Genotypic diversity in *Babesia bovis* field isolates and vaccine strains from South Africa

M.P. Combrink[1a], P. C. Troskie[a], R. Pienaar[a], A.A. Latif[ab], B.J. Mans*[1abc]

[a]*Parasites, Vectors and Vector-borne Diseases (PVVD), Agricultural Research Council-Onderstepoort Veterinary Institute, Private Bag X05, Onderstepoort 0110, South Africa*

[b]*Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa*

[c]*Department of Life and Consumer Sciences, University of South Africa, South Africa*

*Corresponding author: mansb@arc.agric.za; [1]These authors contributed equally

Abstract

Genotypic diversity in *Babesia bovis* (cause of Asiatic redwater in cattle) vaccine strains and field isolates from South Africa were investigated using the Bv80 gene as well as microsatellites. The S11 vaccine strain possessed both A and B alleles of the Bv80 gene, as well as genotypic diversity within each allele type as defined by repeat variation resulting in different amplicon sizes. Rapid serial passage of vaccine strain from passage S10 to S24 resulted in loss of genotypic diversity that yielded a single allele A genotype with an amplicon size of 558 bp. This suggested that clonal selection occurred during rapid passaging. Extensive genotypic diversity exists in 44 field isolates characterized with both Bv80 A and B alleles, but can be readily distinguished from the S24 vaccine strain using either the Bv80 allele specific PCR assays or using multi-locus micro-satellite typing. This indicated that no recent documented clinical

cases of Asiatic redwater were caused by the reversion to virulence of the current vaccine strain.

Keywords: *Babesia bovis*, vaccine, genotypic diversity, Bv80 alleles

1. Introduction

Babesia bovis is the causative agent of Asiatic redwater in cattle, considered to be a globally important disease (Bock et al., 2004). It is transmitted by *Rhipicephalus (Boophilus) microplus*, a tick introduced into South Africa in the late 1900's and now endemic in high rainfall areas. More than 80% of cattle in South Africa occur within the geographic range of the vector tick and it has been estimated that 9% of all cattle mortalities are due to *Babesia* infection (de Waal, 1996). Current control methods include acaricide dipping for the tick vector and the use of a deep-frozen live monovalent vaccine for *B. bovis* (De Waal and Combrink, 2006). Concerns regarding the use of frozen vaccine remain, either as vaccine failure to protect animals against field strain challenge (vaccine breakthrough) or as vaccine related mortality in unmonitored block treatment after vaccination or vaccine reversion to virulence (Bock et al., 1992; Bock et al., 2000; Combrink et al., 2012; Lew et al., 1997b). Characterization of the vaccine strain and comparison to circulating field strains may address these concerns.

The current South African vaccine was derived from two series of repeated blood syringe passage of *B. bovis* in splenectomized calves with the aim of reducing virulence (Callow et al., 1979). The first series was by 10 times rapid syringe passage of the "S isolate" in splenectomized calves resulting in the S10 vaccine strain blood stabilate that was less virulent than the S isolate (De Vos, 1978; De Vos et al., 1982). This isolate was used since 1978 as S11 in the commercial vaccine. Severe reactions in a notable number of calves were,

however, observed following vaccination and a further 13 rapid blood syringe passages of S10 in 1981 resulted in blood stabilate S23 that when tested as S24 vaccine showed lower virulence compared to S11 (Combrink, unpublished research, November 1981). The S24 vaccine replaced the S11 in a chilled bivalent (also containing *Babesia bigemina*) live blood vaccine, and in April 1998 the bivalent chilled vaccine was replaced by deep-frozen monovalent live blood vaccines of *B. bovis* and *B. bigemina*, which is still in use.

Rapid passaging of *B. bovis* vaccine strains through splenectomized calves resulted in phenotypic as well as genotypic changes. Phenotypically, passaged vaccine strains become less virulent or become non-transmissible by the tick vector (Callow et al., 1979; Dalgliesh and Stewart, 1977). While reversion to virulence occurred when re-passaging through intact cattle, non-transmissible strains could not regain infectivity (Callow et al., 1979; Dalgliesh and Stewart, 1977). In the case of the S24 vaccine, both loss in virulence and non-transmissibility has been observed (de Vos et al., 1982; Mason et al., 1986). In regard to genotypic changes, it was observed that passaged populations may become clonal or less genetically diverse, the latter being linked with vaccine breakthroughs (Gill et al., 1987; Lau et al., 2011; Baravalle et al., 2012). Conversely, avirulent vaccines may harbor virulent strains (Timms et al., 1990). Genotypic characterization of vaccine strains in regard to their passage history may therefore be important to understand the genetic composition of current vaccines.

Genotypic diversity is common among *B. bovis* isolates across the globe. Markers for diversity include mini- and microsatellites (Wilkowsky et al., 2009; Perez-Llaneza et al., 2010), merozoite surface antigen 1 (Suarez et al., 2000; Lau et al., 2010), the Bv80/ Bb-1 gene (Lew et al., 1997a; Lew et al., 1997b; Hines et al., 1995; Mazuz et al., 2012) and

BvVA1 (Lew et al., 1997a; Lew et al., 1997b). Most markers have been used to investigate genetic diversity and genotypic differences between vaccine strains and field isolates. In the current study, the S24 vaccine strain from South Africa was compared with field isolates using the Bv80 gene, as well as micro-satellites. A high level of genetic diversity were detected in South African field isolates for *B. bovis* and could be accurately distinguished from the vaccine strain using allele specific primers of the Bv80 gene as markers.

Materials and Methods

Collection of *Babesia* isolates and DNA extraction

South African *Babesia bovis* vaccine strains S11, S16, S18, S22 and S24 were used from deep frozen vaccine stocks. A number of field isolates were collected by veterinarians in 2008-2012 from animals that exhibited clinical symptoms for babesiosis into EDTA-blood tubes. Other isolates were collected from commercial herds in 2011-2012, from regions known for clinical redwater outbreaks into EDTA-blood tubes (Fig. 1; Table 2). One sample (F strain) was obtained from an infected tick (de Vos, 1978). In each case microscopical analysis of Giemsa stained blood smears confirmed the identity as *B. bovis*, as well as the absence of *B. bigemina*. In addition, all samples were confirmed negative for *B. bigemina* by PCR amplification using primers specific for *B. bigemina* (BBIA: CAT CTA ATT TCT CTC CAT ACC CCT CC; BBIB: CCT CGG CTT CAA CTC TGA TGC CAA AG) as described (Figueroa et al., 1992). Genomic DNA was extracted as previously described (Mans et al., 2011), using automated MagNAPure technology (Roche). In all cases, 200 ul of whole blood were used and DNA eluted into 100 ul elution buffer (50mM Tris-HCl, pH 8.0). For all *B. bovis* assays, the *B. bigemina* vaccine strain used at Onderstepoort Veterinary Institute as well as an animal kept under tick-free quarantine conditions were used as negative controls.

Amplification, cloning, sequencing and phylogenetic analysis of the Bv80 gene

Initial characterization of the vaccine strains using the Bv80 gene was performed as described (Combrink et al., 2012) using the 1Bf and 2Br primers (Lew et al., 1997a; Lew et al., 1997b). Amplified products were analyzed using 1.2% agarose gels or the QIAxcell high resolution capillary gel electrophoresis system (Dean et al., 2013). For cloning and sequencing, amplified products were resolved using 1.2% agarose gels and bands were excised from the gels and cleaned before cloning into the pGEM T-Easy vector. Positive clones were screened using the M13F and M13R vector primers and amplified products cleaned up before sequencing using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). In all cases at least three clones were sequenced from both sides and a consensus sequence derived. Consensus sequences were translated into their open reading frames and aligned using the default parameters for ClustalX (Jeanmougin et al., 1998). Alignments were trimmed and gapped regions were removed. Phylogenetic analysis was performed in Mega5 using Neighbor-joining analysis (Tamura et al., 2011). The Jones-Taylor-Thornton (JTT) amino acid substitution model was used assuming uniform rates among sites and homogenous pattern along lineages. Gaps were treated as pairwise deletion and 10000 bootstrap analysis steps were performed to estimate nodal support.

Amplification of the Bv80 alleles

Primers were designed for allele A (BbAF: GTA GTG GAG CCC ACT GAA GAG CCG GCT GGC; BbAR: GCC ACA TTT GGG TAC AAG ATT ACA AGA AGC) and allele B (BbBF: GAG CAG CCA GTT GCT GAA GAA CCA TCT GAT; BbBR: TTC ACC TTT GCG ACC ACC GTA ACA AGG TCT). Amplification was performed using a touch-down procedure that included an initial denaturation at 95°C (2 minutes) followed by denaturation at 95°C (30s), annealing at 65-55°C (30s), extension at 72°C (2 minutes) for 10 cycles,

followed by 40 cycles using an annealing temperature of 55°C. Samples were analyzed using the High Resolution Cartridge of the QIAxcell system (Qiagen) (Dean et al., 2013). As standard, the 100 bp O'Gene Ruler ladder was used (Fermentas) and the peaks integrated using the QIAxcell software.

Amplification of the mini- and micro-satellites

Micro-satellites developed by Perez-Llaneza et al. (2010) were used and included MS1, MS2, MS4, MS5, MS8, MS10, MS11 and MS12. Amplification conditions were as described (Perez-Llaneza et al., 2010). Amplified products were analyzed using the QIAXcell system as described above.

Results

Sampling of clinical and field survey samples of Asiatic redwater

All clinical and field survey samples were confirmed to be *B. bovis* positive by microscopy as well as PCR assay. Samples were obtained from regions that cover the current geographic distribution of *R. (B.) microplus* as well as regions known for clinical *B. bovis* outbreaks (Fig. 1). Several samples were derived from the same general region, while two clinical cases occurred outside the known vector distribution area (Fig. 1).

Characterization of the Bv80 gene for the vaccine strains

Analysis using agarose gel electrophoresis indicated that the banding patterns became less complex with passaging, with the S11 strain showing a large diffuse band at ~700-800 bp that seem to be composed of multiple amplification products, while the S22 and S24 strains only showed one band at ~700 bp (Fig. 2A). A significant change seems to have occurred between passage S16 and S18, where the ~800 bp bands are less prominent. High resolution

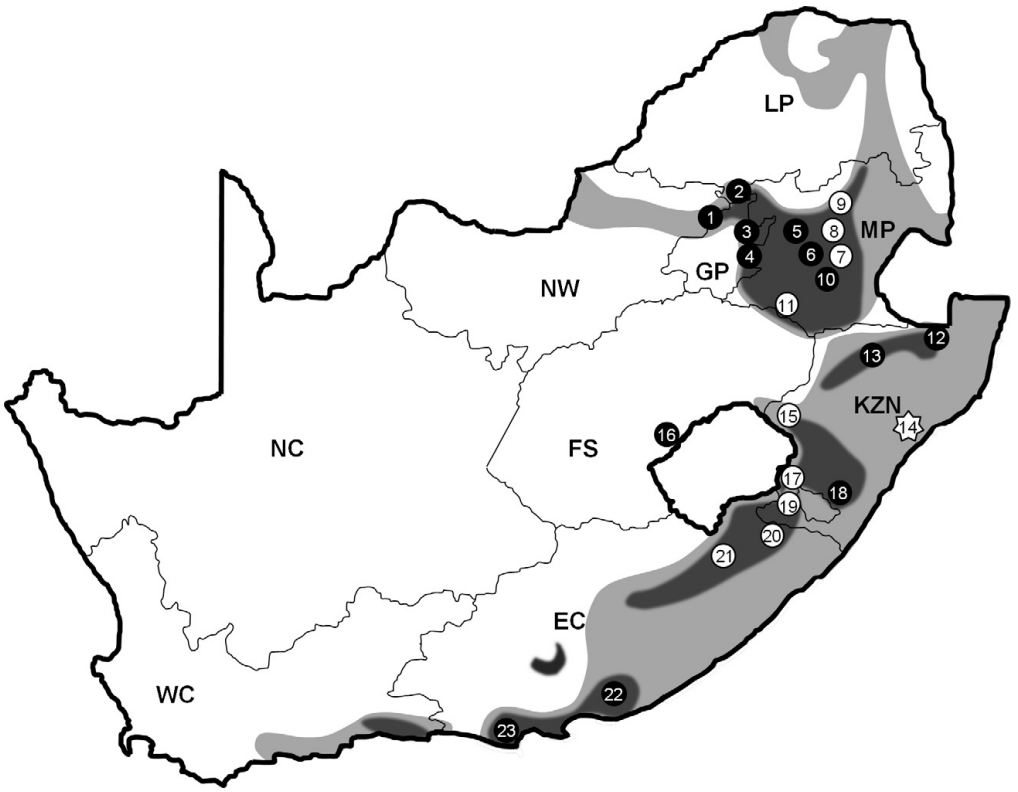

Fig. 1. Geographic distribution of the B. bovis field isolates characterized in the current study. A map of South Africa with the distribution of R. (B.) microplus indicated in light gray (Arthur Spickett, unpublished data). Areas of dark gray indicate historical reported incidents of redwater outbreaks from 2002-2013 (Combrink, personal communication). Black dots with white numbering indicate sites where clinical isolates were sampled. White dots with black numbering indicate sites where field survey isolates were sampled and the star indicate the origin of the infected tick strain F. Numbers indicate regions sampled: 1-Pretoria, 2-Bela-Bela (Warmbaths), 3-Bronkhorstspruit, 4-Delmas, 5-Middelburg, 6-Hendrina, 7-Carolina, 8-eMakhazeni (Belfast), 9-Dullstroom, 10-Ermelo, 11-Standerton, 12-Pongola, 13-Vryheid, 14-Eshowe, 15-Bergville, 16-Ladybrand, 17-Underberg, 18-Ixopo, 19-Swartberg (Kokstad), 20-Kokstad, 21-Maclear, 22-Grahamanstown, 23-Humansdorp. Provinces are indicated by WC (Western Cape), EC (Eastern Cape), NC (Northern Cape), FS (Free State), NW (North West), GP (Gauteng), MPL (Mpumalanga), LP (Limpopo), and KZN (KwaZulu-Natal).

Table 1: Repeats found in different clones from various vaccine passages.

Repeats	S11 1	S11 6	S11 9	S16 2	S16 7	S16 8	S18	S22	S24	S11 7	S16 1
EEPVA	8	8	8	8	8	16	16	16	16	0	0
EEPIA	7	9	8	9	8	4	4	4	4	0	0
EEPIV	1	1	1	1	1	0	0	0	0	2	2
EEPVE	0	0	0	0	0	1	1	1	1	0	0
EEPVV	0	0	0	0	0	0	0	0	0	1	1
PAEK	5	5	5	5	5	1	1	1	1	15	15
PAET	7	6	7	6	7	0	0	0	0	12	12

Table 2: Summary of the analysis of Babesia bovis field isolates and vaccine strains using various genotypic markers.

Region	Sample	Bv80A	Bv80B	MS1	MS2	MS4	MS5	MS8	MS 10	MS11	MS12
Pretoria	5	NEG	597, 681	155, 179	NEG	121, 133	222, 234, 246	324	NEG	241, 265, 277	277, 288
Pretoria	6	690	543, 681	131	NEG	121	222	324	229	193	277, 288
Pretoria	7	NEG	522, 681	131	NEG	121, 133	222, 234, 246	324	NEG	270, 324, 336, 348	277, 288
Pretoria	8	681	465	155, 179, 191	NEG	109, 121	222, 234, 252	336	NEG	253, 313	277, 288
Pretoria	9	429, 798	483	151, 167	245, 275, 285	109, 133, 145	222, 234, 240, 252	336, 348	NEG	265, 277, 289, 301, 313, 325	288, 299
Bela-Bela (Warmbaths)	3	681	579, 624	155, 179	255, 279	121, 133	222, 234, 252, 258, 270	336, 348	229	253, 277, 289, 301	NEG
Bronkhorstspruit	1	NEG	600	155	NEG	133, 145	234	336	NEG	325	NEG
Bronkhorstspruit	07–68	687	NEG	155, 179	275	109, 121, 133	222	336	NEG	277, 313, 361	NEG
Bronkhorstspruit	06–183	648	NEG	131	275	121	222, 234, 240, 252	276, 336	NEG	249	299
Bronkhorstspruit	05–18	675	576	155	275	145	222, 234, 240, 246	336	NEG	277, 325, 373	NEG
Bronkhorstspruit	05–100	NEG	615	155	275, 285, 295	109, 121, 133, 145	234	336	NEG	277, 325, 337, 361	310
Bronkhorstspruit	05–323	NEG	651	143, 155	255	121	222, 246	276, 324	229	241	277, 288
Delmas	2	729	564, 669	155, 179	285	NEG	222, 234, 240, 270	336	229	265	310
Middelburg	10	663	474	155, 167, 179	275, 295	121, 133, 145	222, 234, 246	NEG	229	229, 241, 253, 265, 277, 289, 301, 325	288, 299
Hendrina	12	NEG	603, 633	143, 155	NEG	121	222	336, 360	NEG	241, 277, 289	NEG
Carolina	39	381, 699	630	155	NEG	NEG	NEG	288	NEG	241	NEG
Carolina	40	858	423, 540	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
eMakhazeni (Belfast)	41	NEG	519, 636	NEG	NEG	NEG	NEG	NEG	NEG	281	NEG
Dullstroom	44	NEG	525, 651	167, 179	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Dullstroom	45	720	500, 687	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
Ermelo	36	816	504, 576	155, 179	NEG	NEG	NEG	NEG	NEG	253, 325, 361	NEG
Standerton	15	NEG	594, 693	155, 191	255	NEG	222, 234, 240, 252	336	NEG	289, 325	NEG
Pongola	4	636	549	155	NEG	109, 121, 133, 145	222, 234, 252, 270	288, 336, 348	229	289, 301	NEG
Vryheid	11	687	NEG	155	NEG	121, 133	222	324	NEG	241	NEG
Eshowe	F	771	639	155	285	121	222	336	229	253, 277, 289	277, 288
Bergville	16	NEG	462, 561, 669	155	285	NEG	222	336	NEG	246, 258, 282, 306	NEG
Bergville	17	816	468	143	NEG	NEG	222, 234, 252	336	229	241	NEG
Ladybrand	65	675	NEG	NEG	275	109, 121	234	324, 336	229	277	288
Ladybrand	66	675	NEG	155	275	109, 121, 133	222	324, 336	NEG	277	NEG
Underberg	24	645, 684	492, 603	155, 191	NEG	121	222, 234, 246	336	229	289, 301, 325, 337, 349	288
Underberg	25	645, 690	483	155, 179	285	121, 133	222, 246	336, 360	229	265, 277, 313, 325, 337	NEG
Ixopo	33	693	453, 579	155, 179	285	109,121, 133	222	324	NEG	241, 265, 277, 289	288
Swartberg (Kokstad)	H	NEG	612	143, 155	NEG	109, 121, 145	222, 234, 240, 252	336	229	277, 325, 349, 361	277
Kokstad	27	654, 744	510, 594	155	265	NEG	NEG	NEG	229	253, 325	NEG
Kokstad	29	729	567	143, 155, 191	NEG	NEG	222, 252	336, 348	NEG	253	NEG
Maclear	30	642	456, 696	143, 155	255	157	222, 234, 240, 252	NEG	NEG	241, 253, 277, 289, 301, 313, 325, 337, 349	288, 299
Maclear	31	642	537	143, 155	NEG	NEG	NEG	NEG	229	229, 253, 265, 277, 289	NEG
Grahamstown	14	558	NEG	155	NEG	109, 121	222	336	NEG	241	NEG
Humansdorp	55	690	597	131	NEG	121, 133	NEG	NEG	229	241, 277, 289	288
Humansdorp	58	660	462	NEG	275	121, 157	NEG	276, 336	NEG	277, 325, 337	288, 299
Humansdorp	59	660	459, 786	NEG	275	121	222, 234, 246	336	NEG	277, 325, 337	NEG
Humansdorp	60	675	NEG	NEG	275	121, 133	222, 234, 246	NEG	NEG	301, 325, 349, 361	NEG
Humansdorp	61	NEG	474	NEG	NEG	133	234, 270, 276	324	NEG	241, 325, 349, 361	NEG
Humansdorp	62	675	NEG	NEG	NEG	133	222	336	NEG	241, 325	NEG
Vaccine strain	S11	645	585	155, 167	NEG	121, 133	222	336	229	241	222, 277, 288, 321, 332
Vaccine strain	S16	645	585	155	NEG	121, 133	222	336	229	241	222, 277, 288, 321, 332
Vaccine strain	S18	558	574	155	NEG	121, 133	222	336	229	241	222, 277, 288, 321, 332
Vaccine strain	S22	558	NEG	155	NEG	121, 133	222	324	NEG	241	222
Vaccine strain	S23	558	NEG	155	NEG	121, 133	222	324	NEG	241	222
Alleles		24	44	6	6	5	5	4	1	13	7

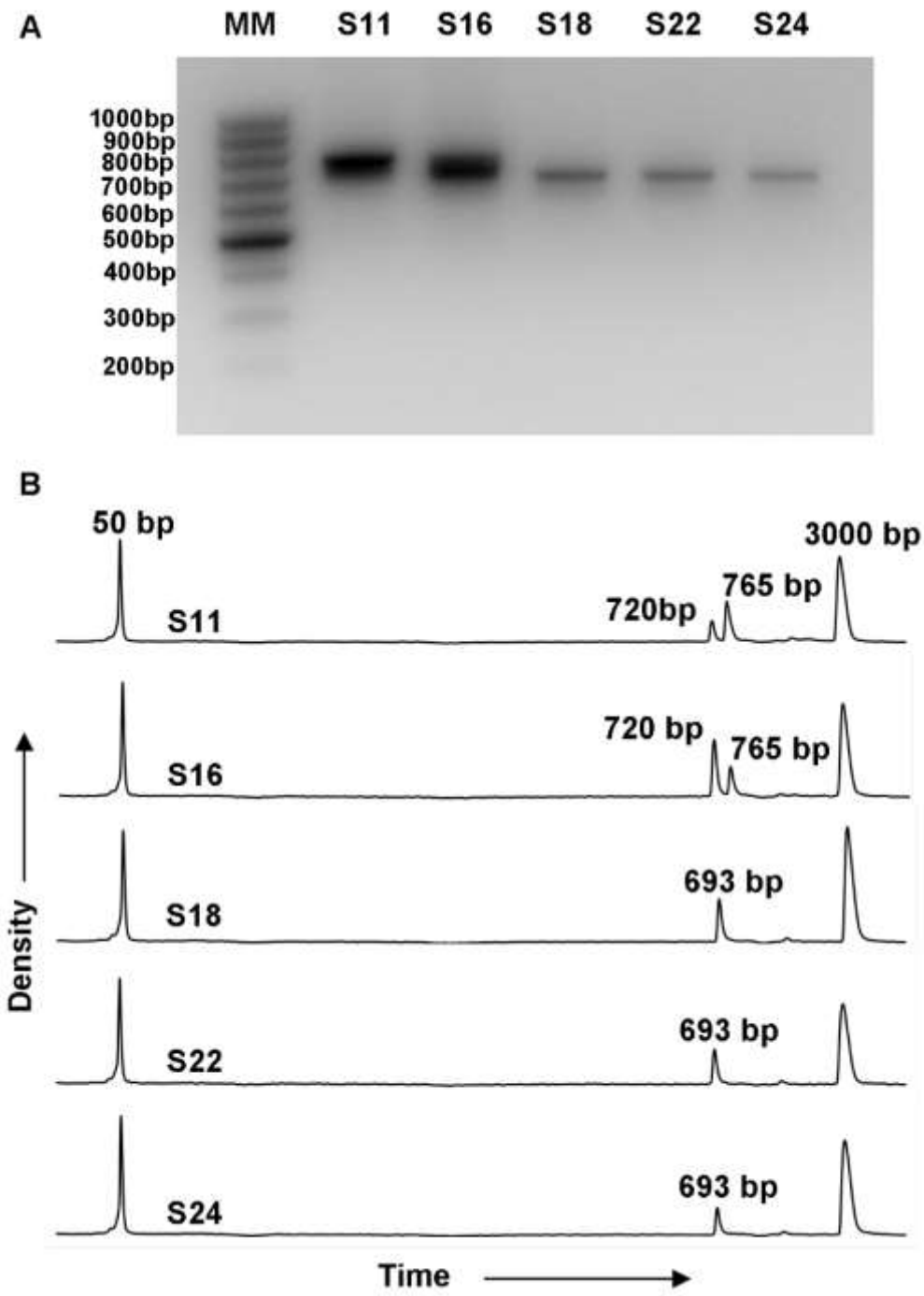

Fig. 2. Characterization of the Bv80 gene for various vaccine passaged strains. Strains were amplified using the 1Bf and 2Br primers of Lew et al. (1997a; 1997b). A) Agarose gel electrophoresis of amplified products. B) Analysis by high resolution capillary gel electrophoretic. Indicated are electrophoretic traces various passaged vaccine strains and their calculated molecular sizes based on a 100 bp control ladder. The 50 bp and 3000 bp alignment markers are also indicated.

electrophoretic analysis indicated that two major peaks are found for S11 and S16, of 720 and 765 bp, respectively. In contrast, S18, S22 and S24 only showed a single peak of 693 bp (Fig. 2B).

Cloning and sequencing indicated that the vaccine strain was composed of a variety of Bv80 genotypes with different known repeats for the Bv80 gene and could be distinguished based on two dominant genotypes designated as allele A and allele B (Fig. 3). Phylogenetic analysis

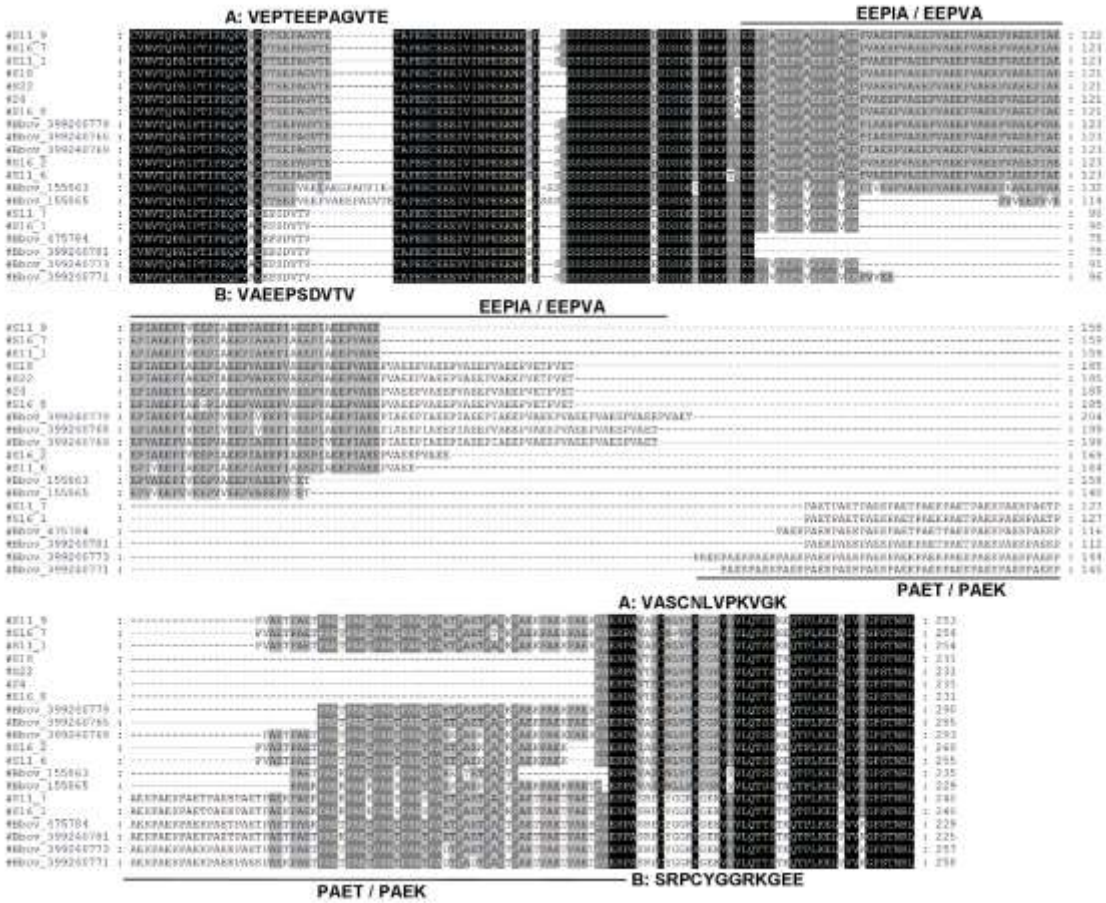

Fig. 3. Alignment of the Bv80 gene for vaccine strains. The conserved regions for Allele A and B, is boxed in gray and white, respectively. Various repeat types are indicated by different types of lines.

indicated that the sequences grouped into two major clades that corresponded with previously characterized strains from Argentina, Australia and Mexico (Fig. 4). The clades represent the

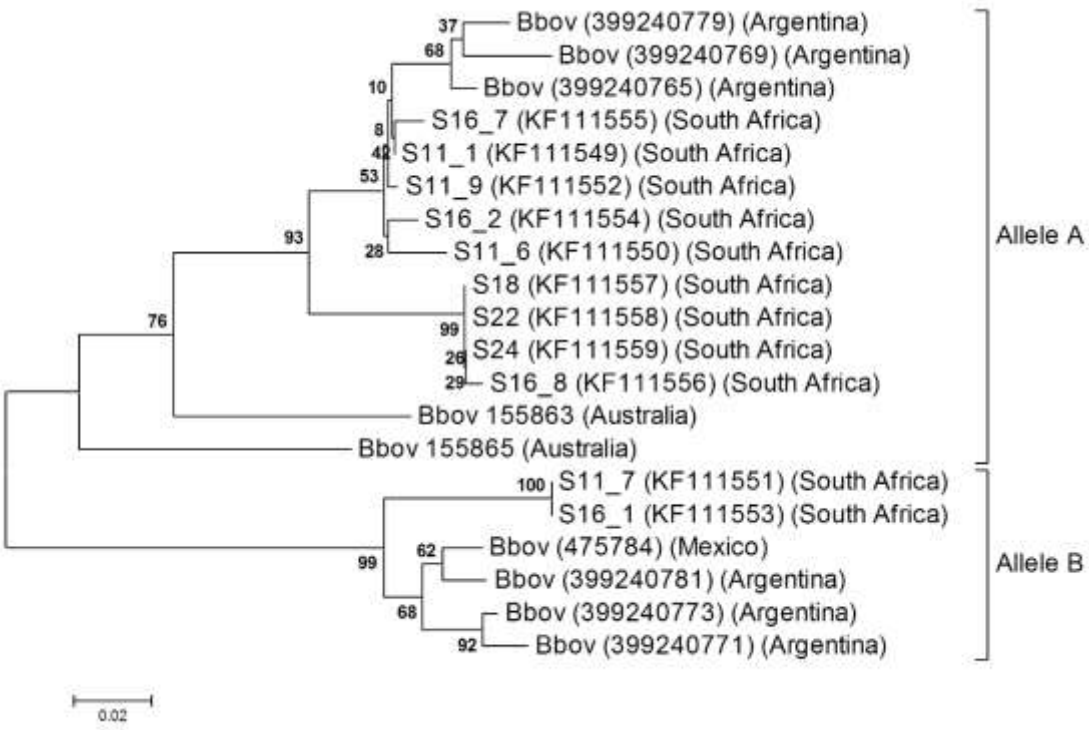

Fig. 4: Phylogenetic analysis of the Bv80 gene for vaccine strains. Indicated are clades for Allele A and B. South African vaccine strains are indicated by the number S followed by the passage, followed by the sequenced clone number. Numbers in parenthesis indicate GenBank accession numbers while the country of origin is also indicated.

A and B alleles for the Bv80 gene and indicated that passages S11 and S16 possessed both alleles while passages S18, S22 and S24 only possess allele A with identical sequences. Of interest is the fact that repeats EEPIA/EEPVA predominate in genotypes with the A alleles, while PAET/PAEK repeats are predominantly found in genotypes from the B allele (Table 2). In the case of S18, S22 and S24 the dominant repeat is EEPVA with only one PAEK repeat present.

Development of Bv80 allele specific assays

Primers specific for the different Bv80 alleles were designed based on conserved sequences, which will specifically amplify the conserved regions of allele A or allele B as well as the

central repeat region. These primers were able to detect the A and B alleles in the various vaccine passaged strains using high resolution capillary gel electrophoresis (Fig. 5). It confirmed that S11 and S16 possess both alleles, while S22 and S24 possess only a single genotype for allele A. The passaged S18 strain show evidence of transition in genotype populations, with the allele A genotype for S22 and S24 present, but also low level of an allele B genotype. The possibility therefore existed to use these assays as means to differentiate vaccine strains from field isolates.

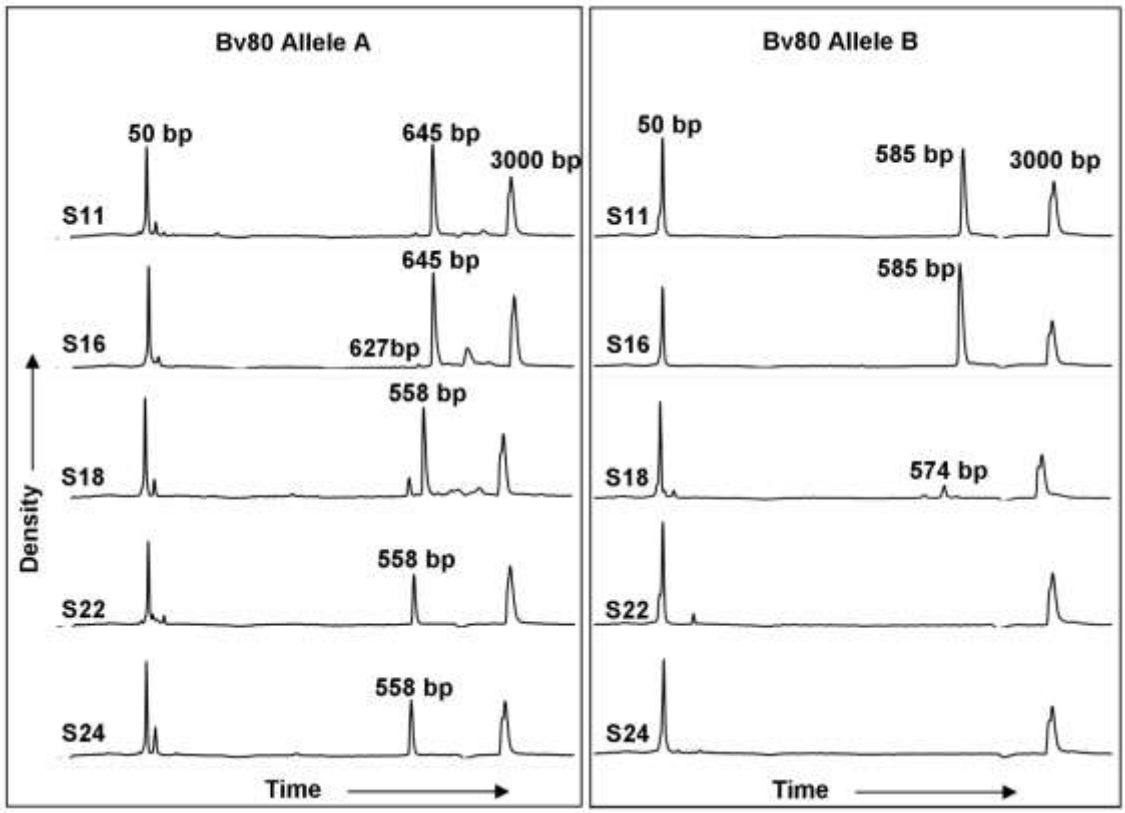

Fig. 5. Characterization of the Bv80 alleles for the vaccine strains. Indicated are electrophoretic traces various passaged vaccine strains and their calculated molecular sizes based on a 100 bp control ladder. The 50 bp and 3000 bp alignment markers are also indicated.

Screening of field isolates using Bv80 allele specific assays as well as micro-satellites

A total of 44 field isolates that derived from various *B. bovis* endemic regions of South Africa were screened using the Bv80 allele specific primers. In addition 8 different micro-satellite

markers were also tested (Table 3). The majority of field isolates were infected with genotypic diverse populations of *B. bovis* that possessed different or both Bv80 alleles as well as a variety of micro-satellites.

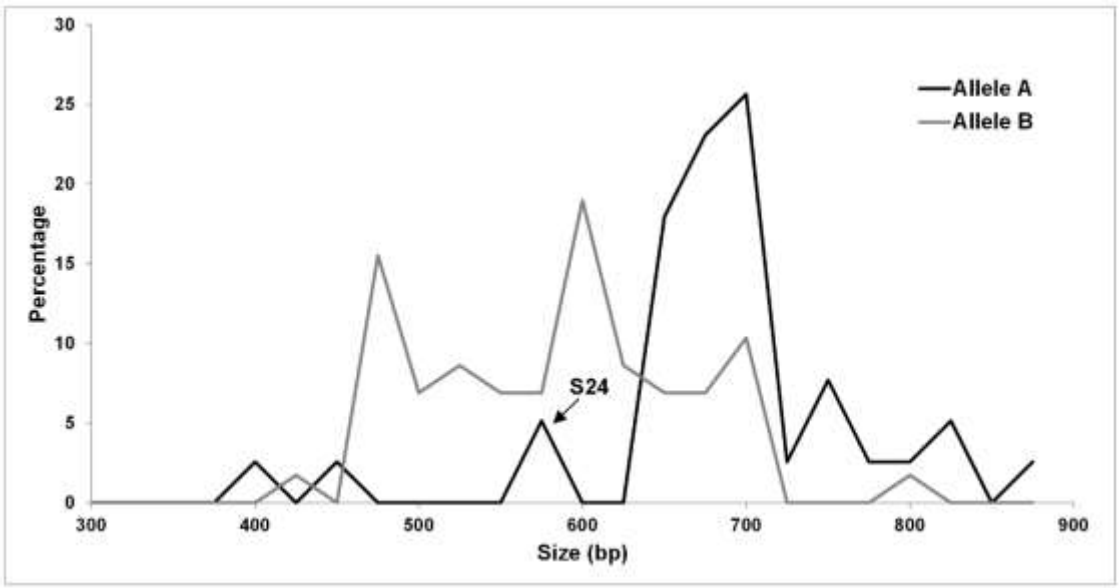

Fig. 6. Frequency distribution of allele sizes for the Bv80 gene from field isolates in South Africa. The size of allele A for S24 vaccine strain is indicated with an arrow.

In the case of the Bv80 allele A band sizes ranged from 381-858 bp (Fig. 6). A frequency distribution indicated that the majority of genotypes (89%) for allele A were above 585 bp. The majority of these genotypes (87%) ranged from 650-835 bp, indicating that the vaccine strain S24 was unique in its composition and could be easily distinguished from field strains. Only one animal (sample 14) possessed a band of 585 bp similar to the vaccine strain, but this animal was inoculated 19 days prior to blood sample collection with the commercially available S24 vaccine (Nick Fischër personal communication, February 2011). The Bv80 allele B band sizes ranged from 423-786 bp with 90% falling between 475-700 bp. This would indicate that definitive differences exist in the repeat composition of allele A and B

genotypes, with allele A genotypes being on average larger than allele B genotypes. Many field isolates possessed both Bv80 A and B alleles, while a number also possessed multiple genotypes for each allele.

For most of the micro-satellites tested, a variable number of alleles were found ranging from 4-13. The only uninformative micro-satellite was MS-10 that either gave a band at 229 bp or was negative. No differences existed in micro-satellite profiles of the vaccine strains to enable distinction between different passages. The alleles for vaccine strain S24 were also found in numerous field isolates, while many field isolates possessed multiple alleles. Using a multi-locus approach, the vaccine strains may be distinguished from field isolates. Of interest, was that the micro-satellite profile of sample 14, the animal recently vaccinated with the S24 vaccine strain differed from that of the vaccine strain.

Discussion

A previous study indicated that S11 and S24 strains differed in their Bv80 composition, but was only analyzed according to size differences for which the allelic information was not known (Olds, 2008). Genotyping of the vaccine strains (S11 – S16) using allele specific primers suggested that the earlier more virulent strains (de Vos, 1978; de Vos et al., 1982), were composed of a higher genotypic diverse *B. bovis* population as exhibited by the presence of multiple bands and alleles for the Bv80 gene. The S11 strain was composed of at least four populations that possessed both alleles of the Bv80 gene. Since this passage already indicated loss of virulence (de Vos, 1978; de Vos et al., 1982), it may be assumed that the original parental strain was composed of an even more diverse and virulent *B. bovis* population. Unfortunately these parental stocks were not available for analysis. It is possible that the loss of virulence and tick-transmissibility in the S24 strain is related to loss in genetic

diversity. This would have to be confirmed, since it is not known whether the S11 strain can still be transmitted by the tick vector.

The avirulent and non-tick transmissible S24 strain is composed of a *B. bovis* population that is less genotypic diverse as exhibited by the single allele A for the Bv80 gene. The S24 genotype was not observed in the S11 strain, but were detected in the S16 strain, suggesting a shift in dominant populations during attenuation (Mazuz et al., 2012; Baravalle et al., 2012). This population remained stable from passage S18 up to the current passage (S24) used for live vaccine production. Inoculation of the S23 vaccine strain into intact cattle to produce the S24 strain, resulted in the same genotype on different occasions (Combrink et al., 2013; unpublished results). This contrasts with reversion to virulence of a non-virulent phenotype upon sub-inoculation into intact steers that was interpreted as possible selection of different sub-populations (Timms et al, 1990). The possibility therefore exist that a clonal *B. bovis* population were derived due to rapid passage in splenectomized animals with the loss of virulent and tick-transmissible parasite populations (Gill et al., 1987). Similar observations were previously made using other geographically distinct *B. bovis* isolates that were attenuated by rapid passage (Lau et al., 2011; Mazuz et al., 2012; Baravalle et al., 2012).

The Bv80 gene, also known as Bb-1 is a merozoite protein localized to the apical spherical body and is secreted during invasion of the host erythrocytes (Hines et al., 1995). Bv80 is composed of conserved flanking regions characterized by either allele A or allele B signatures with the N-terminal regions associated with Th1 epitopes (Brown et al., 1993; Hines et al., 1995). Allele A was previously found in Australia, while the B-allele was found in Mexico and Israel (Dalrymple et al., 1993; Hines et al., 1995; Mazuz et al., 2012). It has not been determined yet whether both alleles occur in Northern America, Australia and Israel

and whether current distributions are due to sampling bias. In contrast, both A and B alleles have been demonstrated to circulate in Argentina (Baravalle et al., 2012), similar to the current study. The vector tick *R. (B.) microplus* and *B. bovis* was presumably introduced into southern Africa during the 1900s from an unknown origin (Norval et al., 1992). It is possible that multiple introductions led to establishment of both A and B alleles from various geographic areas, or from a region where both alleles occurred.

The Bv80 gene also possesses a central variable region composed of repeats (Hines et al., 1995). Two main repeat structures may be observed with local variation that includes the signatures EEPIA/EEPVA or PAET/PAEK (Hines et al., 2009; Wilkowsky et al., 2009). In the case of vaccine strain S24 the PAET/PAEK signature was almost completely lost, yielding a strain with one of the lowest repeat lengths observed compared to field isolates. In field isolates a wide variety of repeat lengths were observed. No correlation was previously found between virulence and Bv80 size (repeat length) or the presence or absence of allele A and B (Lau et al., 2011; Baravalle et al., 2012). The Bv80 gene or the S24 genotype can therefore not be used as a marker for virulence per se, but could be used to distinguish vaccine strains from field strains.

The vaccine strain S24 could be distinguished from field isolates using the combined Bv80 allele and micro-satellite data, or using the Bv80 allele data alone. This could be useful within a southern African setting to investigate and determine the role that the vaccine strain plays in *B. bovis* outbreaks. To date none of the clinical field cases indicated that the vaccine strain played a role in outbreaks.

Acknowledgements

This study was supported by Red Meat Research and Development South Africa (Project: OV14/06/C226). We thank Arthur Spickett for unpublished data on the geographic distribution of *R. (B.) microplus*. We would also like to thank the following persons for their collaborative support during surveys and for the samples sent in from clinical cases: Drs Graham Carr, Nick Fischër, Neil Fourie, Droughty Hartley, Gavin King, Andy Lund, Craig Macfarlan, Mits Morford, Riaan Mulder, Jubie Muller, Frikkie Nel, Dieter Nischk, Silke Pfitzer, Ben Potgieter, Thys Potgieter, Ruan Scheepers, Gustaf Trümpelmann, Nienke van Hasselt, Francois van Niekerk, Dirk Verwoerd, Mr Olivier Matthee and Sr Dinlie Smith.

References

Baravalle, M.E., Thompson, C., Valentini, B., Ferreira, M., Torioni de Echaide, S., Christensen, M.F., Echaide, I., 2012. *Babesia bovis* biological clones and the inter-strain allelic diversity of the Bv80 gene support subpopulation selection as a mechanism involved in the attenuation of two virulent isolates. Vet. Parasitol. 190, 391-400.

Bock, R.E., de Vos, A.J., Kingston, T.G., Shiels, I.A., Dalgliesh, R.J., 1992. Investigations of breakdowns in protection provided by living *Babesia bovis* vaccine. Vet. Parasitol. 43, 45-56.

Bock, R.E., Lew, A.E., Minchin, C.M., Jeston, P.J., Jorgensen, W.K., 2000. Application of PCR assays to determine the genotype of *Babesia bovis* parasites isolated from cattle with clinical babesiosis soon after vaccination against tick fever. Aust. Vet. J. 78, 179-181.

Bock, R., Jackson, L., De Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasit. 129, S247-S269.

Brown, W.C., Zhao, S., Woods, V.M., Tripp, C.A., Tetzlaff, C.L., Heussler, V.T., Dobbelaere, D.A., Rice-Ficht, A.C., 1993. Identification of two Th1 cell epitopes on the

Babesia bovis-encoded 77-kilodalton merozoite protein (Bb-1) by use of truncated recombinant fusion proteins. Infect. Immun. 61, 236-244.

Callow, L.L., Mellors, L.T., McGregor, W., 1979. Reduction in virulence of *Babesia bovis* due to rapid passage in splenectomized cattle. Int. J. Parasitol. 9, 333-338.

Combrink, M.P., Carr, G., Mans, B.J., Marais, F., 2012. Blocking *Babesia bovis* vaccine reactions of dairy cattle in milk. Onderstepoort J. Vet. Res. 79, E1-E4.

Dalgliesh, R.J., Stewart, N.P., 1977. Failure of vaccine strains of *Babesia bovis* to regain infectivity for ticks during long-standing infections in cattle. Aust. Vet. J. 53, 429-431.

Dalrymple, B.P., Peters, J.M., Goodger, B.V., Bushell, G.R., Waltisbuhl, D.J., Wright, I.G., 1993. Cloning and characterisation of cDNA clones encoding two *Babesia bovis* proteins with homologous amino- and carboxy-terminal domains. Mol. Biochem. Parasitol. 59, 181-189.

Dean, D.A., Wadl, P.A., Hadziabdic, D., Wang, X., Trigiano, R.N., 2013. Analyzing Microsatellites Using the QIAxcel System. Meth. Mol. Biol. 1006, 223-243.

De Vos, A.J., 1978. Immunogenicity and pathogenicity of three South African strains of *Babesia bovis* in *Bos indicus* cattle. Onderstepoort J. Vet. Res. 45, 19-124.

De Vos, A.J., Bessenger, R., Fourie, C.G., 1982. Virulence and heterologous strain immunity of South African and Australian *Babesia bovis* strains with reduced pathogenicity. Onderstepoort J. Vet. Res. 49, 133-136.

De Waal, D.T., 1996. Vaccination against babesiosis. Acta Parasit. Turcica 20, 487-500.

De Waal, D.T., Combrink, M.P., 2006. Live vaccines against bovine babesiosis. Vet. Parasitol. 138, 88-96.

Figueroa, J.V., Chieves, L.P., Johnson, G.S., Buening, G.M., 1992. Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30, 2576-2582.

Gill, A.C., Cowman, A.F., Stewart, N.P., Kemp, D.J., Timms, P., 1987. *Babesia bovis*: Molecular and biological characteristics of cloned parasite lines. Exp. Parasitol. 63, 180-188.

Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., Suarez, C.E., Vidotto, O., Rice-Ficht, A.C., 1995. Genetic and antigenic characterization of *Babesia bovis* merozoite spherical body protein Bb-1. Mol. Biochem. Parasitol. 69, 149-159.

Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G., Gibson, T. J., 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23, 403–405.

Lau, A.O., Cereceres, K., Palmer, G.H., Fretwell, D.L., Pedroni, M.J., Mosqueda, J., McElwain, T.F., 2010. Genotypic diversity of merozoite surface antigen 1 of *Babesia bovis* within an endemic population. Mol. Biochem. Parasitol. 172, 107-112.

Lau, A.O., Kalyanaraman, A., Echaide, I., Palmer, G.H., Bock, R., Pedroni, M.J., Rameshkumar, M., Ferreira, M.B., Fletcher, T.I., McElwain, T.F., 2011. Attenuation of virulence in an apicomplexan hemoparasite results in reduced genome diversity at the population level. BMC Genomics 12, 410.

Lew, A.E., Dalrymple, B.P., Jeston, P.J., Bock, R.E., 1997a. PCR methods for the discrimination of *Babesia bovis* isolates. Vet. Parasitol. 71, 223-237.

Lew, A.E., Bock, R.E., Croft, J.M., Minchin, C.M., Kingston, T.G., Dalgliesh, R.J., 1997b. Genotypic diversity in field isolates of *Babesia bovis* from cattle with babesiosis after vaccination. Aust. Vet. J. 75, 575-578.

Mans, B.J., Pienaar, R., Latif, A.A., Potgieter, F.T. 2011. Diversity in the 18S SSU rRNA V4 hyper-variable region of *Theileria* in bovines and African buffalo (*Syncerus caffer*) from southern Africa. Parasitol. 138, 766-779.

Mason, T.E., Potgieter, F.T., Van Rensburg, L., 1986. The inability of a South African *Babesia bovis* vaccine strain to infect *Boophilus microplus*. Onderstepoort J. Vet. Res. 53, 143-145.

Mazuz, M.L., Molad, T., Fish, L., Leibovitz, B., Wolkomirsky, R., Fleiderovitz, L., Shkap, V., 2012. Genetic diversity of *Babesia bovis* in virulent and attenuated strains. Parasitol. 139, 317-323.

Norval, R.A.I., Perry, B.D., Young, A.S., 1992. The Epidemiology of Theileriosis in Africa. Academic Press, London.

Olds, C.L. 2008. The isolation and characterization of a *Babesia bovis* stock from outbreaks in the Swartberg region of KwaZulu-Natal, South Africa. MSc dissertation, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, South Africa.

Perez-Llaneza, A., Caballero, M., Baravalle, E., Mesplet, M., Mosqueda, J., Suarez, C.E., Echaide, I., Katzer, F., Pacheco, G.M., Florin-Christensen, M., Schnittger, L., 2010. Development of a tandem repeat-based multilocus typing system distinguishing *Babesia bovis* geographic isolates. Vet. Parasitol. 167, 196-204.

Suarez, C.E., Florin-Christensen, M., Hines, S.A., Palmer, G.H., Brown, W.C., McElwain, T.F., 2000. Characterization of allelic variation in the *Babesia bovis* merozoite surface antigen 1 (MSA-1) locus and identification of a cross-reactive inhibition-sensitive MSA-1 epitope. Infect. Immun. 68, 6865-6870.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol. Biol. Evol. 28, 2731-2739.

Timms, P., Stewart, N.P., De Vos, A.J., 1990. Study of virulence and vector transmission of *Babesia bovis* by use of cloned parasite lines. Infect. Immun. 58, 2171-2176.

Wilkowsky, S.E., Moretta, R., Mosqueda, J., Gil, G., Echaide, I., Lía, V., Falcón, A., Florin Christensen, M., Farber, M., 2009. A new set of molecular markers for the genotyping of *Babesia bovis* isolates. Vet. Parasitol. 161, 9-18.