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The isolation and characterisation of a *Babesia bovis* stock from outbreaks on a farm in the Swartberg region of KwaZulu-Natal, South Africa

by

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DECLARATION

Apart from the assistance received, which has been reported as such in the text and in the Acknowledgements, this Dissertation is the original work of the author. The investigations reported in this Dissertation have not been presented for any other degree at any other University.

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ABBREVIATIONS

%	Percentage
A	Adenine
ACD	acid-citrate-dextrose
APS	Ammonium persulphate
bp	base pairs
C	Cytosine
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetracetic acid
et al.	and others
G	Guanine
g	Gravitational force
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulphonic Acid
IFAT	Indirect fluorescent antibody test
kDa	KiloDalton
Kg	Kilogram
L	Litre
M	Molar
MASP	Microaerophilous stationary phase
mg	milligram
$MgCl_2$	Magnesium chloride
ml	millilitre
mm	millimeter
mM	millimolar
N_2	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram
NK	Natural killer cells
NO	Nitrous oxide
O_2	Oxygen
ºC	Degrees Celsius
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate buffered saline
pg	Picogram

pmole	Picomole
PCR	Polymerase chain reaction
PCV	Packed cell volume
PVP	Polyvinylpyrrolidone 40
RLB	Reverse Line Blot
RNA	Ribonucleic acid
rpm	revolutions per minute
rRNA	Ribosomal ribonucleic acid
SPA	Soluble parasite antigen
T	Thymine
TAE	Tris acetate EDTA
TAPSO	3-[N-tris(Hyrdoxymethyl)methylamino]-2-Hydroxypropanesulphonic Acid
TBE	Tris borate EDTA
TE	Tris-EDTA buffer
TEMED	N,N,N',N' tetramethylethylenediamine
TES	N-Tris(Hyrdoxymethyl)methyl-2-Amino-Ethanesulphonic Acid
µg	Microgram
µl	Microlitre
VESA 1	Surface-variable erythrocyte surface antigen

SUMMARY

Babesia bovis outbreaks were reported in cattle alleged to be immunised with the commercially available live-blood vaccine in the Swartberg region of KwaZulu-Natal, South Africa and an investigation into the nature of parasites causing the outbreaks was carried out. The H isolate was obtained from a clinically ill animal on the Haistings farm and characterised using BvVA1 and Bv80 size analysis coupled with Bv80 and 18S rRNA V4 hypervariable region sequence analysis. In total, four South African *B. bovis* isolates were analysed: the vaccine stock (S) at passage 11 and 23 and field isolates H and F. The S23 strain used to infect vaccine donor animals could not be detected in the H isolate and could not be responsible for the severe disease symptoms observed in the field animals. Sequence profiles of the Bv80 and 18S rRNA V4 hypervariable regions for all detectable strains were compiled and now serve as a basis for the investigation of future babesiosis outbreaks. It was determined that the Bv80 PCR is not able to detect animals at the carrier stage of infection and that non-specific primer binding to *Boophilus microplus* and *Boophilus decoloratus* tick DNA occurs. For this reason, the Bv80 PCR is not suitable for investigating the nature of *B. bovis* infections in ticks. The BvVA1 PCR reaction required extensive optimisation and did not detect all strains present in the isolates and was therefore not used as a basis for strain discrimination. Microaerophilous stationary phase cultures of the vaccine strain at passage 24 (S24) and the H strain were initiated as a potential source of soluble parasite antigens. Continuous cultivation was not possible despite the alteration of a number of conditions. Currently there is no culture adapted *B. bovis* strain in South Africa and the availability of such a strain would form the basis of studies on the development of alternative vaccines.

LITERATURE REVIEW

I THE BEEF INDUSTRY

The beef and dairy industry of South Africa is a multi-million Rand industry which forms an important part of the country's agricultural sector as well as ensuring food security. The South African National Department of Agriculture estimates that there are approximately 13.8 million cattle farmed in South Africa, as reported in their annual survey of agricultural produce for 2005/6. The majority of these cattle form part of the beef industry with approximately 2.6 million cattle slaughtered in 2005/6. All sectors of the cattle farming industry are at risk of experiencing severe losses as a result of the different diseases affecting cattle (National Department of Agriculture-South Africa, 2006). Continued research into the prevention and treatment of these diseases is crucial to minimise as far as possible, the losses of livestock due to the diseases transmitted by ticks including heartwater, babesiosis, anaplasmosis and theileriosis. Tick-borne diseases affect approximately 80% of the world's cattle population and it is estimated that the global cost incurred by tick-borne diseases and their control is between US$ 13.9 and 18.7 billion (de Castro, 1997).

II BOVINE BABESIOSIS

Bovine babesiosis, also known as redwater fever, is an economically important disease of cattle both in South Africa and worldwide. Outbreaks can result in substantial losses of livestock, impacting on all sectors of the cattle farming industry including the production of beef, dairy and leather. Babesiosis is caused by intra-erythrocytic protozoan parasites belonging to the genus *Babesia.* Originally named after Babes who discovered the parasite in 1888, the parasite was only associated with disease in 1893 by Smith and Kilborne (Mahoney, 1977). *Babesia* parasites fall under the phylum Apicomplexa, class Sporozoasida, order Eucoccidiorida, suborder

Piroplasmorina and the family Babesiidae (Levine, 1971; Allsopp *et al.,* 1994). In South Africa, economically significant cases of bovine babesiosis are caused by *Babesia bigemina* (causing a disease locally referred to as African redwater) and *Babesia bovis* (causing a disease locally referred to as Asiatic redwater) (de Vos *et al.,* 2004). The only other known *Babesia* species infecting cattle in South Africa are *Babesia occultans* and an unnamed *Babesia* species, both of which cause only mild disease symptoms and are not considered to be economically important (Gray and de Vos, 1981; de Waal *et al*., 1990).

Due to slightly different clinical pathogenesis, infection with *B. bovis* results in higher mortalities than *B. bigemina*. In acute *B. bovis* infections, parasitised erythrocytes collect in the microcapillary beds of the brain and lungs resulting in cerebral babesiosis and respiratory distress syndrome (Mahoney, 1977; de Vos *et al*., 2004). Despite this difference, symptoms of infection with either parasite have similarities and as a result of this, diagnosis is made through the use of blood smear examination. *Babesia bovis* and *B. bigemina* can be distinguished from each other using the angle that forms between the paired merozoite bodies within the erythrocyte. *Babesia bigemina* merozoites form an acute angle while *B. bovis* forms an obtuse angle. The most noticeable symptom of infection with *B. bovis* is the presence of a fever (above 40ºC), which may persist for a few days. Other symptoms include depression, weakness, anemia, a red discoloration of the urine due to heamoglobinuria (hence the name redwater fever), diarrhea, and in cases where cerebral babesiosis occur, tremors, muscle wasting, paddling of the limbs and coma are observed. Cerebral babesiosis is normally short in duration and fatal (Mahoney, 1977).

III GEOGRAPHIC DISTRIBUTION

In Africa, *B. bovis* is transmitted by two species of *Boophilus* namely *Boophilus annulatus* and *Boophilus microplus*. The geographical distributions of *B. bovis* and *B. bigemina* within South Africa are determined by the distribution of the tick vectors responsible for their transmission. Due to the common abbreviation of genus names for *Babesia* and *Boophilus* both being *B.*, for the purpose of clarity; *Boophilus* will be abbreviated as *Bo.* and *Babesia* as *B.* The *Bo. microplus* tick is the only known vector of *B. bovis* in South Africa and is restricted to areas of high rainfall including the Eastern Cape, KwaZulu-Natal and Mpumalanga provinces (Potgieter, 1977; de Vos, 1979). Figure 1 shows the distribution of both *B. bovis* and *B. bigemina* within South Africa by the distribution of the vectors *Bo. microplus* and *Boophilus decoloratus*. From the Figure, it can be seen that *B. bovis* has a more limited distribution than *B. bigemina* as *B. bigemina* is transmitted by both *Bo. microplus* and *Bo. decoloratus* (Potgieter, 1977; de Vos, 1979; de Waal *et al.*, 1998).

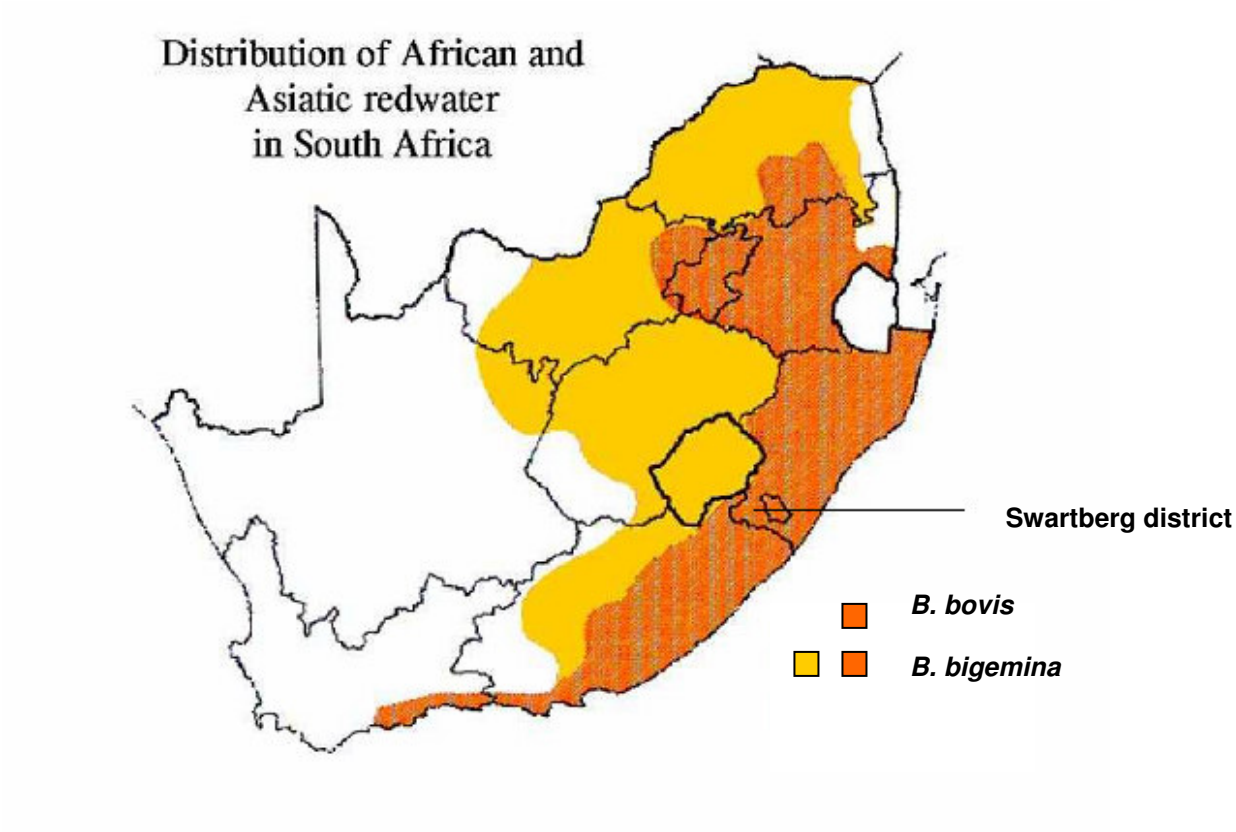

Figure 1: A map of South Africa showing the distribution of *B. bovis* and *B. bigemina* within South Africa by the distribution of *Bo. microplus* and *Bo. decoloratus* (de Waal *et al.*, 1998).

Boophilus microplus is commonly referred to as the Asian blue tick and is difficult to distinguish from *Bo. decoloratus*. *Boophilus microplus* ticks are slightly more red in colour and larger than *Bo. decoloratus*; however the most common method of differentiation employed is mouthpart analysis using a dissection microscope (Walker *et al.*, 2003).

Cattle are the preferred host of *Bo. microplus* but the tick has been reported to be found on other herbivore species (Walker *et al.*, 2003; Norval and Horak, 2004; Nyangiwe and Horak, 2007). *Boophilus microplus* undergoes a one-host life cycle, which implies that it is able to complete one life cycle by attaching to a single host. It attaches to the cattle host at the larval stage, moults twice to form the nymph and

adult stages after which it detaches and in the case of engorged females, lays eggs (Walker *et al*., 2003). In South Africa, it has been noticed with increasing alarm that there is displacement of *Bo. decoloratus* by *Bo. microplus* . This has resulted in a wider distribution of *Bo. microplus* ticks which has been associated with *B. bovis* outbreaks in areas where the disease has no history of occurrence (Tønnesen *et al*., 2004).

IV INFECTION AND IMMUNITY TO *B. BOVIS*

The life cycles of *B. bovis* and *B. bigemina* are similar and are characterised by multiple stages of growth and division in both the bovine host and the tick vector. The parasite is taken up by the adult *Bo. microplus* female while feeding on a bovine infected with *B. bovis*. The parasites enter the basophilic epithelial cells of the tick gut lumen where development of the parasite is initiated. Fusion of gametes occurs in a process called syngamy followed by schizogeny or multiple fission. This results in the formation of large merozoites (also referred to as vermicules or sporokinetes). Further schizogony occurs within different tick cell types including the female reproductive cells (oocytes) (Potgieter, 1977). The vermicules enter the eggs resulting in the transovarial transmission of the parasite to the following generation. In the larval stage, the parasites undergo a final stage of schizogony within the salivary gland cells forming the infectious small merozoites, also referred to as sporozoites. Infection of cattle occurs through the feeding of infected larvae on susceptible cattle (Potgieter and Els, 1976; Potgieter, 1977). Potgieter (1977) determined that the larval stage of *Bo. microplus* tick was solely responsible for the transmission of *B. bovis* with neither the nymph nor adult stages being infective. Parasite proliferation occurs in the bovine erythrocytes only by repeated cycles of fission. The merozoite enters the erythrocyte with the aid of the apical complex (Potgieter and Els, 1977 a; Potgieter and Els, 1979), once inside the erythrocyte, it transforms into a trophozoite, which develops to form two paired merozoites through

merogony. Finally they detach from each other and lyse the erythrocyte to allow for the parasitism of another erythrocyte. This cycle ends and begins again when an infected erythrocyte is taken up as part of the blood meal by a tick (Potgieter and Els, 1977 a; Potgieter and Els, 1979).

Symptoms of infection with *B. bovis* in cattle normally develop 8 to 15 days post infection, depending on the route (by needle or tick feeding) and dose of parasites inoculated (de Vos *et al.*, 2004). Cattle that do not succumb to the infection remain parasite carriers for prolonged periods of time (up to several years). This carrier state is accompanied by long-term immunity against infection but periods of increased parasitemia may occur (Mahoney, 1977). Not all cattle are at equal risk of infection with *B. bovis*, even in babesiosis risk areas. *Bos taurus* breeds of cattle are seen to be more susceptible to infection than *Bos indicus* breeds and animals raised in babesiosis endemic regions acquire natural resistance to infection at a young age (de Vos, 1979; Bock *et al.*, 1999). This is an important factor which can be used to minimise potential losses by farming with *Bos indicus* and *Bos indicus* cross *Bos taurus* cattle breeds with increased resistance to ticks and babesiosis. Calves born to cows previously exposed to *B. bovis* receive maternal antibodies against the parasite in the milk, with protection lasting about six months. This immunity is boosted by natural field exposure to the parasite resulting in long term immunity against disease (Trueman and Blight, 1978).

Immunity to *B. bovis* infection is thought to be similar to that of other babesial parasites and is hypothesised to involve both the innate and adaptive immune response. A strong innate immune response has been proposed to allow for the resolution of acute infections in naïve calves. The response to infection occurs in the spleen via the activation of macrophages by parasite antigens. This activation results in removal of *B. bovis* parasites by phagocytosis and the production of nitrous

oxide (NO). NO is produced by the macrophages themselves and *in vitro* studies have shown that the introduction of NO kills *B. bovis* parasites (Johnson *et al.*, 1996; East *et al.*, 1997; Shoda *et al.*, 2000). The importance of the spleen in controlling *B. bovis* infection is highlighted when an animal is splenectomised. Splenectomy results in a reduction of the animal's ability to control the infection, an increase in parasitemia and severity of clinical disease with a persistent infection (Wright and Goodger, 1988).

It is seen that young animals (below one year of age), show an increased resistance to infection, even if they have not received maternal antibodies against *B. bovis* (Trueman and Blight, 1978; Goff *et al.*, 2001). This age-related natural resistance to infection is due to the earlier expression of interleuken-12 and natural killer (NK) cell mediated interferon-γ response than in adult animals. This expression results in macrophages producing enough NO to kill parasites early enough in the infection to prevent the effects of the disease and the adaptive immune response can then target future infections (Goff *et al.*, 2001; Goff *et al.*, 2003).

Stimulation of the adaptive immune response to infection with *B. bovis* relies on the presentation of parasite antigens to CD 4^+ lymphocytes by antigen presenting cells. The actual control of the infection is most likely due to parasitised erythrocytes being targeted by neutralising antibodies which target extracellular merozoites or the surface-variable erythrocyte surface antigen (VESA1) resulting in the destruction of infected erythrocytes and free merozoites by splenic macrophages (Allred and Al-Khedery, 2004).

V CONTOL METHODS

V.I VECTOR CONTROL

Control of *Bo. microplus* is primarily through the use of acaricides and farming with *Bos indicus*-type cattle breeds more resistant to tick infestation and disease (de Vos, 1979; Bock *et al.*, 1999). Recently, an anti-tick vaccine against *Bo. microplus* has been developed. Two marketed commercial vaccines, TickGARD Plus™ (Intervet) and Gavac™ (Heber Biotech), are available, both based on the Bm86 glycoprotein, present on *Bo. microplus* gut cells. The anti-tick vaccine is effective as the host antibodies taken up in the blood meal bind to and damage the tick gut cells, which then inhibits the successful feeding of the tick. As a result, the number of engorged female ticks in each generation is reduced, the net reproductive capacity is decreased and an overall fall in the tick numbers present in the following generation occurs. The Bm86 antigen is termed a 'concealed' antigen as it would not normally be exposed to the host's immune system and this characteristic has been credited with the success of the vaccine (Rodriguez *et al.*, 1994; Willadsen *et al.*, 1995; Willadsen and Kemp, 1998; de la Fuente *et al.*, 1998).

V.II DISEASE CONTROL

Current vaccination against bovine babesiosis in South Africa (as well as in many other countries) makes use of a frozen live-blood vaccine containing attenuated *B. bovis* parasites. Splenectomised donor animals used for vaccine production are bred and reared under strict tick free conditions at the Onderstepoort Veterinary Institute (OVI). Animals are infected with the vaccine strain parasites by injection directly into the jugular vein. When the parasitemia reaches a high level in the donor animals, blood is collected into an anticoagulant and diluted so that a standard number of parasites can be bottled into the vaccine dose (de Waal *et al.*, 1998). The vaccine strain was originally obtained by tick transmission of *B. bovis* by *Bo. microplus* collected in the Pretoria region of Gauteng, South Africa and has a recorded history

of needle passages through susceptible cattle at OVI for over 30 years (de Vos, 1978; Mason *et al.,* 1986).

Animals of any age can be vaccinated using the *B. bovis* vaccine but it is found that calves (between the ages of three and nine months) show the highest resistance to infection and seldom develop disease symptoms. Though the vaccine strain has been attenuated through rapid passage through splenectomised cattle, some animals may develop disease symptoms and require treatment. If a reaction develops the symptoms will be noticeable between seven and 21 days following vaccination (de Waal *et al.*, 1998).

The vaccination and subsequent treatment method chosen by a farmer depends largely on the number of animals to be vaccinated at any given time. A small number of animals can be easily vaccinated and monitored whilst the vaccination of a larger herd can be more problematic. In cases where large numbers of animals are to be vaccinated, block treatment of the herd can be carried out. If this method is followed, it is suggested that one third of the recommended dose of diminazine aceturate is administered to each animal in the herd on the seventh day after the date of vaccination. Treatment with diminazine aceturate will not inhibit the development of immunity against *B. bovis* and can thus be used safely as a treatment method. This treatment will minimise the development of disease symptoms while still allowing the development of immunity. Treatment of animals with a dose over one third of the recommended dose will prevent the development of immunity resulting in the animals being susceptible to future *B. bovis* challenge. Likewise, treatment with imidocarb should be avoided as it will sterilise the infection (de Waal *et al.*, 1998).

The actual immunity against further infection develops four to six weeks post vaccination and animals being moved from a babesiosis free area to an area where

they are at risk of contracting the disease should be vaccinated at least six weeks prior to the relocation event (de Waal *et al.*, 1998).

Several problems with the live blood vaccine exist and research is focused on developing alternative vaccine options which would be safer and easier to produce. Some of the major problems associated with the live blood vaccine are partly related to the number of viable organisms present in each dose, implying that a standard level of protection is difficult to achieve. Stringent quality control measures are required both during the production and storage stages of vaccine manufacture. Incorrect handling of the vaccine or disruption of the cold chain can result in a loss of parasite viability rendering the vaccine unsuitable for use. The use of cattle donors for the production of the vaccine presents the risk of inadvertently transmitting other pathogens from the donor animals to the vaccine recipient (Schetters and Montenegro-James, 1995; de Vos and Bock, 2000).

VI *IN VITRO* CULTIVATION OF *B. BOVIS*

Initial attempts to adapt *B. bovis* to growth *in vitro* were largely unsuccessful despite numerous attempts using different methods. By 1976, *Plasmodium falciparum* had been successfully cultivated using the candle jar method by Trager and Jensen (1976) but attempts to adapt *B. bovis* to this same method proved unsuccessful (Erp *et al.*, 1978). Limited culture growth of *B. bovis* was attained using the suspension spinner flask method but unfortunately, the growth was not continuous and cultures needed to be constantly replenished with fresh infected erythrocytes. After the extensive manipulation of the spinner flask method, continuous growth of *B. bovis* could be achieved but the percentage of parasitised blood cells was low (Erp *et al.*, 1978, Erp *et al.*, 1980 a, Erp *et al.,* 1980 b). Following these attempts to culture *B. bovis in vitro*, the microaerophilous stationary phase (MASP) culture technique developed by Levy and Ristic (1980) supported continuous growth of *B. bovis*

parasites and yielded large numbers of parasites with the advantage of cultivation being possible in any sample volume with very limited manipulation.

After the development of the MASP culture technique, many efforts have been made to optimise culture conditions. The culture system itself consists of a blood layer containing the parasitised erythrocytes, a layer of medium which forms above the erythrocytes and the external environment defined by the temperature of the culture and the gas system used. Initial reports stated that the use of anticoagulants such as heparin, acid-citrate-dextrose (ACD) and ethylenediaminetetraacetic acid (EDTA) were toxic to the parasites as they altered the bovine erythrocytes, inhibiting the multiplication and colonisation of new cells, thereby inhibiting culture growth. Because of this reported inhibition, blood collected for the initiation and maintenance of cultures was defibrinated by shaking with glass beads, a laborious task compared to the use of anticoagulants (Levy and Ristic, 1980). Despite the claim of inhibition, *in vitro* cultivation of other *Babesia* species using blood collected with the use of the anticoagulants mentioned above has been achieved (Zweygarth, personal communication). This would suggest that while these chemicals may have an effect on the bovine erythrocytes, this negative effect can be minimised with immediate and effective washing, and parasite growth and multiplication can be achieved.

Cultures grow optimally at low parasitemia levels (between 0.5 and 1% of erythrocytes being parasitised) with sub-culturing every three to four days to maintain parasitemia levels below 1% (Levy and Ristic, 1980). The erythrocyte layer settles to the bottom of the culture vessel and darkens as a result of low oxygen tension due to the depletion of oxygen by the metabolic activities of the parasites. Resuspension of the erythrocytes with fresh medium causes the bright red colour to return almost instantaneously. Cultures containing a very low percentage of parasitised cells (below 0.5%) fail to darken and the darkening of the erythrocyte layer can be used as

a rough visual basis to determine the growth of cultures. Cultures failing to darken within three to four days after initiation normally indicate the death of parasites. Failure to grow after subculturing is normally as a result of too low initiation parasitemias or incorrect depth of medium (Levy and Ristic, 1980).

The depth of the culture has been shown to be one of the critical factors affecting *B. bovis* parasite growth. *B. bovis* parasites cannot survive under high oxygen concentrations and the medium layer above the erythrocytes acts as an effective barrier to oxygen exchange. It was found that cultures with a depth of under 0.16 cm did not support growth, with the optimum depth (irrespective of the size and volume of the culture vessel) found to be 0.62 cm (Levy and Ristic, 1980). Levy and Ristic (1980) noted that growth under premixed gas conditions failed (5% CO_2, 10% O_2, 85% N_2 and 5% CO_2, 5% O_2, 90% N_2) whilst Jackson *et al.* (2001) analysed growth of cultures under varying gas conditions and determined that 5% CO_2, 5% O_2 and 90% N_2 supported optimum growth. Irrespective of gas conditions, culture temperatures should be kept between 37 and 38 ºC.

The medium layer is typically made up of roughly 60% tissue culture base and 40% adult bovine serum. The addition of antibiotics (Penicillin and Streptomycin) and antimycotics (Fungizone) should inhibit the growth of any contaminating bacteria or fungi. Buffer systems may vary but buffers such as N-Tris(Hyrdoxymethyl)methyl-2-Amino-Ethanesulphonic Acid (TES), N-2-Hydroxyethylpiperazine-N'-2-Ethanesulphonic Acid (HEPES) and 3-[N-tris(Hyrdoxymethyl)methylamino]-2-Hydroxypropanesulphonic Acid (TAPSO) have been successfully used. It has been shown that irrespective of the buffering system used, parasite growth occurs best under slightly alkaline conditions (pH 7.02 to 7.79) with the optimal pH being between 7.31 and 7.39. *B. bovis* parasites are seen to be intolerant of acidic conditions of pH 6.86 and below (Goff and Yunker, 1988). The natural growth of parasites does seem

to increase the alkalinity of the culture system slightly but it is considered negligible (Levy and Ristic, 1980). When comparing buffer types, each of the above mentioned buffers has been shown to support growth but TAPSO was found to support growth better than TES (Goff and Yunker, 1988). The addition of growth supplements like L-glutamine and hypoxanthine to the culture medium can be carried out, but a publication by Jackson *et al.* (2001) determined that the addition of growth supplements had no effect on the growth of parasites.

The above mentioned conditions mimic, as far as possible, the conditions that *B. bovis* parasites would encounter in the blood capillaries. Despite this, variations in culture conditions to suit each strain may be required. Nonetheless, the MASP culture technique allows for the continuous cultivation of *B. bovis* parasites and has been used as a source of soluble parasite antigens (SPA) (reviewed by Montenegro-James *et al*., 1995). One of the main advantages of this technique is that it allows for the isolation of large amounts of parasite antigen with very limited contamination with bovine products (in the case of serum free cultures). This benefit has resulted in a large number of studies being undertaken focusing on the use of culture-derived antigens as potential vaccine candidates.

VII CULTURE-DERIVED ANTIGENS AS VACCINE CANDIDATES

One approach for the identification of potential vaccine targets would be to physically identify and analyse exoantigens and their effect on the host immune system, an approach which is typically used when characterising culture-derived antigens. SPA are localised to different organelles having a number of potential roles in the parasite's life cycle and could serve as possible vaccine candidates. In 1979, Smith *et al.* used corpuscular and soluble antigen fractions harvested from *in vitro B. bovis* cultures. Both fractions were seen to be able to induce the production of antibodies against *B. bovis* using Indirect Fluorescence Antibody Test analysis (IFAT). Upon

tick challenge, all animals developed reactions but the reaction observed was less severe and the recovery from infection was more rapid in the 'vaccinated' group than the control group (Smith *et al.*, 1979).

In 1981, 100% protection of *Bos taurus* cattle was reported three months after vaccination with a *B. bovis* culture supernatant when challenged using *Bo. microplus* larvae infected with a homologous *B. bovis* strain (Smith *et al.*, 1981). Kuttler *et al.* (1982) described further success using whole culture supernatant as a vaccine. Using homologous needle challenge, protection of the vaccinated cattle was shown. A reaction to the challenge was observed but without any losses being experienced.

Good protection against heterologous challenge is essential for a vaccine candidate as it is heterologous challenge that the animals would experience under field conditions. Limited success against heterologous challenge has been achieved using culture derived immunogens. In 1985, Montenegro-James *et al.* used whole culture supernatant which was fractionated, filtered and used to vaccinate *Bos taurus* cattle, 18 months of age. Full protection against homologous challenge was achieved but only a low degree of protection was achieved against heterologous challenge. Timms *et al.* (1983) showed partial heterologous protection using culture derived immunogens. Most recently, 8000 cattle were vaccinated using a culture-derived *B. bovis* and *B. bigemina* inactivated vaccine in a field trial covering seven states in Venezuela. The investigation spanned five years with a total of 16 clinical trials being undertaken. The results of this trial indicated that this method of vaccination offers a safe and reliable option for the control of bovine babesiosis in Venezuela (Montenegro-James *et al.*, 1992).

In addition to whole culture supernatants, the use of defined culture antigens has also been investigated. Patarroyo *et al.* (1995) used exoantigens (at least four

proteins ranging between 30 and 160 kDa in size) from an attenuated *B. bovis* strain cultured *in vitro* as vaccination material. The proteins were found to afford a high degree of protection against virulent heterologous challenge.

The development of a recombinant vaccine against a virulent *B. bovis* isolate was investigated by Hope *et al.* (2005). Two antigens (designated 12D3 and 11C5) were expressed in *E. coli* and recombinant proteins purified. Animals were vaccinated with recombinant 11C5, 12D3 or a mixture of both proteins. Challenge with the highly virulent W strain caused a severe reaction but animals that received either the single or mixed antigen prior to infection showed a 2.5 to 5 fold reduction in parasitemia with a number of animals observed to be able to control the parasitemia (Hope *et al.*, 2005).

Another protein that has been investigated as a potential vaccine target is the Bv80 protein. The Bv80 protein has also been referred to as the Bb-1 protein and most recently, it has been called the spherical body protein-1 (SBP-1). This protein is highly variable and is not an ideal vaccine candidate due to this variability (Suarez *et al.*, 1991; Goodger *et al.*, 1992; Hines *et al.*, 1995).

A number of culture-derived antigens used to vaccinate animals have afforded protection against both homologous and heterologous challenge (reviewed by Montenegro-James *et al.*, 1995). However, it has recently been suggested that a combined genomic and proteomic approach would enable the identification of subdominant antigens as vaccine candidates. Subdominant antigens are those that illicit little or no immune response during natural infection. The rationale behind the selection of subdominant antigens is that the expression of highly immunogenic antigens would naturally be a target of host immune defense and are likely to be highly variable within populations of the target organism (Brown *et al.*, 2006).

Widespread acaracide resistance has resulted in dipping as a method of disease control becoming increasingly unsuitable. Anti-tick vaccines targeting the *Bo. microplus* gut antigen Bm86 (TickGARD and Gavac) have been developed and are commercially available (Rand *et al.*, 1989; Rodriguez *et al.*, 1994; Odongo *et al.*, 2007). Vaccination with Bm 86 reduced the number of required acaracide treatments by 60% and reduced the transmission of babesiosis, resulting in a saving of $23.4 per animal per annum where investigated (Rand *et al.*, 1989; Rodriguez *et al.*, 1994)

Coupling anti-tick vaccines with culture-derived vaccines may be a viable *B. bovis* vaccination alternative. An assortment of antigens that provide protection against a wide range of heterologous challenges would serve as a viable alternative to the live blood vaccine currently used. The vaccine would need to be stable under storage conditions, easy to produce and administer, produce no deleterious side effects, contain no contaminants and provide long term immunity to disease (Montenegro-James *et al.*, 1995; Schetters, 1995).

VIII OBJECTIVES OF THE STUDY

The acquisition of a virulent field isolate which maintains its virulence irrespective of prolonged cultivation could serve as the basis for the development of an alternative *B. bovis* vaccine. Virulent strains are often associated with cases of babesiosis outbreaks and the isolation of such a strain would allow for the analysis of SPA as possible vaccine targets. Babesiosis outbreaks have been reported on farms in the Swartberg district of KwaZulu-Natal, South Africa (Figure 1) resulting in the deaths of several animals (Combrink, personal communication). Cattle on these farms were allegedly vaccinated against both Asiatic and African redwater using the commercial vaccine but when these animals were moved to other farms in the surrounding area (Haistings and Killrush), outbreaks occurred.

The cause of babesiosis outbreaks is often difficult to determine and in this case, as the animals were vaccinated prior to the outbreaks, the efficacy of the blood vaccine was questioned. A number of possibilities may have resulted in the outbreaks and the isolation of parasites from infected animals would allow the nature of the outbreak to be investigated. If the vaccine strain had regained virulence thereby causing the disease symptoms, the vaccine strain parasites should be present at high numbers. If the outbreaks were as a result of a virulent field isolate which was able to break through the protection afforded by the vaccine strain, it should be possible to isolate the field strain from infected animals.

Identification of the different *B. bovis* genotypes circulating in the field animals would be the first step in determining the nature of the outbreaks and if confirmed to be as a result of field isolate breakthrough, the isolate could be initiated into *in vitro* cultures. These cultures could then serve as a platform for later studies into the development of a safer, more reliable *B. bovis* vaccine.

IX SPECIFIC STUDY OBJECTIVES

The specific objectives of this study were as follows:

I. To obtain *B. bovis* isolates from outbreaks reported in cattle from the study farms in the Swartberg region of KwaZulu-Natal.
   a. To assess under controlled conditions, the virulence of the field isolate in susceptible cattle.
   b. To attempt *B. bovis* transmission using *Boophilus* ticks collected from the study farms.

II. Molecular characterisation of the *B. bovis* isolate collected from the field as well as the vaccine strains.

III. To initiate *in vitro* cultures of a virulent field isolate for future immunisation studies.

CHAPTER ONE: THE ISOLATION OF A *B. BOVIS* STOCK FROM THE SWARTBERG REGION OF KWAZULU-NATAL, SOUTH AFRICA

1.1 INTRODUCTION

The Haistings and Killrush farms are situated in the Swartberg region of KwaZulu-Natal, South Africa. Losses of cattle were experienced when animals were moved from the surrounding farms to the Haistings farm, this despite alleged vaccination against redwater (Combrink, personal communication). Sick animals were reported to show symptoms suggestive of infection with *B. bovis* (red urine, fevers, rough coats and nervous symptoms) and the presence of *B. bovis* parasites in the blood confirmed these suspicions.

The Haistings and Killrush farms are located within the *Bo. microplus* distribution range but the severe winters experienced in the region were believed to have affected the development of the ticks. A river valley passing through the farms was suspected to be acting as an environmentally suitable pocket for the development of *Bo. microplus* ticks. Winter temperatures drop below freezing in the Swartberg region and this valley may experience less severe temperatures, more amenable to the development of *Bo. microplus* ticks. Figure 1.1 shows the river valley passing through the Haistings farm, where cattle were allowed to graze.

Figure 1.1: River valley on the Haistings farm, which may serve as a pocket for developing *Bo. microplus* ticks.

A study was carried out to investigate the *B. bovis* parasite populations circulating in the sick animals in the Swartberg region speculating on a number of possibilities. Vaccine failure in the animals resulting in no cross-protection against field strains may have resulted in the severe reactions observed. Alternatively, the vaccine strain

itself may have regained virulence resulting in outbreaks of disease or a highly virulent field strain may be breaking through the vaccine barrier thereby causing the outbreaks. Collection and analysis of the *B. bovis* populations circulating within animals on the Haistings farm could aid in establishing the nature of the outbreaks. If the outbreaks were as a result of a virulent field strain breaking through, the parasites could be isolated and adapted to grow in MASP cultures. Soluble parasite antigens could then be isolated from these cultured parasites for the development of a recombinant vaccine. For the purpose of this study, virulence will be defined as the infectiousness of an organism and its ability to cause disease in the host animal (http://www.medterms.com/script/main/art.asp?articlekey=6911). An isolate will be defined as a sample of *B. bovis* parasites collected from the field and a strain as a stock of *B. bovis* parasites that have been characterised biologically and molecularly. In literature, a stock is defined as "all the populations of a parasite derived from an isolate without any implication of homogeneity or characterisation. Populations comprising a single stock thus include cell lines and tick stabilates and subsequent parasite preparations derived from them". An isolate is then defined as "viable organisms, isolated on a single occasion from a field sample, in experimental hosts or culture systems, or prepared as a stabilate" and a strain as "a population of homogeneous organisms possessing a set of defined characteristics" (Dolan, 1988).

The specific objectives of this chapter were to attempt to isolate *B. bovis* from clinical cases in the field and to assess the virulence of the isolate in susceptible cattle.

1.2 MATERIALS AND METHODS

1.2.1 *BABESIA BOVIS* ISOLATE FROM HAISTINGS FARM

Blood was collected from field cattle showing disease symptoms such as fever, lethargy and fluffy coats. The field isolate obtained from the Haistings farm was obtained from a blood sample collected from an animal on the farm exhibiting classical symptoms of *B. bovis* infection (persistent temperature elevated above 39ºC, lethargy and fluffy coats and the presence of parasites in the blood smears). Thin blood smears were made from blood collected by piercing the tip of the animal's tail, fixed in methanol and stained with Giemsa stain for 30 minutes.

The infected animal was bled from the vein under the tail into vaccutainer tubes containing either EDTA or heparin as an anticoagulant. Blood samples were kept refrigerated at 4ºC until arrival at OVI where they were used as challenge material.

1.2.2 EXPERIMENTAL INFECTION OF SUSCEPTIBLE BOVINE WITH HAISTINGS *BABESIA BOVIS* ISOLATE

A Hereford splenectomised adult bovine 9469/1 was kept free from tick-borne diseases and other pathogens. A total of 10 ml of blood obtained from the clinically sick bovine (number 80-Haistings farm) was used to infect animal 9469/1 by injection directly into the jugular vein.

Daily rectal temperatures, thin blood smears and the percentage packed cell volume (PCV) of the blood were taken and recorded daily. The PCV was prepared by centrifugation of a micro-capillary tube filled with blood at 630 g for 3 minutes in a IEC MB Centrifuge-Micro Hematocrit (Damon/IEC Division) and the PCV reading determined using a Micro-Capillary Reader (Damon/IEC Division). Percentage parasitemia was obtained by counting the number of parasitised cells per field of 500

erythrocytes and the average of ten fields calculated. The PCV, temperature and parasitemia readings were taken by trained animal technicians at OVI. Treatment with diminazene aceturate (3.5 mg/kg) and blood transfusions (4 L from uninfected blood donor animals) was given when required. All animals involved in this study were fed commercial pellets, autoclave sterilised hay and had free access to water.

Infected blood stabilates (H strain) were made from blood collected from animal 9469/1 at peak parasitemia following the method described by de Vos *et al.* (1982).

1.3 RESULTS

Blood collected from clinically ill bovine 80 on the Haistings farm was used to infect animal 9469/1. The PCV, daily temperatures and percentage parasitemia levels are summarised in Table 1.1. The animal exhibited symptoms associated with the infection of cattle with *B. bovis* including a drop in the PCV to 8% by day 16, a fever for five consecutive days (from day six to ten and for another two days on days twelve and thirteen). This was accompanied with clinical symptoms including lethargy and stumbling.

Table 1.1: Daily temperature, PCV and parasitemia readings for animal 9469/1, experimentally infected with the H isolate.

Day Post Infection	Temperature (ºC)	PCV (%)	Parasitemia (%)
1	39.1	28	Negative
2	not taken	29	Negative
3	39	30	Negative
4	38.6	29	VR*
5	39.2	28	0.01
6	39.7	27	0.0002
7	40.3	26	0.0012
8	40.2	24	0.6
9	40.6	25	3
10	40.8	22	7.2
11	39.4	18	4
12	39.6	14	1.5
13	40.3	12	2
14	39.4	15	1.5
15	38.8	10	4
16	38.2	8	8

* VR: 1 parasite observed in thick smear examination

Animal 9469/1 required treatment with diminazene aceturate by day thirteen when the parasitemia level reached 2% and the PCV fell to 12%. After treatment, the animal showed a slight increase in PCV to 15% and a decrease in the fever (40.3 to 39.4ºC). After an initial decrease in parasitemia there was a consistent rise to 8% by day 16. The animal also received supportive treatment of a blood transfusion (4L on

day 13), which increased the PCV to 15% but the following day the PCV dropped down to 10% and further to 8% at which point the animal succumbed to the infection. These results are represented graphically in Figure 1.2 showing the relationships between temperature, PCV and parasitemia changes.

The H strain (stabilate derived from animal 9469/1) was used to infect four intact susceptible *Bos indicus* cattle as part of another study. It produced only mild reactions in all animals infected. The reaction index for the unvaccinated group challenged with the H strain was calculated to be 15.6 +/- 7.0 while the reaction index for animal 9469/1 was calculated to be 158.02 (Combrink, personal communication 2008). Reaction indices were calculated according to the method set out by Combrink and Troskie (2004).

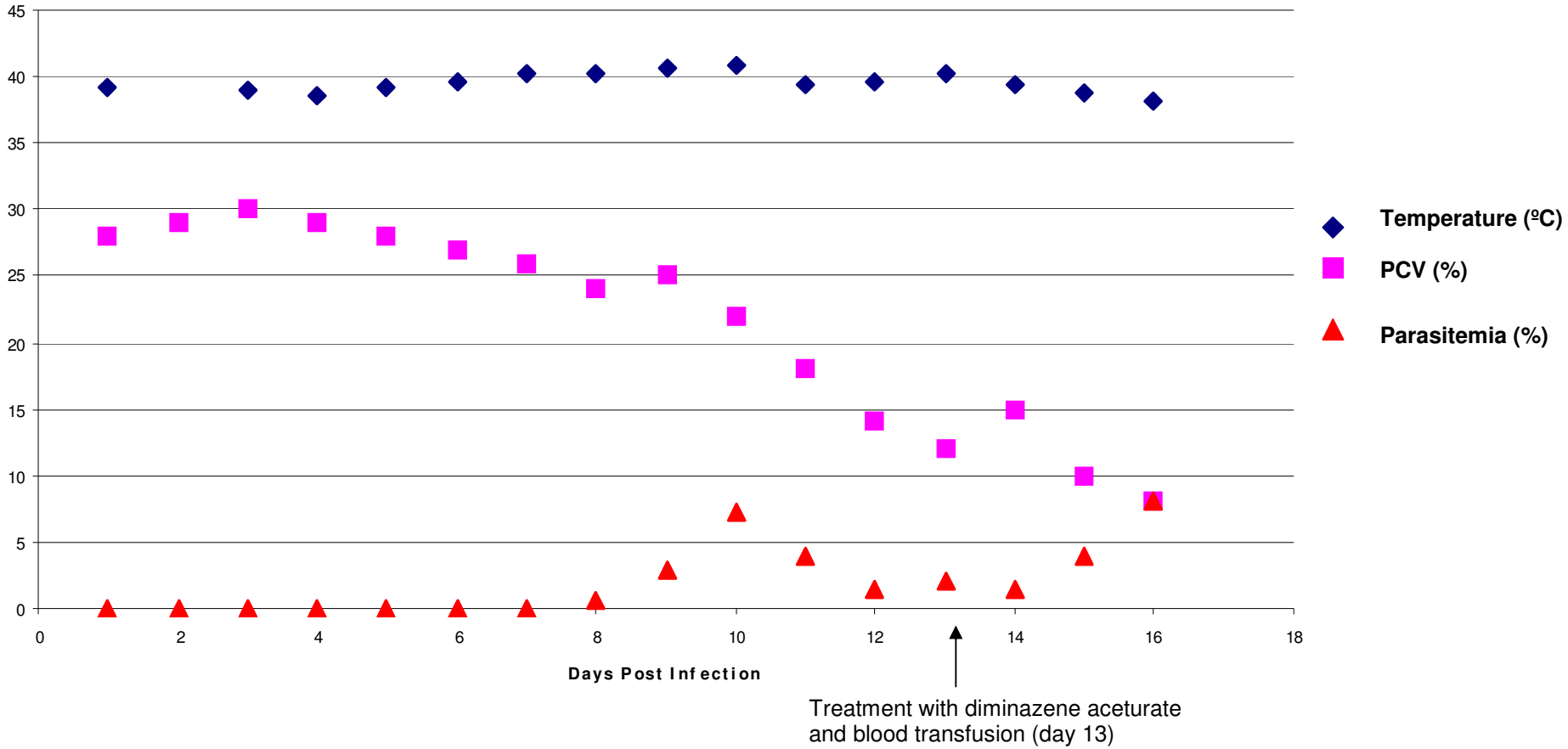

Figure 1.2: Daily temperature (ºC), PCV (%) and parasitemia (% parasitised erythrocytes) for animal 9469/1, experimentally infected with the H isolate.

1.4 DISCUSSION AND CONCLUSIONS

Determining the virulence of the H strain was not clear due to the difference in reactions observed in intact and splenectomised animals. In intact susceptible animals, the H strain seems to be less virulent, causing mild or no disease symptoms. In a splenectomised animal or an animal that had other factors compromising its immune response the H strain may cause more severe disease symptoms as observed in animal 9469/1 and the Haistings field animals. There may also have been a change in the parasite population during the passage through splenectomised animal 9469/1 resulting in the selection of non-virulent parasites.

Given its low virulence in intact cattle, it is most likely that the H strain could not be entirely related to the outbreaks being experienced in the Swartberg region. On the other hand, other external compounding factors may have impacted on the field animals' overall well being and the H strain parasites may indeed have caused the outbreaks.

Infection and disease state is dependant on a number of factors both intrinsic as well as extrinsic. Recent studies, focused on the relationship between the vector, host and parasite, have suggested that the method of infection can play a role in the establishment of a disease state as well as the severity of disease. Likewise, the overall state of health of the animal may influence the severity of disease experienced by the animal. This applies to the infection of intact bovines which, under controlled conditions received excellent nutrition and had free access to water during the course of the infection and only exhibited mild disease symptoms.

Tick modulation of the host immune system is now considered as an important factor in establishing a state of infection in the host animal. The relationship between the tick and pathogen is complex as many pathogens undergo one or more

developmental stages within the vector and in the case of *B. bovis*, a number of important developmental stages occur within the tick (Potgieter and Els, 1976; Potgieter, 1977).

The immune modulating components that the tick has developed are primarily responsible for securing the blood meal. Ixodid ticks feed over a period of days and avoiding host detection while securing the blood meal is of paramount importance. Tick saliva is secreted into the host vascular system and contains components able to secure the blood meal as well as a number of molecules which enable the tick to evade the host immune response (Wikel, 1999). Reducing the host ability to respond to the agents present in tick saliva is essential to the feeding of the tick both for the immediate meal as well as to avoid long term host immunity to ticks (Wikel, 1996). Tick mediated immunosuppression involves a number of complex activities. Agents present in the saliva inhibit the binding of various complement components, thereby inhibiting the cascade reaction essential in recognising foreign material to be taken up by phagocytosis. The blocking of complement components inhibits the inflammatory response which has a number of roles. The inflammatory response is responsible for attracting leukocytes to the site of injury, it increases blood flow to the area, increases the permeability of capillaries to plasma molecules as well as the release of inflammatory mediators from the mast cells. There is also an enzyme-mediated inhibition of the itch response thereby avoiding manual removal of the tick by the host grooming itself. Impairment of the function of antigen presenting cells, required for initiating of immune response in animals, allows the tick to further evade detection by the host. The function of natural killer (NK) cells is also inhibited, antibodies are cleaved by tick enzymes and there is an overall reduction of the antibody response, decreasing the chance of detection and destruction by host phagocytic cells. T lymphocyte function as well as cytokine response are essential for the mediation of the adaptive immune response and are involved with the cellular

immune response to intracellular pathogens and the activity of both are down regulated by the tick. (Wikel and Bergman, 1997; Male *et al*., 2006)

This down regulation of the host immune response by the tick strongly favours pathogen establishment within the host. It stands to reason then that an animal with a higher tick burden would have an immune system less able to respond to infection than one with a lower burden. The association between *Amblyomma variegatum* and dermatophilosis in cattle is a clear demonstration of the 'down regulation' of the host's immune response by tick saliva and its effect on the establishment of disease (Lloyd and Walker, 1993; Walker and Lloyd, 1993). Whilst vectors rarely suppress the immune system of the host to the extent that the animal dies, the cumulative effect of a number of ticks feeding on a single animal cannot be ignored (Wikel, 1999). Studying infection by needle inoculation only neglects the potential effects of the vector on the transmission of pathogens, the establishment of disease and the extent to which the disease develops. Under laboratory controlled conditions, the study of a disease using vector transmission is more difficult to carry out than by needle challenge but is nonetheless important.

The effect of the tick vector coupled with other external pressures on the field animals, such as food quality and quantity, water availability and herd dynamics, can influence an animal's overall well-being, making it more or less susceptible to infection with *B. bovis* and determining the extent to which the disease develops. Another factor that may have affected the difference in the observed parasite virulence could be related to the stage at which the parasite is in its lifecycle. Cattle in the field would have been infected through natural tick transmission by sporozoites. The parasite at this stage of development would probably have activated metabolic pathways and be expressing antigens important for the establishment of infection resulting in the formation of a disease state within the

animal. In the present study, the trial animals were artificially injected with *B. bovis* by using erythrocytes infected with merozoites derived from an infected animal. These merozoites would probably express antigens and activate different metabolic pathways, responsible for the maintenance of the state of infection, which may result in the less severe reactions observed. This difference in the parasite life stage at the point of infection and method of infection (intrinsic) coupled with external pressures on the animal (extrinsic) may explain why cattle in the Swartberg region showed severe disease symptoms while intact laboratory trial animals showed only mild reactions. It is therefore concluded that the H strain is of low virulence under controlled conditions.

CHAPTER TWO: *BABESIA* TICK TRANSMISSION

2.1 INTRODUCTION

The effects of immune suppressing factors present within tick saliva have been well documented, relating both to the effect on the host immune system as well as the effect on disease transmission (Wikel, 1996; Wikel, 1999). The immune modulating components that the tick has developed are primarily responsible for securing the blood meal as Ixodid ticks feed over a period of days. Tick mediated immunosuppression involves the inhibition of the binding of various complement components, inhibiting the inflammatory response. There is also an enzyme-mediated inhibition of the itch response thereby avoiding the manual removal of the tick by host grooming. The function of natural killer (NK) cells is also inhibited, antibodies are cleaved by tick enzymes and there is an overall reduction of the antibody response, decreasing the chance of detection and destruction by host phagocytic cells. T lymphocyte function as well as cytokine response are essential for the mediation of the adaptive immune response and are involved with cellular immune response to intracellular pathogens and both are down regulated by the tick (Wikel and Bergman, 1997; Male *et al.*, 2006).

The low virulence shown by the H strain in intact susceptible animals under controlled conditions may be due to the method of infection used, namely needle challenge. The objective of this study was to attempt to transmit through tick feeding, a more virulent strain of *B. bovis* in intact cattle. Molecular detection techniques were used to investigate parasite infections in both ticks and cattle. Two PCR methods were used, a conventional PCR and a nested PCR. The *B. bovis* nested PCR primers target the Bv60 gene which encodes a 60 kDa merozoites protein (Suarez *et al.*, 1991) and the *B. bigemina* nested PCR primers were designed from plasmids containing *B. bigemina* genomic DNA inserts (Figueroa *et al.*, 1992). The nested

PCR is able to detect carriers of *B. bovis* and *B. bigemina* and would thus be suitable to detect low levels of parasite DNA present in the tick samples. The Bv80 PCR targets the *B. bovis* variable Bv80 gene and has been described as a method of identifying *B. bovis* strains (Lew *et al*., 1997 a). To identify any other parasite DNA donors samples were submitted to the Department of Veterinary Tropical Diseases, University of Pretoria for Reverse Line Blot (RLB) analysis (Gubbels *et al*., 1999; Isogen Life Science). In this assay, an 18S rRNA V4 hypervariable region amplicon is exposed to multiple probes specific for different parasite species. The specific binding of probes to the amplicon determines the parasite populations within a sample. Whilst able to detect DNA from several species simultaneously, the RLB is not able to distinguish between different strains of the same species within a sample.

2.2 MATERIALS AND METHODS

2.2.1 Tick collection

Engorged female ticks were collected from cattle on the Haistings and Killrush farms focusing on the belly, dewlap, shoulders and flanks, the possible sites of attachment for *Boophilus* ticks. Female ticks were placed individually into standard 20 ml pill vials with holes punched in the lid using an eighteen gauge needle. Gauze was placed between the vial and lid to allow for ventilation whilst ensuring no larvae could escape. Ticks were placed in the acaridarium with constant temperature (25°C) and relative humidity (90%) and allowed to oviposit. After completing oviposition, ticks were identified and screened for the presence of parasites in the haemolymph.

2.2.2 Tick identification

Ticks were preserved in 70% ethanol and identified by experienced personnel (H. Heyne, OVI) using mouthpart analysis. As shown by Figure 1 in the literature review, *Bo. microplus* and *Bo. decoloratus* co-exist in many areas and it was necessary to correctly identify the female ticks using mouthpart analysis. *Boophilus microplus* has 4 rows of teeth on either side of its hypostome whereas *Bo. decoloratus* has 3 rows on either side (Walker *et al.* 2003).

2.2.3 Parasite detection in the tick

Female ticks collected were screened for the presence of parasites in the haemolymph and in the eggs. For each female tick, two to three hind legs were severed from the body and gentle pressure applied causing the clear hemolymph to be extruded. The hemolymph was smeared onto a clean glass microscopic slide and spread. 15-20 eggs were aligned in rows next to the haemolymph smear of the corresponding tick and examined under a dissection microscope to ensure that the eggs were not desiccated. The eggs were crushed with a clean slide to form a

smear. Slides were fixed in methanol and stained with 10% Giemsa stain for 30 minutes and examined using a compound microscope under 1000 X oil immersion.

2.2.3.1 DNA isolation from ticks

Parasite DNA was isolated from ticks using an adapted protocol from the Puregene DNA Purification System (Gentra Systems). Ticks eggs (15 ± 1) and larvae (10 ± 1) were collected and frozen overnight at –20 °C. Newly moulted nymphs were collected (12 days after application) from the animals and kept at 37°C to harden for two days after which they were frozen overnight at -20°C. Tick stage samples were then crushed using the blunt end of a sterile toothpick. Homogenised samples were suspended in 300 µl of Cell Lysis Buffer and inverted 20 times to mix. 10 µl of Proteinase K (20 mg/ml) (Qiagen) was added, the sample mixed by inversion 25 times and incubated at 55°C overnight. The sample was cooled to room temperature before the addition of 1.5 µl of RNase A solution and incubated at 37°C for 1 hour. Samples were then cooled on ice for 1 minute and 100 µl of protein precipitation solution added and the sample vortexed for 20 seconds. Centrifugation at 16 000 g for three minutes pelleted the insoluble fraction and the supernatant was added to 300 µl of isopropanol to precipitate any DNA present in the sample. The reaction was mixed by inverting 50 times and the DNA pelleted by centrifugation at 16 000 g for 5 minutes. The pellet was washed in 300 µl of 70% ethanol and centrifuged at 16 000 g for one minute. The resulting pellet was air dried at room temperature and resuspended in 50 µl of DNA hydration buffer. Incubation at 65°C for one hour and overnight at room temperature fully resuspended the DNA pellet.

2.2.3.2 Parasite detection in ticks using molecular techniques

Original K 4192 tick batches showing the presence of parasite bodies in the haemolymph and egg smears were used for further analysis. Samples were

submitted to the University of Pretoria (Department of Veterinary Tropical Diseases) to identify any parasite DNA present using Reverse Line Blot analysis (RLB, Isogen Life Science). 18S rRNA V4 hypervariable region PCR using the *Theileria* and *Babesia* specific PCR primers (section 3.2.4) was used to confirm that parasite DNA isolation had been successful.

All tick DNA samples were subjected to Bv80 PCR analysis (described further in chapter 3) to detect the presence of *B. bovis* DNA. A multiplex, nested PCR, described by Figueroa *et al.* (1993) was used to detect both *B. bigemina* and *B. bovis* DNA. Primers for this PCR can either be used together in a multiplex PCR or separately to identify the presence of specific parasite DNA. Table 2.1 shows the primer sequences for the multiplex nested PCR reaction and Table 2.2 shows the PCR cycling parameters. 20 pmoles of each primer was added to the reaction along with 2.5 µl template DNA (isolated tick samples) for the external (primary) reaction and 2 µl of the external product for the internal (secondary or nested) reaction. Bv80 PCR reactions were set up and carried out as in section 3.2.3.

Table 2.1: Primer sequences for the external and internal nested PCR reaction developed by Figueroa *et al.* (1993) for the detection of *B. bigemina* and *B. bovis* infections in tick samples.

Primer name	Species	Reaction	Sequence 5'-3'
BilA	*B. bigemina*	External	CATCTAATTTCTCTCCATACCCCTCC
BilB		External	CCTCGGCTTCAACTCTGATGCCAAAG
BilAN		Internal	CGCAAGCCCAGCACGCCCCGGTGC
BilBN		Internal	CCGACCTGGATAGGCTGTGTGATG
BoF	*B. bovis*	External	CACGAGGAAGGAACTACCGATGTTGA
BoR		External	CCAAGGAGCTTCAACGTACGAGGTCA
BoFN		Internal	TCAACAAGGTACTCTATATGGCTACC
BoRN		Internal	CTACCGAGCAGAACCTTCTTCACCAT

Table 2.2: Cycle parameters for the PCR detection of *B. bigemina* and *B. bovis* DNA in tick samples, adapted from Figueroa *et al.* (1993).

External Reaction			Internal Reaction		
Temp (°C)	Time (min)	Number of cycles	Temp (°C)	Time (min)	Number of cycles
95	5	1	95	5	1
95	0.5	30	95	0.5	35
60	0.5		60	0.5	
72	0.75		72	0.75	
72	7	1	72	7	1
10	Hold		10	Hold	

Bv80 PCR products were resolved on either 1.5% agarose gels (using TAE buffer with electrophoresis at 100V for 1-2 hours) stained with ethidium bromide (10 µg/ml) or on 15% polyacrylamide gels stained using Silver Stain Plus Kit (Bio-Rad). *Babesia bigemina* and *B. bovis* nested PCR products were resolved on a 2% agarose gel (using TAE buffer with electrophoresis at 100V for 1 hour) stained with ethidium bromide (10 µg/ml).

2.2.4 Tick transmission experiments

2.2.4.1 Tick feeding on cattle

Two 13 months old Nguni cattle (0674 and 0643) as well as two adult Friesian cattle (1173 and 1197) were dipped with Zeropar acaricide (Bayer) eight weeks prior to entering the tick feeding facility. Animals 0674 and 0643 tested negative on the IFAT for the presence of antibodies against *B. bigemina* and *B. bovis* while animals 1173 and 1197 both tested negative for the presence of antibodies against *B. bovis* and positive for antibodies against *B. bigemina.* Experimental cattle were kept in concrete tick free stables, surrounded by moats filled with detergent. The back area of each animal was shaved using sheering clippers and tick feeding bags placed over the shaved region. Bags were made of canvas rectangles measuring approximately 50 X 70 cm and attached using contact adhesive (GenKem). The adhesive was allowed to dry for a day before the release of ticks into the tick feeding area. *Boophilus decoloratus* ticks collected from the Killrush farm (K 4192) were used for the tick feeding experiment. Adult female ticks collected from the farm were allowed to oviposit and tested for the presence of parasites (sections 2.2.1 and 2.2.3). The larvae of egg batches infected with parasites were allowed to feed on the four bovines. Batches 1 and 5 were fed on animal 1173, batch 3 on animal 1197, batches 9 and 11 on animal 0674 and batch 7 on animal 0643 (summarised in table 2.1). A linear incision was made in the bag using scissors and the pill vial containing larvae (200 individuals) placed in the bag, the incision stitched closed with cotton and once closed, the lid of the vial removed. Larvae were allowed to attach and feed until fully engorged females detached. Five ticks at the nymph stage of development were removed from the back of each animal and the remainder of the ticks were allowed to develop. Fully engorged females were collected from the bags and any loose ticks from the back of the animal. Females were placed individually into pill vials and

allowed to oviposit as in section 2.2.1 and tested for the presence of parasites as in section 2.2.3. The cattle were dipped after the completion of tick feeding.

2.2.4.2 Monitoring of cattle

All cattle used in the experiments described in this section were monitored for the presence of parasites in the blood and daily rectal temperatures were taken and recorded (as described in section 1.2.1).

2.2.4.3 Tick feeding on goats

The purpose of this experiment was to attempt to obtain tick eggs free from *Babesia* species infecting cattle for use as controls. Three batches of *Bo. microplus* J 4199 (Generation 2, batches 1, 3 and 4) larvae originally obtained from the University of the Free State and *Bo. decoloratus* K 4192 (Generation 3, batches 10, 11 and 14) larvae (50 individuals in each batch) were fed in tick feeding bags on two adult boer goats separately, one batch on each ear and one at the base of the neck. Larvae were allowed to attach and feed until fully engorged females detached. Engorged females and any loose ticks were collected from the bags. Females were placed individually into pill vials and allowed to oviposit as in section 2.2.1 and tested for the presence of parasites as in section 2.2.3. The goats were dipped after the completion of tick feeding.

2.2.4.4 Pick up of *B. bovis* by *Bo. microplus* and *Bo. decoloratus* under controlled conditions

Two batches of *Bo. decoloratus* K 4192 larvae (Generation 2, batches 29 and 30) and *Bo. microplus* J 4199 larvae (Generation 1, batches 3 and 5) shown to contain no *B. bovis* DNA using the Bv80 PCR test (section 2.2.6) were fed on two Nguni cattle (13 months old). After feeding with *B. decoloratus* ticks from the Kilrush farm (2.2.4.1), bovine number 0674 became a carrier of *B. bigemina*, while bovine 0657

remained free from infection with either *B. bovis* or *B. bigemina.* Animal 0674 received one batch of J 4199 *Bo. microplus* larvae and after two days, a second batch of larvae was added. The same procedure was carried out on animal 0657 using batches from the K 4192 *Bo. decoloratus* strain. Ten days following the attachment of the second larval batch, animal 0674 was administered a standard dose of the commercial *B. bovis* vaccine (1 ml containing 2 $x10^7$ parasites before freezing, administered intramuscularly). Eleven days following the attachment of the second K 4192 *Bo. decoloratus* larval batch, animal 0657 was infected with a stabilate of the H *B. bovis* strain, generation 1 (5 $x10^7$ parasites before freezing, administered intramuscularly). The peak reaction was calculated to be between ten and seventeen days post infection and engorged females detaching over this period were collected and used for further analysis. Individual females were placed in vials for oviposition and subsequent hatching.

Eggs were collected from eight J 4199 *Bo. microplus* females and eight K 4192 *Bo. decoloratus* females, the DNA isolated (described in section 2.2.5) and the levels of infection with both *B. bovis* and *B. bigemina* determined using the nested PCR approach (see section 2.2.6).

Table 2.3 shows a summary of all the tick feeding experiments undertaken in this study highlighting the tick strain, origin, generation, the animal it was fed on and the section where it is further described.

Table 2.3: Summary of tick feeding experiments conducted using *Bo. decoloratus* K 4192 and *Bo. microplus* J 4199 tick on cattle and goats.

Tick strain	Species	Origin	Generation	Larvae batch	Fed on animal	Corresponding nymph batch	Corresponding egg batch oviposited by adult females collected	Section
K 4192	*Bo. decoloratus*	Killrush farm	1	9 and 11	Bovine 0674	N0674	Sample 29, Fig 2.18	2.2.4.1
				7	Bovine 0643	N0643	Samples15 and 27, Fig 2.18	
				3	Bovine 1197	N1197	Samples 10 and 17, Fig 2.18	
				1 and 5	Bovine 1173	N1173	Sample 30, Fig 2.18	
K 4192	*Bo. decoloratus*	Killrush farm	2	29 and 30	Bovine 0657	n/a*	Egg batch F1-F8	2.2.4.4
J 4199	*Bo. microplus*	University of Free State	1	3 and 5	Bovine 0674	n/a	Egg batch E1-E8	
K 4192	*Bo. decoloratus*	Killrush farm	2	1, 3, 4	Goat 8024	n/a	no adults collected	2.2.4.3
J 4199	*Bo. microplus*	University of Free State	3	10, 11, 14	Goat 7098	n/a	Egg batch G1-G3	

* not applicable

2.2.5 DNA sequence analysis from tick eggs and larvae

Bv80 PCR products obtained from tick egg batch 55 and larval batch 11 (both from *Bo. decoloratus* field strain K 4192) were cloned into the pGEM-T Easy vector system (as described in section 3.2.7). Sequences were analysed using pregap 4 and gap4 from the Staden software package (Bonfield *et al*., 1995 a; Bonfield *et al*., 1995 b) and aligned using Clustal X version 1.81 (Thompson *et al*., 1997).

2.3 RESULTS AND DISCUSSION

2.3.1 Tick identification

A total of 300 engorged females were collected from the Killrush and Haistings farms, all of which were identified microscopically as *Bo. decoloratus* which is not known to be a vector of *B. bovis*. *Boophilus microplus* ticks must have been present on the farms as suggested by the presence of *B. bovis* parasites in the Swartberg cattle. It is suspected that seasonal displacement of *Bo. decoloratus* by *Bo. microplus* may be occurring in the region but has not been confirmed. Of the ticks collected, only thirteen showed the presence of vermicules in the haemolymph or egg smears. Morphologically, the majority of vermicules were identified as *B. bigemina* while some could not be identified to species. Despite the ticks all being identified as *Bo. decoloratus* and the parasites as *B. bigemina* the investigation was continued to eliminate the possibility of mixed infections. Figure 2.1 shows an image of the vermicules present in the tick haemolymph.

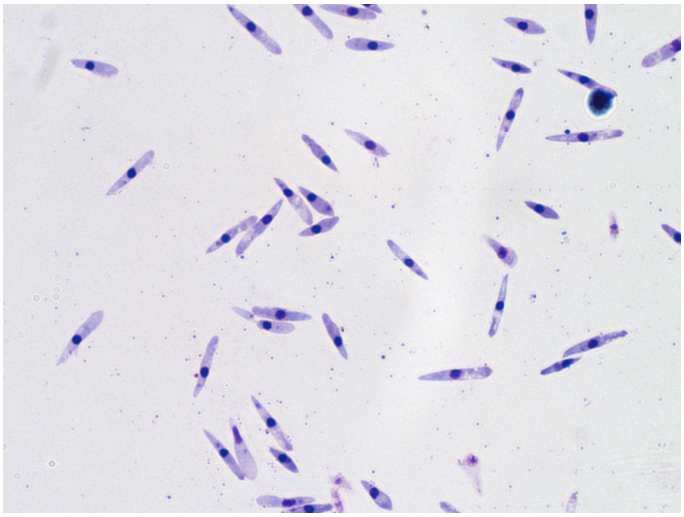

Figure 2.1: Giemsa stained vermicules present in *Bo. decoloratus* haemolymph viewed using 1000 X oil immersion.

2.3.2 Parasite detection in ticks using molecular techniques

Parasite DNA was successfully isolated from the thirteen samples positive for the presence of vermicules using microscope analysis, confirmed by the amplification of the 18S rRNA V4 hypervariable region. All samples were tested for the presence of *B. bigemina* DNA using the nested PCR reaction using the *B. bigemina* primers only and subjected to reverse line blot analysis.

2.3.2.1 RLB results

To identify any other parasite DNA donors, the thirteen egg DNA samples collected from the Killrush females were submitted to the Department of Veterinary Tropical Diseases, University of Pretoria for Reverse Line Blot (RLB) analysis (Gubbels *et al.*, 1999; Isogen Life Science). Table 2.4 shows the RLB results for each of the thirteen samples as well as the two *B. bigemina* controls tested. Tick sample numbers were given new egg and larvae batch numbers one to thirteen for convenience.

Table 2.4: RLB analysis of thirteen tick egg DNA samples indicating parasite DNA populations present within each sample.

Tick sample no.	Egg Batch no.	Sample Origin	RLB result
260	1	Killrush Farm	-
257	2	Killrush Farm	***B. bovis***, *B. bigemina*
241	3	Killrush Farm	*B. bigemina*
264	4	Killrush Farm	***B. bovis***, *B. bigemina, B. rossi*
222	5	Killrush Farm	*T/B*
266	6	Killrush Farm	***B. bovis***
259	7	Killrush Farm	T/B, ***B. bovis***
251	8	Killrush Farm	T/B**, *B. bovis***
254	9	Killrush Farm	-
262	10	Killrush Farm	-
249	11	Killrush Farm	T/B, *B. bigemina*
229	12	Killrush Farm	-
156	13	Haistings farm	-
B. bigemina G	na	Australian Vaccine strain	T/B
B. bigemina P	na	South African Vaccine strain	T/B

T/B = *Theileria-Babesia* genus specific probe (*Theileria-Babesia* catch all)

- = no detection of parasite DNA

na: not applicable

The RLB test detected the presence of *B. bovis* DNA within five of the thirteen egg samples submitted (egg batch numbers 2,4,6-8).

2.3.2.2 Attempts to confirm the presence of *B. bovis* DNA using the Bv80 PCR

2.3.2.2.1 Bv80 results for egg stage

To confirm RLB test results, which suggested the presence of *B. bovis* DNA within the tick egg samples, the Bv80 PCR test was carried out on all thirteen samples. The RLB results seemed to be confirmed by the Bv80 analysis, with five of the thirteen samples showing amplification using the Bv80 PCR. Figure 2.2 shows the resolution of the Bv80 PCR reactions for the thirteen egg samples on an agarose gel. Of interest is the size of the PCR product, in all the tick samples tested it is

substantially larger (around 1900 bp) than the blood derived parasite DNA amplification products (around 700 bp). As this gene is a variable gene, one would expect some degree of difference in the size of the gene between strains but the difference between amplification from tick samples and blood stabilates is over two fold. The large variation in the size of the Bv80 PCR products between the tick and blood samples may indicate that the PCR products are a result of non-specific amplification rather than the amplification of *B. bovis* DNA within *Bo. decoloratus* ticks.

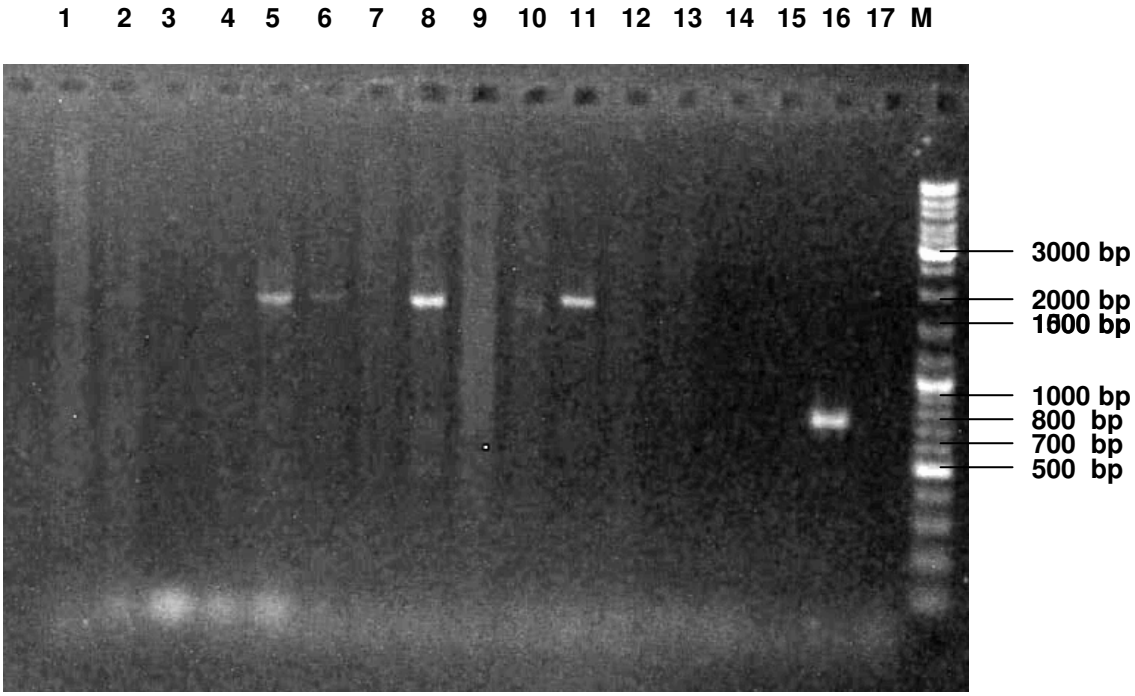

Figure 2.2: Bv80 PCR amplification products obtained using 12 K 4192 and 1 H 4195 *Bo. decoloratus* tick egg DNA as the template. 10 µl of PCR product was loaded in each well of a 1.5 % agarose gel. Lane 1-12: K 4192 egg samples 1-12, lane 13: H 4195 egg sample 13, lane 14-15: *B. bigemina* strains P and G, lane 16: *B. bovis* positive control S23, lane 17: negative control and lane M: 5µl O' GeneRuler DNA Ladder Mix (Fermentas).

2.3.2.2.2 Bv80 results for the larval stage

To determine if the same PCR results could be obtained in the larval stage, the Bv80 PCR reaction was carried out using larval DNA corresponding to the egg batches used as template DNA in the previous experiment. Figure 2.3 shows the Bv80 PCR detection for the larval stage of development. DNA amplification can no longer be detected in sample numbers 5 and 6 whilst in samples 8, 10 and 11 amplification products can still be detected. Samples 1-4, 7 and 12-13 show PCR products in the larval stage but not in the egg stage of development.

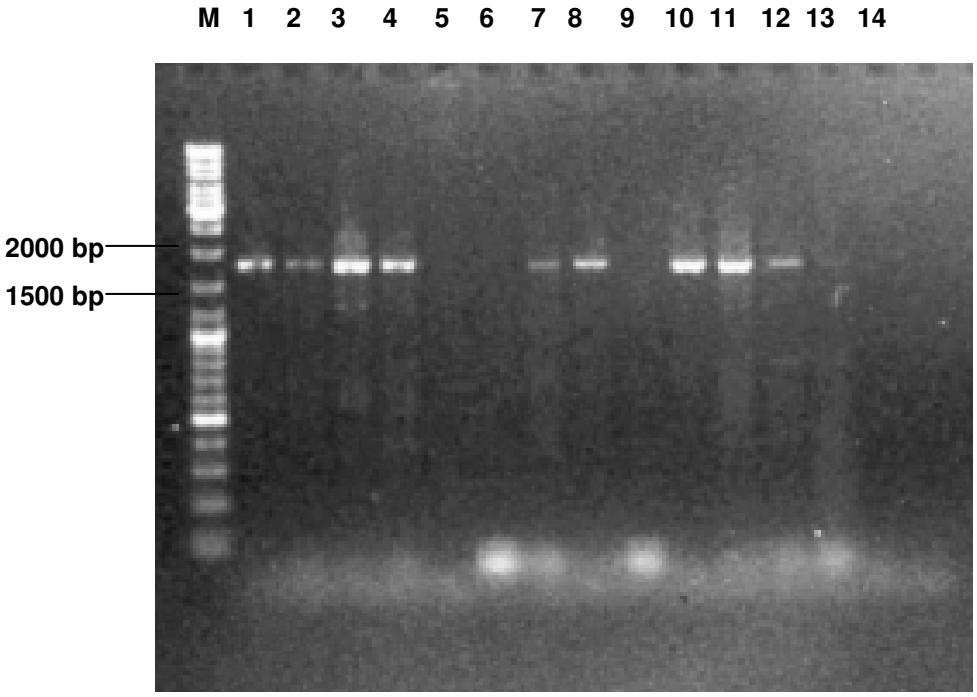

Figure 2.3: Bv80 PCR amplification products obtained using twelve K 4192 and one H 4195 *Bo. decoloratus* tick larvae DNA as template. 10 µl of the reaction was loaded in each well of a 1% agarose gel. Lane M: 5µl O' GeneRuler DNA Ladder Mix (Fermentas), Lane 1-12: K 4192 larval samples 1-12, Lane 13: H 4195 larval sample 13, lane 14: Negative control.

Table 2.5 shows a summary of the Bv80 PCR amplification reactions for each of the tick samples at the egg and larval stages of development. In total, five samples

tested positive in the egg stage whilst ten tested positive in the larval stage. Of interest are samples 5 and 6 where amplification occurred in the egg stage but not the larval stage.

Table 2.5: Summary of the detection of *B. bovis* DNA in the egg and larval stage of tick development for samples one to thirteen.

		Bv80 PCR	
Batch number	**Sample number**	**Egg stage**	**Larval stage**
K 4192	1	Negative	Positive
K 4192	2	Negative	Positive
K 4192	3	Negative	Positive
K 4192	4	Negative	Positive
K 4192	5	Positive	Negative
K 4192	6	Positive	Negative
K 4192	7	Negative	Positive
K 4192	8	Positive	Positive
K 4192	9	Negative	Negative
K 4192	10	Positive	Positive
K 4192	11	Positive	Positive
K 4192	12	Negative	Positive
H 4195	13	Negative	Positive

2.3.2.2.3 Bv80 results for the nymph stage

The *Bo. decoloratus* K 4192 larvae were allowed to develop till the nymph stage (section 2.3.3.2) at which point, five flat nymphs were collected for each batch, the DNA extracted and analysed for the presence of *B. bovis* and *B. bigemina* parasite DNA. The Bv80 PCR again resulted in the detection of non-specific PCR products in the nymph stage, shown below in Figure 2.4.

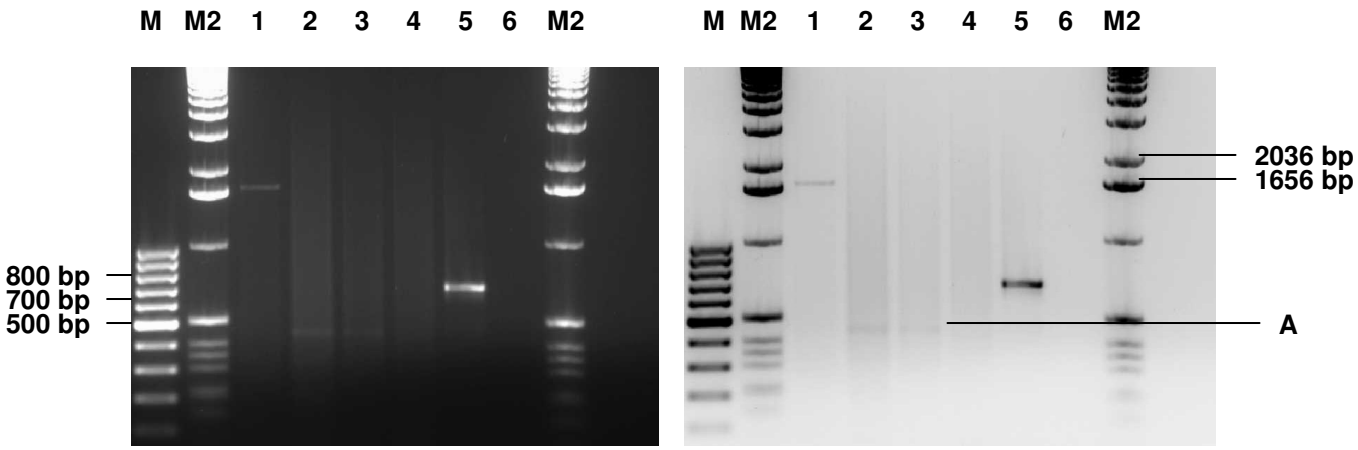

Figure 2.4: Bv80 PCR amplification results using template DNA isolated from nymph samples collected from animals 0674, 0643, 1173 and 1197. 10 µl of PCR product was loaded in each well of a 1.5% agarose gel. Lane M: 5 µl O' GeneRuler 100bp DNA ladder (Fermentas), lane M2 and 9: 5 µl Marker X (Roche), lane 1: nymph batch N0674, lane 2: nymph batch N0643, Lane 3: nymph batch N1173, lane 4: nymph batch N1197, lane 5: 5 µl positive *B. bovis* control S23, Lane 6: Negative control.

Nymph batch N 0674 (larval batches 9 and 11) fed on animal 0674 shows a faint band of 1700 bp (lane 1, Figure 2.4). Nymph batch N0643 (larval batch 7) fed on animal 0643 shows a faint band around 450 bp (which is clearer in the inverted image on the right) (lane 2, Figure 2.4). Nymph batch N1173 (larval batches 1 and 5) fed on animal 1173 shows an even fainter 450 bp band, labeled A (lane 3, Figure 2.4). The only nymph batch to not show the presence of an amplification product was N1197 (larval batch 3) fed on animal 1197 (lane 4, Figure 2.4).

2.3.2.3 Attempts to confirm the presence of *B. bovis* DNA using the BvVA1 PCR

BvVA1 PCR amplification (described in section 3.2.3) was carried out to determine if the Bv80 PCR amplification was as a result of non-specific primer binding to tick DNA. PCR amplification using several batches of *Bo. decoloratus* K 4192 tick DNA was unsuccessful. The BvVA1 PCR is a sensitive test requiring a very high DNA

template quality and concentration (see section 3.3.4). The isolation of DNA using the PureGene isolation kit makes use of an ethanol precipitation step, so the template DNA quality was most probably suitable but, as there was a limited sample volume (only 50 µl in total) the concentration of DNA by a further ethanol precipitation was not possible. As a result the template DNA was probably not concentrated enough to allow successful BvVA1 amplification even if *B. bovis* DNA had been present. Therefore, this test could not be used to confirm the hypothesis that non-specific primer binding to tick DNA was the cause of the Bv80 amplification seen.

2.3.2.4 Attempts to confirm the presence of *B. bovis* DNA using nested PCR

The nested PCR reaction developed by Figueroa *et al.* (1993) was used to determine if *B. bovis* DNA was being amplified in the *Bo. decoloratus* ticks by the RLB and Bv80 PCR reactions. Amplification of *B. bovis* DNA from the egg and larval stages of the *Bo. decoloratus* tick samples K 4192 1-12 and H 4195 13 are shown in Figure 2.5 and 2.6 respectively.

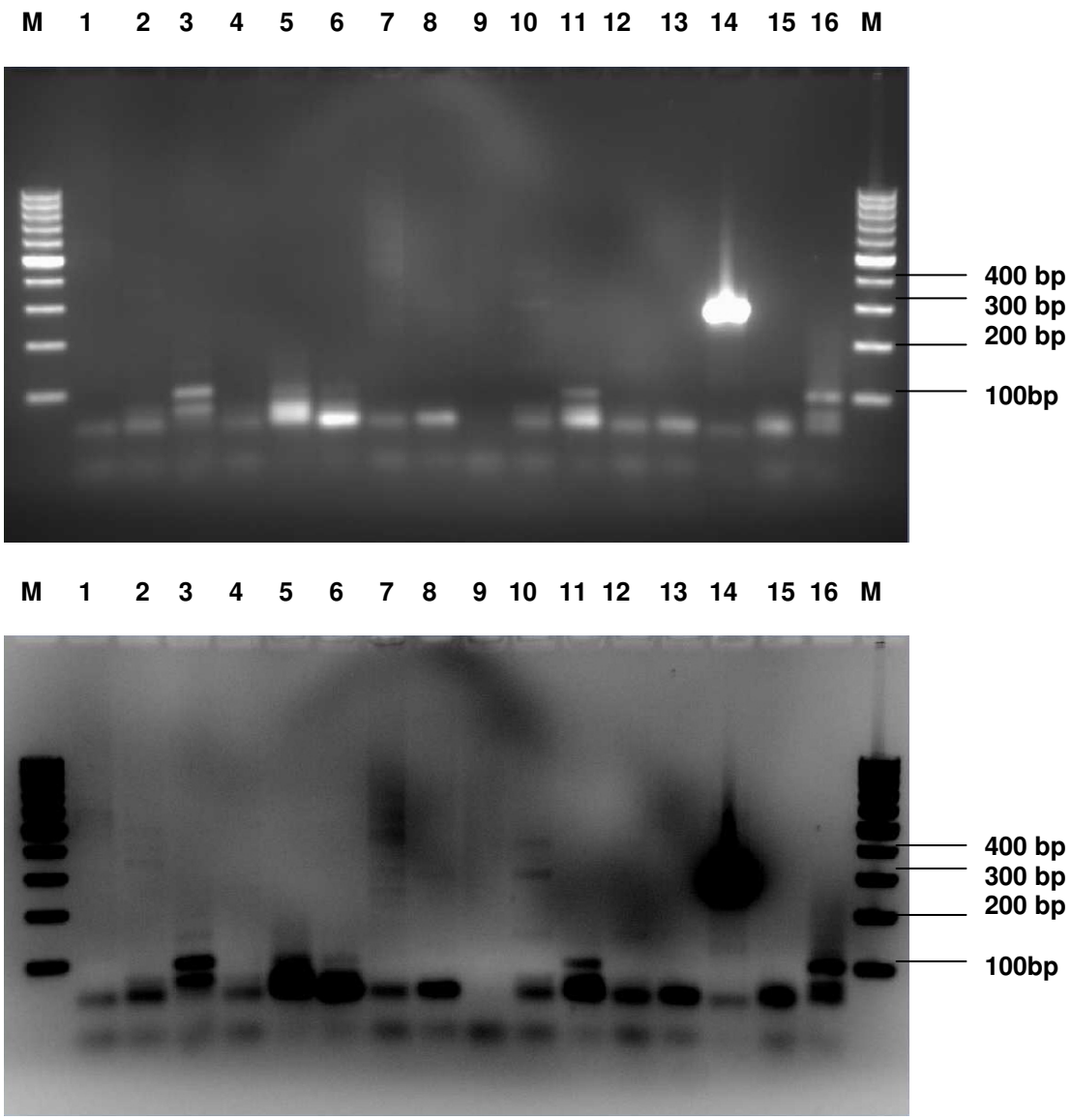

Figure 2.5: PCR amplification of the *B. bovis* Bv60 gene using twelve K 4192 and one H 4195 *Bo. decoloratus* tick egg DNA as template. 10 µl of PCR product was loaded in each well of a 1.5 % agarose gel. Lane 1-12: K 4192 egg samples 1-12, Lane 13: H 4195 egg sample 13, Lane 14: *B. bovis* positive control S23, Lane 15: Negative control, Lane 16: *B. bigemina* strain P and lane M: 5µl O' GeneRuler DNA Ladder Mix (Fermentas).

Faint bands can be observed in lanes 2, 7 and 10, which may represent non-specific primer binding. Figure 2.6 shows the *B. bovis* nested PCR results for the larval stages for tick samples K 4192 1-12 and H 4195 13, confirming that there was no *B.*

bovis DNA present in the *Bo. decoloratus* ticks and that the Bv80 PCR amplification was therefore probably as a result of non-specific primer binding to tick DNA.

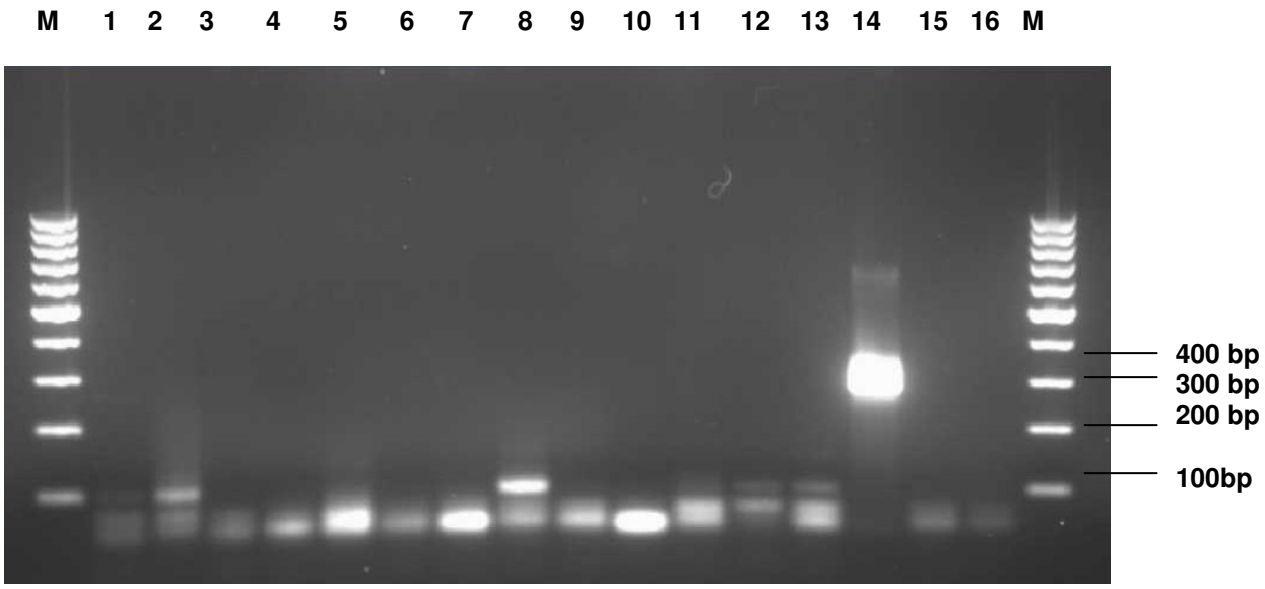

Figure 2.6: PCR amplification of the *B. bovis* Bv60 gene using twelve K 4192 and one H 4195 *Bo. decoloratus* tick larvae DNA as template. 10 µl of PCR product was loaded in each well of a 1.5 % agarose gel. Lane 1-12: K 4192 larval samples 1-12, Lane 13: H 4195 larval sample 13, Lane 14: *B. bovis* positive control S23, Lane 15: Negative control, Lane 16: *B. bigemina* strain P and lane M: 5µl O' GeneRuler DNA Ladder Mix (Fermentas).

2.3.2.5 PCR test specificities

The specificity of the Bv80 and *B. bigemina* nested PCR reactions were tested against two other *Babesia* species known to infect cattle in South Africa, namely *Babesia occultans* and *Babesia* sp. (Gray and de Vos, 1981; de Waal *et al.*, 1990). The specificity testing for the Bv60 primers set was not conducted together with the Bv80 and *B. bigemina* nested PCR tests as the PCRs were performed at a later date. The primers however were found to be specific for *B. bovis* (data not shown).

DNA was isolated from blood stabilates (*B. occultans* stabilate 5/12/06 and *Babesia* sp. stabilate 5/12/06) using the QIAamp DNA Blood Mini Kit (see chapter 3.2.2). The 18S rRNA V4 hypervariable region was amplified to confirm that DNA isolation from the stabilates was successful.

Figure 2.7 shows the 18S rRNA V4 hypervariable region PCR analysis, confirming the successful isolation of DNA from each of the blood stabilates. As expected, the PCR product band intensity for the concentrated *B. occultans* and *Babesia* sp. (lanes 1 and 3) is higher than for the un-concentrated samples (lanes 2 and 4).

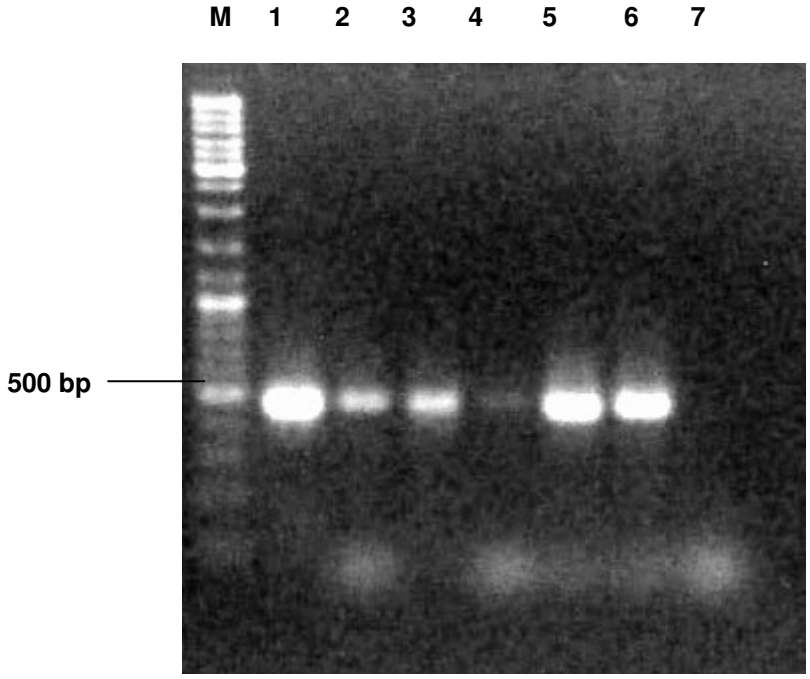

Figure 2.7: 18S rRNA V4 hypervariable PCR amplification, confirming successful DNA isolation from blood stabilates. 5 µl of PCR product was loaded in each well and resolved on a 1.5% agarose gel. Lane M: 5 µl O' GeneRuler DNA Ladder Mix (Fermentas), lane 1: *B. occultans* (concentrated DNA template), lane 2: *B. occultans*, lane 3: *Babesia* sp. (concentrated DNA template), lane 4: *Babesia* sp., lane 5: *B. bovis* S23, lane 6: *B. bigemina* P, lane 7: Negative control.

The Bv80 PCR reaction did not amplify DNA from the three *B. bigemina* strains P (de Vos *et al*., 1982), K (Combrink *et al*., article in press) shown in Figure 2.8 and G (Dalgliesh *et al*., 1981) shown in Figure 3.1, *B. occultans* or *Babesia* sp. Likewise, the *B. bigemina* nested PCR only detected *B. bigemina* DNA and not DNA from the other *Babesia* species. This result confirms that the DNA being amplified in the *Bo. decoloratus* Bv80 PCR is not cross-reaction with DNA from *B. bigemina, B. occultans* or *Babesia* sp. Figure 2.8 shows the *B. bovis* specificity test results with only *B. bovis* DNA being amplified by the Bv80 PCR reaction. Figure 2.9 illustrates the specificity of the *B. bigemina* nested PCR reaction with only *B. bigemina* DNA being amplified.

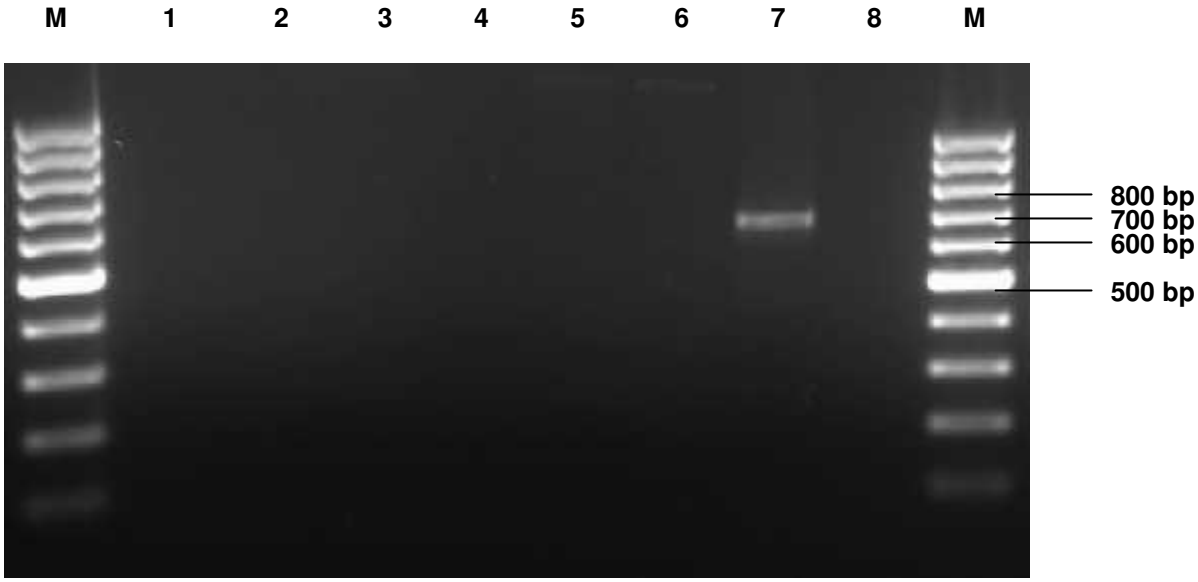

Figure 2.8: Bv80 PCR specificity testing with 5 µl of PCR product loaded in each well of a 1.5% agarose gel. Lanes M: O' GeneRuler 100 bp DNA ladder (Fermentas), lane 1: *B. occultans* (concentrated DNA template), lane 2: *B. occultans*, lane 3: *Babesia* sp. (concentrated DNA template), lane 4: *Babesia* sp., lane 5-6: *B. bigemina* strains P and K, lane 7: *B. bovis* positive control S23, lane 8: Negative control.

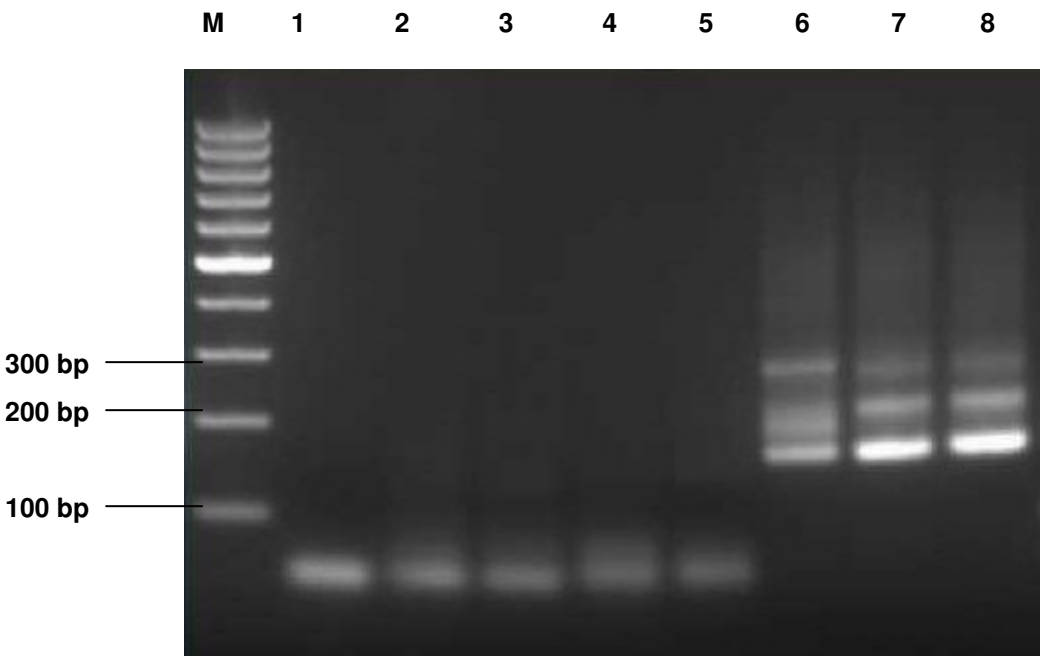

Figure 2.9: *Babesia bigemina* nested PCR specificity testing with 5 µl of PCR product loaded in each well of a 1.5% agarose gel. Lanes M: O' GeneRuler 100 bp DNA ladder (Fermentas), lane 1: *B. occultans* (concentrated DNA template), lane 2: *B. occultans*, lane 3: *Babesia* sp. (concentrated DNA template), lane 4: *Babesia* sp., lane 5: *B. bovis* S23, lane 6-8: *B. bigemina* strain P, K and G positive controls.

2.3.2.6 Feeding of ticks on goats to produce uninfected *Boophilus* ticks for use as controls

Uninfected ticks were required as controls to ensure that the amplicons observed with the Bv80 PCR were not non-specific PCR products caused by non-specific priming on tick DNA. Uninfected *Bo. microplus* and *Bo. decoloratus* tick lines are no longer available at OVI and it was decided to begin such lines for use in future experiments. Attempts to obtain uninfected tick strains were unsuccessful as all ticks obtained were infected with either *B. bigemina* or *B. bovis*. Experiments on the life cycle and transmission of *B. bovis* by *Bo. microplus* by Potgieter (1977) established that *B. bovis* parasites are cleared from the tick in the larval stage as no transmission by the nymph or adult stages was possible. It was speculated that the feeding of ticks on a non-bovine host would clear ticks from infection with *B. bovis*.

It has been found that goats can be infested with *Bo. microplus* ticks under field conditions and due to the presence of a large number of adult females (above 5 mm in length), it was suggested that *Bo. microplus* were successfully completing their life cycle on goats (Nyangiwe and Horak, 2007). Neither *B. bovis* nor *B. bigemina* are infective to goats, and thus goats would be considered as refractory hosts to infection; however, it is important to note that vertical transmission (the transmission of *B. bigemina* parasites to the eggs without re-infection of the female through the blood meal) may occur in the case of high *B. bigemina* infections (Gray and Potgieter, 1981). As a result, even completing a feeding cycle on goats may not produce ticks free from infection with *B. bigemina* parasites.

Three *Bo. microplus* J 4199 and *Bo. decoloratus* K 4192 larval batches were fed separately on two adult boer goats, one batch on each ear and the third on the base of the neck. There was no attachment of either *Bo. microplus* or *Bo. decoloratus* ticks on the back of the goats and only limited attachment of *Bo. microplus* occurred on the ears. A total of four engorged females were collected out of the 150 J 4199 *Bo. microplus* larvae applied to goat 7098. The ticks collected were smaller in size than had been previously collected from cattle and only three of the four females laid eggs. Two of the egg batches hatched to produce approximately 50 larvae per batch while the third batch failed to hatch.

No *Bo. decoloratus* K 4192 larvae were able to feed to engorgement on the ears of the second goat, 8024. Tick probing lesions were observed on the ears as pink spots.

The results of the artificial tick feeding indicated that goats were not a preferred host for *Boophilus* species. The partially fed *Boophilus* ticks collected by Nyangiwe and Horak (2007) on their survey may not represent ticks able to feed to full engorgement

or that only a small percentage of those attached, completed their life cycle. Ticks did not attach on the back and generally, attachment and feeding on the ears was very poor on both goats.

DNA was isolated from the eggs of three *Bo. microplus* J 4199 females collected on goat 7098. *B. bovis* and *B. bigemina* nested PCR reactions were carried out on each of the isolated egg DNA samples using the multiplex approach. Figure 2.10 shows the nested PCR results for the three tick control DNA samples. No amplification occurred using the *B. bovis* and *B. bigemina* nested primers for two of the three tick samples (lanes 1 and 3, Figure 2.10). There was amplification of *B. bigemina* DNA in the third egg DNA sample (lane 2, Figure 2.10) suggestive of the vertical transmission mentioned earlier. Smeenk *et al.* (2000) previously determined that the nested PCR primers did not react with DNA isolated from *Anaplasma* spp., *Theileria* spp., nor *Boophilus* spp. un-infected with *B. bigemina* or *B. bovis*. These results confirm that the nested PCR primers do not react with *Bo. microplus* tick DNA uninfected with *B. bigemina* or *B. bovis* used in this study.

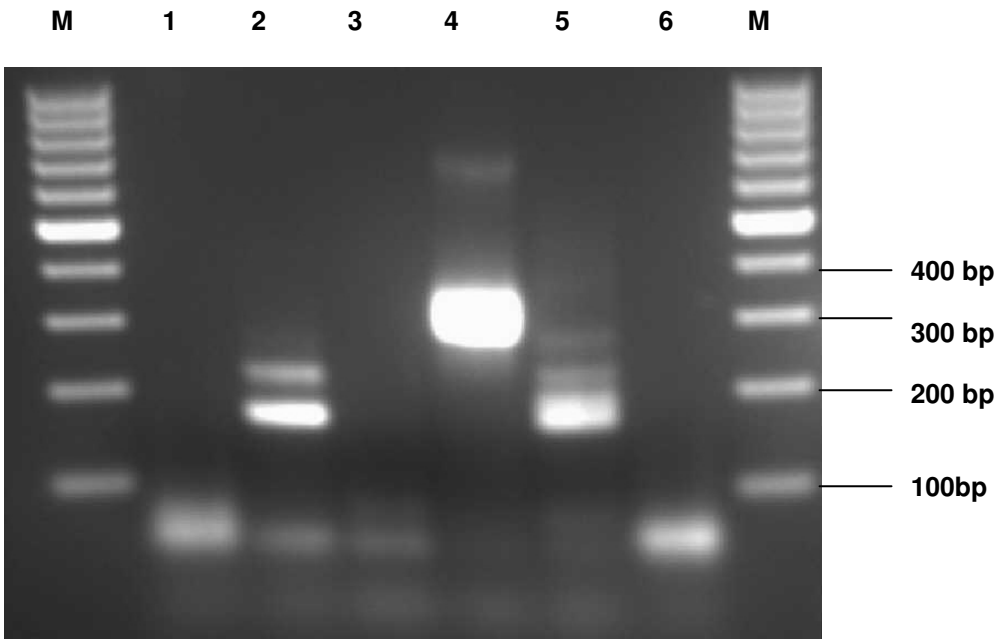

Figure 2.10: Detection of *B. bovis* and *B. bigemina* DNA in eggs laid by adult J 4199 *Bo. microplus* ticks collected after feeding on an adult goat. 5 µl of PCR product was loaded in each well of a 2% agarose gel. Lane M: 5 µl O' GeneRuler 100 bp DNA ladder (Fermentas), lane 1-3: J 4199 egg samples 1-3, lane 4: *B. bovis* positive control S23, lane 5: *B. bigemina* positive control G, lane 6: Negative control.

2.3.2.7 Sequencing of non-specific PCR products

The Bv80 PCR products obtained from *Bo. decoloratus* egg batch K 4192 55 and larvae batch K 4192 11 were cloned into the pGEM-T Easy vector system, the DNA sequenced and data analysed. In total, four clones were analysed, two from each batch. Three of the four clones had regions which overlapped enough to permit the joining of contigs. The fourth clone, 11_5 did not have enough overlap to provide a consensus sequence but as the sequence quality was very good, the forward and complement of the reverse read were analysed independently. Figure 2.11 shows the consensus sequence for clone 55_2. As can be seen on the right, the size calculation for the sequence product is 1702 bp, which is roughly 200 bp smaller than

the size of the PCR products calculated using gel analysis. The forward and reverse Bv80 primers are highlighted in blue.

```
--TGTGTTAATGTAACTCAGCCCGCCTACGAATCGTACGTAATCGCTCAAGTTCTACATC   58
AAACTCTTTGCGTTTTGATGTGTACGTTATGGTGCTCTTAGCCGCTTCCATTACATTTCC
GATTACCTCCTCTAGGCCATATGTGACACCTTCGCATGCCTCATCATCCTTCGTCTTATA
CATTGACCAATTGACTTTTCTGTGAACTGAATAAGGAGAATTTGCGAAACCTGTGACATT
CAAATACATTGGAATATGGTCACTGCCGTGAGTCTCAATATCCGTGAACCATCGCACTTC
TTTAGTGAATTCCCGTGACACCAGTGCCAAATCCAAACAACTGCTGTAATTCAAACCACG
TATAACGTCGGACTGCCGTCATTCATAATGTGAAGTTCATGGCGTGAGGCGAAGTGAGCA
AGTTTCCGGCCCCTGGAATCAATTTTGGTGCTGCCCCAAAGGAAGTGATGAGCATTAAAA
TCCCCAGTGATAATCCAGGGACCAGGTGTAGCCATCAAGATGTCATGCAGTCTTTCACTG
ATGAACTGAACAGAAGGAGAGATGTAAGCTCCGATAAGTGTGAAAGCTACCTTCTGTTTT
ATAACACGGAGGCACACGTACTGATTTCCGTTGTGGGGTTGCACCGACTGCGGAATGTAG
GTTAGATCTGAGCGAATATATACAATTACTTTGCTTGGTTCACCACAAGTCGATGAAGTG
AAGGATTCGTAGCCAGAGAGCTTAATCGGTTCTGATAGGTTTGGTTCGCATATTACTAAT
ATAGGAAATCTACTGAAGAAAACATACCGGCGGAAACAGGAAATCCGTGATTTCAGTCCT
CTGGCATTCCACTGGAAAACCGTCGCACTACGCACCTCTTCACGGAATGAAGGCGGCGGA
CGAGCCATTGTGTCTATGCAAGGTTGGCAACTGGACTCAGGGCATCCAATACTTGCACTG
CGCATCGTGCTGCTGGGGTATTCATATCACCTACAAGGGTGCGTATTGCATCGATGAGAG
ACTTGATCATCGTCAAAATTTCACGGTCACGCTCTCGGGGCTCATCTGGGCGAGATGCGG
AGCGTGGTGCTACCGGTGCGCTTTGCACGTTATCCACAGTAGTTATTCTTGGAAGTGATG
GCCACGCTTCTTCCGCAGTCTCCTTTGCGGGTATAGTATTCGCAGTGCACGTGTTAGTCT
TCGGCGTTGCTAACGATGTAGATTCCACCAAAACGGGCTTTGCTGCAAGCATGCTTCGGT
AGGTAGACTTTCTTGATGAGCGTCTGCGACGAGACCGATGTCGCCTGACGATAGCAGCTG
CCTCCCGGTGAGTCGAATGGTCCCTGACAATTTTCTTCAAAATTGAAATTTCCTGCCTTA
TTTTCGGACACTCCTTCGATGAGGCGTCATGGGGTCCTTGGCAATTCGGGCAGTTGTAAG
ATGCAGCCTTACAATGTTCAGCCAAATGTGGTGCACCGCAACGGGAACATGTTGCAGAAT
TTCCACAGACGGCGCTCACATGTCCAAATTTCTGACATTTTCGGCACTGAAGCGGTTTAG
GAACAAAGGGCCGCACCGCATGTCGGAAATGACCCACCTTTACATGTGACGGCAGGCAAT
CACTCTTAAAGTCAATCTTCACGCATCGGGACTTTCCTAGATGGCATACCTGAAGGATTT
CGGGTCCATCAACTAACAGGCTTT--                                    1702
```

Figure 2.11: Consensus sequence for clone 55_2 showing the binding positions for the Bv80 forward and reverse primers.

Consensus sequences were compared to published sequences using the BLAST program, blastn using the nt/nr database (Altschul *et al.* 1990). Samples 55_2, 11_3, 11_5_forward and 11_5_reverse all showed 100% sequence identity over the primer binding sites to *B. bovis* 85 kDa merozoite protein gene (complete coding sequence) (Accession Number: M99575.1), *B. bovis* Bv80 merozoite protein gene (partial coding sequence) (Accession Number: AY727909.1), *B. bovis* 80 kDa merozoite protein (C6A allele, complete coding sequence) (Accession Number: M93125.1) and *B. bovis* 80 kDa merozoite protein (C1B allele, complete coding sequence) (Accession Number: M93126.1). When the E values for the entire sequence were analysed, it was seen that the values were high (average of 1.4). After the first round of amplification, the primer sequence would be incorporated into the amplicon sequence and thus, the matching using blast analysis was as a result of the PCR primer incorporation rather than actual *B. bovis* sequence. When the clone sequences were compared to the *B. bovis* genome (preliminary data was obtained from http://www.vetmed.wsu.edu/research_vmp/program-in-genomics), no other regions of similarity between the clones and the *B. bovis* genome could be identified.

The fourth sample 55_1 did not show any sequence identity to any known *B. bovis* or *B. bigemina* genes. It did however show some identity to two *Bo. microplus* genes (80 and 82% sequence identity with E values of 3e-20 and 2e-16). Further sequence matches were sequences obtained from other insects, confirming the likelihood that the PCR products seen in the *Bo. decoloratus* samples were as a result of non-specific primer binding to tick DNA. Figure 2.12 shows a multiple alignment of clones, 55_2, 11_3 and 55_1 indicating regions of similarity with *. Areas in blue represent the primer sequences for the amplification of the *B. bovis* Bv80 gene and the purple text indicates what could represent a partial binding site of the forward primer for clone 55_1.

As the amplification of a PCR product hinges on the binding of the 3' end of the primer and the availability of its hydroxyl group, amplification can occur with only partial binding of the primer. After the first round of amplification, the primer sequence is incorporated into the template and any subsequent amplification cycles occur normally, resulting in the amplification of a product. The triple C (region A) may have allowed sufficient binding between the template and primer for amplification of a PCR product to occur. At 1620 bp, in blue text, there is identical sequence for the binding of the reverse primer, thereby allowing for complete amplification. The sequence itself shows a lesser identity number to the confirmed Bv80 coding sequences again confirming that this amplification was due to non-specific primer binding to tick DNA.

```
11_3          ----------TTTGTGTTAATGTAACTCAGCCCGCCTACGAATCGTACG   60
55_2          ----------TTTGTGTTAATGTAACTCAGCCCGCCTACGAATCGTACG
55_1          ----------TTAAAGCC--TGTTAGTTGATGGACCCATTGAAAGTATA
                        **   *    *** * *      **|*   *  ***
                                               A
11_3          TAATCGCTCAAGTTCTACAT--CAAACTCTGTGCGTT-TTGATGTGTACGTTATGGTGCT  120
55_2          TAATCGCTCAAGTTCTACAT--CAAACTCTTTGCGTT-TTGATGTGTACGTTATGGTGCT
55_1          ATAATATTCGGGGTTTACGTGCCGAAGCCAGGATATGACTGAGGCGCGCGGTAG------
                *    **  * * *** *  * **  *     *   *** * *  ** **

11_3          CTTAGCCGCTTCCATTACATTTCCGATTACCTCCTCTAGGCCA-TACGTGACACCTTCGC  180
55_2          CTTAGCCGCTTCCATTACATTTCCGATTACCTCCTCTAGGCCA-TATGTGACACCTTCGC
55_1          --TAGGGGGCTCCG--GAAATTTTGACTACCAGGTGTGCTTTAATGTGCACTGACATTGC
              ***  *  ***     * **  ** ****   * *    * *  *     * * **
              ----------------------------//----------------------------
```

```
                                                                      1500
11_3        ATTCCACCAAAACGGGCTTTGCTGCAAGCATGCTTCGGTAGGAAGACTTTCTTGATGAGC
55_2        ATTCCACCAAAACGGGCTTTGCTGCAAGCATGCTTCGGTAGGTAGACTTTCTTGATGAGC
55_1        GGTCAGC--AGTCTAGCTCCATATCAATAAAACTACCGTGGCGGG--TACCGTGATGCTC
              **  *  *  *  ***      ***  *  ** * ** *   *  *  * *****  *

11_3        GTCTGCGACGAGAACGATGTCGCCTGACGATAGCAGCTGCCTCCCGGTGAGTCGAATGGT
55_2        GTCTGCGACGAGACCGATGTCGCCTGACGATAGCAGCTGCCTCCCGGTGAGTCGAATGGT
55_1        TGCT--CAGGAAGAGGGTGTTTCTT--CGATTCC--CTGCCTT--GTCAGGTCCATCAA-
              **   * **    * ***  * *  ****  *  ******   *    *** *

11_3        CCCTGACCATTTTCTTCAAAATTGAAATTTCCTGCCTTATTTTCGGACACTCCTTCGATG 1620
55_2        CCCTGACAATTTTCTTCAAAATTGAAATTTCCTGCCTTATTTTCGGACACTCCTTCGATG
55_1        --CTAACAGGCTTT-----AATCGA----------------------------------
              ** **    **      *** **
```

Figure 2.12: Clustal 1.81X multiple sequence alignment of clones 55_1, 55_2 and 11_3 with primer sequences in blue, conserved sequences and partial primer binding positions in purple.

When comparing the two consensus sequences from clones 55_2 and 11_3, the sequences are near identical showing only one or two base differences per 60 bp of sequence and one region of insertion (at 940 bp). Figure 2.13 shows the sequence alignment for both clones showing the high degree of sequence identity with some point differences seen. Forward and reverse Bv80 primers are indicated in blue and point differences highlighted.

```
55_2        --------------------TTTGTGTTAATGTAACTCAGCCCGCCTACGAATCGTACGT
11_3        --------------------TTTGTGTTAATGTAACTCAGCCCGCCTACGAATCGTACGT   40
                                ****************************************

55_2        AATCGCTCAAGTTCTACATCAAACTCTTTGCGTTTTGATGTGTACGTTATGGTGCTCTTA
11_3        AATCGCTCAAGTTCTACATCAAACTCTGTGCGTTTTGATGTGTACGTTATGGTGCTCTTA
            **************************** ********************************

55_2        GCCGCTTCCATTACATTTCCGATTACCTCCTCTAGGCCATATGTGACACCTTCGCATGCC
11_3        GCCGCTTCCATTACATTTCCGATTACCTCCTCTAGGCCATACGTGACACCTTCGCATGCC
            **************************************** ******************

55_2        TCATCATCCTTCGTCTTATACATTGACCAATTGACTTTTCTGTGAACTGAATAAGGAGAA
11_3        TCATCCACCTTCGTCTTATACATTGACCAATTGACTTTTCTGTGAACTGAATAAGGAGAA
            *****  ************************************************************

55_2        TTTGCGAAACCTGTGACATTCAAATACATTGGAATATGGTCACTGCCGTGAGTCTCAATA
11_3        TTTGCGAAACCTGTGACATTCAAATACGTTGGAATATGGTCACTGCCGTGAGTCTCAATA
            **************************** ********************************

55_2        TCCGTGAACCATCGCACTTCTTTAGTGAATTCCCGTGACACCAGTGCCAAATCCAAACAA
11_3        TCCGTGAACCATCGCACTTCTTTAGTGAATTGCCGTGACACCAGTGCCAAATCCAAACAA
            ****************************** ****************************
```

```
55_2        CTGCTGTAATTCAAACCACGTATA-ACGTCGGACTGCCGTCATTCATAATGTGAAGTTCA
11_3        CTGCTGTAATTCAAACCACGTATATTACGTCGGACTGCCGTCATTCATAATGTGAAGTTCA
            ************************ ************************************

55_2        TGGCGTGAGGCGAAGTGAGCAAGTTTCCGGCCCCTGGAATCAATTTTGGTGCTGCCCCAA
11_3        TGGCGTGAGGCGAAGTGAGCAAGTTTCCGGCCCCTGGAATCAATTT-GGTGCTGCCCCAA
            ******************************************** ************

55_2        AGGAAGTGATGAGCATTAAAATCCCCAGTGATAATCCAGGGACCAGGTGTAGCCATCAAG
11_3        AGGAAGTGATGAGCATTAAAATCCCCAGTGATAATCCAGGGACCTGGTGTAGCCATCAAG
            ******************************************** ***************

55_2        ATGTCATGCAGTCTTTCACTGATGAACTGAACAGAAGGAGAGATGTAAGCTCCGATAAGT
11_3        ATGTCATGCAGTCTTTCACTGATGAACTGAACAGAAGGAGAGATGTAAGCTCCGATAAGT
            ************************************************************

55_2        GTGAAAGCTACCTTCTGTTTTATAACACGGAGGCACACGTACTGATTTCCGTTGTGGGGT
11_3        GTGAAGGCTACCTTCTGTTTTGTAACACGGAGGCACACGTACTGATTTCCGTTGTGGGGT
            ***** *************** ************************************

55_2        TGCACCGACTGCGGAATGTAGGTTAGATCTGAGCGAATATATACAATTACTTTGCTTGGT
11_3        TGCACCGACTGCGGAATGTAGGTTAGATCTGAGCGAATATATACAATTACTTTGCTTGGT
            ************************************************************

55_2        TCACCACAAGTCGATGAAGTGAAGGATTCGTAGCCAGAGAGCTTAATCGGTTCTGATAGG
11_3        TCACCACAAGTCGATGAAGTGAAGGATTCGTAGCCAGAGAGCTTAATCGGTTCTGATAGG
            ************************************************************

55_2        TTTGGTTCGCATATTACTAATATAGGAAATCTACTGAAGAAAACATACCGGCGGAAACAG
11_3        TTTGGGTCGCATATTACTAATATAGGAAATCTATTGAAGAAAACATACCGGCGGAAACAG
            ***** ************************** ************************
```

```
55_2        GAAATCCGTGATTTCAGTCCTCTGGCATTCCACTGGAAAACCGTCGCACTACGCACCTCT
11_3        GAAATCCGTGATT-CAGTCCTCTGGCATTCCACTGGAAAACCGTCGCACTACGCACCTCT
            ************ ************************************************

55_2        TCACGGAATGAAGGCGGCGGACGAGCCATTGTGTCTATGCAAGGTTGGCAA---CTGGAC  940
11_3        TCACGGAATGAAGGCGGCGGACGAGCCATTGTGTCTATGCAAGGTTGGCAAGCACTGGAC
            ************************************************    ******

55_2        TCAGGGCATCCAATACTTGCACTGCGCATCGTGCTGCTGGGGTATTCATATCACCTACAA
11_3        TCAGGGCATCCAATACTTGCACTGCGCATCGTGCTGCTGGGGTATTCATATCACCTACAA
            ************************************************************

55_2        GGGTGCGTATTGCATCGATGAGAGACTTGATCATCGTCAAAATTTCACGGTCACGCTCTC
11_3        GGGTGCGTATTGCATCGATGAGAGACTTGATCATCGTCAAAATTTCACGGTCACGCTCTC
            ************************************************************

55_2        GGGGCTCATCTGGGCGAGATGCGGAGCGTGGTGCTACCGGTGCGCTTTGCACGTTATCCA
11_3        GGGGCTCATCTGGGCGAGATGCGGAGCGTGGTGCTACCGGTGCGCTTTGCACGTTATCCA
            ************************************************************

55_2        CAGTAGTTATTCTTGGAAGTGATGGCCACGCTTCTTCCGCAGTCTCCTTTGCGGGTATAG
11_3        CAGTAGTTATTCTTGGAAGTGATGGCCACGCTTCTTCCGCAGTCTCCTTTTCGGGTATAG
            ************************************************* *********

55_2        TATTCGCAGTGCACGTGTTAGTCTTCGGCGTTGCTAACGATGTAGATTCCACCAAAACGG
11_3        TATTCGCAGTGCACGTGTTAGTCTTCGGCGTTGCTAACGATGTAGATTCCACCAAAACGG
            ************************************************************

55_2        GCTTTGCTGCAAGCATGCTTCGGTAGGTAGACTTTCTTGATGAGCGTCTGCGACGAGACC
11_3        GCTTTGCTGCAAGCATGCTTCGGTAGGAAGACTTTCTTGATGAGCGTCTGCGACGAGAAC
            *************************** ****************************** *
```

```
55_2        GATGTCGCCTGACGATAGCAGCTGCCTCCCGGTGAGTCGAATGGTCCCTGACAATTTTCT
11_3        GATGTCGCCTGACGATAGCAGCTGCCTCCCGGTGAGTCGAATGGTCCCTGACCATTTTCT
            ********************************************************  *******

55_2        TCAAAATTGAAATTTCCTGCCTTATTTTCGGACACTCCTTCGATGAGGCGTCATGGGGTC
11_3        TCAAAATTGAAATTTCCTGCCTTATTTTCGGACACTCCTTCGATGAGGCGTCATGGGGTC
            ************************************************************

55_2        CTTGGCAATTCGGGCAGTTGTAAGATGCAGCCTTACAATGTTCAGCCAAATGTGGTGCAC
11_3        CTTGGCAATTCGGGCAGTTGTAAGATGCAGCCTTACAATGTTCAGCCAAATGTGGTGCAC
            ************************************************************

55_2        CGCAACGGGAACATGTTGCAGAATTTCCACAGACGGCGCTCACATGTCCAAATTTCTGAC
11_3        CGCAACGGGAACATGTTGCAGAATTTCCACAGACGGCGCTCACATGTCCAAATTTCTGAC
            ************************************************************

55_2        ATTTTCGGCACTGAAGCGGTTTAGGAACAAAGGGCCGCACCGCATGTCGGAAATGACCCA
11_3        ATTTTCGGCACTGAAGCGGTTTAGGAACAAAGGGCCGCACCGCATGTCGGAAATGACCCA
            ************************************************************

55_2        CCTTTACATGTGACGGCAGGCAATCACTCTTAAAGTCAATCTTCACGCATCGGGACTTTC
11_3        CCTTAAACATGTGACGGCAGGCAATCACTCTTAAAGTGAATCTTCACGCATCGGGACTTTC
            ****  ******************************  ************************

55_2        CTAGATGGCATACCTGAAGGATTTCGGGTCCATCAACTAACAGGCTTTAA----
11_3        CTAGGCGGCACATCTGAAGGATTTCGGGTCCATCAACTAACAGGCTTTAATCGA
            ****   ***************************************************
```

Figure 2.13: Clustal 1.81X multiple sequence alignment of clones 55_2 and 11_3 with primer sequences in blue and point sequence differences highlighted in green (T), yellow (C), red (G) and purple (A).

2.3.3 Detection of *B. bigemina* in K 4192 and H 4195 *Bo. decoloratus* ticks

2.3.3.1 Levels of infection of egg and larval samples with *B. bigemina*

Levels of infection of the tick egg and larval samples with *B. bigemina* were determined using the nested PCR approach with the *B. bigemina* primers only. Figure 2.14 shows the detection of *B. bigemina* DNA in egg samples one to thirteen.

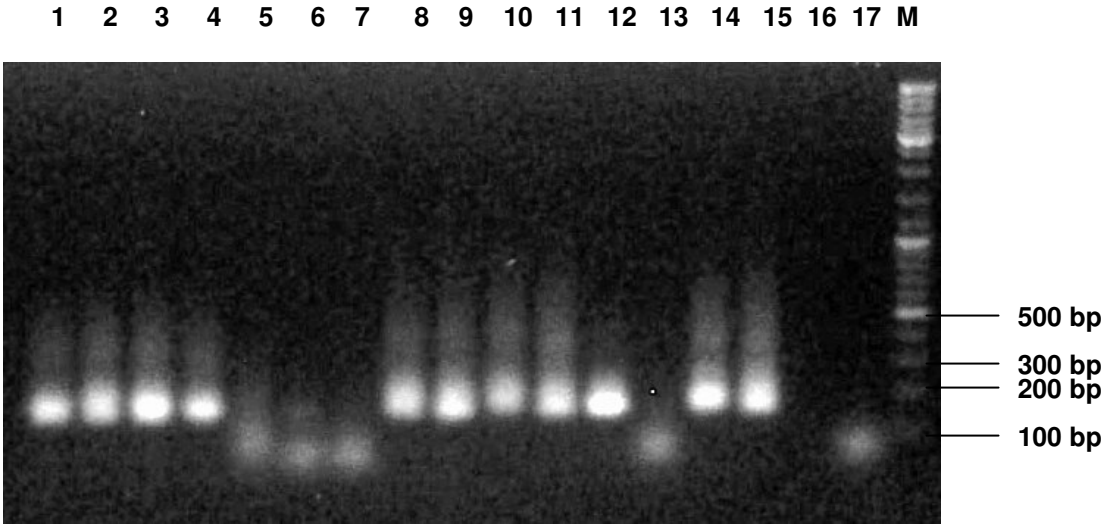

Figure 2.14: PCR detection of *B. bigemina* DNA in twelve K 4192 and one H 4195 *Bo. decoloratus* tick egg batches resolved on a 2% agarose gel. Lane 1-12: 5 µl of nested PCR reaction for egg samples K 4192 1-12, Lane 13: 5 µl of nested PCR reaction for egg samples H 4195 13, lane 14-15: 5µl *B. bigemina* strains G and P positive controls, Lane 16: no sample, Lane 17: Negative control, Lane M: 5µl O' GeneRuler DNA Ladder Mix (Fermentas)

From Figure 2.14, it can be seen that nine of the thirteen egg samples contained *B. bigemina* DNA. Samples K 4192 1-4 and 8-12 all showed the presence of *B. bigemina* DNA in the egg stage as seen by the presence of a 200 bp PCR product. The original publication determined that the PCR product obtained should be 278 bp but all the samples tested in this study showed a PCR product of around 200 bp. The width of the band stretches from below the 200 bp marker band to just below the

300 bp marker band and could reflect more than one PCR band. As the *B. bigemina* controls (known to be pure *B. bigemina* isolates) showed the same profile as the test samples, the bands were considered to reflect a positive result. Multiple bands can be clearly seen in the lanes 6-8 of Figure 2.9 which indicates that the smears described in Figure 2.14 were as a result of three bands migrating together as one. The larval tick stage was also tested for the presence of *B. bigemina* parasite DNA and the results are shown in Figure 2.15. Some samples that did not show the presence of *B. bigemina* DNA in the egg stage showed the presence of DNA in the larval stage, namely H 4195, and K 4192 5-7. This is probably due to the parasite replication cycle which occurs within the tick larvae (Potgieter, 1977; Potgieter and Els, 1977 b) resulting in an increase in the number of parasites within each tick and consequently the concentration of *B. bigemina* DNA is higher. All thirteen samples tested positive for the presence of *B. bigemina* in the larval stage, indicating that each of the tick batches were infected with *B. bigemina* parasites.

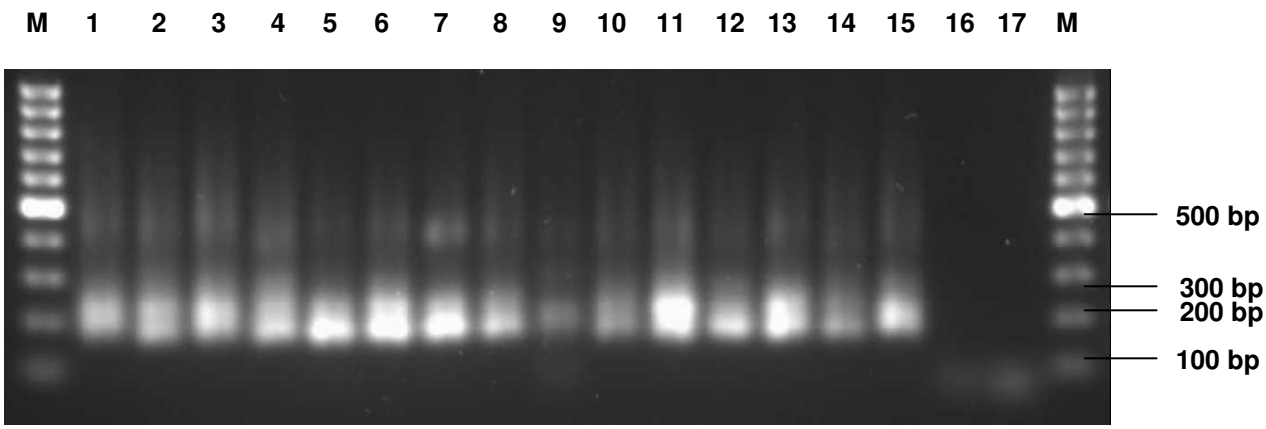

Figure 2.15: PCR detection of *B. bigemina* DNA in twelve K 4192 and one H 4195 *Bo. decoloratus* tick larvae batches resolved on a 2% agarose gel. Lane 1-12: 5 µl nested PCR reaction for larval samples K 4192 1-12, Lane 13: 5 µl nested PCR reaction for larval samples H 4195 13, lane 14-15: 5µl *B. bigemina* strains G and P positive controls,

Lane 16: *B. bovis* negative control, Lane 17: Negative control and Lane M: 5µl O' GeneRuler 100bp Ladder (Fermentas).

2.3.3.2 Detection of *B. bigemina* in nymphs

As *B. bigemina* is transmitted by both the nymph and adult stages, it would be expected that *B. bigemina* DNA would still be detected in the nymph stage. Figure 2.16 illustrates the detection of *B. bigemina* DNA in each of the nymph batches collected from animals 1173, 1197, 0643 and 0674. Positive PCR results for all nymph batches confirm the expected results, that *B. bigemina* DNA is still present in the nymph stage representing parasites that have yet to be transmitted to the cattle host.

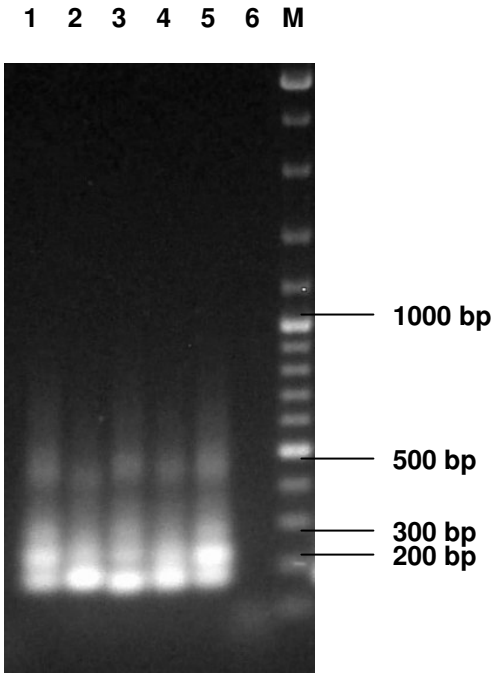

Figure 2.16: Detection of *B. bigemina* DNA in *Bo. decoloratus* nymph samples collected from animals 0674, 0643, 1173 and 1197. 5 µl of each PCR reaction was loaded in the well of a 2% agarose gel. Lane 1: nymph batch N0674, lane 2: nymph batch N0643, Lane 3: nymph batch N1197, lane 4: nymph batch N1173, lane 5: Positive

***B. bigemina* control, lane 6: Negative control, lane M: 5 µl O' Gene Mass Ruler Mix (Fermentas).**

2.3.4 Infectivity of ticks in cattle

Babesia bigemina transmission by the ticks fed on animals 0674, 0643, 1197 and 1173 was confirmed using IFAT, smear examination and PCR detection. Table 2.6 shows the results of *B. bigemina* transmission following the feeding of *Bo. decoloratus* K 4192 tick batches on four cattle all of which subsequently became *B. bigemina* carriers.

Table 2.6: Infectivity of *Bo. decoloratus* K 4192 larvae batches fed on animals 0674, 0643, 1197 and 1173.

	Animal number			
	1173	**1197**	**0674**	**0643**
Tick batch	1 and 5	3	9 and 11	7
Days to temperature	No temperature increase	No temperature increase	No temperature increase	No temperature increase
Days to parasitemia	26	24	20	25
Max parasitemia (%)	0.001	0.002	0.002	0.001

Figure 2.17 shows the detection of *B. bigemina* DNA in the blood of the four animals every second day covering the peak reaction period. The first sample was taken two days before the identification of parasites using blood smear examination and samples were taken every second day thereafter. *Babesia bigemina* DNA was detected on days when piroplasms could be detected in the blood smears and on the days before the identification of parasites using blood smear examination. This result confirms the reported sensitivity of the PCR reaction being able to detect low levels of infection. The only sample that did not show a positive PCR result was from animal 1173 (designated by the white arrow) taken two days before the first detection of parasites in blood smears.

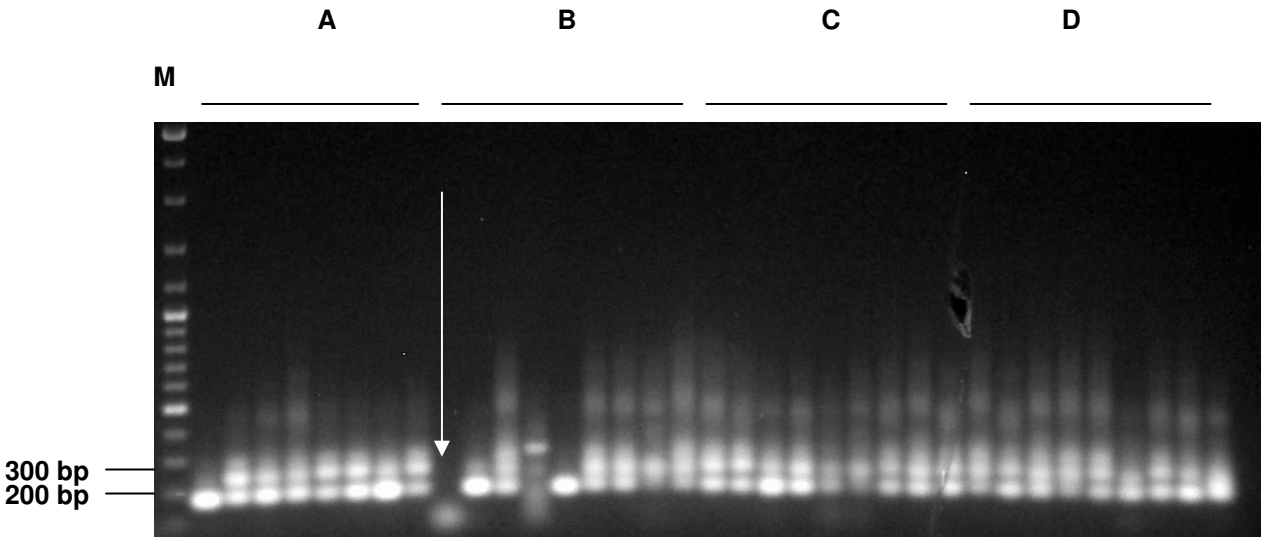

Figure 2.17: Detection of *B. bigemina* DNA in the blood of animals 1197 (A), 1173 (B), 0674 (C) and 0643 (D) using PCR analysis before and during the detection of piroplasms in blood smears. Lane M: 5 µl O' Gene Mass Ruler Mix (Fermentas).

As transmission of *B. bigemina* to the cattle host was successful it was expected that *B. bigemina* DNA would also be detected in the tick eggs laid by the females collected from the four cattle. Figure 2.18 shows the detection of *B. bigemina* DNA in the tick eggs from the female ticks collected from animals 0674, 0643, 1173 and 1197 over the peak parasitemia. Four of the six egg batches were infected with *B. bigemina* suggesting that not all ticks fed on a parasitemic animal will become infected with parasites but as only a few eggs were examined per egg batch, underestimation is to be expected.

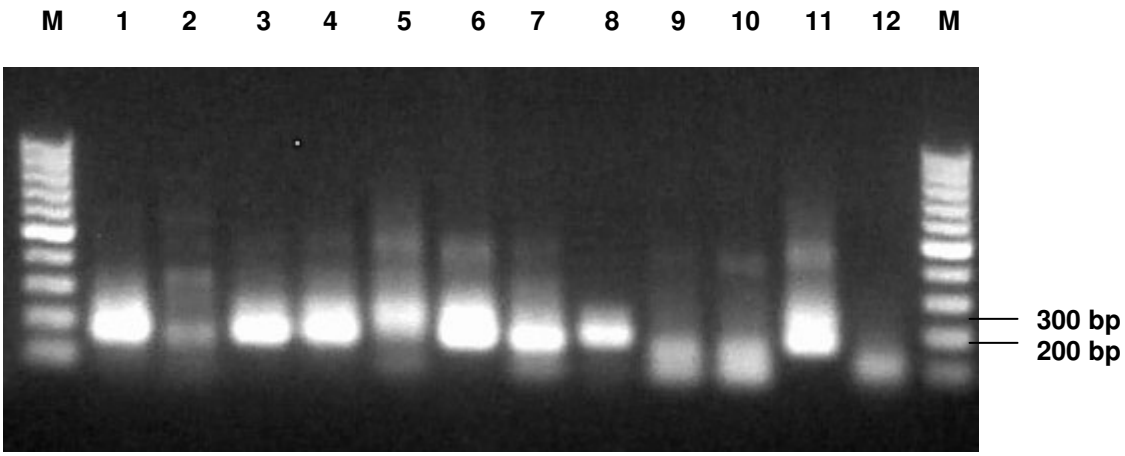

Figure 2.18: Detection of *B. bigemina* DNA in K 4192 *Bo. decoloratus* egg samples oviposited from female ticks collected from animals 0674, 0643, 1197 and 1173. Included is the detection of *B. bigemina* DNA in *Bo. microplus* J 4199. 5 µl of PCR product was loaded in each well of a 2% agarose gel. Lanes M: 5 µl O' GeneRuler 100bp DNA ladder (Fermentas), lane 1: J 4199 sample 3, lane 2: J 4199 sample 5, lane 3: J 4199 sample 7, lane 4: J 4199 sample 8, Lane 5: K 4192 sample 10, lane 6: K 4192 sample 15, lane 7: K 4192 sample 17, lane 8: K 4192 sample 27, lane 9: K 4192 sample 29, lane 10: K 4192 sample 30, lane 11: *B. bigemina* positive control P, Lane 12: Negative control.

Figure 2.18 also includes the results for the detection of *B. bigemina* in the original J 4199 *Bo. microplus* ticks, obtained from the University of the Free State as an uninfected negative tick DNA control. The four egg batches were tested using PCR analysis to confirm that the ticks were not infected with either *B. bovis* or *B. bigemina*. All four showed the presence of *B. bigemina* DNA (lanes 2-5, Figure 2.18) and consequently, these ticks therefore could not be used as an uninfected tick DNA control or as a uninfected tick strain.

2.3.5 *B. bovis* pick up by *Bo. microplus* and *Bo. decoloratus* ticks

To determine if the nested PCR could detect *B. bovis* infections in tick eggs, *Bo. decoloratus* K 4192 and *B. bovis* uninfected *Bo. microplus* J 4199 larvae were fed on

two bovines, which were then infected with *B. bovis* parasites (H strain or vaccine passage S24 respectively). Female ticks that engorged over the peak parasitemia period were collected and examined.

Babesia bigemina vermicules were observed in either the haemolymph or egg smears of three *Bo. decoloratus* K 4192 batches (of the 32 collected) but in none of the eight *Bo. microplus* J 4199 batches screened. The DNA was isolated and levels of infection with *B. bovis* and *B. bigemina* determined for eight *Bo. decoloratus* K 4192 and eight *Bo. microplus* J 4199 egg batches laid from the females collected. Figure 2.19 shows the *B. bovis* nested PCR results for the pick up experiment.

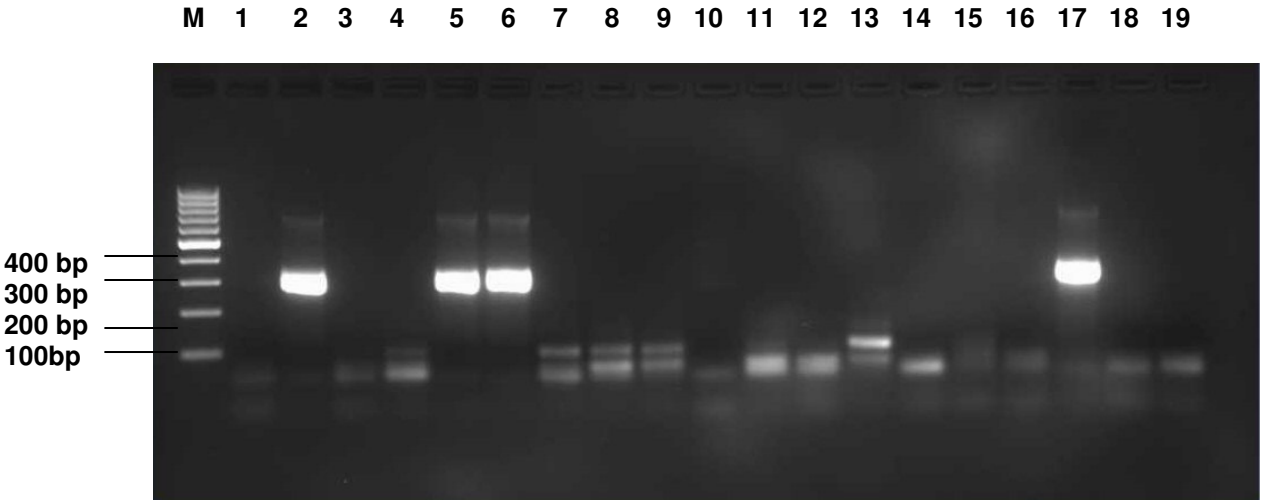

Figure 2.19: Detection of *B. bovis* DNA in *Bo. microplus* J 4199 and *Bo. decoloratus* K 4192 tick eggs oviposited by females fed on bovines infected with S24 or H *B. bovis* strain using *B. bovis* nested primers. 10 µl of PCR product was loaded in each well of a 2% agarose gel. Lane M: 5 µl O' GeneRuler 100 bp DNA ladder (Fermentas), lane 1-8: J 4199 egg samples E1-E8, lane 9-16: K 4192 egg samples F1-F8, lane 17, *B. bovis* S23 positive control, lane 18 *B. bigemina* control P, lane 19: Negative control.

Vermicules were not seen in the eight *Bo. microplus* J 4199 batches examined yet *B. bovis* DNA was detected in three of the egg batches, indicating that the sensitivity of the PCR is superior to convential methods. These results also demonstrated the possibility of the *B. bovis* vaccine strain being picked up from vaccinated animals by *Bo. microplus* ticks and is probably transmissible to another animal through natural tick transmission. Mason *et al.* (1986) originally reported that the blood vaccine, S24 was not picked up by *Bo. microplus* ticks when fed on vaccinated cattle as no vermicules could be detected in the haemolymph of engorged females. They concluded that the vaccine strain was not tick transmissible due to the absence of vermicules and a lack of transmission to a susceptible host.

In the case of *B. bigemina* infections (Figure 2.20), only one sample (*Bo. microplus* J 4199 egg sample E4, lane 4) did not show the presence of *B. bigemina* DNA at the egg stage.

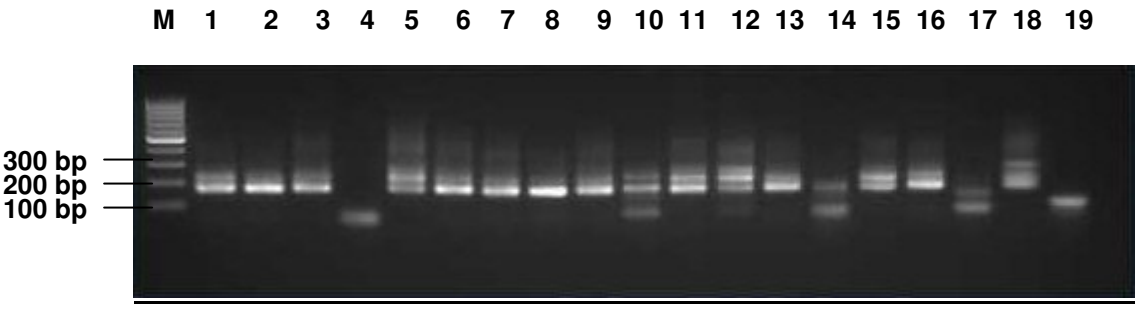

Figure 2.20: Detection of *B. bigemina* DNA in *Bo. microplus* J 4199 and *Bo. decoloratus* K 4192 tick eggs oviposited by female ticks fed on bovines infected with S24 and H *B. bovis* strain using *B. bigemina* nested PCR primers. 10 µl of PCR product was loaded in each well of a 1.5% agarose gel. Lane M: O' GeneRuler 100 bp DNA ladder (Fermentas), lane 1-8: J 4199 egg samples E1-E8, lane 9-16: K 4192 egg samples F1-F8, lane 17: *B. bovis* negative control, lane 18: 5 µl positive *B. bigemina* control P, lane 19: Negative control.

Potgieter (1977) identified *B. bovis* bodies in the gut cells of replete female *Bo. decoloratus* ticks. He noted irregularities in ultrastructure of the parasite cells between days 1 and 2. Projections similar to those seen when high infections of *B. bovis* occur in *Bo. microplus* ticks (representing, presumably dead parasites) were identified, but as no transmission was detected and no further developmental stages encountered, no further investigations were made.

Smeenk *et al.* (2000) detected *B. bovis* DNA in the haemolymph of adult female *Bo. decoloratus* ticks in Zimbabwe using PCR analysis (in as many as 60% of the ticks examined) and suggested that the tick may become infected with *B. bovis* parasites. *Babesia bovis* DNA could not be detected in the *Bo. decoloratus* K 4192 ticks using the nested PCR approach, this despite the RLB indicating the presence of DNA in the eggs of five of thirteen field ticks. *Boophilus microplus* and *Bo. decoloratus* ticks are closely related tick species which coexist in the same habitat and mate to produce sterile offspring (Spickett and Malan, 1978). *Babesia bigemina* and *B. bovis* are also closely related parasite species, both causing bovine babesiosis with similar symptoms. The two parasites share very similar life cycles and the limited development of *B. bovis* parasites within the *Bo. decoloratus* tick is therefore not unlikely. However, in our study, no *B. bovis* DNA could be detected in the K 4192 and H 4195 *Bo. decoloratus* ticks using the nested PCR approach.

The presence and development of parasites in vectors other than those responsible for their transmission have been documented in other species. The infection of different *Hyalomma* tick species with *Trypanosoma*-like flagellates has been reported several times varying from limited survival as seen with the detection of *Trypanosoma lewisi, Trypanosoma evansi* and *Trypanosoma vivax* in *Hyalomma aegyptium excavatum* (Kirmse and Taylor-Lewis, 1976) to successful transstadial transmission in a calf with *Trypanosoma theileria*-like flagellate by *H. a. anatolicum*

(Morzaria *et al.*, 1986). In the present study, a female *Bo. microplus* J 4199 tick collected from the field showed the presence of a *Trypanosoma*-like flagellate in the haemolymph (results not shown), confirming earlier studies that parasites may develop in hosts that are not necessarily their vectors. Though *B. bovis* DNA could not be detected in the *Bo. decoloratus* ticks by the nested PCR method, previous reports have identified the parasite in the gut and haemolymph, indicating that this tick species is able to acquire the *B. bovis* parasite but it is unlikely that it is able to facilitate its life cycle beyond this point. It is possible that *B. bovis* DNA might have been detected in *Bo. decoloratus* ticks that had fed on animals infected with *B. bovis* but it is unlikely that *B. bovis* DNA would be detected in the eggs, larvae or nymphs from these ticks.

The RLB results suggest that there was *B. bovis* DNA in the eggs of the *Bo. decoloratus* K 4192 and H 4195 field ticks. Although at first glance, the Bv80 PCR test results also appeared to be positive, these PCR products were shown to be as a result of non-specific binding of the Bv80 primers to tick DNA rather than amplification of *B. bovis* DNA. The false positive RLB results may have been due to non-specific probe binding if the hybridisation conditions were not sufficiently stringent. The RLB probe sequences for *B. bovis, B. bigemina* and *B. rossi* are indicated below in Table 2.7. One of the samples indicated the presence of *Babesia rossi* DNA (Table 2.4, K 4192 egg batch no. 4), which is one of the causative agents of babesiosis in canids. The presence of this DNA isolated from ticks collected from cattle is more likely due to non-specific binding than the actual presence of *B. rossi* DNA.

Table 2.7: RLB probe sequences for *B. bigemina, B. bovis* and *B. rossi* (Isogen Life Science).

Species	RLB probe sequence 5'-3'
B. bigemina	CGTTTTTTCCCTTTTGTTGG
B. bovis	CAGGTTTCGCCTGTATAATTGAG
B. rossi	CGGTTTGTTGCCTTTGTG

The areas of similarity can be seen highlighted in yellow (C), red (G) and green (T). Under less stringent washing steps, the probes for *B. rossi* and *B. bovis* may have bound to *B. bigemina* DNA resulting in the detection of *B. rossi* and/or B. *bovis* DNA in the *Bo. decoloratus* ticks.

2.4 CONCLUSIONS

Only *Bo. decoloratus* ticks could be collected from the Haistings and Killrush farms but *Bo. microplus* ticks must have been present in the area at some stage for there to be *B. bovis* parasites circulating in the cattle. Seasonal changes in tick distribution are known to occur and the absence of *Bo. microplus* ticks may have been as a result of seasonal climate changes or dipping regimes.

RLB and Bv80 PCR tests suggested the presence of *B. bovis* DNA in the egg and larval stages of *Bo. decoloratus* ticks, but a nested PCR reaction indicated that these results were probably caused by non-specific Bv80 primer binding to *Bo. decoloratus* DNA. These results were confirmed by sequence analysis of cloned Bv80 PCR products and consequently, the Bv80 PCR should not be used to identify *B. bovis* infections in *Boophilus* ticks or if used, the results should be confirmed using another PCR protocol such as the nested PCR used in this study. In the case of the RLB, the positive *B. bovis* results were most likely as a result on non-specific probe binding.

Boer goats were determined to be an unsuitable experimental host for the feeding of both *Bo. decoloratus* and *Bo. microplus* ticks. *Bo. microplus* fed better on the goats than the *Bo. decoloratus* ticks which showed no attachment or feeding, but even so, the number of engorged *Bo. microplus* ticks collected was very low. The ears were determined to be the better site for feeding compared to the back of the animal.

CHAPTER THREE: HAISTINGS *B. BOVIS* STRAIN CHARACTERISATION

3.1 INTRODUCTION

Outbreaks of babesiosis were suspected in herds of cattle on the Haistings and Killrush farms which had allegedly been vaccinated against *B. bovis* using the vaccine commercially available (produced by Onderstepoort Biological Products in collaboration with the OVI) (Combrink, personal communication). The vaccine produced is a live frozen blood vaccine and is sensitive to changes in temperature. The vaccine needs to be thawed, kept on ice and administered within a certain period of time (30 minutes) otherwise the parasites lose viability (de Waal *et al.*, 1998). Any disruption to the cold chain during production, storage and administration can cause a loss of parasite viability and as a result, the product becomes ineffective. If this had been the case, the farmer may not have been aware of the loss in viability of the product and administered the product none-the-less. The farmer would be under the impression that his animals were vaccinated and thus protected from field challenge when in essence, they received no exposure to viable parasites and acquired no immunity to future challenge.

The redwater outbreaks in the Swartberg region in 2005 and 2006 could be as a result of two possibilities. A virulent field isolate breaking through the protection offered by the vaccine strain (either due to inefficient vaccination practices or due to true breakthrough) or the vaccine could have reverted to a more virulent form causing severe disease symptoms. It should be noted that throughout the past twenty years of production, no claims of vaccine breakthroughs could be substantiated for the vaccine strain currently in production (Combrink, personal communications).

In addition to determining the populations of *B. bovis* parasites circulating in the Haistings animals, there was a need to characterise the isolate associated with the outbreaks to ensure that the field strain to be cultured was not the vaccine strain collected from vaccinated field animals. No previous characterisation of South African *B. bovis* isolates has been carried out and it was thus necessary to characterise the vaccine strain as well as any other reference strains available at the OVI including the strain collected from the Haistings farm.

The PCR based tests chosen for the characterisation have previously been used to discriminate between several Australian *B. bovis* strains based on two variable genes, namely the Bv80 and BvVA1 genes (Lew *et al.*, 1997 a). First identified in 1992, the BvVA1 gene was referred to as the BoVA1 gene and described as a single copy gene comprised of variable length tandem repeats flanked by conserved regions. The space between the conserved regions could be used as a physical basis to discriminate between *B. bovis* strains (Dalrymple *et al.*, 1992). It was concluded that multiple bands per sample were indicative of several genotypes present within a strain. Original investigations suggested that the variable region is stable over an extended period of time showing no size difference between passages through cattle hosts or in continuous culture systems (Dalrymple *et al.*, 1992). The Bv80 gene is structurally similar to the BvVA1 gene but is smaller in size. The BvVA1 gene encodes a 170-320 kDa protein (the variation in size is a result of the variation of the repeated coding sequences, between 3.6 and 7.6 kb, depending on the strain), where the Bv80 gene encodes a substantially smaller 80 kDa protein (Goodger *et al.*, 1992; Dalrymple *et al.*, 1993). Both proteins show homology at their carboxy- and amino-terminal domains, in the case of Bv80 it has a 30% amino acid identity between the carboxy- (215 amino acids) and the amino- (149 amino acids) terminal ends with the region between consisting of short repeat sequences rich in proline and glutamic acid residues (Dalrymple *et al.*, 1993). The carboxy-terminal

end of the BvVA1 protein seems to be a diverged copy of the amino-terminal sequence of 170-171 amino acids and as with the Bv80 gene, the carboxy and amino domains are separated by repeated sequences. The 73 amino acid BvVA1 repeat is comprised of a number of shorter repeating units (Dalrymple *et al*., 1992; Dalrymple *et al*., 1993). Both the Bv80 and BvVA1 genes belong to variable gene families. The Bv80 gene shows a 79% sequence identity with another *B. bovis* protein named Bb-1. This protein was investigated for its use as a possible recombinant vaccine but the high degree of sequence variation between strains made it an unfavorable target (Hines *et al.,* 1995).

Lew *et al.* (1997 a) developed a PCR based assay targeting the Bv80 and BvVA1 genes as a method of discriminating between Australian *B. bovis* strains based on the length of the tandem repeats. The BvVA1 PCR amplifies a substantially larger repeat (around 7.5 kb) and makes use of a long template PCR protocol. They were successfully able to discriminate between ten Australian strains using the Bv80 PCR assay and thirteen using the BvVA1 assay. Further analysis of the BvVA1 repeats using *Acc* I restriction allowed for increased discrimination between strains. Using this *Acc* I restriction, the authors concluded that the BvVA1 PCR method used in conjunction with *Acc* I profiling was a more sensitive method of strain identification than the Bv80 PCR assay but admitted it showed some bias towards some genotypes of BvVA1 repeats. The test was sensitive enough to amplify different genotypes from mixed infection and did not amplify host DNA nor *B. bigemina* parasite DNA (Lew *et al*., 1997 a).

The Bv80 and BvVA1 PCR assays appeared to be suitable to determine the nature of the outbreaks in the Swartberg region. If successful, these tests could be used routinely to determine the nature of *B. bovis* outbreaks in South Africa by isolating and characterising the circulating parasites. To do this, the tests would need to be

adapted and optimised to suit South African strains and the sensitivity of the tests would need to be further investigated as well as the specificity.

The Bv80 and BvVA1 genes seem to be stable over a period of two to three passages in cattle (Lew *et al.*, 1997 b) but no investigations have been conducted into whether changes in the Bv80 gene are reflected in changes in another independent gene which is known to be both stable over time yet able to reflect strain differences. The V4 hypervariable region of the 18S ribosomal ribonucleic acid (rRNA) gene was therefore chosen as a point of reference. In *Babesia* there are three to four RNA genes and as in other eukaryotes, they may be repeated a number of times depending on the species. When repeated, these genes are presumed to be highly homogenised under the concerted evolution model (reviewed by Rooney, 2004). Under this model, when any mutation arises in one gene in a multi gene family, the mutation spreads to all other members of the family. In this model, members of a multi gene family (such as the rRNA genes) do not evolve independently but rather together. This would suggest that irrespective of the number of copies of a certain rRNA gene, the sequence would be identical (Brown *et al.*, 1972; Dover and Coen, 1981; Ohta, 1989; Ohta, 2000).

Despite this, there are cases where divergence from the concerted evolution model have taken place. For example, divergence from this model has been noted in the apicomplexan phylum, under which *B. bovis* falls (reviewed by Rooney, 2004). The structure and function of apicomplexan rRNA genes has been most extensively studied in *Plasmodium* species. Responsible for causing malaria, extensive research has been conducted on the various aspects of this parasite's biology. Through the sequencing of the genome and studies on the rRNA genes, it has arisen that *Plasmodium* has functionally distinct rRNA 'types'. It is believed that the evolution of different types of rRNA molecules was in response to the complex multi host life

cycle of the malaria parasite (Gunderson *et al.*, 1987; McCutchan *et al.*, 1988; Rogers *et al.*, 1995; Mercereau-Puijalon *et al.*, 2002). Having different 'types' of the same rRNA molecule allows for a certain rRNA type to be expressed during a specific stage of the life cycle, facilitating the optimum growth of the parasite under varying environmental conditions (Gunderson *et al.*, 1987; Mercereau-Puijalon *et al.*, 2002).

Determining changes in the 18S rRNA gene would allow a relationship to be drawn between changes in the Bv80 sequence and changes in the 18S rRNA sequence. In most species the rRNA genes themselves are relatively homogenous between strains with few sequence differences even at the species level but the intergenic regions flanking the rRNA genes show more variable sequence. Focusing on these variable regions can aid in identification of strain differences (Long and Dawid, 1980; Dams *et al.*, 1988; Gonzalez *et al.*, 1988; Guttell and Fox, 1988). If the *B. bovis* rRNA genes are similar to those of *Plasmodium*, there may be different 'types' of 18S rRNA genes. If this is the case, the relationship between changes in 18S rRNA sequence and strain differences would be more difficult to establish, as two rRNA sequences might not necessarily represent two *B. bovis* strains, but rather, two different copies present in the same strain. Factoring in the different number of copies for each rRNA type, a major 'type' donor may be picked up using PCR and sequencing whilst the minor 'type' donor may be overlooked.

Dalrymple (1990) cloned and characterised the rRNA genes of *B. bovis* as well as the flanking regions to be used as a basis for strain discrimination. Dalrymple analysed four *B. bovis* strains and concluded that the organism has three sets of rRNA genes. Jones *et al* (1997) confirmed that the *B. bovis* genome contains three, probably single copy rRNA genes, all located on the third chromosome. The three units are organised as the small subunit, 5.8S and large subunit rRNAs and the

spacing of the genes seems to be identical in all strains (Dalrymple, 1990). As *B. bovis* also has a multi host life cycle, it was reasonable to suspect that *B. bovis* may also have different 'types' of rRNA genes but there is evidence to suggest that *B. bovis* does not contain distinctly different 'types' of rRNA genes (Jones *et al.,* 1997). Whilst *Plasmodium* and *B. bovis* both have complex life cycles, having 'types' of rRNA molecules may not be as important in hemoparasites (Dalrymple, 1990). Variation in the polymorphic flanking regions enables strain discrimination confirming the potential use of this sequence as a strain indicator (Dalrymple, 1990).

Confirmation of the single rRNA 'type' in the *B. bovis* genome allows for the use of the 18S rDNA as a point of reference for strain discrimination. Sequencing of both the Bv80 gene products as well as the variable regions of the 18S rRNA gene provides not only a confirmation of strain differences but also clarity on the relationship between size and sequence difference of the Bv80 gene with respect to changes in the 18S rRNA gene.

The Bv80 and BvVA1 PCR tests have been previously used to investigate the nature of *B. bovis* outbreaks in Australia and this project aimed to adapt these techniques to determine the parasite populations in animals in the Swartberg region of KwaZulu-Natal. In conjunction, the sequence of the Bv80 gene for each strain would be analysed and compared to the sequence of all the genotypes present in the other strains. The 18S rRNA V4 hypervariable region sequences from each strain would be analysed to determine if changes in the Bv80 gene correlate with changes in the 18S rRNA V4 hypervariable region sequences.

3.2 MATERIALS AND METHODS

3.2.1 *B. bovis* strains

The H strain was obtained as described in chapter one. The vaccine strain 'S' has been used for the past 30 years and has been syringe passaged through splenectomised animals. The strain was originally obtained by tick transmission experiments carried out with ticks collected in the Pretoria district of Gauteng, South Africa. It was rapidly passaged ten times in splenectomised calves and then a further 13 times in splenectomised calves to produce the S23 passage (Mason *et al.*, 1986; de Vos, 1978). The F strain was obtained by tick transmission experiments carried out with ticks collected from the Eshowe district of KwaZulu-Natal in 1978 (de Vos, 1978).

3.2.2 DNA isolation from whole blood

Parasitised blood was obtained from infected animals bled from the vein under the tail into EDTA vaccutainer tubes and DNA was isolated using either the QIAamp DNA Blood Mini Kit (Qiagen) or the MagNA pure automated DNA extraction machine (Roche). DNA was isolated using the QIAamp DNA Blood Mini Kit as set out in the Blood and Body Fluid Spin Protocol with elution into either 100 µl of elution buffer or nuclease free water. Blood was stored at 4°C until use and at -20°C for prolonged storage. MagNA pure DNA was prepared using the standard DNA LV Blood 20_200 protocol and the Large Volume DNA Extraction Kit with 200 µl of whole blood input, eluted in 100 µl of elution buffer.

Template DNA for the BvVA1 PCR was further prepared by ethanol precipitation of the isolated genomic DNA. Double the volume of 95% ethanol was added to the eluted DNA and allowed to stand at room temperature for 2 minutes. The samples were centrifuged at 12 000 g for 10 minutes and the supernatant discarded. The

pellet was then washed by the addition of 1 ml of 70% ethanol, inverted and the centrifugation step repeated. The pellet was allowed to dry at room temperature and resuspended in a quarter of the initial volume using nuclease free water.

3.2.3 Bv80 and BvVA1 PCR

Reaction parameters of the Bv80 and BvVA1 PCR reactions were carried out as described in the original publication (Lew *et al.*, 1997 a) except for the annealing temperature for the Bv80 PCR, which was increased to 53ºC. The Bv80 PCR was carried out in a 25 µl reaction volume using the High Fidelity PCR Master (Roche) with 20 pmoles of forward and reverse primer per reaction. 2.5 µl of genomic DNA was used as template for the Bv80 PCR and 2.5 µl of ethanol-precipitated DNA in the case of the BvVA1 PCR. BvVA1 PCR was carried out in a 25 µl reaction volume using buffer 2 of the Expand Long Template PCR System (Roche). 20 pmoles of each primer were added along with 7.5 pmoles of each dNTP (Fermentas). Table 3.1 shows the primer sequences for both Bv80 and BvVA1 PCRs and Tables 3.2 A and B show the cycle parameters.

Table 3.1: Primer sequences for Bv80 and BvVA1 PCR reactions from Lew *et al.* (1997 a).

Primer name	Gene	Primer sequence 5'-3'
1Bf	Bv80	TGTGTTAATGTAACTCAGCCCG
2Br		AAAGCCTGTTAGTTGATGGACC
BVA7lf	BvVA1	TGCTGAACTCAAAAGAAGAGATGTTGTGCGC
BVA8lr		GAGCGGCTTTCAGTTCATTAGCAAAATCAGACG

Table 3.2: Cycling parameters for Bv80 (A) and BvVA1 (B) PCR reactions adapted from Lew *et al.* (1997 a).

A

Bv80 cycle parameters		
Temperature (°C)	**Time (minutes)**	**Cycles**
94	3	1
94	0.5	30
53	0.5	
72	2	
72	10	1

B

BvVA1 cycle parameters		
Temperature (°C)	**Time (minutes)**	**Cycles**
92	2	1
92	10 sec	10
65	0.5	
68	6	
92	10 sec	20
65	0.5	
68	6 minutes + 20 sec per cycle	

BvVA1 PCR fragments were further analysed using *Acc* I restriction analysis. BvVA1 PCR products (50 µl total volume) was cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the protocol outlined by the manufacturer and eluted in 25 µl of nuclease free water. One unit of *Acc* I (Roche) was used to restrict 10 µl of DNA by incubating at 37°C for three hours.

3.2.4 18S rRNA V4 hypervariable region PCR

The amplification of the 18S rRNA V4 hypervariable region was adapted from the Reverse Line Blot (RLB) hybridisation assay (Gubbels *et al.*, 1999; Isogen Life Science). PCR reactions were carried out using the High Fidelity Master kit in 25 µl volume using 2.5 µl of template genomic DNA and 20 pmoles of each primer. Primer sequences are set out below in Table 3.3 and reaction parameters in Table 3.4.

Table 3.3: 18S rRNA V4 hypervariable region PCR primer sequences (Gubbels *et al.*, 1999; Isogen Life Science).

Primer name	Primer sequence 5'-3'
RLB-F2	GACACAGGGAGGTAGTGACAAG
RLB-R2	CTAAGAATTTCACCTCTGACAGT

Table 3.4: 18S rRNA V4 hypervariable region PCR cycling parameter conditions (Gubbels *et al.*, 1999; Isogen Life Science).

Bv80 cycle parameters		
Temperature (ºC)	**Time (minutes)**	**Cycles**
92	2	1
92	0.5	40
58	0.5	
72	1	
72	7	1

3.2.5 Visualisation of PCR products

All PCR products were resolved on agarose gels (between 1 and 2%) using TAE buffer by electrophoresis at 100V for 1-2 hours and visualised with ethidium bromide staining (10 µg/ml).

Bv80 and BvVA1 PCR products were also resolved on polyacrylamide gels to obtain better resolution. Polyacrylamide gels for the resolution of Bv80 PCR products were cast using a 10% stacking and a 15% resolving gel. Polyacrylamide gels for the resolution of BvVA1 PCR products were cast using a 4% stacking and 10% resolving gel. Table 3.5 shows the reagent volumes for each polyacrylamide gel.

Table 3.5: Reagent volumes for casting of polyacrylamide gels for the resolution of Bv80 and BvVA1 PCR products.

Bv80		BvVA1	
10% Stacking	**15% Resolving**	**4% Stacking**	**10% Resolving**
0.3 ml glycerol^	0.3 ml glycerol^	1 ml glycerol^	0.3 ml glycerol^
3.9 ml water	2.2 ml water	5.2 ml water	3.9 ml water
3.3 ml acrylamide*	5 ml acrylamide*	1.3 ml acrylamide*	3.3 ml acrylamide*
2.5 ml 1X TBE buffer	2.5 ml 1X TBE buffer	2.5 ml 1XTBE buffer	2.5 ml 1XTBE buffer

*30% Acrylamide/Bis Solution, 29:1 (3.3% C) (Bio-Rad)
^ Saarchem

Gels were polymerised using 50 µl of 10% ammonium persulfate (APS) (Bio-Rad) and 5 µl or 10 µl of N, N, N', N' Tetramethylethylenediamine (TEMED) (Bio-Rad) for the resolving and stacking gels respectively.

DNA bands were visualised with the silver staining technique using the Silver Stain Plus Kit (Bio-Rad) according to the manufacturer's guidelines. Agarose and polyacrylamide gels were imaged using the GelDoc XR Imaging System (Bio-Rad) and analysed using the Quantity One Software package.

3.2.6 Detection of carrier animals using the Bv80 PCR

Blood from 12 animals that were experimentally infected with the *B. bovis* vaccine (as part of a separate research project) was used to determine if the Bv80 PCR could detect *B. bovis* DNA in carrier animals. Animals were determined to be in the carrier state of infection if one parasitised cell per 15 fields of approximately 500 erythrocytes or one to three infected cells after five minutes of examination were observed.

DNA was extracted using the QIAamp DNA Blood Mini Kit and concentrated to twenty times the initial volume of DNA. Bv80 PCR reactions were carried out in triplicate and PCR products resolved on a 1.5 % agarose gel.

3.2.7 Cloning and sequencing of Bv80 and 18S rRNA V4 hypervariable region PCR products

Due to the mixed infections in three of the four strains, PCR products were cloned into a suitable vector to enable the sequencing of individual strain products.

50 µl PCR product for each strain was cleaned using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the protocol outlined by the manufacturer. Samples were eluted into half the initial reaction volume and stored at 4°C until use or –20 °C for prolonged periods.

3 µl of each cleaned PCR product was used in a ligation reaction with 50 ng of pGEM-T Easy vector (Promega) and 3U of T4 DNA ligase in a total reaction volume of 10 µl. Ligation reactions were incubated at room temperature for one hour and overnight at 4°C. The ligation reactions (10 µl) were transformed into 50 µl of competent JM109 *E. coli* cells (Promega) using the heat shock method. Cells were incubated with the DNA ligation mix on ice for 20 minutes before being heat shocked at 42°C for 45 seconds exactly. Samples were then incubated on ice for a further two minutes before the addition of 950 µl of Luria Broth and incubation at 37°C for one and a half hours. Cells were pelleted by centrifugation at 10 000 g for two minutes, the supernatant decanted and the cell pellet resuspended in the remaining 50 µl of medium. Transformants were plated on imMedia Amp Blue (Invitrogen) plates to allow for blue/white colony screening. Plates were incubated at 37°C overnight and white colonies picked for the inoculation of starter cultures. Cultures

were grown overnight in 5 ml of imMedia Amp liquid medium (Invitrogen) at 37°C with shaking at 2 000 rpm in a shaking incubator.

Plasmid DNA was extracted from 3 ml of starter cultures using the Wizard *Plus* Minipreps DNA purification System (Promega) as per the manufacturer's instructions. DNA was eluted in 50 µl of nuclease free water and stored at -20°C.

Clones were screened for the presence of Bv80 or 18S rRNA V4 hypervariable region inserts using restriction endonuclease enzyme analysis with *Eco* RI or *Acc* I. 2 µl of isolated clone DNA was restricted with 1U *Eco* RI (Fermentas) in a total reaction volume of 20 µl or with 1U *Acc* I (Roche) in a total volume of 25 µl. The entire restriction reaction was resolved on a 1% agarose gel and stained with ethidium bromide.

DNA concentrations were measured using the DNA quantification program on the Nanodrop spectrophotometer.

DNA was sequenced using the Big Dye terminator mix and resolved using the ABI Big Dye V3.1.1. 3.2 pmoles of the pUC/M13 forward and reverse sequencing primers were used along with 500 ng DNA of template in a total volume of 12 µl. Sequencing reactions were either carried out at the OVI sequencing facility or at Inqaba Biotech.

3.2.8 Sequence analysis

AB1 sequence files were evaluated using the pregap4 program from the Staden package (Bonfield *et al*., 1995 a, Bonfield *et al*., 1995 b). Vector sequences were removed using the *Eco* RI restriction sites at either end of the vector cloning site.

Forward and reverse reads were assembled using the gap4 program (Bonfield *et al.*, 1995) from the Staden package, the resulting contigs edited and consensus sequences saved. Consensus sequences were aligned using ClustalX version 1.81 (Thompson *et al.*, 1997) and similarities and differences identified. Samples were compared both within and between strains.

3.3 RESULTS AND DISCUSSION

3.3.1 Bv80 size analysis

It was essential to characterise the H strain obtained from the Haistings farm for two reasons, firstly to ensure that the isolated field strain was not the vaccine strain and secondly, to investigate the nature of the outbreak. As the animals on the farm were allegedly vaccinated, and if effective, the vaccine strain should not be detected in the blood stream of the animals after the peak reaction. In the unlikely event that the strain had regained virulence and was present at higher concentrations, it would be possible to isolate the vaccine strain in the field strain.

3.3.1.1 Optimisation of the Bv80 PCR reaction

The annealing temperature of the Bv80 PCR reaction was increased from the published 50°C to 55°C to reduce non-specific binding of the primers resulting in smearing. Whilst this increment did eliminate the smearing, the concentration of PCR product was substantially reduced. The annealing temperature was lowered to 53°C, which increased PCR product concentration while still eliminating the non-specific binding.

3.3.1.2 Analysis of Bv80 PCR products

Bv80 PCR products for the H, F and vaccine strains at both the present passage (23) and at passage 11 were resolved on a 2% agarose gel. It was expected that the size difference between the Bv80 bands of the different strains would be substantial enough to allow for easy discrimination between the genotypes present in each strain. From Figure 3.1 it can be seen that the size difference between bands is relatively small. It can also be seen that though faint in some cases, there seems to be more than one genotype donor present per strain except for S23, which contains only one band. The Bv80 PCR does not amplify bovine host DNA as illustrated in

lanes 5 and 6 of Figure 3.1. The S11 (lane 1) vaccine passage is seen to contain two bands of sizes 807 and 721 bp. S23 (lane 2) has one detectable band of 718 bp and the H strain (lane 3) seems to have two bands, a larger faint band at 804 bp, and a slightly smaller, bright band at 753 bp. The F strain (lane 4) has three detected bands of 905 bp, 782 bp and a very faint band at 704 bp.

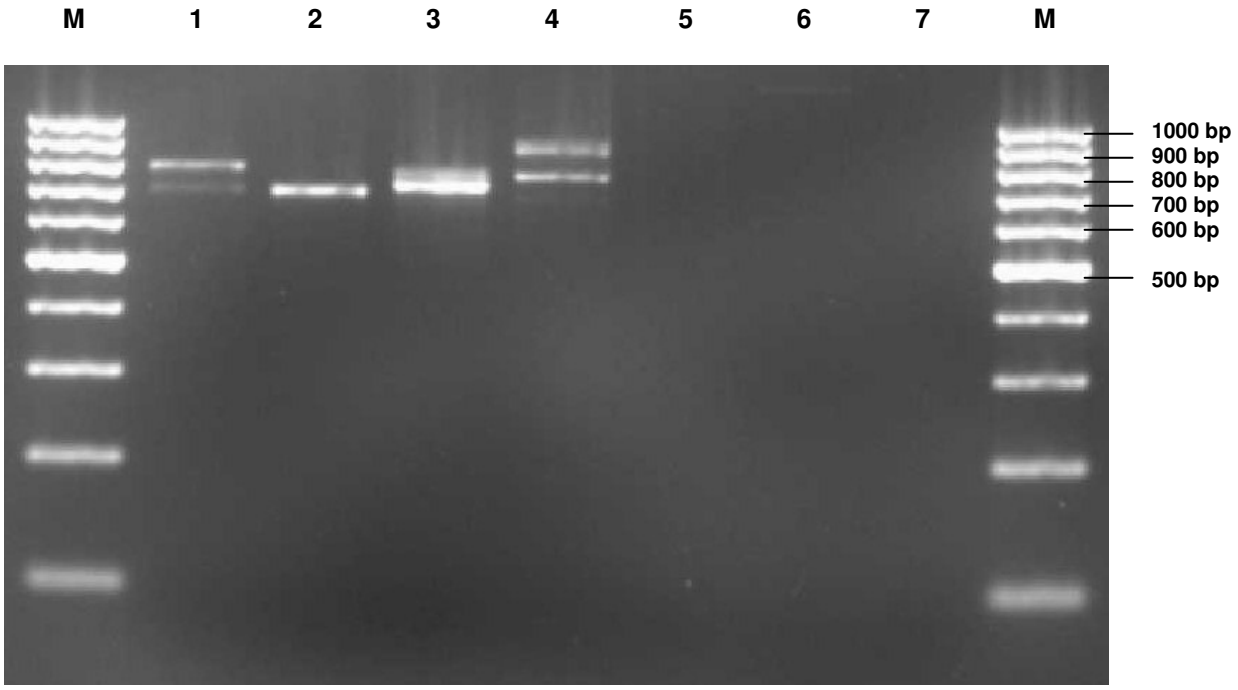

Figure 3.1: Resolution of Bv80 PCR products for vaccine passages S11 and S23, and the H and F strains. 5 µl of PCR product was loaded in each well of a 2% agarose gel. Lane M: 5 µl O' GeneRuler 100 bp DNA Ladder (Fermentas), lane 1: vaccine passage S11, lane 2: vaccine passage S23, lane 3: strain H, lane 4: strain F, lane 5: *B. bigemina* strain G, lane 6: uninfected bovine control, lane 7: negative control.

Due to the cross linking matrix, polyacrylamide gels have smaller pore sizes than agarose gels, enabling better resolution of PCR products. Figure 3.2 shows the resolution of the Bv80 PCR products from each of the four strains on a 15% polyacrylamide gel.

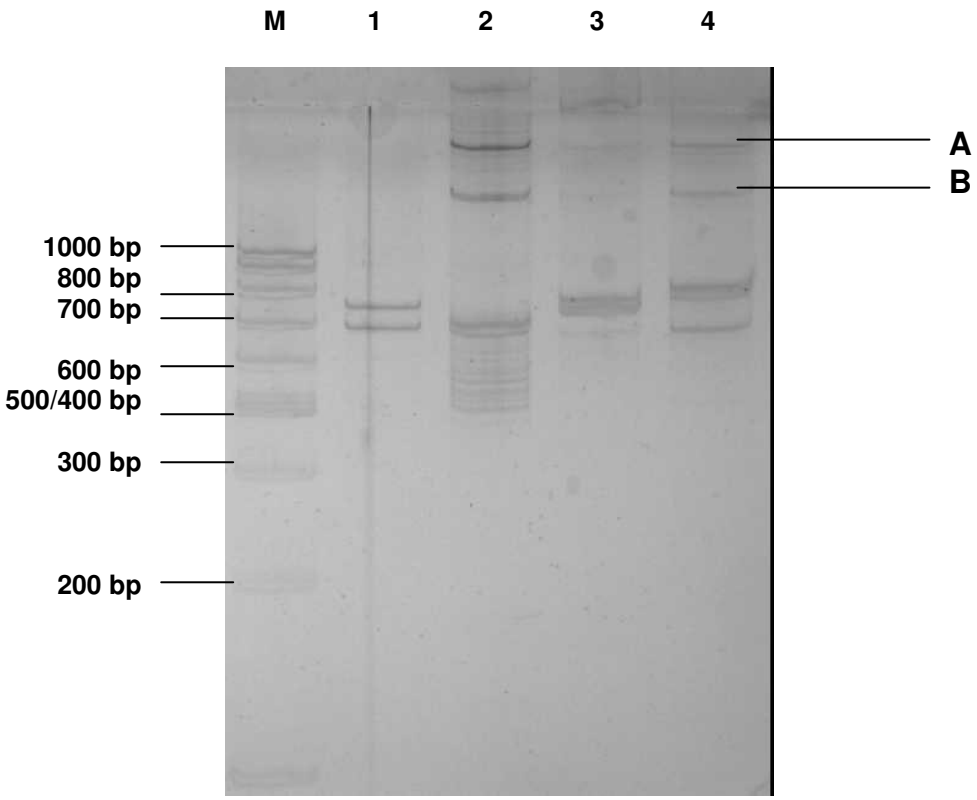

Figure 3.2: Polyacrylamide resolution of Bv80 PCR products for vaccine passages S11 and S23, and the H and F strains. 1 µl of PCR product was loaded in each well of a 15% polyacrylamide resolving gel and visualized using silver staining. Lane M: 0.5 µl O' GeneRuler 100bp DNA ladder (Fermentas), lane 1: vaccine passage S11, lane 2: vaccine passage S23, lane 3: strain H, lane 4: strain F.

From Figure 3.2 it can be seen that again, strains F, H and the S11 passage show more than one Bv80 PCR product per strain. The S11 vaccine passage (lane 1 of Figure 3.2) contains two Bv80 bands, one of which seems to be the same size as the S23 Bv80 band (lane 2). The vaccine from passage 11 to 23 was produced by rapid passage through cattle and it is likely that through this procedure, the most rapidly growing strain was selected for, whilst any slower growing strain comprised less and less of the total population with each passage step till the point where it could no longer be detected. The H strain seen in lane 3 of Figure 3.2 seems to have three donor genotypes, two major genotypes with Bv80 bands close in size and one smaller band which is present at a lower concentration. The F strain contains three

Bv80 bands, one of which seems to be, upon visual analysis, similar to one of the Bv80 bands in the H strain.

The silver staining technique enables minute quantities of DNA to be detected (as little as 15 pg of DNA per well) (Bassam *et al.*, 1991). The advantage of using this staining technique is that minor genotype donors in the strain can be detected. Unfortunately, as a result of this increased sensitivity comes an increase in the detection of non-specific binding products. This non-specific binding can be seen as the double banding seen at points A and B, which represents binding to host DNA. Bands of identical sizes were observed in the silver stained PCR products of uninfected negative animal controls (data not shown). These bands are not detected using ethidium bromide staining due to the low concentration and thus are likely to represent non-specific products rather than Bv80 products of different sizes.

The sizes and numbers of bands present in each strain are difficult to calculate accurately due to the small size differences between bands. Decreasing the volume of DNA loaded per sample would cause the bands of similar sizes to separate from each other instead of merging together into one band. Although decreasing the volume of sample loaded would allow better resolution, diluting too much would cause the fainter Bv80 bands to be undetectable. Three, two fold serial dilutions of the PCR products obtained from each strain were carried out and was determined to be sufficient to allow the bands of similar sizes to separate from each other whilst not eliminating any of the less concentrated Bv80 bands.

Figure 3.3 shows the serial dilution for the S11 and S23 samples. The dilution confirms that the S11 vaccine passage is visually composed of two *B. bovis* strains with Bv80 PCR fragments of 758.66 (759 bp) and 687.86 (688 bp). The S23 passage shows one Bv80 band, implying one detectable strain. The Quantity One

software program (Bio Rad) places the band measure in the center of what it determines to be a band. As the concentration of a band decreases, the center of the band shifts and the size of the S23 Bv80 band changes from 709.74 (710 bp) to 732.9 (733 bp).

As concentration of the band has an effect on the size, a set concentration of PCR product should be loaded for each strain. As three of the four strains contain more than one genotype, loading equal amplicon concentrations would not necessarily result in bands of equal concentrations. Bv80 bands present in lower concentrations may not be detected if a constant concentration is loaded and conversely, if the concentration is increased to include the minor donors then the bands of similar sizes would merge into single bands.

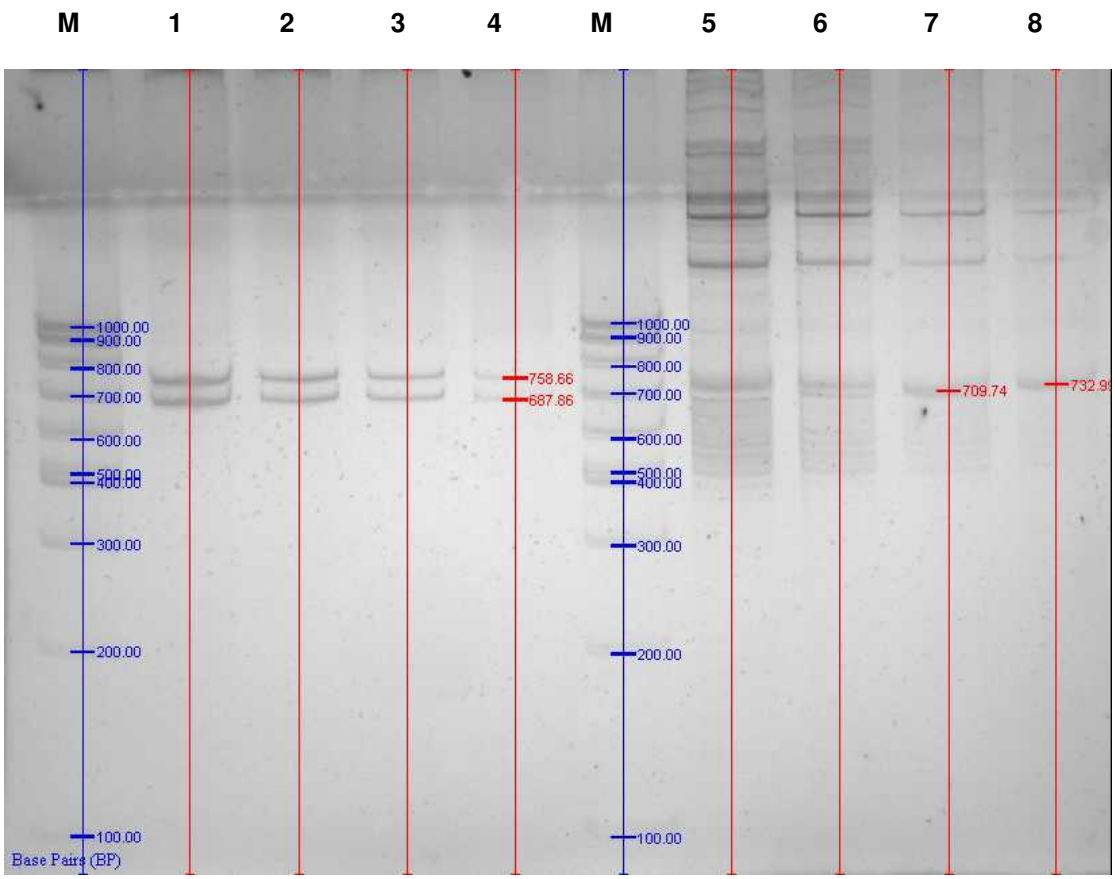

Figure 3.3: Two fold serial dilutions of S11 and S23 vaccine passage Bv80 PCR products. 1 µl of each dilution was loaded into each well of a 15% polyacrylamide gel and visualized using the silver staining technique. Lane M: 0.5 µl O' GeneRuler 100bp DNA ladder (Fermentas), lane 1: S11 2^0, lane 2: S11 2^1, lane 3: S11 2^2, lane 4: S11 2^3, lane 5: S23 2^0, lane 6: S23 2^1, lane 7: S23 2^2, lane 8: S23 2^3.

The size difference between the 'carry through' Bv80 product from S11 and S23 is 22 bp if one takes the smallest band values for each strain. Irrespective of the change in the size of the band in the S23 passage with concentration, the size of the 'carry through' band is not the same in the S11 and S23 vaccine passages at any dilution. Either the PCR product does not represent Bv80 bands from the same strain or the gene is not stable over prolonged periods of time as previously thought. As the strains were syringe passaged through animals, there was no chance for a sexual reproduction cycle, which occurs in the tick vector (Potgieter, 1977) to take place and

therefore the size difference could not be as a result of genetic recombination with another strain. Another possibility could be that the S23 strain was present at such a low concentration in the S11 vaccine passage that it was not detected. The relative concentration could then have increased with each successive passage till it became the major genotype donor in the S23 passage.

It must also be noted that in addition to size, separation on polyacrylamide gels is affected by the composition of the sequence, with Adenine (A) and Thymine (T) rich sequences migrating at a slower rate than Guanine (G) and Cytosine (C) rich sequences (Westermeier, 1997). This may explain the observed difference in band sizes using agarose and polyacrylamide gels. This affect on migration may also be the cause of the three bands observed for the F strain using agarose gel analysis to merge into two using polyacrylamide analysis. Lew *et al.* (1997 b) did also note discrepancies in the sizes and separation of Bv80 bands between gels but decided that the Bv80 and BvVA1 PCR assays used in conjunction offered a greater ability to distinguish between populations than either assay being used as the sole method of identification.

Figure 3.4 shows the serial dilutions for strains H and F. As expected, the less concentrated band in the H strain is not detected as the sample is diluted. The three bands are estimated to be 677, 743 and 790 bp respectively. The F strain is comprised of two Bv80 products of 703 and 828 bp with the 828 bp band showing a higher band intensity and thus concentration. The hypothesis that there was a shared Bv80 band between the H and F strains could not be confirmed using size analysis on polyacrylamide gels.

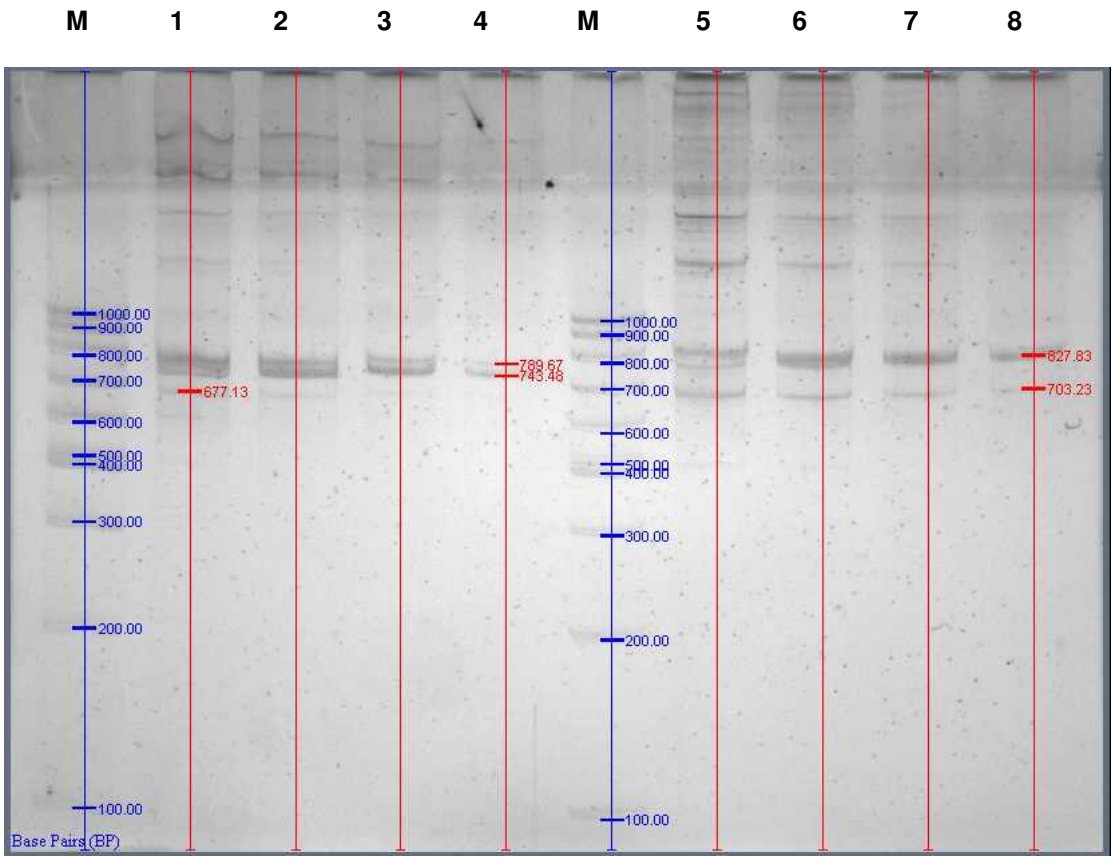

Figure 3.4: Two fold serial dilutions of H and F strain Bv80 PCR products. 1 µl of each dilution was loaded into each well of a 15% polyacrylamide gel and visualized using the silver staining technique. Lane M: 0.5 µl O' GeneRuler 100bp DNA Ladder (Fermentas), lane 1: H 2^0, lane 2: H 2^1, lane 3: H 2^2, lane 4: H 2^3 Bv80, lane 5: F 2^0, lane 6: F 2^1, lane 7: F 2^2, lane 8: F 2^3.

Table 3.6 summarises the number and sizes of each of the Bv80 bands for S11, S23, H and F using both polyacrylamide and gel electrophoresis. In the case of the S23 vaccine passage, the Bv80 band showed a change in size depending on the concentration of the Bv80 PCR product and the 710 bp measurement was taken as the band size.

Table 3.6: Summary of Bv80 band sizes calculated for vaccine passages S11, S23 and the H and F strains using agarose and polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis		
Strain	**Number of Bv80 bands**	**Sizes of Bv80 bands (bp)**
S11	2	759, 688
S23	1	710
H	3	677, 743, 790
F	2	703, 828
Agarose gel electrophoresis		
Strain	**Number of Bv80 bands**	**Sizes of Bv80 bands (bp)**
S11	2	807, 721
S23	1	718
H	2	804, 753
F	3	905, 782, 704

Despite the variation in the size of the S23 Bv80 band through the serial dilutions, a band of the same or similar size cannot be seen in the H strain, indicating that the vaccine strain is not present at high concentration within the H strain. This implies that the S23 strain could not be responsible for the outbreaks in the region with the hypothesis that the strain reverted to a more virulent form. If the vaccine strain had increased in virulence to an extent sufficient enough to cause disease symptoms, it would be detected using the Bv80 PCR due to the high parasitemia levels. The two remaining possible causes for the outbreaks are a virulent field strain breaking through the vaccine protection or true vaccine failure. As the H strain was deemed non-virulent in susceptible intact cattle using needle challenge, it is not likely that it was as a result of virulent field isolate break through. As highlighted in chapter one, there are a number of factors involved in the virulence of an isolate such as the method of infection and the overall well being of the animal. Under stressful conditions, the H strain may induce more severe disease symptoms mimicking those seen in the infected animals in the field.

The other scenario to be investigated was whether the vaccine was transported and administered correctly to allow for sufficient protection to be achieved. Any disruption to the cold chain during transport, storage and administration would render the vaccine useless. Figure 3.5 shows a vaccine sample upon arrival at Swartberg, KwaZulu-Natal with a compromised box structure and depleted dry ice. This vaccine batch should not be administered and should instead be discarded but farmers often use vaccine samples such as these.

Figure 3.5: *Babesia bovis* vaccine batch on arrival at Swartberg region showing depleted dry ice and compromised box structure (pictures courtesy of M.P. Combrink).

During a visit to the Haistings and Killrush farms, treatment of entire herds with diminazine (irrespective of the presence of disease symptoms or circulating parasites) by farm laborers was observed. If vaccination of animals with viable parasites was followed by indiscriminate treatment with babesiacides, the animals would not receive sufficient exposure to parasites to allow the development of immunity and a seemingly non-virulent field isolate could cause disease symptoms of increased severity.

The difference in size of the Bv80 bands between Australian genotypes ranged from 500 bp to 820 bp (Lew *et al*., 1997 a), which was larger than was seen for the four South African genotypes analysed where the difference only ranged from 703 to 828 bp. The small size differences, coupled with the difficulty in accurately determining band sizes lead to the conclusion that the Bv80 PCR test cannot be used as sole proof that the vaccine strain was not present in the H strain. Sequence analysis of both the Bv80 gene and 18S rRNA V4 hypervariable region would shed light not only on the presence of the vaccine strain in the H strain but also the relationship between size and sequence differences.

3.3.2 Clone and sequence analysis

As seen from the polyacrylamide gels in Figures 3.3 and 3.4, three of the four strains contain more than one *B. bovis* genotype. The S11 vaccine passage and F strain contain two genotypes whilst the H strain is seen to have three. Direct sequencing of the PCR product was not possible as there was a mixed template present in the PCR reaction. In addition to this, unlike PCR primers, sequencing primers have to bind at least 50 bp upstream of the region to be sequenced otherwise the smaller, initial fragments cannot be detected by the machine. The PCR products were therefore cloned into the pGEM-T Easy vector.

Despite the presence of blue/white screening, an effect caused by the interruption of the *Lac* Z gene as a result of the presence of an insert in the vector, the formation of white colonies may occur without the presence of a insert. Incomplete PCR products can be ligated to the vector backbone and as competent cells preferentially take up smaller DNA fragments, the presence of clones consisting of vector without an insert or only a partial insert is a possible occurrence. To detect which clones contained inserts of the correct size (between 500 and 700 bp depending on the nature of the

insert), all clones were restricted in two separate reactions with *Eco* RI and *Acc* I restriction endonucleases. *Eco* RI recognition sequences are located either side of the insertion site (at positions 52 and 70) of the vector. Restriction with *Eco* RI causes the insert, if present, to be released from the vector backbone. When resolved on an agarose gel, two bands should be present, one between 500 and 700 bp representing the insert and another at 3015 bp representing the linearised vector.

As the ligation of the vector to itself, producing white colonies, is a possibility, the clones are restricted with an enzyme which has only one recognition sequence located within the vector sequence and is unlikely to be found within the insert sequence. If the insert is of unknown sequence, the presence of recognition sites may not be known but can be determined to a certain degree by looking at closely related gene sequences. In this case *Acc* I was chosen as it has only one recognition site within the pGEM-T Easy vector sequence (at position 91) and analysis of available Bv80 gene sequences indicated that it was not present within the gene sequence.

Plasmid DNA occurs in three structural forms, linear, circular and supercoiled and three pieces of DNA of the same size will not migrate through a gel matrix at the same rate in each of these forms. Supercoiled DNA is more compact and migrates faster than circular DNA which in turn migrates faster than the more bulky linear DNA. Resolution of the supercoiled clone DNA would give an inaccurate representation of its size, being smaller than its actual size. For this reason, all clones were linearised with *Acc* I to be able to accurately determine the size of each of the clones.

Figure 3.6 A and B shows the *Eco* RI restriction for vaccine passage S11. A 3kb fragment can be seen as well as a faint 800 bp insert. As fluorescent intensity is

related to the size of the DNA fragment, large fragments show stronger fluorescent intensity than smaller fragments. The 800 bp inserts cannot be readily seen, due to their size and concentration, except for those marked in the block. Figure 3.6 B shows the inverted version of Figure 3.6 A with the insert being slightly more visible.

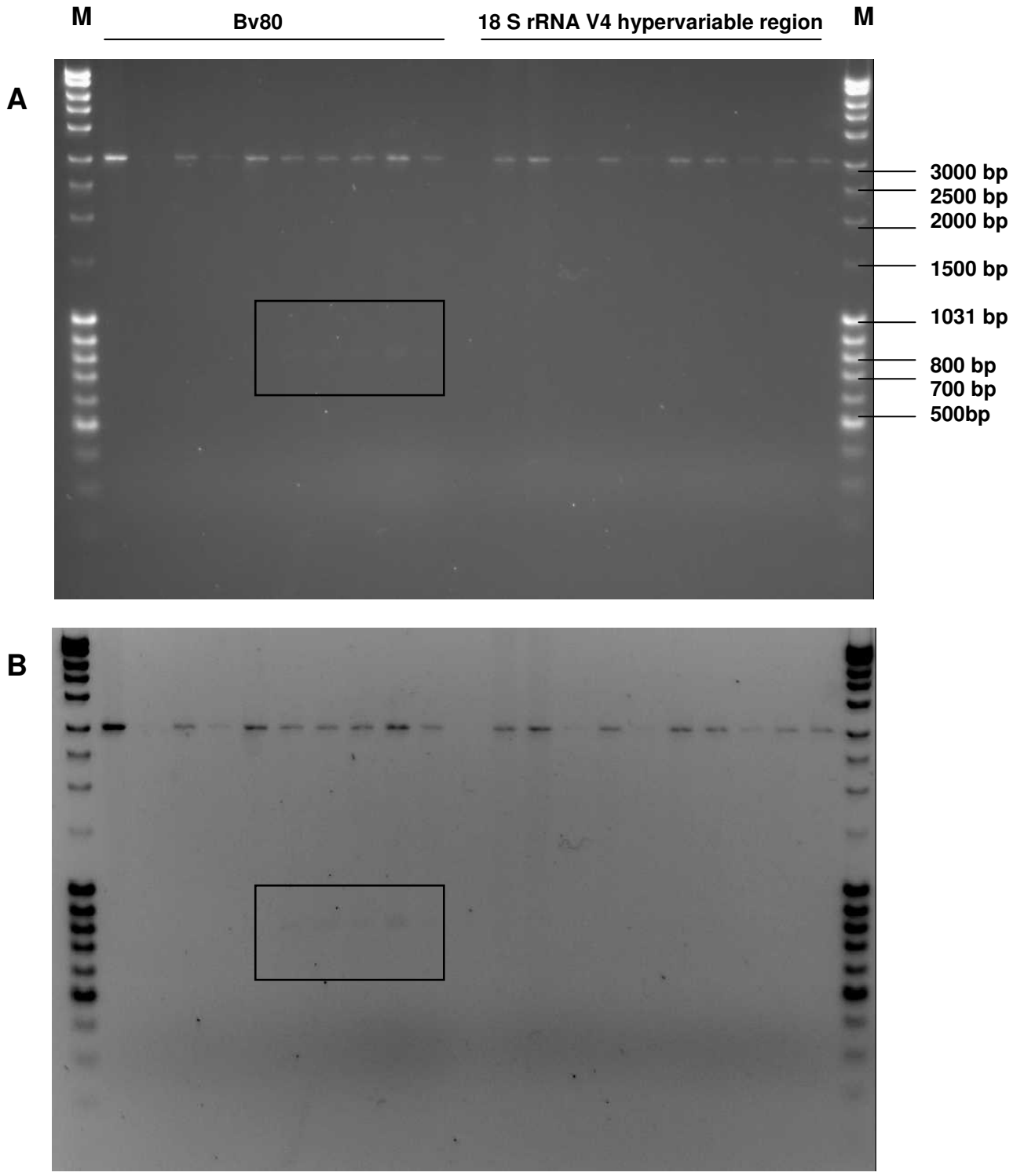

Figure 3.6: Resolution of S11 vaccine passage clones with Bv80 and 18S rRNA V4 hypervariable region inserts restricted with *Eco* RI. Lanes M: 5 µl MassRuler DNA Ladder Mix (Fermentas).

Figure 3.7 shows the *Acc* I restriction digests of 18S rRNA V4 hypervariable region or Bv80 clones for the H strain. The difference between clones with and without inserts can be seen in lanes 1 and 2 of Figure 3.7 A and B. The vector in lane 1 is 3kb while the clone in lane 2 is just over 4kb. The 3kb band represents a vector that had ligated to itself whilst the 4 kb clone indicates the vector with a 700 bp insert.

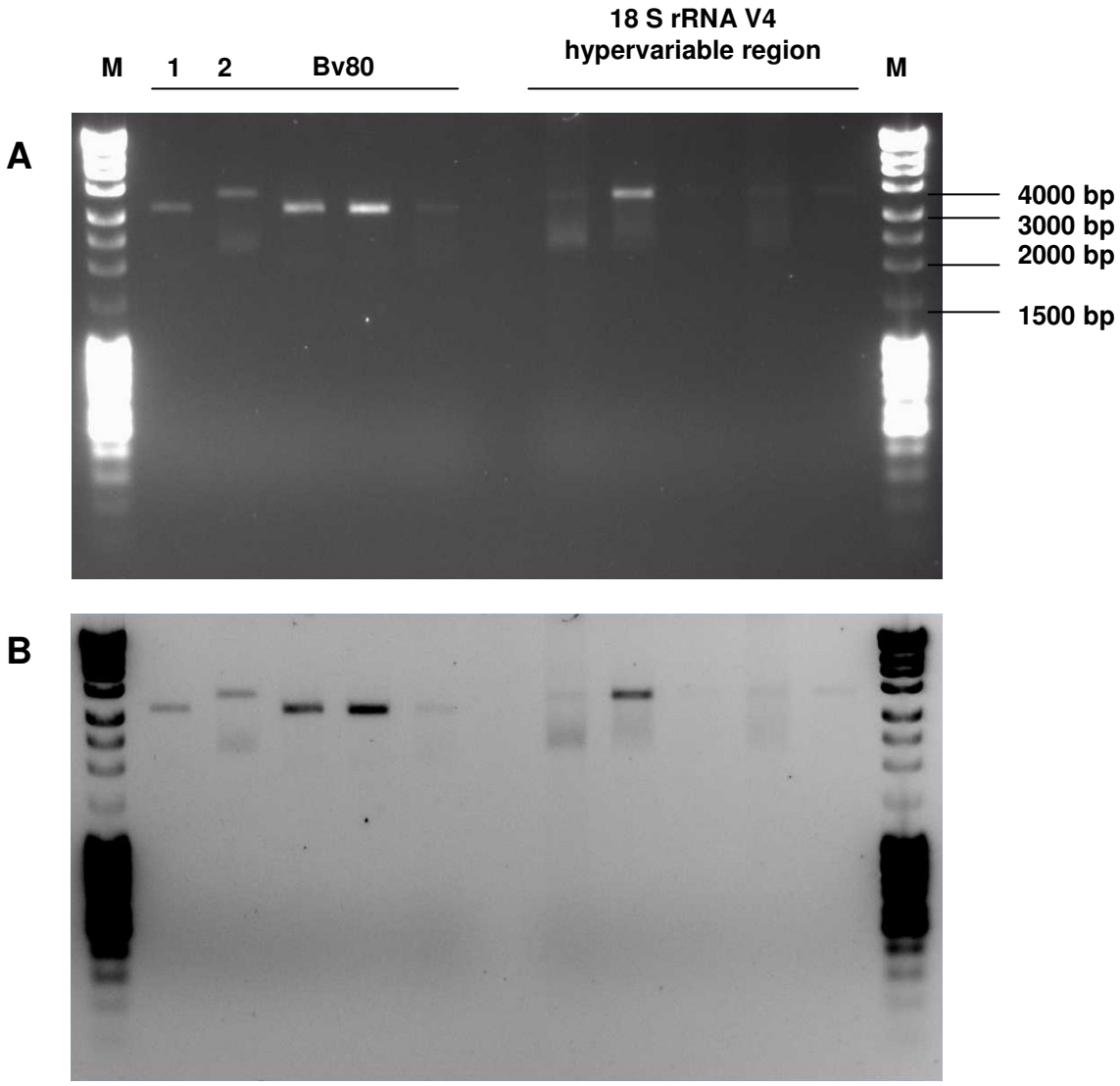

Figure 3.7: Resolution of H strain clones with 18S rRNA V4 hypervariable region or Bv80 inserts restricted with *Acc* I. Lane M: 5 µl MassRuler DNA Ladder Mix (Fermentas), lane 1: clone H_18S_1 restricted with *Acc* I, lane 2: clone H_18S_2 restricted with *Acc* I.

Clones for each strain with a 3 kb *Eco* RI (with or without a visible insert) and a 4 kb *Acc* I profile were chosen for DNA sequencing.

Consensus sequences were obtained for each of the clones and the orientation of the insert checked using the positions of the forward and reverse PCR primers. In the event that sequences were inverted, the reverse compliment sequences were obtained using BioEdit. Sequences were checked against the Blast database of gene sequences using blastn (Altschul *et al.*, 1990). All 18S rRNA V4 hypervariable region inserts were most similar to published *B. bovis* 18S ribosomal RNA sequences (with an average of 98.5% sequence identity with an E value of 0.0) and all Bv80 inserts were most similar to *B. bovis* 85 kDa merozoite protein gene (complete coding sequence) (M99575.1), *B. bovis* Bv80 merozoite protein gene (partial coding sequence) (AY727909.1), *B. bovis* 80 kDa merozoite protein (C6A allele, complete coding sequence) (M93125.1) and *B. bovis* 80 kDa merozoite protein (C1B allele, complete coding sequence) (M93126.1) with an average of 83% sequence identity and an E value of 0.0. This implies that the sequences being analysed have been amplified from the correct organism and target genes.

3.3.2.1 Vaccine passage S11 and S23 Bv80 and 18S rRNA V4 hypervariable region sequence analysis

Analysis of the 18S rRNA V4 hypervariable region of the vaccine strain at passage 11 and 23 indicated that there were three genotypes present in the S11 passage, none of which were identical to the S23 passage. Figure 3.8 shows the multiple sequence alignment of the 18S rRNA V4 hypervariable region for the S11 and S23 passages. Primer sequences are indicated in blue and sequence differences are highlighted. The strains seem to be closely related with only a few point differences between the sequences identified. The S23 strain must have come from one of the

parasite populations present in the S11 passage as it has a known history of passage through animals. The relative concentrations of the parasite populations present at each stage of the vaccine strain may explain why the S23 strain is not detected in the S11 passage.

Using Bv80 size analysis, only two PCR products could be detected on a gel for S11 vaccine passage. Likewise, two Bv80 sequence variants could be detected but three 18S rRNA V4 hypervariable region sequences were found. The third Bv80 gene sequence may be the same size as one of the other Bv80 sequences and thus not recognisable using gel analysis. The detection of only two Bv80 sequences may be as a result of the third strain donor being present at a very low concentration, remaining undetected on a gel and making detection using the screening of clones difficult.

The genotypes represented by clones S11_2 and S11_4 seem to be very closely related with only three point differences. Interestingly, most of the regions of difference show a division of the four genotypes between two base choices. This occurs for all the regions of difference except for the region between 283 and 286 bp which shows more variation. All three S11 clone sequences seem similar to each other and markedly different from the S23 clone which shows ten points where it does not show any identity with any of the S11 clones (marked with ^).

```
S11_3   ---------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
S11_4   ---------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
S11_2   ---------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
S23_2   ---------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
                                   **********************************

S11_3   GGGGCCACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
S11_4   GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
S11_2   GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
S23_2   GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
        ***** ************************************************************

                                                    ^
S11_3   GAGGGCAAGTCTGGTGCCAGCGGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
S11_4   GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
S11_2   GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
S23_2   GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGATCCAATAGCGTATATTAAACT
        ********************** ********** ********** **********

                                                  ^^^ ^  ^^      ^ ^^
S11_3   TGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCCCCGCAGTGTCCTTTCCTCGCC
S11_4   TGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCCCCGCAGTGTCCTTTCCTCGCC
S11_2   TGTTGCAGTTAAAAAGCTCGTAGTTGTACTTCACGTCCCCCGCTTGGTCCTTTCCTCGCC
S23_2   TGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCGCC--CTAGTGTTTTCCACTAC
        ************************** *  ******** **     **   ***** *  *

         ^
S11_3   G-GGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
S11_4   G-GGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
S11_2   G-GGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
S23_2   GCGGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
        * ************************************************************

S11_3   TGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTAATGGTT
S11_4   TGAGCATGGAATAGCCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTAATGGTT
S11_2   TGAGCATGGAATAACCTTGTATGACCCTGTCGTGCCGTTGGTTGGCTTTGGGTAATGGTT
S23_2   TGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGGCTTTGGGTAATGGTT
        ************* ****************** ********** **************
```

```
S11_3          AATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG-------
S11_4          AATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG-------
S11_2          AATAGGAACGGTCGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG-------
S23_2          AATAGGAACGGTCGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG-------
               ************ ****************************************
```

Figure 3.8: Clustal 1.81X multiple sequence alignment of the 18S rRNA V4 hypervariable regions for vaccine passages S11 and S23. Primer sequences are shown in blue and base differences in green (T), yellow (C), red (G) and purple (A).

Bv80 analysis indicated that the S11 passage contained two strains, none of which were the same as the S23 vaccine passage sequence. Bv80 sequence analysis revealed both point differences between the clones as well as size differences. It is important to note that changes in the sequences as well as the size of the gene would indicate a strain difference as it is impossible for a single copy gene to have two different sequences in a haploid organism. If two parasite populations had Bv80 genes of the same size with different repeat sequences, they would only be detected as one genotype by size discrimination. Using both size and sequence differences, two Bv80 genotypes were identified in the S11 passage and one in the S23 passage. Again, the S23 sequence was different from the S11 passage sequences both in size and sequence. The S23_2_8 clone showed repeat similarity to clone S11_3_8 but had a different size. Figure 3.9 shows the multiple alignment of the Bv80 sequences for vaccine passages S11 and S23. Unfortunately, despite repeated sequencing of multiple clones, the full length sequence of the S23 Bv80 clone could not be obtained. In general, sequencing of Bv80 clones was problematic with the insert appearing to change in size with each sequence reaction. Possible formation of secondary structures due to the repeats could explain why sequencing of the Bv80 PCR products was difficult.

```
S11_3_8     ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
S11_5_8     ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
S23-2-8     ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
                                               * ************************

S11_3_8     TCCCACCATCCCTGAGCAGCCAGTAGTGGAGCCCACTGAAGAGCCGGCTGGCGTCACCGA
S11_5_8     TCCCACCATCCCTGAGCAGCCAGTTG---------CTGAAGAACCATCTGATGTCACCGT
S23-2-8     TCCCACCATCCCTGAGCAGCCAGTAGTGGAGCCCACTGAAGAGCCGGCTGGCGTCACCGA
            ************************ *          ******* **  ***  *******

S11_3_8     AACTGCTCCGGAGGAGTGTGAGGAAGAAATTGTTATCAATCCAGAGGAAGAAAACAAATC
S11_5_8     AACTGCTCCCGAAGAGTGTGAGGAAGAAGTTGTTATTAATCCAGAGGAAGAAAACAAACC
S23-2-8     AACTGCTCCGGAGGAGTGTGAGGAAGAAATTGTTATCAATCCAGAGGAAGAAAACAAATC
            ********* ** *************** ******* ******************** *

S11_3_8     TGACTCCTCCTCCTCTTCTTCTTCTTCATCATCATCTTCCTCAGATTCTGATTCAGA
S11_5_8     TGACTCCTCCTCCTCCTCATCTTCTTCTTCATCATCGTCTTCTTCAGAATCGGATTCAGA
S23-2-8     TGACTCCTCCTCCTC------TTCTTCTTCATCATCATCTTCCTCAGATTCTGATTCAGA
            ***************      *************** ***** ***** ** ********

S11_3_8     TGAAGAGGATAGGGAACCCATTGTTGAGGAGCCCATTGCTGAGGAGCCCGTTGCTGAGGA
S11_5_8     TGAAGAGGATAGGGAACCCATTGTTGAGGAGCCAGTTGTTGAGGAGCCAGTTGTTGAGGA
S23-2-8     TGAAGGGGATAGGGAACCCATTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGGA
            ***** ***************** *********  *** ********* **** ******

S11_3_8     GCCCATTGCTGAGGAACCCGTTGCTGAGGAACCCGTTGCTGAGGAACCCGTTGCTGAGGA 360
S11_5_8     GCCAGTTGTTGAGGAGCCAGTTGTTGAGGAGCCAGTTGTTGAGGAACC---TGCTGAGAA
S23-2-8     GCCCGTTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGGAGCC---CGTTGAGAC
            ***  *** ****** ** **** ****** ** **** ****** **    * ****

S11_3_8     ACCCGTTGCTGAGGAACCCGTTGCTGAGGAACCCATTGCTGAGGAACCCATTGCTGAGGA
S11_5_8     ACC---TGCTGAGAAACC---TGCTGAGAAACC---TGCTGAGACTCCC---GCTGAGAA
S23-2-8     TCC---CGTTGAGACTCC---CGCTGAGAAGCC---TGCAGTCACTTCT-------TGCA
             **    * ****   **    ****** * **   *** *      *         * *

S11_3_8     ACCCATTGTTGAGGAGCCCATTGCTGAGGAGCCCATTGCTGAGGAGCCCATTG------C
S11_5_8     ACC---TGCTGAGAAACC---TGCTGAGAAACC---TGCTGAGAAACCCGCTGAGACTCC
S23-2-8     ATCTTGTACCCAAATG-----TGGCAAAGTGCTCGTTCTCCAAACTACCAGTA------C
            * *   *    *         **   *    *    *    *     **  *       *
```

```
S11_3_8       TGAGGAGCCCATTGCTGAGGAGCC--CATTGCTGAGGAGCCCGTTG------CTGAGGAG
S11_5_8       CGCTGAGACTCCCGCTGAGAAACC--C---GCTGAGAAACCCGCTGAGAAACCCGCTGAG
S23-2-8       GAAACAAACACCCCTTAAGGAGTTAGCTATAGTGACAGGTCCATCAACTAACAGGCTTTA
                    *   *       * ** *      *      ***      **            *

S11_3_8       CCCGTTGCTGAGGAGCCCGTTGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCTGCT
S11_5_8       ACTCCCGCTGAGAAACCCG---CTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCT
S23-2-8       ATCACT------------------------------------------------------

S11_3_8       GAGACTCCCGCTGAGACTCCCGCTGAGACTCCTGCTGAGACTCCCGCTGAGAAACCTGCT
S11_5_8       GAGACTCCCGCTGAGACTCCCGCTGAGAAACCCGCTGAGAAACCCGCTGAGAAACCTGCT
S23-2-8       ------------------------------------------------------------

S11_3_8       GAGAAACCTGCTGAGAAACCCGCTGAGAAACCCGCTGAGAAGCCTGCAGTCGCTTCTTGT
S11_5_8       GAGACTCCCGCTGAGAAACCTGCTGAGACTCCCGCTGAGAAACCCGCATCCAGACCTTGT
S23-2-8       ------------------------------------------------------------

S11_3_8       AATCTTGTACCCAAATGTGGCAAAGTGCTCGTTCTCCAAACTAGCAGTAAGGAGCAAACA
S11_5_8       TACGGTGGTCGCAAAGGTGAAGAAGTGGTCGTCCTTCAAACTACCAGTACAAAGCAAACA
S23-2-8       ------------------------------------------------------------

S11_3_8       CCCCTTAAGGAGTTAGCTATAGTGACAGGTCCATCAACTAACAGGCTTT---------
S11_5_8       CCACTTAAGGAGTTACCTGTAGTGAAAGGTCCATCAACTAACAGGCTTT---------
S23-2-8       ------------------------------------------------------------
```

Figure 3.9: Clustal 1.81X multiple sequence alignment of Bv80 sequences for vaccine passages S11 and S23 with deviations in the repeats highlighted. Primer sequences are shown in blue.

Despite the inability to obtain the full length S23 Bv80 sequence, it can still be seen that there is a substantial difference between the Bv80 sequences obtained from passages S11 and S23. The size difference observed on a gel is seen here as regions of sequences either absent or present (dependant on the genotype), rather

than point insertions or deletions. These regions of insertions are scattered through the gene and vary in size.

In the repeat regions, the repeats are very similar with only point differences being observed. If differences occur in the repeats, these differences are carried through successive repeats, indicating that it is not a result of incorrect sequence amplification and analysis. Clone S11_5_8 shows a repeat different from all the other clones, highlighted in Figure 3.9 (around 360bp). These repeats form part of larger repeating units, characteristic of the gene.

3.3.2.2 H strain Bv80 and 18S rRNA V4 hypervariable region sequence analysis.

Analysis of the H strain 18S rRNA V4 hypervariable region showed a high degree of sequence homology within genotype sequences, with point differences indicating the presence of three genotypes in the strain. Compared to the S23 strain 18S rRNA V4 hypervariable region, there is a high degree of deviation between the sequences, confirming that the S23 strain is not present in the H strain. Figure 3.10 shows the multiple sequence alignment of the 18S rRNA V4 hypervariable regions for the H strain and vaccine passage S23. If the vaccine strain had increased in virulence, one would expect it to be present at higher levels and detectable in the H strain using PCR analysis. The absence of the vaccine strain in the H strain indicates that the vaccine strain had not reverted back to a virulent state and provides assurance of the isolation of a true field strain.

```
H_5       ------------------------------GACACAGGGAGGCAGTGACAAGAGATACC
S23_2     ------------------------------GACACAGGGAGGTAGTGACAAGAAATACC
H_2       ------------------------------GACACAGGGAGGTAGTGACAAGAAATACC
H_3       ------------------------------GACACAGGGAGGTAGTGACAAGAAATACC
                                         ************ ********** *****

H_5       AATACGGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACC
S23_2     AATACGGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACC
H_2       AATACGGGGCTACTGTTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACC
H_3       AATACGGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACC
          *************** ****************************************

H_5       CATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT
S23_2     CATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGATCCAATAGCGTATATT
H_2       CATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCGGCTCCAATAGCGTATATT
H_3       CATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATT
          **************************************** * ****************

H_5       AAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCC-CCGCGTGGTCCTTTC
S23_2     AAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCG-CCC--TAGTGTTTTC
H_2       AAACTTGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCCGCCCCAGTGTCCTTCA
H_3       AAACTTGTTGCAGTTAAAAAGCTCGTAGTTGTACTTTATGTCCC-CCGCAGTGTTCTTCA
          ******************************** *  * * ****  **     **  **

H_5       CTCGCCG-GGACGCCTCGTTACTTTGAGAAGATTAGAGTGTTTCAAGCAGGTTTCGCCTG
S23_2     CACTACGCGGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTG
H_2       CTTGGCG--GACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTG
H_3       CTTGGCG--GACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTG
          •     **  ********************* ******************************

H_5       TATAATTGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTA
S23_2     TATAATTGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGGCTTTGGGTA
H_2       TATAATTGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGGCTTTGGGTA
H_3       TATAATTGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTA
          ************************************************* **********
```

```
H_5             ATGGTTAATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
S23_2           ATGGTTAATAGGAACGGTCGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
H_2             ATGGTTAATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
H_3             ATGGTTAATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
                ****************** ****************************************
```

Figure 3.10: Clustal 1.81X multiple sequence alignment of 18S rRNA V4 hypervariable regions for strain H and S23. Primer sequences are shown in blue and base differences in green (T), yellow (C), red (G) and purple (A).

Bv80 sequence analysis indicated the presence of three Bv80 sequences in the H strain, two near identical sequences (clones H_2_8 and H_4_8) which differ at only one base point (indicated as ^, Figure 3.10) and a different sized sequence (clone H_5_8) while gel analysis indicated the presence of three bands of different sizes. The S23 Bv80 sequence does not match any of the H strain Bv80 clones analysed, again confirming that it is not present in the H strain. Figure 3.11 shows the Bv80 multiple alignment for the H strain compared to S23.

```
H_2_8           ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
H_4_8           ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
H5-8            ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
S23-2-8         ----------------------------------TGTGTTAATGTAACTCAGCCCGCCAT
                                                  *    ****************************

H_2_8           TCCCACCATCCCTGAGCAGCCAGTTG---------CTGAAGAACCATCTGATGTCACCGT
H_4_8           TCCCACCATCCCTGAGCAGCCAGTTG---------CTGAAGAACCATCTGATGTCACCGT
H5-8            TCCCACCATCCCTGAGCAGCCAGTTG---------CTGAAGAACCATCTGATGTCACCGT
S23-2-8         TCCCACCATCCCTGAGCAGCCAGTAGTGGAGCCCACTGAAGAGCCGGCTGGCGTCACCGA
                ************************ *          ******* **   ***   *******

H_2_8           AACTGCTCCCGAAGAGTGTGAGGAAGAAGTTGTTATTAATCCAGAGGAAGAAAACAAACC
H_4_8           AACTGCTCCCGAAGAGTGTGAGGAAGAAGTTGTTATTAATCCAGAGGAAGAAAACAAACC
H5-8            AACTGCTCCCGAAGAGTGTGAGGAAGAAGTTGTTATTAATCCAGAGGAAGAAAACAAACC
S23-2-8         AACTGCTCCGGAGGAGTGTGAGGAAGAAATTGTTATCAATCCAGAGGAAGAAAACAAATC
                ********* ** *************** ******* ********************** *
```

```
H_2_8       TGACTCCTCCTCCTC---ATCTTCTTCTTCATCATCGTCTTCTTCAGAATCGGATTCAGA
H_4_8       TGACTCCTCCTCCTC---ATCTTCTTCTTCATCATCGTCTTCTTCAGAATCGGATTCAGA
H5-8        TGACTCCTCCTCCTCCTCATCTTCTTCTTCATCATCGTCTTCTTCAGAATCGGATTCGGA
S23-2-8     TGACTCCTCCTCCTC------TTCTTCTTCATCATCATCTTCCTCAGATTCTGATTCAGA
            ***************      *************** ***** ***** ** ***** **

H_2_8       TGAAGAGGATAGGGAACCCATTGTTGAGGAACCCATTGTTGAGGAGCCCATTGTTGAGGA
H_4_8       TGAAGAGGATAGGGAACCCATTGTTGAGGAACCCATTGTTGAGGAGCCCATTGTTGAGGA
H5-8        TGAAGAGGATAGGGAACCCATTGTTGAGGAGCCAGTTGTTGAGGAGCCAGTTGTTGAGGA
S23-2-8     TGAAGGGGATAGGGAACCCATTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGGA
            ***** ***************** ****** **  *** *********  *** ******

H_2_8       GCCCGTTGTTGAGGAGCCCGTTGTTGAGGAACCAGTTGTTGAGGAACCTGCTGAGACTCC
H_4_8       GCCCGTTGTTGAGGAGCCCGTTGTTGAGGAACCAGTTGTTGAGGAACCTGCTGAGACTCC
H5-8        GCCAGTTGTTGAGGAGCCAGTTGTTGAGGAGCCAGTTGTTGAGGAACCTGCTGAGAAACC
S23-2-8     GCCCGTTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGAGACTCC
            *** **** ********* **** ****** ** **** ****** ** * *****  **

                                                                 ^
H_2_8       TGCTGAGACTCCTGCTGAGAAACCTGCTGAGAAACCTGCTGAGAAACATGCTGAGAAACC
H_4_8       TGCTGAGACTCCTGCTGAGAAACCTGCTGAGAAACCTGCTGAGAAACCTGCTGAGAAACC
H5-8        TGCTGAGAAACCTGCTGAGAAACCTGCTGAGACTCCCGCTGAGAAACCTGCTGAGAAACC
S23-2-8     CGTTGAGACTCCCGCTGAGAAGCCTGCAGTCA---CTTCTTGCAATCTTGTACCCAAATG
             * *****  ** ******** ***** *  *   *  **   ** * **     ***

H_2_8       CGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACC
H_4_8       CGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACC
H5-8        TGCTGAGAAACCTGCTGAGAAACCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGAAACC
S23-2-8     TGGCAAAGTGCTCGTTCTCCAAACTACCAGTA---CGAAACAAACACCCCTTAAGGAGTT
             *   *    *  * *    ** *  *    *   *     * *  ***  * ** *

H_2_8       CGCTGAGAAACCCGCTGAGAAACCTGCTGAGA----------AACCTGCTGAGACTCC
H_4_8       CGCTGAGAAACCCGCTGAGAAACCTGCTGAGA----------AACCTGCTGAGACTCC
H5-8        CGCTGAGAAACCCGCTGAGAAACCCGCTGAGACTCCCGCTGAGAAACCCGCTGAGACTCC
S23-2-8     AGCTATAG--------TGACAGGTCCATCAACT----------AACAGGCTTTAATCAC
             ***           *** *   *     *              ***  ***   *   *
```

```
H_2_8          CGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGAAACC
H_4_8          CGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGAAGCC
H5-8           CGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGAAACC
S23-2-8        T-----------------------------------------------------------

H_2_8          CGCTGAGAAACCCGCTGAGAAACCCGCTGAGACTCCCGCTGAGAAACC----------
H_4_8          CGCTGAGAAACCCGCTGAGAAACCCGCTGAGACTCCCGCTGAGAAACC----------
H5-8           CGCTGAGAAACCCGCTGAGAAACCTGCTGAGACTCCCGCTGAGAAACCTGCTGAGACTCC
S23-2-8        -----------------------------------------------------------

H_2_8          ----------CGCATCCAGACCTTGTTACGGTGGTCGCAAAGGTGAAGAAGTGGTCGT
H_4_8          ----------CGCATCCAGACCTTGTTACGGTGGTCGCAAAGGTGAAGAAGTGGTCGT
H5-8           CGCTGAGAAACCCGCATCCAGACCTTGTTACGGTGGTCGCAAAGGTGAAGAAGTGGTCGT
S23-2-8        -----------------------------------------------------------

H_2_8          CCTTCAAACTACCAGTACAAAGCAAACACCACTTAAGGAGTTACCTGTAGTGACAGGTCC
H_4_8          CCTTCAAACTACCAGTACAAAGCAAACACCACTTAAGGAGTTACCTGTAGTGACAGGTCC
H5-8           CCTTCAAACTACCAGTACAAAGCAAACACCACTTAAGGAGTTACCTGTAGTGAAAGGTCC
S23-2-8        -----------------------------------------------------------

H_2_8          ATCAACTAACAGGCTTT
H_4_8          ATCAACTAACAGGCTTT
H5-8           ATCAACTAACAGGCTTT
S23-2-8        -----------------
```

Figure 3.11: Clustal 1.81X multiple sequence alignment of Bv80 sequences for vaccine passage S23 and the H strain with primer sequences shown in blue and deletions highlighted in cyan.

3.3.2.3 F strain Bv80 and 18S rRNA V4 hypervariable region sequence analysis

18S rRNA V4 hypervariable region analysis of the F strain indicated the presence of three genotypes. As seen with the other strains, there is a high degree of sequence identity. Point differences are highlighted in Figure 3.12.

```
F_3     --------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
F_4     --------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
F_5     --------------------------GACACAGGGAGGTAGTGACAAGAAATACCAATAC
                                  **********************************

F_3     GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
F_4     GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
F_5     GGGGCTACTGCTCTGTAATTGGCATGGGGGCGACCTTCACCCTCGCCCGAGTACCCATTG
        ************************************************************

F_3     GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
F_4     GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
F_5     GAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACT
        ************************************************************

F_3     TGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCCCCGCGCGTG-GTCCTTTCCTCGC
F_4     TGTTGCAGTTAAAAAGCTCGTAGTTGTACTTCACGTCCCCCGCGCGTG-GTCCTTTCCTCGC
F_5     TGTTGCAGTTAAAAAGCTCGTAGTTGAATCTCACGTCCCGCCCTAGTGTTCTTCACTAGG
         ************************** *  ********* * *  * ** ***  ** *

F_3     CGGGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
F_4     CGGGACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
F_5     CGG-ACGCCTCGTTACTTTGAGAAAATTAGAGTGTTTCAAGCAGGTTTCGCCTGTATAAT
        *** ************************************************************

F_3     TGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGGCTTTGGGTAATGGTT
F_4     TGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTAATGGTT
F_5     TGAGCATGGAATAACCTTGTATGACCCTGTCGTACCGTTGGTTGACTTTGGGTAATGGTT
        ******************************************** ***************
```

```
F_3     AATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
F_4     AATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
F_5     AATAGGAACGGTTGGGGGCATTCGTACTCGACTGTCAGAGGTGAAATTCTTAG
        **************************************************
```

Figure 3.12: Clustal 1.81X multiple sequence alignment of 18S rRNA V4 hypervariable regions for the F strain. Primer sequences are shown in blue and base differences in green (T), yellow (C), red (G) and purple (A).

Figure 3.13 shows the Bv80 multiple sequence alignment for the F strain. The number of detectable strains calculated with the different methods varies greatly for the F strain with four Bv80 sequences, three 18S rRNA V4 hypervariable region sequences and only two Bv80 bands being detected using size discrimination. The forward primer sequence could not be detected for clone F_3_8, which showed a high degree of sequence homology with clone F_1_8. A multiple alignment of only F_1_8 and F_3_8 reveals point differences scattered through the sequence, indicating that they are not the same genotype. There is also a high degree of deviation before the beginning of the F_3_8 repeats, confirming that they are not the same genotype. As mentioned before, sequencing the full length of the Bv80 fragments was problematic and may have resulted in the truncated sequence for F_3_8. The large size differences between the F strain genotypes was caused by differing numbers of repeats in region A, with clones F_1_8 and F_3_8 showing fewer repeats than clones F_4_8 and F_5_8.

```
F_5_8          TGTGTTAATGTAACTCAGCCCGCCATTCCCACCATCCCTGAGCAGCCAGTAGTG
F_4_8          TGTGTTAATGTAACTCAGCCCGCCATTCCCACCATCCCTGAGCAGCCAGTTG--
F_1_8          TGTGTTAATGTAACTCAGCCCGCCATTCCCACCATCCCTGAGCAGCCAGTAGTG
F_3-8          ----------------------------------------------------

F_5_8      GAGCCCACTGAAGAGCCGGCTGGCGTCACCGAAACTGCTCCGGAGGAGTGTGAGGAAGAA
F_4_8      -------CTGAAGAACCATCTGATGTCACCGTAACTGCTCCCGAAGAGTGTGAGGAAGAA
F_1_8      GAGCCCACTGAAGAGCCGGCTGGCGTCGCCGAAACTGCTCCGGAGGAGTGTGAGGAAGAA
F_3_8      ------------------------------------------------------------

F_5_8      ATTGTTATCAATCCAGAGGAAGAAAACAAATCTGACTCCTCCTCCTCTTCTTCTTCTCT
F_4_8      GTTGTTATTAATCCAGAGGAAGAAAACAAACCTGACTCCTCCTCCTCATCTTCTTCTTC-
F_1_8      ATTGTTATCAATCCAGAGGAAGAAAACAAATCTGACTCCTCCTCCTCTTCCTCTTCTTCT
F_3_8      ----------------------------------------------------------AG

F_5_8      TCATCATCATCTTCCTCAGATTCTGATTCAGATGAAGAGGATAGGGA---ACCCATTGTT
F_4_8      --ATCATCGTCTTCTTCAGAATCGGATTCAGATGAAGAGGATAGGGA---ACCCATTGTT
F_1_8      TCATCATCATCTTCCTCAGATTCTGATTCAGATGAAGAGGATAGGGA---ACCCATTGTT
F_3_8      TGATTAAAGCCTGTTAGTTGAT-GGACCGGGCTGAGTTACATTAACACATGCCCATTGCT
             ** *    **          *  **    * ***     **    *    ******* *

F_5_8      GGGGAGCCCATTGCTGAGGAGCCCGTTGCTGAGGAGCCCATTGCTGAGGAACCCGTTGCT
F_4_8      GAGGAACCCATTGTTGAGGAGCCCATTGTTGAGGAGCCCGTTGTTGAGGAGCCCGTTGTT
F_1_8      GAGGAGCCCATTGCTGAGGAGCCCGTTGCTGAGGAACCCGTTGCTGAGGAGCCCATTGCT
F_3_8      GAGGAGCCCATTGCTGAGGAGCCCGTTGCTGAGGAACCCGTTGCTGAGGAGCCCGTTGCT
           * *** ******* ********** *** ****** *** *** ****** *** *** *

F_5_8      GAGGAACCCGTTGCTGAGGAACCCGTTGCTGAGGAACCCGTTGCTGAGGAACCCGTTGCT
F_4_8      GAGGAACCAGTTGTTGAGGAACC---TGCTGAGACTCC---TGCTGAGACTCC---TGCT
F_1_8      GAGGAACCCGTTGCTGAGGAGCCCGTTGCTGAGACTCC---CGCTGAGACTCC-------
F_3_8      GAGGAGCCCGTTGCTGAGGAGTCCGTTGCTGAGACTCC---CGCTGAGACTCC-------
           ***** ** **** ******  *   *******   **    ******   **
```

A

```
F_5_8    GAGGAACCCATTGCTGAGGAACCCATTGCTGAGGAACCCATTGTTGAGGAGCCCATTGCT
F_4_8    GAGAAACC---TGCTGAGAAACC---TGCTGAGAAACC----------TGCT
F_1_8    ------------------------------------------------------------
F_3_8    ------------------------------------------------------------

F_5_8    GAGGAGCCCATTGCTGAGGAGCCCATTGCTGAGGAGCCCATTGCTGAGGAGCCCATTGCT
F_4_8    GAGAAACCC---GCTGAGAAACC---TGCTGAGAAACCC---GCTGAGAAACC---TGCT
F_1_8    ------------------------------------------------------------
F_3_8    ------------------------------------------------------------

F_5_8    GAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGGAGCCCGTTGCTGAGACTCCCGCTGAG
F_4_8    GAGAAACCCG---CTGAGAAACCCG---CTGAGAAACCCG---CTGAGAAACCTGCTGAG
F_1_8    ----------------------CG----------CTGAGACTCCTGCTGAG
F_3_8    ----------------------CG----------CTGAGACTCCTGCTGAG
                              **          ******  ** ******

F_5_8    ACTCCCGCTGAGACTCCTGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCTGCTGAG
F_4_8    AAACCTGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAGACTCCCGCTGAG
F_1_8    ACTCCCGCTGAGACTCCCGCTGAGACTCCTGCTGAGACTCCCGCTGAGACTCCCGCTGAG
F_3_8    ACTCCCGCTGAGACTCCCGCTGAGACTCCTGCTGAGACTCCCGCTGAGACTCCCGCTGAG
         *  ** ********** ********** ********************** ******

F_5_8    ACTCCCGCTGAGAAACCTGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACCCGCTGAG
F_4_8    ACTCCCGCTGAGAAGCCCGCTGAGAAACCCGCTGAGAAACCCGCTGAGACTCCCGCTGAG
F_1_8    ACTCCTGCTGAGACTCCCGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACCCGCTGAG
F_3_8    ACTCCTGCTGAGACTCCCGCTGAGAAACCTGCTGAGAAACCCGCTGAGAAACCCGCTGAG
         ***** *******  ** ********** ******************  *********

F_5_8    AAGCCTGCAGTCGCTTCTTGTAATCTTGTACCCAAATGTGGCAAAGTGCTCGTTCTCCAA
F_4_8    AAACCCGCATCCAGACCTTGTTACGGTGGTCGCAAAGGTGAAGAAGTGGTCGTCCTTCAA
F_1_8    AAGCCTGCAGTCGCTTCTTGTAATCTTGTACCCAAATGTGGCAAAGTGCTCGTTCTCCAA
F_3_8    AAGCCTGCAGTCGCTTCTTGTAATCTTGTACCCAAATGTGGCAAAGTGCTCGTTCTACAA
         ** ** ***  *    ***** *   **  * **** ***   ***** **** ** ***
```

A

```
F_5_8       ACTAGCAGTAAGGAGCAAACACCCCTTAAGGAGTTAGCTATAGTGACAGGTCCATCAACT
F_4_8       ACTACCAGTACAAAGCAAACACCACTTAAGGAGTTACCTGTAGTGACAGGTCCATCAACT
F_1_8       ACTAGCAGTAAGGAGCAAACACCCCTTAAGGAGTTAGCTATAGTGACAGGTCCATCAACT
F_3_8       ACTAGCAGTAAGGAGCAAACACCCCTTAAGGAGTTAGCTATAGTGACAGGTCCATCAACT
            **** *****    ********** *********** ** ********************

F_5_8       AACAGGCTTT
F_4_8       AACAGGCTTT
F_1_8       AACAGGCTTT
F_3_8       AACAGGCTTT
            **********
```

Figure 3.13: Clustal 1.81X multiple sequence alignment of Bv80 sequences for the F strain with primers shown in blue and deletions highlighted in cyan.

The number of genotypes identified in each strain using size analysis, 18S rRNA V4 hypervariable and Bv80 sequence analysis did not always correlate. One genotype was identified in the S23 vaccine passage for each analysis method, but S11 showed two Bv80 bands, two Bv80 sequences and three 18S rRNA V4 hypervariable sequences. Though extensive cloning and sequencing reactions were performed, it is possible that the third Bv80 sequence could not be detected due to low levels. The H strain showed two 18S rRNA V4 hypervariable sequences but three Bv80 bands and sequences and as with the S11 vaccine passage, it is possible that the third 18S rRNA V4 hypervariable sequence was missed or otherwise, that two strains have the same 18S rRNA V4 hypervariable sequence. For the F strain two Bv80 bands were detected visually but four Bv80 sequences were obtained and three 18S rRNA V4 hypervariable sequences. There is only a 60 bp difference between clones F_4_8 and F_5_8, and at high DNA concentrations, these may resolve as one band.

Whilst useful for visual analysis and comparison, it can be seen that the number of Bv80 bands detected using gel analysis can be less than really exist in the sample. In the same light, two strains having the same Bv80 band size may not necessarily have the same Bv80 gene sequence and thereby represent two different genotypes. The size difference observed on a gel was as a result of sections of sequence either present or absent. The sizes of these inserts range from 3bp to the larger inserts (over 100 bp) seen in region A, Figure 3.13 for the F strain.

In general, the 18S V4 hypervariable region analysis showed a high degree of sequence homology. Point differences served as a good marker for the detection of genotype differences. The use of different techniques to determine the number and nature of *B. bovis* genotypes present in each strain highlighted how different tests and methods of detection can result in a different number of genotypes determined for each strain. This is important when considering that many studies are carried out using one method of detection only, which may under represent the number of genotypes present in a strain. The ambiguous nature of size discrimination can be further clarified using sequence analysis. Though time consuming and costly, sequence analysis has been proven in this case, essential in determining the compositions of the different *B. bovis* strains.

Through the sequence analysis, it can be confirmed that the vaccine strain could not be detected in the H strain. Therefore, the lack of protection achieved may be as a result of vaccine failure due to inappropriate handling and administration coupled with indiscriminate treatment post vaccination of animals.

3.3.3 Detection of *B. bovis* carrier animals using Bv80 PCR

The Bv80 PCR was shown to be able to determine the nature of outbreaks in Australia (Lew *et al.*, 1997 b; Bock *et al.*, 2000) and coupled with sequence analysis has been shown to be able to distinguish between four South African strains. The Bv80 PCR protocol could be adapted and used as a routine diagnostic tool to determine the nature of future *B. bovis* infections and mortalities in South Africa. For the Bv80 PCR to be used as a diagnostic tool, the test would have to be sensitive enough to be able to detect carrier animals.

DNA was extracted from blood collected from bovines infected with the vaccine strain (as part of another study), confirmed to be *B. bovis* carriers using blood smear analysis. DNA was concentrated using ethanol precipitation to 1/20th of the original volume. PCR reactions were carried out in triplicate to maximise the likelihood of detection. Of the 12 animals tested, none showed the presence of Bv80 bands. Non-specific binding can be seen in Figure 3.14 as the double bands A and B. This non-specific amplification is most likely due to mispriming on the highly concentrated bovine host DNA resulting from the ethanol precipitation procedure.

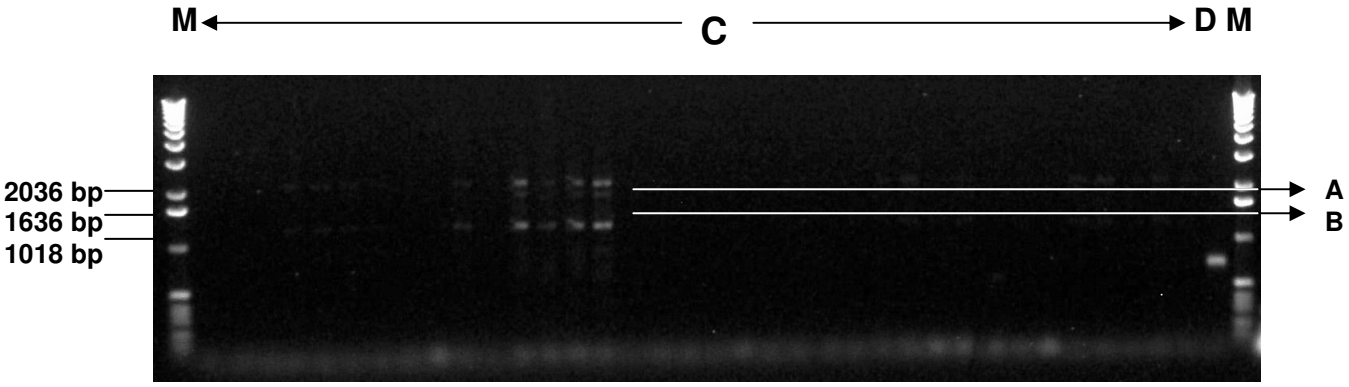

Figure 3.14: Detection of *B. bovis* DNA in carrier animals using the Bv80 PCR. Lane M: 5 µl of Marker X (Roche), lane D: 5 µl positive *B. bovis* control, region C: 5 µl of Bv80 PCR reaction for each of the 12 carrier animals carried out in triplicate.

From Figure 3.14 it can be seen that the Bv80 PCR is not sensitive enough to detect carrier animals, even when the DNA is substantially concentrated. This inability to detect carrier animals as well as the need to resolve PCR products on a polyacrylamide gel to obtain sufficient resolution implies that the test requires two to three days to obtain results. Polyacrylamide gels are time consuming to prepare and run and not suited to the high throughput required of diagnostic services. Therefore, whilst theoretically suitable, the practical requirements of the Bv80 test do not lend themselves to use in routine diagnostic testing. The test does however serve as a tool to investigate the nature of outbreaks, while the profiling of the vaccine strain allows for a point of reference where vaccine efficacy is called into question.

Current identification of *B. bovis* infected animals at OVI makes use of the IFAT, whereby antibodies against *B. bovis* are detected in the serum of infected animals. Antibodies against infectious particles circulate in the vascular system of an individual for a longer period of time than visible parasites and hence, a test based on antibody detection will be more sensitive than a PCR test, particularly if the PCR focuses on a single copy gene. While the IFAT does allow for the detection of carrier animals, it is limited in that it makes no distinction between prior and current infections with *B. bovis*, nor is it able to distinguish between exposure to vaccine *B. bovis* parasites and field acquired infections. PCR based tests targeting multiple copy genes theoretically have a higher degree of sensitivity (Fahrimal *et al.*, 1992; Figueroa *et al.*, 1993; Patarapadungkit *et al.*, 2004) and are better able to detect carrier animals and should be used for PCR detection if animals are suspected to be in the carrier stage of infection.

3.3.4 BvVA1 PCR optimisation

The optimisation of the BvVA1 PCR was originally carried out using vaccine passages S11, S23 and the H strain with the High Fidelity Long Template PCR System (Roche). This kit is supplied with three buffer options with varying $MgCl_2$ concentrations, buffer 1 contains 17.5 mM $MgCl_2$, buffer 2 contains 27.5 mM $MgCl_2$ and buffer 3 contains 27.5 mM $MgCl_2$ with added detergents. Figure 3.15 shows the BvVA1 PCR products for the three strains using each of the three buffers.

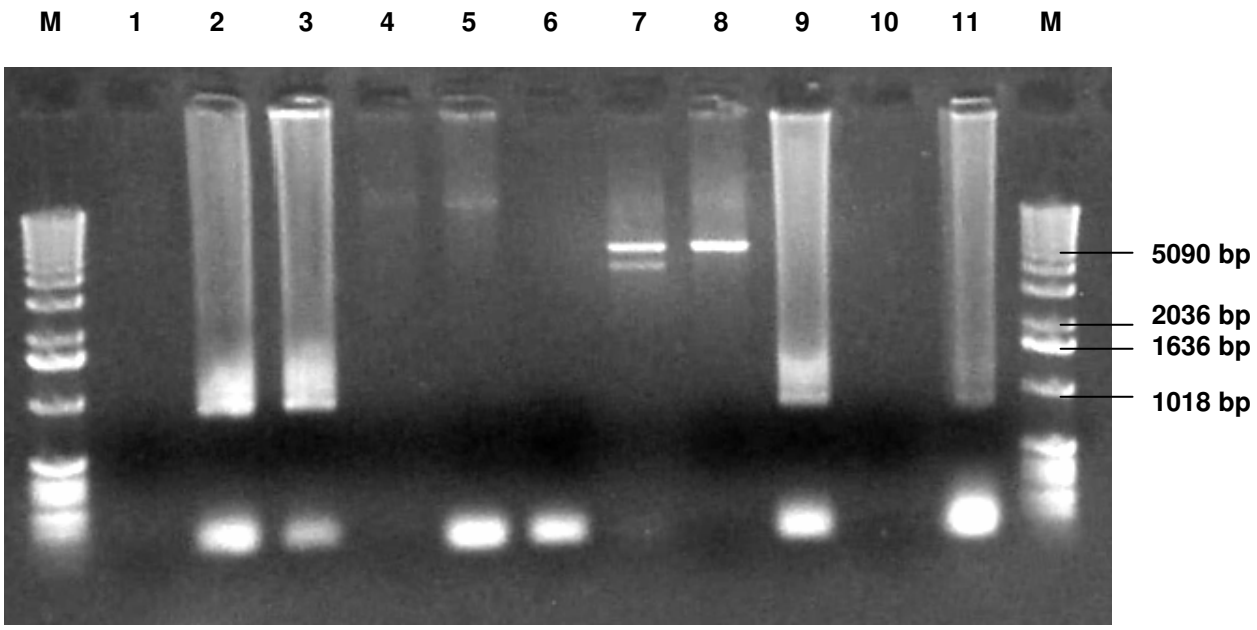

Figure 3.15: BvVA1 PCR amplification of vaccine passages S11, S23 and the H strain with Long Template PCR buffers 1, 2 and 3 (Roche). 5 µl of PCR product was loaded in each well of a 1.5% agarose gel. Lane M: 5 µl Marker X (Roche), lane 1: uninfected bovine control with buffer 2, lane 2: S23 using buffer 1, lane 3: S23 using buffer 2, lane 4: S23 using buffer 3, lane 5: H using buffer 1, lane 6: negative control using buffer 2, lane 7: S11 using buffer 1, lane 8: H using buffer 2, lane 9: H using buffer 3, lane 10: S11 using buffer 2, lane 11: S11 using buffer 3.

In many lanes, a large amount of smearing can be seen from the well to below the 1018 bp mark. Despite the use of different buffers, optimum results could not be

obtained for each of the strains with any one buffer type. Buffer 2 was continued with for further amplification reactions as it produced an overall lesser degree of smearing. Despite the clear BvVA1 bands in lanes 7 and 8, the results of this experiment could not be duplicated when the experiment was repeated. A series of reactions were undertaken altering the reaction parameters until smearing was removed.

The smearing was initially thought to be as a result of non-specific primer binding. The annealing temperature for the reaction was therefore increased from 65°C to 70°C. As the smearing was so extensive, a large increase in the annealing temperature was carried out to allow for a maximum change to be observed but despite the increase, no change in the degree of smearing was observed.

Taq polymerase enzymes have a 5'-3' exonuclease activity and with the long extension times, the generation of artifacts resulting in smearing is possible (Roche Long Template Troubleshooting guide). For this reason the amount of enzyme added per reaction was decreased from 2.5 U to 1.6 U but still no change in the extent of the smearing could be observed. The Fermentas Long PCR Enzyme Mix (Fermentas) was used under identical reaction conditions to determine if the Roche enzyme was amplifying the template correctly but the Fermentas enzyme produced the same smearing effect.

The amount of $MgCl_2$ in a PCR reaction has an effect on the amplification, the positively charged Mg ions bind to the negatively charged DNA backbone and stabilise the DNA when it is in the single strand form (Roche Long Template Troubleshooting guide). $MgCl_2$ is present in the buffer solutions as well as in the dNTPs and any excess ions can cause overstability resulting in the disruption of

cycling forms essential for amplification. The amount of each dNTP was decreased from 10 pmoles to 5 pmoles in increments of 2.5 pmoles, but again, no change in the extent of smearing was seen. Figure 3.16 shows the effect of dNTP concentrations on the BvVA1 PCR reaction.

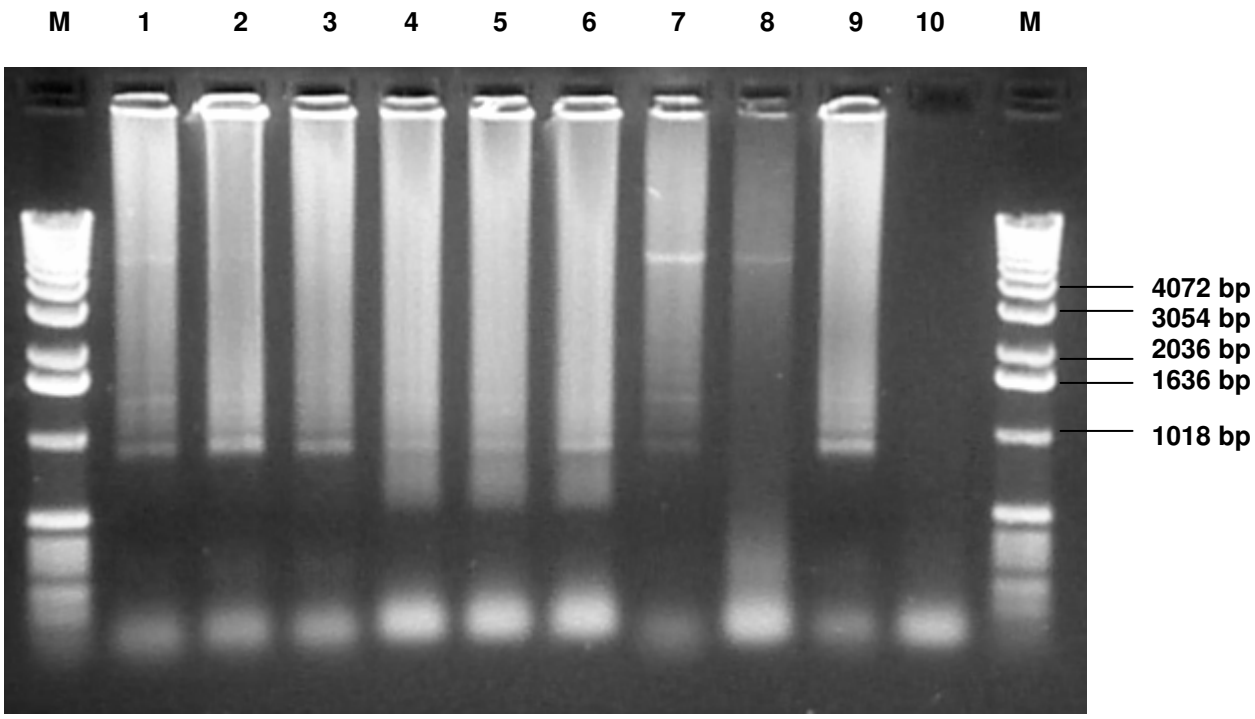

Figure 3.16: BvVA1 PCR amplification of vaccine passages S11; S23 and the H strain with varying concentrations of dNTPs. 5 µl of PCR product was loaded in each well of a 1.5% agarose gel. Lane M: 5 µl Marker X (Roche), lane 1: S11 with 10 pmoles of each dNTP, lane 2: S11 with 7.5 pmoles of each dNTP, lane 3: S11 with 5 pmoles of each dNTP, lane 4: S23 with 10 pmoles of each dNTP, lane 5: S23 with 7.5 pmoles of each dNTP, lane 6: S23 with 5 pmoles of each dNTP, lane 7: H with 10 pmoles of each dNTP, lane 8: H with 7.5 pmoles of each dNTP, lane 9: H with 5 pmoles of each dNTP, lane 10: 5 µl negative control.

The concentration of template DNA was decreased as it was thought that perhaps there was too much template DNA. The primer concentration was also increased

from 20 pmoles to 40 pmoles of each primer per reaction but neither produced any effect.

Though the dilution of the template DNA did not produce an effect it was possible that the template DNA was degraded. However, using DNA freshly extracted with the QIAamp DNA Blood Mini Kit produced no effect, whilst ethanol precipitation of the template DNA and resuspension in water did decrease the smearing. Comparisons made between Qiagen and MagNA Pure prepared DNA showed that Qiagen prepared DNA produced less smearing. The washing step in the MagNA Pure machine involves the binding of DNA to magnetic beads and moving them through the buffer in a pipette tip. This mechanical process would cause more sheering than would occur in the Qiagen spin columns which make use of the downward gravitational force created by the motion of a centrifuge rotor to pull the liquid and DNA through the column. Ethanol precipitation is based partly on the concept that heavier molecules will sediment faster under a centrifugal force than lighter ones. The heavier DNA molecules being insoluble after the addition of ethanol would sediment faster than the lighter molecules, thereby partially eliminating the lighter, sheered DNA molecules. These smaller DNA fragments would cause the formation of different sized products seen as the smearing on the agarose gels. Figure 3.17 shows the results of BvVA1 PCR using ethanol precipitated template DNA. Whilst some degree of smearing can still be seen, it has been substantially reduced.

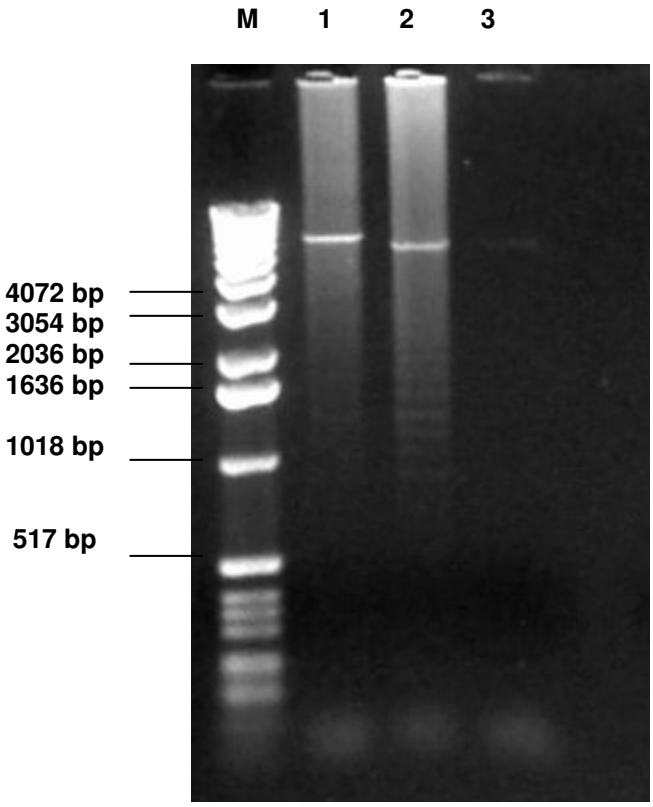

Figure 3.17: PCR amplification of the BvVA1 gene using ethanol precipitated template DNA. 5 µl of PCR product was loaded in each well of a 1.5% agarose gel. Lane M: 5µl Marker X (Roche), lane 1: S11, lane 2: S23, lane 3: H strain

3.3.5 BvVA1 analysis of *B. bovis* strains

Lew *et al.* (1997 a) described the BvVA1 PCR method as more sensitive method than the Bv80 PCR but did note that with the BvVA1 PCR not all parasites within an strain may be represented as the reaction favours the generation of smaller fragments. Figure 3.18 shows the BvVA1 PCR products for the S11 and S23 passages, and the H and F strains resolved on a 10% polyacrylamide gel.

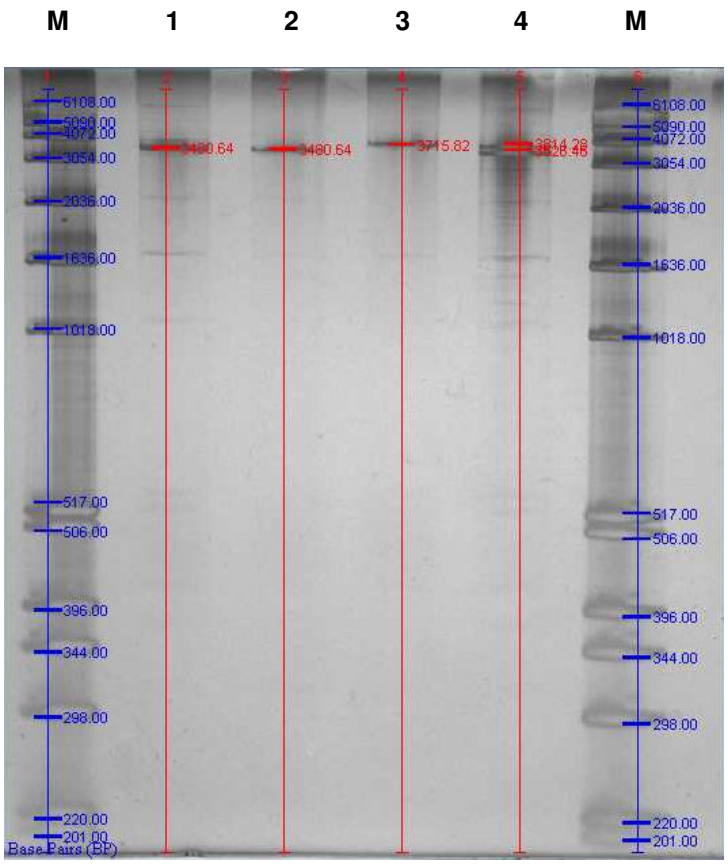

Figure 3.18: Polyacrylamide resolution of BvVA1 PCR products for S11, S23, H and F. 1 µl of PCR product was loaded in each well of a 10% polyacrylamide gel and visualised using silver staining. Lane M: 0.5 µl Marker X (Roche), lane 1: S11, lane 2: S23, lane 3: H, lane 4: F strain.

Both the S11 and S23 vaccine passages (lanes 1 and 2) have BvVA1 products of 3480.64 (3481 bp) confirming the 'carry through' theory that one strain from the original S11 passage was selected through the passage of the vaccine strain till passage 23. If this S23 strain was carried through it should have the same sized BvVA1 PCR product as S11, which is indeed shown in Figure 3.18. The H strain (lane 3, Figure 3.18) shows one slightly larger band of 3745.82 (3746 bp) and the F strain (lane 4) shows two bands, one of 3528.48 (3528 bp) and 3814.28 (3814 bp).

The *Acc* I profile of the BvVA1 products does yield data that is easier to interpret as described in the original publication (Lew *et al.*, 1997 a). Figure 3.19 shows the *Acc* I restricted BvVA1 PCR products generated for vaccine passages S11 and S23, and the H and F strains.

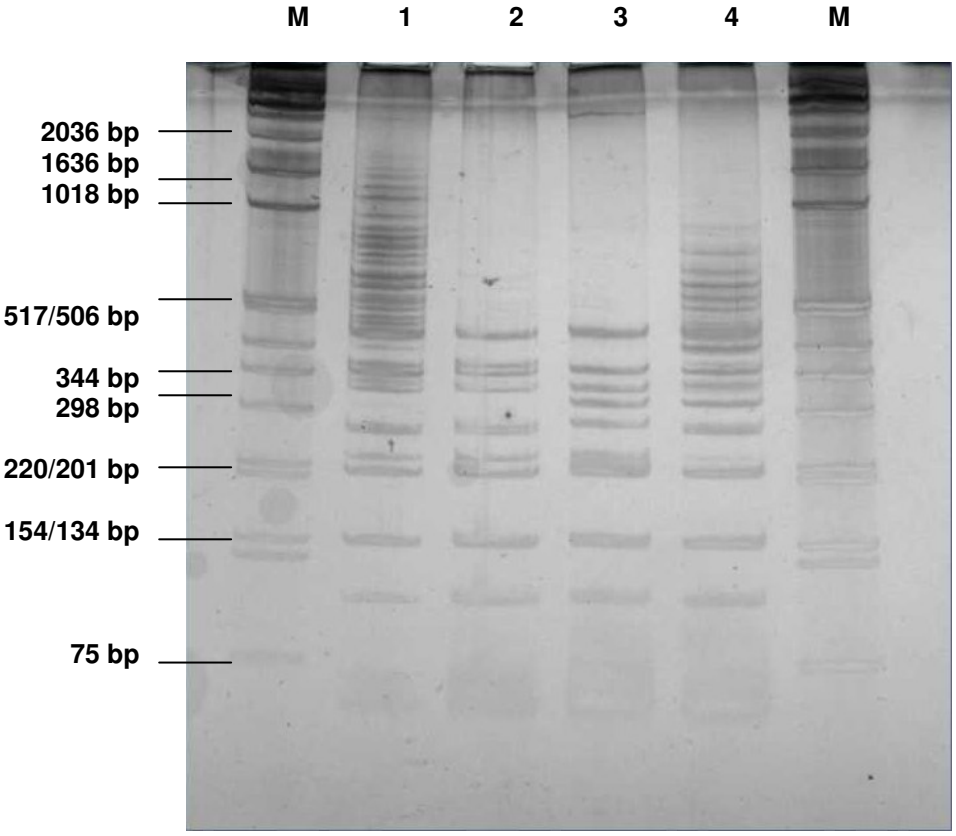

Figure 3.19: Resolution of *Acc* I restricted BvVA1 PCR products on a 15% polyacrylamide resolving gel visualised using silver staining. 2 µl of restricted PCR product was loaded in each well. Lane M: 0.5 µl Marker X (Roche), lane 1: S11, lane 2: S23, lane 3: H strain, lane 4: F strain.

The sizes of each of the *Acc* I restricted BvVA1 PCR products were calculated using the Quantity One program and are shown below in Figure 3.20.

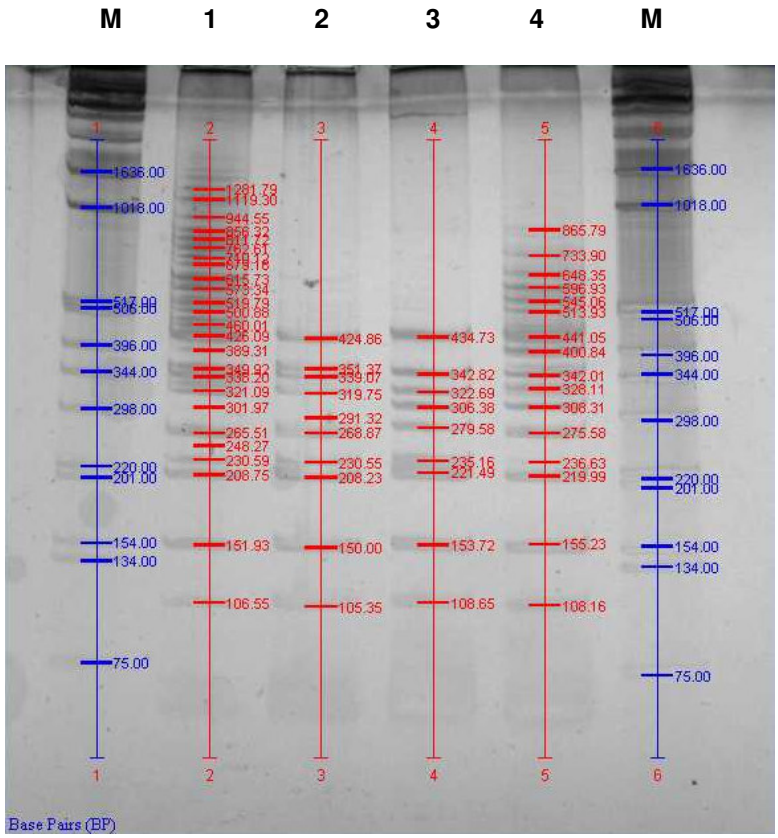

Figure 3.20: Resolution of *Acc* I restricted BvVA1 PCR products on a 15% polyacrylamide resolving gel visualised using silver staining with Quantity One generated band sizes. 2 µl of PCR product was loaded in each well. Lane M: 0.5 µl Marker X (Roche), lane 1: S11, lane 2: S23, lane 3: H strain, lane 4: F strain.

Table 3.7 shows a summary of the sizes of each of the bands created by the *Acc* I digest for each of the strains. Some bands have very similar sizes and can be seen in more than one strain, particularly between the S11 and S23 passages and the H and F strains respectively. Bands that differed by less than 5 bp were classified as similar in size and can be seen as either in bold face or underlined text.

Table 3.7: Summary of the sizes of *Acc* I restricted BvVA1 PCR products for vaccine passage S11 and S23, the H and F strains.

	BvVA1 *Acc* I profile band sizes (bp)
S11	1282,1119, 845, 856, 812, 763, 740, 678, 520, 501, 460, 426, 389, 350, 338, **321**, 302, 266, 248, **231, 209, 107**
S23	**425**, **351**, 339,**320**, 291, 269, **231,208, 105**
H	435, 343, 323, 306, 280, 235, 221, 154, **109**
F	866, 734, 648, 597, 545,520, 441, 401, 342, 328, 308, 276, 237, 220, 155, **108**

Only a single BvVA1 band is seen for the S11 vaccine passage suggesting one donor genotype but a number of *Acc* I bands are created. It is likely that there are multiple BvVA1 products of the same size within this sample, which are only revealed by *Acc* I profiling.

The 'carry through' of vaccine passage S23 strain from passage S11 is confirmed by the *Acc* I profiles of the two passages. It is clear that the S23 strain is present in the mixed profile produced for S11. Neither Bv80 size nor sequence analysis could confirm that the S23 strain was present in the S11 vaccine passage and it is possible that the BvVA1 gene is more stable over time and a better reflection in changes in genotype. Despite this, *Acc* I generated profile for the BvVA1 PCR products is not a true reflection of the genotypes present in each of the strains. The protocol requires that each BvVA1 band be restricted independently to obtain a profile for each genotype in the strain. This is problematic in the case of these four strains for two reasons, firstly because the number of bands (representing the number of genotypes) for each of the strains using the Bv80 and BvVA1 PCR reactions do not correlate with one another. The only strains to show the same number of Bv80 and BvVA1 amplification products are the F strain and the S23 passage showing two and

one band respectively. The S11 passage shows only one BvVA1 band where it showed two Bv80 bands, and the H strain also only shows one BvVA1 band where there were three Bv80 bands.

The apparent amplification of a limited number of the genotypes in the strains may be either because the templates were not amplified or because the BvVA1 genes for each of the strains are identical in size. Lew *et al.* (1997 a) did note that if BvVA1 PCR products are of the same size they should none-the-less show different *Acc* I restriction profiles, but if the fragments are the same size within an strain it would not be possible to separate and restrict them independently and thus no differentiation between strain profiles could be made.

The second limitation of the *Acc* I restriction analysis is that where more than one BvVA1 gene (of different sizes) did exist, the size difference was too small to allow the bands to be separated from each other without contaminating each band sample. This was evident for F and S11 which both contained more than one BvVA1 band too similar in size to allow them to be separated and restricted independently. Instead, a restriction of the mixed population of amplicons was conducted for each strain.

The inability of the reaction to amplify BvVA1 genes from each of the genotypes present in the strain as well as its inability to excise and restrict individual BvVA1 bands suggests that the BvVA1 PCR is not suitable for discriminating between the South African *B. bovis* strains investigated in this study.

3.4 CONCLUSIONS

Discrimination between the vaccine strain and the *B. bovis* strain collected from the Haistings farm was necessary to determine the nature of the outbreak as well as to ensure that the H strain was not merely the vaccine strain isolated from field animals. Using the Bv80 PCR test discriminatorily, profiles were successfully created for the S11 and S23 vaccine passages, and the H and F strains.

Confirmation of the observed changes in size of the Bv80 PCR products were reflected by differences in the cloned Bv80 sequences. Bv80 sequence analysis showed repeats that make up the variable region of the gene. Large size differences were as a result of differences in the number of repeats while smaller size differences were caused by small inserts in the sequence. Sequence changes in the repeats were observed and where they occurred, were carried through successive repeats. The use of Bv80 PCR product size analysis, while convenient, may produce misleading results with not all parasite genotypes in a strain being detected. This method should be coupled with sequence analysis to obtain a more accurate reflection of the genotypes present in a strain. The inability of the Bv80 PCR to detect infections in carrier animals was seen as a limiting factor in the use of the test for routine diagnostic practices. This, in addition to requiring resolution on polyacrylamide gels, means that the test is too time consuming to be used for routine diagnoses.

The 18S rRNA V4 hypervariable region sequence analysis showed a high degree of sequence homology, with observed point differences being sufficient to be used as a distinguishing factor between *B. bovis* strains. Point differences in the 18S rRNA V4 hypervariable region confirmed that the Bv80 variable region could reflect *B. bovis* strain differences. The number of genotypes in each strain calculated using 18S

rRNA V4 hypervariable region sequence analysis and Bv80 size and sequence analysis did not correlate. This may be due to the difficulty in detecting minor strain donors and the fact that Bv80 bands of the same size would be indistinguishable from each other using visual analysis. Through coupled visual and sequence analysis, it was determined that the S23 strain was not present in the H strain and thus not responsible for the outbreaks in the region. The poor vaccination strategy in addition to animal stress may have caused the outbreaks.

BvVA1 size analysis indicated that there was a 'carry through' of one strain from the S11 passage to S23. However, these results could not be confirmed using 18S rRNA V4 hypervariable region sequence analysis, Bv80 size or Bv80 sequence analysis. It is possible that the S23 strain carried through was present at a very low level in the S11 passage. With each passage step, it may have comprised more and more of the total population till it became the sole parasite population present. BvVA1 PCR product size analysis showed that not all genotypes are represented for each strain. The *Acc* I restriction analysis did yield easier to interpret data but the combination of the PCR method's inability to amplify all genotypes in a strain and the problems associated with restricting of each BvVA1 fragment independently implies that this PCR is not suitable for discriminating between the South African *B. bovis* strains investigated.

As a result of this characterisation, the profiles of four South African *B. bovis* strains have been generated, and most importantly, the vaccine strain has been profiled. These profiles can now be used as a point of reference when investigating future outbreaks.

CHAPTER FOUR: *IN VITRO* CULTIVATION OF *B. BOVIS* STRAINS

4.1 INTRODUCTION

The first successful continuous *in vitro* culture technique for *B. bovis* was reported by Levy and Ristic in 1980. This technique, known as microaerophilous stationary phase (MASP) culture, supports the continuous growth of *B. bovis* parasites and yields large numbers of parasites with the advantage of cultivation being possible in any sample volume with very limited manipulation. The culture system consists of a blood layer containing the parasitised erythrocytes, a layer of medium which forms above the erythrocytes and the external environment defined by the gas system and the temperature of the culture.

Cultures grow optimally at low parasitemia levels (between 0.5 and 1% of erythrocytes being parasitised) with sub-culturing every three to four days to maintain parasitemia levels below 1% (Levy and Ristic, 1980). The erythrocyte layer settles to the bottom of the culture vessel and darkens as a result of the depletion of oxygen by the metabolic activities of the parasites. Resuspension of the erythrocytes with fresh medium causes the bright red colour to return almost instantaneously.

It has been shown that parasite growth occurs best under slightly alkaline conditions (pH 7.02 to 7.79) with the optimal pH between 7.31 and 7.39. Buffer systems may vary with buffers such as TES, HEPES and TAPSO being successfully used. *B. bovis* parasites are seen to be intolerant of acidic conditions of pH 6.86 and below (Goff and Yunker, 1988).

The depth of the culture has been shown to be one of the critical factors affecting *B. bovis* parasite growth. *B. bovis* parasites cannot survive under high oxygen concentrations and the medium layer above the erythrocytes acts as an effective

barrier to oxygen exchange. It was found that cultures with a depth of under 0.16 cm did not support growth, with the optimum depth (irrespective of the size and volume of the culture vessel) found to be 0.62 cm (Levy and Ristic, 1980). Levy and Ristic (1980) noted that growth under premixed gas conditions failed (5% CO_2, 10% O_2, 85% N_2; 5% CO_2, 5% O_2, 90% N_2) whilst Jackson *et al.* (2001) analysed growth of cultures under varying gas conditions and determined that 5% CO_2, 5% O_2 and 90% N_2 supported optimum growth. Irrespective of gas conditions, culture temperatures should be kept between 37 and 38 ºC.

While MASP culture conditions mimic, as far as possible, the conditions that *B. bovis* parasites would encounter in blood capillaries, variations in culture conditions to suit each strain may be required. The MASP culture technique has enabled the continuous cultivation of *B. bovis* parasites for the first time, and allows for the isolation of large amounts of parasite antigen. This benefit has resulted in a large number of studies being undertaken focusing on the use of culture-derived antigens as potential vaccine candidates. A culture derived vaccine would need to be effective against homologous and heterologous challenge, should be stable under storage conditions, easy to produce and administer, produce no deleterious side effects, contain no contaminants and provide long term immunity to disease (Montenegro-James *et al.,* 1995; Schetters, 1995).

One approach for the identification of potential vaccine targets is to identify and analyse exoantigens and determine their effect on the host immune system, an approach which is typically used when characterising culture derived antigens. A number of studies using both whole culture supernatant and purified antigens as vaccine candidates have been undertaken. Most showed strong homologous protection whilst others have shown good heterologous protection (reviewed by Montenegro-James *et al.*, 1992).

Although the H strain has been determined to be non-virulent using needle challenge, it could still be used as a control to identify potential vaccine candidate proteins by comparing its soluble parasite antigen profile to that of a virulent strain. In this chapter, attempts to obtain an *in vitro* MASP culture of the H strain and vaccine passage S24 are described.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of blood

Clean and infected *B. bovis* blood (vaccine S24 and H strain generation 1) were obtained from animals by bleeding from the vein under the tail into EDTA vaccutainer tubes. Blood was immediately washed with phosphate buffered saline (PBS) (Onderstepoort Biological Products) in a ratio of 1:1. Blood was centrifuged at 770 g (Megafuge 1.0R, Heraeus) for ten minutes. The braking system was deactivated to ensure that the blood/serum interface was not disturbed.

After centrifugation, the serum and leukocyte layer (visible as a white film that settles above the packed erythrocyte layer) were removed and discarded. The packed erythrocytes were then resuspended in an equal volume of PBS and centrifuged. This process was repeated four to five times and washed uninfected blood was stored at 4 ºC for periods of no longer than one week. Washed infected blood was initiated into cultures immediately and the remainder discarded.

4.2.2 Blood stabilates

Stabilates of *B. bovis* infected blood (vaccine passage S24) were prepared by washing the blood four times in PBS. The packed erythrocytes were resuspended in a final concentration of 10% (v/v) Polyvinylpyrrolidone 40 (PVP) (Sigma). 500 µl aliquots of the washed erythrocytes were frozen at -70 ºC.

4.2.3 Preparation of culture media

The culture medium was made of 40% serum (either equine or bovine, not heat inactivated), 10mM Buffer (TAPSO or HEPES) (Sigma), 1mM Hypoxanthine, 1µM L-glutamine, 100 µg/ml Streptomycin and 100 U/ml Penicillin (following procedures by

Dr Zweygarth) in either Medium M199 (Highveld Biological Products) or HL-1 (Gibco).

The pH of the medium was adjusted to pH of 7.3 with 2M NaOH and the medium filtered through a 0.2 µm acrodisc syringe filter (Pall corporation).

ALBUMAX II based medium was made using the same components and volumes as above but the serum was removed and replaced with 1% w/v ALBUMAX II (Gibco).

4.2.4 Initiation of cultures

Cultures were initiated using 50 µl of washed erythrocytes with a 1-2% parasitemia and 950 µl culture medium, ensuring that the depth of the culture medium was at least 0.62 cm in total. Where the parasitemia of the collected blood was above 2%, the infected erythrocytes were diluted to reach a parasitemia between 1-2% with fresh uninfected erythrocytes.

4.2.5 Maintenance of cultures

Cultures were maintained by changing the culture medium daily. Erythrocyte smears were made every second day using 5 µl of settled erythrocyte layer. Where culture parasitemias increased above 2%, the cultures were subinoculated into empty rows of the same plate using fresh washed erythrocytes to give a parasitemia of 1%. Cultures were placed into a sealed vessel and filled with a gas mix (90% N_2, 5% O_2 and 5% CO_2) and the chamber was placed into a 37ºC incubator. Alternatively, if the cultures were to be incubated in CO_2, they were placed directly into CO_2 incubators set at 37ºC.

4.3 RESULTS AND DISCUSSION

The effects of a number of *in vitro* culture parameters were investigated in this study in an effort to determine the optimal growth conditions for the H and S24 *B. bovis* strains. The parameters investigated were serum type, atmospheric gas conditions, the addition of growth supplements, initiation parasitemia as well as the temperature of the medium upon daily replacement.

4.3.1 Serum type

The first attempts to initiate *B. bovis* cultures were carried out using a 40% serum supplement with a 60% medium base. The aim was to slowly decrease the serum concentration in the medium after establishment of cultures till eventually no serum was required for the growth of parasites. The reason for the removal of serum from the media was that it would contaminate any *B. bovis* SPA collected, making the differentiation between host and parasite proteins difficult. Two serum types were examined, specifically adult bovine serum and equine serum (sera were prepared by Dr Erich Zweygarth). One equine serum batch (prepared from equine 320) and six bovine batches (prepared from bovines 9333, 9334, 9439, 9414, 9442 and 9462 respectively) were tested.

Initial attempts to establish *in vitro* cultures were done using the H strain (initiation parasitemia of 1%), six bovine serum batches mentioned above and M199 medium base. None of the six bovine serum batches supported growth of *B. bovis* parasites as no proliferation could be detected and the cultures were terminated after 7 days at which point no parasites could be detected.

Both bovine and equine sera were used in attempts to establish cultures of the S24 vaccine passage. One batch of bovine serum (prepared from animal 9334) and one

equine serum batch (prepared from equine 320) were used in conjunction with M199 base. Cultures were initiated at a parasitemia of 1% from blood with an initial parasitemia of 20%. The growth of parasites could not be supported using bovine serum while the use of equine serum showed an increase in culture parasitemia from 1 to 3% from days one to three. At this point, sub-culturing was performed but no further growth could be observed from day three onwards. By the tenth day, no parasites could be detected in the erythrocyte layer and the cultures were terminated. Figure 4.1 A and B show the S24 *B. bovis* parasites on day two of growth in equine serum supplemented medium M199.

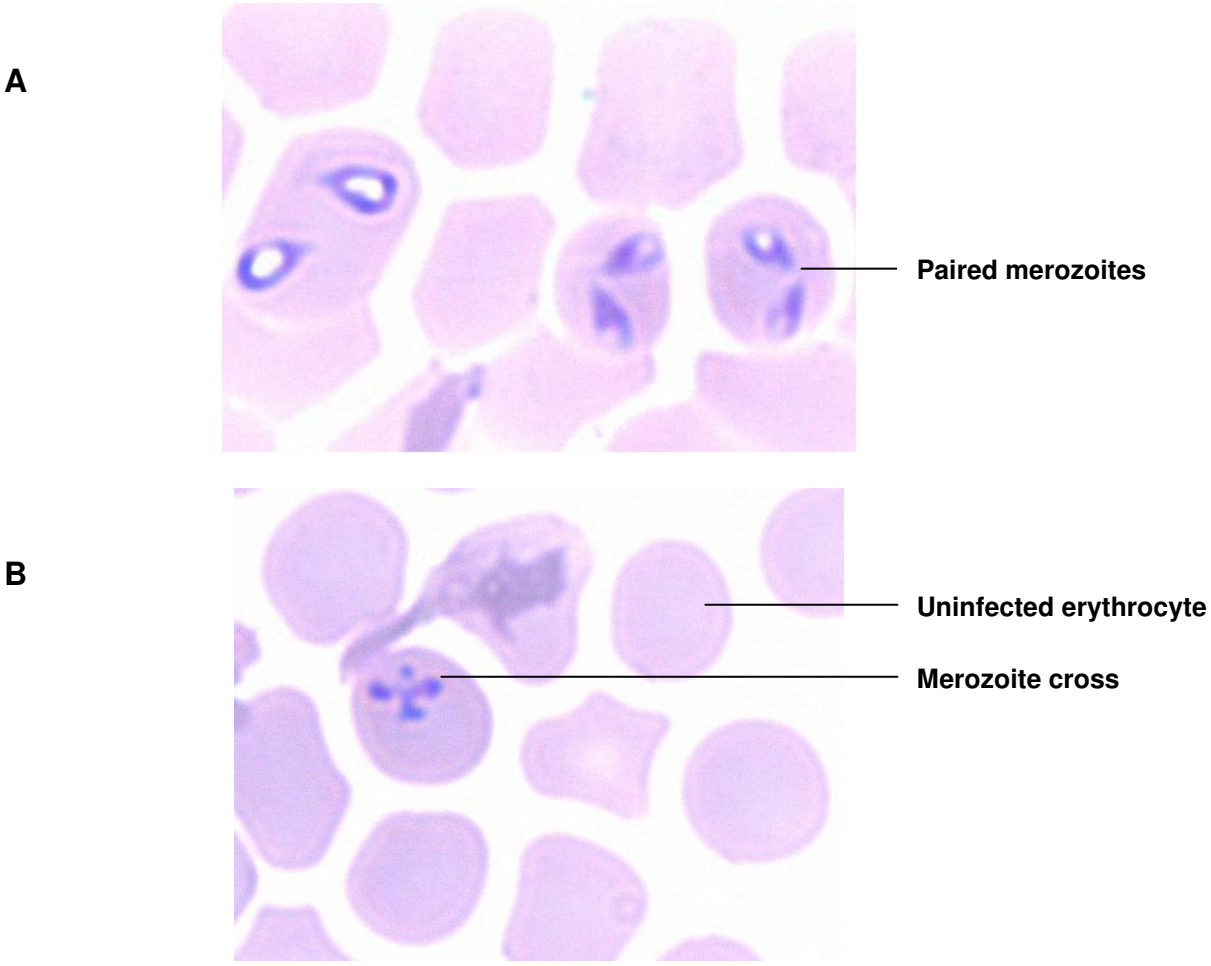

Figure 4.1 A and B: S24 *B. bovis* vaccine passage parasites on day two of growth in 40% equine serum and M199 medium, 1000 X magnification.

The shape of the pairing bodies in Figure 4.1 A are slightly irregular, specifically in the erythrocyte where the two merozoite bodies have disassociated from each other. These bodies do not represent healthy parasites capable of active growth and metabolism. The obtuse angle between the merozoites can be clearly seen. This angle can be used to distinguish between *B. bovis* and *B. bigemina*-infected erythrocytes, as *B. bigemina* parasites pair with an acute angle while *B. bovis* shows an obtuse angle. This characteristic is particularly useful where mixed infections of *B. bovis* and *B. bigemina* occur. Figure 4.1 B shows a merozoite cross which is commonly seen when *Babesias* are rapidly growing and dividing. However, this body is more likely a relic from the strain during its rapid growth cycle within the animal rather than as a result of growth of cultured parasites.

It was concluded that though equine serum supported growth, the level of growth achieved was limited, as evident by the shape of parasite bodies as well as the limited growth period seen. Bovine serum would seem a more natural choice as it comes from the host animal and has been used previously with success, but in the case of the strains cultured in this study, it did not support the growth of parasites.

4.3.2 Atmospheric gas conditions

Two gas conditions were investigated, 90% CO_2; 5% N_2 and 5% O_2 as well as 90% N_2, 5% O_2 and 5% CO_2. The limited growth of the equine serum supplemented S24 vaccine passage was achieved under 90% N_2, 5% O_2 and 5% CO_2 gas conditions whilst no growth could be supported using either equine or bovine serum under 90% CO_2; 5% N_2 and 5% O_2 gas conditions. These results suggest that equine serum coupled with 90% CO_2; 5% N_2 and 5% O_2 gas conditions supported the limited growth of the S23 vaccine passage parasites.

4.3.3 Addition of growth supplements

The addition of growth supplements such as hypoxanthine and L-glutamine did not show any effect on the growth of the H strain nor did the use of M199 or HL-1 based medium make any difference to parasite growth.

4.3.4 Initiation parasitemia

The initiation parasitemia of the *B. bovis* infected blood used to initiate *in vitro* cultures did seem to have an effect on the successful establishment of cultures. Cultures of the S24 vaccine passage were initiated using both blood of a low initial parasitemia (1%) and a high initiation parasitemia (20% diluted down to 1% with fresh uninfected erythrocytes) and it was seen that no growth occurred with a low initiation parasitemia whilst limited growth (10 days) was achieved from the high parasitemia blood diluted to 1%. Parasites growing in the bovine host at a high parasitemia would be rapidly growing and dividing whilst at a low parasitemia they would be growing less rapidly as they would still be in the process of establishing the state of infection. This difference in the 'growth stage' of the parasite may account for the establishment of cultures initiated with blood of a higher initial parasitemia whilst cultures initiated with a lower initial parasitemia failed.

4.3.5 Heating of media

The effect of the temperature of the medium used for daily medium changes on culture growth was investigated to ascertain if there was any difference between adding medium at 4℃ and medium at 37℃ to parasites growing at 37℃. Medium was divided into 800 µl aliquots, placed in sterile 1.5 ml tubes and stored at 4℃. Prior to addition, medium aliquots were removed from 4℃ and heated to 37℃ before addition to the first culture plate. The second plate received medium at a temperature of 4℃. A stabilate of S24 was used to initiate these cultures but growth

of parasites using either the 4℃ or 37℃ medium could not be achieved. The failure of cultures to establish meant that the effect of the medium temperature on the growth of parasites could not be established. However, since equine serum supplemented medium that supported growth of the H strain was added at 4℃, it can be extrapolated that the addition of cold medium can still support parasite growth. None-the-less, it is possible that while the addition of cold medium may not inhibit parasite growth, the addition of medium equilibrated to the temperature of the culture sample may further encourage parasite growth.

4.3.6 ALBUMAX II supplemented medium

Jackson *et al.* (2001) reported that with the use of ALBUMAX II supplemented medium, frozen as well as previously non culture adapted *B. bovis* stocks could be cultured for prolonged periods of time. Other than facilitating the growth of the parasites that fail to grow in serum supplemented media, the lipid nature of ALBUMAX II means that it does not interfere with the analysis of SPA, thus eliminating the necessity of weaning cultures from the serum additive. Attempts were made to culture frozen stocks of the S24 strain using ALBUMAX II as a serum replacement.

Frozen stabilates were thawed in a 37ºC water bath and washed once in PBS as described in section 4.2.1. A 5 µl suspension of packed erythrocytes of the original frozen stock diluted in 95 µl of freshly washed uninfected erythrocytes (implying a parasitemia of 1%) was used for the initiation of cultures. Despite repeated attempts, S24 *in vitro* cultures from frozen stocks could not be established. This may have been due to the strain itself not adapting well to the ALBUMAX II culture systems coupled with the source of parasites being from a frozen stock rather than directly from an infected animal.

There are no culture adapted *B. bovis* strain stabilates available at OVI or in South Africa to determine if the unsuccessful culture attempts were as a result of the strains not adapting to *in vitro* cultivation or if the techniques and culture parameters did not suit the growth of *B. bovis* parasites. A positive control would have shown whether the conditions used were suitable for *B. bovis* and the lack of a positive control makes it difficult to determine which parameters to alter to obtain parasite growth.

Problems were experienced with the contamination of cultures with fungi due to the nature of the culture vessels used. Once contaminated, the vessel had to be cleaned several times by bathing in chlorine bleach and ethanol. Often the darkening of cultures was a result of this contamination rather than parasite growth.

4.4 CONCLUSIONS

The use of bovine serum supplemented media did not support the growth of either the H strain or S24 vaccine passage. Equine serum facilitated the growth of the S24 strain for a maximum period of 10 days. Gas conditions of 90% N_2, 5% O_2 and 5% CO_2 supported the growth of the S24 vaccine passage whilst 90% CO_2, 5% O_2 and 5% N_2 gas conditions did not support growth. Growth supplements did not seem to enhance growth, confirming reports made by Jackson *et al.* (2001). Hypoxanthine was added to all culture media even though it seemed to produce no effect on the growth of parasites.

The effect of heating medium to 37ºC before changing could not be evaluated as no cultures could be established, but it was concluded that the addition of medium at 4ºC did not inhibit growth. The use of ALBUMAX II supplemented medium did not facilitate the revival and growth of the S24 stock from frozen stabilates.

Further attempts to initiate cultures using ALBUMAX II supplemented medium using freshly collected *B. bovis* infected blood should be carried out. The acquisition of a culture adapted strain would serve as a positive control which would help to determine if the failure of culture growth was due to inefficient culture conditions or due to the strains themselves not adapting well to *in vitro* cultivation.

FINAL CONCLUSIONS AND RECOMMENDATIONS

The aim of this project was to investigate the nature of babesiosis outbreaks in the Swartberg region of KwaZulu-Natal, South Africa and initiate the breakthrough strain into *in vitro* MASP *B. bovis* cultures.

Isolation of the field strain H was successful and the virulence of the strain determined. Virulence can be related to a number of variables such as the method of infection, the number of infectious particles introduced, the overall well-being of the host animal and the stage in the life cycle of the pathogen. Infection of splenectomised bovine 9469/1 with the H strain proved to be fatal, despite repeated treatment attempts. Infection of intact animals with stabilates of the H strain prepared from animal 9469/1 caused mild reactions to no disease symptoms. This difference highlights the potential variation in virulence of an organism, depending on the general well-being of the host animal it infects. It is probable that in animals under stressed conditions, the H strain is potentially virulent but under stress free conditions, the strain is not virulent.

Characterisation of the vaccine strain S at passage 11 and 23 and the F and H field strains using PCR based genotyping provided profiles of the genotypes present within each strain. Through the use of the Bv80 PCR, it was established that whilst useful for discriminating between strains, this method has to be used subjectively. The number of genotypes present in each strain using Bv80 size analysis did not consistently correlate with Bv80 and 18S rRNA V4 hypervariable region sequence analysis. It was expected that the S23 vaccine passage would be present in the S11 vaccine passage because of its known passage history. Despite this, neither size nor sequence analysis could confirm this ‘carry through’ theory. The reason for this

may be that the S23 strain is present within the S11 strain but at very low levels and thus remains undetectable using both size and sequence analysis.

Bv80 size and sequence analysis, as well as 18S rRNA V4 hypervariable region sequence analysis, could not detect the S23 vaccine passage in the H strain. The Bv80 PCR could not detect animals in the carrier state of infection and if the vaccinated Haistings animal had become a carrier of the vaccine strain, it would not be detected using Bv80 analysis. However, if the vaccine strain had regained virulence and was responsible for the disease state in the animals, it would be expected to be found at higher concentrations and would be detected with the Bv80 PCR. This rules out a vaccine strain reverting to virulence as the cause of the deaths in the region.

The other strain to be investigated in this study was the F strain. It was shown to be comprised of strains that were different to the vaccine or H strain strains. A minimum number of four genotypes were identified in this strain and it showed the most diversity in Bv80 size and sequence of all the strains analysed.

Non-specific primer binding of the Bv80 PCR primers to *Bo. decoloratus* and *Bo. microplus* tick DNA implies that whilst useful for genotyping the blood stage of the *B. bovis* parasite, this method cannot be used to investigate the nature of infections in ticks. In general, it is suggested that Bv80 size analysis be coupled with another method of discrimination when investigating the genotype composition of strains. If Bv80 size and sequence analysis are used in conjunction with a method such as sequencing the 18S rRNA V4 hypervariable region, this method can be successfully used to investigate the nature of *B. bovis* outbreaks.

Though the size difference between Bv80 PCR products was small, it did allow for strain identification and discrimination. This was in contrast to the BvVA1 PCR which, despite extensive optimisation, could not be used as a method of strain discrimination for the four strains investigated. All the genotypes within a strain could not be detected using the BvVA1 PCR and it is likely that there was amplification of the major genotype donors in each strain only or that all the genotypes within the strain had BvVA1 genes of the same size. When more than one BvVA1 PCR product was amplified, the size difference was too small to allow for individual excision and restriction of the PCR product with *Acc* I. Although original publications stated that the BvVA1 PCR method was more sensitive, it has been concluded that this PCR method does not allow for the easy identification of the genotypes present in the four South African *B. bovis* strains investigated.

Establishment of *in vitro* cultures of both the H and S24 strains proved unsuccessful despite repeated attempts. A number of parameters were investigated, namely the addition of different serum types, atmospheric gas conditions, the addition of growth supplements, the heating of media before application, the initiation parasitemia and the use of ALBUMAX II as a serum replacement agent.

Limited *in vitro* growth of the S24 vaccine passage was achieved using 40% equine serum supplemented medium but growth could not be achieved in bovine serum supplemented medium. Initiation parasitemia seemed to play a role in the successful establishment of cultures and is likely due to the stage of growth of the parasites at the point of initiation. Blood collected at a high initial parasitemia, diluted to a lower parasitemia with uninfected erythrocytes, contains rapidly growing and dividing parasites whilst blood collected at a lower parasitemia would contain slower growing parasites. Optimal gas conditions were established to be 90% N_2, 5% O_2 and 5% CO_2 while the addition of growth supplements such as hypoxanthine and L-glutamine

did not have any effect on the growth of parasites. The effect of heating the medium prior to addition to the culture could not be fully investigated as cultures failed to establish. As medium was not equilibrated to 37ºC before addition in the case of the equine serum supplemented S24 vaccine passage cultures but was added at 4ºC, it can be concluded that while the addition of equilibrated medium may promote growth, the addition of cold medium does not inhibit *B. bovis in vitro* growth.

ALBUMAX II had previously been used successfully as a serum replacement agent and attempts to culture the S24 vaccine passage in medium containing ALBUMAX II was attempted. Failure to achieve culture growth was attributed to the blood used to initiate the cultures being derived from a frozen non-culture adapted *B. bovis* stock rather than that ALBUMAX II failed to support growth. The reason for the failure of cultures to establish is difficult to determine as no positive control *B. bovis* strain that has been previously cultivated is available in South Africa. The procurement of such a strain would help to determine if the culture parameters are not suited to *in vitro B. bovis* growth or if the strains used themselves do not adapt well to *in vitro* cultivation. It is recommended that before any future culture attempts are made, a positive control *B. bovis* strain be obtained.

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APPENDIX

TAE buffer (50X) - Promega

242 g Tris base and 37.2 g EDTA in 900 ml deionised water

Add 57.1 ml Glacial acetic acid and adjust final volume to 1 L

TAE buffer (1X)

Add 20 ml 50X TAE to 980 ml deionised water

TBE buffer (10X) - Promega

108 g Tris base and 55 g boric acid in 900 ml deionised water.

Add 40 ml 0.5 M EDTA (pH 8) and adjust final volume to 1 L with deionised water

TBE buffer (1X)

Add 100 ml 10X TBE to 900 ml deionised water

TE buffer- Promega

10 mM Tris-HCl

1 mM EDTA

Luria Broth

10 g Yeast Extract , 5 g Tryptone and 5 g NaCl in 1 L water

Agarose gel sample buffer (6X)- Promega

4 g sucrose and 2.5 mg bromophenol blue in 10 ml TE buffer

Ethidium Bromide (10 mg/ml)

0.1 g ethidium bromide dissolved into 10 ml distilled water