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Determination of the minimum protective dose for bluetongue serotype 2, 4 and 8 vaccines in sheep

BY

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Declaration

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of Master of Science (Veterinary Tropical Diseases) has not been previously submitted by me 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 acknowledged.

Signed:

Date:

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Abbreviations

BEI	-	Binary ethylenimine
BHK	-	Baby hamster kidney cells
BLP	-	Buffered lactose peptone
BP	-	Beta propriolactone
BTA	-	Blood treptose agar plates
BT	-	Bluetongue
BTV	-	Bluetongue virus
CPE	-	Cytopathic effect
CRI	-	Clinical reaction index
CTL	-	Cytotoxic T lymphocytes
cELISA	-	Competitive enzyme-linked immunosorbent assay
DIVA	-	Differentiatind infected from vaccinated animals
EFSA	-	European Food Safety Authority
FCS	-	Foetal calf serum
IAH	-	Institute for animal health
IV	-	Intravenous
MLV	-	Modified live virus
MOI	-	Multiplicity of infection
OBP	-	Onderstepoort Biological Product
OIE	-	Office International des Epizooties
OVI	-	Onderstepoort Veterinary Institute
PFU	-	Plaque forming unit
P.C.	-	Post challenge
P.V.	-	Post vaccination
QC	-	Quality control
RBC	-	Red blood cells
RR	-	Relative reaction
RT-PCR	-	Reverse-transcriptase polymerase chain reaction
SGIB	-	Salivary glands escape barrier
SOP	-	Standard operating procedure

SPF	-	Specific pathogen free
$TCID_{50}$	-	Tissue culture infective dose (50%)
YS	-	Yolk-sac
UP	-	University of Pretoria
VLP	-	Virus like particles
VMRD	-	Veterinary Medical Research and Development
VP	-	Viral protein
SNT	-	Serum neutralization test
WFI	-	Water for injection
WSV	-	Working seed virus

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Abstract

The bluetongue (BT) live attenuated virus vaccine has been used successfully in the control of BT in southern Africa and Europe. However, concerns about the safety, possible development of viraemia and clinical signs post vaccination (p.v.) presented an opportunity to investigate the possibility of reducing the current bluetongue virus (BTV) vaccine titre to below 10^4 PFU/ml.

A total of 83 merino sheep were used and vaccinated with BTV monovalent vaccines containing either serotypes 2, 4 or 8 with the following titres: 10^2, 10^3 and 10^4 PFU/ml. Positive and negative control sheep were also included. Animals were bled from Day 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 p.v and tested for viraemia. Seroconversion was determined on Day 0, 3, 9, 15, 21, 6 weeks, 3 and 4 months p.v. Vaccinated sheep were then challenged at 6 weeks p.v. using BTV infected blood and at 4 months using cell cultured material and evaluated for 14 days using the clinical reaction index.

Seroconversion was demonstrated p.v. in more than 70% of sheep vaccinated with a low titre 10^2 PFU/ml of BTV serotypes 2, 4 and 8 from day 9 and at 4 months. All three serotypes did not demonstrate any viraemia p.v. at the three different titres (10^2, 10^3 & 10^4 PFU/ml). Viraemia was demonstrated p.c. with cell culture material in sheep vaccinated with low titres (10^2 & 10^3 PFU/ml) of BTV serotypes 2 and 4. Viraemia could not be detected in sheep p.v. and p.c. with BTV serotype 8 in all different titres. Sheep challenged with cell culture material of BTV 2 and 4 showed mild clinical signs compare to those challenged with blood culture material that did not respond as expected as positive controls did not demonstrate any clinical signs of BT.

It was demonstrated in this study that BTV monovalent vaccines containing serotypes 2, 4 and 8 with titres below 10^4 PFU/ml can protect more than 90% of vaccinated

animals against clinical disease. Although certain serotypes failed to protect against viraemia, all serotypes protected against the development of clinical disease when challenged with either BTV-infected blood or cell cultured material.

CHAPTER 1

Literature review

1.1 Introduction

Bluetongue (BT) is a non-contagious, insect-transmitted disease of certain domestic and wild ruminants that is caused by Bluetongue virus (BTV) (Theiler 1908); Bluetongue virus is believed to be of African origin and was described as “malarial catarral fever” and “epizootic catarrhal of sheep” by investigators in South Africa (Theiler 1908). Bluetongue virus is the prototype member of the genus *Orbivirus* in the family *Reoviridae.* The virus shares specific common properties which include segmented genome of double –stranded RNA.

Infection in ruminants occurs throughout much of the temperate and tropical regions of the world and coincides with the distribution of specific species of *Culicoides* biting midges that are biological vectors of the virus. Though the disease is of economic importance not all breeds of sheep are affected by the disease (Erasmus 1975; 1980). Clinical signs of BT are variable but can include fever, increased salivation and lacrimation, watery discharge excessive licking of the movement of the tongue and lameness (Verwoerd and Erasmus 2004). In South Africa BTV has been isolated from a number of *Culicoides* species namely *Culicoides imicola* and *Culicoides bolitinos*. The epidemiology of these vectors makes it difficult to control the disease and farmers rely on vaccination to control the disease.

Sir Arnold Theiler developed the first vaccine against BT by passaging the virus a few times in sheep and then inoculated it into sheep to test its efficacy. Though the vaccine was protective, its side effects resulted in some sheep developing BT symptoms. The vaccine was later adapted into eggs and later into cell cultures. Currently all the serotypes in the cell culture adapted vaccine are released at a titre of 5×10^4 PFU/ml. However, concerns are regularly raised about the long duration of viraemia that may sometimes occur in especially merino sheep, an exotic breed in South Africa, which may later follow with the development of BT- like symptoms.

Not withstanding this, the vaccine has been used with success in Southern Africa and more recently in Europe, using only selected serotypes. However, there was a need to investigate the possibility of using monovalent live attenuated BTV vaccines at lower titres to determine if the vaccine will have a shorter duration or absence of viraemia and still be efficacious.

1.2 The virus

Bluetongue virus is a non-enveloped virus with a double stranded segmented RNA genome consisting of ten segments which each encodes for a specific protein (Els and Verwoerd 1969; Howell and Verwoerd 1971). Seven structural proteins (VP1 – VP7) and four non structural proteins (NS1, NS2, NS3, NS3A) are present. These genome segments are packaged within a two layered protein capsid. The outer capsid layer contains two major proteins VP2 and VP5 (Huismans and Erasmus 1981; Huismans *et al*., 1987). Parts of the VP2 protein contain the major determinants of serotype specificity. The inner layer contains 2 additional major proteins VP3 and VP7 and three minor proteins VPI, VP4 and VP6 (Figure 1) (Mertens *et al.,* 1989; Schwartz-Cornil *et al.*, 2008).

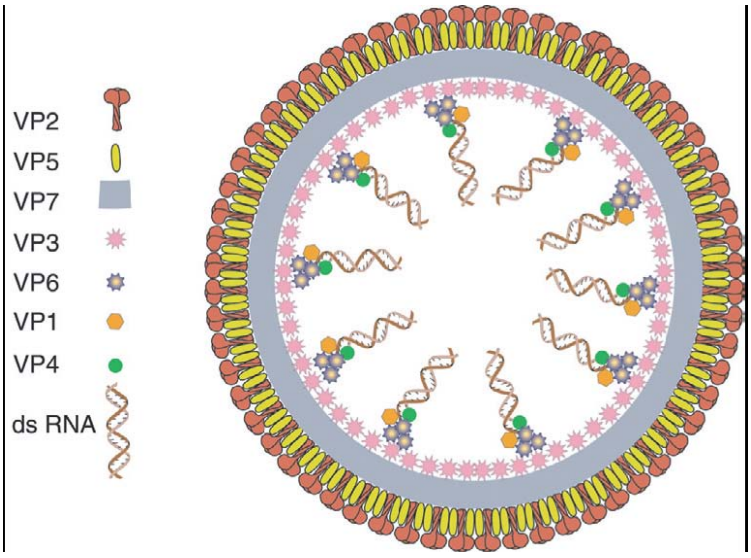


Figure 1. Schematic represention of BTV structural proteins and dsRNA segments.
(Obtained from Schwartz-Cornil *et al.*, 2008)

When BTV is freeze-dried in buffered lactose-peptose, it can survive almost indefinitely at room temperature, however in blood and tissue specimens the virus has been shown to be stable at 20°C for almost a week, 4°C for almost 25 to 30 years if preserved in OCG (anti-coagulant) blood and in blood without coagulant for almost 2 years and -70°C almost indefinitely (Gerdes, *personal communication,* 2008*;* OBP's Research and Development, *personal communication,* 2008). The virus is however not very stable at -20°C (Verwoerd and Erasmus 2004).

The virus is unstable below pH5 (Owen *et al.,* 1964) and it also seems to be unstable at high temperature when all extraneous proteins are removed (Howell *et al.,* 1967). The virus is relatively resistant to ultraviolet gamma irradiation and lipid solvents such as ether and chloroform. It is inactivated by disinfectants containing acid, alkali, sodium hypochlorite and iodophors (Howell and Verwoerd 1971; OIE 2002; Sofer 2003).

1.3 Serological characteristics of bluetongue virus

Howell (1960) grouped 22 BTV isolates into 12 serotypes by using an *in vitro* neutralization test and later other serotypes were identified. To date, 24 known serotypes of BTV have been described worldwide (Gerdes 2004; Batten *et al.,* 2008) that all share common group specific antigens but which are easily distinguishable on the basis of serotype specific virus neutralizing assays. Twenty one of these serotypes are known to occur in South Africa (Dungu *et al.,* 2004; Gerdes 2004) of which 15 of these are considered to be pathogenic for sheep (Erasmus 1975; Verwoerd and Erasmus 2004).

1.4 Epidemiology

1.4.1 Distribution

Although BTV occurs throughout the extensive portion of the tropical and temperate regions of the world, there are regional differences in the occurrence of BTV virulence in livestock. In South Africa the disease is endemic and since 1999 there has been emergence of bluetongue in some European countries most notably Spain, Italy, France, Netherlands, Germany and Portugal. The most prevalent BTV serotypes currently circulating in Europe are serotypes 2, 4, 8, 9 and 16 (EFSA 2007; Batten *et al*., 2008). Serotype 6 recently caused outbreaks in the Netherlands (ProMED 2008; Batten *et al*., 2008). A possible 25th serotype, the so called Toggenburg Orbivirus was also mentioned causing outbreaks in goats in Switzerland (Hofmann *et al*., 2008).

It is well established that the main vectors of BTV are mainly biting midges of the genus *Culicoides* (Erasmus 1980; Mellor 1990). The distribution of the disease is linked to the occurrence of the vector. There are also profound differences in the virus serotypes and or principle species of *Culicoides* vectors that occur within specific regions. The geographical distribution and vector capacity of *Culicoides* therefore determines the spatial and temporal incidence of BTV transmission. Vector population is influenced by climatic factors such as temperature, rainfall, humidity and wind (Mellor 2000). Temperature can influence vector capacity by its impact on adult survival, biting rate and vector competence. Table 1 shows the different countries where different serotypes of the virus occur as well as the species of *Culicoides* midges that are responsible for its transmission.

Approximately 30 *Culicoides* spp. representing at least eight sub-genera are considered to be involved in the transmission of BTV (Venter *et al.,* 2007). In South Africa, at least 10 species representing eight subgenera of more than 120 species of *Culicoides* identified at present have been implicated in the transmission of BTV and related orbiviruses. In South Africa *C. imicola* and *C. bolitinos* play a major role in the transmission of BTV (Venter *et al*., 1998; Paweska *et al*., 2002; Meiswinkel *et al.,* 2004). The prevalence of *C. bolitinos* is 10 times less than *C. imicola* and is abundant in winter and the latter more prevalent in summer (Paweska *et al.,* 2002).

Table 1: Summary of bluetongue serotypes and vectors (Table adapted from EFSA 2007)

Geographical distribution	BTV Serotypes	Vectors
South Africa	1-24 majority in sub-Saharan Africa	*Culicoides imicola and C. bolitinos* are the major vectors of all BTV serotypes. Other *Culicoides* species may be of lesser or local importance
Australia	1, 3, 9, 15, 16, 20, 21 & 23	*Culicoides actoni, C. brevitarsis, C. fulvus, C. Wadai*
China and Taiwan	1-6, 9, 11, 12, 15, 16, 21 & 23	*Culicoides actoni, C. imicola* and *C. fulvus* probably are the major vectors. Other *Culicoides* species may also be involved but data have not been presented to confirm this
Europe	1-2, 4, 8, 9 &16	*Culicoides imicola, C. obsoletus, C. scoticus, C. pulicaris* and, most recently, *C. dewulfi* are proven vectors or have been implicated in the transmission of one or more BTV serotypes
Indian subcontinent and adjacent areas	1-4, 7, 8, 9, 12, 16-18, 23	*Culicoides imicola, C. actoni* and *C. fulvus* are present and are major BTV vectors. Other *culicoides* species may also be involved but data have not so far been presented to confirm this
Japan	4, 11, 13, 20 & 21	*Culicoides brevitarsis,* is the main vector in Australia and has been reported from Japan. Three other orbiviruses, including the BT-related Ibaraki virus have been isolated from blood-free C. *oxystoma* suggesting that this species might also be a potential vector of BTV
Middle East	2, 3, 4, 6, 9, 10, 13, 15 & 16	Not yet determined
North America	10, 11, 13 & 17	*C. sonorensis* is the main vector in continental North America and *C. insignis* is also present in the Gulf of Mexico region (Florida and coastal Mississippi, Louisaiana etc)
South and Central America, Caribbean Basin	1-4, 6, 8, 10, 12, 17, 19 & 20	*Culicoides insignis* and probably *C. pusillus* are the major vectors
Southeast Asia (Malaysia and Indonesia)	1-3, 7, 9, 12, 16, 21 & 23	Vectors are same as those in Australia

1.4.2 Host preference

Bluetongue virus infects all known ruminant species. Due to its economic importance in sheep and the occurrence of major epidemics that cause heavy economic losses, this species is regarded as the most important species affected. However, the outcome of infection varies between different breeds. Severe disease usually occurs only in certain breeds of sheep and in some species of deer such as white –tailed deer (*Odocoileus virginianus*) (MacLachlan 1994). Indigenous breeds of sheep are less severely affected by bluetongue than exotic breeds (Mellor 1994). Various antelope species such as kudu (*Tragelaphus strepsiceros)*, blesbok (*Damasliscus albifrons*) and buffalo (*Syncerus caffer*) of which over 90% have antibodies to BTV, are known to be susceptible. It is possible that most, if not all African antelope species are susceptible to BTV infection (Paweska *et al.,* 2002).

It has also been reported that *C. bolitinos* breeds in buffalo dung and actively follow buffalo herds (Paweska *et al.,* 2002). This now suggests that the *C. bolitinos*-buffalo association could be the primary cycling mechanism of BTV in South Africa with a secondary cycle taking place between *C. bolitinos* and cattle (Venter *et al*., 1998; Paweska *et al.,* 2002).

In Africa *C. imicola* favours moist nutrient rich clay soils exposed to full sunlight leading to its superabundance following widespread rains which will mostly be in summer. This explains the reason why *C. imicola* is the traditional vector and the most important transmitter of BTV over most of South Africa and is the means whereby the virus is amplified so effectively during the summer rainfall season (Paweska *et al*., 2002). Though *C. imicola* is more abundant than *C.bolitonus*, the latter has a higher vectoral competence due to its susceptibility to infection with different BTV serotypes that support replication to higher titres (Venter *et al*., 2006).

1.4.3 Overwintering and climatic influence

Outbreaks of BT, even during seasons that did not support the survival of *Culicoides spp.* have been reported and even during winter seasons. Cold winters often end vector-borne disease episodes by killing virtually all of the adult vectors and thereby preventing transmission for several months of the year (Erasmus 1980; Purse *et al*., 2005). The duration of viraemia in the vertebrate host is also of major importance in BTV overwintering. In this context, cattle, which are the favoured host of many vector species of *Culicoides*, have a prolonged viraemia for up to at least 6 weeks and may be regarded as a reservoir host for BTV (Maclachlan 2004; EFSA 2007; Swarzt- Cornil *et al.,* 2008). In South Africa BT in sheep is known to occur in late summer and early autumn but due to environmental conditions which favours coprophilic breeders such as *C. bolitinos* and *C. imicola*, have now become abundant in cooler zones of South Africa and when the temperature rises replication does occur as a result of transmission (Paweska *et al.,* 2002).

Another form of overwintering is in the vector e.g. *C. bolitinos* which breeds in buffalo dung and also contributes to outbreaks. The biology of *C. bolitinos* suggests that this species plays a very significant role during the winter period (Paweska *et al*., 2002). In some infected ruminants the virus establishes a persistent infection of T-cells. When a vector bites these animals it might be infected, contributing to the overwintering problem (Purse *et al.,* 2005).

1.5 Clinical signs

The pathology of BT can be assigned to vascular endothelial damage resulting in changes to capillary permeability and fragility, with subsequent disseminated intravascular coagulation and necrosis of tissues supplied by damaged capillaries (Pini 1976). These changes result in oedema, congestion, haemorrhage, inflammation and necrosis. Fever is the most usual but not invariable clinical sign.

If fever occurs, sheep first become pyrexic 4 to 10 days after infection. There is a correlation between the duration of fever and severity of the disease (Erasmus 1990). Other common clinical signs include oedema of the lips, nose, face, submandibulum, eyelids and sometimes ears and congestion of the mouth, nose, nasal cavity, conjunctiva, skin and coronary bands (Theiler 1908; Mellor and Boorman 1995). There is also frequently a serious nasal discharge, later becoming mucopurulent. Sores appear on the mouth and the sheep may champ to produce a frothy oral discharge. Sheep are not strictly anorexic but eat less because of oral soreness and will hold food in their mouths to soften before chewing. Affected sheep occasionally have swollen, congested, cyanotic tongues. Lameness, due to coronary band congestion, may occur early in the disease and lameness or torticollis, as a result of skeletal muscle damage, may occur later. The other clinical signs soon follow with acute deaths occurring during the second week following infection. Sheep may die from more chronic disease 3 to 5 weeks after infection and usually have secondary bacterial complications (Mellor 1994). The mortality rate and the severity of the clinical signs vary with the breed and age of the animal. Foot lesions normally develop at the end of the febrile reactions with hyperemia of the coronary band and wool breaking within the wool follicles also occurs (Erasmus 1990). The disease can also result in congenital defects (Barnard and Pienaar 1976; Erasmus 1990)

1.6 Pathogenesis

After cutaneous infection of virus by an insect bite, the virus first replicates in the adjacent lymph nodes and then spreads to infect vascular endothelium and macrophages or dendric cells in many tissues or organs. Vascular induced injury to endothelial cells in small blood vessels leads to vascular thrombosis and ischaemic necrosis of the tissues involved, which eventually result in lesions such as acute oral ulceration, coronitis, muscle and vascular leakage to facial and pulmonary oedema as well as pleural and pericardial effusion (MacLachlan 1994; DeMaula *et al*., 2002). Viraemia in BTV- infected ruminants is cell associated and is prolonged but not persistent. Bluetongue virus is quantitatively associated with cells such as platelets

and most notably red-blood cells which facilitates both prolonged infection and protects virus from immune clearance in ruminants (MacLachlan 1994 and 2004; Forzan *et al*., 2007). This prolonged blood cell associated infection of ruminants is important because it increases the likelihood that feeding insect vectors will acquire the virus. Duration of viraemia in sheep can vary from 14 to 60 days depending on the serotype and breed of sheep (Pini 1970; Maclachlan 1994 and 2004). Viraemia tends to be longer in cattle than in sheep due to the differences in the life span of their red blood cells (MacLachlan 1994; Schwartz-Cornil *et al.*, 2008). Neutralizing antibodies typically appear in blood of infected animals within approximately 14 days post infection (Jeggo *et al* ., 1984; Ghalib *et al* ., 1985).

1.7 Diagnosis

A presumptive diagnosis of BT can usually be made from the clinical signs and lesions in affected sheep, especially in the areas where the disease is endemic. Blood, collected as early as possible during the febrile reaction in anticoagulants such as heparin, sodium citrate, EDTA or OCG, is the most suitable specimen for virus isolation (Erasmus 1990). In fatal cases, specimens of spleen, lymph node or red bone marrow should be collected as soon as possible after death, kept at 4°C and sent to the laboratory for virus isolation. Bluetongue virus can most readily be isolated by the intravascular inoculation of 10 – 12 day-old embryonated chicken eggs with blood or clarified tissue suspensions (Foster and Luedke 1968; Clavijo *et al*., 2000; Goldsmit *et al*., 1975; Hammoumi *et al*., 2003). However, routes of inoculating the egg have effects on the successful isolation of the BTV. It has been demonstrated that intravenous (IV) route of inoculation was more sensitive than the yolk-sac (YS) route (Goldsmit *et al*., 1975; Goldsmit and Barzilai 1985).

For serotyping, virus neutralization tests such as the plaque reduction or plague inhibition tests are commonly used (Howell *et al*., 1970; Clavijo *et al*., 2000; Hambling 2004). Serotype-specific antibodies directed against VP2 are best detected by means of a virus neutralization test such as the plague reduction, or the haemagglutination test (Poli *et al.,* 1982). Currently serum neutralization tests are considered highly sensitive and specific in that they do not cross react, these are done mostly on 96 well plates (Bulut *et al*., 2006).

Techniques to identify BTV without prior isolation have been developed such as the indirect peroxidase test (Cherrington *et al.,* 1985) and the antigen capture ELISA using either infected insect tissue or blood from infected sheep (Hambling 2004). There are many commercially available diagnostic kits e.g. ELISA and Complement fixation test kits which have been used with success which are sero-group reactive assays. However, an ELISA test kit supplied by the Veterinary Medical Research and Development Incorporation (VMRD, Inc.) is one that is commonly used at both OBP and Onderstepoort Veterinary institute (OVI).
Molecular tools such as the reverse- transcriptase polymerase chain reaction (RT-PCR) to identify BTV directly in blood samples or cultured cells (Shad *et al.,* 1997; Aradaib *et al*., 1998) have also been developed. A combination of an enzyme linked oligonucleotide sorbent assay and PCR have been used with success to detect BTV in infected blood (Aradaib *et al.,* 1998).

1.8 Control

Due to the wide host range of BTV, different BTV serotypes and the role played by *Culicoides* spp*.* as vector make the eradication of the disease in endemic areas such as in S.A. difficult to achieve (Erasmus 1975). European sheep mutton and wool-breed are highly susceptible with most African indigenous sheep breeds being more resistant to clinical disease (Erasmus 1980). Consequently, in countries endemically infected, vaccination has been the most effective and practical method used in reducing disease spread (Erasmus 1975 and1980; Verwoerd and Erasmus 2004;

Dungu *et al.,* 2004). Other countries in Europe where the disease is exotic, surveillance, movement control and slaughter has been advocated (EFSA 2007). Due to the economic impact of using other methods of control such as slaughter, most countries consider vaccination as the best tool available to control the spread of the disease.

A variety of vaccines have been developed to prevent BTV infection of ruminants. These include: modified live vaccines (MLV), inactivated whole (killed) virus preparations and virus like particles (VLPs). The latter is produced from recombinant baculoviruses and recombinant vaccinia, capripoxvirus or canarypox virus-vectored vaccines (Murray and Eaton 1996; Boone *et al.,* 2007). Only MLV and inactivated vaccines are commercially available and have been used in recent years in Europe (Table 2).

Table 2. Bluetongue vaccination in Europe (Table obtained from Savini *et al.,* 2007)

		Year of vaccination							
Vaccine		**France**	**Italy**		**Spain**		**Portugal**		**Bulgaria**
		Sheep	**Sheep**	**Cattle**	**Sheep**	**Cattle**	**Sheep**	**Cattle**	**Sheep**
MLV	BTV 3, 8, 9, 11								1999-2000
	BTV 2	2000-2002	2002-2006	2005-2006	2000-2001				
	BTV 4				2004-2006		2005-2006		
	BTV 2 &4	2003-2004	2004-2006	2004-2006	2003 (Balearic Island)				
	BTV 2 &9		2002-2006	2002-2006					
	BTV 16	2004							
	BTV 2, 4 & 16		2004	2004					
	BTV 2,4, 9,16		2004	2004					
	BTV 2, 4, 9		2005-2006	2005-2006					
	BTV 9								
Inactivated	BTV 2	2005							
	BTV 4				2000-2006	2005-2006	2005-2006	2005-2006	
	BTV 2 &4	2006	2005-2006						

1.8.1 Modified live vaccines

At the turn of the last century it was clear that BTV was enzootic in South Africa and vaccination was recognized as a necessary control measure. An attenuated blood vaccine, developed by Theiler (1908) after serial passage in sheep, was used for almost 40 years (Verwoerd and Erasmus 2004). Consequently, other serotypes of BTV were identified in outbreaks (Neitz 1948), and so a quadrivalent vaccine was developed in the early 1950's (Alexander and Haig 1951). The biggest discovery came when further attenuation of the vaccine strain was done through serial passage of the BTV in embryonated chicken eggs and tissue culture aided by plaque purification of the virus (Howell 1969; Erasmus 1975). Polyvalent vaccines produced from this material have been used with success for more than 50 years.

Live attenuated vaccines are easy and economical to produce, and can be administered by a single injection (Erasmus 1975; Murray and Eaton 1996). Stimulation of a strong antibody response by these vaccines is directly correlated with their ability to replicate in the vaccinated host. They replicate in the host without causing significant clinical effects and provide protection against challenge with virulent virus of the same serotype (Howell 1969; Wark *et al*., 1982; Chalib *et al*., 1985; Jeggo *et al*.,1984; Hunter and Modumo 2001; EFSA 2007).

1.8.1.1 Current use of modified live vaccines in SADC

The multiple BTV serotypes occurring in South Africa and the limited cross neutralisation protection between serotypes resulted in the formation of the current polyvalent live attenuated BT vaccine (Howell 1969; Erasmus 1975). The vaccine registration number is Reg. No G 358 Act No. 36/1947 which comprises of 3 bottles (Vaccine A, B & C) and includes the following serotypes (Figure 1).

- Bottle A: Serotypes 1, 4, 6, 12 & 14
- Bottle B: Serotypes 3, 8, 9, 10 & 11
- Bottle C: Serotypes 2, 5, 7, 13 & 19

The combination of BTV serotypes in the vaccine is based on serological cross-neutralisation studies and grouping of BTV serotypes (Figure 2) (Erasmus 1980). This

was earlier confirmed by studies in which sheep previously exposed to BTV 3 and 4 were found to be resistant to challenge by BTV 6 (Jeggo *et al.,* 1983; Schultz and Grieder 1987). This cross reactivity has been confirmed by the analysis of the VP2 and VP5 genes which showed similar cross relationship (Letchworth and Appleton 1983; Mertens *et al.,* 1989; Maan *et al*., 2007). The serotypes in the vaccine therefore support the broad spectrum coverage that exists amongst serotypes occurring in South Africa.

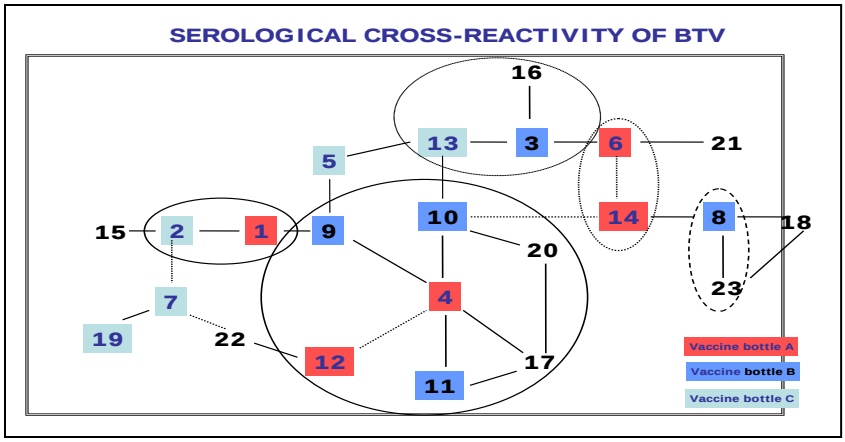


Figure 2: Obtained from Dungu *et al.,* 2004

An average of 8 million doses of BT MLV vaccine is sold and used annually in South Africa to vaccinate sheep. However, this demonstrates that only 27% of the estimated 29 million sheep is vaccinated annually in South Africa (Dungu *et al.,* 2004). Previously around early to mid 1970's an estimated 20 million doses of polyvalent BT vaccine used to be demanded by farmers (Erasmus 1975). The Eastern Cape Province is the biggest consumer of the vaccine as it consists of the largest area of wool-producing sheep. A further one million doses are sold annually to some of the neighbouring countries e.g. Botswana, Namibia, Lesotho and Mozambique. Since 2000 Onderstepoort Biological Product (OBP) has sold several million doses of monovalent, bivalent and pentavalent combinations of BT vaccines to France, Spain, Italy and Portugal to assist in controlling the BTV epidemics. This contributed in reducing loses due to disease and indirect losses due to trade embargoes caused by the presence of BTV.

1.8.1.2 Efficacy *of* Bluetongue MLV

Modified live vaccines are able to elicit both cellular and humoral immune responses, which are able to protect sheep against BTV infection and disease (Maclachlan 1994; Schultz and Grieder 1987; Breard *et al*., 2004). Bluetongue viral proteins that are responsible for inducing neutralising antibodies are VP2 and VP5 with VP2 being the major protein (Huismans and Erasmus 1981; Mertens *et al*., 1989).

Sheep vaccinated with BTV 2 and challenged seven months post vaccination (p.v.) with a virulent homologous field isolate of BTV 2 with a titre of $2x10^{5.8}TCID_{50}$/ml showed no clinical signs and no viraemia in 90% of vaccinated sheep (Savini *et al.,* 2007; Hunter and Modumo 2001). Cellular immune response mediated by cytotoxic T-lymphocytes (CTLs) generally gives heterotypic protection. The use of multiple whole live BTV vaccine allows for a greater and more extensive induction of CTL responses due to the presence of multiple CTL epitopes (Jeggo and Wardly 1985; Kevin *et al*., 1987)

In Italy, the efficacy of MLV monovalent and bivalent BTV serotypes 2 and 4 vaccines has also been demonstrated during mass vaccination against BT in which the disease disappeared almost entirely (Caporale *et al.,* 2005). Over the years the disease has been successfully controlled through the use of MLV (Erasmus 1985 and 1990; Dungu *et al.,* 2004).

1.8.1.3 Quality control and safety of Bluetongue MLV

The BT MLV is currently tested and released at a minimum titre of 5×10^4 PFU/ml**.** Some of the BT monovalent MLV produced at 10^4 PFU/ml offer a very good protection and were proven to be safe in South African Merino sheep (Hunter and Modumo 2001). However, BTV serotype 2 monovalent live vaccine used at a titre of 5×10^4 PFU/ml could be isolated on cell cultures 21 days p.v. due to viraemia (Savini *et al.,* 2004b).

Modified live vaccines have different potential adverse effects depending on the specific serotype combinations used. Replication of the BT 2 in a vertebrate host yielded a viraemia of not more than 10^3 PFU/ml in sheep (Dungu *et al.,* 2004). However, even at this low titre it has been shown that viraemia may be sufficient to establish an infection in a population of biting vectors. In South Africa BTV vaccine serotypes have been demonstrated by isolation from *C. imicola* and *C. bolitinos* and transmission to unvaccinated animals can therefore occur (Venter *et al.,* 2007). Bluetongue virus has also been isolated in other *Culicoides* spp. (Foster *et al*., 1968; Mecham *et al*., 1990; Mellor 1990 and 2000).

A monovalent BT MLV 2 was used in Corsica from 2001 to 2004 and Italy from 2002 on approximately 130,000 -4,000,000 sheep and goats. A negligible number of adverse effects were reported, however when the same vaccine was used in Spain (Menorca and Mallorca) on 320,000 sheep, abortions in sheep were reported (Patta *et al*., 2004; Savini *et al*., 2007; EFSA 2007).

A bivalent BT 2 and BT 4 MLV was used in Tuscany and Sardinia to vaccinate almost 4,000,000 sheep and goats. No side effects were reported p.v. However, in 2004 a BT 16 MVL vaccine was used in Corsica and typical signs of BT were reported in vaccinated sheep several days p.v. On the contrary a polyvalent MLV containing BTV serotypes 2, 4, 9 and 16 was used in Italy in 2004 and approximately 1,700,000 sheep were vaccinated and no side effects were reported (Caporale *et al*., 2005).

Side effects that have commonly been reported include the following:

- Sheep and cattle abortions (Savini *et al*., 2007)
- Vaccinated rams showed lower semen quality after just the first vaccination (Erasmus 1975; Bowen *et al*., 1985; Bréard *et al*., 2007)
- Effect on milk production was slightly lower. This was attributed more to transient perturbation of health induced by the vaccine and not related to the virus itself on the mammary gland (Savini *et al.,* 2004a ; Giovanni *et al*., 2004).
- Possible transmission BT MLV by vectors (Venter *et al*., 2006)

1.8.2 Inactivated Bluetongue vaccines

The first BT inactivated vaccine developed for European countries was BTV serotype 2 and used in the field after the emergence of BT in Europe. Subsequently, vaccines against BTV serotype 4 and bivalent 2 and 4 vaccines have been developed and used in Corsica, Spain, Portugal and Italy (Stott *et al*., 1979; Campbell 1985; Savini *et al.,* 2007)(Table 2). Most of the BTV serotypes used were isolated from clinically affected animals during the outbreak. Currently BTV serotype 8 has also been developed and is being used in European countries experiencing outbreaks. The working seed virus (WSV) for producing the vaccine was expanded from the master seed stock using no more than three passages in BHK cells. This was then followed by purification and concentration using centrifugation or chromatography and inactivation by chemical procedures (binary ethylenimine or beta-propriolactone). The minimum vaccine virus titre ranged from 10^6 to 10^8 PFU/ml per individual dose. Adjuvants used were based in oil (mineral or not) or aluminium hydroxide and saponin or detoxified (Savini *et al.,* 2007).

1.8.2.1 Safety, efficacy and immunogenicity of inactivated Bluetongue vaccines

Several studies have been conducted in sheep to evaluate the safety of a subcutaneous injection of inactivated prototype monovalent vaccines of BTV 2 and 4 and a combination of BTV 2 and 4 by using either single injections or booster 3 to 4

weeks later (Hamers *et al.*, 2006; Di Emidio *et al*., 2004). The majority of sheep showed no systemic reaction and these vaccines are currently being used in Europe. However, a few sheep vaccinated had local reactions of variable severity but signs disappeared within few days p.v. Anaphylactic shock was also reported in those sheep previously vaccinated with BT MLV 4 (Savini *et al.,* 2007).

Assessment studies on efficacy were based on clinical signs, virological data and immunogenicity. The latter was assessed by the analysis of the antibody response induced as measured by ELISA and by titration in a VNT against BTV serotypes 2 and 4. Efficacy of the BTV serotypes 2 and 4 vaccines was evaluated in vaccinated animals using clinical signs and viraemia as indicators . Animals were inoculated with an infective dose of live virulent blood or cell propagated BTV at a dose of 2 x 10^6 $TCID_{50}$/ml. When a monovalent or bivalent vaccine was inoculated using two doses at 24 days interval, it prevented viraemia when challenged with a homologous virulent serotype. A single dose however, did not protect cattle against viraemia 7 months p.v. The inactivation process was confirmed p.v. by viraemia, by either using a BTV-specific quantitative real time RT-PCR assay (Jimenez-Clavero *et al.,* 2006) or by virus isolation (Savini *et al.,* 2007).

1.8.3 Recombinant vaccines

Recombinant vaccines have been found to have numerous inherent potential benefits which include rapid onset of immunity and lack of transmissibility. Bluetongue virus like particles (VLP) derived from multiple baculovirus expression vectors combined with an appropriate adjuvant, elicited an immune response which protected sheep against infectious virus challenge (Roy *et al.,* 1990 & 1992; Frence *et al*., 1990). This was further confirmed when two doses, 10u of VLP's elicited a long lasting immune response in sheep (Roy *et al.,* 1994). When sheep were vaccinated with a recombinant vaccinia virus that expressed both VP2 and VP5 of the Australian BTV serotype 1, the vaccine induced variable titres of neutralising antibodies in sheep and afforded protection against homologous challenge (Lobato *et al.,* 1997).

A recombinant capripoxvirus vaccine expressing two capsid proteins VP2 and VP7 at 10^4 PFU/ml and two non-structural proteins (NS1 and NS3) of BTV serotype 2 was shown to provide partial protection against heterologous BTV challenge (Perrin *et al.,* 2007). Another recombinant canarypox-VP2/VP5 vaccine of BTV serotype 17 has been shown to induce protection when challenged with a field strain of BTV 17 (Boone *et al.,* 2007).

1.9 Conclusion

The bluetongue live attenuated viral vaccine is currently used in endemic countries such as S.A. as a polyvalent vaccine with each serotype in the vaccine at a minimum titre of 5 x 10^4 PFU/ml. It will be to the advantage of both the farmers and OBP, as producer, to investigate the minimum titre where serotypes within the vaccine will still give protection without any adverse effect to the animals.

This will benefit the farmer as the product will be more affordable and OBP, as it will be able to produce more quantities at a lower cost. Bluetongue virus serotypes 2, 4 and 8 were mainly chosen for this study due to the current outbreaks occurring in Europe.

The aims and objectives of this project were:

a) To vaccinate sheep with either monovalent BT MLV 2, 4 and 8 serotypes.
b) To challenge these sheep with a homologous serotype 6 weeks or 4 months p.v.
c) To demonstrate the serological response p.v. using a serum neutralization test and the commercially available competitive ELISA.
d) To monitor the level of virus in the blood p.v. and p.c.
e) To determine the level of protection and safety of the vaccine administered at different titers using the clinical reaction index (CRI) method

CHAPTER 2

Materials and Methods

2.1 Introduction

Sheep susceptible to BTV and above 6 months of age were selected for the study. This ensured that the passive immunity in sheep, which may lasted for up to six months (Erasmus 1985) would not interfere with the use of the BT MLV. The latter was produced at OBP where sheep were challenged 6 weeks p.v. using blood culture material and 4 months p.v. using cell culture material, tested for viraemia and a percentage protection index determined. Two diagnostic tests were selected for serology, an ELISA and SNT. The former is a group specific test targeting mainly VP 7 and is mainly used as a screening test for pre and p.v., while the latter is targeting VP2 which is serotype-specific. Quality control testing on the vaccine for sterility and safety was done according to the SOP of OBP.

2.2 Animals and sampling methods

The experiment was conducted at the premises of Onderstepoort Biological Products (OBP) Ltd in South Africa ($25^0$29'S, 28^0.11 E, 1219 m a.s.1.). The total number of susceptible BT merino sheep used for this experiment was 83 ranging in age from 9 -12 months. Sheep were kept in an insect free isolation stable, fed daily adlib and had continuous access to clean water. Sheep were divided into 2 groups, A and B. Sheep from Group A were challenged at six weeks and those in Group B challenged at 4 months. Three BTV serotypes 2, 4 and 8 vaccines each with the following titres 10^2, 10^3 and 10^4 PFU/ml was produced and injected into 12 sheep per serotype and 4 sheep per titre (Table 3). Positive and negative control sheep were included for each group. Three sheep were used for evaluating and testing of the challenge material.

Table 3: Summary of the total number of sheep used in groups A and B

BTV Serotype (A &B)	Vaccine titre-PFU/ml (Vaccination)				Challenge		Challenge preparation	Negative control
	10^2	10^3	10^4		Day 44	Day 120		
BTV-2	4(x2)	4(x2)	4(x2)		4/titre	4/titre		
Positive Controls					1	1	1	
BTV 4	4(x2)	4(x2)	4(x2)		4/titre	4/titre	1	
Positive Controls					1	1		2
BTV 8	4(x2)	4(x2)	4(x2)		4/titre	4/titre	1	
Positive Controls					1	1		
Total used	83							

(x2) represents group A & B

Group A

A total of 36 sheep was vaccinated using 1 ml of vaccine and injected subcutaneously on the inner side of the thigh. A positive control, which was challenged and a negative control, which was not vaccinated and challenged for each BTV serotype were included. Sheep were clinically monitored p.v., temperature readings recorded for 14 days and post bled using 10 ml vacu-tubes containing lithium heparin as a coagulant and 10 ml vacu tube for serum, without a coagulant. Blood was collected on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 p.v. Heparin blood was tested for viraemia and the serum for neutralizing antibodies indicating seroconversion.

Six weeks p.v., both heparin and serum blood was collected from the controls and vaccinated sheep and sheep were challenged with a homologous BTV serotype. They were then clinically monitored, temperature reactions were recorded for 14 days p.c. and scored using the method of Huismans (1987). Blood was also collected in heparin tubes on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 p.c.

Group B

Sheep from Group B were vaccinated in the same manner and simultaneously with Group A. However, this group was only challenged at 4 months p.v. Prior to challenge sheep were bled for serum, and blood was collected in heparin on days 0, 3, 6, 9, 12, 15, 18, 21, 25 and 28 p.c. Sheep were clinically evaluated, temperature readings recorded for 14 days p.c.

2.3 Vaccine development

All BTV serotypes used for vaccine production were developed from field isolates obtained in South Africa from sheep suffering from BT. Table 4 summarizes the origin, history, name of isolates and number of passages each isolate were passaged to reach the required level of attenuation for it to be immunogenic but not pathogenic..

Table 4: Origin of the attenuated BTV serotypes in the OBP vaccines used in this study (Obtained from OBP Ltd.). All serotypes identified at Onderstepoort (OBP, Faculty of Veterinary Science & OVI).

Type	Strain & origin	Country & year of isolation	Passage level of attenuated vaccine
2	5036 Vryheid	RSA 1958	50E 3P 3BHK
4	79043 Theiler	RSA ~ 1900	60E 3P 9BHK
8	8438 Camp	RSA 1937	50E 3BHK 10P 7BHK

E: embryonated eggs, P: Plague isolation, V: Vero cells, BHK: Baby Hamster kidney cells

Production of the vaccines was done according to the current SOP of OBP (proprietary information). Prior to production, the working seed virus (WSV) (BTV serotypes 2, 4 and 8) was firstly identified using SNT and only serotypes 2 and 4 were tested by RT- PCR (Onderstepoort Veterinary Institute- Biochemistry section) as there were no primers developed for serotype 8. Eagle's medium, containing streptomycin (50 µg/ml), penicillin (50 µg/ml) and fungizone (2 µg/ml) was also prepared and reconstituted in sterile water for injection (WFI).

Bluetongue virus 2, 4 and 8 WSV in freeze dried vials were reconstituted in 1 ml of Eagle's medium and further dilutions were made to get the correct multicity of infection (MOI). About 10 ml of virus dilution was inoculated into confluent baby hamster kidney cells (BHK) in roller bottles and incubated for 1 hr to allow virus adsorption. A further 250 ml of maintenance medium was added and re-incubated at 37 °C. These cells were microscopically examined daily for specific cytopathic effect (CPE). After 100% CPE was reached, the supernatant was collected and tested for sterility using soyabroth and blood treptose agar (BTA). It was observed for 14 days and results recorded.

Five litres of harvested material was produced for each serotype and mixed with 50% stabilizer, buffered lactose peptone (BLP). A volume of 1 ml of the diluent was added to 8 ml glass vials and later freeze dried. Eagles medium and BHK-21 cells were used during production. The original titre for serotype 8 was 5 x 10^6 PFU/ml and the quantity of stabilizer and Eagles medium was adjusted again and freeze dried. Titres after freeze drying for serotype 8 was 3 x 10^4 PFU/ml, serotype 2 and 4 was 1,17 x 10^5 PFU/ml and 1,17 x 10^6 PFU/ml, respectively. Prior to injecting the sheep, the freeze dried material was reconstituted into sterile water to obtain the following titres 10^2, 10^3 and 10^4 PFU/ml. The production of vaccines is illustrated in Figure 3.

(Serotypes after freeze drying)

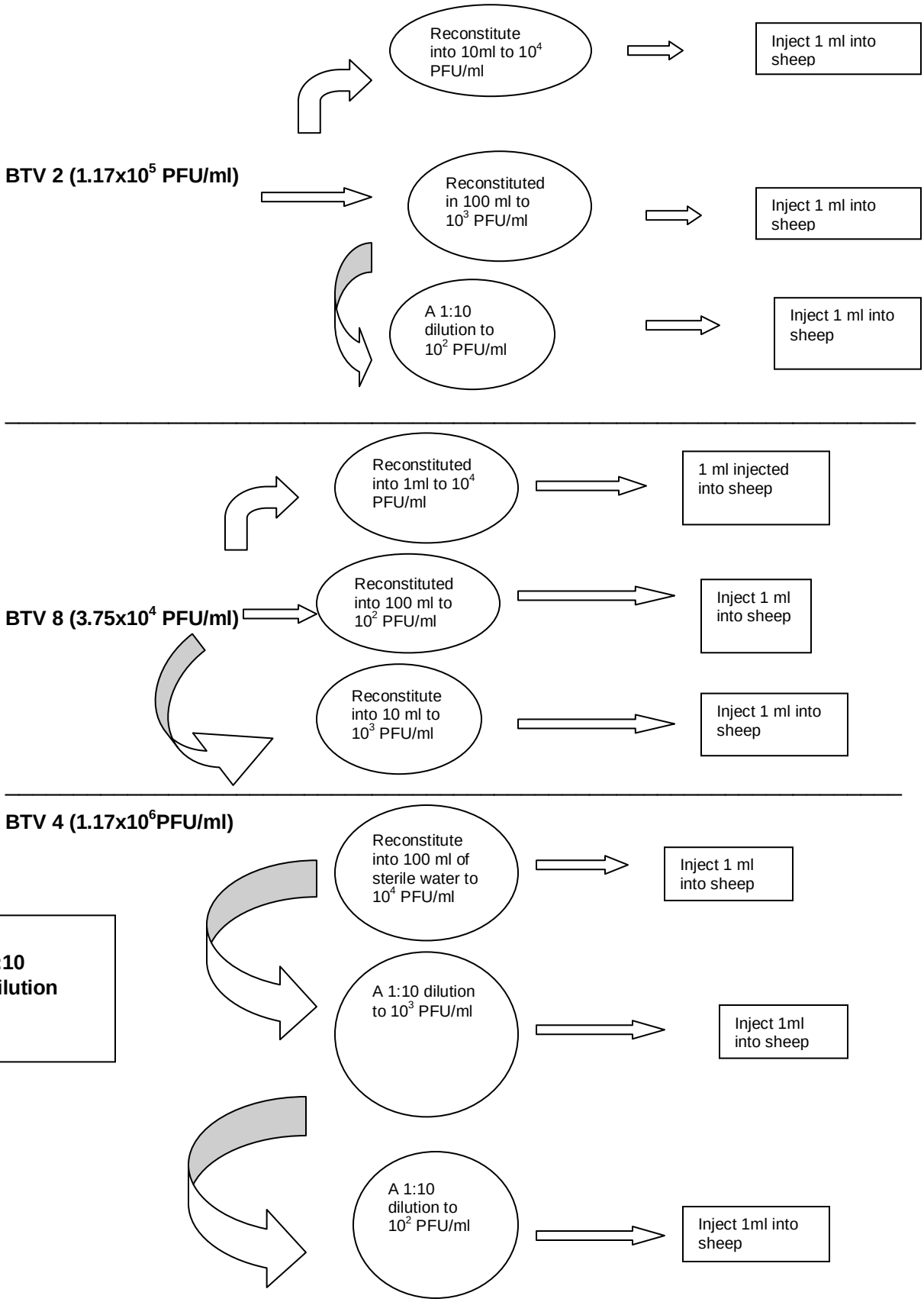


Figure 3: Schematic representation of the reconstitution and dilution of the different vaccines

2.4 Challenge material

Bluetongue virus serotype 2 and 4 used in this study were both isolates obtained from the OVI. To prepare the BTV challenge material, sheep were infected with BTV 2 and 4, and bled when a temperature reaction was observed (>40 °C). Bluetongue virus serotype 8 was an isolate BTV 8(G) 5/10A 2006/01KC 3BHK obtained from the Institute for Animal Health (IAH) Pirbright. The virus was passaged once on BHK cells and 2 ml of this cell culture material was later injected intravenously (i.v.) into sheep and bled when temperature reaction was observed. Blood from these infected sheep was later used as a challenge material for Group A.

The challenge material for Group B was prepared from cell cultures. One Roux flask with Vero cells was inoculated with 10 ml infected sheep blood. The flask was incubated at 37 °C in an incubator containing 5% CO_2 for 15-20 minutes. Using Eagle's medium, blood was rinsed from monolayers of cells. Flask were later filled with Eagles medium and incubated at 37 °C and observed daily for CPE. The material (cell/virus) was harvested when 80-100% CPE was observed, centrifuged at 3000 rpm for 15-20 minutes and the supernatant stored at 4 °C until titrated. The titre of the challenge material was determined by viral plaque assay.

Briefly, a series of 10 fold dilutions were prepared and 1 ml was inoculated on Vero cells in a 6-well plate. The agarose overlay was stained using a 1 % neutral red solution and the titre determined. The decision to use a cell culture challenge material in group B sheep was due to the failure of the positive controls in Group A to develop clinical signs.

2.5 Viraemia testing of blood

Every 3rd day p.v. and p.c. 5-10 ml heparin blood was collected as described in 2.2 above. The method for viral isolation on cell culture was used according to the SOP of OBP as described in the OIE Terrestrial Manual 2008 and also as described by Clavijo *et al*., 2000. Blood was considered negative for viraemia when no CPE was evident even after blind passage of the supernatant done once. Samples that showed the presence of virus were further titrated on confluent Vero 6-well plates. Samples from both Group A and B were treated in the same way.

2.6 Serological assays

Pre- and post vaccination serum collected from sheep as described in 2.2 were tested to demonstrate if they were never exposed to BTV before, for seroconversion and to quantify the level of antibody reaction p.v. Serum samples from both groups A and B from Day 0 to four weeks p.v. were tested using a commercially available competitive ELISA and used according to the method described by the manufacturer (VMRD, Inc.). Serum samples collected 4 and 6 weeks and 4 months p.v. were tested using the SNT test on 96-well plates. Briefly the performance of the test entails the dilution of the test serum in microtitre plates, followed by the addition of an equal volume of virus suspension diluted to contain approximately 100 - 300 infective doses (100 $TCID_{50}$). Following incubation of 1 to 2 h at 37 °C, indicator Vero cell cultures at a certain concentration are added. Plates are sealed, incubated at 37 °C, and observed daily for development of viral CPE (Bulut *et al*., 2006; OIE manual, 2008).

2.7 Quality control

Tests were conducted to confirm the titres of the freeze dried vaccine, safety in laboratory animals and sterility testing to confirm absence of other organisms.

2.7.1 Bluetongue viral titration and sterility testing

To confirm the titre of the vaccines before and after freeze drying, the PFU assay was used on 6-well plates. Media preparation and sterility testing was also done as described in 2.3.

2.7.2 Laboratory animal testing

These vaccines were also tested for safety and contamination from other infectious organism by infecting laboratory animals. Mice were kept in a strictly intensive facility situated at OBP. Twenty animals were injected per serotype which included 6 adult mice inoculated intra-peritoneal, 7 infant mice inoculated using the same route and 7 infant mice kept as controls. A total of 60 laboratory animals were used and observed for 14 days and results recorded.

2.8 Determination of the percentage protection index

The determination of the clinical reaction index (CRI) was calculated using the method described by Huismans *et al*., 1987 (Appendix 1). Briefly: the following 3 scores (a + b+ c) were added:

(a) The fever score: - Cumulative total of fever readings above 40 °C, days 3 to 14 after challenge (maximum score about 12).

(b) The lesion score:- Lesions of the mouth, nose, and feet each scored on a scale of 0-4 and added together (maximum score about 12)

(c) The death score: - an additional 4 points were added to the sum of (a) and (b) if death occurred within 14 days after challenge.

The relative reaction (RR) is the CRI of the test sheep expressed as a percentage of that of the control, and the percentage protection index = 100% minus RR
An example using sheep number 149 (Table 16) is given below.

The CRI of sheep 149 was 2, while that of the positive control was 10.
RR as a percentage of the control will be 2/10= 20%

Therefore using the formula of % protection = 100%- RR
= 100%- 20%
= 80%

CHAPTER 3

Results

3.1 Introduction

This chapter outlines results of both group A and B which firstly summarizes temperature reactions p.v. and p.c. which were both observed clinically for 14 days. Serological assays for viraemia, seroconversion and later calculation of the percentage protection index results are also included. Sheep number 59 which act as a positive control died after challenged with BTV 2 and post mortem was performed at the University of Pretoria (Faculty of Veterinary Science) which did both macroscopic and viral isolation to confirm BTV 2. It was interesting during this study to note the difference of how sheep reacted to blood and cell culture challenge material.

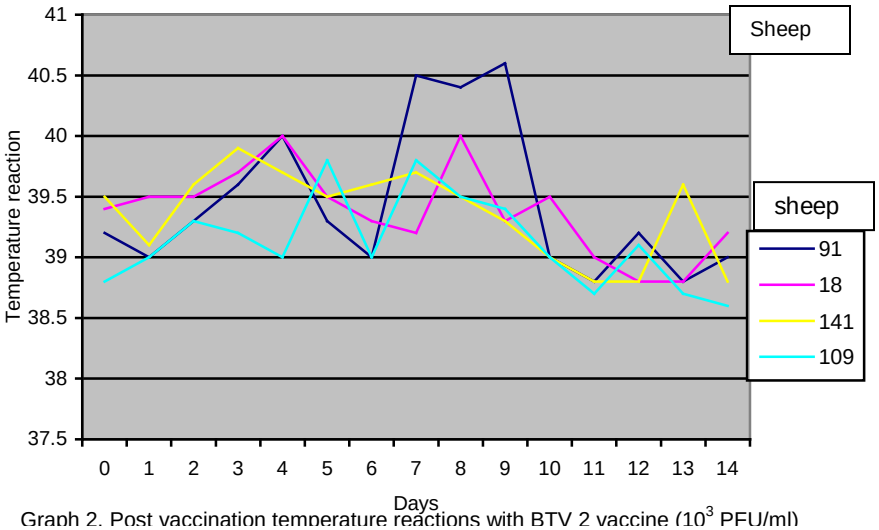
RESULTS GROUP A SHEEP

3.2 Post vaccination temperature reactions

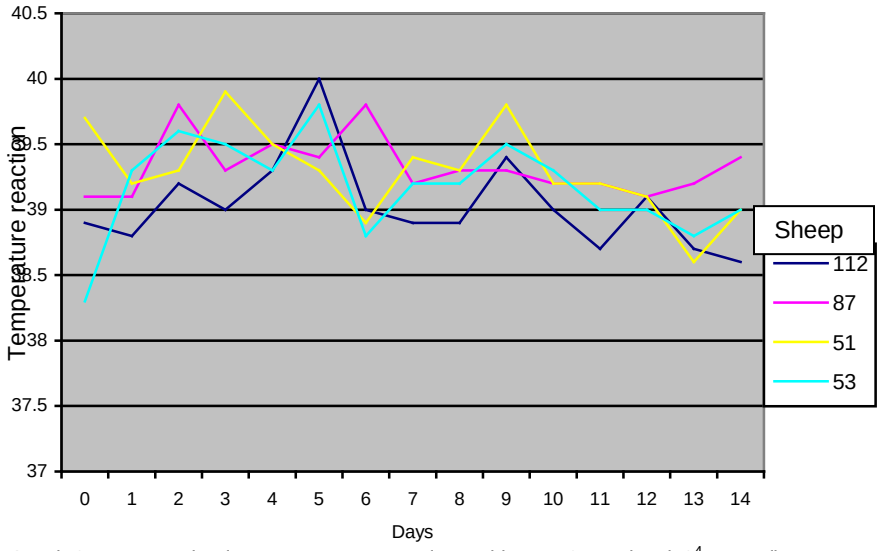
All sheep showed pyrexia within the first 10 days p.v.

BTV 2

All sheep (34, 120, 2 & 54) vaccinated with low titre (10^2 PFU/ml) vaccine had temperatures above 40 °C, with sheep 2 reacting on days 8 and 9, sheep 120 and 54 having pyrexia for a day on days 9 and 12 respectively. It was only sheep 34 which had pyrexia for three days (Graph 1). Only two sheep (91 & 18) vaccinated with 10^3 PFU/ml had pyrexia with sheep 91 reacting for 3 consecutive days on days 7 to 9 (Graph 2). Those vaccinated with the high titre vaccine only sheep 112 had pyrexia on day 5 (Graphs 3).



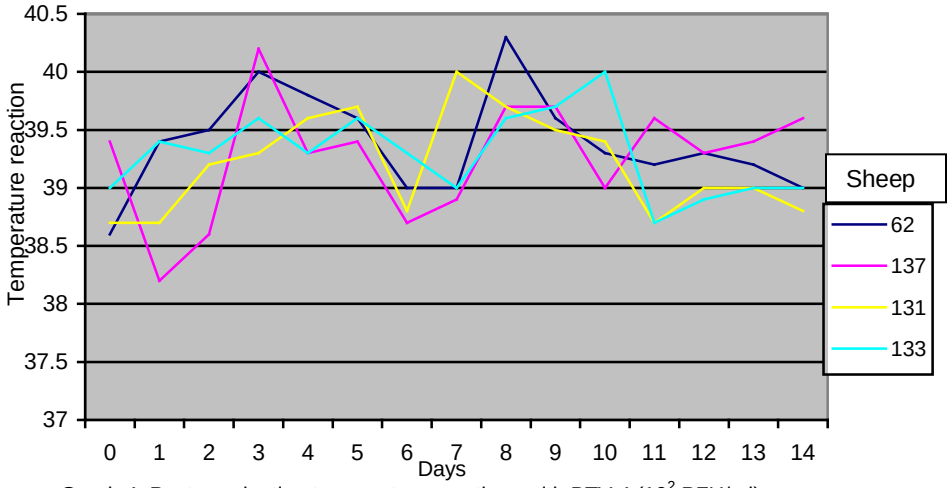
Graph 2. Post vaccination temperature reactions with BTV 2 vaccine (10^3 PFU/ml)



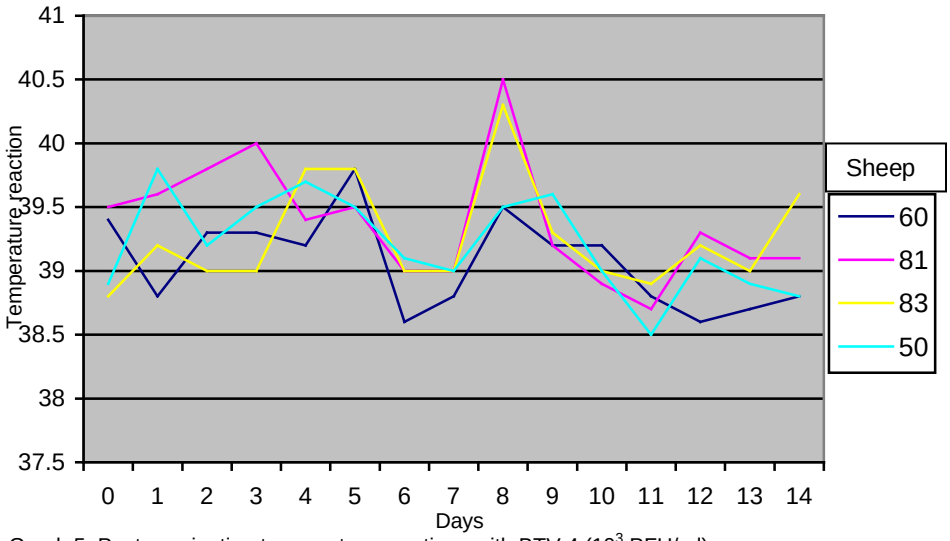
Graph 3. Post vaccination temperature reactions with BTV 2 vaccine (10^4 PFUml)

BTV 4

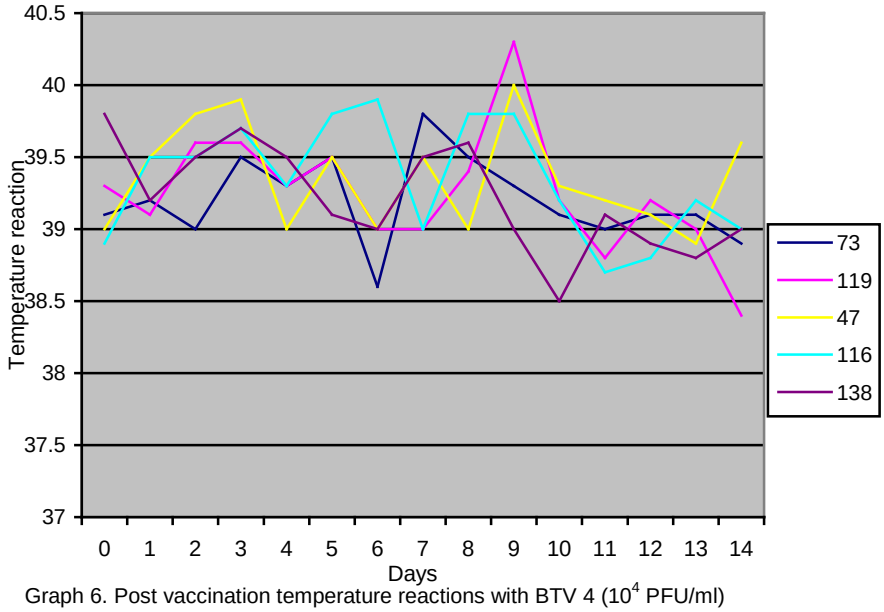
All four sheep vaccinated with BTV 4 low titre vaccine (10^2 PFU/ml) had inconsistent pyrexia on different days. Sheep 137 on day 3 with sheep 131 on day 7, sheep 62 on day 8 and sheep 133 on day 10 (Graph 4). Two sheep (81 & 83) vaccinated with BTV 4 (10^3 PFU/ml) had pyrexia on day 3 (sheep 81) and both on day 7 (Graph 5). Two sheep (47 & 119) vaccinated with a high titre vaccine were pyretic on day 9 (Graph 6). The temperature reaction of the negative control (138) was normal.



Graph 4. Post vaccination temperature reactions with BTV 4 (10^2 PFU/ml)



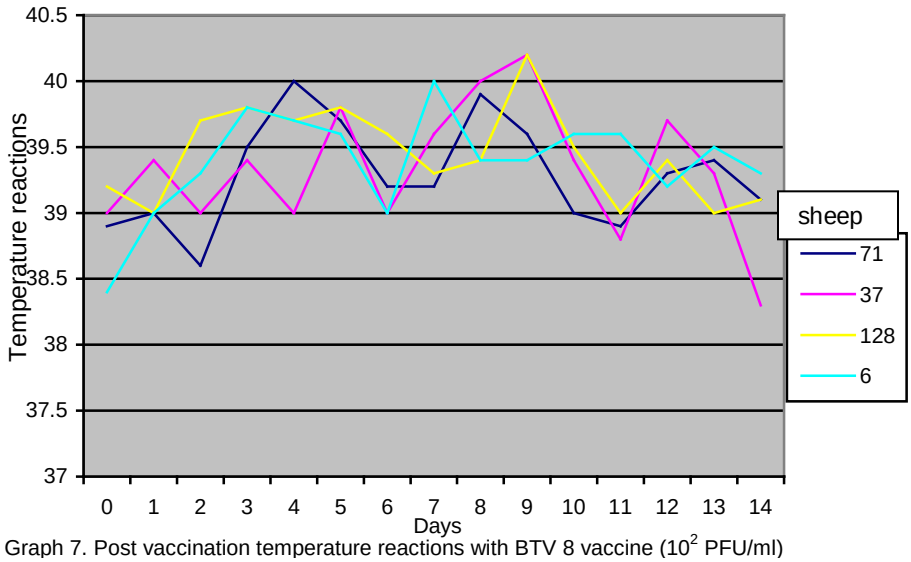
Graph 5. Post vaccination temperature reactions with BTV 4 (10^3 PFU/ml)



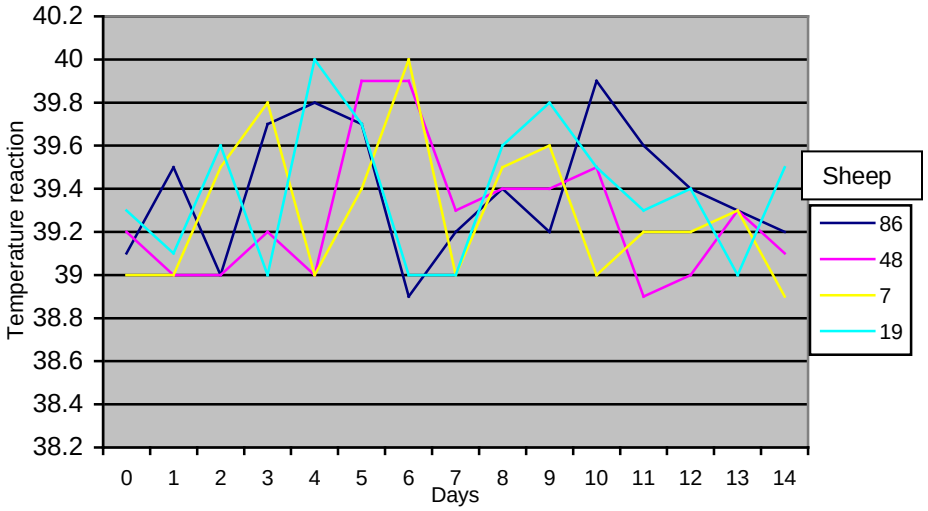
Graph 6. Post vaccination temperature reactions with BTV 4 (10^4 PFU/ml)

BTV 8

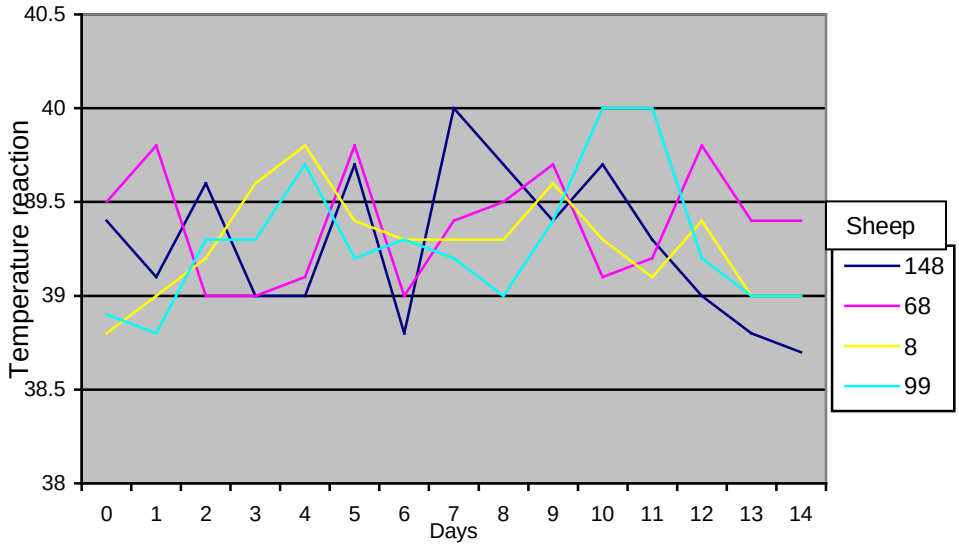
Six sheep (6, 71, 37, 128, 19 & 7) vaccinated with BTV 8 low titre vaccine 10^2 and 10^3 PFU/ml had pyrexia for a day but on different days (4, 7 & 9) (Graph 7 & 8). Two sheep vaccinated with high titre BTV 8 had pyrexia for 1 to 2 days (Graphs 9).



Graph 7. Post vaccination temperature reactions with BTV 8 vaccine (10^2 PFU/ml)



Graph 8. Post vaccination temperature reactions with BTV 8 vaccine (10^3 PFU/ml)



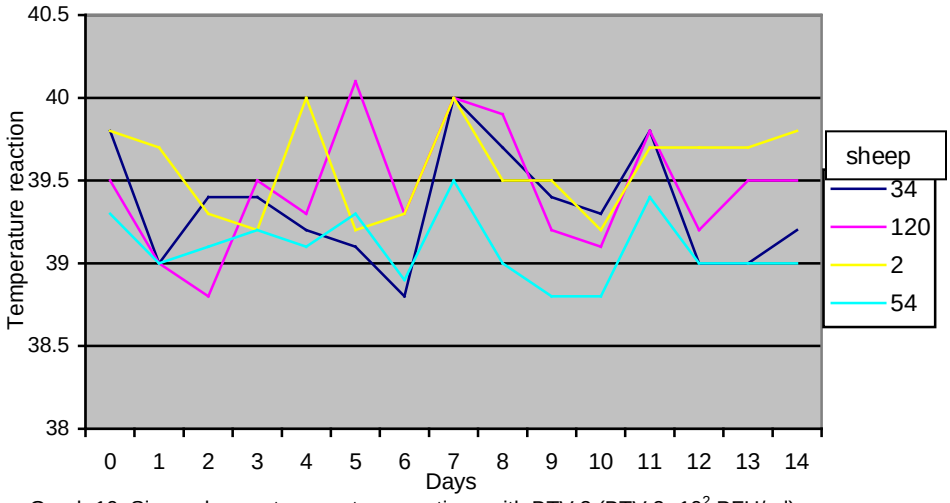
Graph 9. Post vaccination temperature reactions with BTV 8 (10^4 PFU/ml)

3.3 Post challenge temperature reactions

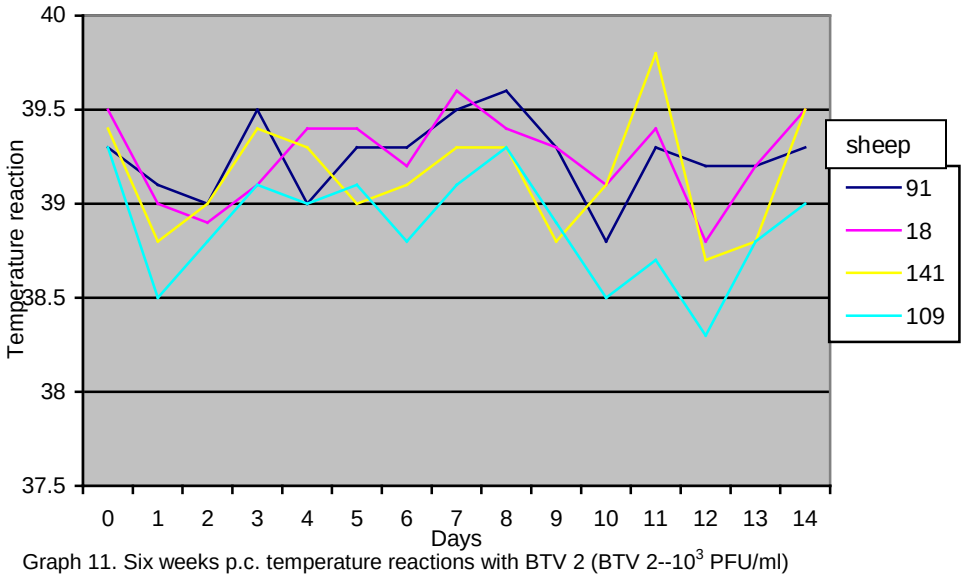
Group A sheep were challenged 4 weeks p.v. using homologous blood challenge material. Sheep 105 act as a positive control for BTV 2, sheep 28 for BTV 4 while sheep 72 was a positive control for BTV 8. Sheep 138 was used a negative control.

BTV 2

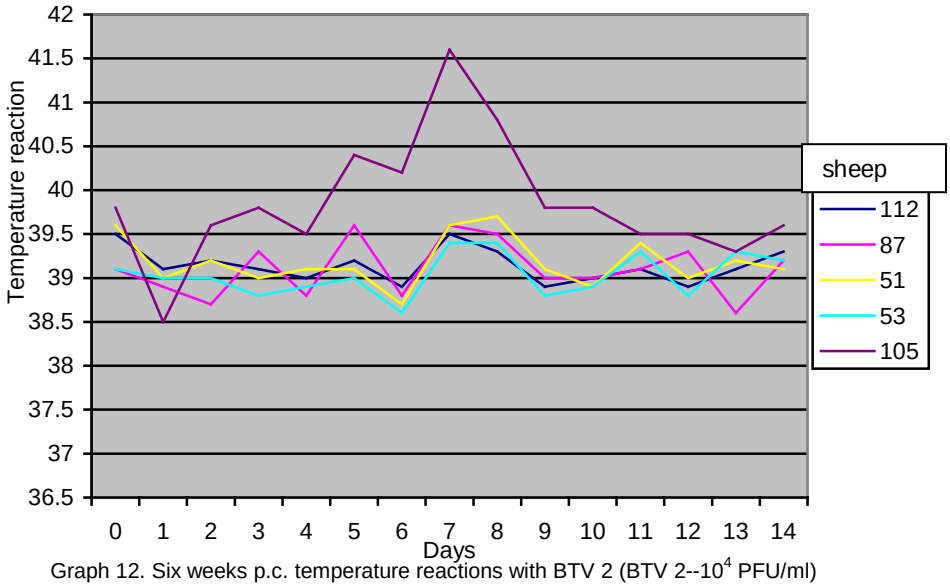
Three sheep (34, 120 & 2) vaccinated with low titre BTV 2 vaccine (10^2 PFU/ml) and challenged with BTV2 (blood) had inconsistent pyrexia from days 3 to 6 (Graph 10). There was no pyrexia p.c. in sheep vaccinated with BTV 2 vaccine (10^3 PFU/ml and 10^4 PFU/ml) (Graph 11 and 12) except for the positive control (105), challenged with BTV 2 blood which had pyrexia from day 5 to 8 (Graphs 12).



Graph 10. Six weeks p.c. temperature reactions with BTV 2 (BTV 2--10^2 PFU/ml)



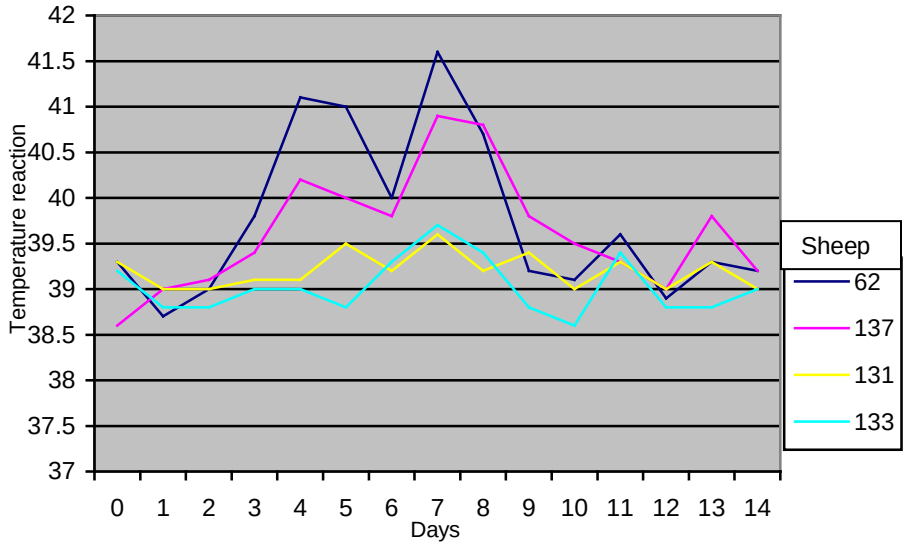
Graph 11. Six weeks p.c. temperature reactions with BTV 2 (BTV 2--10^3 PFU/ml)



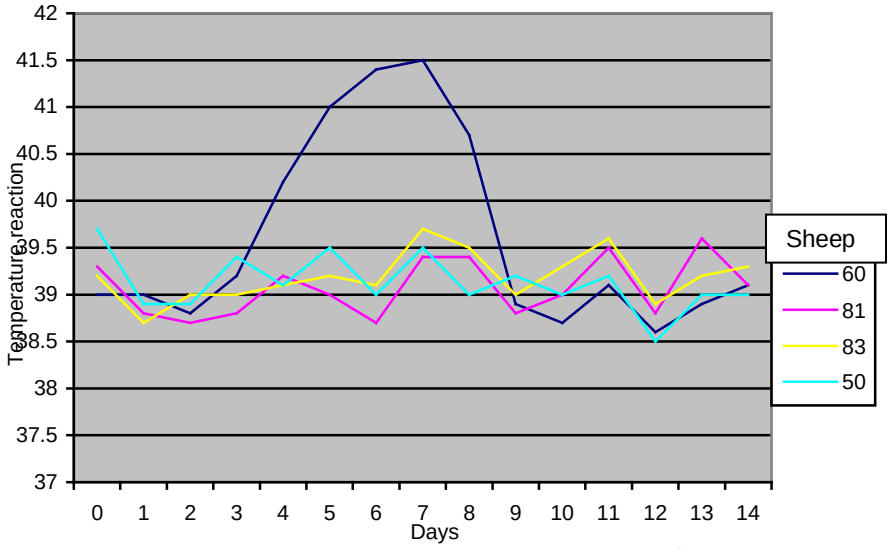
Graph 12. Six weeks p.c. temperature reactions with BTV 2 (BTV 2--10^4 PFU/ml)

BTV 4

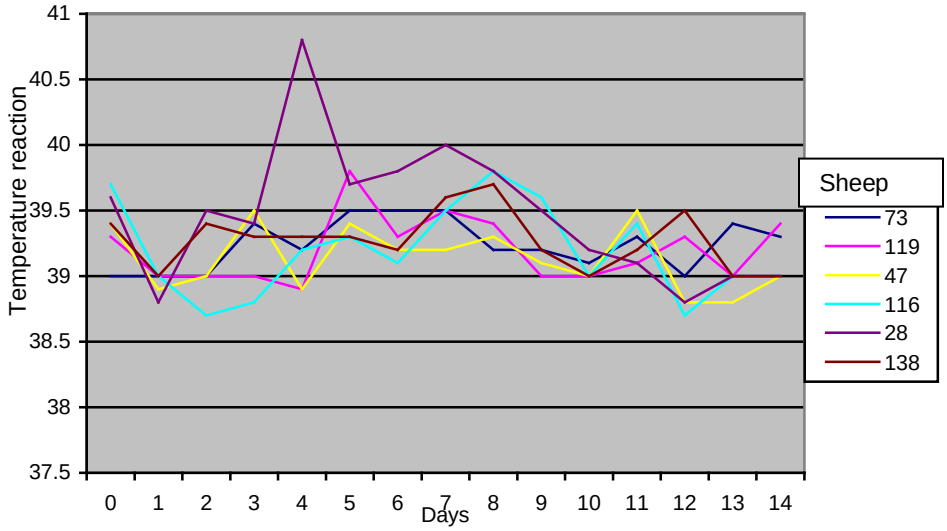
Two sheep (62 & 137) vaccinated with a titre of 10^2 PFU/ml and challenged with BTV 4 blood challenge material had pyrexia from day 4 to 8 with sheep 62 having high temperature (41,5 °C) on day 8 and sheep 137 showing pyrexia on days 8 and 9 (Graph 13). One sheep (60) vaccinated with 10^3 PFU/ml had pyrexia from days 4 to 8 (Graph 14). There was no pyrexia on sheep vaccinated with a high titre BTV 4 vaccine except the positive control (28) on day 4 and again on day 7 and the negative control (138) had a normal temperature reaction (Graphs 15).



Graph 13. Six weeks p.c. temperature reactions with BTV 4 (BTV 4--10^2 PFU/ml)



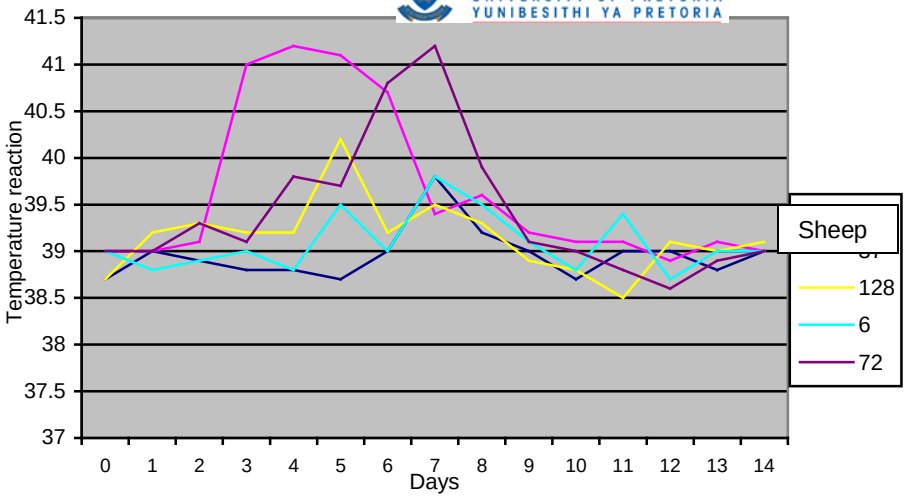
Graph 14. Six weeks p.c. temperature reactions with BTV 4 (BTV 4--10^3 PFU/ml)



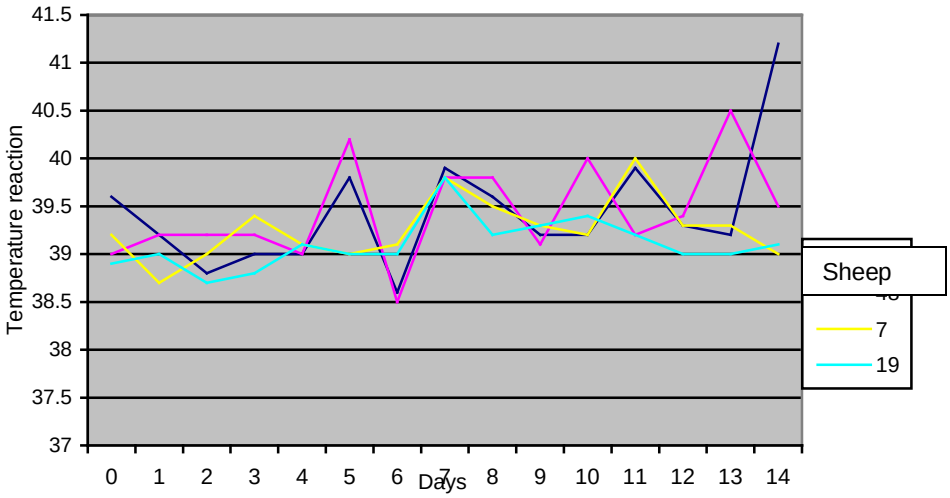
Graph 15. Six weeks p.c. temperature reactions with BTV 4 (BTV 4--10^4 PFU/ml)

BTV 8

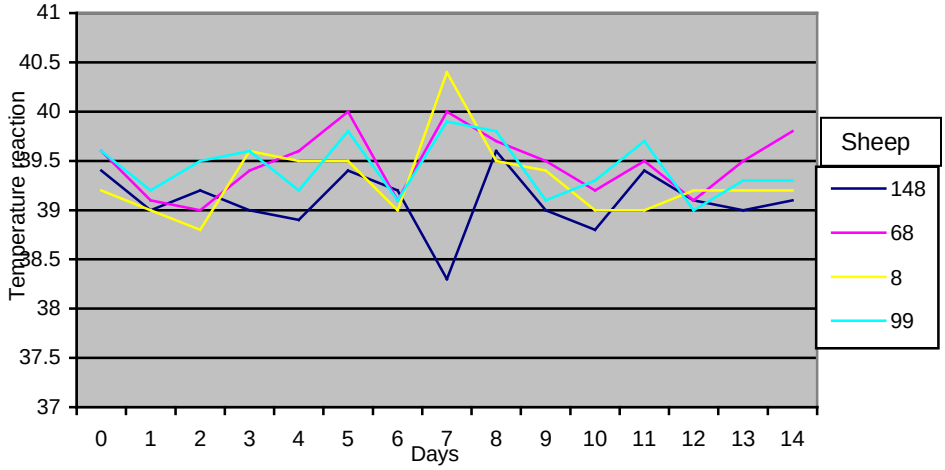
Eight sheep challenged with BTV 8 blood challenge material had inconsistent pyrexia from day 3 to day 10 with two of the sheep (37 & 128) vaccinated with BTV 8 (10^2 PFU/ml) showing pyrexia on day 3 to day 6 (sheep 128) and sheep 37 on day 5 (Graph 16). While sheep vaccinated with BTV 8 vaccine 10^3 PFU/ml had inconsistent pyrexia starting from days 5, 6, 9 and 10 (Graph 17). Sheep 48 and 86 had pyrexia which re-appeared again on days 13 and 14 (Graph 17). Two sheep (8 & 68) had pyrexia on day 7 with sheep 68 reacting on day 5 (Graph 18). The positive control (72) had pyrexia for 3 days also within the first 10 days (Graph 16).



Graph 16. Six weeks p.c. temperature reactions with BTV 8 (BTV --10^2 PFU/ml)



Graph 17. Six weeks p.c. temperature reactions with BTV 8 (BTV 8--10^3 PFU/ml)



Graph 18. Six weeks p.c. temperature reactions with BTV 8 (BTV 8--10^4 PFU/ml)

3.4 Post vaccination and Post challenge viraemia Group A

No viraemia could be detected on cell cultures p.v. for all BTV serotypes and p.c. only the positive control of BTV serotype 2 showed CPE. Some of the blood sample had haemolysed which might have interfered with some of the ELISA results.

3.5 Bluetongue seroconversion

BTV 2

Sheep vaccinated with 10^2 PFU/ml vaccine were positive from Day 9 p.v. using ELISA and on SNT they had neutralising titres of 1:8 to 1:16 at 4 weeks and 6 weeks p.v. (Table 5). Those vaccinated with 10^3 PFU/ml vaccine were positive on ELISA by Day 9 and had neutralising titres of 1:8 to 1:128 by 4 weeks and 6 weeks p.v. Two sheep vaccinated with high titre (10^4 PFU/ml) vaccine had neutralising titres below 1:4 at 4 weeks and up to 1:64 at 6 weeks p.v. (Table 5).

Table 5 : Post vaccination seroconversion- BTV 2 vaccine

Animal no	Titres PFU/ml	ELISA results Days p.v.					SNT- titres	
		0	3	9	15	21	4weeks	6weeks
34	10^2	-	-		-	+	1:8	1:16
120		-	-		+	+	1:32	1:32
2		-	-	-	+	-	1:16	1:16
54		-	-	+	-	+	<1:4	1:128
91	10^3	-	-	-	+	+	1:32	1:128
18		-	-	+	-	-	Na	na
141		-	-	+	-	-	1:128	1:32
109		-	-	-	-	+	<1:4	1:8
112	10^4	-	-	+	+	+	1:64	1:64
87		-	-		-	+	<1:4	1:32
51		-	-	-	-	-	<1:4	1:32
53		-	-	+	+	+	1:16	1:64
138	N- control	-	-	-	-	-	<1:4	<1:4

BTV 4

Sheep vaccinated with the different titres of BTV 4 vaccines were mostly positive on ELISA by Day 9. Three sheep (137, 131 & 133) vaccinated with low titre 10^2 PFU/ml and two sheep (60 & 81) vaccinated with 10^3 PFU/ml vaccines had very low neutralising titres below 1:4. Three sheep (73, 119 & 116) vaccinated with the high titre vaccine also did not demostrate neutralisation titres at 4 weeks and only two sheep (47 & 116) had neutralisation titres after 6 weeks (Table 6).

Table 6: Post vaccination seroconversion- BTV 4 vaccine

Animal no	Titres PFU/ml	ELISA results Days p.v.					SNT-titres	
		0	3	9	15	21	4weeks	6weeks
62	10^2	-	-	-	-	-	<1:4	<1:4
137		-	-	+	-	-	<1:4	<1:4
131		-	-	+	+	+	<1:4	1:16
133		-	--	-	+	-	1:16	1:16
60	10^3	-	-	+	+	-	<1:4	1:8
81		-	-	-	-	-	<1:4	<1:4
83		-	-	+	+	+	1:16	1:16
50		-	-	+	-	-	1:8	1:4
73	10^4	-	-	+	+	+	<1:4	<1:4
119		-	-	-	-	-	<1:4	<1:4
47		-	-	+	+	-	1:4	1:16
116		-	-	+	-	-	<1:4	1:16
138	Neg Cont	-	-	-	-	-	<1:4	<1:4

BTV 8

Sheep were positive on ELISA within the first 21 days p.v. The SNT results showed antibodies reaching neutralisation titres from 1:8 to 1:32 for those vaccinated with low titre BTV 8 vaccine (10^2 & 10^3 PFU/ml) 6 weeks p.v. except for one sheep (37) (Table 7). Those vaccinated with a high titre vaccine were positive on ELISA and had neutralising antibodies on SNT at 6 weeks.

Table 7 : Post vaccination seroconversion- BTV 8 vaccine

Animal no	Titres PFU/ml	Elisa results Days p.v.					SNT-titres	
		0	3	9	15	21	4weeks	6weeks
71	10^2	-	-	n/a	n/a	-	n/a	1:16
37		-	-	n/a	n/a	-	n/a	<1:4
128		-	-	n/a	n/a	+	n/a	1:8
6		-	-	n/a	n/a	+	n/a	1:8
86	10^3	-	-	-	n/a	+	n/a	1:8
48		-	-	+	n/a	+	n/a	1:16
7		-	-	+	n/a	+	n/a	1:16
19		-	-	+	n/a	+	n/a	1:32
148	10^4	-	-	+	n/a	+	n/a	1:16
68		-	-	+	n/a	+	n/a	1:32
8		-	-	-	n/a	+	n/a	1:32
99		-	-	+	n/a	+	n/a	1:64
138	Neg Control	-	-	-	-	-	<1:4	<1:4

n/a= not available (blood sample spoiled)

3.6 Percentage protection index

The protection index was calculated as indicated in 2.8 after sheep had been clinically evaluated for 14 days p.c. The CRI for the control sheep challenged with BTV 2 blood was 5 and for sheep vaccinated with BTV 2 low titre vaccine (10^2 PFU/ml) they had protection index of 68.75%. Sheep vaccinated with BTV 2, 10^3 PFU/ml and 10^4 PFU/ml had 100% protection (Table 8).The CRI of the control sheep (28) challenged with BTV 4 was 2.5 and some of the sheep vaccinated with the BTV 4 low titre (10^2 PFU/ml) vaccine had a higher CRI than the positive control (Table 9). The CRI for the positive control sheep challenged with BTV 8 blood challenge material was 2. The average percentage index for low titre vaccines (10^2 & 10^3 PFU/ml) was 62.5% and 84.3%, respectively while for the high titre vaccine 75% (Table 10).

Table 8: BTV 2 clinical reaction and percentage protection index challenged at 6 weeks p.v.

Animal no	**Titres PFU/ml**	**CRI**	**Protection %**	**Average %**
34	10^2	1	75%	68.75
120		2	50%	
2		2	50%	
54		0	100%	
91	10^3	0	100%	100
18		0	100%	
141		0	100%	
109		0	100%	
112	10^4	0	100%	100
87		0	100%	
51		0	100%	
53		0	100%	
105		5	control	

Table 9: BTV 4 clinical reaction and percentage protection index challenged at 6 weeks p.v.

Animal no	Titres PFU/ml	CRI	Protection %	Average %
62	10^2	5	<0%	50
137		4	<0%	
131		0	100%	
133		0	100%	
60	10^3	6	<0%	75
81		0	100%	
83		0	100%	
50		0	100%	
73	10^4	0	100%	100
119		0	100%	
47		0	100%	
116		0	100%	
28		2.5	control	

Table 10: BTV 8 clinical reaction and percentage protection index challenged at 6 weeks p.v.

Animal no	Titres PFU/ml	CRI	Protection %	Average %
71	10^2	0	100%	62.5
37		2.5	<0%	
128		1	50%	
6		0	100%	
86	10^3	0	100%	84.3
48		0	100%	
7		1.5	37%	
19		0	100%	
148	10^4	0	100%	75
68		1	50%	
8		1	50%	
99		0	100%	
72		2	control	

GROUP B

This group was vaccinated at same time with Group A sheep but was challenged with cell culture challenge material 4 months p.v. Sheep 147 act as a negative control.

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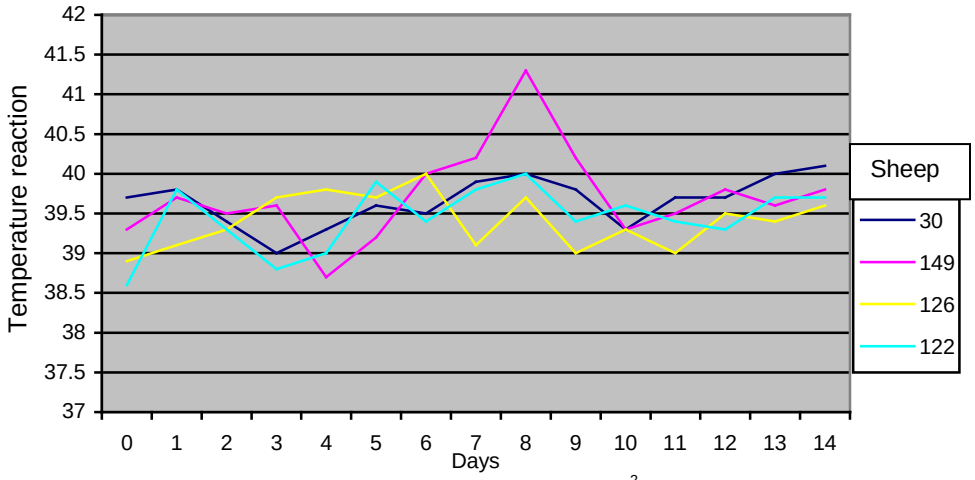
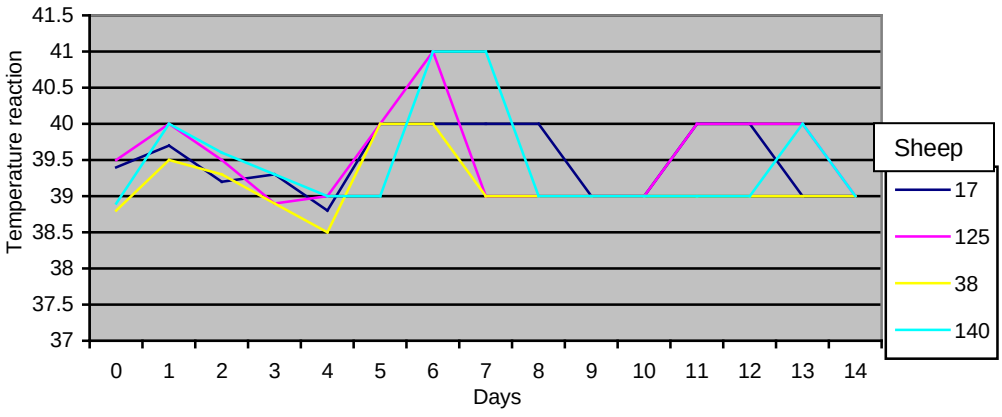
3.7 Post vaccination temperature reactions

BTV 2

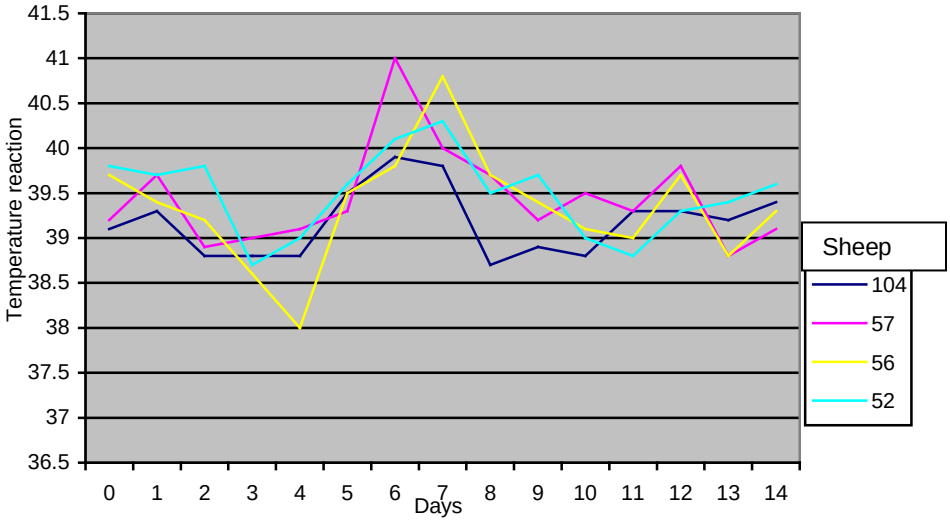
Sheep 149 vaccinated with BTV 2 vaccine titre of 10^2 PFU/ml had pyrexia on different days on day 7 to 9 (Graph 19). All sheep vaccinated with BTV 2 vaccine titre of 10^3 PFU/ml had pyrexia from day 5 to 7 with sheep 17, 125 and 140 having pyrexia late from day 11 to 13. Sheep 140 and 125 had high temperatures reactions of 41°C and 40,7°C, respectively (Graph 20). Three sheep (52, 56 & 57) vaccinated with the high titre BTV 2 vaccine (10^4 PFU/ml) had pyrexia also from day 6 to 8 on different days (Graph 21).



Graph 19. Group B p.v. temperature reactions with BTV 2 (10^2 PFU/ml)



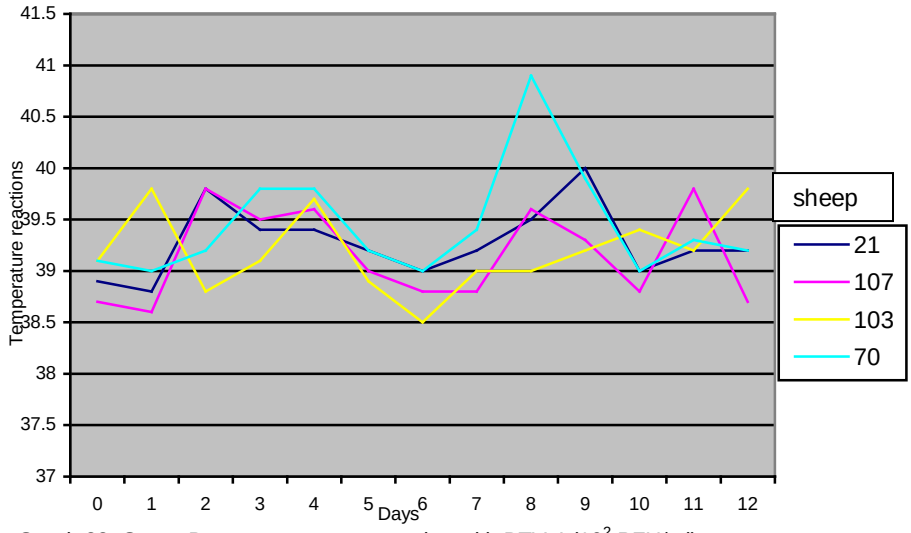
Graph 20. Group B p.v. temperature reactions with BTV 2 (10^3 PFU/ml)



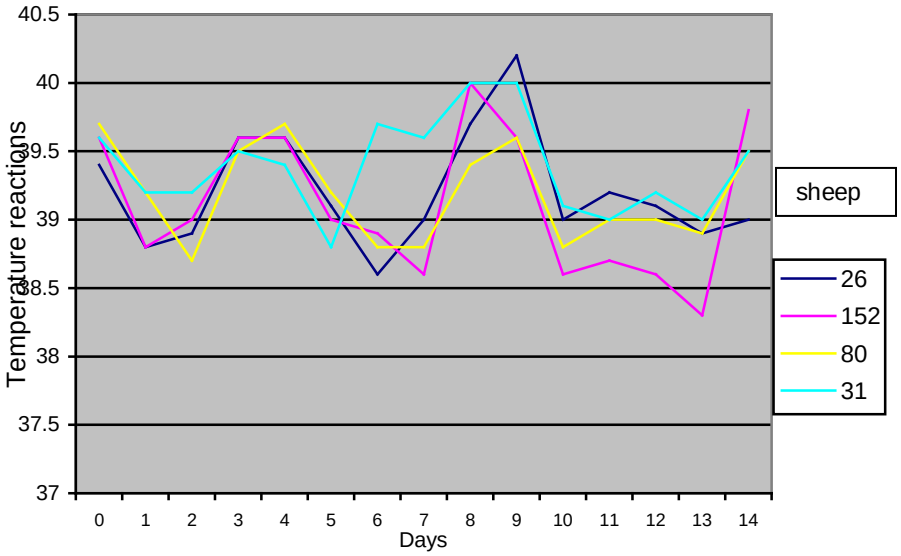
Graph 21. Group B p.v. temperature reactions with BTV 2 (10^4 PFU/ml)

BTV 4

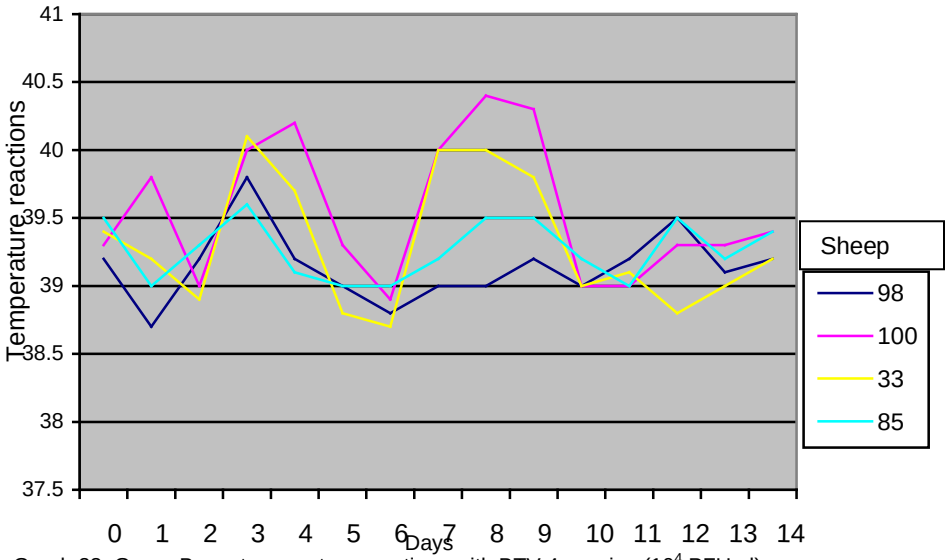
Sheep 21 and 70 vaccinated with low titre BTV4 (10^2 PFU/ml) vaccine had pyrexia for a day on day 8 and 9, respectively (Graph 22) while sheep 70 had a temperature above 40,5 °C. Those vaccinated with 10^3 PFU/ml three sheep (26, 31 & 152) had pyrexia for 1 to 2 days with sheep 31 reacting for two days (8 & 9) and sheep 26 having temperature above 40 °C on day 9 (Graph 23). Those vaccinated with high BTV 4 titre two sheep (33 & 100) had pyrexia which was bi-phasic on days 3 and 4 and later on day 7 to 9 (sheep 100) (Graph 24).



Graph 22. Group B p.v. temperature reaction with BTV 4 (10^2 PFU/ml)



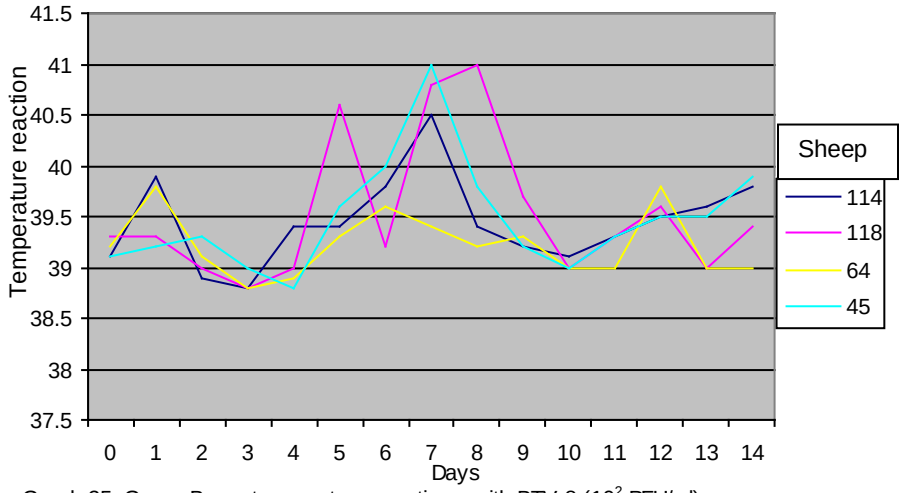
Graph 23. Group B p.v. temperature reactions with BTV 4 vaccine (10^3 PFU/ml)



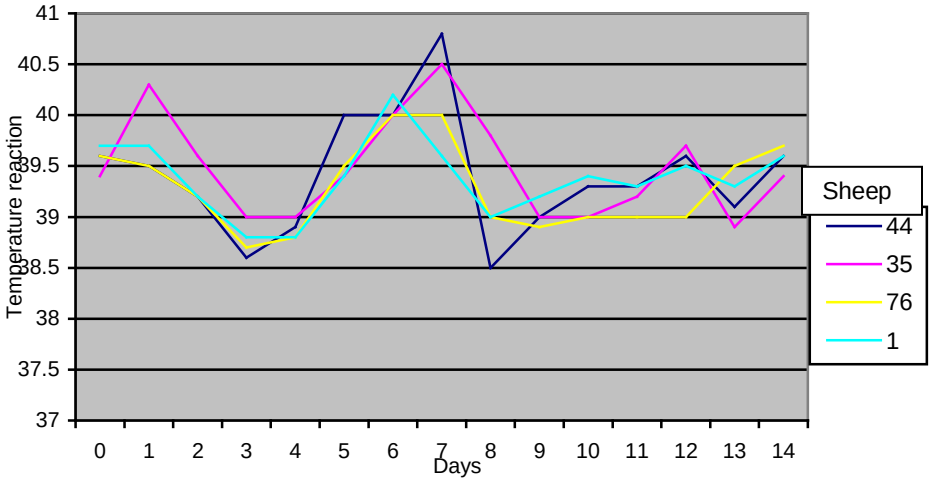
Graph 23. Group B p.v. temperature reactions with BTV 4 vaccine (10^4 PFUml)

BTV 8

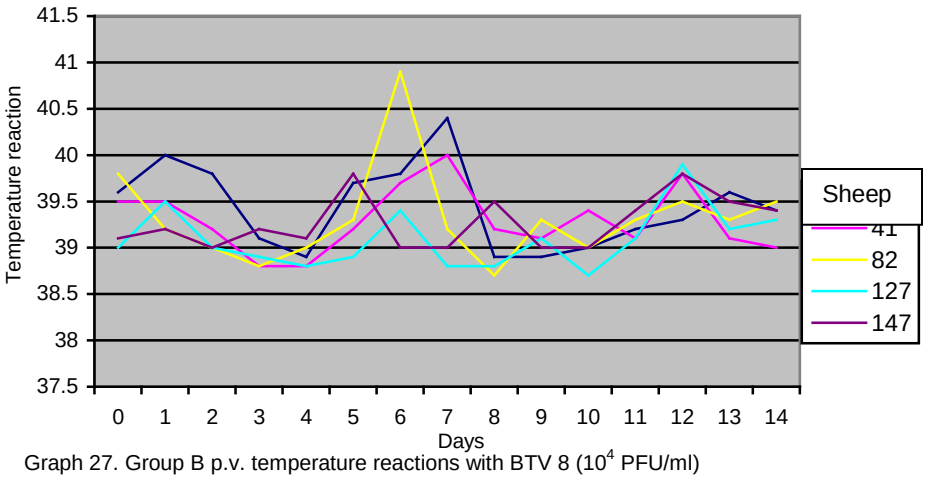
All sheep vaccinated with BTV 8 low titre vaccine 10^2 PFU/ml had pyrexia on different days (5, 7 & 8), sheep 114 and 45 being pyretic on day 7 (Graph 25). All sheep vaccinated with10^3 PFU/ml had pyrexia on different days from day 5 to 8, only sheep 1 reacted on day 6 (Graph 26). All sheep vaccinated with high titre also had pyrexia on different days (6, 7 and 9). Sheep 41 and 5 had pyrexia on day 7, sheep 82 on day 6 while sheep 147 which was a negative control had normal temperature reactions (Graph 27).



Graph 25. Group B p.v. temperature reactions with BTV 8 (10^2 PFU/ml)



Graph 26. Group B p.v. temperature reactions with BTV 8 (10^3 PFU/ml)

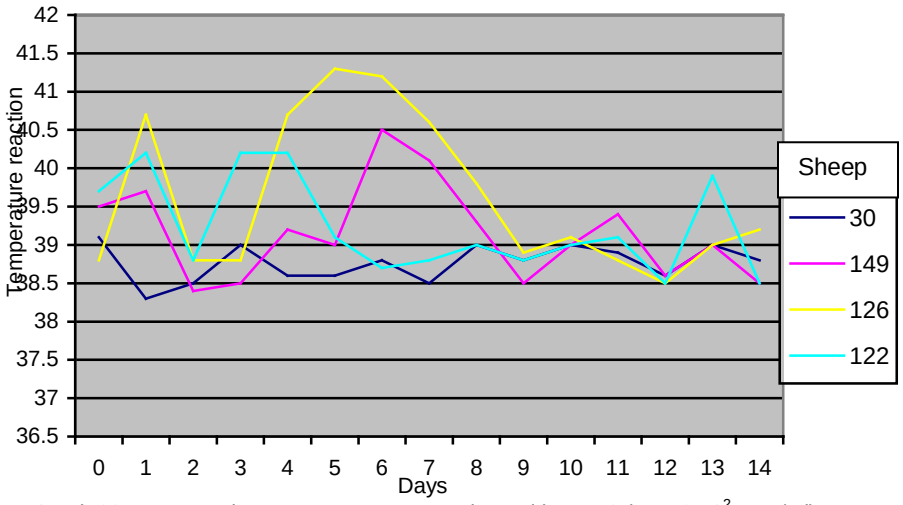


Graph 27. Group B p.v. temperature reactions with BTV 8 (10^4 PFU/ml)

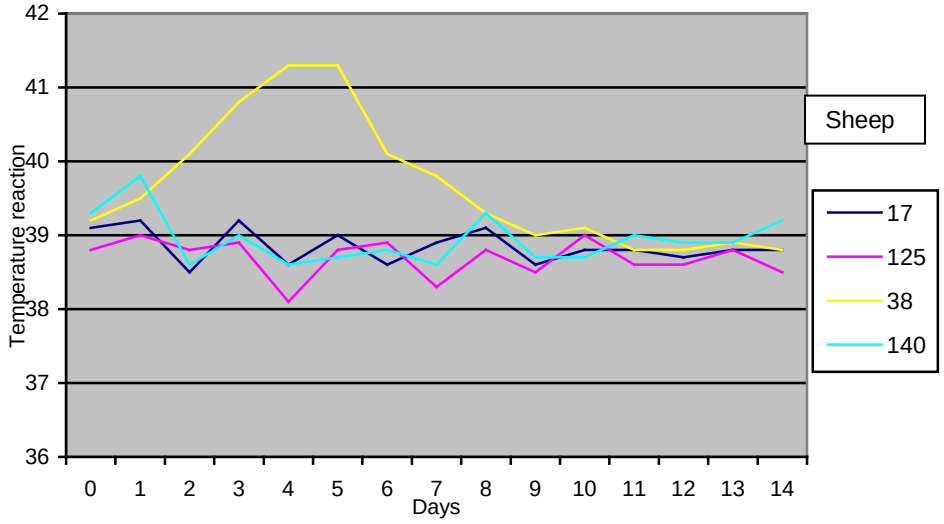
3.8 Post challenge temperature reaction

BTV2

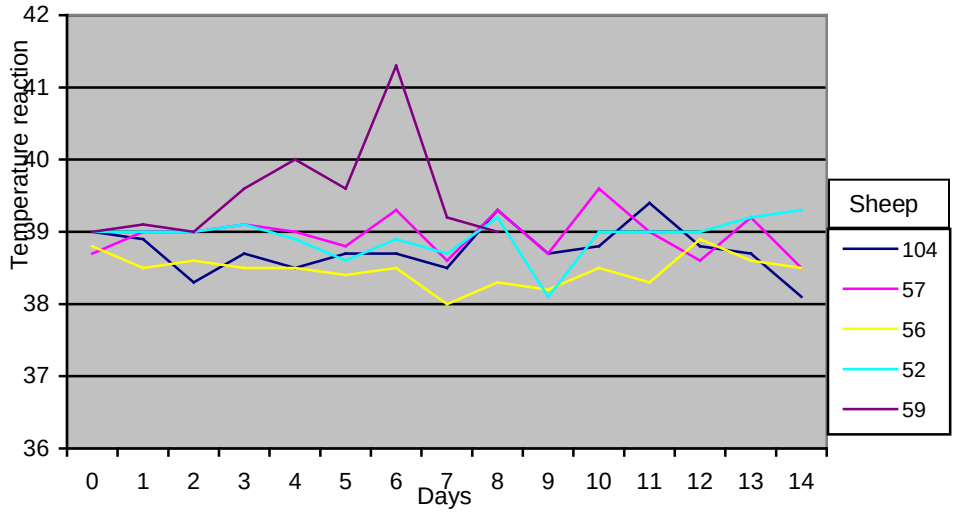
Three sheep (122, 126 & 149) vaccinated with BTV 2 vaccine titre of 10^2 PFU/ml and challenged with BTV 2 cell culture material had pyrexia from day 1 and again on day 3 to 4 p.c. (Graph 28). Only one sheep (38) vaccinated with BTV 2 vaccine (10^3 PFU/ml) had pyrexia from day 2 to 6 p.c. (Graph 29). There was no pyrexia p.c. in sheep vaccinated with the high titer vaccine (10^4 PFU/ml) except positive control sheep (59) which had pyrexia from day 3 to 5 before it died on day 8 (Graph 30).



Graph 28. Four months p.c. temperature reactions with BTV 2 (BTV 2-10^2 PFU/ml)



Graph 29. Four months p.c . temperature reactions with BTV2 (BTV 2-10^3 pfu/ml)

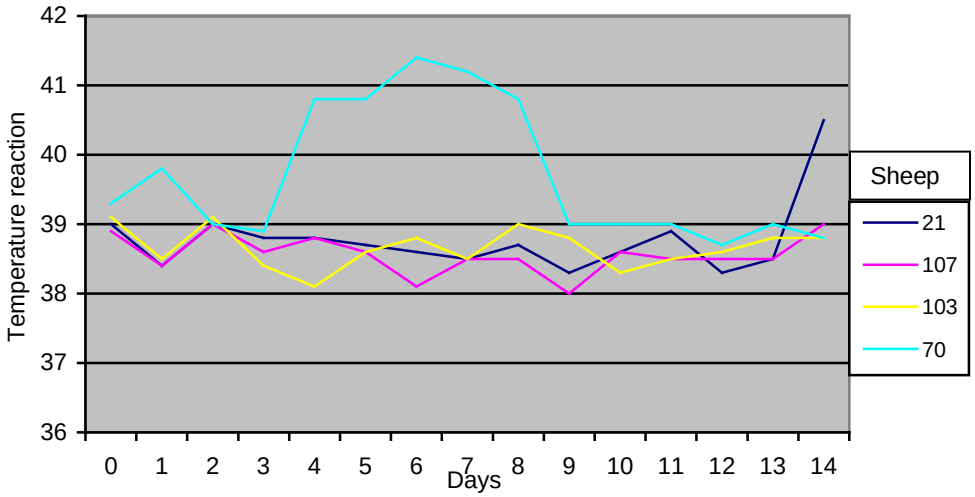


Graph 30. Four months p.c. temperature reactions with BTV2 (BTV 2-10^4 pfu/ml)

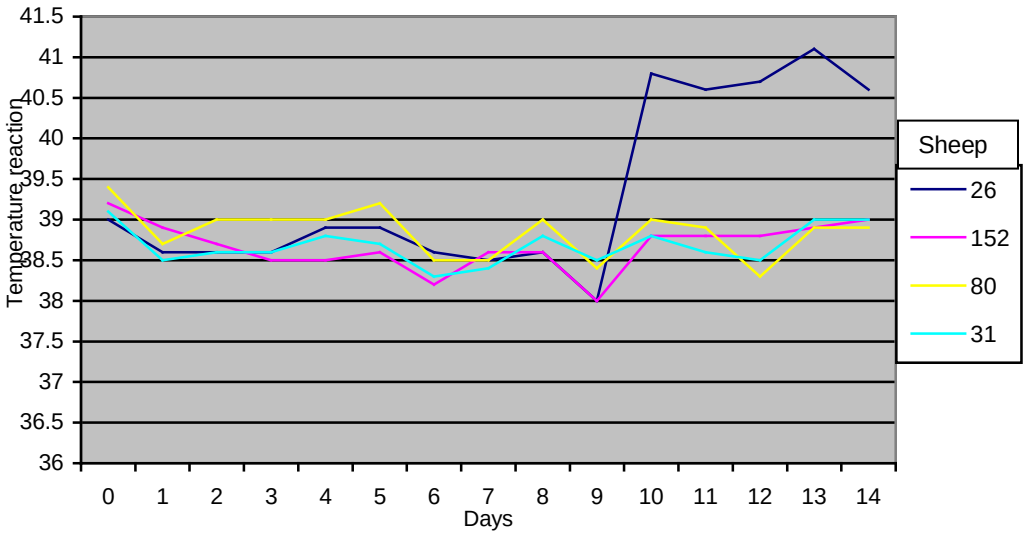
BTV4

Sheep 70 vaccinated with low titre BTV 4 vaccine (10^2 PFU/ml) had pyrexia p.c. from day 3 to 8 with temperature above 41°C (Graphs 31), however within the same group one had pyrexia on day 14 which is unusual. Only one sheep (26) vaccinated with a titre of 10^3 PFU/ml had pyrexia p.c. on day 10 to 14 (Graph 32).

Sheep (100 & 85) vaccinated with the high titre vaccine (10^4 PFU/ml) had pyrexia on day 6 and sheep 85 on day 12 to 14 (Graph 33). One positive control sheep (43) had pyrexia for 7 days p.c. with BTV 4 (Graph 33). The negative control sheep (132) had pyrexia on day 8.



Graph 31. Four months p.c. temperature reactions with BTV 4 (BTV 4-10^2 PFU/ ml)

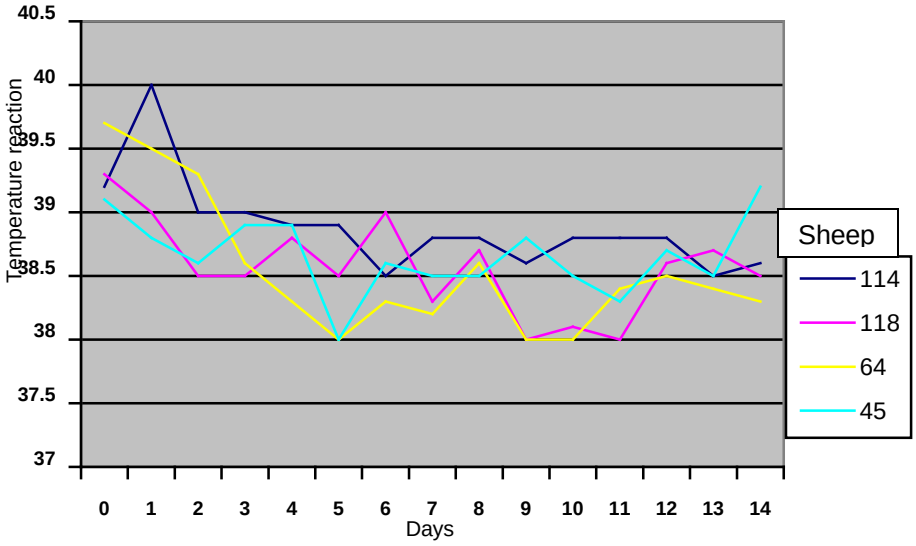


Graph 32. Four months p.c. temperature reactions with BTV 4 (BTV 4-10^3 PFU/ml)

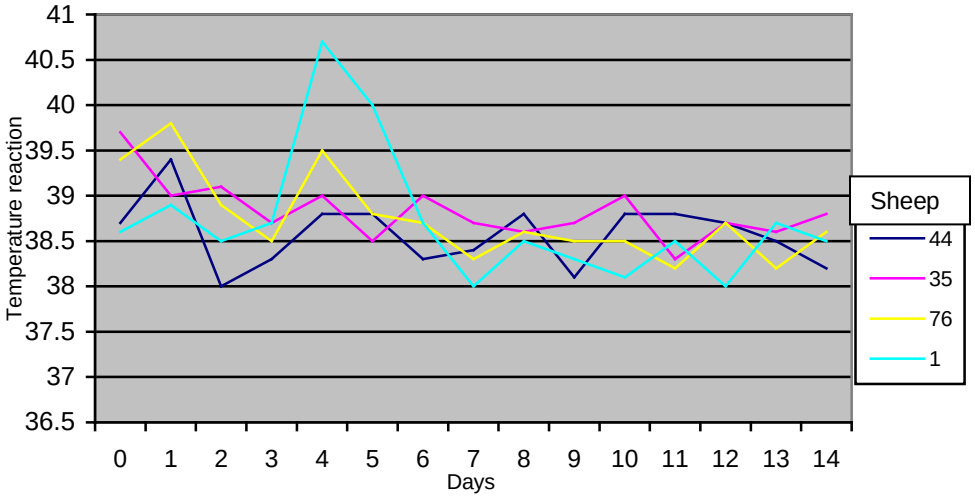
Sheep

BTV 8

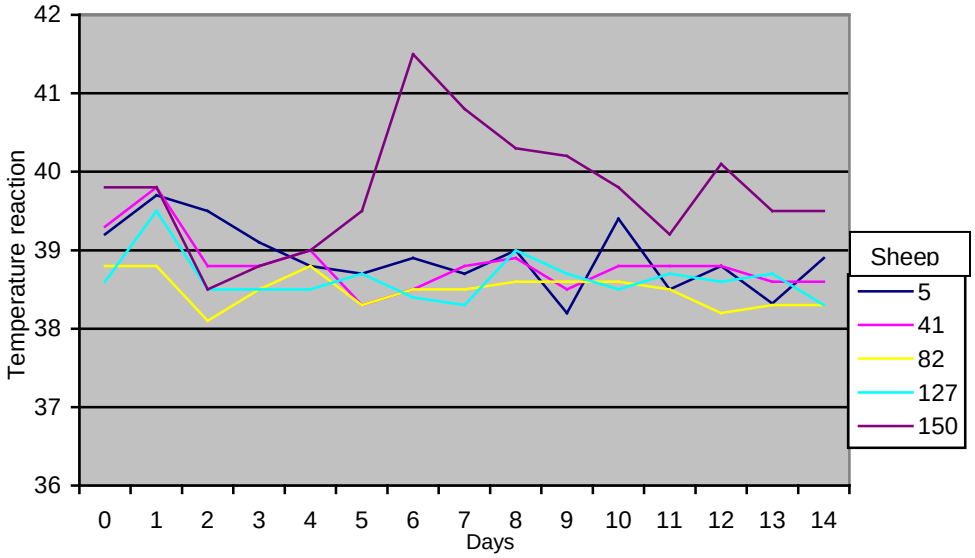
Two sheep (114 & 1) vaccinated with low titre BTV 8 vaccines (10^2 &10^3 PFU/ml) and challenged with BTV 8 cell culture material had pyrexia on day 1 and 4 (Graph 34 & 35). Sheep vaccinated with the high titre vaccine (10^4 PFU/ml) did not demonstrate any abnormal reactions except the positive control sheep (150) which had pyrexia from day 6 -10 (Graph 36).



Graph 34. Four months p.c temperature reactions with BTV8 (BTV 8-10^2 PFU/ml)



Graph 35. Four months p.c. temperature reactions with BTV 8 (BTV 8-10^3 PFU/ml)



Graph 36. Four months p. c. temperature reactions with BTV 8 (BTV 8-10^4 PFU/ml)

3.9 Post challenge viraemia- Group B

Sheep challenged at 4 months p.v. using with BTV 2 cell culture material, showed viraemia was detected in sheep vaccinated with both low titre vaccines (10^2 & 10^3 PFU/ml) except sheep 30 and 17, while only one sheep (59) from those vaccinated with high titre vaccine (10^4 PFU/ml) also showed viraemia (Table 11). Only two sheep (30 & 17) vaccinated with low titre BTV 4 vaccines (10^2 & 10^3 PFU/ml) viraemia was detected p.c.(Table 12). Viraemia was not detected after challenge with BTV 8 cell

culture material in all three groups of sheep vaccinated with different vaccine titres (Table 13).

Table 11: Viraemia results 4 months p.c. with BTV 2

Animal no	Titres PFU/ml	Days p.c.							
		0	3	6	9	12	15	18	21
30	10^2	-	-	-	-	-	-	-	-
149		-	-	+	+	-	-	-	-
126		-	-	+	+	-	-	-	
122		-	-	-	-	-	+		
17	10^3	-	-	-	-	-	-	-	-
125		-	-	-	-	+	-	-	-
38		-	-	+	+	+	-	-	-
140		-	-	-	-	-	+	-	-
104	10^4	-	-	-	-	-	-	-	-
57		-	-	-	-	-	-	-	-
56		-	-	-	+	-	-	-	-
52		-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-
59	Positive control	-	-	+	+	+	Dead		

Table 12:Viraemia results 4 months p.c. with BTV 4

Animal no	Titres PFU/ml	Days p.c.									
		0	3	6	9	12	15	18	21	28	
21	10^2	-	-	+	+	+	-	-	-	-	
107		-	-	-	-	+	-	-	-	-	
103		-		+	-	+	-	-	-	-	
70		-	-	+	+	+	-	-	-	-	
26	10^3	-	-	-	+	+	-	-	-	-	
152		-	-	+	-		-	-	-	-	
80		-	-	-	-	-	-	-	-	-	
31		-	-	+	-	-	+	-	-	-	
98	10^4	-	-	-	-	-	-	-	-	-	
100		-		-	-	-	-	-	-	-	
33		-	-	-	-	-	-	-	-	-	
85		-	-	-	-	-	-	-	-	-	
43	Positive control	-	-	+	+	+	-	-	-	-	
132	Negative control	-	-	-	-	-	-	-	-	-	-

Table 13: Viraemia results 4 months p.c. with BTV 8

Animal no	Titres PFU/ml	Days p.c.								
		0	3	6	9	12	15	18	21	28
114	10^2	-	-	-	-	-	-	-	-	-
118		-	-	-	-	-	-	-	-	-
64		-	-	-	-	-	-	-	-	-
45		-	-	-	-	-	-	-	-	-
44	10^3	-	-	-	-	-	-	-	-	-
35		-	-	-	-	-	-	-	-	-
76		-	-	-	-	-	-	-	-	-
1		-	-	-	-	-	-	-	-	-
5	10^4	-	-	-	-	-	-	-	-	-
41		-	-	-	-	-	-	-	-	-
82		-	-	-	-	-	-	-	-	-
127		-	-	-	-	-	-	-	-	-
150	Positive control	-	+	+	+	-	-	-	-	-

3.10 Bluetongue seroconversion- GROUP B

This group as stated before was challenged with cell culture material at 4 months and clinically evaluated for 14 days p.c.

BTV 2

Sheep vaccinated with low titre vaccine (10^2 & 10^3 PFU/ml) were seropositive on ELISA by day 21 p.v. and showed varied neutralisation titres of 1:16 to 1:64 by 4 weeks and similar titres at 4 months p.v. Those vaccinated with high titre vaccine also had neutralisng titres by 4 weeks and 4 months (Table 14).

Table 14: Post vaccination seroconversion- BTV 2 vaccine

Animal no	Titres PFU/ml	ELISA results(Days)					SNT titres	
		0	3	9	15	21	4 weeks	4 months
30	10^2	-	-	-	-	-	<1:4	1:64
149		-	-	-	+	+	1:64	1:64
126		-	-	-	-	+	1:16	1:4
122		-	-	-	+	+	1:4	1:4
17	10^3	-	-		+	+	1:32	1:32
125		-	-	-	+	+	1:64	1:64
38		-	-		-	+	1:32	1:16
140		-	-	-	+	+	1:32	1:16
104	10^4	-	-	+	-	+	1:16	1:4
57		-	-	-	-	+	1:4	1:16
56		-	-	+	+	+	1:4	1:16
52		-	-	-	+	+	1:4	1:32
132	n-control	-	-	-	-	-	<1:4-	<1:4

BTV 4

Sheep vaccinated with low titre vaccine (10^2 & 10^3 PFU/ml) were seropositive from day 9 and had neutralising titres above 1:4 at 4 weeks and 4 months p.v. except sheep number 80. Sheep 98 and 107 did not seroconvert on ELISA and SNT until after 4 weeks, however it had neutralising titres of 1:128 at 4 months.

Table 15: Post vaccination seroconversion- BTV 4 vaccine

Animal no	Titres PFU/ml	Elisa titres(Days)					SNT-titres	
		0	3	9	15	21	4weeks	4months
21	10^2	-	-	+	+	+	1:32	1:8
107		-	-	-	-	-	1:8	1:4
103		-	-	+	-	+	1:16	1:4
70		-	-	+	+	+	1:32	1:32
26	10^3	-	-	+	+	+	1:32	1:8
152		-	-	+	-	+	1:8	1:32
80		-	-	+	-	+	<1:4	<1:4
31		-	-	+	+	+	1:32	1:8
98	10^4	-	-	-	-	-	<1:4	1:8
100		-	-	+	+	+	1:4	1:16
33		-	-	+	+	+	1:4	1:32
85		-	-	+	+	+	1:4	1:8
132		-	-	-	-	-	<1:4	<1;4

BTV 8

Sheep 1 did not demonstrate detectable antibodies after 4 weeks while sheep 114 and 41 only seroconverted after day 9 p.v. However, all sheep had neutralising titres above 1:16 at 4 months p.v. (Table16).

Table 16: Post vaccination seroconversion- BTV 8 vaccine

Animal no	Titres PFU/ml	ELISA titres(Days)					SNT titres	
		0	3	9	15	21	4 weeks	4 months
114	10^2	-	-	-	+	+	na	1:2048
118		-	-	+	+	+	na	1:16
64		-	-	+	-	+	na	1:512
45		-	-	+	+	+	na	1:4096
44	10^3	-	-	-	+	+	na	1:1024
35		-	-	+	+	+	na	1:32
76		-	-	-	+	+	na	1:572
1		-	-	-	-	-	na	1:128
5	10^4	-	-	+	+	+	na	1:512
41		-	-	-	+	+	na	1:256
82		-	-	+	+	+	na	1:64
127		-	-	+	+	+	na	1:16
147	n control	-	-	-	-	-	<1:4	<1:4

3.11 Percentage protection index

BTV 2

At four months post challenge with BTV 2 cell culture material, sheep vaccinated with low titre vaccine (10^2 PFU/ml) had 72% protection and there were two sheep 126 and 38 which had very high CRI. The positive control sheep (59) had a CRI of 10 and died on d 8 p.c. (Table17).

Table 17: BTV 2 clinical reaction and percentage protection index, challenged at 4 months p.v.

Animal no	Titres PFU/ml	CRI	Protection %	Average %
30	10^2	0	100%	72
149		2	80%	
126		9	10%	
122		0	100%	
17	10^3	0	100%	82.5
125		0	100%	
38		7	30%	
140		0	100%	
104	10^4	0	100%	100
57		0	100%	
56		0	100%	
52		0	100%	
59	Positive control	10	control	

BTV 4

The CRI for BTV 4 positive control after challenge with BTV 4 cell culture material was 8 and sheep vaccinated with low BTV 4 titre (10^2 PFU/ml) vaccine had a 50% protection with one sheep having CRI of 7. Those vaccinated with high titre vaccine had a protection of 75.25% (Table 18).

Table 18: BTV 4 clinical reaction and percentage protection index, challenged at 4 months p.v.

Animal no	Titre PFU/ml	CRI	Protection %	Average %
21	10^2	1	87.5%	50
107		0	100%	
103		0	100%	
70		7	12.5%	
26	10^3	6	25%	81.25
152		0	100%	
80		0	100%	
31		0	100%	
98	10^4	0	100%	75.25
100		3.5	56%	
33		0	100%	
85		5	37%	
43	Positive control	8	Control	

BTV 8

Those sheep challenged with BTV 8 cell culture material and vaccinated with low titre vaccine (10^2 & 10^3 PFU/ml) had a protection of 100% and 90.75%, respectively. Sheep vaccinated with titre of 10^4 PFU/ml had protection of 100% (Table 19).

Table 19: BTV 8 clinical reaction and percentage protection index, challenged at 4 months p.v.

Animal no	Titre PFU/ml	CRI	Protection %	Average %
114	10^2	0	100%	100
118		0	100%	
64		0	100%	
45		0	100%	
44	10^3	0	100%	90.75
35		0	100%	
76		0	100%	
1		2	63%	
5	10^4	0	100%	100
41		0	100%	
82		0	100%	
127		0	100%	
150	Positive control	5.5	Control	

CRI- clinical reaction index

CHAPTER 4

Discussion

Vaccination against bluetongue may be used for different purposes depending on the epidemiological situation of the affected areas. The purpose of vaccination is to:

- Prevent clinical disease
- To limit the regional extension of BTV infection through preventing the spread of the virus and to reduce virus circulation.

In BT endemic areas like SA the BT MLV is predominantly used to control BT disease in sheep (Erasmus 1980; EFSA 2007) and this vaccine has successfully played a major role in controlling the spread of the virus in Europe (Erasmus 1980; Dungu *et al.,* 2004; Caporale *et al.,* 2005). Given the complex interaction of the different serotypes of BTV, *Culicoides* vectors and animal hosts in the life cycle of infection, virus titres induced by BT MLV should be kept to an absolute minimum especially if transmission of viruses used in the vaccine by vectors is a concern (EFSA 2007).

Currently the BT MLV are used more in Africa than any other continent. In SA, the existence of at least 17 serotypes and the presence of the vector that can transmit the disease to different susceptible hosts make the use of BT MLV economical to sheep farmers. The European Union has decided not to use BT MLV due to a variety of disadvantages which include the presence of a long duration of viraemia when BT MLV with titres of or above 10^4 PFU/ml is used. It is also at this titre when susceptible sheep show clinical signs and experience abortions (Young and Condy 1964; Erasmus 1975). From this it is clear that it is necessary to test the use of reduced titres of e.g.10^2 and 10^3 PFU/ml in the vaccine for use in sheep.

European countries such as Italy, Spain, Portugal and France have used BTV 2 and 4 because of outbreaks associated with these serotypes. As this was a live viral vaccine, the use of these two serotypes has therefore created concern and was selected to be tested in this project. Bluetongue virus serotype 8 was also selected due to the current outbreak in Europe and afforded us an opportunity to confirm that OBP's BT MLV is protective at different titres against the BTV 8 strain.

In this experiment 10^4 PFU/ml as well as two lower titres of 10^2 and10^3 PFU/ml were chosen to be tested as vaccines doses of BT MLV. Both blood and cell culture materials were used as challenge material. From previous experiences it was noticed that the reaction in sheep is either late or poor when blood culture material is used (EFSA 2007). In previous studies it was also noted that cell culture material causes sheep to react earlier than blood culture material (Pini 1976; EFSA 2007), and this was also demonstrated in this study by the CRI of the positive controls (Table 17). The CRI of the positive control challenged with BTV 2 cell culture material was 10 (Table 17) compare to the blood culture material in which the CRI of the positive control challenged with BTV 2 was 5 (Table 8) The reason for this might be because BTV is a cell associated virus and binds mainly to red blood cells (RBC), platelets and mononuclear cells (Maclachlan 2004; Forzan *et al.,* 2007; Schwarzt-Cornil *et al.,* 2008). It is thus possible to assume that the early response caused by the cell culture material might be because of the presence of more unbound BTV circulating in cell culture material which elicit an immediate effect as compared to bounded BTV on RBC's which might be the reason for the delayed effect.

In this study there was no local and systemic reaction p.v. and this confirms the results of Hammoumi *et al.,* (2003) who tested stability, safety and level of viraemia in sheep and found BT MLV serotype 2 did not cause local or systemic reactions p.v. However, transient fever was seen in this study within the first 14 days p.v. which generally lasted only one to two days in both groups A and B. The difference in p.c. temperature reactions was evident between blood and cell culture challenge materials.

Though both reacted within the first 14 days p.c. some vaccinated sheep such as those challenged with BTV 2 cell culture material had higher temperature reactions above 40,5 - 41,5 °C and were consistent for 3-4 days as demonstrated by sheep 126 and 38 (Graph 28 & 29). Those vaccinated with blood culture material e.g. BTV 2, the maximum temperature reactions were 40,1 - 40,2 °C and were mostly inconsistent and shorter as were seen in sheep 54 and 2 (Graph 10).

For BT MLV to be immunogenic, stimulation of a strong antibody response is correlated with the ability of the virus to circulate and replicate in vaccinated hosts and it should therefore be possible to detect viraemia p.v. (EFAS 2007). Though viraemia could not be detected p.v. in both groups A and B, temperature reactions could be demonstrated as shown in Graph 1, 2, 19 and 20 and seroconversion was demonstrated within the first 21 days p.v. as shown in Table 5 (sheep 34, 120 & 2) and also Table 14 (sheep 149, 126 &122). This confirms what was previously found by Hammoumi *et al.,* (2003) and Hunter and Modumo (2000). In both these studies, although temperature reactions did occur p.v. viraemia could not be detected. This also correlates with the statement made by Dungu *et al*., (2004) that temperature reactions might not be associated with viraemia.

Viraemia could also not be detected six weeks p.c. with all the serotypes when blood challenge material was used. However, viraemia was detected within the first 15 days challenged with cell culture material of BTV 2 and 4 on sheep vaccinated with low and high BTV 2 and 4 titre vaccines (Graphs 11 & 12).

Seroconversion was seen using ELISA and SNTs in all BTV vaccine serotypes and at all different titres used within the first 21 days, at 4 and 6 weeks and 4 months p.v (Tables 5, 6, 14 & 15). It was also interesting to note that both viraemia and seroconversion occurred in the same period as shown by sheep 149 and 126 and again by sheep 38 and 125 (Tables 11 & 14).

The occurrence of viraemia and the presence of antibodies can be attributed to the fact that BTV is a cell associated virus as indicated earlier. This close association of the virus with cells results in the presence of viraemia even when high titre of neutralising antibodies are circulating (Maclachlan 2004; Schwartz-Cornil *et al.,* 2008). This close association between RBC's and BTV therefore protect the virus from early immune clearance (Schwartz-Cornil *et al.,* 2008). However, it is worth noting that BTV does not replicate in RBC's but might elicit an inflammatory process resulting in pyrexia without severe clinical symptoms (Maclachlan 2004) as was also demonstrated by sheep 1 (Graph 35). It was also not surprising again to notice that there was a correlation between the febrile reaction or pyrexia and viraemia p.c. as shown by sheep 126 and 149 (Graph 28 and Table 11) which confirms the research results of Pini (1976).

The percentage protection index results six weeks p.v. were inconclusive due to the low CRI of the positive controls. Some of the CRI of sheep vaccinated with a low titre BTV 8 (Tables 8 and 9) and BTV 4 vaccines were even higher than the positive controls. However, the positive control sheep challenged at 4 months p.v had a very high CRI. It has previously been established that BT MLV has demonstrated 90.5% protection level against clinical disease (Howell 1969; Erasmus 1980; Hunter and Modumo 2001; Caporale *et al.,* 2005). In this case, serotype 8 has demonstrated 90% protection index with titres 10^2 and 10^3 PFU/ml (Table 19). Though BTV 2 low titre vaccine (10^2 PFU/ml) gave a protection index lower than 90%, the vaccine was able to protect against clinical disease especially taking note that the positive control died (Table 17). Bluetongue virus 4 vaccine had a low protection index (Table 18) below 90% in all titres. It is important to note that infection with a high titre of virus might occur in the presence of high antibodies due to reasons explained above which can result in mild clinical signs such as transient fever, nasal discharge and depression (Schwartz-Cornil *et al.,* 2008).

This study demonstrated that each BTV serotype reacts differently and their immunogenic potential differs from serotype to serotype (Howell 1969). It also demonstrated that low titre PFU/ml BTV 2, 4 and 8 vaccines do offer protection against clinical disease and it is thus recommended that a BTV serotype 2 and 8 vaccine be released after production with a titre at least 10^3 PFU/ml for BTV 2 and 10^2 PFU/ml for BTV 8. It is further recommended that the immunogenicity of BTV 4 vaccine at a titre of 10^4 PFU/ml should further be investigated or reviewed as the protection index was low at 75% (Table 18). It is thus not recommended to use a low titre BT MLV serotype 4 due to its low percentage protection.

This study clearly confirms that BTV 2 and 8 are more immunogenic compared to BTV 4 as demonstrated by their higher protection index (Tables 17 & 19). We can thus recommend that when releasing a multi serotype BTV vaccine, specific serotype titres should be considered rather than the average titres of all the serotypes in a batch as is currently practiced. One should also consider the fact that during the use of the current polyvalent BTV attenuated vaccine serotypes may interfere with each other after vaccination and during development of immunity resulting in e.g. the less dominant serotype not able to elicit good immunity to protect against a homologous field challenge (Erasmus 1980). Titres of different serotypes should therefore also be tested when polyvalent vaccines are used.

Currently BT MLV is a cost effective product to produce with a production time of 6 - 8 weeks (OBP- Research and Development) compare to the BT inactivated vaccine which has a longer lead time (EFSA 2007). In countries such as SA, the use of a live, low titre BTV vaccine will financially benefit OBP with more quantities and vials that can be produced and will eventually be less costly to the farmer.

More work still needs to be done on the current OBP BTV multi serotype vaccine to investigate the effect of combining different serotypes in eliciting immune response. The following also need to be investigated taking note of the outcome this study:

- Identify other serotypes which need to be studied and determine their minimum protective dose
- Quantify the duration and level of viraemia p.v. and p.c. especially when low titres of virus are used also applying other diagnostic tools like PCR
- Reduce and or re-arrange the BTV serotypes in the different bottles of the current OBP BTV vaccine according to their immunogenic potential and level of cross protection
- Assess the cost benefit to the farmer of using the live attenuated BTV vaccine compare to the risk factors associated with it
- Assess response of indigenous European sheep breeds which may differ from SA breeds

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APPENDIX 1

Bluetongue clinical reaction index

CLINICAL EXAMINATION	DEGREE	SCORING
Nasal mucosa:	Slight or suspicious hyperaemia	0.5
	Clear hyperaemia or erosions	1
	Hyperaemia, moderate petechiae and moderate nasal discharge	2
	Intense hyperaemia, petechiae and severe nasal discharge	3
	Extensive petechiae and erosions	4
Mouth (lips and buccal mucosa)	Slight or suspicious hyperaemia	0.5
	Clear hyperaemia	1
	Outspoken hyperemia, with slight oedema, erosions and salivation	2
	Intense hyperaemia with petechiae and severe oedema	3
	Extensive oedema with cyanosis, petchiae and ulcerations	4
Feet	Slight hyperaemia of coronary band	0.5
	Light hyperemia	1
	Clear hyperemia and petchiae	2
	Intense hyperaemia and petechiae	3
	Intense cyanosis and petechiae	4
Temperature	Above 40 ºC from day 3-14	1