EVALUATION OF CROSS PROTECTION OF BLUETONGUE VIRUS SEROTYPE 4 WITH OTHER SEROTYPES IN SHEEP

by

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Submitted in partial fulfilment of the requirements for the degree of

Magister Scientiae (Veterinary Tropical Diseases)

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November 2012

In memory of my beloved late Mum & Dad

DECLARATION

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

Signature

.....

(Gcwalisile Bandliwe Zulu)

Pretoria, ___/____/2012

This dissertation forms part of the requirements for a course work MSc degree research project in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.
These projects carry a weight of approximately 100 credits, and are therefore smaller than projects required for a research-based MSc degree with a weight of 240 credits.
It would be appreciated if reviewers could evaluate the dissertation in that context.

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ABBREVIATIONS

ARC-OVI	Agricultural Research Council - Onderstepoort Veterinary Institute
BT	Bluetongue
BTV	Bluetongue virus
cELISA	Competitive enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immunosorbent assay
LAV	Live attenuated vaccine
MLV	Modified live virus vaccine
NS	Non-structural
OCG	Oxalate –citrate –glycerol anti-coagulant
OBP	Onderstepoort Biological Products
OIE	Organisation International des Epizooties (World Organisation for Animal Health)
PCR	Polymerase chain reaction
PPI	Percentage protection index
RDV	Virology laboratory of Department of Research and Development
RT-PCR	Real-time PCR
SNT	Serum neutralization test
SA	South Africa
SADC	Southern African Development Community
SOP	Standard operating procedure
USA	United States of America
VP	Viral protein

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Bluetongue (BT) is a non-contagious disease of mainly sheep but other ruminants like cattle, goats, and wild ruminants like alpacas, African antelopes and deer can also be affected. It is transmitted by *Culicoides* midges and its occurrence is seasonal, especially after good rains. The disease is subsiding when temperatures drop. The virus is distributed throughout the world in the tropical, subtropical and temperate areas where there are culicoides vectors which can transmit it (Tabachnick *et al.,* 2011). This includes most countries in Africa, the Middle East, India, China, Australia, the United States of America, Canada and Mexico. Until 2008 24 BTV serotypes were known, but from 2008, data on the 25th serotype was published and recently, the 26th serotype has been identified (Hofmann *et al*., 2008; Maan *et al*., 2012a).

In Africa 21 serotypes have been identified and BT is controlled mainly by annual vaccinations using a freeze–dried live attenuated polyvalent BTV vaccine. Currently the vaccine used in the Southern African Development Community (SADC) region is produced by Onderstepoort Biological Products (OBP). The vaccine is constituted of fifteen serotypes of the bluetongue virus (BTV) divided into three separate bottles. Each bottle contains five serotypes. The inoculation procedures are that bottle B is given three weeks after bottle A and bottle C three weeks after bottle B. The full immunity is established three weeks after the last bottle. The vaccine is effective and it induces both humoral and cellular immune response (Dungu *et al.,* 2004). However, the challenge with the vaccine is that during outbreaks, sheep might not have nine full weeks to develop protection against

the disease; and the farmer loses money on treatment and death of animals. Hence the purpose of the study is to determine whether the number of serotypes in the vaccine can be reduced without affecting efficacy; thus shorten the time taken for the full development of immunity after vaccination of animals.

This study is based on previously reported cross-neutralization of specific BTV serotypes in *in vitro* studies by Howell *et al*. (1970) and Dungu *et al*. (2004). Bluetongue virus serotype 4 was selected for this trial and was tested for cross-protection against serotype 4 (control), 1, 8 (unrelated serotypes) 9, 10 and 11 in sheep using the serum neutralization test (SNT).

The unvaccinated animals in all groups reacted to the challenge material. The animals vaccinated with and challenged with BTV-4, showed good immune response. Those animals that were vaccinated with BTV-4 and challenged with BTV-1 which is not directly related to BTV-4 (Howell *et al*., 1970), only 20% of the group was completely protected and did not show clinical signs other than a temperature reaction. The rest showed clinical signs, however the reaction was not as severe as the unvaccinated animal. The animals challenged with BTV-9 and 11 had good protection while those challenged with BTV-10, some showed good protection, some got very sick while others had mild clinical signs.

The results showed that BTV serotype 4 do not only develop a specific immune response but can also protect against other serotypes. Future studies should be done looking at more serotypes but also look at the specific titres used per serotype in the vaccine. The development of cellular immunity should also be taken in consideration. With further studies it should be possible to develop a vaccine with fewer serotypes without compromising the immunity against the disease.

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

Bluetongue (BT) is an insect-borne viral disease of sheep, other domestic ruminants and wild ruminants. It is caused by the bluetongue virus (BTV) that belongs to the genus *Orbivirus* in the family *Reoviridae*. The disease does not only affect southern Africa but in recent years has also spread throughout Europe. The disease has a major impact on the economy and animal welfare within the affected country and has the ability to spread to new geographical areas where naïve animal populations are at risk to suffer severe mortalities**.** Based on the study done by the Scottish government in 2008 (www.scotland.gov.uk/publications/2008), the estimate cost of a BT outbreak could be £100 million per annum including direct and indirect costs. The financial impact of BT in SA is not well established.

The disease is transmitted by *Culicoides* midges and transmission is associated with high activity of these vectors, which commonly takes place after rainy seasons (Elfatih *et al.,* 1987). There is up to 30% mortality in affected animals, lowered production (due to sickness) and temporary infertility in rams (Kirschvink *et al.,* 2009). There is no treatment for the disease but control by vaccination and supportive therapies are used, which have major economic implications. The BTV vaccine is polyvalent and it is consisted of three bottles, each bottle contains five different serotypes which gives 15 BTV serotypes in the vaccine. The disadvantages of the vaccine are: sheep can abort; teratogenic effects on the fetus may be observed when the vaccine is used during pregnancy; and the period from vaccination until the animal achieves immunity against all serotypes is prolonged. Since the farmer needs to vaccinate three times within three weeks, the vaccine is also not practical. In this study the possible cross-protection of different serotypes was investigated in sheep, in order to develop a vaccine with fewer serotypes without compromising the immunity against the disease.

1.2 The disease

1.2.1 Aetiology

Bluetongue virus is a non-enveloped virus which contains a 10-segmented, named 1 – 10, double-stranded RNA genome. Each segment codes for at least one viral protein (VP) which is divided into structural and non-structural (NS) proteins (Verwoerd *et al*., 1979; Gorman *et al.,* 1983). Recently it has been found that segment 9 is coding for a second NS protein, NS4 that plays a role in the virus-

host interaction to counteract the antiviral response by the host (Ratinier *et al.,* 2011). There are seven structural VPs ranging from one to seven of which four (VP2, VP3, VP5 and VP7) are major components and constitute about 94% of the total quantity of proteins. The other three form a minor component of the viral proteins. The VP2 and VP5 form a diffuse outer capsid layer of the virus (Huismans & Erasmus, 1981; Hyatt & Eaton, 1988; Verwoerd *et al.,* 1972) whereas the other five are in the core of the particle. The VP2 is responsible for determining the serotype (Huismans & Erasmus, 1981; Appleton & Letchworth, 1983; Maan *et al.,* 2007).

The three non-structural proteins (NS1, NS2 and NS3 /NS3A) which are produced in infected cells have a role in the assembly of the virion (Roy, 1989). Recently NS4 has been found as discussed above (Ratinier *et al.,* 2011). The NS1 determines the pathogenicity of BTV and it does that by augmentation of virus cell association but it does not transport the virus to the surface, which leads to lysis of infected cells (Mellor *et al.,* 2008) but in another study that was done recently, it was shown that NS1 is a positive regulator of viral protein synthesis by means of translation of viral mRNA (Boyce *et al.,* 2012). The BTV non-structural protein NS2 (encoded by segment 8) is the major component of viral inclusion bodies in infected cells and has been suggested to be involved in virus replication and morphogenesis. It is located in the cytoplasm of the infected host cell (Butan *et al.,* 2010). The NS2 undergoes intracellular phosphorylation and possesses a strong single-stranded RNA binding activity (Kar *et al.,* 2007). The NS3 and NS3A (encoded by segment 10) have been implicated in promoting virus release from infected cells, and affecting cellular functions including protein trafficking and membrane permeability (Celma *et al.,* 2011). The NS4 aggregates throughout the cytoplasm and nucleus. The BTV NS4 prevents degradation of DNA by DNAse (Belhouchet *et al.,* 2011).

A schematic representation of the BTV which shows the position of structural proteins is given in Figure 1 (Roy *et al.,* 2009).

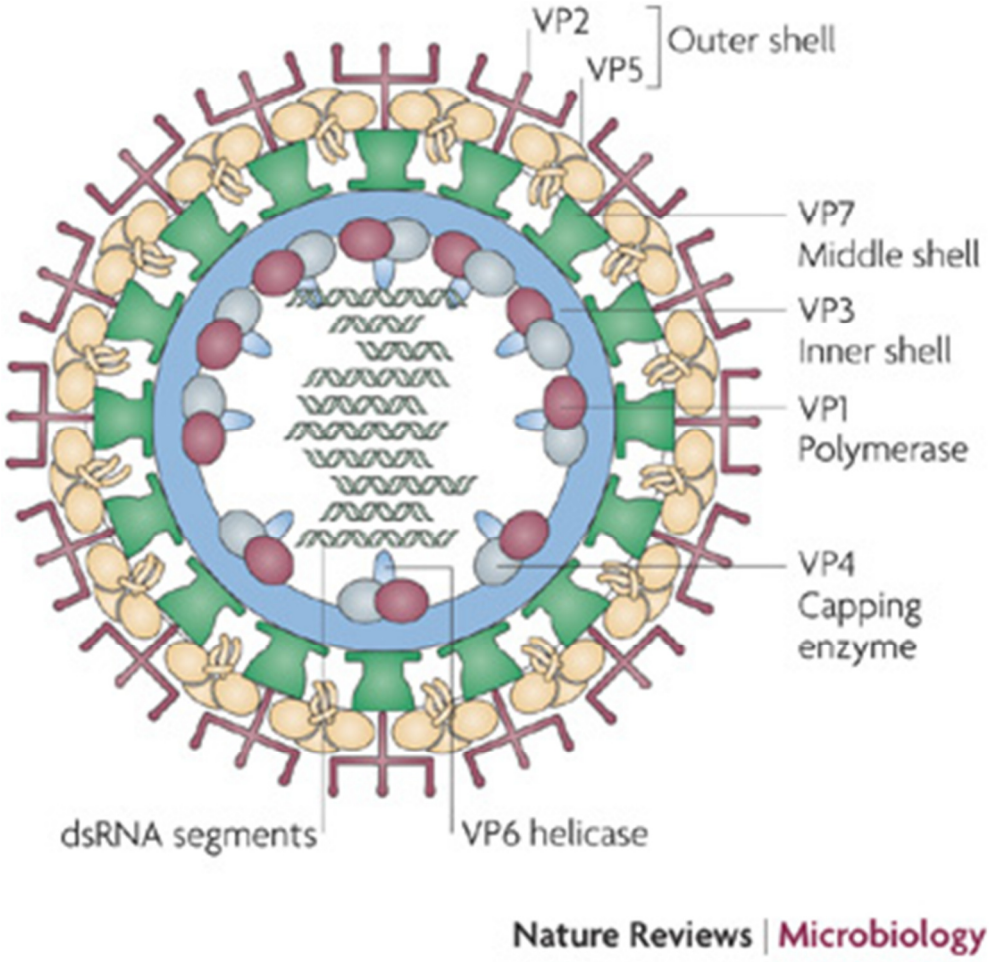

Figure 1: A diagrammatic representation of the intact BTV particle demonstrating the location of the structural proteins of the outer diffuse protein layer (VP2, VP5), the viral core (VP7), sub-core (VP3) and transcriptase complexes (VP1, VP4, VP6) (Roy *et al*, 2009).

Bluetongue virus in blood and tissue samples is stable at 20°C, 4°C and -70°C, and it can survive for years when it is freeze-dried in buffered lactose-peptone (Howell *et al*., 1967). The virus is sensitive to trypsin treatment, extreme pH conditions and purification but readily inactivated by disinfectants containing acid, alkali, sodium hypochlorite and iodophors. It is resistant to ether, ultraviolet, gamma irradiation, chloroform and sodium deoxycholate (Svehang *et al.,* 1966; Verwoerd & Erasmus, 2004).

1.2.2 Vectors

About 17 - 20 of the approximately 1400 species of culicoides are competent BTV vectors (Meiswinkel *et al.,* 2008). Since 1998 there has been a global change in BTV distribution especially in Europe. It has been proposed that global climate change is the cause (Baylis *et al.,* 2005). Different *Culicoides* species are responsible for vectoring the virus in Africa, the Middle East, India, China, North America and Australia. *Culicoides imicola* is the major vector throughout Africa and the Middle East. In other areas in South Africa which are less arid and much cooler, *C. bolitinos* has been implicated in BTV transmission. In the laboratory environment, this species is up to 20 times more susceptible to the virus as compared to *C. imicola* (Venter *et al.,* 1998).

In Australasia the main vectors are *C. brevitalis*, *C. wadaii*, *C. actoni*, and *C. fulvus*. In south eastern America *C. sorensis* is the primary vector (Mellor *et al.,* 2008; Tabachnick *et al.,* 2011) and in central and South America *C. insignis* has been implicated (Tanya *et al.,* 1992; Tabachnick *et al.,* 2011). In Europe *C. obsoletus* and *C. pulicaris* are the main vectors (Savini *et al.,* 2005; Saegerman *et al.,* 2008).

The season and geographic distribution of competent vectors are the keys to the epidemiology of the disease and the areas of BT outbreaks are mostly defined by climate; high rainfall, temperature and humidity favouring the vector cycle with a high increase in the presence of *Culiciodes* spp. The distribution of vectors and the serotypes of BTV circulating in different parts of the world are shown in Figure 2 (Maclachlan & Osburn, 2006). The distribution of BTV is closely linked to the distribution of vector competent midge species. The presence of culicoides is the most important factor in understanding the epidemiology of BT (Gibbs & Greiner, 1994).

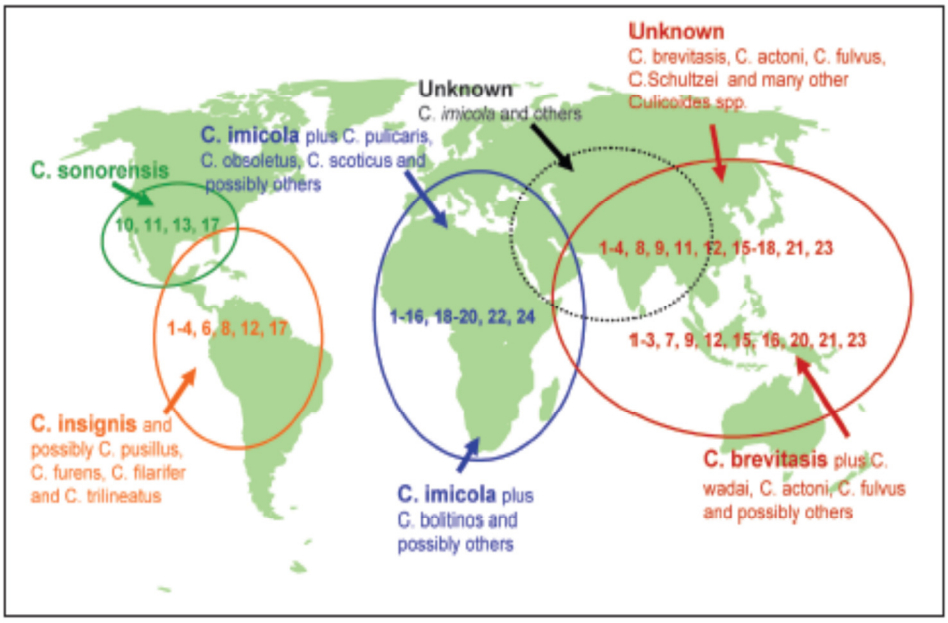

Figure 2: A map demonstrating the distribution of the main *Culicoides* vectors which are responsible for the transmission of different BTV serotypes in different ecological zones around the world (Maclachlan & Osburn, 2006).

1.2.3 Epidemiology

Bluetongue virus has a wide host range mostly affecting sheep, but also goats, cattle and some wild ruminants. Indigenous South African sheep are more resistant compared to European sheep breeds (Spreull, 1905). South Africa has mixed sheep breeds i.e. Merino, Dohne, SA Mutton Merino and Dorper being the most popular breeds (Campher *et al.,* 1998).

Until 2008 24 BTV serotypes were known (Gorman *et al.,* 1983; Knudson & Shope, 1985), a 25th serotype (Toggenburg virus) were identified in 2008 (Hofmann *et al*., 2008) and in 2011, the 26th serotype has been identified (Maan *et al.,* 2012a; Batten *et al.,* 2012). All known serotypes except BTV-20, 21, 25 and 26 are endemic in southern Africa.

Multiple BTV serotypes and strains can co-circulate (Mertens *et al.*, 2007). According to Dr Gerdes, Clinical Virologist at the Agriculture Research Council's Onderstepoort Veterinary Institute (ARC-OVI), (personal communication) BTV serotypes 1 – 9 and 24 are the most prevalent serotypes in SA. The relationship between the 24 BTV serotypes in terms of cross-neutralization *in vitro* by the serum

neutralization test (SNT) is illustrated in Figure 3. From this data it was decided in this study to evaluate the cross-reaction of related serotypes in sheep using BTV-4 as vaccine serotype and challenged with different serotypes as indicated in Table 4. The serotype was selected based on its ability to causing outbreaks and its prevalence in SA.

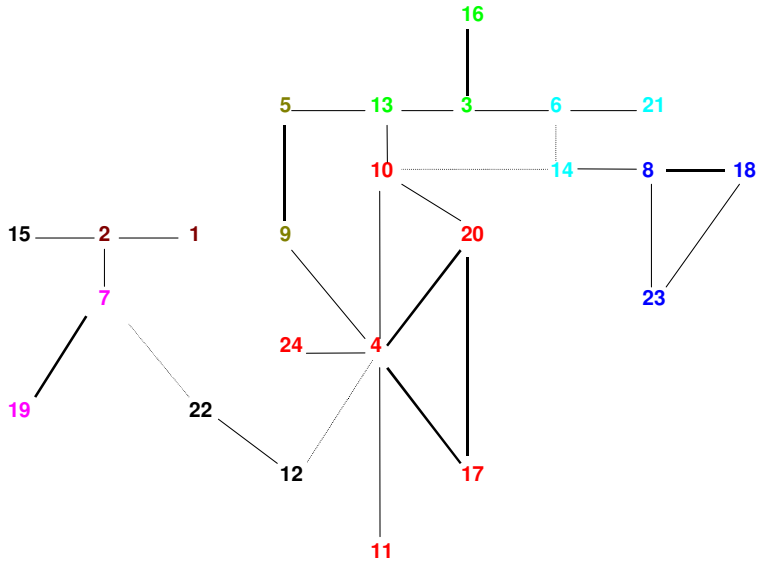

Figure 3: Serological cross-neutralization of bluetongue virus serotypes (Erasmus 1990)

Until 1998 the main disease outbreaks appeared in Africa, Asia, Australia, the USA, the Middle East and the Mediterranean countries. It appears that a fundamental change in the European ecosystem occurred since 1998, possibly linked to climate change (Baylis *et al.,* 2006) which favours the introduction and survival of BTV in Europe. In recent years, northern European countries have experienced BT outbreaks causing substantial economic losses. The first major outbreak of BTV 8 was experienced in August 2006 in North-West Europe affecting The Netherlands, Belgium, Luxembourg, North of France and Germany (Elbers *et al.,* 2008). The BTV-8 strain managed to overwinter in the vector free period of the year, reappearing in summer 2007, and has managed to spread further north as well as westwards affecting additional countries including Denmark, the United Kingdom, Switzerland, the Czech Republic, Spain, Portugal, Italy and Northern Ireland (Saegerman *et al*., 2008). The BT situation in northern Europe continues to evolve with BTV-8 specific antibodies being detected for the first time in Norway, Denmark and Sweden in December 2009 (Tollersrud, 2009).

It is hypothesized that bluetongue virus was introduces into Europe by four possible routes (1) from the east via Turkey / Cyprus; (2) from North Africa (Algeria, Tunisia) into Italy and the eastern Mediterranean Islands; (3) from Morocco into southern Spain and Portugal; and (4) via an unknown route into northern Europe (Mellor *et al.,* 2008). Global warming and environmental adaptation assisted by unknown overwintering mechanisms of the midges in temperate climates (UK Bluetongue Technical Review, 2002) are possible explanations for the spread of the virus (Darpel *et al.,* 2008).

The European Union Member States have demarcated restriction zones per BTV serotypes. These are areas which have serotypes that have been identified to be circulating in different regions. The reason for these zones is to establish protection to the outside areas which are not affected by those serotypes, for proper surveillance and a ban of the susceptible animals leaving those zones in order to control the spread of the disease (http://ec.europa.eu/food/animal/diseases/controlmeasures/bt_restrictedzones_2012).

The distribution of different BTV serotypes is summarized in Table 1 (Mertens *et al.,* 2012). The table illustrates the current situation worldwide in terms of serotype occurrence. It shows the distribution of the 26 serotypes that have been identified. It also indicates if the serotype was isolated (I), or detected using neutralizing antibodies (S) or its presence was detected by Real Time PCR (RT-PCR) for the presence of RNA (R).

Table 1: The geographical distribution of different BTV serotypes (Mertens *et al.,* 2012).

Geographical area	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Africa	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	-	I	I	-	I	-	I	-	-
Middle East	-	I	-	I	-	I	-	-	S	I	-	S	S	S	I	I	S	-	S	S	-	-	-	I	-	-
Pakistan + India	I	I	I	I	S	I	S	S	I	S	S	S	S	S	S	I	I	I	-	S	S	-	-	I	-	I
Australia	I	I	I	-	-	-	I	-	I	-	-	S	-	-	I	I	-	-	-	I	I	-	I	-	-	-
South East Asia and Indonesia	I	I	I	I	-	-	I	-	I	I	S	I	S	-	-	S	I	S	S	S	I	-	I	-	-	-
China	I	I	I	I	-	-	-	-	-	-	-	I	-	-	I	I	-	-	-	-	-	-	I	-	-	-
USA	I	I	I	-	I	I	-	-	-	I	I	-	I	I	-	-	I	-	I	-	-	I	-	-	-	-
Central and South America	I	-	I	I	-	I	-	I	-	-	I	I	I	I	-	S	I	-	-	-	-	-	-	I	-	-
Europe	I*	I*	I	I*	-	I*	-	I*	I*	I	R	-	-	-	-	I*	-	-	-	-	-	-	-	-	-	-

I = virus serotype isolated

S = serological evidence for the presence of each serotype (detection of neutralising antibodies)

R= Detection of relevant serotype specific RNA e.g. by RT-PCR and sequence analysis

I* =BTV types currently in Europe. Prior to 1999 BTV serotypes from Europe had been isolated from relatively short lived (up to 4 years) epizootics and the virus had subsequently been eradicated on each occasion

1.2.4 Pathogenesis and clinical signs

The virus enters the host through the bite of an infected midge. It multiplies in the regional lymph nodes before spreading to the rest of the body (systemic). It replicates primarily in endothelial cells and pericytes of capillaries and venules leading to oedema, necrosis and haemorrhages. The incubation period is usually 4-6 days. Incubation can be shorter or as long as 15 days (Barratt-Boyes & MacLachlan, 1995). All sheep breeds are susceptible, but European breeds and young sheep between 6-12 months of age are more susceptible. The disease can be acute or subacute in presentation. The animal will be pyretic (40°C - 41°C) and all the symptoms associated with damaged of endothelial cells and pericytes will manifest. It is characterized by an erosive – ulcerative stomatitis, gingivitis, and glossitis. Dermatitis, coronitis, necrosis of skeletal muscles and a marked pulmonary oedema is often seen (Vosdingh *et al.,* 1968; Hamblin *et al.,* 1998; MacLachlan *et al.,* 2008). The disease's name stems from the fact that affected sheep begin to develop a mucopurulent nasal discharge after the first few days and the tongue may become cyanotic. Many affected animals become depressed and die while others make a full recovery. Break in the wool and diarrhea are commonly seen. The clinical signs are exacerbated by direct sunlight, and this is demonstrated especially in South Africa. South Africa has the world's highest average daily hours of sunshine - 8.5 compared to 3.8 in London, 6.4 in Rome and 6.9 in New York (www.edusouthafrica.com/south-africa.html). High summer temperatures favour BTV transmission through efficient virus replication (Mullens *et al.,* 1995). Cattle normally carry the disease sub-clinically but they can also have a mild form of the disease and they are important reservoir hosts of the virus (MacLachlan, 1994). Although this is still observed in South Africa, the pattern changed during the outbreak by BTV-8 in Europe, Belgium in 2006, where cattle showed prominent clinical signs (Elbers *et al.,* 2008).

1.2.5 Diagnosis

The disease can tentatively be diagnosed based on clinical signs, epidemiology and pathology. The definitive diagnosis can be made by using laboratory tests like virus isolation, serological tests and serotyping. In order to confirm the diagnosis and if funds allow, both direct and indirect methods should be used by the diagnostic laboratory.

Samples to be submitted for isolation and identification of the virus

Blood samples in heparin and serum should be collected from sick animals. If the animal has recently died or aborted or produced congenitally infected fetuses; spleen, liver, red bone marrow, heart blood, and lymph nodes should be submitted on ice (preserved at 4°C) to the diagnostic laboratory (OIE 2009).

Serological tests

The competitive ELISA (c-ELISA) has proven to be the best serologic test for BTV antibody detection for international trade (OIE 2009), because of increased sensitivity and specificity (Mecham & Wilson, 2004). The c-ELISA for BTV has a sensitivity of 83.83% and specificity of 85.95% (Bhanuprakash *et al.,* 2007). Monoclonal antibodies are also used in the c-ELISA to decrease the chance of cross-reaction. In general, the detection of BTV antibodies is poorly correlated with BTV viraemia. Other BTV antibody detecting tests are the agar gel immunodiffusion (AGID) and SNT which is also one of the serotype-specific tests (Della-Porta *et al.,* 1985). The SNT is sensitive and specific but not used for routine testing because it is time consuming, expensive reagents and the quality of sera may negatively affect the cells (Hamblin, 2004). The complement fixation tests which has largely been displaced by the AGID test, is becoming abandoned because it lacks specificity. It causes cross reaction with other orbiviruses especially with epizootic haemorrhagic disease virus (OIE 2009).

Isolation and identification of the virus

In the laboratory, the virus can be isolated by intravascular inoculation of 10-12-day-old embryonated chicken eggs, and identified by plaque reduction, SNT and polymerase chain reaction (PCR) tests (Afshar, 1994). The PCR is known to be very sensitive and specific in identifying the BTV specific nucleic acid (OIE 2009) but a positive PCR is not always indicative of infectious virus since viral RNA can be detected in some tissues after viraemia has passed which means some false positive of the infectious virus can occur (Alstad *et al.,* 1994). In recent studies, it has shown that real-time (RT)-PCR is a rapid (results can be available within 24 hours), sensitive and reliable method to identify and differentiate all 26 BTV serotypes (Maan *et al.,* 2012a). A study to compare the sensitivity and specificity of RT-PCR to viral isolation of epizootic haemorrhagic disease virus, which is in the same family as BTV, it was shown that the sensitivity and specificity of RT-PCR was 92.3% and 100%, respectively whereas for viral isolation was 75% and 100%, respectively (Clavijo *et al.,*

2010). The RT-PCR is expensive; for one sample the cost of reagents, staff, and sequence analysis followed by phylogenetic analysis can cost up to £3000 without any profit added whereas SNT costs up to £1500 per serum (www.scotland.gov.uk/Publications/2008). The RT-PCR for BTV would cost £107 per plate of samples and an ELISA plate costs £85.

1.2.6 Control

The most effective method of controlling BT in endemic areas (like South Africa) is by prophylactic vaccination using live attenuated and inactivated vaccines (the latter especially in Europe) (Savini *et al.,* 2008).

Another way of control is to protect the animals from being bitten by midges (Ilango, 2006). This could be done by avoiding low-lying wet pastures, stabling the animals from early evenings until late mornings and also shearing in early summer to allow some wool growth before the onset of the BT season which is late summer in SA (Erasmus, 1975).

Live attenuated vaccine

The live attenuated vaccines (LAV) are easy and cheap to produce (Murray & Eaton, 1996). These vaccines (produced by OBP) have shown to provide a safe (in non-pregnant animals) and efficacious means of controlling the disease in regions of southern Africa, as well as other areas of the world (Dungu *et al.,* 2004). The vaccine also protects against 24 BTV serotypes, except those which have not been isolated in the country and recently identified BTV-25 and 26. No studies have been conducted to determine the presence of these two serotypes in SA.

The BTV vaccine is polyvalent and it consists of three bottles, each bottle contains five different serotypes which gives 15 BTV serotypes in the vaccine. The criteria used for the serotypes in the vaccine were their pathogenic index and prevalence in the SA as well as the ability to provide enough cross-protection to the less dominant ones (Dungu *et al*., 2004). Previously published data showed that there is serological cross-neutralization between BTV serotypes (Erasmus, 1990). The cross-protection of serotypes and their presence in the three different bottles of the OBP LAV are shown in Figure 4. The figure illustrates serological cross-neutralization of 23 serotypes. The highlighted (colour coded) serotypes are included in the vaccine and those that are not, are cross-protected by other serotypes in the vaccine.

The disadvantages of the vaccine are; sheep can abort and teratogenic effects on the fetus can be observed when the vaccine is used during pregnancy (Murray & Eaton, 1996) and the time taken before the animal is immune against all the serotypes is long. When the MLV was used in Europe, 0.5% < pregnant ewes which were vaccinated aborted (Savini et al., 2008). The farmer also needs to vaccinate three times, three weeks apart before protection against all BTV serotypes can be obtained. During the vaccination period, animals are still at risk of developing the disease from the serotypes they have not yet been inoculated with (Dungu *et al.,* 2004). The duration of immunity starts appearing from day 10 post vaccination, reaching the maximum four weeks post inoculation. The duration of the immunity lasts for a year (OIE, 2009) once fully vaccinated and the animals are therefore vaccinated annually. Currently the MLV is used since the inactivated vaccines are not available.

During the first outbreaks of BT in Europe in 1998, monovalent modified live virus vaccines (MLV) containing specific serotypes circulating in particular areas of Europe were used. These vaccines produced good immunogenicity, however the problems experienced included prolonged viraemia, and some animals developed typical BT clinical signs especially with the monovalent vaccine of serotype 16 (Savini *et al*., 2008). Inactivated vaccines were then developed by these countries in order to control and eradicate the disease.

The use of MLV also has the disadvantage that viruses could revert back to virulence during replication in and transmission by the midge vectors (Veronesi *et al.* 2005) and by reassortment of their segmented genome segments, BTV can reassort to virulent viruses causing new outbreaks. This was demonstrated in Western India in 1988, where BTV-23 Indian strain (IND1988/2) caused outbreaks. Phylogenetic analyses showed most of its genome belongs to the “eastern” BTV topotype whereas genome segment 5 belongs to the major “western” topotype demonstrating that IND1988/02 is a reassortment virus (Maan *et al.,* 2012b). The outbreak of BTV-6/net08 (BTV-6 which caused outbreaks in Germany and the Netherlands in 2008) is suggested to be due to reassortment of MLV of BTV-6 and BTV-2. The BTV-6/net08 is closely related to BTV-6 of the MLV but genome segment 10 shows homology with BTV-2 of the MLV (Maan *et al.,* 2012b). In the study done in 2010 to prove the above reassortment statement, synthetic reassortants of BTV-6/net08/S7^{8} (BTV-6/net08 and segment 7 of BTV-8/net06) was created (Van Gennip *et al*., 2010) and the study clearly demonstrated that MLV of BTV can reassort into a virulent virus which can cause disease.

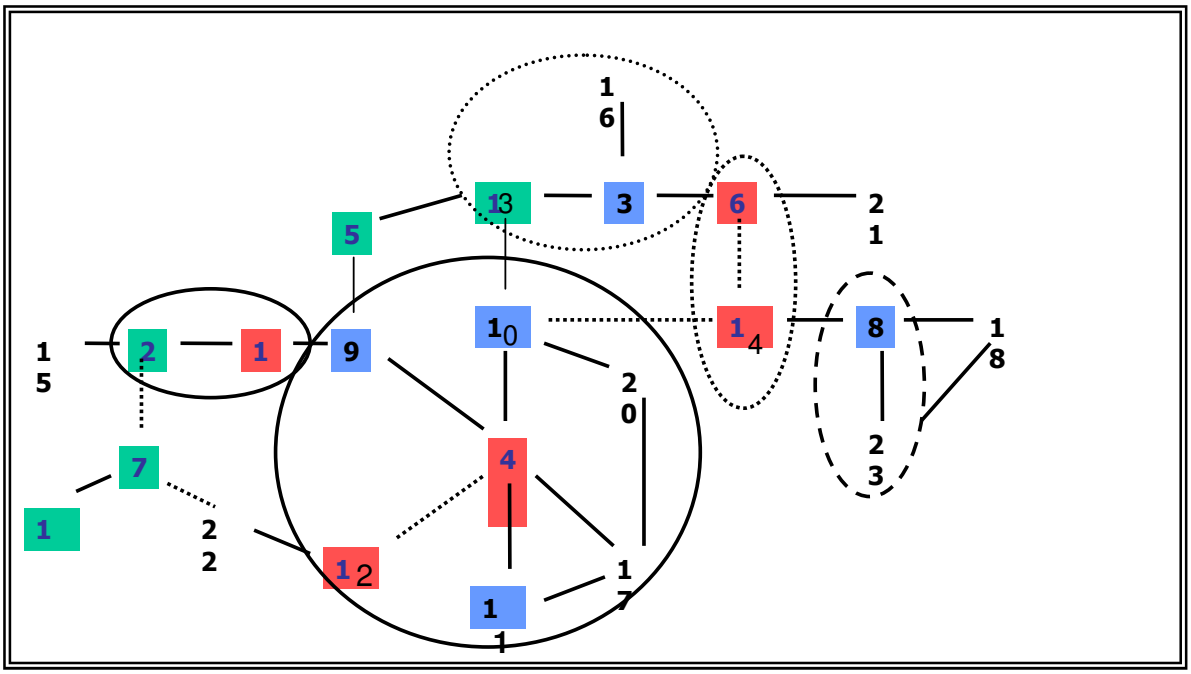

Figure 4: An illustration of the cross-protection of different BTV serotypes using the SNT and the grouping of serotype combinations used in the vaccine (Erasmus 1990; Dungu *et al,,* 2004).

Inactivated vaccines

Inactivated vaccines have been developed for experimental animals and shown good immune response for up to seven months without viraemia and overt clinical signs if the booster dose was given (Savini *et al.,* 2007). During outbreaks in Europe, inactivated BTV-2 and 4 vaccines were developed as monovalent and bivalent vaccines to be used in the field (Savini *et al*., 2008). The disadvantages of some previously mentioned inactivated vaccines are local reactions at the site of injection (Audarya *et al*., 2006; Savini *et al*., 2008) but farmers do not normally complain about these local reactions. The second disadvantage is that it is expensive to produce and it has to be given in two doses for long lasting immunity (Savini *et al*., 2008). This is a disadvantage but long lasting immunity is a good response since animals should be protected from getting the disease. The animals vaccinated with BTV-2 vaccine developed a strong immunity which lasted for at least a year (Hamers *et al.,* 2009). There are BTV-8 commercially available inactivated vaccines (Wackerlin *et al.,* 2010) which are used in Europe to control BTV-8 and they are regarded as efficacious vaccine. Multivalent inactivated vaccines to be used in the field in South Africa are currently under development at OBP.

Recombinant vaccines

Recombinant vaccines are second generation non-infectious subunit vaccines which are produced by extracting subunits responsible for certain gene expression from infectious agents without using the whole genome or intact microorganism. Genes encoding for these proteins are inserted into expression vectors like e.g. baculovirus, expressed and used as vaccines (Sanchez-Vizcaino, 2001). Virus-like particles (VLP) and core-like particles (CLP) are used as recombinant BTV vaccines, the former contains e.g. only four major structural proteins of BTV to elicit an immune response and shown good protective immunogenicity against homologous serotypes (Pearson & Roy 1993). The advantages are that reassortment cannot occur and it has a high immunogenicity. The VLP and CLP recombinant vaccines are safe and efficacious vaccines (Murray & Eaton, 1996). In the study reported by Stewart *et al.,* (2012), animals vaccinated with VLP of BTV-1 developed neutralizing antibodies that when challenged with the same serotype, prevented viraemia and disease. The animals vaccinated with CLP of BTV-1 only developed group-specific VP7 antibodies. The CLP failed to protect the animals from developing viraemia and clinical signs but reduced the severity of the disease.

With recent recombinant DNA technology, recombinant modified vaccinia virus expressing VP2, VP7 and NS1 proteins from BTV-4 has been engineered. This vaccine generated significant specific antibodies of the above mentioned proteins including antibodies that have neutralizing activity against BTV-4, and specific $CD8^{+}$. It also induced cross-protection against BTV-1 and 8 (Calvo-Pinilla, 2012).

1.3 Problem statement and objective of the study

Bluetongue is endemic in southern Africa and the live attenuated polyvalent vaccine produced by the OBP is used to control the disease. It takes about nine weeks for the animal to be fully protected. During this period, the animals can contract the disease from serotypes to which the animal has not established immunity against. It will be economically favorable to develop a vaccine that is only administered once for full protection. It is therefore necessary to conduct studies to determine if fewer serotypes can be used in the live attenuated vaccine, (to achieve suitable immunity) and if cross-neutralization between serotypes will protect against all serotypes.

It was previously shown using the SNT that some serotypes of BTV cross-neutralize with other serotypes (Table 2; Howell *et al.* 1970). In the study by Della-Porta *et al.* (1980), it is also indicated that BTV-20 is closely related to BTV-4 and distantly related to BTV-17.

Table 2: Summary of cross-neutralization of BTV serotypes using the SNT

BTV serotype	**Cross-neutralize the following other serotypes:**
4	9, 10, 11, 12, 17, 24
10	4, 13, 14, 20
2	1, 7, 15
9	1, 4, 5
7	2, 19, 22
3	6, 13, 16
6	14, 21, 3
18	8, 23

It is therefore necessary to establish:

- If the number of serotypes in the current vaccine can be reduced and the vaccine still retains the efficacy which protects the animal from clinical manifestation of the disease.

In this study, if some of the serotypes producing cross-neutralization by SNT could also produce cross-protection in target animals.

Bluetongue virus serotype 4 vaccine was used to show cross-protection against challenges with homologous (BTV-4) and heterologous (BTV-1, 8, 9, 10, 11) virulent viruses under controlled conditions in sheep.

CHAPTER II

MATERIALS AND METHODS

2.1 Facility and animals

The study was conducted at OBP. Thirty-two BTV-specific antibody free mutton-merino sheep older than seven months but younger than 18 months were sourced for the study, and 25% of sheep were bred at OBP (which are considered as an indigenous breed) in insect free stables. Animals were all screened for antibodies against BTV using a BTV-specific c-ELISA which is a test of choice by the Organization International des Epizooties (OIE). The screening was carried out by the OIE **R**eference Virology Laboratory of the ARC-OVI. Only BTV susceptible sheep were selected for the study and they were housed in the insect -free stables at OBP for the duration of the trial. As part of the acclimatization programme, sheep were dewormed, treated for ectoparasites, identified with ear tags, introduced to the diet consisting of concentrates, lucerne and erogrostis grass, and stabled 21 days before the commencement of the trial. The holding pens were cleaned daily, the animals were fed thrice daily (concentrates in the morning, lucerne midday and hay in the afternoon). Water was supplied ad lib. The average room temperature for the duration of the study was kept at 23°C.

2.2 Bluetongue virus vaccine and challenge material

Bluetongue virus serotype 4 was the vaccine serotype for this trial. The BTV-4 vaccine with a titre of 4.2×10^6 log10$TCID_{50}$/ml was formulated according to the standard operating procedure (SOP) of OBP for formulation and titre determination. The standard OBP challenge material for BTV-4, 1, 8, 9, 10 & 11 which is stored in an Oxalate –Citrate –Glycerol Anti-coagulant (OCG) blood was used. Before it was used, it was tested for infectivity using two sheep per above mentioned serotypes but the titre was not determined. The origins of the BTV serotypes used in this study are reflected in Table 3.

Table 3: The origin of the bluetongue virus serotypes that were used in this study

Virus type	Strain Identification	Origin	Passage History
BTV-1	8012 Biggarsberg	RSA 1958	50E 3P 3BHK
BTV-4	Theiler/79043	RSA, ~1900	60E 3Pa 9BHK
BTV-8	Camp/8438	RSA, 1937	50E 3BHK 10 Pa 7BHK
BTV-9	University Farm/2766	RSA, 1942	70E 2BHK pp 3BHK 7P 6BHK
BTV-10	2627 Portugal	Portugal 1956	E81 (Portugal) 1BHK
BTV-11	Nelspoort/4575	RSA, 1944	35E 3P 5BHK

Bluetongue virus 4 was selected based on its ability to causing outbreaks and its prevalence in SA. Serotype 9, 10 and 11 are cross-neutralized by BTV-4 using SNT. Serotype 1 was used since it is indirectly related to BTV-4 through BTV 9 (Figure 3). Serotype 8 is from a different group and, is not related to BTV-4 (Figure 3). It was used in the study as challenge material based on its virulence and its occurrence in European countries (Bruckner *et al.,* 2009).

All serotypes used in this study were isolated from clinical cases in SA except BTV-10 which caused the disease in southern Portugal (Ribeiro *et al.,* 1957). The virus was isolated and identified as BTV-10 by Howell at Onderstepoort (Howell 1960). It was attenuated to be adapted for the vaccine by Howell and is used in the vaccine until today (Dungu *et al*., 2004).

2.3 Animal inoculation

Three days before the start of the trial, sheep were monitored by OBP Animal Production Technicians (Mr Johannes Molomo and Mr Erens Radingwana), for clinical parameters, i.e. body temperature, respiratory rate, heart rate, capillary refill time, mucous membranes (eye, mouth and nostrils), appetite and habitus, and the parameters were used as baseline health status of the animals. Serum was collected by Mr Molomo and Radingwana on Day 0 and sheep were then grouped into vaccinated and unvaccinated; 27 sheep were inoculated subcutaneously with 2 ml live attenuated BTV-4 vaccine and five were left unvaccinated as negative controls. The inoculation was done on the inner part of the thigh, groin area. The parallel experimental design of a randomized

clinical trial was the method used for sampling the animals (Lund *et al*., 1994). The sample size was determined according to the formula below. The sampling formula used to determine the sample size is described by Kadam & Bhalerao (2010). The formula is:

$$n = \frac{2\left(Z_{\alpha}+Z_{1-\beta}\right)^{2}\sigma^{2}}{\Delta^{2}}$$

Where:
n = the required sample size;
Zα, Z = a constant (set by convention according to the accepted α error for one-sided) = 1.65
Z 1-β, Z is a constant set by convention according to power of the study (80%) = 0.8416
The standard deviation (based on the data in the published paper) would be approximately 0.65
For Δ= 100% (in vitro study done SNT)=1

Therefore the sample size = $2(1.65+0.84)^2\ 0.65^2 / 1^2 = 5.2 = 5.0$

Since this is a parallel study, the total number of animals involved should be 5 x 3 (positive and negative controls included). Since there is enough available data on positive and negative control animals, the control animals were reduced to 2 positive control animals for the whole study and one negative control animal parallel to a group with 5 animals each challenged with the different serotype.

The clinical parameters were monitored for 14 days, twice daily and sheep were bled weekly. On Day 28 sheep were further grouped and challenged intravenously via the jugular vein using different BTV serotypes as outlined in Table 4. They were monitored for clinical signs using the relevant clinical parameters mentioned previously. On days 3, 5, 7 and 10 post challenge, blood was collected in heparin tubes. The trial ran for 56 days and the animals were bled from the jugular vein for serum every seven days starting from Day 0 to Day 56. The details of the trial are summarized in Table 4.

Table 4: Inoculation of sheep with BTV-4 and challenged with BTV-4, 1, 8, 9, 10 and 11.

Monovalent <u>Live vaccine:</u> serotype 4 given subcutaneously	**Weekly bleeding from jugular vein**	**Challenge after 4 weeks with 1 ml of a specific BTV serotype**	**Bleeding after challenge –**
<u>32 X Sheep in total:</u> 27 sheep were vaccinated with serotype **<u>4</u>** (dose = 2ml).	8 ml serum was collected from each animal on days 0 (on the day of vaccination), 7, 14, 21, and 28 after vaccination. On Day 28, they were also challenged and continued with bleeding on days 35, 42, 49 and 56.	2 X sheep challenged with serotype **<u>4</u>***. **(Group A -** Vaccinated with BTV-4 and challenged with BTV-4)	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
		5 X sheep challenged with serotype **<u>1</u>**. **(Group B** - Vaccinated with BTV-4 and challenged with BTV-1**)**	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
		5 X sheep challenged with serotype **<u>9</u>**. **(Group C -** Vaccinated with BTV-4 and challenged with BTV-9**)**	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
		5 X sheep challenged with serotype **<u>10</u>**. **(Group D -** Vaccinated with BTV-4 and challenged with BTV-10**)**	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
		5 X sheep challenged with serotype **<u>11</u>**. **(Group E -** Vaccinated with BTV-4 and challenged with BTV-11**)**	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
		5 X sheep challenged with serotype **<u>8</u>**. **(Group F -** Vaccinated with BTV-4 and challenged with BTV-8**)**	6 ml blood in heparin tubes on days 3, 5, 7 and 10 post challenge.
5 sheep were not vaccinated. These were used as negative control animals	They were bled as above	Each sheep was challenged with a different serotype i.e. BTV-1, 8 ,9, 10, and 11	The bleeding was done as above

* → these two sheep were used as positive control animals

2.4 Animal monitoring

After vaccination and challenge the animals were monitored for fever and BT related clinical signs. The percentage protection index (PPI) which is the degree of clinical reaction post challenge per animal was set to be 55%. Below are parameters that were used to monitor the animals:

- Normal rectal temperature ranges between 37 and 40°C;
- Clinical signs for animals reacting to the vaccine and challenge material were fever, depression, dyspneoa, haemorrhages of but not limited to, ears, mucosa of the nose and mouth, glossitis, coronitis, subcutaneous oedema (all clinical signs associated with endothelial cell damage), nasal discharge, etc;
- The PPI by the vaccine after challenge was determined according to the publication by Huismans *et al.* (1987) (**Appendix B).** The PPI for this study was 55%. A PPI above 55% with no overt clinical signs was regarded as protective.
- To calculate the PPI: the clinical reaction index (CRI) is divided by CRI of the negative control animal to get the relative reaction (RR). 100%-RR = PPI.
    - CRI = (a-febrile reaction)+(b-clinical score)+(c-fatality) (**Appendix A**);
    - RR= CRI/CRI (negative control animal of the same group);
    - PPI=100%-RR. (Huismans *et al*., 1987)

The clinical status of each animal was established before they were inoculated to establish the normal clinical status of each animal.

Animals were closely monitored for unnecessary suffering by using the humane end score sheet of the Animal Ethics Committee of the OBP (**Appendix D**) which was designed for the humane treatment of animals involved in research trials, e.g. an animal with a score between 15-20 points must immediately be treated, or even euthanized.

2.5 Laboratory diagnostic test: Serum neutralization

The samples taken were analyzed at OBP in the Virology Laboratory of the Research and Development Department (RDV). All serum samples for antibody response to BTV after vaccination and challenge were tested by the SNT. The SNT was performed according to the OIE Manual of Standards for Diagnostic tests and Vaccines of 2004 and a SOP of OBP (OIE, 2004) by the researcher. Briefly the test was performed in 96-well microtitre plates and the vaccine strain of the BTV-1, 4, 8, 9, 10, and 11 were used as antigens. All sera were inactivated for 30 minutes in a water bath at 56°C

before used. Phosphate buffered saline was used as diluent and two-fold dilutions of the serum were prepared in duplicate in the microtitre plate (1-8 wells, two wells per sample) and 50 µl of each dilution was used. The 50 µl BTV antigen, the titre of each serotype was determined beforehand (**Appendix B**), was added to each well that contains the diluted test serum. Virus and cell control plates were also prepared. The plates were incubated at 37°C for 1h in an incubation containing 5%. CO_2. A volume of 100 µl of Vero cells at a concentration of 480 000 cells/ml were added to each well of the microplates, followed by incubation at 37°C in a 5% CO_2 atmosphere. The monolayers were examined daily for evidence of cytopathic effects using an inverted microscope. After 5 days the results were recorded according to the neutralization pattern as either at a dilution of 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. A positive reaction at a dilution of 1:16 was regarded as the cut-off point that determines the presence of neutralizing antibodies in the serum. The results below the cut-off of 1:16 was regarded as negative, no neutralizing antibodies present.

Onderstepoort Biological Products is an ISO 9001:2004 accredited vaccine producing company, therefore during the laboratory testing, quality monitoring was done according to the SOP for the OBP Virology laboratory.

CHAPTER III

RESULTS

Sheep were vaccinated twice. The first time they did not respond, and on trouble shooting using both c-ELISA and SNT, it showed that the vaccine was not effective and no antibodies against BTV-4 were present. On Day 32 which serves as Day 0, they were inoculated again with BTV-4 vaccine. They were monitored for both clinical and antibody reaction. With this inoculation, they responded to the vaccine and were challenged according to Table 5 on Day 28 after the second inoculation.

The detailed results (raw data) are in **Appendix B and C**. Results obtained by animals challenged with BTV-8 (Group F) did not have BTV-8 antibodies, BTV-8 virus could not be detected in the blood and no temperature reaction post challenge in all the animals in this group including the negative control animal could be determined, and as a result this group was taken out of the study.

3.1 Group A – Vaccinated with BTV-4 and challenged with BTV-4 challenge material

Two sheep were used as a positive control group. Only one sheep (# 72) had mild fever with the first vaccination. After challenge the animals did not have any increase in rectal temperatures except a slight rise in sheep 207 on Day 13 (Figures 5 and 6). On Day 7 after vaccination the SNT results were 1:16 and 1:64 for sheep 72 and 207 respectively. On Day 7 after challenge for both sheep, the SNT results were 1:256 (**Appendix B**). No clinical signs were observed in either of the two control animals.

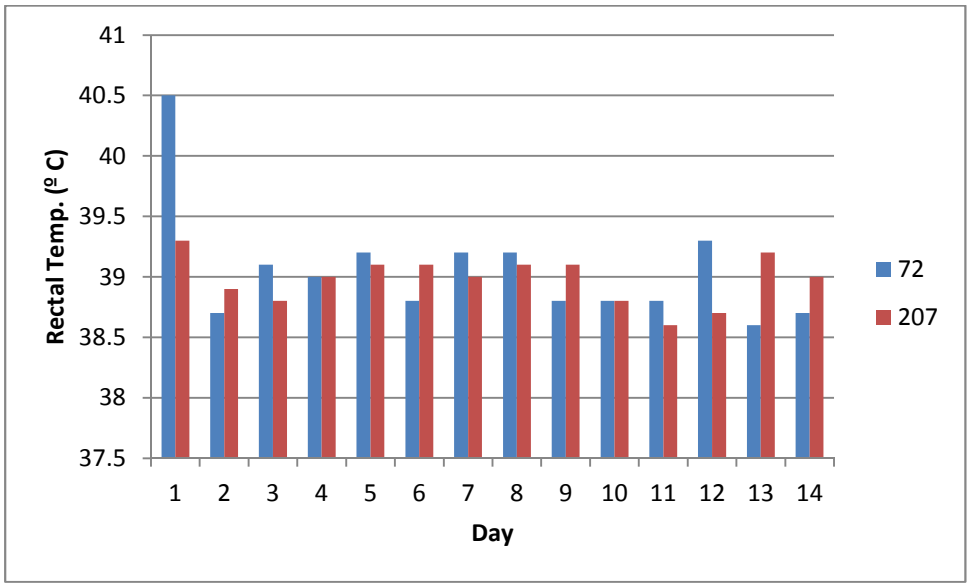

Figure 5: Rectal temperatures of sheep after vaccination with BTV-4 – Group A

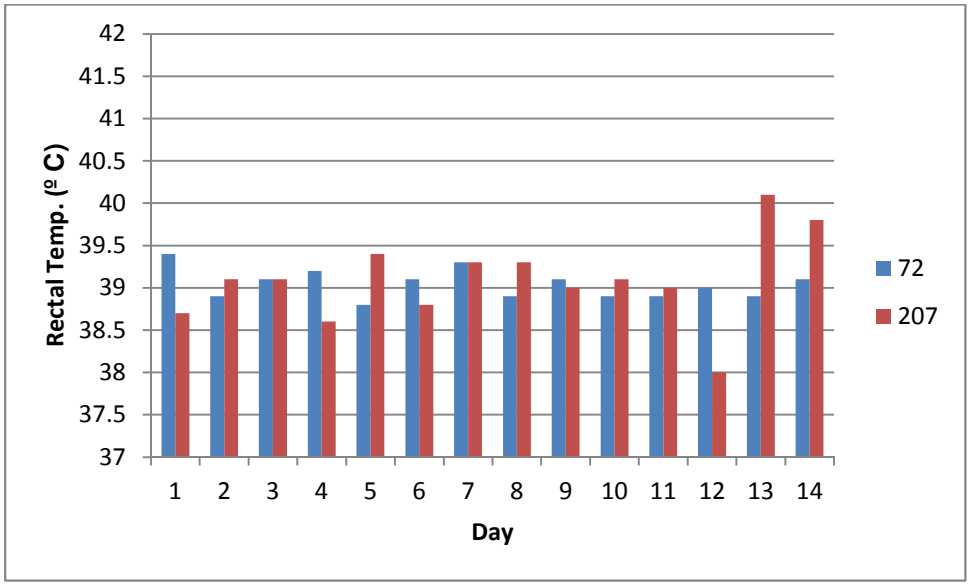

Figure 6: Rectal temperatures of sheep after challenge with BTV-4 – Group A

3.2 Group B - Vaccinated with BTV-4 and challenged with BTV-1

The body temperature of animals after vaccination were normal but sheep 76 (negative control, which was not vaccinated) had mild unexplained fever on Day 1 and 6 (Figure 7).

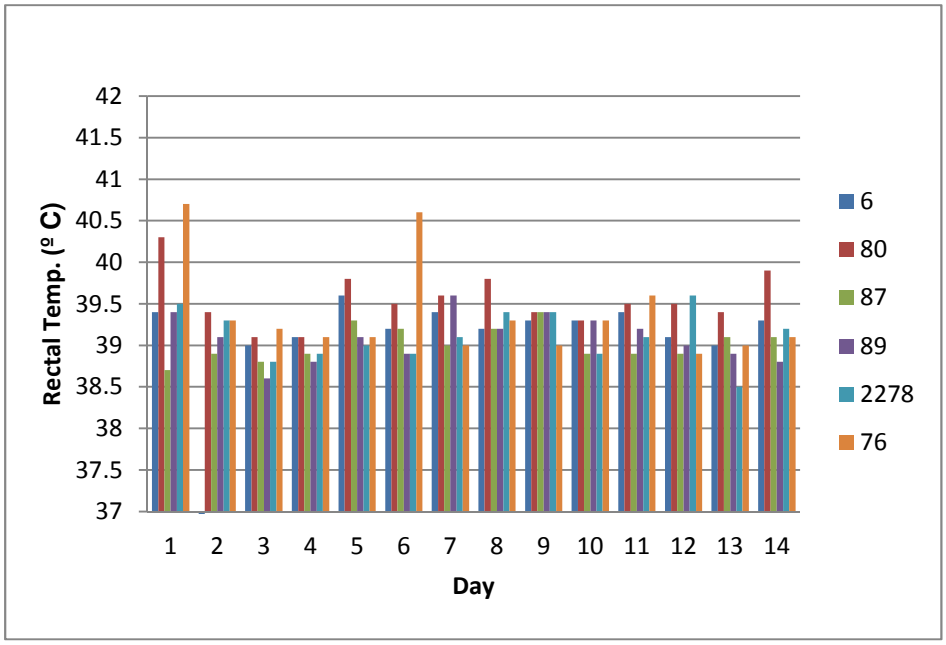

Figure 7: Rectal temperatures of sheep after vaccination with BTV-4 – Group B

After challenge, all animals reacted on different days. Sheep 76 which was not vaccinated (negative control) got very sick and it died before the end of the study. From Day 8 it developed dyspnea, it was depressed and it was on the floor most of the time. On Day 10 it was symptomatically treated for fever, pains and dyspnoea and died on Day 11. No neutralizing antibodies were detected. Post mortem results clearly indicated typical bluetongue lesions.

Sheep 6 had fever on Day 7 until Day 10. On Day 9, it was very high, depressed, hyperpnoea and hyperaemia (1) of the tongue (**Appendix A**- clinical scoring) until Day 14. It was treated on Day 10 and 11. Neutralizing antibodies were only seen from Day 28. The PPI was 48% (Table 5).

Sheep 80 had mild fever from Day 1 until Day 4 and moderate to severe fever on Day 13 (Figure 8). It was treated on Day 13 because of the tongue congestion score of 1 (**Appendix A**) and it could not breathe properly (panting). Even though it showed a PPI of 76% on clinical scoring, no neutralizing antibodies developed (Table 5).

Sheep 87 had fever from Day 5 until Day 10 and the treated was given on Day 10. It had stopped eating, and it was on the floor most of the time, breathing very heavily with the head hanging down, salivating (mildly). It had the average of 39.0°C rectal temperature prior to challenge which was relatively low compared to the rest of the group. It had a PPI of 76% (Table 5).

The average rectal temperature for sheep 89 was 39.3°C. It had fever from Day 7-13. From Day 9, it was symptomatically treated since it could not stand due to the pain on the hooves (grade 2 of coronary band hyperaemia) (**Appendix A**), panting and hyperaemia of the tongue and the ears. It developed neutralizing antibodies as from Day 14 post challenge. The PPI was 44% (Table 5).

Sheep 2278 had average temperature of 39.2°C before challenge. After challenge it had fever from Day 7 until day 11 (Figure 8) but no other clinical signs. The neutralizing antibodies appeared as from Day 21 and the PPI was good at 78% (Table 5).

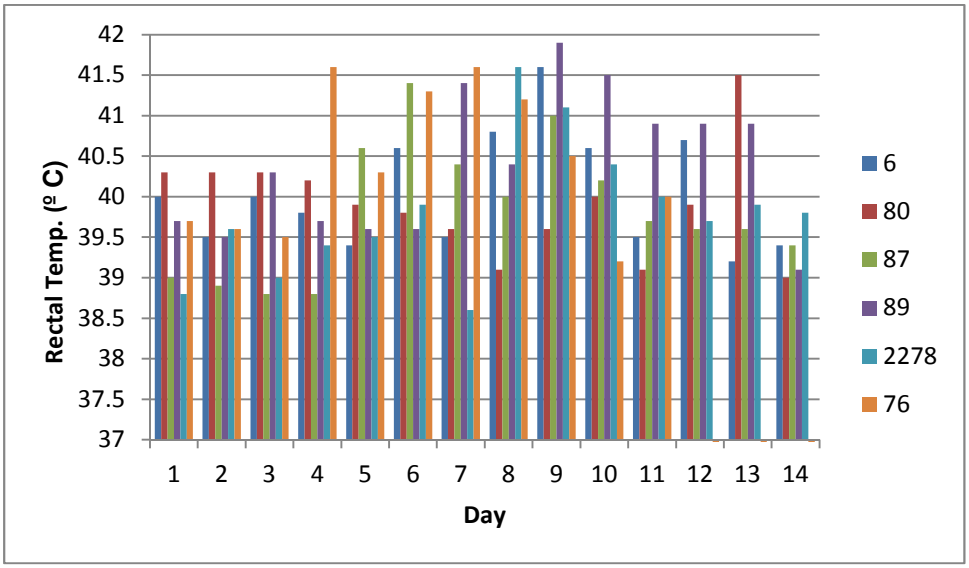

Figure 8: Rectal temperatures of sheep after challenge with BTV 1 – Group B

The summary of results obtained by SNT and the PPI for Group B animals are listed in Table 5. Detail SNT results are presented in **Appendix B**. For SNT results, only the cut-off results are highlighted which indicate protective neutralizing antibodies against the virus. The PPI is the clinical manifestation of the disease post challenge. The higher the percentage, the lesser are the clinical signs.

Table 5: Summary of results obtained by the serum neutralization test and percentage protection index – Group B

Animal number	SNT results post vaccination				SNT results post challenge				PPI
	Day 7	14	21	28	Day 7	14	21	28	
6	1:16	1:64	1:32	1:32	1:2	<1:2	1:4	1:16	48%
80	1:32	1:64	1:64	1:64	<1:2	1:4	1:4	1:4	76%
87	1:64	1:256	1:256	1:128	<1:2	1:2	1:8	1:8	76%
89	1:64	1:64	1:64	1:128	<1:2	1:16	1:16	1:16	44%
2278	1:64	1:64	1:128	1:128	<1:2	1:4	1:16	1:16	78%
76 (unvaccinated)	1:2	<1:2	<1:2	<1:2	<1:2	Dead	Dead	Dead	0%

3.3 Group C - Vaccinated with BTV-4 and challenged with BTV-9

The animals were vaccinated except sheep 2396 (negative control). After vaccination, only sheep 2394 had a mild fever. The rest of the animals were within normal ranges (Figure 9).

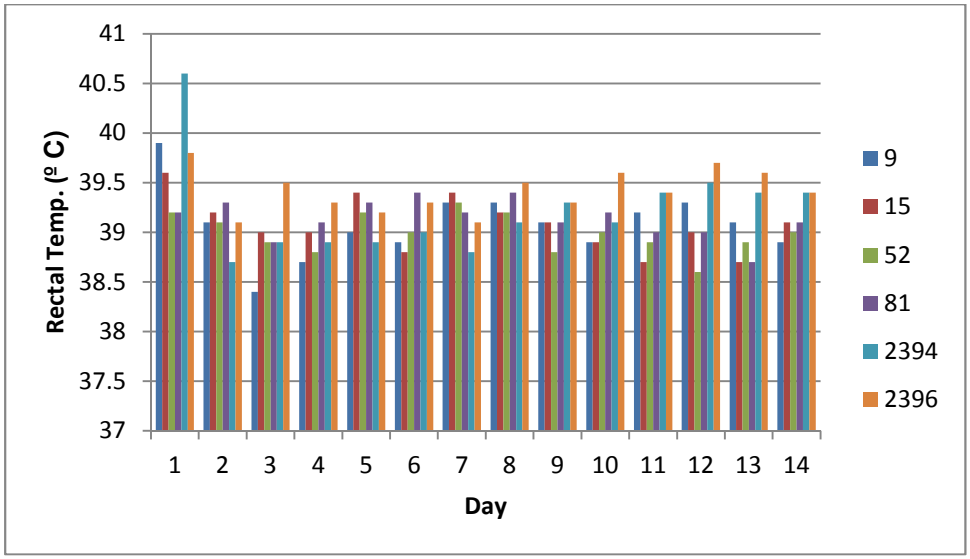

Figure 9: Rectal temperatures of sheep after vaccination with BTV-4 (except unvaccinated sheep 2396)

After challenge with BTV-9, all animals had temperature reactions starting from Day 4. From Day 6 until Day 10, sheep 81 had fever which increased on Day 7 and Day 8. It developed mild hyperaemia of the mouth which cleared without any treatment. From Day 12 until Day 14, sheep 2394 had moderate fever. The unvaccinated sheep 2396 had fever from Day 11 (Figure 10). It developed bluetongue clinical signs which included dyspnoea, depression and anorexia and then hyperaemia of the mouth, hooves manifested. It was symptomatically treated.

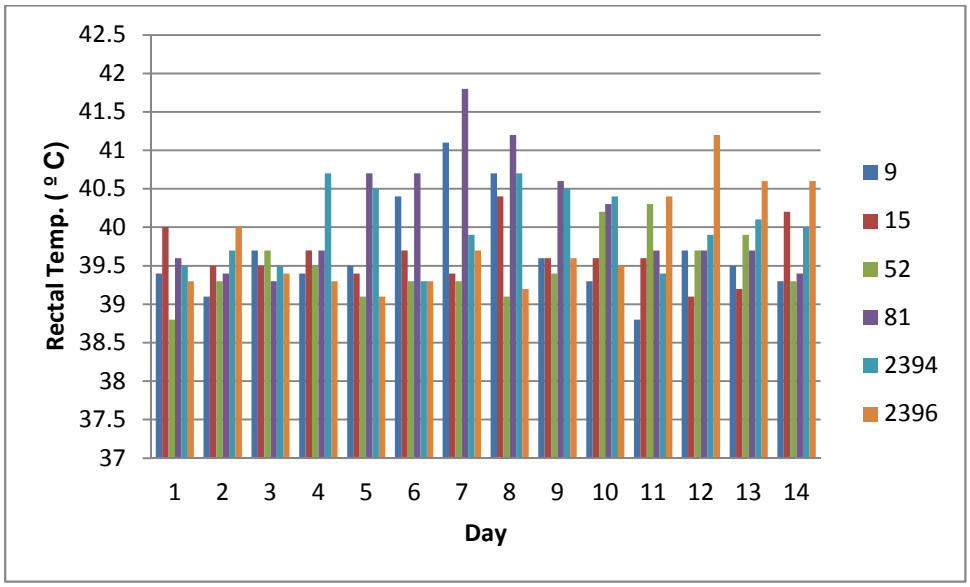

Figure 10: Rectal temperatures of sheep after challenge with BTV-9 – Group C

The SNT and PPI results are listed in Table 6. For SNT results, only the cut-off results are highlighted which indicate protective neutralizing antibodies against the virus. The rest of the results are detailed in **Appendix B**.

Table 6: Summary of results obtained by the serum neutralization test and percentage protection index - Group C

Animal number	SNT results post vaccination				SNT results post challenge				PPI
	Day 7	14	21	28	Day 7	14	21	28	
9	1:64	1:32	1:64	1:64	1:2	1:64	1:128	1:128	58%
15	1:32	1:64	1:64	1:64	1:2	1:16	1:256	1:256	72%
52	1:32	1:64	1:64	1:64	1:2	1:4	1:64	1:128	72%
81	1:32	1:64	1:64	1:32	1:2	1:8	1:32	1:64	-71%
2394	1:32	1:64	1:64	1:64	1:2	1:16	1:64	1:32	0%
2396 (Unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2	1:16	1:32	0%

According to SNT results, the animals were protected as early as Day 14 post challenge but sheep 81 had severe clinical signs even though it showed antibodies from Day 21 post challenge.

3.4 Group D - Vaccinated with BTV-4 and challenged with BTV-10

After vaccination (except 56), only sheep 14, 29, and 2283 had increased rectal temperatures on Day 1. The reaction was only for one day and thereafter the temperatures went back to normal (Figure 11).

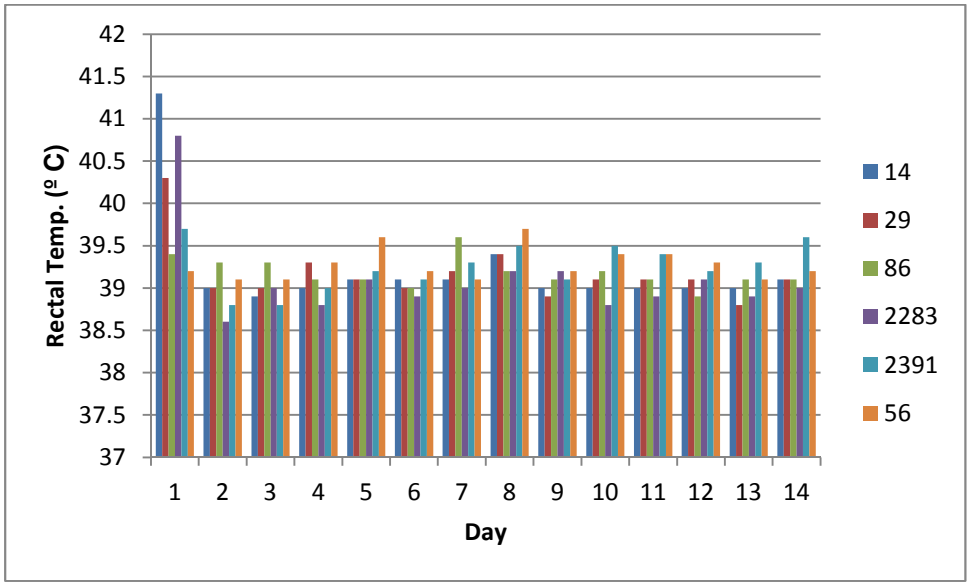

Figure 11: Rectal temperatures of sheep after vaccination with BTV-4 – Group D

Post challenge, all animals reacted on different days starting from Day 5 (Figure 12). Sheep 14 which had a temperature reaction from Day 5-10, sheep 86 with temperature reaction from Day 8 -13 and sheep 2283 from Day 6 until Day 12 had severe clinical signs. Sheep 56, the unvaccinated animal, had fluctuating rectal temperatures from days 1, 10, 11, 12 and 14 and high temperatures from Day 11. There were only two animals (sheep 29 and 2391) that had mild fever without any other clinical signs post challenge. These two were not given any treatment and they recovered. The neutralizing antibodies were significant from Day 14 and Day 21 for sheep 29 and 2391 respectively. The PPI of 43% and 72% for sheep 29 and 2391 respectively were achieved (Table 7). The other sheep (14, 86 and 2283) showed good neutralizing antibodies post challenge but they got very sick, showing typical BT clinical signs and had to be treated, including sheep 56 which was the unvaccinated negative control animal.

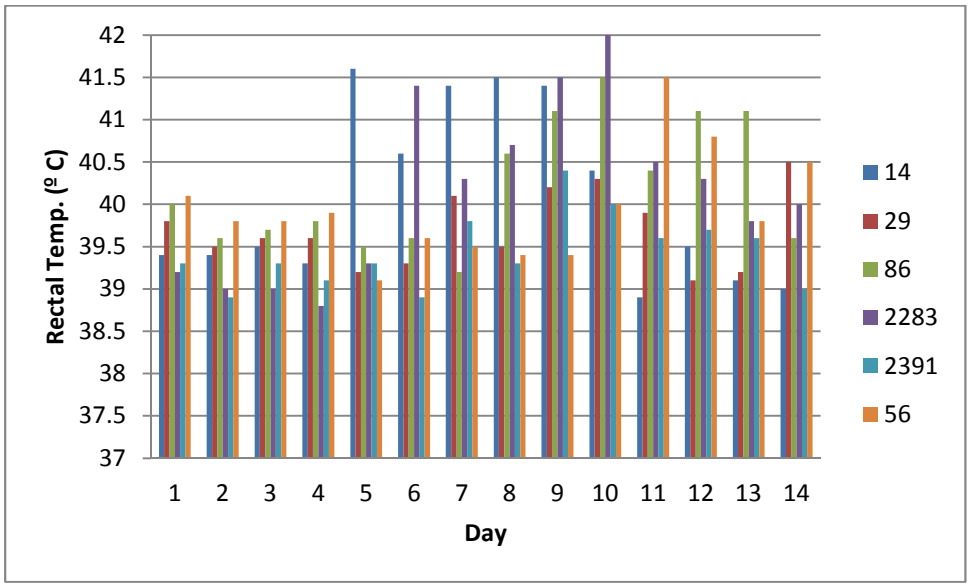

Figure 12: Rectal temperatures of sheep after challenge with BTV-10 – Group D

The SNT and PPI results are listed in Table 7. The highlighted areas indicate the first day neutralizing antibodies were detected. The cut-off point was 1:16. The rest of the results are detailed in **Appendix B**.

Table 7: Summary of results obtained by the serum neutralization test and percentage protection index – Group D

Animal number	SNT results post vaccination				SNT results post challenge				PPI
	Day 7	14	21	28	Day 7	14	21	28	
14	<1:2	<1:2	1:32	1:32	1:16	1:256	1:256	1:256	-57%
29	<1:2	<1:2	1:64	1:64	1:2	1:256	1:256	1:256	43%
86	1:64	1:64	1:64	1:64	1:32	1:256	1:256	1:256	-71%
2283	1:16	1:64	1:32	1:32	1:16	1:16	1:256	1:256	-57%
2391	1:32	1:64	1:64	1:256	1:8	1:2	1:128	1:256	72%
56 (Unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	1:4	1:256	1:256	0%

3.5 Group E - Vaccinated with BTV-4 and challenged with BTV-11

The temperature results post vaccination for sheep in Group E are shown in Figure 13, only sheep 53 and 2286 had mild fever on Day 1. Sheep 84 was not vaccinated (negative control animal).

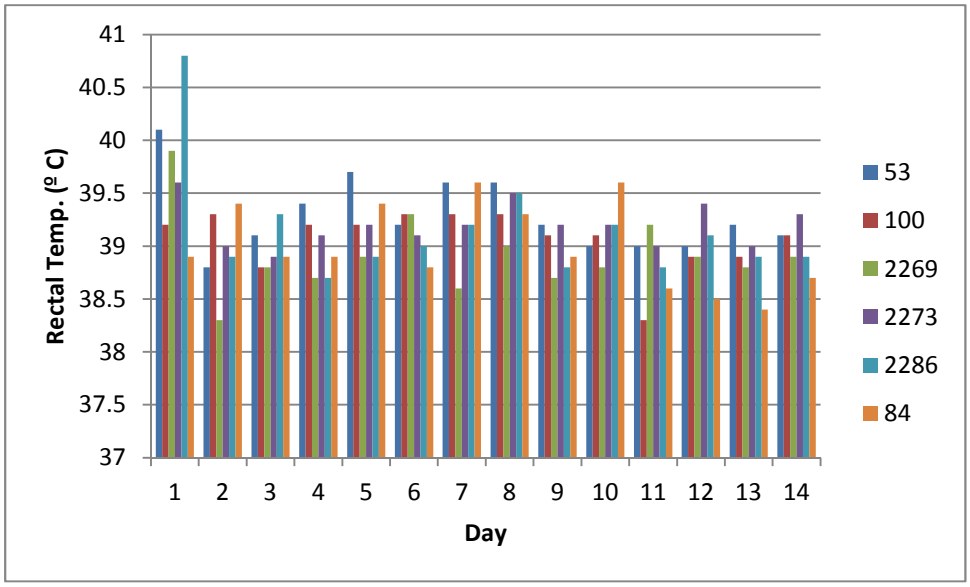

Figure 13: Rectal temperatures of sheep after vaccination with BTV-4 – Group E

After challenge (Figure 14), all animals (sheep 53, 2269, 2273) except sheep 2286 which did not have any temperature reaction, developed mild temperature reactions and other clinical signs. Sheep 2273 had a fever for two days only. Sheep 2269 had fever from Day 4 until Day 8. On Day 7, the temperature was very high (Figure 13 and **Appendix C**). Sheep 84 which was not vaccinated but only challenged with BTV-11 had a fever from Day 4 until Day 14 and developed severe clinical signs. It was symptomatically treated and died on Day 23 post challenge. Sheep 100 was treated when it could not eat and was very depressed. Neutralizing antibodies could be detected from Day 14 in all sheep except sheep 2286 which only developed antibodies from Day 35 (**Appendix B**), but it was 100% protected (Table 8).

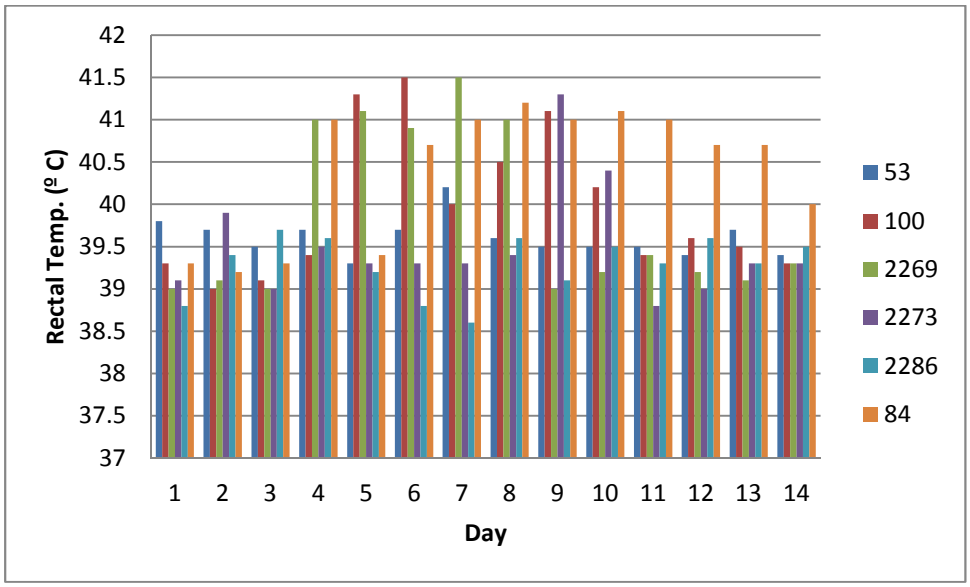

Figure 14: Rectal temperatures of sheep after challenge with BTV-11 – Group E

The SNT and PPI results are listed in Table 8. The highlighted areas indicate the first day neutralizing antibodies were detected. The cut-off point was 1:16. The rest of the results are detailed in **Appendix B**.

Table 8: Summary of results obtained by the serum neutralization test and percentage protection index – Group E

Animal number	SNT results post vaccination				SNT results post vaccination				PPI
	Day 7	14	21	28	Day 7	14	21	28	
53	1:32	1:64	1:128	1:256	1:4	1:16	1:16	1:16	95%
100	1:16	1:128	1:256	1:256	1:16	1:128	1:256	1:128	55%
2269	1:256	1:256	1:256	1:256	1:4	1:64	1:64	1:64	64%
2273	1:128	1:128	1:256	1:64	1:2	1:64	1:128	Did not bleed	91%
2286	1:32	1:64	1:64	1:64	1:2	1:2	1:4	1:4	100%
84 (Unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2	1:8	dead	0%

CHAPTER IV

4. DISCUSSIONS AND CONCLUSION

Serological cross neutralization among BTV serotypes within specific groups using the SNT is well described (Erasmus 1990). However, only a few studies exist where sheep were used to determine cross-protection. Jeggo (1986) did a study on target animals (sheep) and cattle showed, no cross protection between two serotypes but only when vaccinated at least 2 times using 2 different serotypes, the broad heterotypic antibodies were produced which protected the animal when challenged with the third different serotype. However Randall (2005) showed a limited and variable cross protection between BTV-10 with other serotypes. Using a recombinant vaccine, Perez De Diego (2011) and Calvo-Pinilla (2012) showed that BTV protection is serotype-specific, and over and above this finding Calvo-Pinilla (2012) described that cross protection only occurs if the NS1 encoding gene is included in the recombinant vaccine. Cross protection between serotypes is therefore still not clear and with this background, this study was conducted using five BTV serotypes to determine their cross protection in sheep, the natural host of BTV. Since at least 21 BTV serotypes are present in southern Africa and live attenuated vaccines are used, the outcome of the study might improve the use of the currently used vaccine; to make it more affordable by using fewer serotypes, and to minimize the pathogenic effects of the disease.

Animals were first vaccinated using BTV-4 and challenged with BTV-4 (positive control) and BTV-1, 8, 9, 10 and 11. The serotypes were selected following the cross neutralization study done by Erasmus in 1990 (Figure 3), which shows the relationship between serotypes. The BTV-4 group was chosen because of its prevalence and virulence in southern Africa, and BTV-17, 20 and 24 were not considered because they are not included in the current vaccine. The selection of BTV-8 was done because of its prevalence and different pathogenesis in Europe. It was unfortunate that the results obtained for this serotype were inconclusive, see the discussion below for Group F.

Animals were divided into five groups. Two animals were involved in Group A, the positive control group, but for the experimental Groups B to E, six animals were used in each group. In general all animals in all groups had a slight fever after vaccinations. This is well known that transient fever and mild clinical signs are observed in most of the experiments conducted when animals are vaccinated with the BTV MLV (Savini *et al.,* 2008).

The clinical reaction of the animals post challenge was monitored serologically using SNT in which the cut-off neutralizing antibody titre was 1:16. The BTV-4 cross-protection clinical reaction post

challenge was measured using PPI values with a cut-off value of 55%. Previous published studies similar to this one did not indicate a specific cut-off value. A cut-off value where the PPI was not very high but acceptable was decided on which also can lead as a benchmark value for future studies. The use of PPI values is well published (Huismans *et al.,* 1987) but no specific cut-off value stated (Van Gennip *et al.,* 2012; Modumo & Venter, 2012).

All animals reacted to the infection post challenge except Group A, the positive control group (Figures 5 and 6). The reaction of Group A demonstrated sufficient protective immunity against BTV-4 challenge (Patta *et al.,* 2004; Dungu *et al.,* 2004). The animals in other groups reacted differently to the infection post challenge. Some animals demonstrated significant clinical signs like animals in Group B (BTV-1) with low neutralizing antibodies (below 1:16) (Table 5), which indicated the lack of protection by BTV-4. Animals in Group D (BTV-10) had neutralizing antibody titres above 1:16 (Table 7) but the animals had overt clinical signs and only a few had mild reactions post challenge. This indicates that BTV-4 neutralizing antibodies were not sufficient to provide protection to animals against BTV-10.

Some animals did not show any clinical signs although SNT titres were above the cut-off value. These animals could have been subclinically infected (Sperlova, 2011). Subclinical infection of animals by viruses, experimentally or naturally infected is well described *(*MacLachlan *et al*., 2008; Lee *at al.,* 2010).

One animal in Group E (Table 8) had a 100% PPI value but the SNT results were below the cut-off value of 1:16. A possible explanation to this is that BTV-4 does not only elicit a humoral immune response for BTV-11 but there were other factors like cell-mediated immune response that played a role in the protection of the animal. This has been recorded for BTV that T-cells directed to the NS1 non-structural and inner core proteins are stimulated (Schwartz-Cornil *et al*., 2008).

A few unique findings in the different groups are discussed per group:

Group A (BTV-4): This group did not show any clinical abnormalities post challenge. This showed that BTV-4 vaccine was protective against BTV-4 challenge material as expected.

Group B (BTV-1): Before the study, one sheep (unvaccinated) had a mild fever according to the normal ranges of rectal temperatures for this study, but it was eating and behaving normally. The possible cause could be that animals are unique and some have a higher body temperature in general. This has been observed (by the researcher and Animal Production Technicians mentioned above) with other trials at OBP that some animals will have a higher normal body temperature. This

animal (negative control) developed typical bluetongue clinical signs and died before the end of the study. This was proof that the challenge material used was efficacious.

In general this group had low neutralizing antibodies (Table 5) that improved from Day 35 post vaccination, **Appendix B**. By Day 21, only three animals had seroconverted (1:16) and the other two had very low seroconversion until Day 35. As a result, all of them developed moderate to severe clinical signs with the exception of one animal which did not react to the infection. This animal had a good PPI of 78% (Table 5, sheep 2278). Two other animals also had high PPI values of 76% (Table 5, sheep 80 and 87) but this was due to symptomatic treatment which masked the clinical signs; lowering of rectal temperature which is one of clinical reactions which forms part of CRI (Figure 8 and **Appendix A**). Therefore these animals had basically no protection. Only one animal was protected by BTV-4, 20% of the group, therefore BTV-4 vaccine should not be used as the primary vaccine for protection to BTV-1.

Group C (BTV-9): All animals in this group (n=5) had mild fever post challenge except one animal (Figure 10, sheep 81) which had moderate to high fever and mouth lesions which cleared without treatment. Overt clinical signs were observed in the negative control animal. This animal was treated following the guidelines of the Animal Ethics humane score sheet. The clinical signs for the negative control animal were used as base line to determine the PPI values for all the animals in the group. But since the clinical signs of this animal were masked by treatment, PPI values of two animals (Table 6, sheep 81 and 2394) did not reflect the true picture. Although these animals had very low PPI values, they only had mild fever, but did not get sick. The PPI results were skewed because their CRI was higher or equal to the CRI of the negative control animal (sheep 2396) (**Appendix B**). In general the animals were protected against BTV-9 by BTV-4 neutralizing antibodies. From the five animals (excluding negative control animal) that were involved, only one developed mild clinical signs with challenge, and the rest (80%) were protected from the effects of the virus.

Group D (BTV-10): As discussed above.

Group E (BTV-11): A total of 80% of animals developed good neutralizing antibodies post challenge and as a result the animals were protected to BTV-11 by antibodies to BTV-4 (Table 8). One animal had a good PPI value of 100% but no neutralizing antibodies could be detected as discussed earlier (Table 8).

Results obtained from animals challenged with BTV-8 (**Group F**) were inconclusive. No clinical signs were observed after challenged. No neutralizing antibodies could be detected and no virus could be

isolated on cell cultures from the material used to challenge the animals. The challenge material was therefore not adequate. The results obtained for this group were taken out of the study.

The BTV-4 vaccine used in this study at a titre of 4.2 x 10^6 log10$TCID_{50}$/ml showed good cross-protection against BTV-9 and 11. The vaccine had very limited cross-protection against BTV-1 and therefore BTV-4 vaccine at the above mentioned titre should not be used as primary vaccine against BTV-1. According to Figure 3, BTV-1 is only distantly related to BTV-4 which might be the reason why there was poor cross-protection. Results obtained in this study also indicated that BTV-4 cannot be used as primary vaccine for BTV-10. It must be highlighted though that results obtained for BTV-10 were disappointing, one would have expected a good cross-protection between these two serotypes in target animals since according to SNT results (Figure 3) there is a strong relationship between them. The reason could be that the degree of protection does not always correlate with the levels of neutralizing antibodies (Jeggo, 1986).

Future studies should include the investigation of cross protection between BTV serotypes using more and different serotypes e.g. BTV 8, 3 and 2 according the groups in Figure 3. In the study done in 1983 (Jeggo *et al.,* 1983), it was established that if animals infected serially with three different BTV serotypes, the animals were protected when challenged with different serotypes. Those animals which were exposed to BTV-3 had protection against BTV-6 as well as those that infected with BTV-3 and challenged with BTV-4.

In vivo studies should therefore include the above serotypes and their combinations looking into humoral and cellular immune response together with clinical signs. *In vitro* studies on the different serotypes should also include the kinetics of cell death, apoptosis and necrosis of these strains firstly as individual serotypes and then in combination with other serotypes. It is well known that the immunogenicity of the different BTV serotypes is different. Those which have the ability to replicate well in the host elicit strong humoral immune response (Modumo & Venter, 2012) and some replicate faster than others (Dungu *et al.,* 2004) and their virulence might be different in sheep. The use of different titres for each serotype used within the vaccine should therefore also be considered especially for BTV-2 and 8 since they are more immunogenic (Modumo & Venter, 2012).

Following this study, it is clear that there is a huge potential to improve the BTV MLV to benefit the manufacturer as well as the farmers. Basic scientific data pertaining to the different BTV isolates circulating in the field should however be determined; why do some serotypes cross the placenta, the potential secretion of the virus in semen, the difference in susceptibility to *Culicoides* midges and the production of clinical signs compare to virulence and immunogenicity.

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APPENDIX A

OBP - Bluetongue standard operating procedure

Clinical scoring (Post Challenge)

The severity of clinical BT after challenge with a virulent virus is expressed numerically as a clinical reaction index (CRI) that is obtained by adding the following 3 scores (a+b+c):

a) Febrile reaction- The cumulative total fever readings above 40°C from day 3 to 14.
b) Clinical lesion score – Lesions of the nose, mouth and feet are scored on a scale of 0-4
c) Fatality- an additional 4 points are added if death occurs within 14 days.

Clinical lesion score:

In order to perform clinical examination it is often necessary to clean the nose and mouth as nasal discharge is common with clinical BT. For this clean water and a soft sponge are used.
Nose and mouth lesions usually appear within one or two days after the onset of fever.
Foot lesions usually develop towards the end of the febrile reaction. The hind feet are most frequently affected.

Numerical Score	Nose	Mouth	Hooves
0	Normal	Normal	Normal
1	Slight hyperaemia	Slight hyperaemia	Slight hyperaemia of the coronary band.
2	Hyperaemia & slight erosion	Slight hyperaemia & erosions at mucocutaneous junction of upper lip and swelling of mouth	Hyperaemia of the coronary band with slight petechiae
3	Erosion & petechiae	Hyperaemia, erosion and slight cyanosis of the mucosa	Hyperaemia of the coronary band with petechiae
4	Severe erosion, hyperaemia & haemorrhages	Severe erosion and cyanosis	Severe hyperaemia of the coronary band with petechiae (might become streaky)

Clinical score sheet

Project:___________________________________

Start date (Day3):_________________________

Ovine no	Clinical score	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												
	Temp.												
	Nose												
	Mouth												
	Hooves												

APPENDIX B

Huismans *et al*., 1987

Group A– Positive control animals

SNT results

Cell types: Vero
Original virus titre: 5.6X10 log 5
Virus titre used: 3.1x 10 log 2
Positive control serum: 1:128
Cut-off point (protection): 1:16

Animal identification number	Day 0 (of the study)	Day 7	Day 14	Day 21	Day 28	Day 35 (D7 post challenge with BTV 4)	Day 42 (D14 post challenge with BTV 4)
72	<1:2	1:64	1:128	1:128	1:256	1:256	1:256
207	<1:2	1:8	1:64	1:64	1:128	1:128	1:256

Clinical score index per animal in Group A

SHEEP 72 - Vaccinated and challenged with the same serotype

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI =0					RR = 0			PPI % =100%						

SHEEP 207 - Vaccinated and challenged with the same serotype

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI =0		RR = 0						PPI =100%						

Group B

SNT results

(i) After vaccinations – BTV 4 antigen

Cell types: Vero
Original virus titre: 5.6 x10 log 5
Virus titre used: 5.6 x10 log 2
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35 (D7 post challenge with BTV 1)
6	<1:2	1:16	1:64	1:32	1:32	1:16
80	<1:2	1:32	1:64	1:64	1:64	1:64
87	<1:2	1:64	1:256	1:256	1:128	1:128
89	<1:2	1:64	1:64	1:64	1:128	1:128
2278	<1:2	1:64	1:64	1:128	1:128	1:128
76 (unvaccinated)	<1:2	1:2	<1:2	<1:2	<1:2	1:4

(ii) After challenge with BTV 1 Antigen

Cell types: Vero
Original virus titre: 1.7 x10 log 4
Virus titre used: 7.5 x10 log 3
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0 (Challenged) / D28 post vaccinations	Day 7	Day 14	Day 21	Day 28	Day 35
6	<1:2	1:2	<1:2	1:4	1:16	1:32
80	<1:2	<1:2	1:4	1:4	1:4	1:8
87	<1:2	<1:2	1:2	1:8	1:8	1:16
89	<1:2	<1:2	1:16	1:16	1:16	1:32
2278	<1:2	<1:2	1:4	1:16	1:16	1:32
76 (unvaccinated)	<1:2	<1:2	Dead	Dead	Dead	Dead

Clinical score index per animal in Group B

SHEEP 76 -Unvaccinated

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	1	1	1	1	1	1	1	1	1	0	0	4	13
(b)	0	0	0	0	1	1	1	1	1	1	0	0	4	10
(c)	0	0	0	0	0	0	0	0	0	0	4	0	0	0
CRI = 23			RR =N/A			PPI = 0%								

SHEEP 6

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	1	0	0	1	0	1	1	1	1	1	0	0	0	7
(b)	0	0	0	0	0	0	0	1	1	1	1	1	0	5
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 12					RR = 12/23 =52%			PPI = 100% -52% =48%						

SHEEP 80

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	1	1	0	0	0	0	0	1	0	0	1	0	0	4
(b)	0	0	0	0	0	0	0	0	0	0	1	1	0	2
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 6					RR = 12/23 =26%			PPI = 100% -26% =76%						

SHEEP 87

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	1	1	1	1	1	1	0	0	0	0	0	6
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 6					RR = 6/23 =26%			PPI = 100% -26% =76%						

SHEEP 89

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	1	0	0	0	1	1	1	1	1	1	1	0	0	7
(b)	0	0	0	0	0	0	1	1	1	1	1	1	0	6
(c)	0	0	0	0	0	0	0	0	0		0	0	0	0
CRI = 13			RR = 13/23 =56%				PPI = 100% -56%=44%							

SHEEP 2278

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	1	1	1	1	1	0	0	0	0	5
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 5					RR = 5/23 =22%			PPI = 100% -22% =78%						

Group C

SNT results

(i) After vaccinations – BTV4 antigen

Cell types: Vero
Original virus titre: 5.6 x10 log 5
Virus titre used: 5.6 x10 log 2
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35 (D7 post challenge with BTV 9)
9	<1:2	1:64	1:32	1:64	1:64	1:128
15	<1:2	1:32	1:64	1:64	1:64	1:256
52	<1:2	1:32	1:64	1:64	1:64	1:128
81	<1:2	1:32	1:64	1:64	1:32	1:64
2394	<1:2	1:32	1:64	1:64	1:64	1:64
2396 (unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2

(ii) After challenge with BTV9 Antigen

Cell types: Vero
Original virus titre: 5.6X10 log 5
Virus titre used: 3.1x 10 log 2
Positive control serum: 1:128
Cut-off point (protection): 1:16

Animal identification number	Day 0 (Challenged) / D28 post vaccinations	Day 7	Day 14	Day 21	Day 28	Day 35
9	<1:2	1:2	1:64	1:128	1:128	1:128
15	<1:2	1:2	1:16	1:256	1:256	1:256
52	<1:2	1:2	1:4	1:64	1:128	1:256
81	<1:2	1:2	1:8	1:32	1:64	1:64
2394	<1:2	<1:2	<1:2	1:16	1:32	1:64
2396 (unvaccinated)	<1:2	1:2	1:16	1:64	1:32	1:32

Clinical score index per animal in Group C

SHEEP 2396 - Unvaccinated

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	0	1	1	1	1	0	4
(b)	0	0	0	0	0	0	0	0	0	1	1	1	0	3
(c)	0	0	0	0	0	0	0	0	0	0	0	0		
CRI = 7					RR =N/A			PPI = 0%						

SHEEP 9

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	1	1	1	0	0	0	0	0	0	0	3
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 3					RR = 3/7 =42%			PPI = 100% -42% =58%						

SHEEP 15

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	1	0	0	0	0	0	1	0	2
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 2				RR = 2/7 =28%			PPI = 100% -28% =72%							

SHEEP 52

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	1	1	0	0	0	0	2
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 2					RR = 2/7 =28%			PPI = 100% -28% =72%						

SHEEP 81

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	1	1	1	1	1	1	0	0	0	0	0	6
(b)	0	0	0	0	1	1	1	1	1	1	0	0	0	6
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 12			RR = 12/7 =171%					PPI = 100% -171% = -71%						

SHEEP 2394

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	1	1	0	0	1	1	1	0	0	1	1	0	7
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 7				RR = 7/7 =100%				PPI = 100% -100% =0%						

Group D

SNT results

(i) After vaccinations with BTV4 Antigen

Cell types: Vero
Original virus titre: 5.6 x10 log 5
Virus titre used: 5.6 x10 log 2
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35 (D7 post challenge with BTV 9)
14	<1:2	<1:2	<1:2	1:32	1:32	1:64
29	<1:2	<1:2	<1:2	1:64	1:64	1:128
86	<1:2	1:64	1:64	1:64	1:64	1:128
2283	<1:2	1:16	1:64	1:32	1:32	1:32
2391	<1:2	1:32	1:64	1:64	1:256	1:256
56(unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2

(ii) After challenge with BTV10 Antigen

Cell types: Vero
Original virus titre: 5.6 x10
Virus titre used: 5.6 x10 log 2
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0 (Challenged) / D28 post vaccinations	Day 7	Day 14	Day 21	Day 28	Day 35
14	<1:2	1:16	1:256	1:256	1:256	1:256
29	<1:2	1:2	1:256	1:256	1:256	1:256
86	<1:2	1:32	1:256	1:256	1:256	1:256
2283	<1:2	1:16	1:16	1:256	1:256	1:256
2391	<1:2	1:8	1:2	1:128	1:256	1:256
56 (unvaccinated)	<1:2	<1:2	1:4	1:256	1:256	1:256

Clinical score index per animal in Group D

SHEEP 56 - Unvaccinated

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	1	1	1	0	1	0	4
(b)	0	0	0	0	0	0	0	0	0	1	1	1	0	3
(c)	0	0	0	0	0	0	0	0	0	0	0	0		
CRI = 7					RR =N/A			PPI = 0%						

SHEEP 14

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	1	1	1	1	1	1	0	0	0	0	0	6
(b)	0	0	0	0	0	1	1	1	1	1	0	0	0	5
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 11					RR = 11/7 =157%			PPI = 100% -157% = -57%						

SHEEP 29

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	1	0	1	1	0	0	0	1	0	4
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI =4				RR = 4/7 =57%			PPI = 100% -57% = 43%							

SHEEP 86

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	1	1	1	1	1	1	0	0	6
(b)	0	0	0	0	0	0	1	1	1	1	1	1	0	6
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 12					RR = 12/7 =171%			PPI = 100% -171% = -71%						

SHEEP 2283

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	1	1	1	1	1	1	1	0	1	0	8
(b)	0	0	0	0	0	0	0	1	1	1	0	0	0	3
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 11					RR = 11/7 =157%			PPI = 100% -157% = -57%						

SHEEP 2391

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	1	1	0	0	0	0	0	2
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 2					RR = 2/7 =28%			PPI = 100% -28 = 72%						

Group E

SNT results

(i) After vaccinations with BTV4 Antigen

Cell types: Vero
Original virus titre: 5.6 x10 log 5
Virus titre used: 5.6 x10 log 2
Positive control serum: 1:64
Cut-off point (protection): 1:16

Animal identification number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 35 (D7 post challenge with BTV 9)
53	<1:2	1:32	1:64	1:128	1:256	1:256
100	<1:2	1:16	1:128	1:256	1:256	1:256
2269	<1:2	1:256	1:256	1:256	1:256	1:256
2273	<1:2	1:128	1:128	1:256	1:64	1:128
2286	<1:2	1:32	1:64	1:64	1:64	1:64
84(unvaccinated)	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2

(ii) Post challenge with BTV11 Antigen

Cell types: Vero
Original virus titre: 2.3x10 log 5
Virus titre used: 1x10 log 2
Positive control serum: 1:128
Cut-off point (protection): 1:16

Animal identification number	Day 0 (challenged)	Day 7	Day 14	Day 21	Day 28	Day 35
53	<1:2	1:4	1:16	1:16	1:16	1:64
100	<1:2	1:16	1:128	1:256	1:128	1:256
2269	<1:2	1:4	1:64	1:64	1:64	1:256
2273	<1:2	1:2	1:64	1:128	missed bleeding	1:256
2286	<1:2	1:2	1:2	1:4	1:4	1:16
84 (unvaccinated)	<1:2	<1:2	<1:2	1:8	dead	dead

Clinical score index per animal in Group E

SHEEP 84 -Unvaccinated

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	1	0	1	1	1	1	1	1	1	1	1	0	10
(b)	0	0	0	0	0	0	1	1	1	1	5	3	0	12
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 22			RR = N/A			PPI % = 0%								

SHEEP 53

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	1	0	0	0	0	0	0	0	0	1
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 1			RR = 1/22 =5%					PPI = 100% -5 = 95%						

SHEEP 100

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	1	1	1	1	1	1	0	0	0	0	0	6
(b)	0	0	0	0	0	0	1	1	1	1	0	0	0	4
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 10					RR = 10/22 =45%			PPI = 100% -45 = 55%						

SHEEP 2269

Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	1	1	1	1	1	0	0	0	0	0	0	0	5
(b)	0	0	0	0	1	1	1	0	0	0	0	0	0	3
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 8					RR = 8/22 =36%			PPI = 100% -36 = 64%						

SHEEP 2273

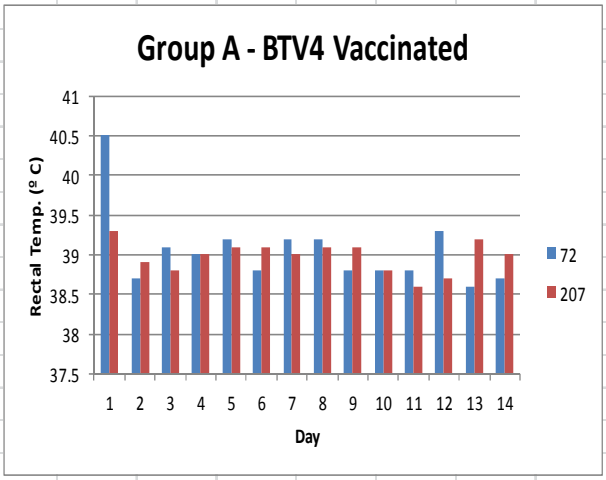
Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	1	1	0	0	0	0	0	2
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 2					RR = 2/22 =9%			PPI = 100% -9 = 91%						

SHEEP 2286

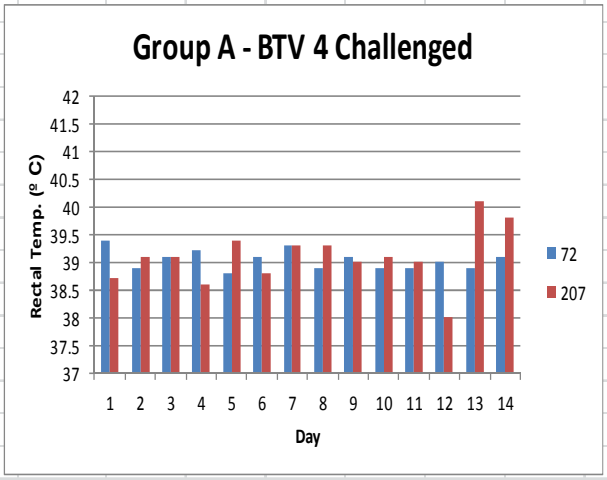
Day	3	4	5	6	7	8	9	10	11	12	13	14	Plus death	Total
(a)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(b)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
(c)	0	0	0	0	0	0	0	0	0	0	0	0	0	0
CRI = 20					RR = 0/22 =9%			PPI = 100% -0 = 100%						

APPENDIX C – Temperature results

		GROUP A	
	Temp results post vaccinations		
		72	207
Day	*A.M. Rectal temperature (Degrees Celsius)*		
-3		39	39.5
-2		39.4	38.8
-1		39.1	39.7
0			
1		40.5	39.3
2		38.7	38.9
3		39.1	38.8
4		39	39
5		39.2	39.1
6		38.8	39.1
7		39.2	39
8		39.2	39.1
9		38.8	39.1
10		38.8	38.8
11		38.8	38.6
12		39.3	38.7
13		38.6	39.2
14		38.7	39



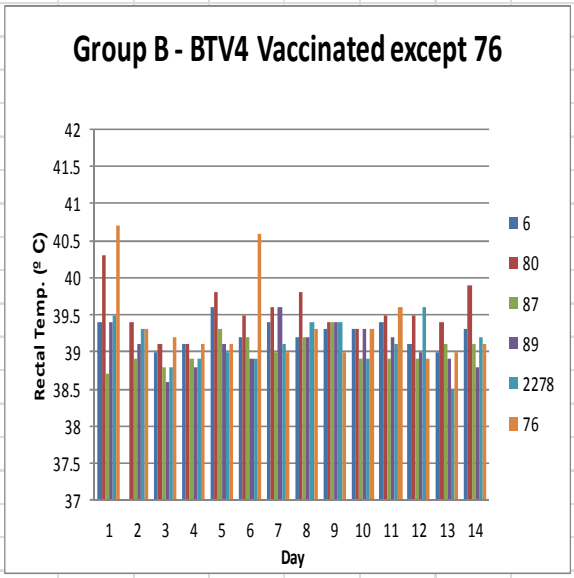
	Temp results post challenge		
		72	207
Day	*A.M. Rectal temperature (Degrees Celsius)*		
-3		39	38.8
-2		39.6	39.1
-1		38.9	38.9
0		39.6	38.9
1		39.4	38.7
2		38.9	39.1
3		39.1	39.1
4		39.2	38.6
5		38.8	39.4
6		39.1	38.8
7		39.3	39.3
8		38.9	39.3
9		39.1	39
10		38.9	39.1
11		38.9	39
12		39	38
13		38.9	40.1
14		39.1	39.8



GROUP B

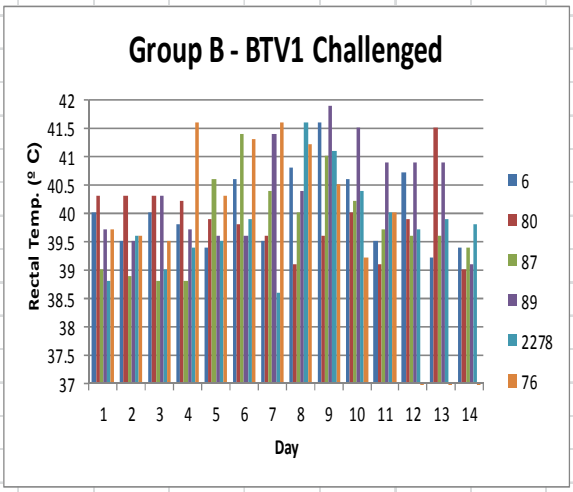
Temperature results post vaccinations

	6	80	87	89	2278	76 (Control)
Day	*A.M. Rectal temperature (Degrees Celsius)*					
-3	39.5	39.8	38.9	39.1	39.2	39.2
-2	39.4	39.5	38.7	39	39.5	39.1
-1	39.8	40	38.9	39	39.2	39.7
0						
1	39.4	40.3	38.7	39.4	39.5	40.7
2	0	39.4	38.9	39.1	39.3	39.3
3	39	39.1	38.8	38.6	38.8	39.2
4	39.1	39.1	38.9	38.8	38.9	39.1
5	39.6	39.8	39.3	39.1	39	39.1
6	39.2	39.5	39.2	38.9	38.9	40.6
7	39.4	39.6	39	39.6	39.1	39
8	39.2	39.8	39.2	39.2	39.4	39.3
9	39.3	39.4	39.4	39.4	39.4	39
10	39.3	39.3	38.9	39.3	38.9	39.3
11	39.4	39.5	38.9	39.2	39.1	39.6
12	39.1	39.5	38.9	39	39.6	38.9
13	39	39.4	39.1	38.9	38.5	39
14	39.3	39.9	39.1	38.8	39.2	39.1



Temperature results post challenge

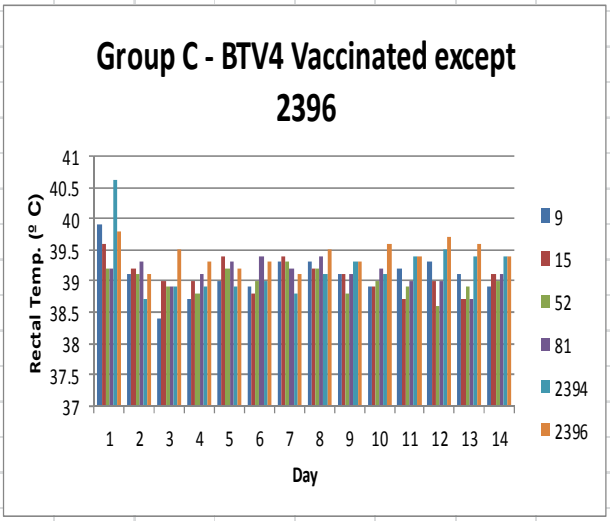
	6	80	87	89	2278	76 (Control)
Day	*A.M. Rectal temperature (Degrees Celsius)*					
-3	39.1	39.6	38.7	38.9	39.2	39.1
-2	39.9	40.1	39.1	39.7	39	39.7
-1	39.1	40.4	39.2	39.3	39.3	39.4
0	39.5	38.8	38.9	39.3	39.1	39.5
1	40	40.3	39	39.7	38.8	39.7
2	39.5	40.3	38.9	39.5	39.6	39.6
3	40	40.3	38.8	40.3	39	39.5
4	39.8	40.2	38.8	39.7	39.4	41.6
5	39.4	39.9	40.6	39.6	39.5	40.3
6	40.6	39.8	41.4	39.6	39.9	41.3
7	39.5	39.6	40.4	41.4	38.6	41.6
8	40.8	39.1	40	40.4	41.6	41.2
9	41.6	39.6	41	41.9	41.1	40.5
10	40.6	40	40.2	41.5	40.4	39.2
11	39.5	39.1	39.7	40.9	40	40
12	40.7	39.9	39.6	40.9	39.7	0
13	39.2	41.5	39.6	40.9	39.9	0
14	39.4	39	39.4	39.1	39.8	0



GROUP C

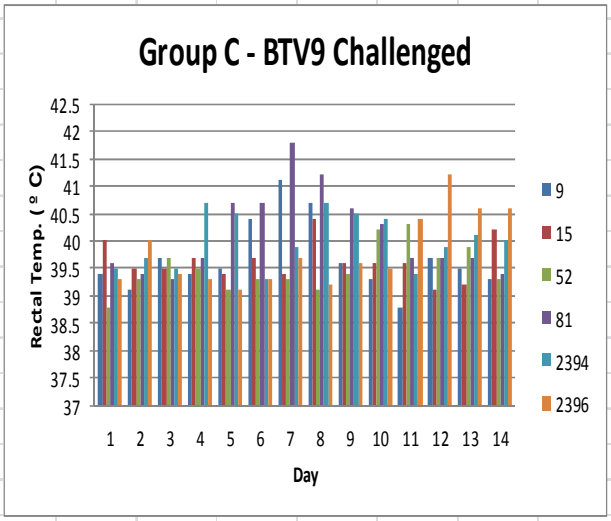
Temperature results post vaccinations

Day	9	15	52	81	2394	2396 (Control)
	A.M. Rectal temperature (Degrees Celsius)					
-3	39.6	39.7	39.5	39.3	39.6	39.5
-2	39	39	39.2	39.3	39	39.7
-1	39.2	39.2	39.4	39.2	39.7	39.8
0						
1	39.9	39.6	39.2	39.2	40.6	39.8
2	39.1	39.2	39.1	39.3	38.7	39.1
3	38.4	39	38.9	38.9	38.9	39.5
4	38.7	39	38.8	39.1	38.9	39.3
5	39	39.4	39.2	39.3	38.9	39.2
6	38.9	38.8	39	39.4	39	39.3
7	39.3	39.4	39.3	39.2	38.8	39.1
8	39.3	39.2	39.2	39.4	39.1	39.5
9	39.1	39.1	38.8	39.1	39.3	39.3
10	38.9	38.9	39	39.2	39.1	39.6
11	39.2	38.7	38.9	39	39.4	39.4
12	39.3	39	38.6	39	39.5	39.7
13	39.1	38.7	38.9	38.7	39.4	39.6
14	38.9	39.1	39	39.1	39.4	39.4



Temperature results post challenge

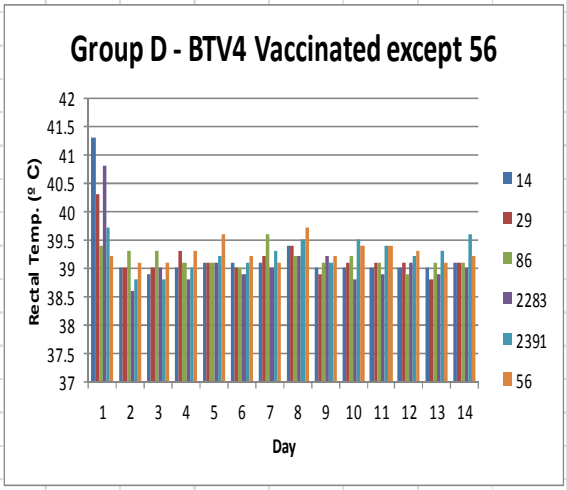
Day	9	15	52	81	2394	2396 (Control)
	A.M. Rectal temperature (Degrees Celsius)					
-3	39.4	39.3	39.3	39.9	38.6	38.8
-2	39.7	39.8	39.8	39.8	39.3	39
-1	39.3	39.6	39.1	39.3	39.4	39
0	38.9	39.2	39.3	39.1	39	39.2
1	39.4	40	38.8	39.6	39.5	39.3
2	39.1	39.5	39.3	39.4	39.7	40
3	39.7	39.5	39.7	39.3	39.5	39.4
4	39.4	39.7	39.5	39.7	40.7	39.3
5	39.5	39.4	39.1	40.7	40.5	39.1
6	40.4	39.7	39.3	40.7	39.3	39.3
7	41.1	39.4	39.3	41.8	39.9	39.7
8	40.7	40.4	39.1	41.2	40.7	39.2
9	39.6	39.6	39.4	40.6	40.5	39.6
10	39.3	39.6	40.2	40.3	40.4	39.5
11	38.8	39.6	40.3	39.7	39.4	40.4
12	39.7	39.1	39.7	39.7	39.9	41.2
13	39.5	39.2	39.9	39.7	40.1	40.6
14	39.3	40.2	39.3	39.4	40	40.6



GROUP D

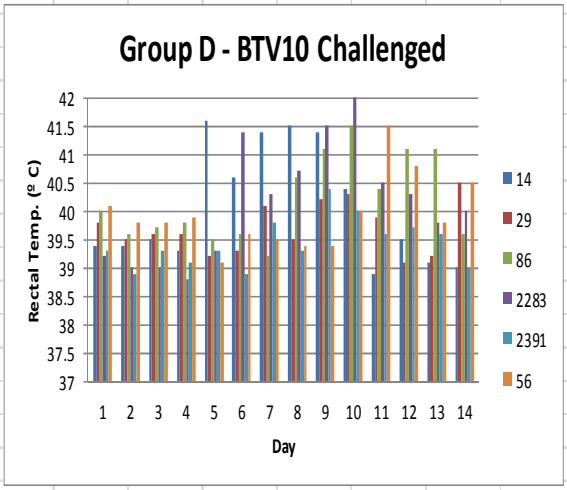
Temp results post vaccinations

		14	29	86	2283	2391	56 (Control)
Day	*A.M. Rectal temperature (Degrees Celsius)*						
-3		39.5	39.5	39.3	39.3	39.4	39.5
-2		39.2	39	39.5	38.9	38.8	39.2
-1		39.3	39.5	39.4	39.1	39.4	39.6
0							
1		41.3	40.3	39.4	40.8	39.7	39.2
2		39	39	39.3	38.6	38.8	39.1
3		38.9	39	39.3	39	38.8	39.1
4		39	39.3	39.1	38.8	39	39.3
5		39.1	39.1	39.1	39.1	39.2	39.6
6		39.1	39	39	38.9	39.1	39.2
7		39.1	39.2	39.6	39	39.3	39.1
8		39.4	39.4	39.2	39.2	39.5	39.7
9		39	38.9	39.1	39.2	39.1	39.2
10		39	39.1	39.2	38.8	39.5	39.4
11		39	39.1	39.1	38.9	39.4	39.4
12		39	39.1	38.9	39.1	39.2	39.3
13		39	38.8	39.1	38.9	39.3	39.1
14		39.1	39.1	39.1	39	39.6	39.2



Temp results post challenge

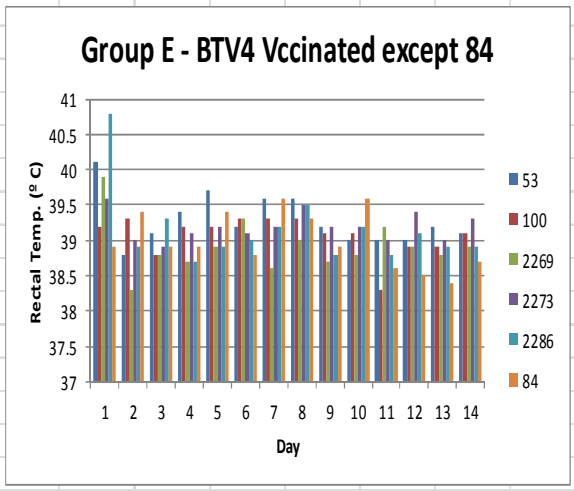
		14	29	86	2283	2391	56 (Control)
Day	*A.M. Rectal temperature (Degrees Celsius)*						
-3		39.2	39.3	39.6	38.9	39.1	39.8
-2		39.7	39.5	40.1	38.7	39.1	40
-1		39.3	39.3	39.7	39.1	39.6	39.8
0		39.6	39	39.5	39.1	39.3	39.1
1		39.4	39.8	40	39.2	39.3	40.1
2		39.4	39.5	39.6	39	38.9	39.8
3		39.5	39.6	39.7	39	39.3	39.8
4		39.3	39.6	39.8	38.8	39.1	39.9
5		41.6	39.2	39.5	39.3	39.3	39.1
6		40.6	39.3	39.6	41.4	38.9	39.6
7		41.4	40.1	39.2	40.3	39.8	39.5
8		41.5	39.5	40.6	40.7	39.3	39.4
9		41.4	40.2	41.1	41.5	40.4	39.4
10		40.4	40.3	41.5	42	40	40
11		38.9	39.9	40.4	40.5	39.6	41.5
12		39.5	39.1	41.1	40.3	39.7	40.8
13		39.1	39.2	41.1	39.8	39.6	39.8
14		39	40.5	39.6	40	39	40.5



GROUP E

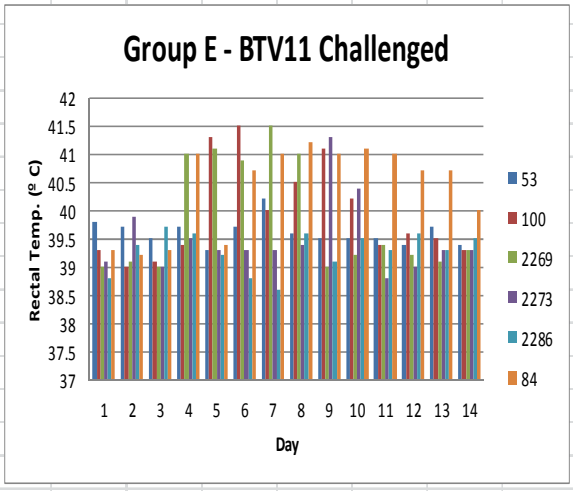
Temp results post vaccinations

Day	A.M. Rectal temperature (Degrees Celsius)					
	53	100	2269	2273	2286	84 (Control)
-3	39.7	39.2	39	39.6	39.1	39.2
-2	39.1	39.2	38.5	39.4	39.3	39
-1	39.7	39.2	39	39.5	39	39
0						
1	40.1	39.2	39.9	39.6	40.8	38.9
2	38.8	39.3	38.3	39	38.9	39.4
3	39.1	38.8	38.8	38.9	39.3	38.9
4	39.4	39.2	38.7	39.1	38.7	38.9
5	39.7	39.2	38.9	39.2	38.9	39.4
6	39.2	39.3	39.3	39.1	39	38.8
7	39.6	39.3	38.6	39.2	39.2	39.6
8	39.6	39.3	39	39.5	39.5	39.3
9	39.2	39.1	38.7	39.2	38.8	38.9
10	39	39.1	38.8	39.2	39.2	39.6
11	39	38.3	39.2	39	38.8	38.6
12	39	38.9	38.9	39.4	39.1	38.5
13	39.2	38.9	38.8	39	38.9	38.4
14	39.1	39.1	38.9	39.3	38.9	38.7



Temp results post challenge

Day	A.M. Rectal temperature (Degrees Celsius)					
	53	100	2269	2273	2286	84 (Control)
-3	39.3	39.1	38.8	39	39.3	39.3
-2	39.9	39.7	38.9	39.1	38.9	39.5
-1	39.6	39.2	38.9	39.5	39.1	39.3
0	39.1	39.3	39.3	38.8	39.2	39.3
1	39.8	39.3	39	39.1	38.8	39.3
2	39.7	39	39.1	39.9	39.4	39.2
3	39.5	39.1	39	39	39.7	39.3
4	39.7	39.4	41	39.5	39.6	41
5	39.3	41.3	41.1	39.3	39.2	39.4
6	39.7	41.5	40.9	39.3	38.8	40.7
7	40.2	40	41.5	39.3	38.6	41
8	39.6	40.5	41	39.4	39.6	41.2
9	39.5	41.1	39	41.3	39.1	41
10	39.5	40.2	39.2	40.4	39.5	41.1
11	39.5	39.4	39.4	38.8	39.3	41
12	39.4	39.6	39.2	39	39.6	40.7
13	39.7	39.5	39.1	39.3	39.3	40.7
14	39.4	39.3	39.3	39.3	39.5	40



APPENDIX D

HUMANE END SCORE MONITORING SHEETS

From OBP Animal Ethic Committee - Humane end score sheet: Infectious disease research

EXPERIMENT NO:				**ANIMAL NO:**			
WEIGHT (Kg)							
	DATE/TIME						
APPEARANCE	**SCORE**						
Normal							
Diminished grooming							
Piloerection, discharege nose/eyes							
Soiled, poorly groomed coat							
BODY WEIGHT							
Normal < 5%							
Body weight 5-10%							
Body weight 10-15%							
Body weight 15-20%							
CLINICAL SIGNS							
Food and water intake							
Stool normal – slightly sort							
Diarrhoea							
Increased abdominal dimension, soft on palpation, no stool							
RESPONS TO HANDLING							
Normal							
Slightly decrease or increased response							
Strongly decreased or increased response/vocalization on abdominal palpation							
Decrease or increased response							
TOTAL							
SIGNATURE							

CONDITION SCORING:

- Breathing: R = rapid; S = Shallow; L = laboured; N = normal.
- Condition: 4 = Normal, 0 = emaciated Condition: 4 = Normal, 0 = emaciated.

SPECIAL HUSBANDRY REQUIREMENTS:

Assess animals 2x per day of challenge until end of the experiment.

ASSESMENT:

0 - 4	Normal
5 - 9	Increase frequency of assessment and observe the animal more closely.
10 - 15	Clear distress present. Treat the animal if possible. Increase the frequency of observation. Consult with principal investigator/veterinarian/head animal technologist. Consider euthanizing the animal.
15 - 20	Severe distress. If there are no pressing scientific reasons, the animals should be euthanized.

(OBP SOP)

HUMANE ENDPOINTS AND ACTIONS:

1. In case of central nervous signs such as ataxia or convulsions the animals may be euthanized.
2. The same is true of low body temperature (<34.5 ˚C).
3. Validation studies have shown that a drop in body weight is not always predictive of a lethal outcome.
4. The decision to terminate is taken by the responsible veterinarian.
5. For each animal group, the number of animals that dies per day is recorded.

(OBP SOP)

Animal Welfare Monitoring Sheet

ANIMAL IDENTIFICATION:	Score	Date / Time	Date / Time	Date / Time	Date / Time	Date / Time	Date / Time	Date / Time
APPEARANCE								
Normal	0							
General lack of grooming	1							
Coat staring, ocular and nasal discharges	2							
Piloerection, hunched up	3							
FOOD AND WATER INTAKE								
Normal	0							
Uncertain: body weight ↓ <5%	1							
↓ intake: body weight ↓ 10-15%	2							
No food or water intake	3							
NATURAL BEHAVIOUR								
Normal	0							
Minor changes	1							
Less mobile and alert, isolated	2							
Vocalization, self-mutilation, restless or very still	3							
PROVOKED BEHAVIOUR								
Normal	0							
Minor depression or exaggerated response	1							
Moderate change in expected behavior	2							
Reacts violently, or very weak and precomatose	3							
CLINICAL SIGNS								
Normal cardiac and respiratory rates, hydration	0							
Slight changes, slight dehydration	1							
C/R rates ↕ 30%, 10-20% dehydrated	2							
C/R rates ↕ 50% or very ↓, >20% dehydrated	3							
SCORE ADJUSTMENT								
If you have scored a 3 more than once, add an extra point for each 3	2 - 5							
TOTAL	**0 - 20**							
Signature (initials)								

JUDGEMENT

0 - 4 Normal

5 - 9 Monitor carefully, consider analgesics

10 - 14 Suffering; provide relief, observe regularly. Seek second opinion from day-to-day care person and/or veterinary surgeon. Consider termination.

15 - 20 Severe pain. Does your experimental protocol need rethinking