria, South Africa, www.inqababiotec.co.za).

Fastq files (Format) containing the sequence data and quality values were imported into the CLC genomics software (V5.5, http://www.clcbio.com/) and quality trimmed using default settings. Sequence tags (barcodes) were also removed at this stage. Sequence reads were assembled with the CLC software by either mapping reads to reference sequences of BTV that were downloaded from the National Centre for Biotechnology (NCBI, http://www.ncbi.nlm.nih.gov/) web-site or assembled using shotgun assembly (de novo) as appropriate, using default parameters. Consensus and/or contig sequences (>500 nt in length) were subjected to a multi-nucleotide BLAST (Basic Local Alignment Search Tool, NCBI), in order to determine the sequence identities.

The BTV serotype 11 sequence had small gaps in segment 1 while BTV serotype 9 had small gaps in 2 of the segments (i.e. segment 2 and 6). Gaps in the sequences were filled by means of RT-PCR with primers that were designed to flank regions that were incomplete (Table 2.2). Double stranded RNA was prepared as described in Section 2.6.2 and a One *Taq*RT-PCR kit (BioLabs) was used.

The dsRNA were denatured at 70°C for 5 minutes with random primers that were included in the One *Taq*RT-PCR kit (BioLabs) and snapped cooled on ice. The cDNA synthesis reaction was prepared by adding 10 µl M-MuLV mix and 2 µl M-MuLV enzyme (both enzymes are included in the kit), and then incubated at 25°C for 5 minutes followed by 42°C for one hour. The RT-enzyme was inactivated by heating at 80°C for 4 minutes. The cDNA was diluted to 50 µl by adding 30 µl PCR quality water.

The PCR samples were prepared by mixing 12.5 µl One *Taq* Hot start 2X master mix, 1µl forward and reverse primers (10 pmol/µl), 5 µl cDNA and 5 µl nuclease free water. The total PCR volume was

25 µl per sample. The PCR parameters were: initial denaturation at 95°C for 30 seconds, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 68°C for 2 minutes. The final extension was at 68°C for 5 minutes and the amplicons were held at 4°C until samples were placed on ice. The amplicons were analyzed using agarose gel electrophoresis and sent for cloning and sequencing by Inqaba Biotec. Sequencing of cloned amplicons were done using plasmid specific forward and reverse primers, the final chromatograms manually edited as appropriate, and assembled together with Miseq data as previously described.

Table 2.2: Primers designed to fill the gaps in sequence data of the BTV parental sequences

BTV-9 segment 2		Nucleotide positions
Forward primer	5' TAA GCA TGG AAA CCCC GGA3'	720 -739
Reverse primer	5' ATA TCC ACT TTC CCA TGA CG3'	1275 -1292
BTV 9 segment 2		
Forward primer	5'ATC CAG CAA TAA AGC GAA CG3'	1696 – 1715
Reverse Primer	5'CGA CCT GAA ACT GAT ACT TA 3'	2739 – 2759
BTV 9 segment 6		
Forward primer	5'CGA GTT GGT TCG TCT TAA AT3'	349 – 368
Reverse primer	5'GAG CGT CCC AAA TAA ATG3'	1363 – 1383
BTV 11 segment 1		
Forward primer	5' GGC TAA GGG ATA AAG ATT	1144 – 1163

	TG3'	
Reverse primer	5' GGG TCA TCT TCG TTC TTC TC3'	2264 – 2284

2.9 Characterization of viral plaques

The electrophoretic migration profiles of viral isolates obtained from cattle used in this study were compared to viral isolates from the vaccine bottle and the BTV-4 isolate. Genetic profile comparisons were firstly done by means of PAGE and then confirmed using phylogenetic analysis.

2.9.1 Polyacrylamide gel electrophoresis

Double stranded RNA extractions were done on every sample including the 13 buffy coat samples, the 136 first cell culture passage samples and the 6 parental viruses as described in Section 2.6.2. Electrophoresis was carried out using criterion TGX 12% precast gels (Bio-rad) in a BioRad Mini Protean cell. Electrophoresis was carried out at 120V and a current of 30mA using a PowerPac 300 power supply (Bio-rad). The running time was 6 hours and 15 minutes. Immediately following electrophoresis the gels were stained with 5 µl ethidium bromide (stock 10 mg/ml) (Invitrogen) and gently shaken for 20 minutes with a Bench waver shaker (MIDSci). The gels were visualized with a Chemdoc XRS (Bio-rad).

BioNumerics analysis

Polyacrylamide gels were photographed with a Chemdoc XRS (Biorad) and compared with each other using a cluster analysis software program (BioNumerics,Applied Math).The program is able to align the gels and compare all the samples to each other in order to detect electrophoretic mobility shifts. The analysis was therefore band based, with a 2% band matching tolerance. The dendogram was build using the UPGMA method. No changes between the bands were tolerated and all uncertain bands were excluded.

The UPGMA method was selected with the degeneracy handling option off, and with cophonetic correlation. The bands were then compared and a phylogenetic tree used to select the isolates that were the least identical to the parental viruses.

On the basis of PAGE and BioNumeric results, a total of 11 isolates were selected as possible reassortants and sent for sequencing (Table 2.3).

Table 2.3: Suspected reassortant virus samples selected for further analysis

Sample number:	Origin of plaques	Trial day sample was collected	Animal sample was collected from
2b	Buffy coat (BC)	Day 12 post infection	Animal 1
3b	Buffy coat	Day 7 post infection	Animal 5
4b	Buffy coat	Day 5 post infection	Animal 4
6b	Buffy coat	Day 8 post infection	Animal 1
8b	Buffy coat	Day 9 post infection	Animal 1
9b	Buffy coat	Day 7 post infection	Animal 1
11b	Buffy coat	Day 8 post infection	Animal 5
17	First passage of BC	Day 5 post infection	Animal 5
47	First passage of BC	Day 3 post infection	Animal 4
89	First passage of BC	Day 14 post infection	Animal 4
105	First passage of BC	Day 7 post infection	Animal 1

Phylogenetic analysis of genome sequences

The samples listed in Table 2.3 were sequenced and analyzed as described in Section 2.9.1. Geneoius version 8.1.6 (Genious R8) was used to draw a neighbour-joining tree for each genome segment of each of the isolates. Statistical support for each node of the phylogenetic tree was calculated using bootstrap analysis (1000 replicates).

2.9.2 Cell viability assay

To evaluate whether reassortment was associated with changes in viral phenotype, viruses in Table 2.3 and the parental vaccine strains were characterized as to their effect on cell viability. Two different assays were used: or two assays measuring different parameters were used:

CellTiter-Blue Cell Viability Assay

The CellTiter-Blue Cell Viability assay can be used to measure a reduction in cell viability in virally infected cells. The assay is based on the principle that metabolically active cells are able to convert rezasurin to resorufin, both of which have different excitation and absorbance spectra. Cell that are no longer viable are no longer metabolically active, and as a consequence the conversion of rezasurin to resofurin is reduced. The 11 samples (Table 2.3) and the 6 parental viruses were titrated and the $TCID_{50}$ calculated for each sample as described in Section 2.4. All samples were then diluted to the titre of the virus isolate that had the lowest concentration ($TCID_{50}$: $10^{4.7}$). Two negative controls were included in the assay. These included a background fluorescent control as a non-infected cell culture control. In order to prepare the background fluorescent control wells were set up without cells and only 100 µl of MEM added. Untreated cell controls were prepared similarly to infected well controls using the same amount of non-virus containing MEM

The plate was set up with 50 000 Vero cells per well which was suspended in 70 µl MEM with 10% foetal calf serum (Highveld biological). The virus was then added to a final volume of 100 µl. A volume of 20 µl of CellTiter-Blue Reagent (Promega) was added to each well. The plate was then incubated at 37°C for 4 hours, shaken for 10 seconds and absorbance measured using a Powerwave HT Microplate Spectrofotometer (Biotek) at 570/600nm. The plate was further incubated at 37°C and readings repeated at 22, 46 and 72 hours after virus addition.

CellTox Green Cytotoxicity assay

The CellTox Green Cytotoxicity assay is used to measure the cytotoxic effect a virus will have in cell culture. The assay uses changes in cell membrane integrity that occurs as a result of cell death. The cyanine dye that is included in the kit reaction mix cannot enter the intact cell membrane, but as

soon as the membrane integrity is compromised due to cell lysis as a result of viral infection, the cyanine can bind to the DNA and the dye fluoresces. Fluorescence is measured at 484 nm (excitation) and 520 nm (emission). Vero cells were dispensed in 5 ml MEM (Sigma) supplemented with 5% foetal calf serum (Highveld biological) at a density of 200 000 cells per ml. A volume of 10 µl of the CellTox Green dye was dispensed into the cell solution at 10 µl to every 5 ml of cells. The solution was gently vortexed to ensure dye homogeneity. The cells were pipetted into a 96-well plate, 50 µl of the cell/dye solution added per well and the plates incubated in a CO_2 until they attached to the plate surface. The viruses, 50 µl per well, were then added to the plate. An untreated cell control was added with a final volume of 100 µl. Fluorescence was measured with a GloMax-Multi-detection system (Promega) at 4, 24, 28 and 72 hours, while the plates were incubated at 37°C.

2.10 Reference

1. Kärber, G. (1931). Beitrag zur kollektiven behundlung pharmakologisher reihenversuche. *Archiv Für Experimetelle Pathologie und pharmakologie,* 162, 480-483.

2. Mertens, P.P.C., Maan, N.S., Prasad, G., Sameul, A.R., Shaw. A.E., Potgieter, A.C., Anthony, S.J. and Maan, S. (2007). Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *Journal of General Virology*, 88, 2811-2823.

3. Potgieter, A.C., Page, N.A., Liebenberg, J., Wright, I.M., Landt, O. and Van Dijk, A.A. (2009). Improved strategies for sequence-independent amplification and sequencing of viral double stranded RNA genomes. *Journal of General Virology,* 90,1-11. DOI 10.1099/vir.o.oo9381-0.

4. Toussaint, J.F., Sailleau, C., Breard, E., Zientara, S., De, C.K. (2007). Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segment. *Jounal of Virology Methods*, 140, 115-123.

Chapter 3

Results

3.1 Before infection

3.1.1 Prescreening of cattle for BTV group-specific antibodies and viral RNA

In order to confirm that the cattle were free of antibodies specific to BTV and viral RNA both a group-specific cELISA and a BTV-specific qRT-PCR targeting segment 5 of the viral genome (Figure 3.1) were conducted on cattle blood prior to the onset of the study. The cELISA test was conducted 3 days before the animals were transported to the UPBRC, and again on Day 0 of the animal trial. The BTV-4 infected cell culture sample that was included as a positive control demonstrated a Ct-value of 32, whereas no amplification was observed for the negative controls. The cELISA results demonstrated inhibition values below 50% for all animals and were therefore considered negative for BTV group-specific antibodies (Table 3.1). The positive controls demonstrated average inhibition values of 78,46% and the inhibition values of the negative controls were 3.84%. The qRT-PCR contained the following samples; a positive control (total RNA extracted from BTV-4 infected cell culture) that demonstrated a Ct-value of 32, whereas the Ct-value of the negative control sample (RNA free water) was undetermined. All the cattle samples were negative for BTV RNA as no amplification using PCR was observed.

3.2 Post infection

3.2.1 Animal trial

After the inoculation of the cattle they were monitored daily for any signs of distress. The rectal temperatures were taken daily. Only two animals showed slightly elevated temperatures on Day 1 post inoculation. Animal 4 showed a temperature of 39.1°C and Animal 5 a temperature of 39.4°C. Both animals also showed a loss of appetite. All the animals demonstrated normal temperatures (between 38.3°C and 38.7°C) and a healthy appetite on Day 2 post inoculation and showed no further signs of distress throughout the remainder of the trial.

3.2.2 Serological results

In order to monitor sero-conversion following infection, the cattle were tested for BTV group-specific antibodies on days 13, 15, 17 and 21. The cattle tested sero-negative on Day 0 and seropositive (inhibition vales >50%) on days 13, 15, 17, 19 and 21 as expected (Table 3.1).

Table 3.1: The results of the cELISA on cattle serum that was taken from the animals on days 0, 13, 15, 17, 19 and 21. The values below 50% indicate no BTV-specific antibodies present (green) and above 50% indicate the presence of BTV-specific antibodies as expected after inoculation.

	Day 0	Day 13	Day 15	Day 17	Dag 19	Day 21
Animal 1	31.71	67.45	60.43	64.69	62.10	54.03
Animal 2	33.49	67.81	60.12	73.21	77.17	70.33
Animal 3	36.53	58.99	52.51	70.02	67.43	49.17
Animal 4	35.90	83.27	76.86	66.36	72.75	68.34
Animal 5	31.75	87.95	78.54	75.80	67.73	76.26
Animal 6	27.03	63.30	76.71	67.89	61.04	65.30

3.3 Plaque purification from infected blood

In order to isolate possible reassortant strains from infected animals, viruses were isolated directly from the separated buffy coats using the PFU assay. Very few of the buffy coat samples yielded plaques and in total only 13 plaques could be successfully isolated and amplified in cell cultures. A total of 136 plaques were however obtained from the buffy coats of samples after first passaging once on Vero cells and then isolated by using the PFU assay.

3.4 Isolation of parental viruses from BTV Bottle B vaccine

Plaques representing each of the parental viruses were successfully isolated from Bottle B of the OBP vaccine. Plaques were prepared directly from the vaccine, randomly picked and amplified in cell cultures. Plaques corresponding to serotypes 3, 8, 9, 10 and 11 were subsequently identified by the plaque neutralization assay (Figure 3.1). Serotype 8 proved to be more difficult to isolate and subsequently a conventional RT-PCR instead of the plaque inhibition assay was used to screen plaques for specifically BTV 8 (Figure 3.2).

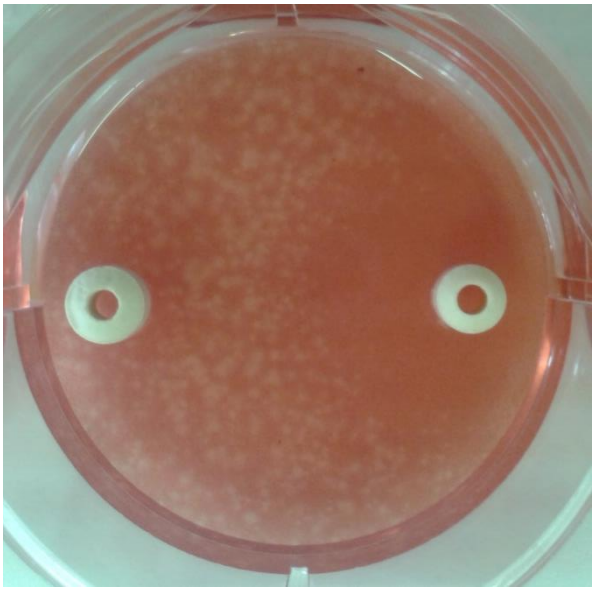


Figure 3.1 Plague neutralization assay used to serotype the parental vaccine strains on Vero cells. Anti-serum against a specific serotype, placed in ceramic beads, inhibits the formation of plaques in adjacent zones in the cell culture monolayer if the antiserum used was raised against the homologous virus (i.e. the same serotype).

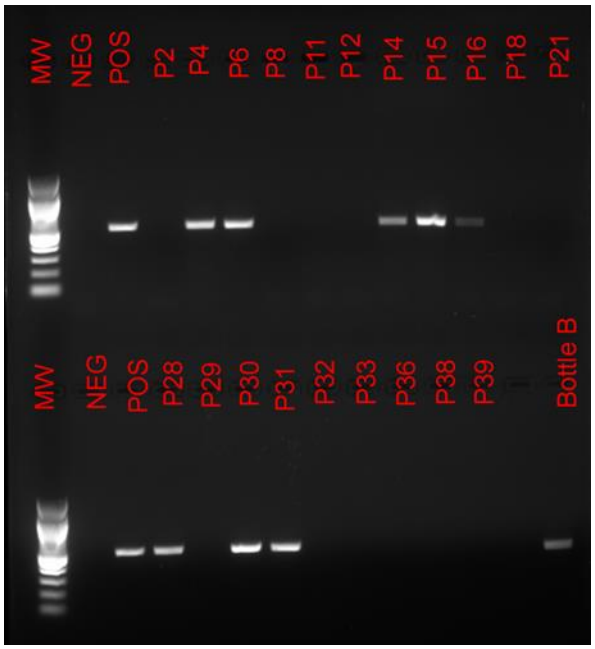


Figure 3.2: RT-PCR products on an agarose gel (1%) showing individual plaques isolated from the vaccine. A BTV 8 infected cell cultures were used as the positive control and RNA free water as a negative control. Total RNA obtained from Bottle B of the BTV vaccine was included to confirm the presence of BTV 8 in the vaccine bottle. Samples P4, 6, 14, 15, 16, 28, 30 and 31 tested positive as well as the vaccine confirming that BTV 8 is present in the vaccine.

3.5 Sequencing of parental viruses

In general sequencing yielded nearly full length sequences for each of the genome segments of all the vaccine serotypes and BTV-4. The sequencing data including the total read count, and the average coverage are illustrates in Table 3.2. All viruses further demonstrated 97–100% nucleotide sequence identity to the corresponding genome segments of the MLV sequences available on GenBank (Table 3.2). All the parental strains from the vaccine Bottle B and the wild type strain of BTV serotype 4 were submitted to GenBank (Bioproject number: PRJNA287219) and the accession numbers are listed in Table 3.3

Table 3.2: Sequencing information used to determine the quality of the each of the parental sequences as well as the reassorted sequence.

	BTV-3	BTV-4	BTV-8	BTV-9	BTV-10	BTV-11	Reassorted virus
Total read count	1571299	684634	2430533	411644	1574360	1735017	1998556
Total reference length	19186	19156	18076	18076	19184	19185	19432
Total consensus length	19186	15032	18070	18209	19188	18650	18885
Average coverage	18115.7	6661.29	32438.47	5068.6	12004.82	11822.88	18435.42

Table 3.3: Accession numbers of all parental viruses and the reassortant virus submitted to GenBank. The isolated parental viruses were compared to the MLV sequences on GenBank and the similarity indicated.

Wild type strain BTV serotype 4	Accession number	GenBank sequences	Similarity to sequences on GenBank
BTV4-wildtype-Rustenburg VP1(Pol)	KT317665	JX272369.1	97%
BTV4-wildtype-Rustenburg VP2	KT317666	KP821071.1	97%
BTV4-wildtype-Rustenburg VP3(T2)	KT317667	JX272441.1	97%
BTV4-wildtype-Rustenburg VP4(CaP)	KT317668	KP821313.1	97%
BTV4-wildtype-Rustenburg VP5	KT317669	KP821460.1	98%
BTV4-wildtype-Rustenburg VP6(Hel)	KT317670	KP821553.1	97%

BTV4-wildtype-Rustenburg VP7(T13)	KT317671	JX272575.1	99%
BTV4-wildtype-Rustenburg NS1(TuP)	KT317672	KP821760.1	98%
BTV4-wildtype-Rustenburg NS2(ViP)	KT317673	KP821915.1	98%
BTV4-wildtype-Rustenburg NS3	KT317674	KP822001.1	99%
Vaccine strain BTV serotype 3			
BTV-3 vac VP1(Pol)	KT317675	JX272589.1	99%
BTV-3 vac VP2	KT317676	AJ585124.1	99%
BTV-3 vac VP3(T2)	KT317677	JX272591.1	99%
BTV-3 vac VP4(CaP)	KT317678	JX272592.1	99%
BTV-3 vac VP5	KT317679	KP821400.1	99%
BTV-3 vac VP6(Hel)	KT317680	KP821520.1	100%
BTV-3 vac VP7(T13)	KT317681	JX272595.1	100%
BTV-3 vac NS1(TuP)	KT317682	JX272596.1	99%
BTV-3 vac NS2(ViP)	KT317683	JX272597.1	99%
BTV-3 vac NS3	KT317684	KP822003.1	100%
Vaccine strain BTV serotype 8			
BTV-8 vac VP1(Pol)	KT317685	JX272539.1	99%
BTV-8 vac VP2	KT317686	KP821076.1	99%
BTV-8 vac VP3(T2)	KT317687	KP821198.1	99%
BTV-8 vac VP4(CaP)	KT317688	JX272542.1	99%
BTV-8 vac VP5	KT317689	KP821381.1	99%
BTV-8 vac VP6(Hel)	KT317690	KP821558.1	99%
BTV-8 vac VP7(T13)	KT317691	KP821680.1	99%

BTV-8 vac	NS1(TuP)	KT317692	KP821800.1	99%
BTV-8 vac	NS2(ViP)	KT317693	JX272547.1	99%
BTV-8 vac	NS3	KT317694	KP822041.1	100%
Vaccine strain BTV serotype 9				
BTV-9 vac	VP1(Pol)	KT885055	KP820963.1	99%
BTV-9 vac	VP2	KT885056	JN255963.1	100%
BTV-9 vac	VP3(T2)	KT885057	KP821211.1	99%
BTV-9 vac	VP4(CaP)	KT885058	KP821331.1	100%
BTV-9 vac	VP5	KT885059	KP821451.1	100%
BTV-9 vac	VP6(Hel)	KT885060	KP821571.1	100%
BTV-9 vac	VP7(T13)	KT885061	KP821693.1	100%
BTV-9 vac	NS1(TuP)	KT885062	KP821813.1	98%
BTV-9 vac	NS2(ViP)	KT885063	KP821933.1	100%
BTV-9 vac	NS3	KT885064	KP821054.1	100%
Vaccine strain BTV serotype 10				
BTV-10 vac	VP1(Pol)	KT317695	JX272519.1	99%
BTV-10 vac	VP2	KT317696	JX272520.1	99%
BTV-10 vac	VP3(T2)	KT317697	JX272521.1	99%
BTV-10 vac	VP4(CaP)	KT317698	JX272522.1	99%
BTV-10 vac	VP5	KT317699	JX272523.1	99%
BTV-10 vac	VP6(Hel)	KT317700	AJ586709.1	100%
BTV-10 vac	VP7(T13)	KT317701	JX272525.1	100%
BTV-10 vac	NS1(TuP)	KT317702	JX272526.1	99%

BTV-10 vac	NS2(ViP)	KT317703	JX272527.1	99%
BTV-10 vac	NS3	KT317704	JX272528.1	99%
Vaccine strain BTV serotype 11				
BTV-11 vac	VP1(Pol)	KT885065	JQ972841.1	99%
BTV-11 vac	VP2	KT885066	JQ972842.1	99%
BTV-11 vac	VP3(T2)	KT885067	JQ972843.1	99%
BTV-11 vac	VP4(CaP)	KT885068	JQ972844.1	99%
BTV-11 vac	VP5	KT885069	JQ972845.1	99%
BTV-11 vac	VP6(Hel)	KT885070	JQ972846.1	100%
BTV-11 vac	VP7(T13)	KT885071	JQ972847.1	100%
BTV-11 vac	NS1(TuP)	KT885072	JQ972848.1	100%
BTV-11 vac	NS2(ViP)	KT885073	JQ972849.1	100%
BTV-11 vac	NS3	KT885074	JQ972850.1	100%
Reassortant BTV9 and BTV 8				
BTV-11 vac	VP1(Pol)	KT885075		
BTV-11 vac	VP2	KT885076		
BTV-11 vac	VP3(T2)	KT885077		
BTV-11 vac	VP4(CaP)	KT885078		
BTV-11 vac	VP5	KT885079		
BTV-11 vac	VP6(Hel)	KT885080		
BTV-11 vac	VP7(T13)	KT885081		
BTV-11 vac	NS1(TuP)	KT885082		

BTV-11 vac	NS2(ViP)	KT885083		
BTV-11 vac	NS3	KT885084		

3.6. Characterization of parental viruses

3.6.1 Polyacrylamide gel electrophoresis

The double stranded RNA from the parental viruses (Figure 3.3) and the isolated viruses were run on 12% precast PAGE gels (Section 2.9.1). The parental viruses each demonstrated a unique electrophoretic profile. In general it was possible to distinguish between the majorities of genome segments based on the PAGE results. The more conserved segments e.g. segment 8 and 9 were however difficult to distinguish. Gel analysis of the blood isolated viruses did not show prominent genome segment shifts. Two viruses that were directly isolated from the buffy coat showed a clear shift in segment 8 (Figure 3.5). Plaques 2b and 6b overall resemble BTV serotype 9, but there is a visible shift in segment 8 as regards to the BTV-9 electropherotype. The samples from the first cell culture passage appeared identical to each other and corresponded with the profile of BTV serotype 4 (Figure 3.4). The gels containing the results of the other 119 sample are not shown since they are all identical resembling BTV4.

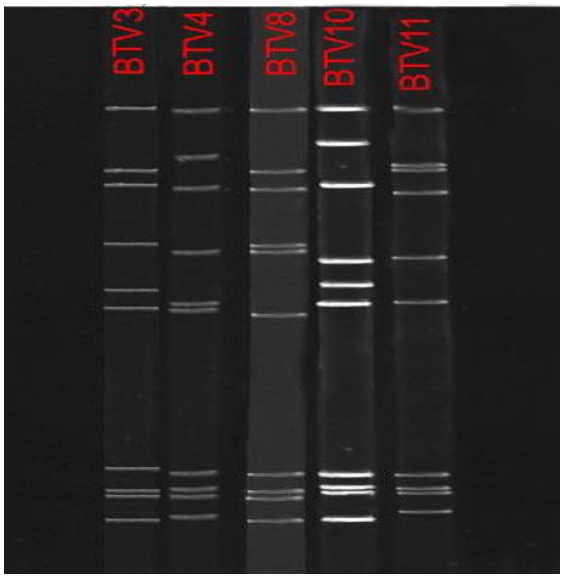


Figure 3.3: Electrophoretic profiles of the parental viruses using PAGE. All 10 segments are visible and each serotype has a unique profile.

BTV serotype 4

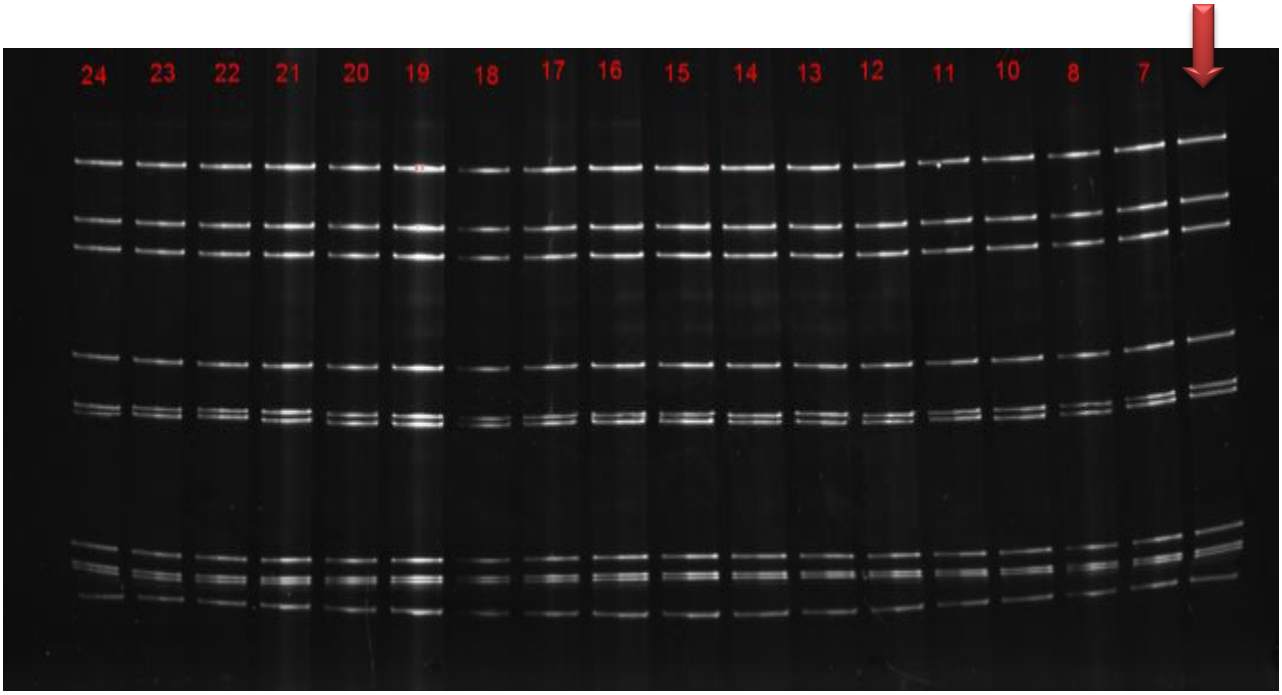


Figure 3.4: Double stranded RNA of viruses purified from plaques (Samples 7 – 24) from the first passage of the buffy coat all resemble BTV serotype 4.

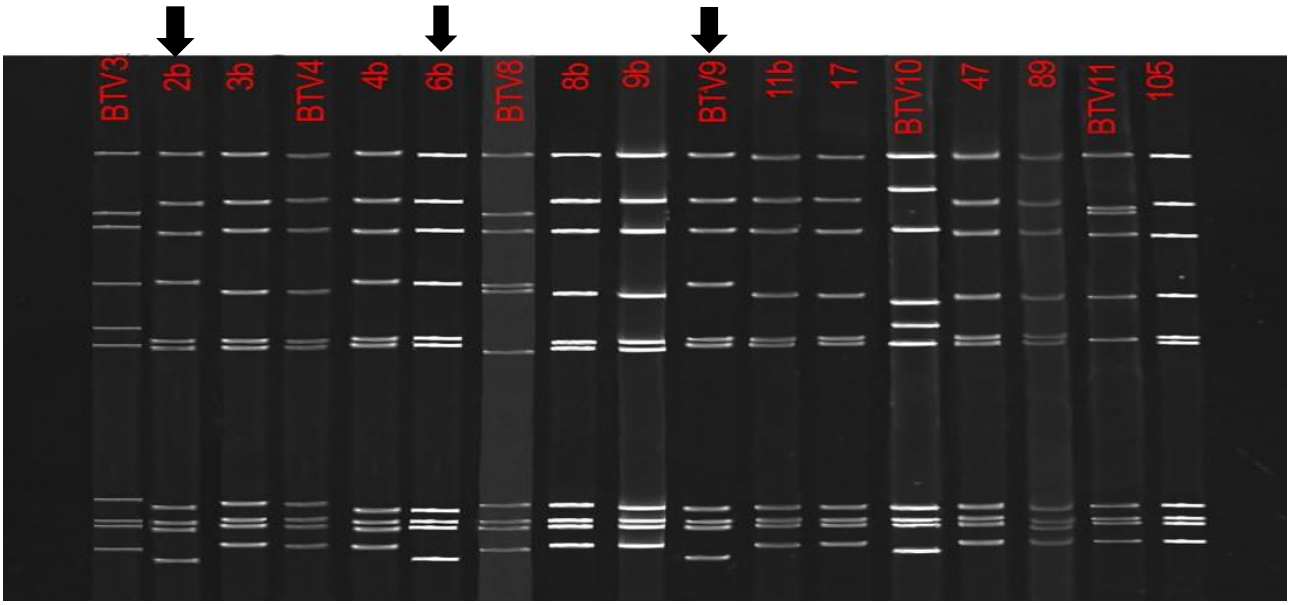


Figure 3.5: Double stranded RNA of plaque purified viral samples directly from the buffy coat of animal numbers 1, 4 and 5 compared to the parental viruses. The majority of the sample resembles BTV serotype 4, while virus 2b and 6b resembles BTV serotype 9.

3.6.2 BioNumerics

The PAGE gels from all the samples were compared to each other using the BioNumerics computer program. According to the results there were multiple groups of isolates that were not identical to the parental viruses (Figure 3.6).

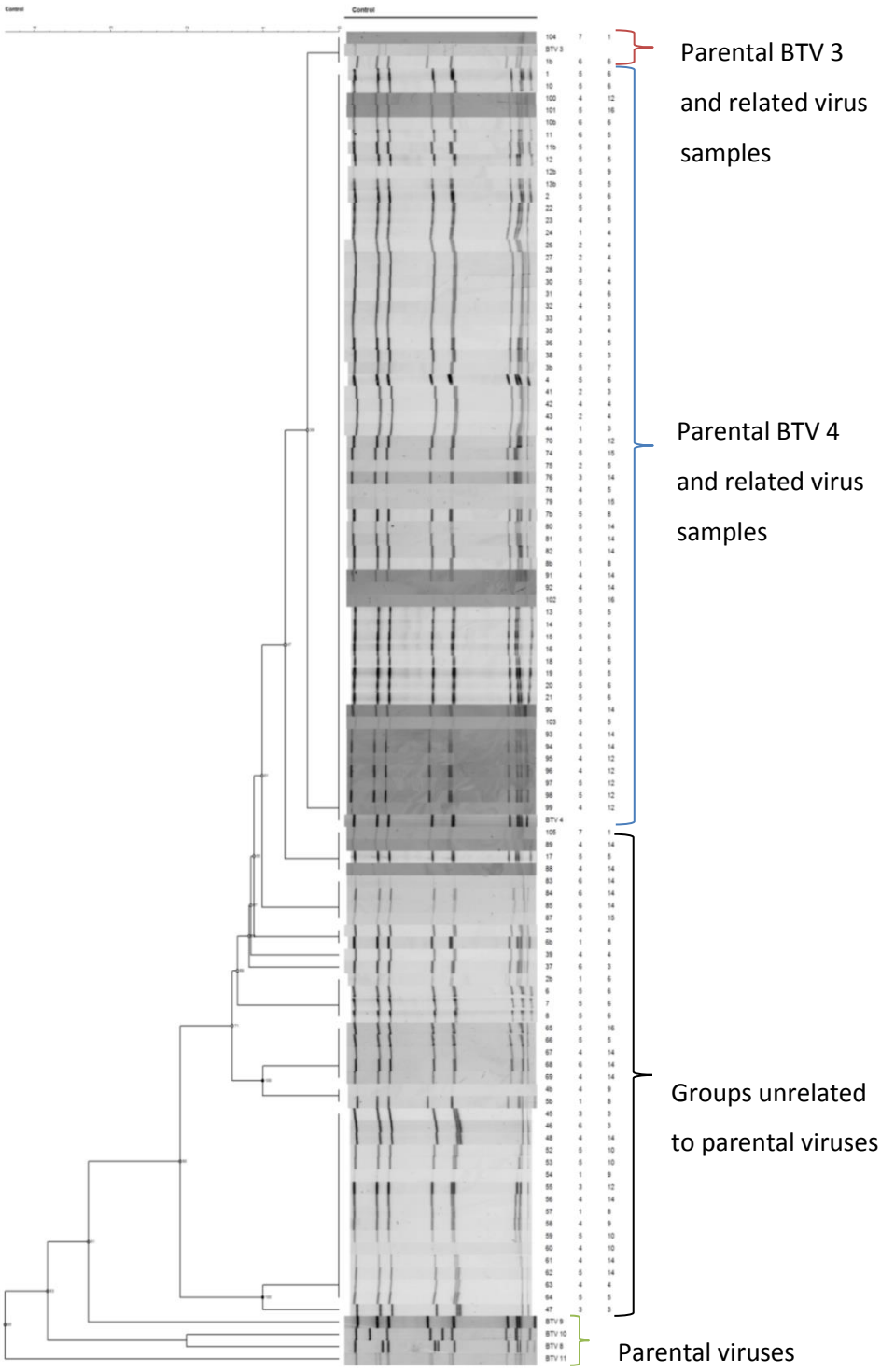


Figure 3.6: RFLP cluster analysis using Pearson correlation and unweighted pair group method using arithmetic averages. Parental viruses are illustrated, with related isolates. Isolate groups are identified that are not related to the parental viruses.

3.7 Sequence analysis

The genomic sequences of each of the suspected reassortant isolates (Table 2.3) were compared with parental strains using Geneious (version 8.1.6, Geneious R8). Phylogenetic trees for each of the 10 segments of the BTVs were drawn and the relationship between the isolates and parental stains evaluated on a segmental basis. The phylogenetic trees are illustrated in Figures 3.7 to 3.18. Bootstrap replicates of 1000 were used in each of the phylogenetic tree analysis.

Phylogenetic analysis of segment 1 using the neighbour joining tree indicated that the segment 1 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 9 and samples 2b and 6b. Both clades were supported by bootstrap values of 100%. Segment 1 of BTV-10, BTV-3 and BTV-8 clustered separately from Clades 1 and 2, AHSV segment 1 was included as an out-group (GenBank accession number is AKP39977.1). It was observed that the majority of segment 1 sequences of viruses obtained directly from the buffy coat, grouped with BTV-4, whereas two sequences grouped with BTV serotype 9 i.e. samples 2b and 6b (Figure 3.7).

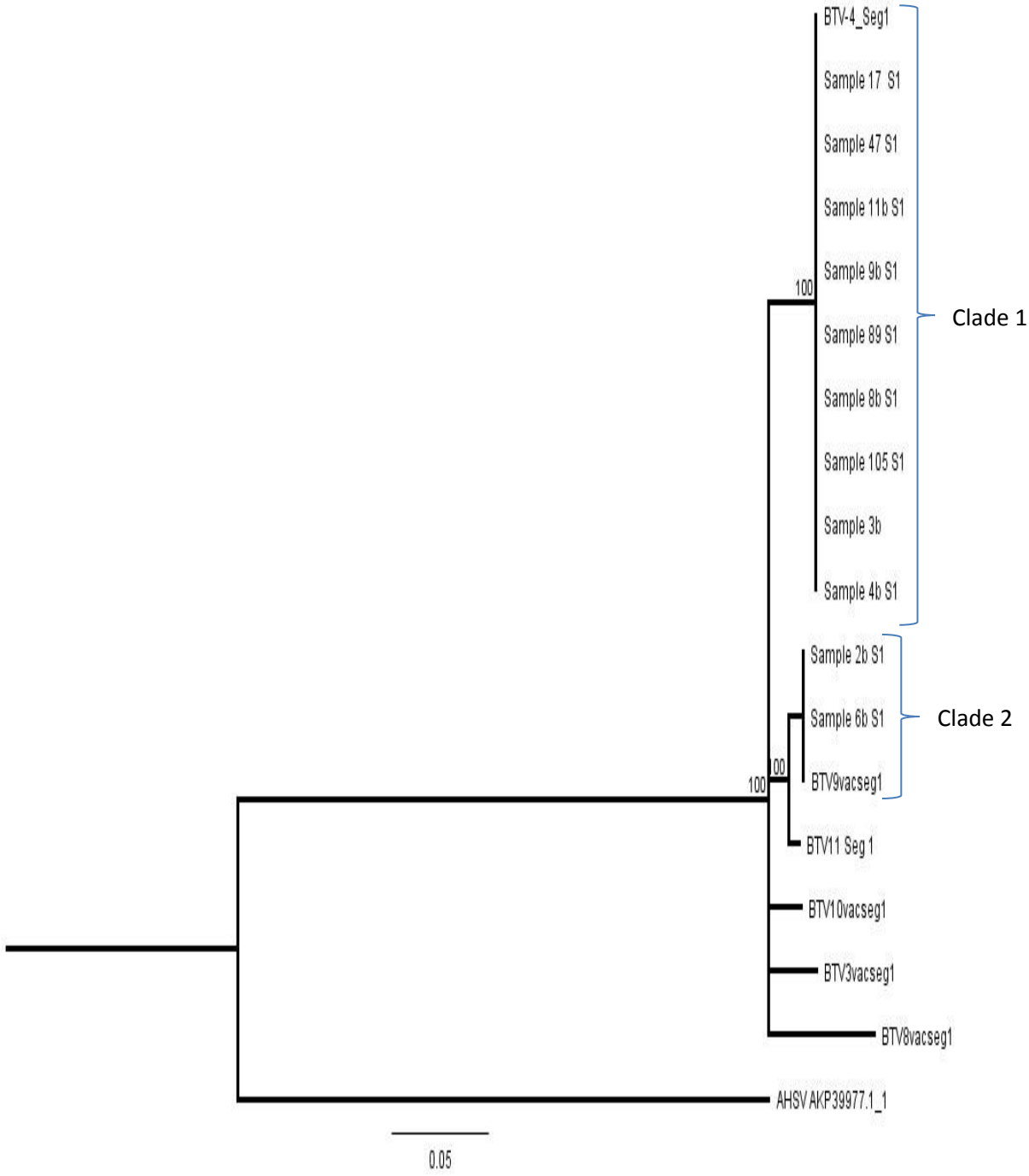


Figure 3.7: Neighbour-joining tree constructed using nucleotide sequence data of segment 1 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 2 using the neighbour joining tree indicated that the segment 2 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. Segment 2 of BTV-10, BTV-3 and BTV-8 clustered separately from the clades 1 and 2, AHSV segment 2 was included as an out-group (GenBank accession number is JQ796725.1). It was observed that the majority of segment 2 sequences of viruses obtained directly from the buffy coat, grouped with BTV-4, whereas two sequences grouped with BTV serotype 9 i.e. samples 2b and 6b (Figure 3.8).

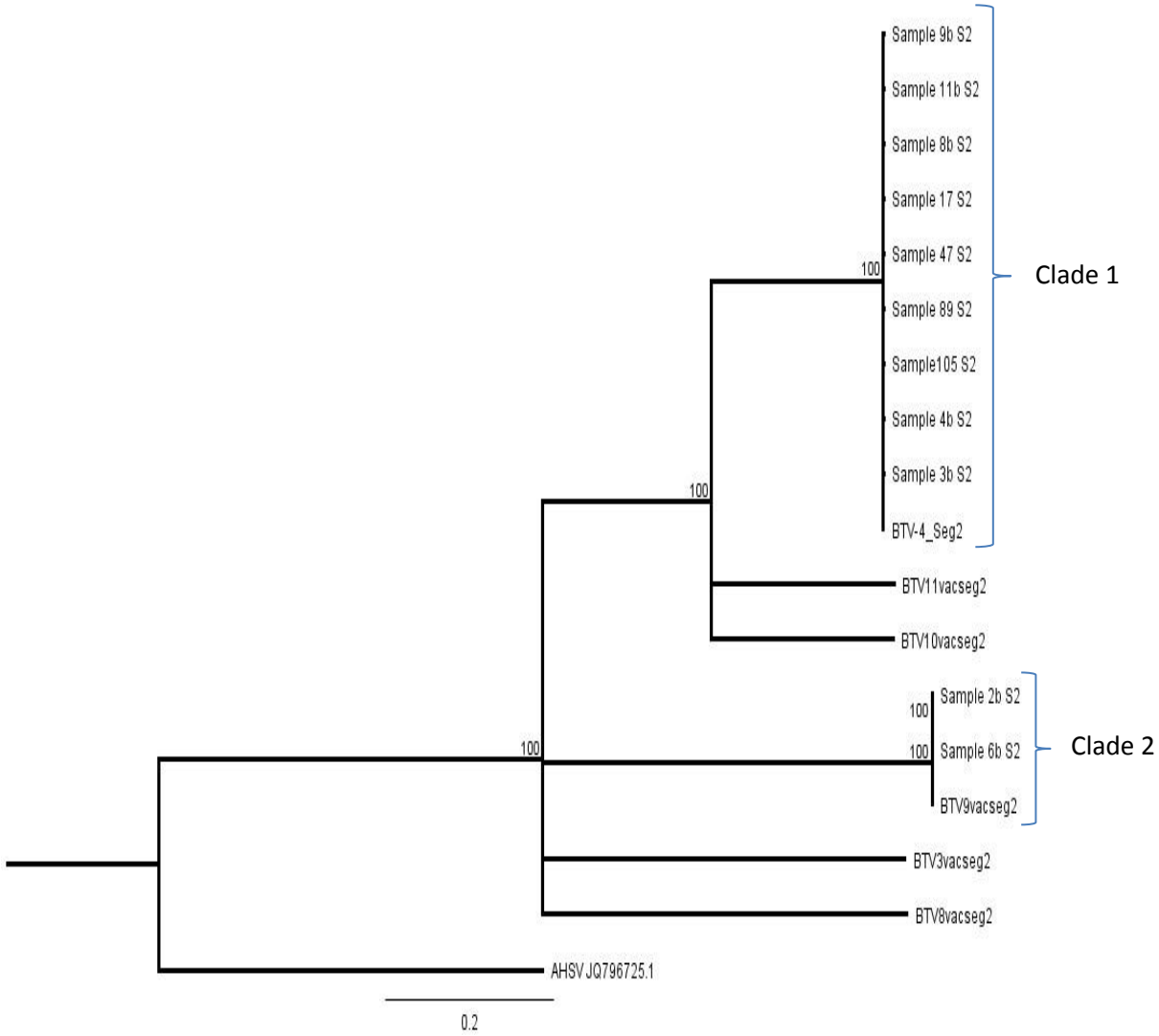


Figure 3.8: Neighbour-joining tree constructed using nucleotide sequence data of segment 2 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 3 using the neighbour joining tree construction method indicated that the segment 3 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. BTV-10 and BTV-3 clustered together with a bootstrap value of 100% and BTV-8 segment 3 clustered separately from the clades 1 and 2. AHSV segment 3 was included as an out-group (GenBank accession number is NC7006017.1). It was observed that the majority of segment 3 sequences of viruses, obtained directly from the buffy coat, grouped with BTV-4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.9).

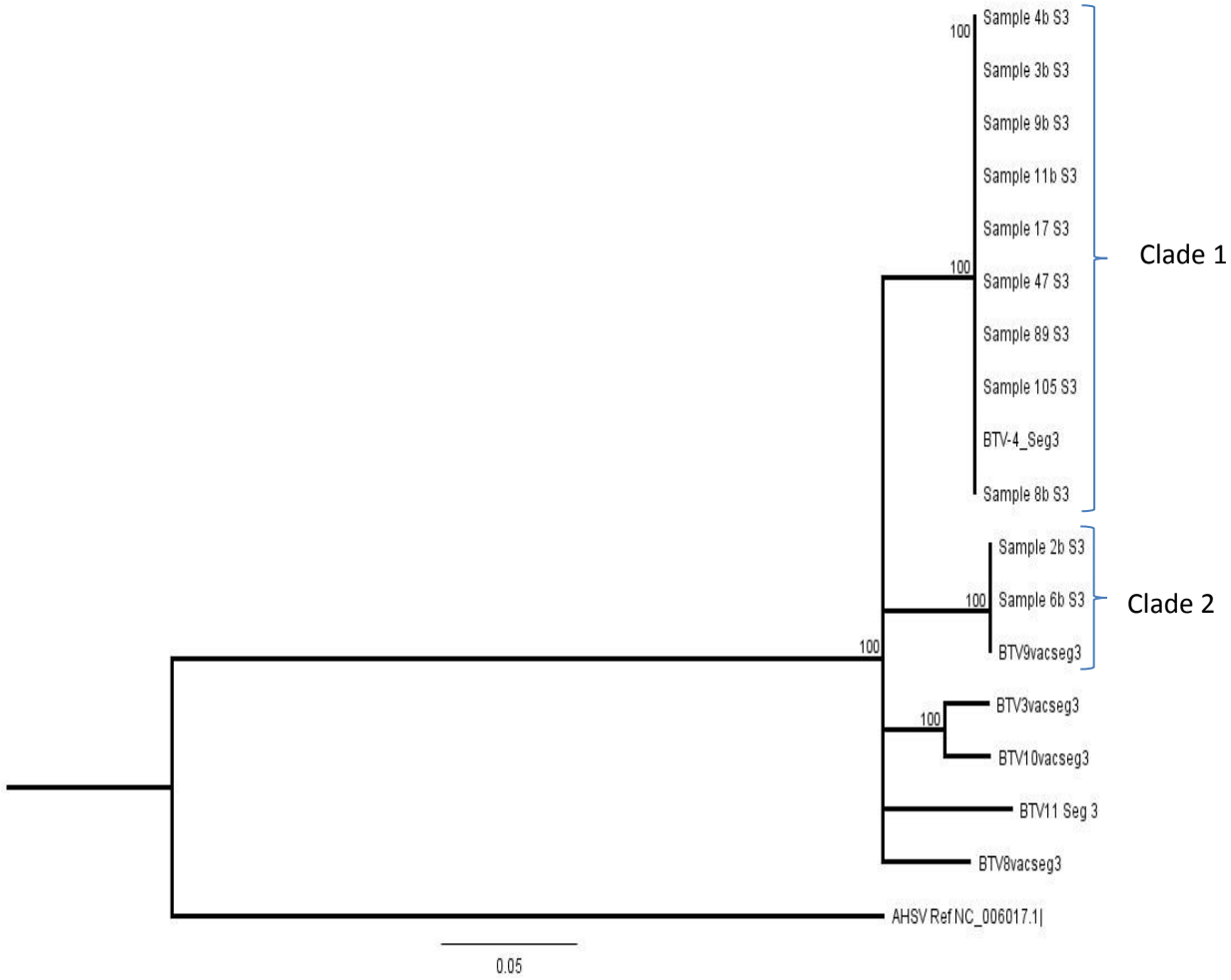


Figure 3.9: Neighbour-joining tree constructed using nucleotide sequence data of segment 13sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 4 using the neighbour joining tree construction method indicated that the segment 4 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. Segent 4 of BTV-10 and BTV-11 are clustered together with a bootstrap value of 91.6 and BTV-8 and BTV 11 segment 4 clustered separately from the clades 1 & 2, AHSV segment 4 was included as an out-group (GenBank accession number is KT007171.1.). It was observed that the majority of segment 4 sequences of viruses, obtained directly from the buffy coat, grouped with BTV-4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.10).

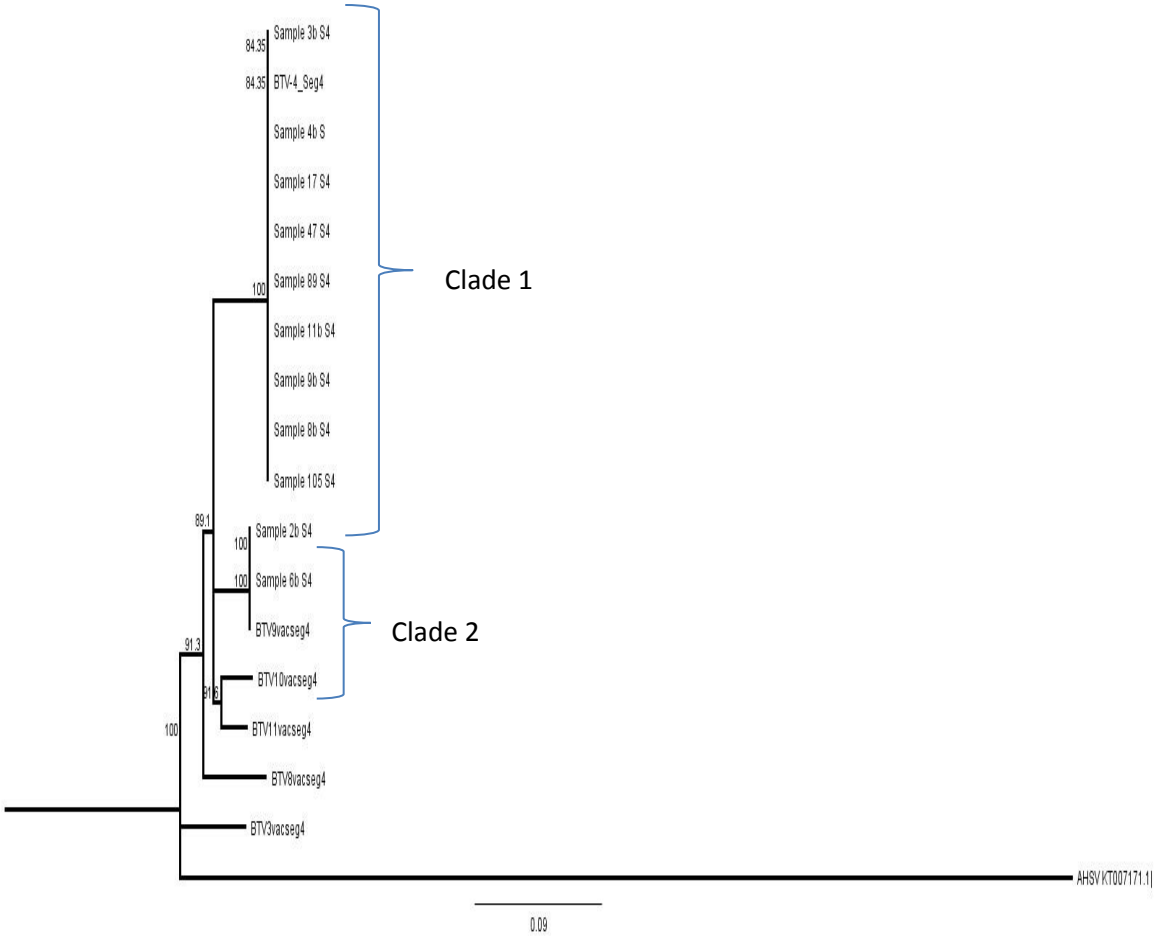


Figure 3.10: Neighbour-joining tree constructed using nucleotide sequence data of segment 4 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 5 using the neighbor joining tree construction method indicated that the segment 5 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. Segment 5 of BTV-10 and BTV-11 are clustered together with a bootstrap value of 100% and BTV-8 and BTV-3 segment 5 clustered separately from the clades 1 & 2, AHSV segment 5 was included as an out-group (GenBank accession number is JQ796728.1). It was observed that the majority of segment 5 sequences of viruses, obtained directly from the buffy coat, grouped with BTV-4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.11).

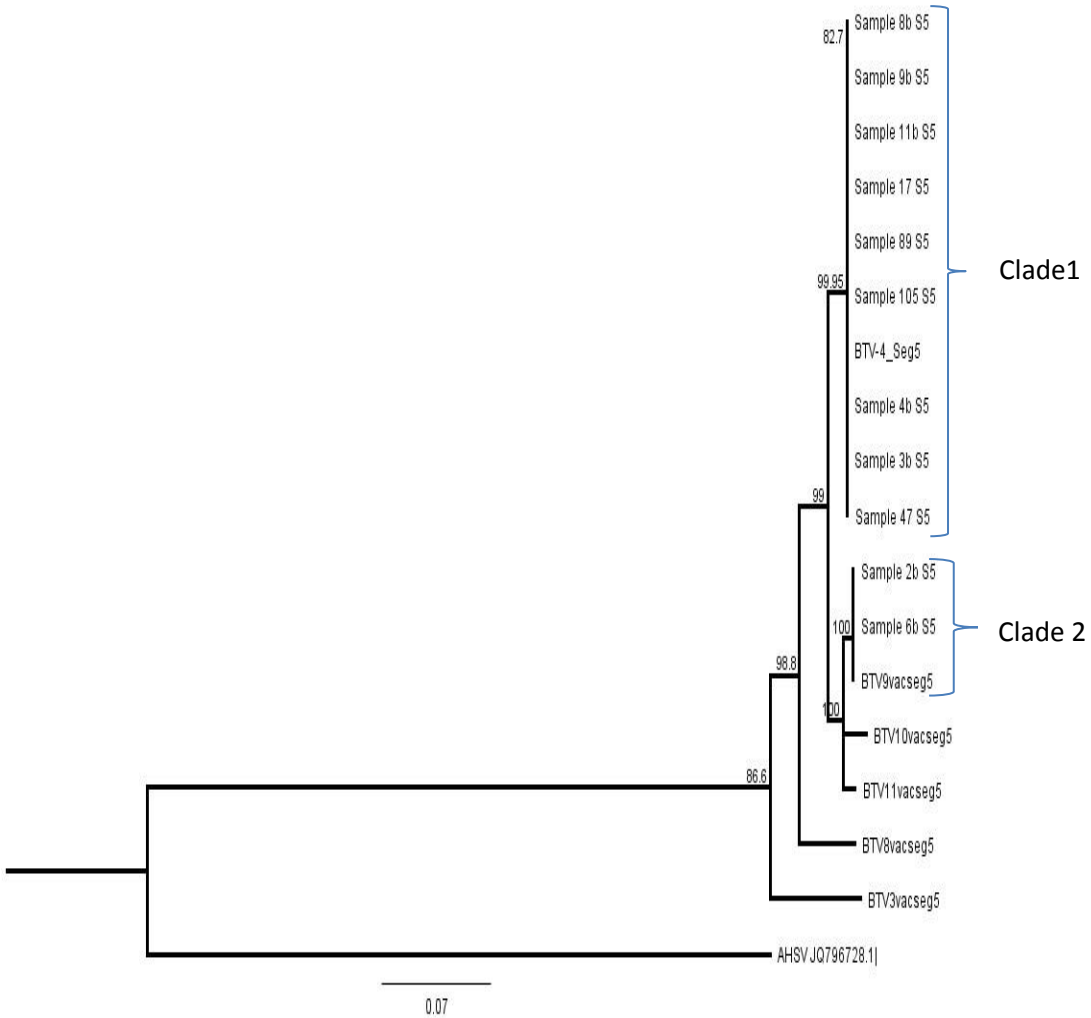


Figure 3.11: Neighbour-joining tree constructed using nucleotide sequence data of segment 5 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 6 using the neighbour joining tree construction method indicated that the segment 6 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105 Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. BTV-10 and BTV-11, BTV-8, BTV-3 segment 6 clustered separately from the clades 1 & 2, AHSV segment 6 was included as an out-group (GenBank accession number is JQ796729.1). It was observed that the majority of segment 2 sequences of viruses, obtained directly from the buffy coat, grouped with BTV 4, whereas two sequences grouped with BTV serotype 9 i.e sample 2b and 6b (Table 3.12).

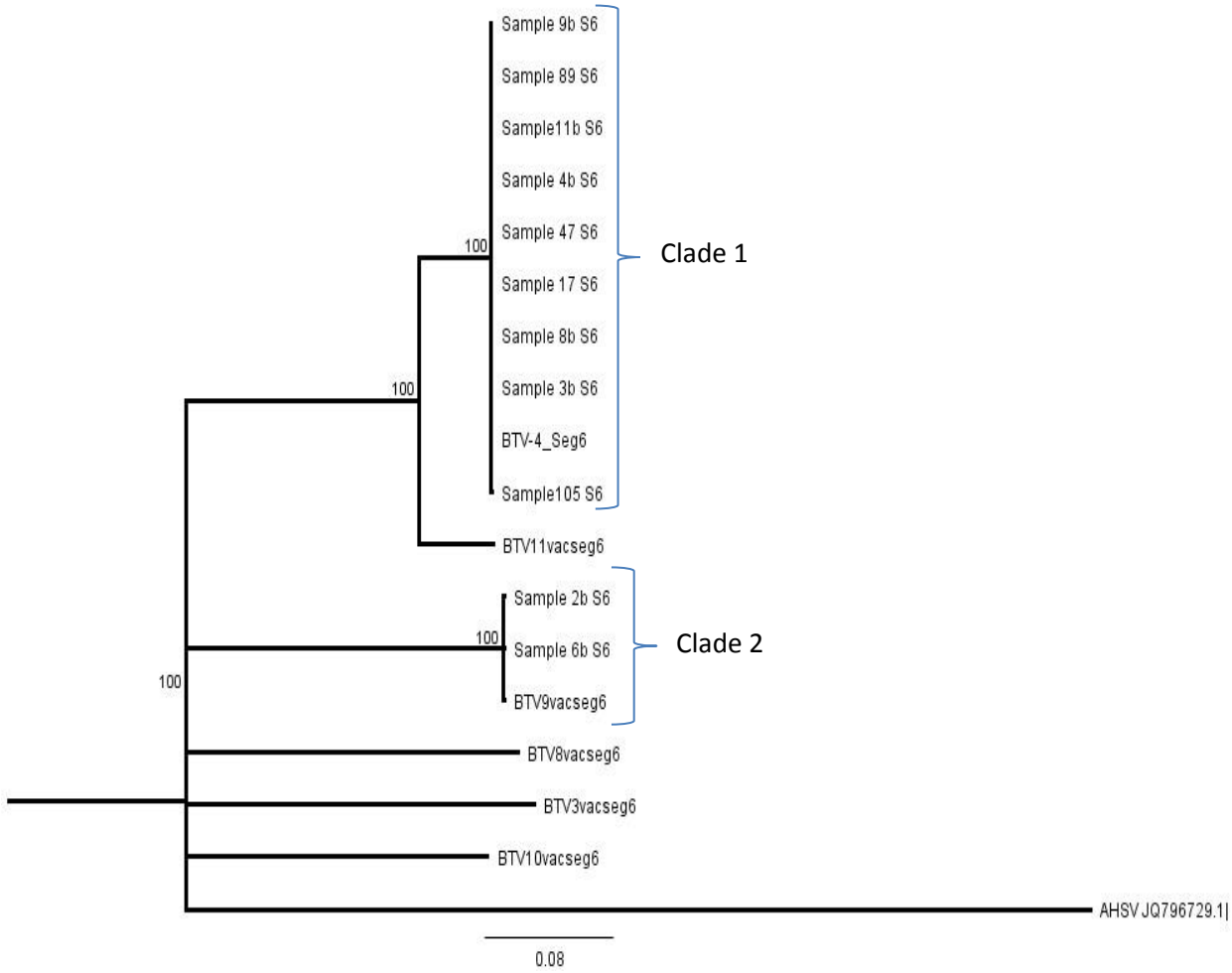


Figure 3.12: Neighbour-joining tree constructed using nucleotide sequence data of segment 6 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 7 using the neighbour joining tree construction method indicated that the segment 7 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105 Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 100%. Segment 7 of BTV-10 , BTV-11, BTV-8, clustered together with a bootstrap value of 100% while BTV-3 clustered separately from clades 1 and2, AHSV segment 7 was included as an out-group (GenBank accession number is AJU57338.1). It was observed that the majority of segment 2 sequences of viruses, obtained directly from the buffy coat, grouped with BTV 4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.13).

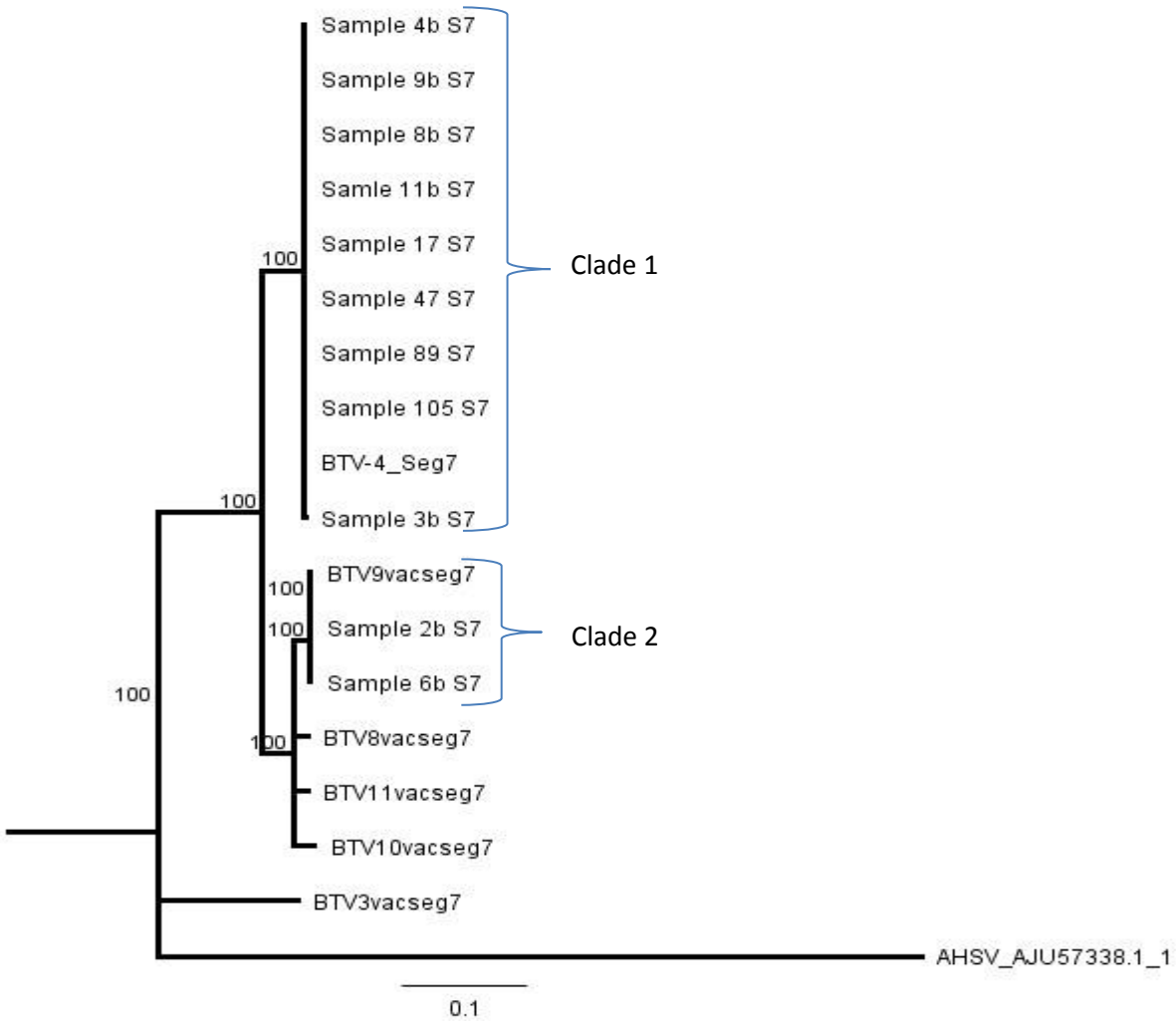


Figure 3.13: Neighbour-joining tree constructed using nucleotide sequence data of segment 8 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 8 using the neighbour joining tree construction method indicated that the segment 8 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105. Clade 2 consisted of BTV serotype 8 and sample 2b and 6b showed a bootstrap value of 87.7%, while Clade 1 was supported by bootstrap values of 99.95%. Segment 8 of BTV-10 , BTV-11, BTV-9 and BTV-3 clustered separately from the clades 1 & 2, AHSV segment 8 was included as an out-group (GenBank accession number is AJU57349.1). As regards to the viruses that were isolated from the buffy coat, it was observed that the majority of the segment 8 sequences grouped with BTV 4, whereas two sequences grouped with BTV serotype 8 i.e. sample 2b and 6b (Figure 3.14).

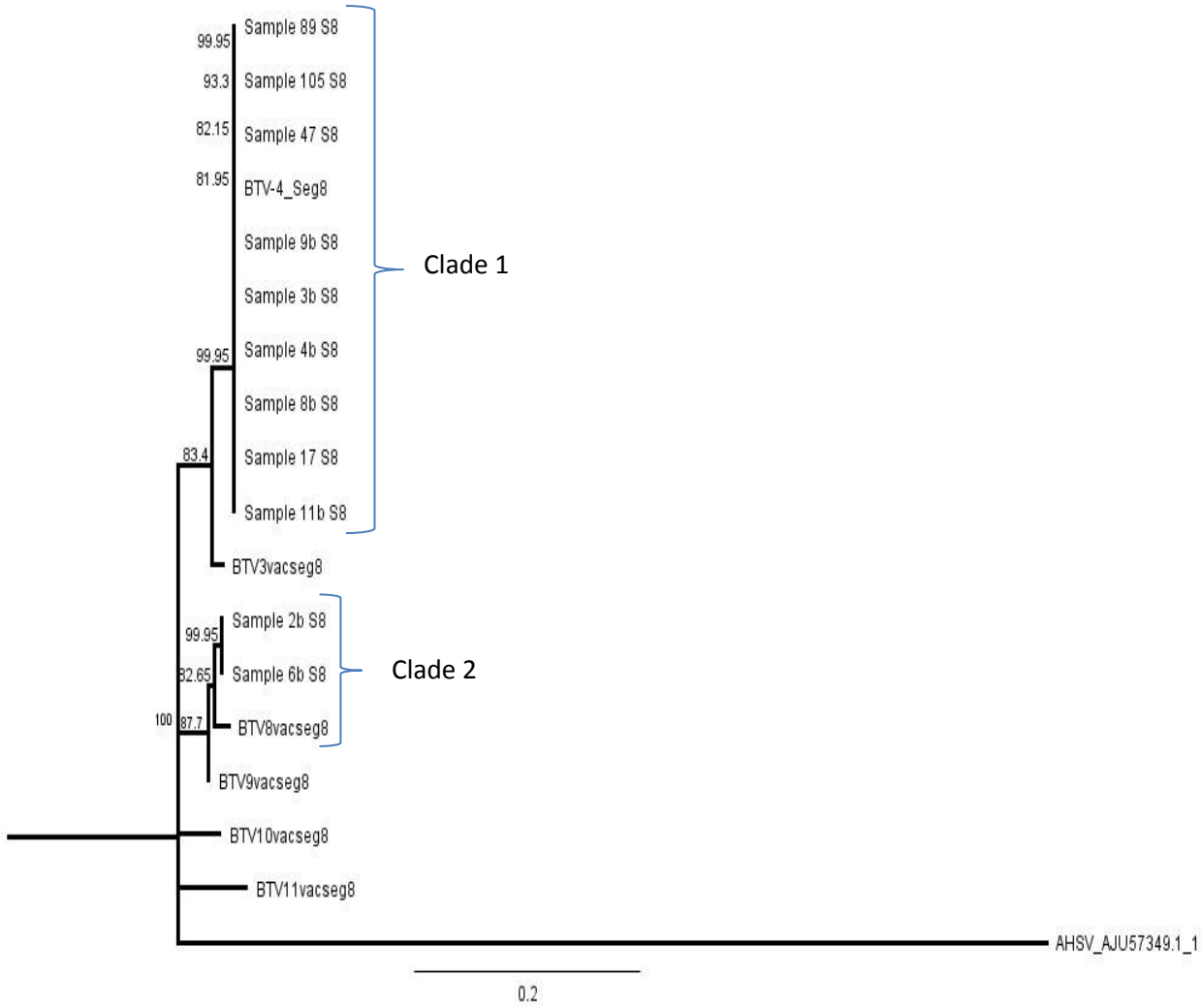
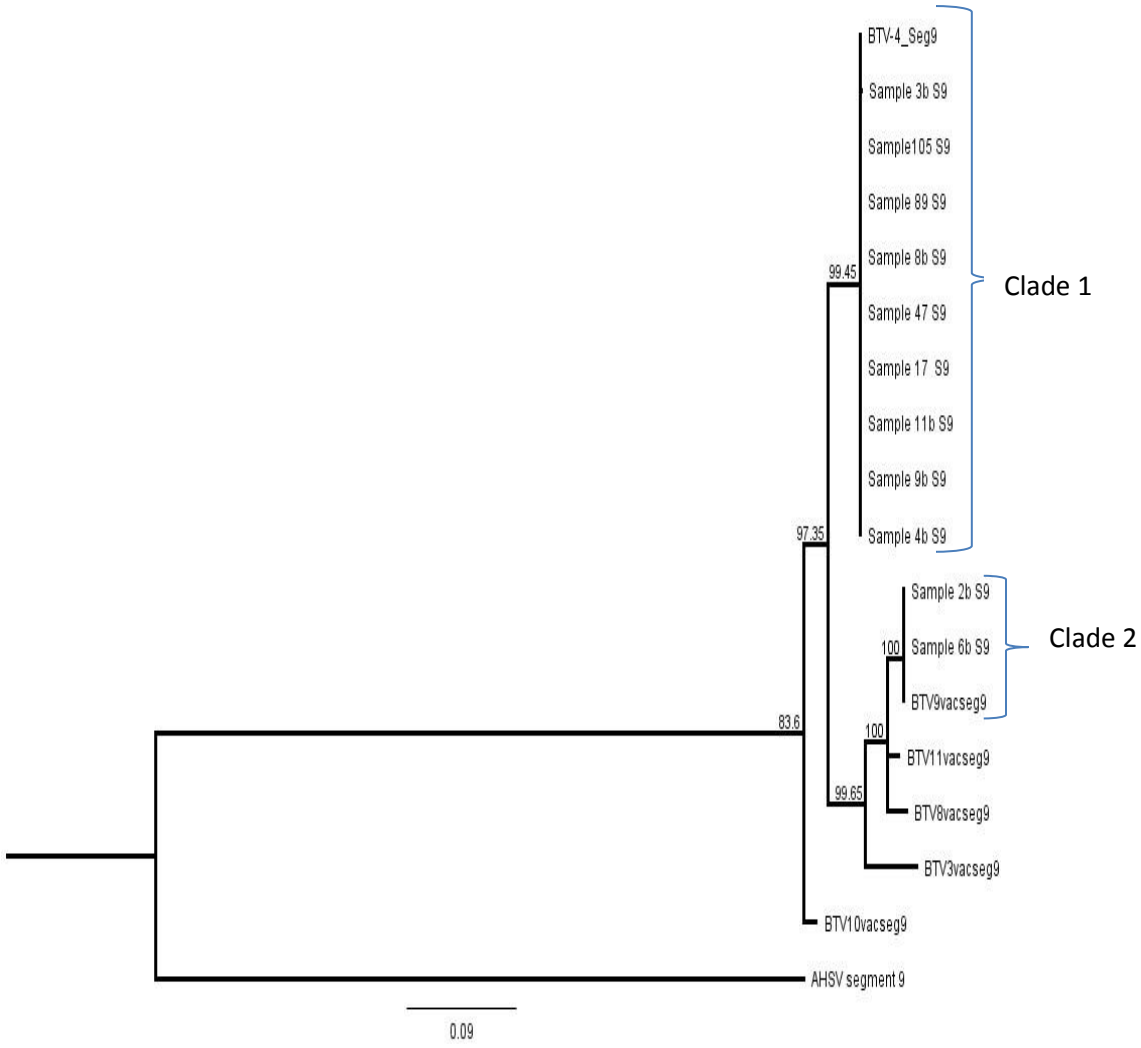


Figure 3.14: Neighbour-joining tree constructed using nucleotide sequence data of segment 8 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 9 using the neighbour joining tree construction method indicated that the segment 9 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105 Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 99.94% and 100% irrespectively. Bluetongue virus serotype 10, BTV-11, BTV-8, BTV-3 segment 9 clustered separately from the clades 1 & 2, AHSV segment 9 was included as an out-group (GenBank accession number is ADI87403.1). As regards to the viruses that were isolated from the buffy coat, it was observed that the majority of the segment 9 sequences grouped with BTV 4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.15)



.

Figure 3.15: Neighbour-joining tree constructed using nucleotide sequence data of segment 9 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

Phylogenetic analysis of segment 10 using the neighbourjoining tree construction method indicated that the segment 10 sequences mainly clustered into two clades. Clade 1 consisted of BTV serotype 4 and samples 3b, 4b, 8b, 9b, 11b, 17, 47, 89 and 105 Clade 2 consisted of BTV serotype 9 and sample 2b and 6b. Both clades were supported by bootstrap values of 96% and 100% irrespectively. Segment 10 of BTV-10 and BTV-3 clustered together with a bootstrap value of 100 while BTV-8 and BTV-11 clustered separately from the clades 1 & 2, AHSV segment 10 was included as an out-group (GenBank accession number is HG779569.1). It was observed that the majority of segment 10 sequences of viruses, obtained directly from the buffy coat, grouped with BTV 4, whereas two sequences grouped with BTV serotype 9 i.e. sample 2b and 6b (Figure 3.16).

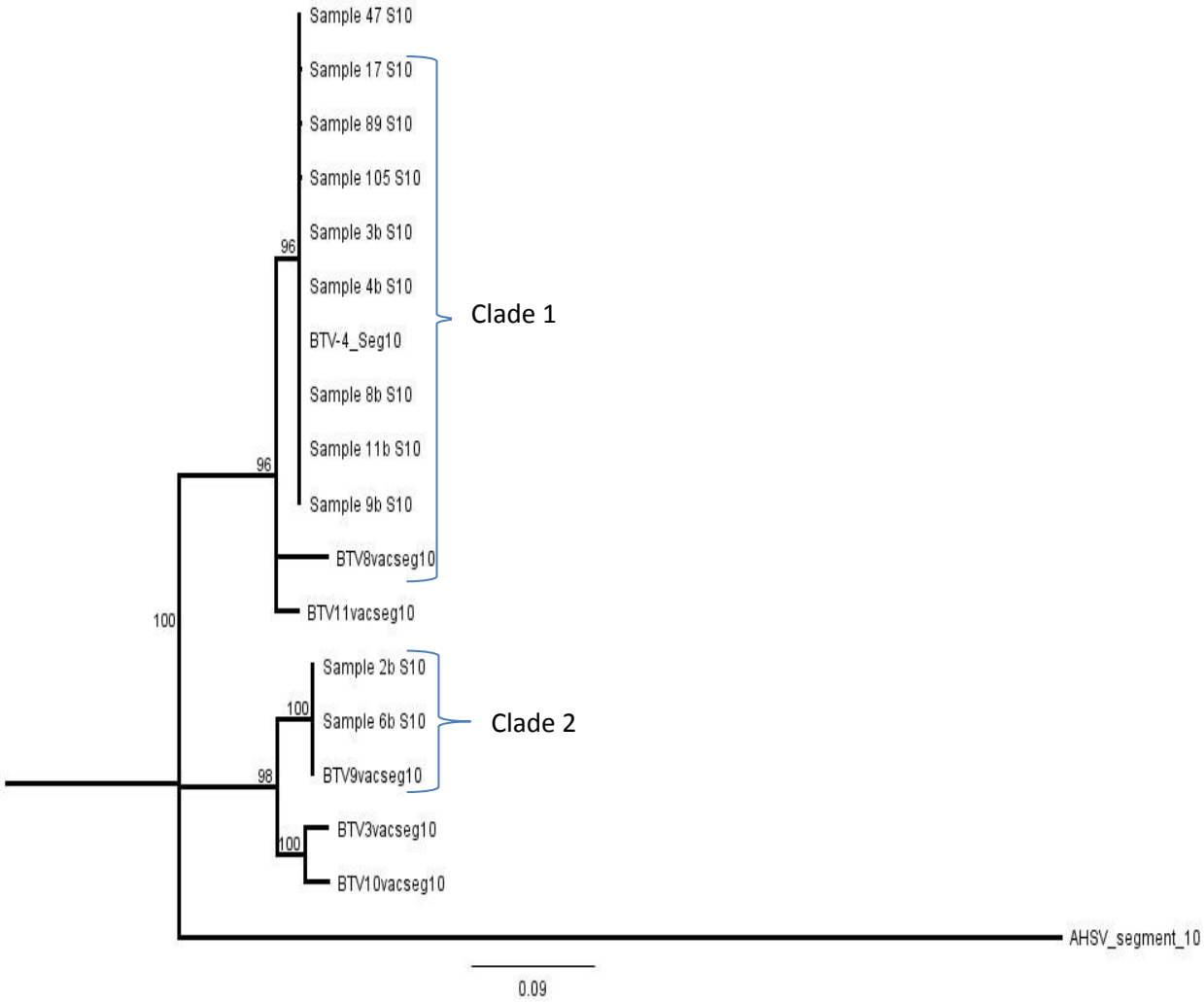


Figure 3.16: Neighbour-joining tree constructed using nucleotide sequence data of segment 10 sequences of the 11 suspected reassortant isolates and the 6 parental viruses.

3.8 Colour map

The cumulative results of the phylogenetic trees were compiled into a colour map. Each of the parental viruses is represented with a colour in the map for example BTV-4 is represented with green, and the segments that belong to BTV serotype 4 are green(Table 3.4?). All of the segments from the isolated viral strains were compared to each of the parental viruses. The colour map illustrates similarity in the genome segments. Segment 2 of BTV serotype 10 (dark blue) and BTV-11 (light blue) clustered together Segments 3 and 10 of BTV serotype 10 (dark blue) were very similar to the same segments in BTV serotype 3 (yellow). Segment 7 BTV serotype 11 (light blue), BTV-8 (red) and BTV-10 (dark blue) clustered together.

Table 3.4: Colour map with the cumulative results from the phylogenetic trees. Each parental virus is represented by an individual colour.

	SEG 1	SEG 2	SEG 3	SEG 4	SEG 5	SEG 6	SEG 7	SEG 8	SEG 9	SEG 10
Sample 2b										
Sample 3b										
Sample 4b										
Sample 6b										
Sample 8b										
Sample 9b										
Sample 11b										
Sample 17										
Sample 47										
Sample 89										
Sample 105										
BTV3										
BTV4										
BTV8										
BTV9										
BTV10										
BTV11										

Yellow: BTV-3

Green: BTV-4

Red: BTV-8

Orange: BTV-9

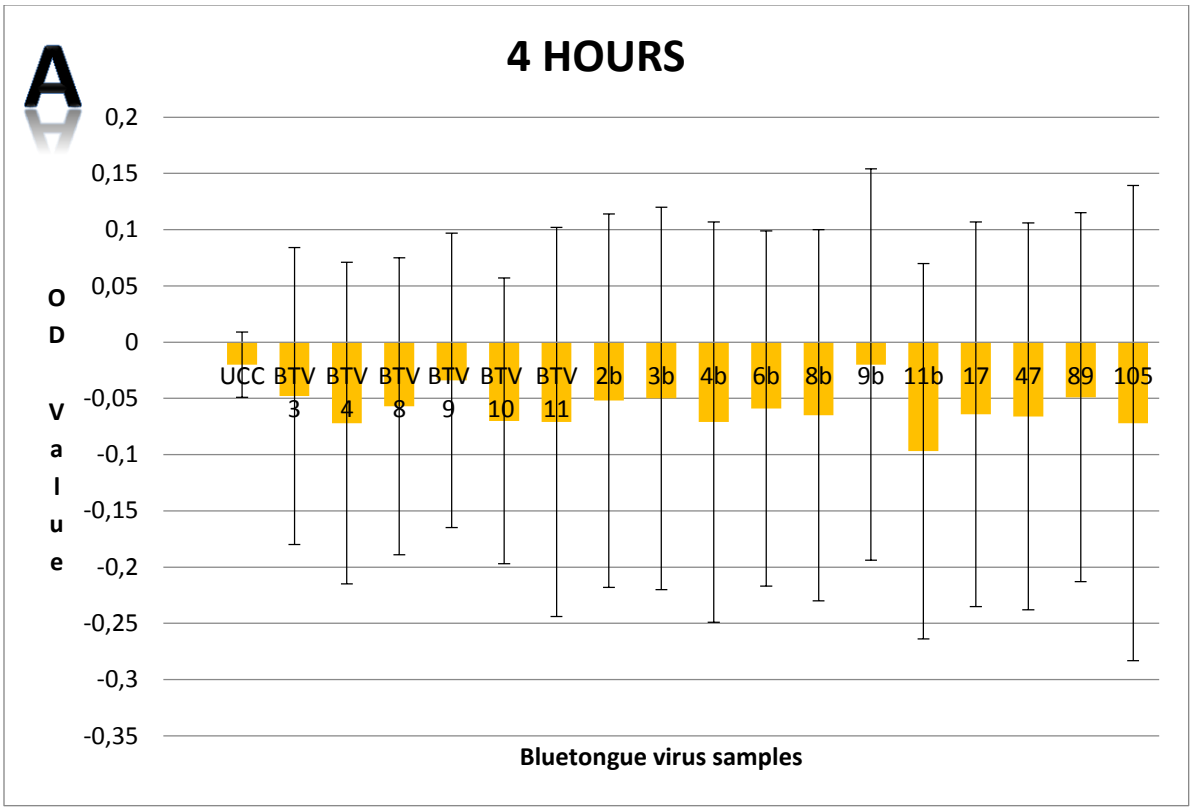
Dark Blue: BTV-10

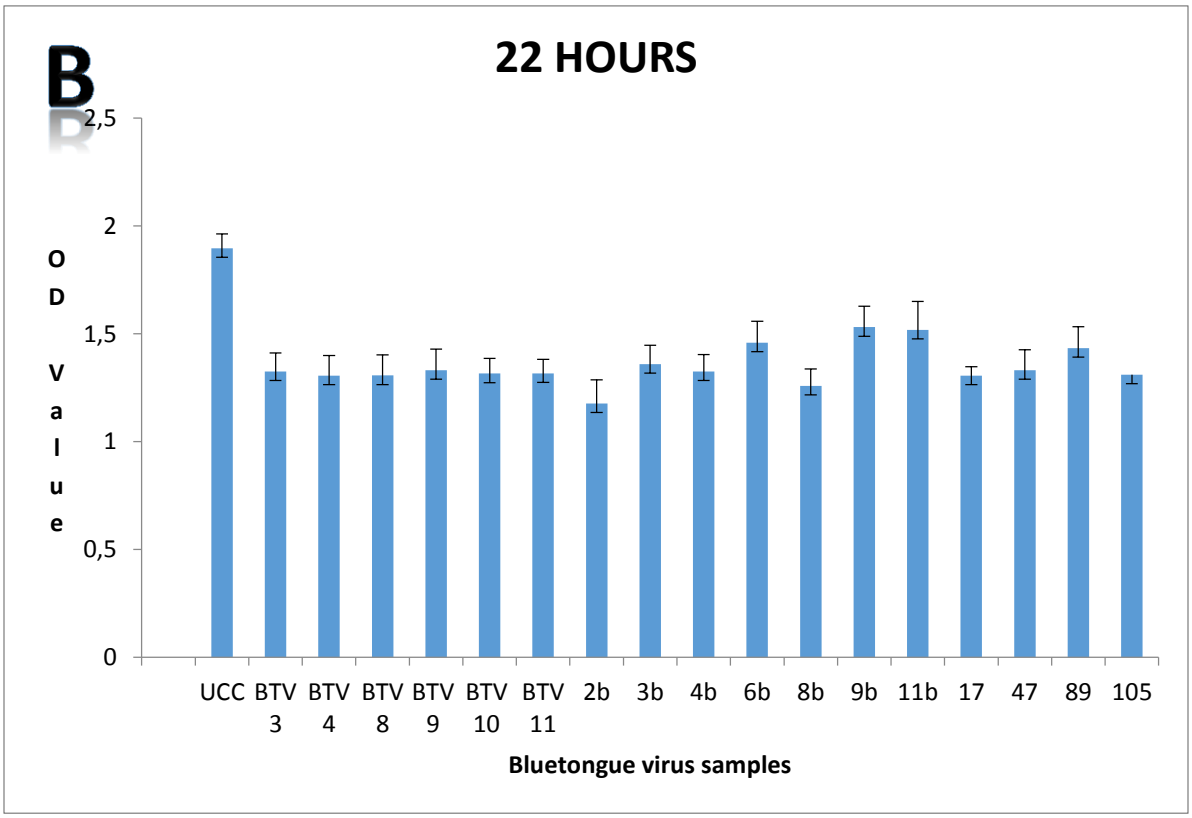
Light Blue: BTV-11

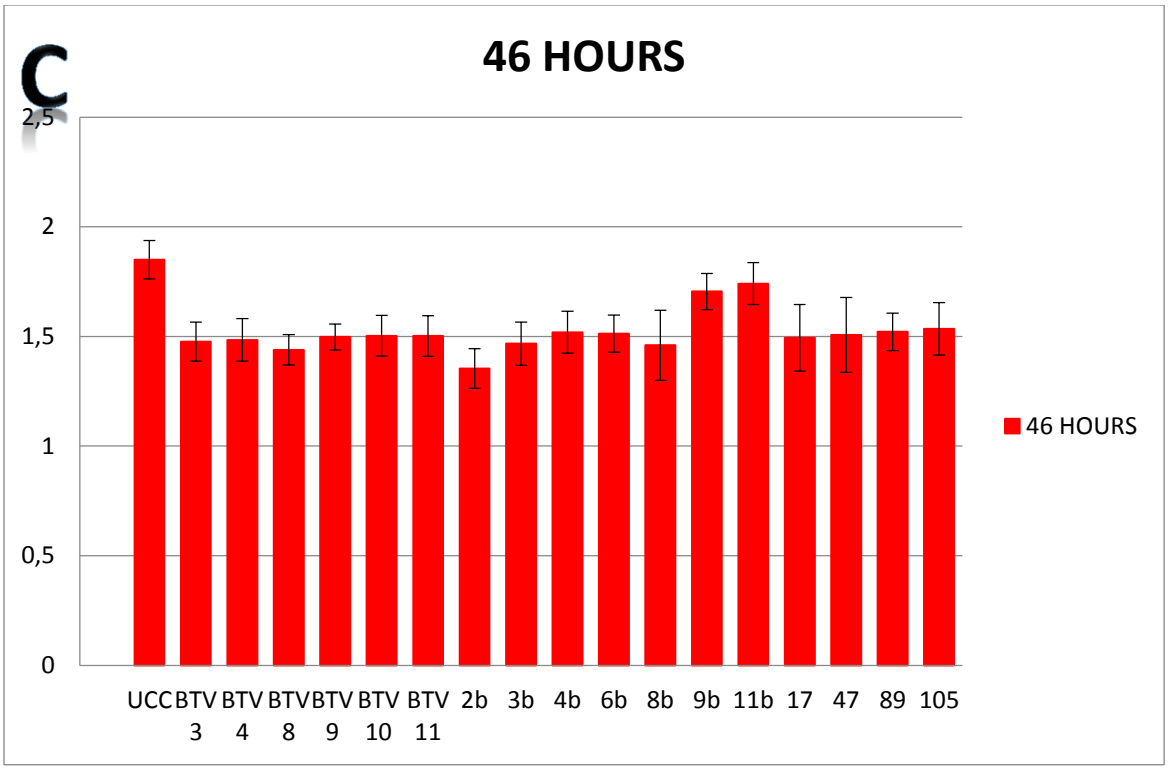
3.9 Cell Viability and cytotoxicity assays

3.9.1 CellTiter-Blue Cell Viability Assay

Four repeats of each of the samples were done using a 96-well plate and an average of the results was used to draw Graphs A-D. The viral isolates were all diluted to the viral $TCID_{50}$ with the lowest concentration i.e.$TCID_{50} = 10^{4.7}$. Graph A represents the metabolic activity of the Vero cells 4 hours post infection with each of the viral isolates. The OD values represents the metabolic activity of each of the cells illustrated in Figure 3.17, high metabolic activity will be measured as a high OD value. All the samples were below 0 with the untreated cell control (UCC). The untreated cell control (OD reading: 1.847) in Graph B are much higher than any of the other cells indicating the validity of the test as the cells in the control were not affected by the dye and the metabolic activity were normal. Samples 9b (OD reading: 1.53) and 11b (OD reading: 1.518) show higher OD values than the rest of the samples, therefore it is assumed that they have the least cytotoxic effect, while sample 2b (OD reading: 1.176) have the lowest OD values at this time point and seems to be the most cytotoxic. In Graphs C at 46 hours after infection there is little variation between the samples. There is no visible variation between the parental viruses. Samples 9b and 11b however are the outliers and seem to be the least cytotoxic. Graph D illustrates that sample 2b is the most cytotoxic at 72 hours post infection as the cells in this well have the lowest metabolic activity (OD reading: 1.029), while sample 9b (1.589) is the least cytotoxic (OD reading: 1.589) thereby representing the highest metabolic activity.







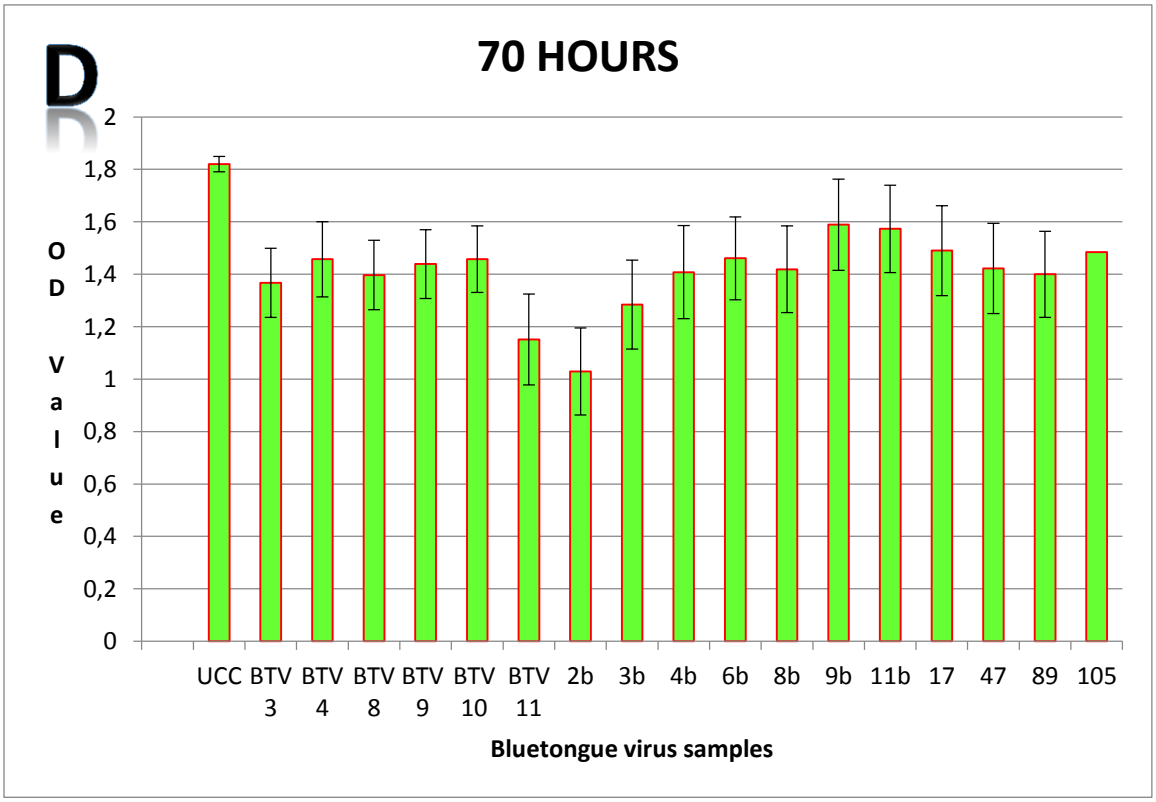
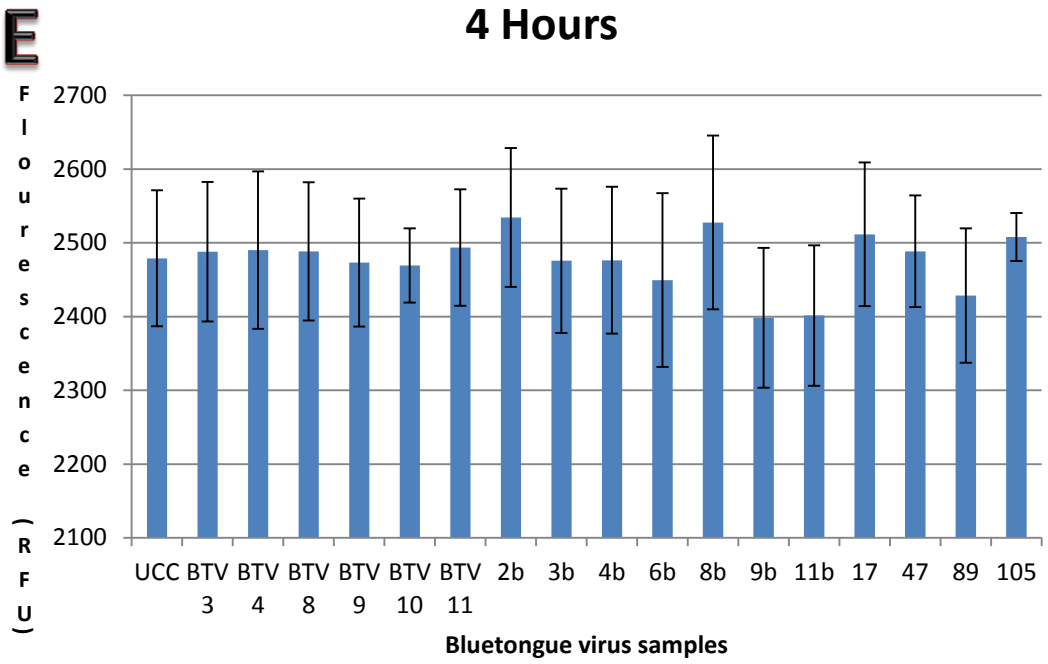


Figure 3.17: Graphs A-D represent the metabolic activity of Vero cells at 4, 22, 46 and 72 hours after infection with the parental viruses and the 11 viral isolates.

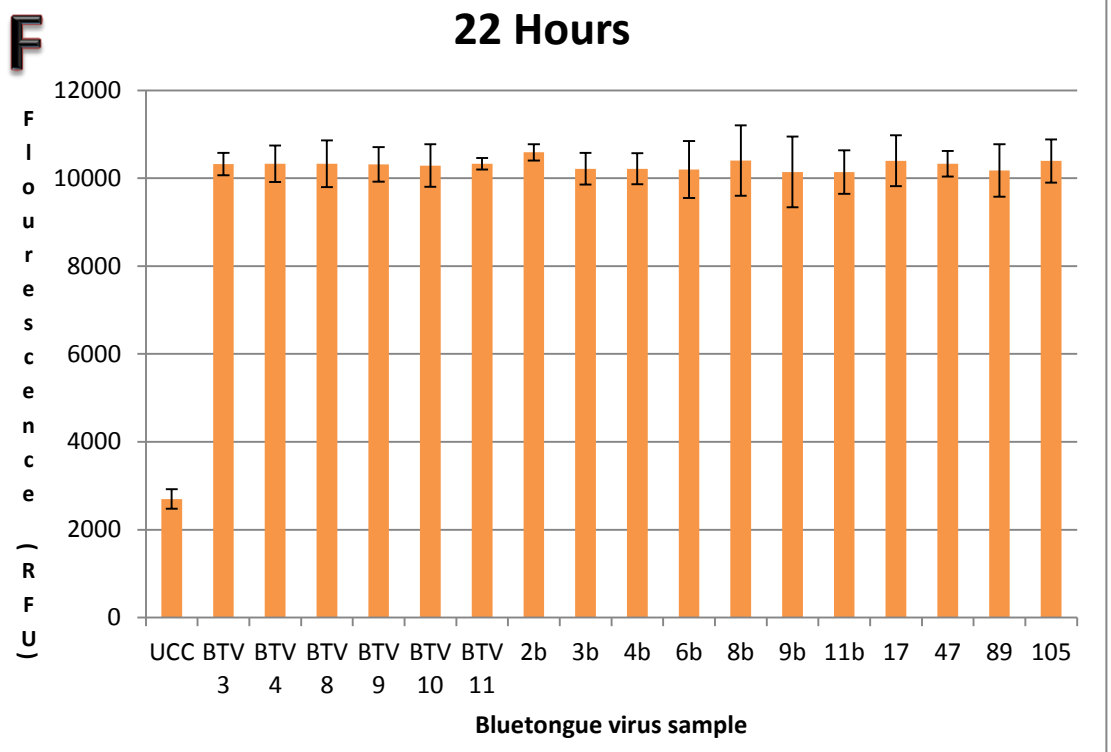
3.9.2 CellTox-Green Cytotoxicity assay

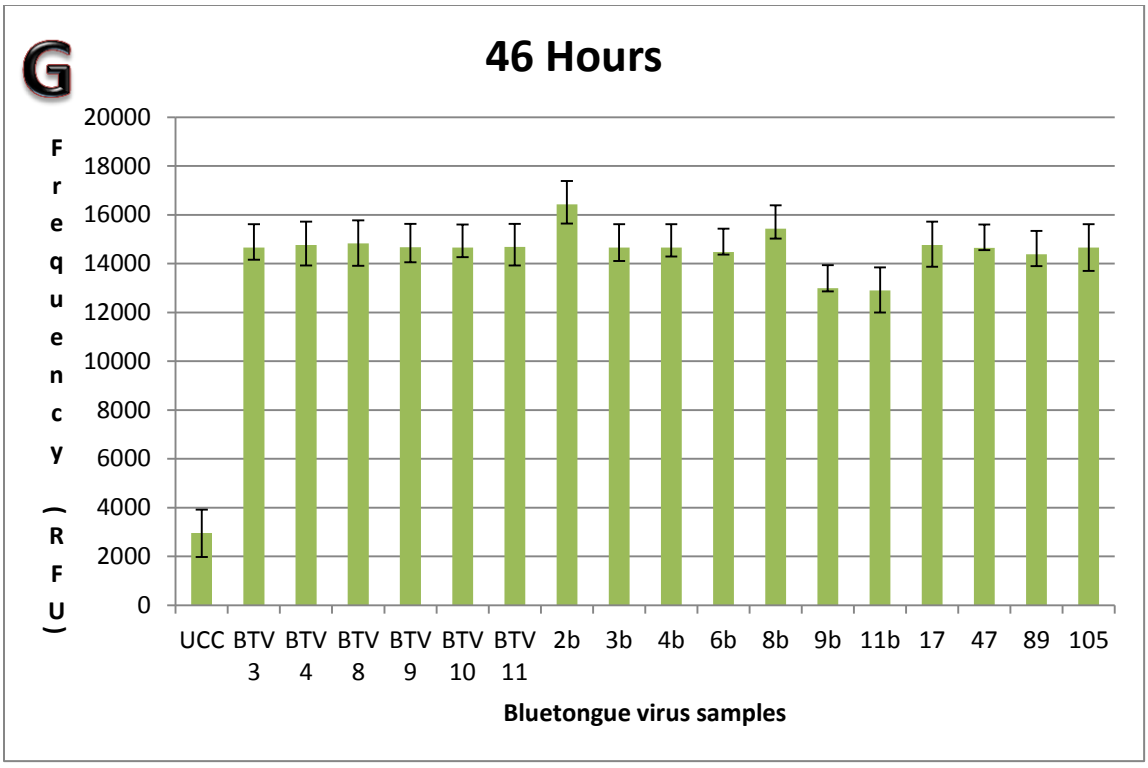
Four repeats of each of the samples were done using a 96-well plate and an average of the results was used to draw Graphs E-H. The viral isolates were all diluted to the viral $TCID_{50}$ with the lowest concentration. All the viral isolates including the parental viruses had a $TCID_{50} = 10^{4.7}$. Graph E represents the cytotoxic effect the viral isolates had on the Vero cells 4 hours after infection. The fluorescence reading represents the dead cell, cells that have lost membrane integrity are permeable to the dye, and it can then bind the DNA. It seems that the effect of the virus on the cells can still be negligible at this point. The untreated cell control's OD reading in Graph F (22 hours post infection) is much lower than any of the other cells. There was very little variation between the samples and very little variation between the parental viruses and the virus isolates at 22 hours. In Graphs G (46 hours post infection) there is also little variation between the parental viruses but samples 9b and 11b have the lowest fluorescent readings (9b: 12986,63 and 11b: 16434.56 fluorescence (RFU)) and therefore seem to be the least cytotoxic, while sample 2b had the highest fluorescence reading indicating that it is the most cytotoxic. At 72 hours post infection virus 2b was the most cytotoxic with a fluorescence reading of 28953.35 RFU as the cells infected by the virus had the highest fluorescence reading. Sample 9b (20356 RFU) and 11b (20857.56 RFU) were the least cytotoxic as wells infected with these viruses had the lowest fluorescence reading at this time point (Figure 3.18).

E



F





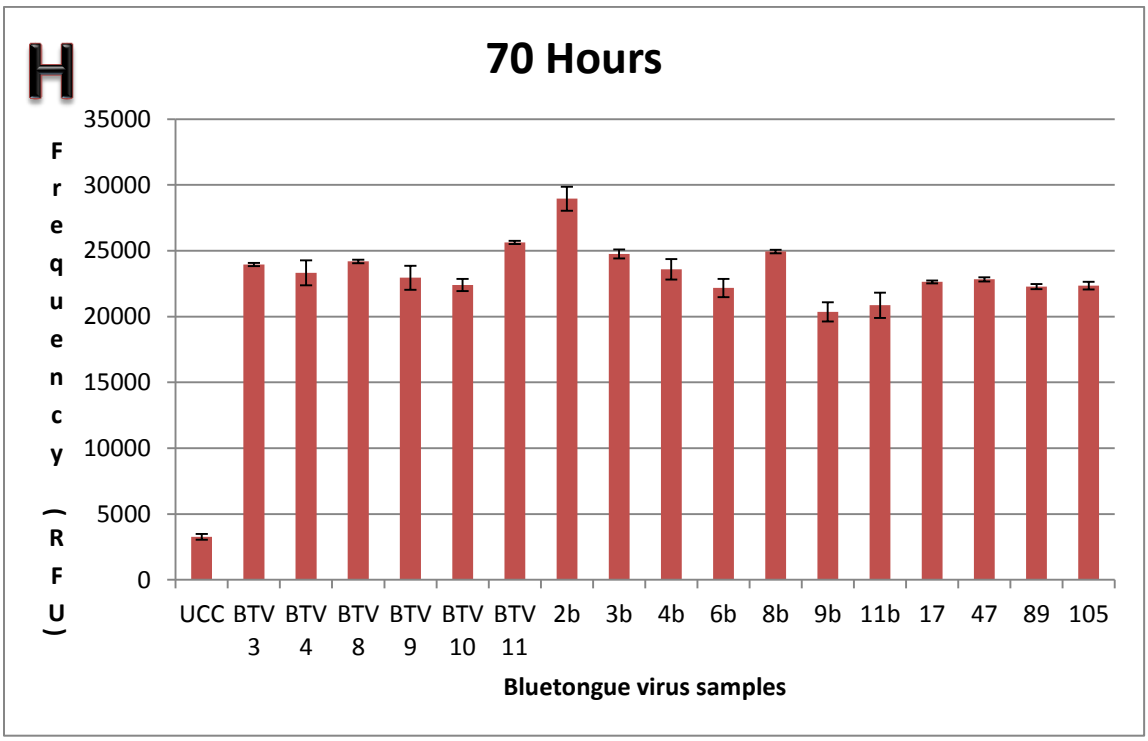


Figure 3.18: Graph E-H represents the florescence reading after the CellTox green cells binds to the genomic material after apoptosis. The readings were taken at 4, 22, 46 and 70 hours after cells were infected with each of the viral samples.

Chapter 4

Discussion

Bluetongue has been reported globally and affects hosts including sheep, goats and cattle. Sheep are normally severally affected while goats and cattle shown mild or no clinical signs. Severe clinical signs and fatalities were however recorded between 1955 and 1980 in cattle that had not been exposed to BTV before in areas such as Israel, Spain and Portugal (Lopez & Sanchez, 1858; Parsonson, 1990). During the recent outbreaks in north-west Europe in 2006, 10% of cattle, infected with BTV serotype 8, were affected and the mortality rate reached a high of 10% (Darpel *et al*., 2007). In the Netherlands in 2006 BTV serotype 8 caused disease in sheep and some wild ruminants but no clinical signs were observed among cattle and goats. However a study on Dutch dairy goats infected with BTV serotype 8 recorded mild symptoms including apathy, dysphagia, lameness and diarrhea while one of the goats with no clinical signs were also positive for BTV using PCR (Backx *et al.,* 2007). However, during outbreaks in Europe in 2008, cattle were severely affected and showed overt clinical signs (Wouda *et al*., 2009; Elbers *et al*., 2008).

Cattle in South Africa are normally not affected by BTV outbreaks and are considered amplification and/or maintenance hosts of the virus (Metcalf & Luedke, 1979). The prevalence of BTV in cattle in South Africa is however not known. Outbreaks of BT were reported in 1996/7 in the Delareyville District in the Northern Province and in Donkerhoek in Bronkhorstspruit District, Gauteng Province where clinical signs including coronitis, lacrimation, high temperature, haemorrhagic diarrhoea and a 42% decrease in milk yield have been recorded (Barnard *et al.,* 1998; Coetzee *et al*, 2012). No other outbreaks amongst cattle have been reported in South Africa. In this study cattle were bled (n=2168) in different geographical regions in South Africa e.g. Gauteng, Mpumalanga and Northwest Province and a total of 96.5% of the cattle tested positive to BTV antibodies.

Sheep are vaccinated annually while cattle and goats are not vaccinated in South Africa. A modified live attenuated vaccine is used and it has been reported that vaccine virus replicates in vaccinated sheep to titers that are high enough to permit infection of midges and allow subsequent infection

transmission to other animals (Ferrari *et al*., 2005; Veronesi *et al*., 2005). This contributes to a pool of BTV serotypes circulating in the field in South Africa which includes both vaccine and wild virus.

The segmented dsRNA genome of BTV gives the virus the ability to reassort and can recombine its genome segments in vertebrate hosts and vectors if these are simultaneously infected with more than one strain or serotype of the virus (Bodewes & Worobey, 2011; Saegerman *et al*., 2007). There are multiple known hosts susceptible to BTV including wildlife and when infected with more than one strain of the virus, cattle and these hosts could provide opportunities for reassortment of the virus genome. The consequences and the rate of reassortment are impossible to predict. However, Janowicz and co-workers (2015) have identified different segments that can determine the virulence of the virus. The primary pathogenetic determinants illustrated to be NS3 and VP2 but multiple segments including VP1, VP5, VP4, VP6 and VP7 contribute to the virulence of the BTV. No markers have been identified and no information on the role of the exchange of different segments in the phenotypic expression of the virus is available (Nomikou., *et al* 2015; Shaw *et al.,* 2013).

Cattle negative to BTV antibodies were used in the study and were inoculated subcutaneously using Bottle B of the vaccine and intravenously (IV) in the jugular vein using a wild BTV serotype 4. The reason for the two different routes of inoculation was that the vaccine was administered in the way prescribed by the manufacturer and the researchers were aware of the fact that it might cause anaphylactic reaction in the animals if given IV. The IV inoculation of the wild type virus was motivated by previous studies that were done on cattle (MacLachlan *et al*,. 1990, Oberst *et al*., 1985). In retrospect it is realized that it would have been more ideal if the cattle were inoculated subcutaneously with both the wild type virus and the vaccine. The route of administration would then mimic the transmission of the virus from *Culicoides* midges to the animals in field situations.

The cattle showed no adverse effects or severe stress during the trial. Two of the cattle that were inoculated with the vaccine and the wild type virus showed a mild temperature with no appetite one day post infection, but both temperatures and appetite returned to normal on Day 2 post infection. The cattle showed no signs of distress and all of the cattle gained weight during the trial. No virus could be detected in four animals (Bovines 1, 2, 3 and 6) on Day 33 post infection using

virus isolation but two of the animals (Bovines 4 and 5) were viraemic for up to 39 days post infection. A previous study reported that cattle can be viraemic for any time between 14 and 100 days post infection (Sellors & Taylor, 1980; Singer *et al*., 2001) while viraemic periods of up to 54 days post infection have been reported in sheep (Richards *et al.,* 1988; Kambouti *et al.*, 1999). The long viraemic periods in both sheep and cattle provide ideal circumstances for viral reassortment to occur in the hosts and for *Culicoides* vectors to become infected and to transmit the virus to susceptible hosts.

The isolation and characterization of the vaccine serotypes (BTV serotypes 3, 8, 9, 10 and 11) using the plaque neutralization test was previously described by Howell *et al*., (1970) and Poli *et al*., (1982). Type specific antiserum was used to neutralize a specific serotype could be isolated and plaque purified. Serotype 8 was however a problem to isolate. This could be due to a low concentration of virus in the vaccine or that BTV serotype 8 was slower to form plaques on Vero cells and that the other serotypes were purely more dominant in replicating and causing CPE. Bluetongue virus serotype 8 was identified by means of a RT-PCR from selected plaques using primers designed by Mertens *et al*, (2007) (Figure 3.2). Sequence data confirmed the identity of the parental/vaccine virus.

Although the vaccine/parental viruses illustrated unique profiles when they were compared to each other using PAGE (Figure 3.3) and the correct serotype were present, sequence data obtained from vaccine strains and the analysis by phylogeny showed that not all the vaccine/parental viruses were pure. The results confirmed that segment 3 and 10 were similar using bootstrap values in both BTV serotypes 3 and 10 (Figure 3.9). Segment 7 of BTV serotype 11, serotype 8 and serotype 10 were similar to each other (Figure 3.13). Since the vaccine serotypes were isolated directly from the vaccine bottle using plaque forming units and the original seed vaccine serotypes to produce the vaccine were not used, it is not clear what the status of the original vaccine serotypes is. However it is possible but not most likely that reassortment of these segments could have happened within the one passage on cell culture.

Virus isolation from animal blood samples were done using two methods; directly from the buffy coat of collected blood samples and after one passage of blood samples on cell cultures. The direct isolation from the buffy coat was the preferred method as it would minimize the possibility of genome segment reassortment occurring between two or more parental viruses on cell culture (Stott *et al.*, 1987; Bonneau *et al.,* 2002). This method was however extremely difficult to conduct since most of the buffy coat samples that were inoculated onto Vero cells coagulated and when they were washed with PBS, the cells detached. Plaques were also very slow to form and in many cases no plaques formed before the cells died from natural processes. This might have been due to the low concentration of virus in blood samples. Only 13 plaques were picked successfully from the buffy coat using this method.

The next option was to passage the buffy coat once on Vero cells before performing the plaque forming assay. This was not an ideal method because the buffy coat contains many different serotypes of the virus and the exchange of segments could occur when they are passaged once on cell cultures (Ramig *et al*., 1989). Thus the number of passages was limited to one, in order to minimize the chance of this to happen. Another 136 plaques were successfully picked using this method.

When the samples were compared to each other using PAGE gels (Figure 3.4) all the samples obtained from the cell culture passaged buffy coats were from the same serotype and that resembled the electropherotype of BTV serotype 4. The reason for this can be due to the fact that the fastest multiplying viruses were first selected while the rest of the strains might have been too slow in causing CPE for the formation of plaques. Another reason could also be due to the fact that BTV serotype 4 was inoculated IV while all the vaccines strains were inoculated subcutaneously, resulting in the high titers of BTV serotype 4 compared to the rest of the vaccine serotypes. It is also expected that BTV serotype 4 being the wild type virus should be more virulent and faster in replicating than vaccine viruses.

The majority samples that were picked directly from the buffy coat were also very similar to BTV serotype 4 except for two samples. Samples 2b and 6b illustrated unique profiles (Figure 3.5) and

confirmed genome segment shifts. This confirms that genomic shifts could only have happened within the animal during the viraemic period and not during replication on cell cultures. The electrophoretic profiles from all the samples were then analyzed with restricted fragment length polymorphism RFLP cluster analysis software; BioNumerics. This program eliminates all gel errors and detects possible segment shifts. The results obtained using this software illustrated sets of samples that were not related to any of the parental viruses and BTV 4 strains, and it's assumed that they had unique profiles (Figure 3.6).

The samples that were selected based on the data obtained by Page profiles and the BioNumeric program for further characterization were 5 isolates that were directly picked from the buffy coat; they were identical to each other but not identical to any of the parental viruses. Another 3 samples from the passaged buffy coats were selected that resembled no other samples, including the parental viruses. The last selected sample was unique and did not resemble any other sample or any of the parental viruses. Two samples showing unique profiles using PAGE were also included into the sample set (Table 2.3). Next generation sequencing was done on 11 samples and sequence data demonstrated that all the selected samples that were identified to be unrelated to the parental viruses using the Bionumerics software were similar to BTV serotype 4.

It is clear that the data obtained by the BioNumerics software is not discriminative enough. Although there is no evidence of the analysis of dsRNA from viruses using BioNumerics, one of the reasons the non-discriminative data could be due to the fact that no RNA size ladders were used in any of the PAGE gels. It is not possible to eliminate gel errors properly without a ladder. Parental vaccine viruses were used as reference material instead of a RNA size ladders. This is therefore not a valid technique and other methods should be considered to determine band shifts using PAGE. Previous studies using BioNumerics to analyze band patterns have been conducted on yeast strains for the production of wine (Vaudano & Garcia-Moruno, 2008), various bacteria e.g. *Pseudomonas aeruginosa* (Pirnay *et al*., 2002), human probiotic bacteria (Temmerman *et al.,* 2003), *Mycobacterium tuberculosis* (Ferdinand *et al*., 2003; Hasan *et al*., 2006) and *Bacillus anthracis* (Lindler *et al*., 2005). No research regarding the analysis of band patterns of dsRNA viruses could be found.

Full genome sequence data of the 11 selected samples were compared to each other using neighbour joining trees (Figure 3.8-3.17). Each of the ten segments was compared to each other and to the segments of the 6 parental viruses. Each of the segments in the trees had similar results. The majority of the samples were grouped with BTV serotype 4. These remains constant with all 10 segments and no segment exchange were noticed with the samples (Clade 1, Figures 3.7-3.16). However, all segments of two samples namely 2b and 6b (Clade 2, Figures 3.7-3.13 and 3.15-3.16) grouped with BTV serotype 9 except segment 8, both samples 2b and 6b grouped with serotype 8 (Figures 3.14). A colour map was used to illustrate all the cumulative results of the phylogenetic analysis (Table 3.3). The map represents genome segments of each of the parental viruses and the 11 isolated viruses and clearly illustrated the exchange of segments. This is however not a heatmap; heatmaps have been used before by other studies to illustrate the intensity or frequencies of reassortment of the different segments but this was not possible since the reassortment frequency of this study was too low (Galindo *et al.,* 2012).

In order to demonstrate whether reassortment viruses may demonstrate unique properties as compared to the parental viruses, cell viability and cytotoxicity assays were conducted. In these assays the ability of reassortment viruses to reduce cell viability and/or damage the cell membrane was compared to that of the parental viruses at different time points post infection, following infection of cells with the parental and reassortant viruses at the same titer. The sample set was used in a cell viability assay. This assay mainly determines their ability of interfering with the metabolic activity of cell culture (i.e. Vero cells). The CellTiter-Blue Cell viability test indicates whether the Vero cells were able to maintain viable metabolic activity when infected with each of the virus serotypes and has been used before to test the effect of inhibitors and drugs on different cancer cells (Heuckmann *et al*., 2011; Prohallad *et al.,* 2012). The cell viability test were valid since the untreated cell control was unaffected by the dye. Graph B (Figure 3.19) illustrates that little variation between the metabolic activity of the samples were noticeable at 22 hours. Variation in the metabolic activity of the BTV virus infected cells however became noticeable at 46 and 72 hours (Graph C and D). Two of the samples 9b and 11b were outliers showing cells with a higher metabolic activity than the rest of the isolated viruses and the parental viruses. Another outlier was sample 2b showing cells with the lowest metabolic activity (Figure 3.17).

Variations in cytotoxicity between the parental viruses were distinguishable and BTV serotype 11 proved to be the most cytotoxic while BTV serotype 10 was the least cytotoxic. Sample 2b was more cytotoxic than any other virus isolate and was even more cytotoxic than BTV serotype 11. Sample 2b is one of the two reassortant viruses and the segment exchange might be the cause of the more cytotoxic characteristic.

Another cytotoxicity test was used to determine the rate if cytotoxicity. The CellTox-Green Cytotoxicity assay measures the changes in the membrane activity of each cell and has been used to test the toxic effect of chemicals and drugs on cell culture (Bradlaw, 1986). The Cyanine dye only binds to genomic material when the membrane integrity has been compromised. The cytotoxicity assay is valid since the UCC remained unaffected by the Cyanine dye. Graph E and F (Figure 3.18) illustrates the cytotoxic effect of the parental and isolated viruses on Vero cells at 4 and 22 hours and it is very difficult to make any conclusions at this point in time. Graph G and H illustrates that at 72 hours post infection sample 2b was the most cytotoxic and samples 9b and 11b were the least cytotoxic. At 72 hours (Graph H) it is noticeable that BTV serotype 11 was the most cytotoxic and BTV serotype 10 was the least cytotoxic parental viruses. Sample 2b was the most cytotoxic when compared to the other viruses including the parental viruses. The results of the CellTox-Green Cytotoxicity Assay were conclusive with the results of the CellTiter-Green Cell Viability assay. The results were conclusive with both assays and it was clearly demonstrated which viruses were more cytopathic.

Samples 2b and 6b were unique by PAGE profile analysis and also showed reassortment using sequence data and phylogeny. Sample 2b was also the outlier in the cell viability assay and proved to be the most cytotoxic. This confirms that virus reassortment of a single genome segment can lead to change in phenotypic expression which can lead to e.g. a modification in viral virulence (O'Hara *et al*., 1998). These results must however be confirmed *in vivo,* since there is no guarantee what type of phynotypic expression will be evident/seen in the field because of variable selective pressures (Nomikou *et al*., 2015).

In this study the generation of reassortant viruses between vaccine strains of BTV was clearly demonstrated in cattle. Segment 8 was exchanged between two vaccine serotypes 8 and 9. This segment encodes the non-structural protein NS2 which is a highly charged hydrophilic protein and is the only protein to undergo phosphorylation (Kar *et al.,* 2007*;* Huismans *et al.,* 1987: Devaney *et al.,* 1988). The protein has a single stranded RNA binding activity as well as phosphatase activity suggesting that the protein has a role in the recruitment of RNA for replication (Horstcroft & Roy, 2000).

The influence of segment preference during packaging was studied before by Shaw and co-workers (2013) and illustrated that there is no structural constraints when the 10 segments of BTV are packaged as well as no favors influencing the packaging. The segment exchange is more likely influenced by factors such as host immunity, vector specificity and the existing distribution of field and vaccine serotypes. The reassortment of segment 8 in sample 2b clearly demonstrated faster growth in cell culture as well as an increase in cytotoxic effect on Vero cells. In both the CellTiter-Blue Viability assay (Figure 3.19) and CellTox- Green cytotoxicity assay (Figure 3.20) it was evident that this sample is overall more cytopathic than any of the parental /vaccine or wild type virus that were initially used to vaccinate the cattle.

Reassortment between viruses is not limited to vaccine serotypes or closely related field isolates. Maan and co-workers (2012) illustrated that reassortment could occur between unrelated viruses from e.g. the Western and Eastern lineages. A full genome analysis of a BTV serotype 2 isolate from India illustrated that 9 segments were unique and belonged to the Eastern topotype while segment 5 belonged to the Western topotype. This was the first study to illustrate reassortment between the two topotypes. Another example from India was the reassortment between field isolates of BTV 21 and 16. Whole genome sequencing confirmed segment 6 of BTV 21 clustered with BTV 16 and showed a 97.6% nucleotide similarity (Shafiq *et al*, 2013). A study in 1987 illustrated reassortment between 2 virus isolates after a Holstein Bull was inoculated with BTV 11 and 17. The isolated viruses were compared with electrophoretic profiles and 16 unique profiles were identified (Stott *et al*., 1987). A more recent study in Europe illustrated how BTV 16 a field isolates from Italy and vaccine strain BTV serotype 2 exchanges segment 5 (Batten *et al*., 2008). Whole genome sequences were done on 116 BTV isolates from Europe, the Mediterranean region and African countries

collected during 1958 -2012. The isolates were compared to 4 MLV serotypes and the 26 serotypes available on Genbank and revealed reassortment between field isolates and vaccine strains as well as reassortment between field isolates (Nomikou *et al*., 2015). This study confirmed previous reports that there is no way of predicting how the virus will reassort or how frequent the virus will reassort.

Kinetic studies relating to BTV reassortment have illustrated different frequencies in different hosts; an approximate 5% of reassortant progeny were isolated from viraemic sheep (Samal *et al.,*1987b) while in bovines it was significantly higher (89%) (Stott *et al*., 1987). Reassortment studied in a mixed group of *Cullicoides* midges reported reassortment between 7-78% (Samal *et al.,* 1987a). This is concerning since female *Culicoides* takes up multiple blood meals with the possibility of multiple serotypes and stays infected for most of their life span (Mellor *et al.,* 2000). A high frequency of reassortant viruses have been isolated from *Culicoides* midges (Samal *et al*., 1987a) and BT vaccine virus serotype 2 (a live attenuated vaccine produced by OBP) has been shown to circulate in cattle and *Culicoides* midges (Ferrari et al. 2005).

The low percentage reassortment in this study might be due to the limited amount of viruses that were isolated directly from the buffy coat. The study was limited to a small animal group with one cycle of interaction between viruses. The animals in the study had a short viraemic period (33-39 days) when compared to other studies with viraemic periods up to 100 days (Sellors & Taylor; 1980). The low numbers may also be due to the fact that the wild type strain were administered IV resulting in high titers of BTV serotype 4 in a very short period of time while the vaccine serotypes were administered SC and as a result they were slower to replicate to high titers.

In a study by Dungu and co-workers (2004) on the production and circulation of BT MLV strains in South Africa the authors implied that reassortment is unlikely to occur between vaccine serotypes and circulating field serotypes. Reassortment was described as a rare event unless it was driven by environmental factors (Dungu *et al*., 2004). This was largely based on the hypothesis that the period between the recommended vaccination period and the actual disease period was long enough to prevent the co-circulation of the vaccine and the field serotype, by assuming that enough time

would pass for the MLV titers to drop low enough before the animals become infected with the field strains thus implicating that the occurrence of reassortment is unlikely (Dungu et al., 2004; Saegerman et al., 2007). Full genome sequencing was not yet available to test the theory and the ability of vaccine and field serotype strains to reassort was now demonstrated by multiple studies (Batten et al. 2008, De Mattos et al., 1994; Heidner *et al*, 1991; Maan *et al*. 2010). Recently Nomikou *et al*., (2015) illustrated that circulating vaccine serotypes contributed segments to the circulating field serotypes in Europe resulting in phenotypic and genotypic variation among the circulated reassorted progeny. The study used full genome sequences of 116 isolates as previously mentioned. No recent studies were however conducted in South Africa to prove this hypothesis and no studies have been done in cattle in South Africa before. The current study is the first to evaluating the effect of reassortment of vaccine strains in experimentally infected cattle.

Reassortment assists virus to overcoming any evolutionary constraints (Bonneau *et al*., 2001) e.g. BTV serotypes that remain unselected because of poor replication ability could undergo reassortment with another serotype and acquire segments to facilitate the ability to replicate or spread easier in an ecological niche. The emergence of reassortment strains assist in the formation of dominants serotypes in outbreaks or regions with advantage over the original field and vaccine strains (Batten *et al*., 2008).

The live attenuated vaccine serotypes introduced by the annual vaccination of BTV and the presence of 22 endemic BTV serotypes that circulates in the field in South Africa increase the chance of co-circulation of viruses and provides endless opportunities for the generation of BTV reassortants (Gerdes, 2003). This phenomenon provides opportunities for different serotypes to simultaneously infect the same animal. Reassortment therefore contributes to the high heterogeneity of BTV's and might be responsible for the large variation in virulence between field strains. This might also lead to the possibility of emerging viruses of unknown characteristics and a more structured surveillance strategy is required to identify these viruses in the field.

4.1 References:

1. Backx, A., Heutink, G., Van Rooij, E.M.A. and Van Rijn, P.A. (2007). Clinical signs of bluetongue virus serotype 8 infections in sheep and goats. *Veterinary Record*, 161(17), 591 – 593.

2. Barnard, B.J.H. (1998). Epidemiology of African horse sickness and the role of the zebra in South Africa. *Archives of Virology* (Suppl 14) pp.13-19.

3. Batten, C.A., Maan, S., Shaw, A.E., Maan, N.S. and Mertens, P.C. (2008). A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. *Virus Research*, 137(1), 56-63.

4. Bodowes, R. and Worobey, M. (2011). Homologous recombination in negative sense RNA viruses. *Viruses,* 3, 1358-1373.

5. Bonneau, K.R., Mullens, B.A. and MacLachlan, N.J. (2001). Occurrence of genetic drift and founder effect during quasispecies evolution of the VP2 and NS3/NS3A genes of bluetongue virus upon passage between sheep, cattle and *Culicoides sonorensis*. *Journal of Virology*, 75 (17), 8298-8305.

6. Bradlaw, J. A. (1986). Evaluation of drug and chemical toxicity with cell culture systems. *Toxicological Sciences*, 6(4), 598-606.

7. Coetzee, P., Van Vuuren, M., Stokstad, M., Myrmel, M. and Venter, E.H. (2012). Bluetongue virus reassortment, an overlook aspect of viral evolution with potentially serious implications. *Veterinary Microbiology*, 161, 1-12.

8. Darpel, K.E., Batten, C.A., Veronesi, E., Shaw, A. E., Anthony, S., Bachanek-Bankkowska, K., Kgosana, Bin-Tarif, A, Carpenter, S., Muller-Doblies, U.U., Takamatsu, H-H., Mellor, P.S., Mertens, P.P.C. and Oura, C.A.L. (2007). Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. *The Veterinary Record*, 161(8), 253-261.

9. De Mattos, C.C., de Mattos, C.A., Osburn, B.I. and MacLachlan, N.J. (1994). Heterogeneity of the L2 gene of field isolates of bluetongue virus serotype 17 from the San Joaquin Valley of California. *Virus Research*, 31, 67-87.

10. Devaney, M.A., Kendall, J. and Grubman, M.J. (1988). Characterization of a non-structural phosphoprotein of two orbiviruses. *Virus Research,* 11(2), 151-164.

11. Dungu, B., Gerdes, T. and Smit, T. (2004). The use of vaccination in the control of bluetongue in Southern Africa. *Veterinaria Italiana*, 40 (4), 616-622.

12. Elbers, A. R., Backx, A., Mintiens, K., Gerbier, G., Staubach, C., Hendrickx, G., & van der Spek, A. (2008). Field observations during the Bluetongue serotype 8 epidemic in 2006: II. Morbidity and mortality rate, case fatality and clinical recovery in sheep and cattle in the Netherlands. *Preventive veterinary medicine*, 87(1), 31-40.

13. Ferdinand, S., Sola, C., Verdol, B., Legrand, E., Goh, K. S., Berchel, M., Aubery , A., Timothee, P.J., Pape, J.W. and Rastogi, N. (2003). Molecular characterization and drug resistance patterns of strains of *Mycobacterium tuberculosis* isolated from patients in an AIDS counseling center in Port-au-Prince, Haiti. *Journal of clinical microbiology*, 41(2), 694-702.

14. Ferrari, G., De Liberato, C., Scavia, G., Lorenzetti, R., Zini, M., Farina, F., Magliano, A., Cardet, G.,Scholl, F guidoni, M., Scicluna, M.T., Amaddeo , D., Scaramozzino, P. ans Autorino, G.L. (2005). Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. *Preventative Veterinary Medicine*, 68(2), 103-113.

15. Galindo, R. C., Falconi, C., Lopez-Olvera, J.R., Jimenez-Clavero, M.A., Fernandez-Pacheco, P., Fernandez-Pinero, J., Sanchez-Vizcaino, J.M., Gortazar, C. and De la Fuente, J. (2012). Global gene expression analysis in skin biopsies of European red deer experimentally infected with bluetongue virus serotypes 1 and 8. *Veterinary Microbiology*, 161(1), 26-35.

16. Gerdes, G.H. (2003). A South African overview of the virus, vectors, surveillance and unique features of bluetongue. *Veterinalia Italiana* 40(3): 39-42.

17. Hasan, Z., Tanveer, M., Kanji, A., Hasan, Q., Ghebremichael, S. and Hasan, R. (2006). Spoligotyping of *Mycobacterium tuberculosis* isolates from Pakistan reveals predominance of Central Asian Strain 1 and Beijing isolates. *Journal of Clinical Microbiology*, 44(5), 1763-1768.

18. Heidner, H.W., Iezzi, L.G., Osburn, B.I. and MacLachlan, N.J. (1991). Genetic variation and evolutionary relationships amongst bluetongue viruses endemic in the United States. *Virus Research*, 21(2), 91-109.

19. Heuckmann, J. M., Hölzel, M., Sos, M. L., Heynck, S., Balke-Want, H., Koker, M., Peifer, M., Weid, J., Lovly, C.M., Grutter, C., Rauh, D. & Thomas, R. K. (2011). ALK mutations conferring differential resistance to structurally diverse ALK inhibitors. *Clinical Cancer Research*, *17*(23), 7394-7401.

20. Huisman, H., van Dijk, A.A. and Bauskin, A.R. (1986). In vitro phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. *Journal of Virology* 61, 3589-3595.

21. Huismans, H. E. N. D. R. I. K., van Dijk, A. A., & Bauskin, A. R. (1987). In vitro phosphorylation and purification of a nonstructural protein of bluetongue virus with affinity for single-stranded RNA. *Journal of virology*, *61*(11), 3589-3595.

22. Horscroft, N. J., & Roy, P. (2000). NTP binding and phosphohydrolase activity associated with purified bluetongue virus non-structural protein NS2. *Journal of General Virology*, *81*(8), 1961-1965.

23. Howell P.G., Kümm, N.A. and Botha, M.J. (1970). The application of improved techniques to the identification of strains of bluetongue virus*. Onderstepoort Journal of Veterinary Research,* 37, 59–66.

24. Kar, A.K., Bhattacharya, B. and Roy, P. (2007). Bluetongue virus RNA binding protein NS2 is modulator of viral replication and assembly. *BCM Molecular Biology*, 8 (1), 4. Doi: 10.11186/1471-2199-8-4.

25. Kambouti, M., Mangano, O., Nomikou, K., Mellor, P.S. and Papadopoulos, O. (1999). Duration of Bluetongue vireamia and serological responses n experimentally infected European breeds of sheep and goats. *Veterinary Microbiology*, 64(4).

26. Lindler, L. E., Huang, X. Z., Chu, M., Hadfield, T. L. and Dobson, M. (2005). Genetic fingerprinting of biodefense pathogens for epidemiology and forensic investigation. *Biological Weapons Defense* (pp. 453-480). Humana Press.

27. Lopez, A.C. & Sanchez, C.B. (1958). L'eipootie de fievre catarrhale en espagne (bluetongue). *Bulletin de l'Office Internasionale des Epizooties*, 50, 65-93.

28. Maan, N.S., Maan, S., Nimikou, K., Guimeram M., Pullinger, G., Singh, K.P., Belganahalli, M.N. and Mertens, P.P.C. (2012) The genomic sequences of Bluetongue virus type 2 from India: Evidence for reassortment between Eastern and Western Topotype field strains. *Journal of Virology*, 86 (10), 5967-5968.

29. Maan, S., Maan, N.S., Van Rijn, P.A., Van Gennip, R.G., Sanders, A., Wright, M., Batten, C., Hoffman, B., Eschbaumer, M., Oura, C.A.L., Potgieter, A.C., Nomikou, K. and Mertens, P.P.

(2010). Full genome characterization of bluetongue virus serotype 6 from Netherlands 2008 and comparison to other field and vaccine strains. *PLoS One*, 5(4), e10323.

30. MacLachlan, N.J., Jagels, G., Rossito, P.V., Moore, P.F. and Heidner, H.W. (1990). The pathogenesis of experimental bluetongue virus infection in calves. *Veterinary Pathology Online,* 27(4) 223-229.

31. Mellor, P. S., Boorman, J. and Baylis, M. (2000). Cullicoides biting midges: Their role as arbovirus 515 vectors. *Annual Review of Entomology* 45, 307-340

32. Mertens, P.C.C., Maan, N.S., Prasad, G., Sameul, A.R., Shaw, A.E., Potgieter, A.C., Anthony, S.J. and Maan, S. (2007). Design of primers and use of RT-PCR assays for typing bluetongue virus isolates; differentiation of field and vaccine strains. *Journal of General Virology*, 88(10), 2811-2823.

33. Metcalf, H.E. and Luedke, A.J. (1979). Bluetongue and related diseases. *Proceedings of the Foreign Animal Disease Symposium.* University of Florida

34. Nomikou, K., Hughes, J., Wash, R, Kellam, P., Breard, E., Zientara, S., Palmirini, M., Biek, R. and Mertens, P. (2015). Widespread reassortment shapes the evolution and epidemiology of Bluetongue Virus following European Invasion. *PLOS Pathogens,* 11(8), e1005056. DOI:10.1371/ journal.ppat.1005056.

35. Oberst, R.D., Squire, K.R.E., Stott, J.L. Chuang, R.Y. and Osburn, B.I. (1985). The coexistence of multiple bluetongue virus electropherotypes in individual cattle during natural infection. *Journal of General Virology*, 66(9), 1901-1909.

36. O'Hara, R.S., Meyers, A.J., Burroughs, J.N., Pullens, L, Martins L.A. and Mertens, P.P. (1998). Development of a mouse model system, coding assignments and identification of the genome segments controlling virulence of African horse sickness virus serotype 3 and 8. *Archives of Virology Supplement,* 14:259-279.

37. Parsonson, I.M. (1990). Pathology and pathogenesis of BT infections. *Bluetongue viruses* (pp 119-141). Springer Berlin Heidelberg.

38. Pirnay, J.P., De Vos, D., Mossialos, D., Vanderkelen, A., Cornelis, P. and Zizi, M. (2002). Analysis of *Pseudomonas aeruginosa* oprD gene from clinical and environmental isolates. *Environmental Microbiology* 4(12), 872-882.

39. Poli, G., Stott, J. and Manning, J.S. (1982). Bluetongue virus: Comparative evaluation of enzyme-linked immunosorbent assay, immunodiffusion and serum neutralization for detection of viral antibodies. *Journal of Clinical Microbiology,* 15(1), 159-162.

40. Prahallad, A., Sun, C., Huang, S., Di Nicolantonio, F., Salazar, R., Zecchin, D., Beijersbergen, R.L., Bardeli, A. & Bernards, R. (2012). Unresponsiveness of colon cancer to BRAF (V600E) inhibition through feedback activation of EGFR. *Nature*, 483(7388), 100-103.

41. Ramig, R.F., Garrison, C., Chen, D. and Bell-Robinson, D. (1989). Analysis of reassortment and superinfection during mixed infection of vero cells with Bluetongue virus serotype 10 and 17. *Journal of Virology*, 70(10), 2595-2603.

42. Richards, R.G., MacLachlan, N.J., Heidner, H.W. and Fuller, F.J. (1988). Comparison of virological and serological responses of lambs and calve infected with bluetongue virus serotype 10. *Veterinary Microbiology*, 18(3-4), 277-285.

43. Ruiz-Fons, F., Reyes-Garcia, A.R., Alcaide, V. and Gortazar, C. (2008). Spatial and temporal evolution of bluetongue virus in wild ruminants, Spain. *Emerging Infectious diseases*, 14(6), 951-953.

44. Saergerman, C., Berkvens, D. and Mellor, P.S. (2008). Bluetongue epidemiology in the European Union. *Emerging Infectious Diseases*, 14(4), 539-544.

45. Saegerman, C., Hubaux, M., Urbain, B., Lengele, L. and Berkvens, D. (2007). Regulatory issues surrounding the temporary authorization of animal vaccination in emerging situations: the example of bluetongue in Europe. *Revue scientifique et technique-Office International des Epizooties*, 26(2), 395.

46. Samal, S.K., El Hussein, A., Holbrook, F.R., Beaty, B.J. and Ramig, R.F. (1987a). Mixed infection of *Culicoides variipens* with bluetongue virus serotype 10 and serotype 17: evidence of high frequency reassortment in the vector. *Journal of General Virology*, 68, 2319-2329.

47. Samal, S.K., Livingstone, C.W.J., McConnel, S. and Ramig, R.F. (1987b). Analyses of mixed infection of sheep with bluetongue serotype 10 and 17: evidence for genetic reassortment in the vertebrate host. *Journal of Virology*, 61(4), 1086-1091.

48. Sellers, R.F. and Taylor, W.P. (1980). Epidemiology of bluetongue and the import and export of livestock, semen and embryos. *Bulletin de l'Office International des Epizooties*, 92, 587-592.

49. Shafiq, M., Minakshi, P., Bhateja, A., Ranjan, K. and Prasad, G. (2013). Evidence of genetic reassortment between Indian isolate of bluetongue virus serotype 21 (BTV-21) and bluetongue virus serotype 16 (BTV -16). Virus Research, 173(2), 336 -343.

50. Shaw, A.E., Ratinier, M., Nunes, S.F., Nomikou, K., Carporale., Carporale, M., Goldner, M., Allan, K., Hamers, C., Hudelet, P., Zientara, S., Breard, E., Mertens, P. and Palmarini, M. (2013). Reassortment between two serologically unrelated Bluetongue virus strains is flexible and can involve any genome segment. *Journal of Virology,* 87(1), 543-557.

51. Singer, R.S., MacLachlan, N.J. and Carpenter, T.E. (2001). Maximal predicted duration of vireamia in bluetongue virus-infected cattle. *Journal of Veterinary Diagnostic Investigation*, 13(1), 43-49.

52. Stott, J.L., Oberst, R.D., Channell, M.B. and Osburn, B.I. (1987). Genome segment reassortment between two serotypes of Bluetongue virus in a natural host. *Journal of Virology,* 61(9), 2670-2674.

53. Temmerman, R., Scheirlinck, I., Huys, G and Swings, J. (2003). Culture-independent analysis of probiotic products by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology, 69* (1), 220-226.

54. Vaudano, E., & Garcia-Moruno, E. (2008). Discrimination of *Saccharomyces cerevisiae* wine strains using microsatellite multiplex PCR and band pattern analysis. *Food microbiology*, 25(1), 56-64.

55. Veronesi, E., Hamblin, C., & Mellor, P. S. (2005). Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). *Vaccine*, 23(48), 5509-5516.

56. Wouda, W., Peperkamp, N.H., Roumen, M.P., Muskens, J., Van Rijn, A and Vellema, P. (2009). Epizootic congenital hydranencephaly and abortion in cattle due to bluetongue virus serotype 8 in the Netherlands. *Tijdschr Diergeneeskd*, 134(10), 422-427.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**To evaluate the generation of Bluetongue virus vaccine reassortants in cattle**
PROJECT NUMBER	**V005-14 (Amendment 2)**
RESEARCHER/PRINCIPAL INVESTIGATOR	**Prof. EH Venter**

STUDENT NUMBER (where applicable)	----------
DISSERTATION/THESIS SUBMITTED FOR	**Academic**

ANIMAL SPECIES	**Bovine**	
NUMBER OF ANIMALS	**6**	
Approval period to use animals for research/testing purposes		**July 2014-July 2015**
SUPERVISOR	**Prof. EH Venter**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 28 July 2014
CHAIRMAN: UP Animal Ethics Committee	Signature

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

ANIMAL ETHICS COMMITTEE (AEC)

APPLICATION FOR AMENDMENT (2013)

Amend 2.

Submission Date	24-7-2014	For Administrative Purposes	Project No.	V005/14
AEC approval Date	24-7-2014	Signature (only on approval)		

TITLE	TO EVAULATE THE GENERATION OF BLUETONGUE VIRUS VACCINE REASSORTANTS IN CATTLE		
AMENDMENT	Added bleed to confirm that cattle are still negative for Bluetongue		
RESEARCHER	Prof E Venter	SIGNATURE	EHVenter
UPBRC signature (where applicable)		Application date	24/7/2014

This application form is for obtaining prior approval by the AEC for deviations from the approved application for any desirable or necessary significant changes that may need to be made in the methods and procedures used which may affect the welfare of the animal subjects.

A departure from the approved application that was necessitated by an urgently required response due to a clinical situation to an individual animal is not considered a significant change as long as it remains a once-off event. *A report shall however be submitted to the AEC for noting.*

Significant changes: any act or omission that will increase the impact a procedure or method will have, or is expected to have, on the welfare of animals. This includes, but is not limited to, any increase in animal numbers, any increase in the severity of procedures, any increase in the duration of holding and usage time of the animals, converting terminal surgery to survival surgery, etc. Furthermore, any changes that may decrease the statistical power of an experiment are also considered significant changes.

1. **Explanation and justification**
(Explain in detail why the proposed amendment is required, what events led up to the decision and justify the amendment against the principles of Replacement, Reduction, and Refinement.)

Added bleeds to confirm that cattle are still negative for Bluetongue

2. **Proposed amendment**
*(State in detail the proposed amendment. Refer to the appropriate sections in the previous protocol. Should the amendment affect any section of the original application that requires signatures of the relevant authorised persons, such consent and signatures shall be provided. **In case of extensive amendments, consider submitting a revised proposal.**)*

Animals can't be release from BSL2 unless isolations are negative