Characterization of *Anaplasma marginale* subsp. *centrale* Strains by Use of *msp1aS* Genotyping Reveals a Wildlife Reservoir

Zamantungwa T. H. Khumalo,[a] Helen N. Catanese,[b] Nicole Liesching,[a] Paidashe Hove,[a,c] Nicola E. Collins,[a] Mamohale E. Chaisi,[a] Assefaw H. Gebremedhin,[b] Marinda C. Oosthuizen,[a] Kelly A. Brayton[a,d]*

Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa[a];
School of Electrical Engineering and Computer Science, Washington State University, Pullman, Washington, USA[b];
Biotechnology Platform, Agricultural Research Council, Onderstepoort, Pretoria, South Africa[c];
Program in Vector Borne Diseases, Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington, USA[d]

*Address correspondence to Kelly A. Brayton, kbrayton@vetmed.wsu.edu.

Bovine anaplasmosis caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* is endemic in South Africa. *Anaplasma marginale* subspecies *centrale* also infects cattle; however, it causes a milder form of anaplasmosis and is used as a live vaccine against *A. marginale*. There has been less interest in the epidemiology of *A. marginale* subsp. *centrale*, and, as a result, there are few reports detecting natural infections of this organism. When detected in cattle, it is often assumed that it is due to vaccination, and in most cases, it is reported as coinfection with *A. marginale* without characterization of the strain. A total of 380 blood samples from wild ruminant species and cattle collected from biobanks, national parks, and other regions of South Africa were used in duplex real-time PCR assays to simultaneously detect *A. marginale* and *A. marginale* subsp. *centrale*. PCR results indicated high occurrence of *A. marginale* subsp. *centrale* infections, ranging from 25 to 100% in national parks. Samples positive for *A. marginale* subsp. *centrale* were further characterized using the *msp1aS* gene, a homolog of *msp1*α of *A. mar-ginale*, which contains repeats at the 5′ ends that are useful for genotyping strains. A total of 47 Msp1aS repeats were identified, which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo, and wildebeest. RepeatAnalyzer was used to examine strain diversity. Our results demonstrate a diversity of *A. marginale* subsp. *centrale* strains from cattle and wildlife hosts from South Africa and indicate the utility of *msp1aS* as a genotypic marker for *A. marginale* subsp. *centrale* strain diversity.

Bovine anaplasmosis (gallsickness) is a tick-borne disease caused by the intraerythrocytic rickettsial pathogen *Anaplasma marginale* (1). *A. marginale* is globally prevalent and results in anemia, with mortality rates of up to 30% (2). *Anaplasma marginale* subspecies *centrale* is a less virulent subspecies detected by Sir Arnold Theiler, who recognized its potential as a vaccine against anaplasmosis; 100 years later this live vaccine is still in use in South Africa, Israel, South America, and Australia (3, 4). The strain that is used as a vaccine originated from Theiler's original isolation and was exported at various times to other countries where it has been propagated in the laboratory; the strain known as the "Israel strain" or the "vaccine strain" was sent to Israel in the 1950s and was used to generate the complete genome sequence for *A. marginale* subsp. *centrale* in 2010 (5). *A. marginale* subsp. *centrale* does not provide complete protection against *A. marginale* infection but does protect against severe anaplasmosis (6, 7).

A. marginale infects a wide range of ruminants including buffalo (*Bubalus bubalis* and *Syncerus caffer*), wildebeest (*Connochaetes gnou* and *Connochaetes taurinus*), American bison (*Bison bison*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) (8–11). Cattle are naturally susceptible to *A. marginale* (4). There has not been much interest in the epidemiology of *A. marginale* subsp. *centrale*, with few reports detecting natural infections of this organism; most often, when detected in cattle it is assumed that it is due to vaccination and is reported as coinfection with *A. marginale* without characterization of the strain (12). Reported *A. marginale* subsp. *centrale* single infections were detected by the reverse line blot (RLB) hybridization assay in Italy without characterization of the strain. More recently, the first known case of bovine anaplasmosis caused by *A. marginale* subsp. *centrale* in Europe was reported (13). While this study described genetic heterogeneity of *A. marginale* subsp. *centrale* strains from different geographic areas in Italy, it is not clear how these are related to the vaccine strain.

For *A. marginale*, the Msp1a protein/gene (*msp1*α) has been used as a genotypic marker to differentiate strains (14). Msp1a is encoded by the single-copy gene, *msp1*α and differs among strains due to variable sequence and numbers of an 84/87-bp repeat sequence (28 or 29 amino acids) located near the amino terminus of the protein (14). A number of studies have examined Msp1a repeats in the United States, South America, Australia, the Philippines, Europe, Israel, China, and Mexico, resulting in identification of more than 200 repeats (14–16). In South Africa, two studies have been conducted to genetically characterize strains using *msp1*α (17, 18), revealing that the repeat structure is common between South African, American, and European strains of *A. marginale*; in fact, some of the repeat sequences that were detected were identical to ones that were detected in the United States. Not surprisingly, there were also new repeat sequences de-tected that are, thus far, unique to South Africa.

A. marginale subsp. *centrale* was thought not to have a homolog of *msp1*α; however, complete genome sequencing of the Israel vaccine strain revealed that there is a gene that resides in a position syntenic to *A. marginale msp1*α (5). This gene was named *msp1aS* (S for syntenic; a gene flanked by the same set of genes in two genomes) and has 31 to 36% amino acid sequence identity de-pending on the *A. marginale* strain compared. Importantly, there are structural similarities, including repeats near the amino termi-nus and two sets of transmembrane domains near the carboxy terminus that indicate that these proteins are likely homologs (Fig. 1). The repeats in *A. marginale* subsp. *centrale* strain Israel Msp1aS are longer (47 amino acids in length) than the *A. marginale* Msp1a repeats, and there is no sequence identity between the repeats in the two organisms. The vaccine strain has four repeats with an *msp1aS* genotype of Ac1 Ac1 Ac1 Ac2.

In the present study, we have used a duplex quantitative real-time PCR (qPCR) assay to screen for the presence of *A. marginale* subsp. *centrale* and *A. marginale* in vaccinated and unvaccinated cattle and wildlife, indicating that these infections are common and often occur as mixed infections. Samples that tested positive using this screen were then further analyzed for the *msp1aS* genotype, demonstrating that the vaccine strain genotype is prevalent in cattle herds that practice vaccination, while other more divergent genotypes are present in wildlife species.

MATERIALS AND METHODS

Blood collection and DNA extraction. A total of 380 blood samples from wild ruminant species including African buffalo (*Syncerus caffer*, $n = 97$); waterbuck (*Kobus ellipsiprymnus*, $n = 14$); eland (*Taurotragus oryx*, $n = 23$); black wildebeest (*Connochaetes gnou*, $n = 54$); and blue wildebeest (*Connochaetes taurinus*, $n = 23$), together with 86 cattle samples, were obtained from the Wildlife Biological Resource Center (WBRC) and Biobank South Africa (SA) under the auspices of the National Zoological Gardens of South Africa (NZG) and from the South African National Parks (SANParks) Biobank. The remaining buffalo blood samples ($n = 41$) were made available to us by Dave Cooper from Hluhluwe-iMfolozi Park. Additionally, 42 blood samples from vaccinated cattle were obtained from two commercial farms in Bergville, KwaZulu-Natal, South Africa (Table 1). Standard techniques were followed in collecting blood samples for laboratory examination. Genomic DNA was extracted using the QIAmp DNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA was eluted in 100 μl of elution buffer and stored at −20°C.

The study was approved by the Animal Ethics Committee of the University of Pretoria, South Africa (V085-14), and permission to use wildlife samples was given by SANParks Biobank under reference number LARBJ1118 Conservation Genetics, by the WBRC, and by Biobank SA under the auspices of the NZG of South Africa and the Johannesburg Zoo with project number NZG/P13/05. Collection of cattle samples was approved by the Department of Agriculture, Forestry and Fisheries under section 20 of the Animal Diseases Act of 1984 with reference 12/11/1/1~~/6~~.

Duplex real-time PCR assay. Quantitative real-time PCR (qPCR) for simultaneous detection and quantification of *A. marginale* and *A. marginale* subsp. *centrale* DNA was performed as described previously (19) with some modifications for use on a LightCycler real-time machine (28) (Roche Diagnostics, Mannheim, Germany). The qPCR was performed in a final reaction volume of 20 μl, containing 2 μl of DNA template (100 to 200 ng of DNA), 12.5 μl of FastStart DNA Master hybridization mix (Roche Diagnostics, Mannheim, Germany), 600 nmol/liter of *A. marginale*-specific primers AM-For (5′ TTG GCA AGG CAG CAG CTT 3′) and AM-Rev (5′-TTC CGC GAG CAT GTG CAT-3′), 900 nmol/liter of *A. marginale* subsp. *centrale*-specific primers AC-For (5′-CTA TAC ACG CTT GCA TCT C-3′) and AC-Rev (5′-CGC TTT ATG ATG TTG ATG C-3′), and 200 nmol/liter of probes AM-Pb (5′-6FAM-TCG GTC TTA ACA TCT CCA GGC TTT CAT-BHQ1-3′) and AC-Pb (5′-LC610-ATC ATC ATT CTT CCC CTT TAC CTC GT-BHQ2-3′). Thermal cycling conditions were as follows: UDG activation at 40°C for 10 min, preincubation at 95°C for 10 min, 40 cycles of denaturation at 95°C for 1 min and annealing-extension at 60°C for 1 min, and a final cooling step at 40°C for 30 s. The results were analyzed using LightCycler software ver-

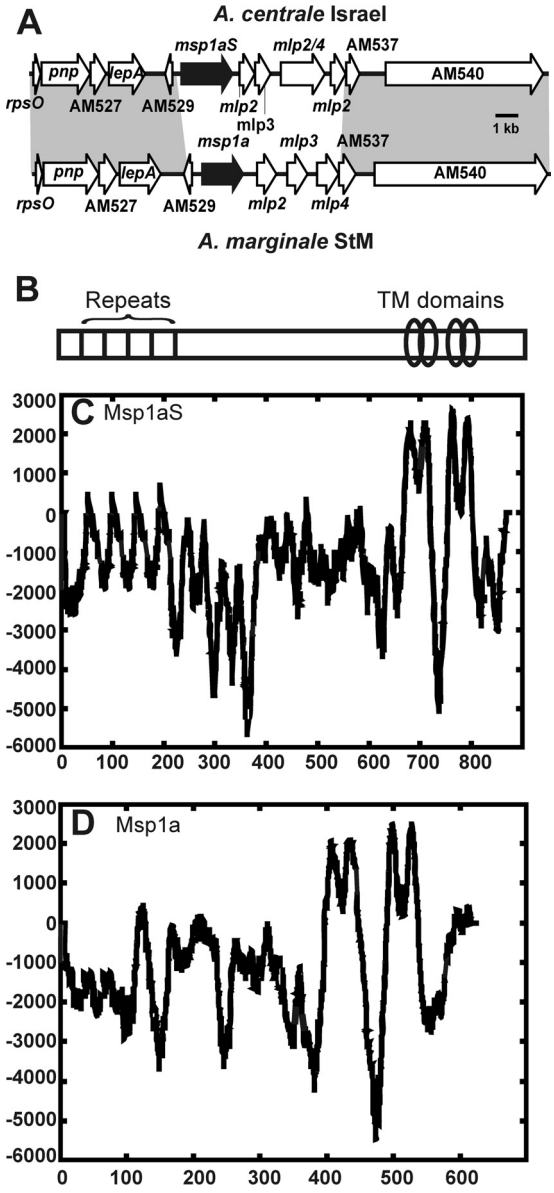

FIG 1 Schematic representation and TMpred plots of Msp1aS. (A) Genomic positioning of Msp1aS of *A. marginale* subsp. *centrale*, also showing that it is syntenic to Msp1a of *A. marginale* St. Maries strain (StM), which suggests that these proteins are homologs. (B) While there is little sequence conservation, these proteins have similar structures: both have a set of repeats near the amino terminus and two sets of transmembrane (TM) domains toward the carboxy terminus. (C and D) TMpred plots show the transmembrane prediction profile for both molecules (Msp1aS from the fully sequenced Israel strain of *A. marginale* subsp. *centrale* and Msp1a from the fully sequenced St. Maries strain of *A. marginale*). Values greater than 500 (*y* axis) indicate transmembrane domains. The repeats of Msp1aS are almost twice as long as those of Msp1a.

TABLE 1 Host samples used in this study

Sample no.	Species	No. of samples	Sample type	Collection site	Origin[a]	Province
565–614	Buffalo	50	EDTA-blood	SANParks[b]	KNP	Mpumalanga
974–987	Buffalo	14	EDTA-blood	SANParks	CNP	Eastern Cape
1002–1016	Buffalo	15	EDTA-blood	SANParks	AEP	Eastern Cape
988–995 and 66/13	Buffalo	9	EDTA-blood	SANParks	GNP	Northern Cape
998–1001 and 1017–1021	Buffalo	9	EDTA-blood	SANParks	MNP	Northern Cape
1–41	Buffalo	41	EDTA-blood	HiP	HiP	KwaZulu-Natal
924–937 and 947–955	Black wildebeest	23	EDTA-blood	SANParks	MTNZNP	Eastern Cape
938–939	Black wildebeest	2	EDTA-blood	SANParks	TMNP	Western Cape
942, 944–953 and 955–972	Black wildebeest	29	EDTA-blood	SANParks	MNP	Northern Cape
1036–1056	Blue wildebeest	21	EDTA-blood	SANParks	MNP	Northern Cape
1057–1058	Blue wildebeest	2	EDTA-blood	SANParks	WCNP	Western Cape
1022–1031	Eland	10	EDTA-blood	SANParks	MNP	Northern Cape
1032–1035	Eland	4	EDTA-blood	SANParks	AEP	Eastern Cape
459–467	Eland	9	FTA filter paper	WBRC, NZG[c]	NZG	Gauteng
1059–1062	Waterbuck	4	EDTA-blood	SANParks	MNP	Northern Cape
468–470	Waterbuck	3	FTA filter paper	WBRC, SA, NZG	Rietvlei NR, JHB Zoological Gardens, Mohale Gate (Gauteng area)	Gauteng
543, 549	Waterbuck	2	EDTA-blood	WBRC, SA, NZG	KNP	Mpumalanga
544–548	Waterbuck	5	EDTA-blood	WBRC, SA, NZG	MaNP	Limpopo
WC103–WC128	Cattle	26	EDTA-blood	NZG collection	WC F3[d]	Western Cape
KZN129–KZN158	Cattle	30	EDTA-blood	NZG collection	KZN F4	KwaZulu-Natal
FS1–FS30	Cattle	30	EDTA-blood	NZG collection	FS F5	Free State
Berg 1–Berg 21	Cattle	21	EDTA-blood	Bergville farm	Bergville F1	KwaZulu-Natal
Berg 22–Berg 42	Cattle	21	EDTA-blood	Bergville farm	Bergville F2	KwaZulu-Natal

[a] Origin, the park/farm from where the sample originates: Kruger National Park (KNP), Cambedoo National Park (CNP), Graspan National Park (GNP), Mokala National Park (MNP) Addo Elephant Park (AEP), Hluhluwe-iMfolozi Park (HiP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), Marakele National Park (MaNP).
[b] SANParks, South African National Parks.
[c] WBRC, Wildlife Biological Research Center; NZG, National Zoological Gardens, South Africa.
[d] F, farm.

sion 4.0 (Roche Diagnostics). The software indicates a positive result by a C_q value (quantification cycle, synonymous with the C_p, crossing point, value given by the LightCycler instrument), at which fluorescence from amplification exceeds the background fluorescence, and a score of 1 to 5. Negative samples have a score of −1 to −5 and no C_p values. A lower C_q correlates with a higher starting concentration of target DNA in a sample, which then indicates a positive infection. FAM fluorescence (530 nm) was generated in *A. marginale*-positive samples and LC-610 (610 nm) signals were generated in *A. marginale* subsp. *centrale*-positive samples. DNA extracted from the *A. marginale* subsp. *centrale* vaccine strain (Onderstepoort Biological Products [OBP], Pretoria, South Africa) was used as a positive control, and samples C14, C57, or F48 (originating from cattle in the Mnisi Community area, Mpumalanga, South Africa) were used as positive controls for *A. marginale*. The presence of *A. marginale* in these samples was confirmed by sequencing of the *msp1*β genes. A negative and a positive control were included in each set of PCRs that was performed. The analytical specificity of the assay was determined by analyzing DNA from closely related species such as *Anaplasma* sp. Omatjenne and *A. phagocytophilum* (20). The efficiency of the assay was determined from 10-fold serial dilutions of plasmid DNA from clones 9410c (*A. marginale* subsp. *centrale*) and F48a (*A. marginale*).

Analysis of the *msp1aS* gene. *A. marginale* subsp. *centrale*-positive samples which had low C_q values as detected by qPCR were selected for analysis of the *msp1aS* gene. Primers MSP1asFZ (5′-CAA GGT CAA GAG TCA GCA TCA TCA GAT G-3′) and MSP1asRZ (5′-CTC CGC GCA CAA TAC TTT CAA CCT CC-3′) were designed based on the *A. marginale* subsp. *centrale* genome sequence (GenBank accession CP001759) to target tandem repeats within the *msp1aS* gene. PCR was performed in a final reaction volume of 25 μl containing Phusion Flash high-fidelity PCR master mix (Thermo Fisher Scientific), 10 pM of each primer, and genomic DNA. Thermal cycling was carried out in a Veriti thermal cycler (Thermo Fisher Scientific) and consisted of an initial denaturation at 98°C for 10 s, followed by 30 cycles of denaturation at 98°C for 1 s, annealing at 67°C for 30 s and extension at 72°C for 15 s, and a final extension at 72°C for 1 min. DNA extracted from the *A. marginale* subsp. *centrale* vaccine obtained from OBP (Pretoria, South Africa) was used as a positive control.

Purified PCR amplicons were cloned into the pJET vector (Thermo Fisher Scientific). Recombinant plasmids were isolated using a High Pure plasmid isolation kit (Roche Diagnostics, Mannheim, Germany) and sequenced using 1 μl of 2 μM M13 primers with an ABI BigDye v3.1 kit on an ABI 3500xL genetic analyzer at Inqaba Biotec (Pretoria, South Africa).

Sequences were assembled, edited, and translated to amino acids using CLC Main Workbench 7.0.3 (Qiagen, Denmark). Tandem repeats were identified using Tandem Repeats Finder (https://tandem.bu.edu/trf/trf.html) (21). The repeats were named A*cn*, to distinguish them from *A. marginale* Msp1a repeats. Truncated repeats were designated with a T at the end of the name. Repeats were curated and analyzed using RepeatAnalyzer (29). Repeat sequences were aligned using the AlignX module of Vector NTI (Invitrogen).

Diversity measures. RepeatAnalyzer calculates four genetic diversity metrics, each of which captures the diversity of repeats in a geographic region in a different way. Broadly, they fall into two groups, those that measure the amount of different repeats and those that measure the distribution of those repeats. Within each of these categories there is a global and a local formulation. The local version of a metric calculates the score independently on each genotype and averages these together to get the final score, while the global version looks at all genotypes together. Specifically, the GDM1-L score can be interpreted as the percentage of unique repeats in each genotype in the region, while the GDM1-G score is the percentage of unique repeats across all genotypes in the region. The

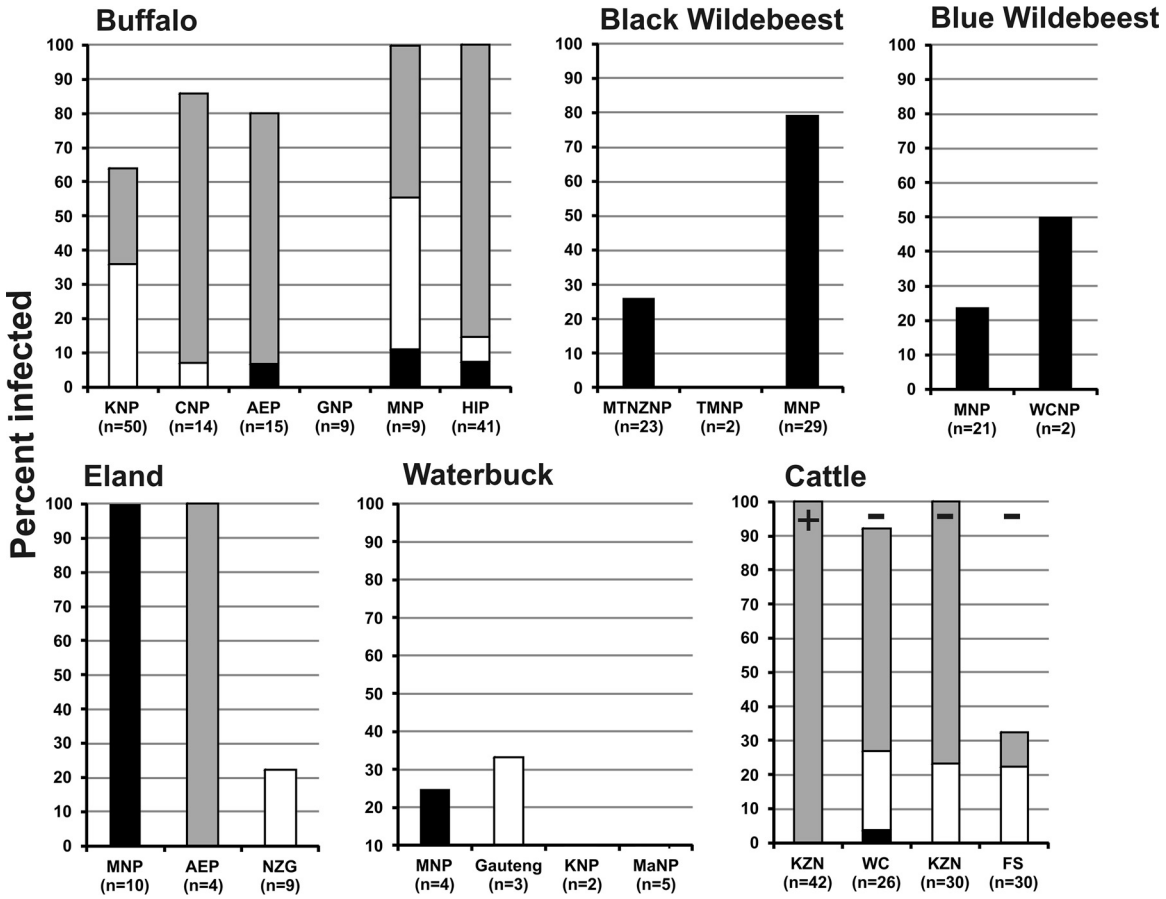

FIG 2 Stacked bar graphs showing occurrence of *Anaplasma* species in wild ruminants and cattle. Buffalo, black and blue wildebeest, eland, waterbuck, and cattle were analyzed by duplex real-time PCR. Animals were sampled from the following national parks and provinces: Kruger National Park (KNP), Cambedoo National Park (CNP), Addo Elephant Park (AEP), Graspan National Park (GNP), Mokala National Park (MNP), Hluhluwe-iMfolozi Park (HIP), Mountain Zebra National Park (MTNZNP), Table Mountain National Park (TMNP), West Coast National Park (WCNP), National Zoological Gardens of South Africa (NZG), Marakele National Park (MaNP), KwaZulu-Natal (KZN), Western Cape (WC), and Free State (FS). Numbers in parentheses indicate the total numbers of animals sampled from that park/province. Samples were collected from vaccinated (+) and unvaccinated (−) cattle. Black indicates animals positive for *A. marginale* subsp. *centrale*, gray indicates animals with mixed infections, and white indicates animals positive for *A. marginale*.

GDM2-L score can be interpreted as the amount of variation (measured as standard deviation) in the number of occurrences of the repeats in a genotype, while the GDM2-G score is the amount of variation in the number of occurrences of all the repeats in all genotypes in the region. A high GDM1 score means that there are more unique repeats, with 0 as the minimum (when all repeats are the same) and 1 being the maximum (when each repeat is unique). A high GDM2 score means that the repeats are distributed more unevenly, with a minimum of 0 (when all repeats occur the same number of times) and values ranging up to but not including 0.5 as the unevenness of repeat distribution increases.

RESULTS

Occurrence of *Anaplasma* species in wild ruminants and cattle in South Africa. Duplex qPCR results indicated that *A. marginale* subsp. *centrale* single infections are common among black wildebeest (Mokala National Park [MNP], 79.3%), blue wildebeest (West Coast National Park [WCNP], 50%), waterbuck (MNP, 25%), and eland (MNP, 100%). Wildebeest did not harbor any *A. marginale* infections. Mixed infections were frequently found in both buffalo and cattle, ranging from 28% to 100% of animals from a given area being positive for both *A. marginale* and *A. marginale* subsp. *centrale* infections. Buffalo samples had high rates of mixed infections and also had lower rates of single infections with *A. marginale* subsp. *centrale* than with *A. marginale*. Interestingly, single infections of both species predominated in sets of animals from specific parks (see eland and waterbuck in Fig. 2), indicating that environment plays a role in exposure to the two pathogens.

Characterization of MSP1aS. Because the sequenced Israel vaccine strain was removed from South Africa more than 60 years ago, we obtained a batch of the vaccine currently produced at OBP in Pretoria, South Africa, and sequenced the *msp1aS* gene. The sequence of the OBP vaccine strain Msp1aS tandem repeat from 2014 was identical to that of the Israel strain (5) with four tandem repeats: Ac1 Ac1 Ac1 Ac2.

Based on the duplex qPCR results, *A. marginale* subsp. *centrale*-positive samples ($n = 25$) were selected for further analysis. *Msp1aS* primers amplified at least one single strong product from all samples tested. Some samples exhibited multiple bands which demonstrated mixed infection (Fig. 3). The *msp1aS* PCR products were cloned and sequenced, and sequence analyses confirmed the presence of tandem repeats similar to those of the vaccine strain (Table 2). The first five columns of Table 2 would combine to

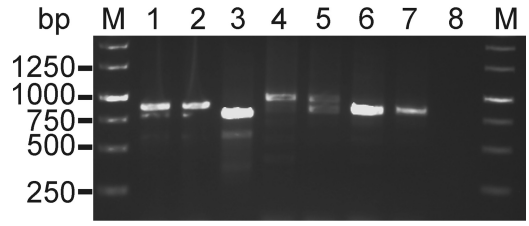

FIG 3 Gel image showing amplicons of *msp1aS*. Lanes 1 and 2, vaccine strain (814 bp); lane 3, animal FS 383 (790 and 637 bp); lane 4, animal Berg10 (922 and 814 bp); lane 5, animal Berg12 (937 and 814 bp); lane 6, animal Berg20 (814 bp); lane 7, animal WC_108 (799 bp); lane 8, negative control. Note that for some samples only a subset of the amplicons were successfully sequenced, while for others, clones with different sequences were obtained from what appeared as a single band. Lanes marked "M" have a 1-kb molecular weight marker.

provide the full strain and sample designation as suggested previously (29), i.e., Ac11 Ac8_ZA, EC_2007_CNP_986; however, we have used shorter names for some of the genotypes for ease of discussion. The strains tested in this study yielded one to five repeat units as predicted from the PCR product sizes; however, there were strains that did not correspond with their PCR products (data not shown). Altogether, 47 different Msp1aS tandem repeats were identified. The repeats ranged from 45 to 51 amino acids with seven truncated repeats ranging from 31 to 33 amino acids (Fig. 4). The most common repeat length was 46 amino acids (Fig. 5A). The Ac1 and Ac2 tandem repeats, contained in the vaccine strain, were detected in cattle, buffalo, and wildebeest.

The vaccine strain was detected in cattle from Bergville which were previously vaccinated with *A. marginale* subsp. *centrale* vaccine. We tested six cattle from Bergville farm 2 which yielded 15 *msp1aS* sequences. The vaccine genotype was detected in five of the six cattle (Table 2). Interestingly, two "vaccine variant" genotypes were detected that were closely related to the vaccine strain genotype and differed by only a single amino acid (VV1 and VV3). Another vaccine variant genotype, VV2 (Ac1 Ac1 Ac1 Ac2 Ac2), that had one additional Ac2 repeat but was otherwise identical to the vaccine strain genotype was noted. Two additional genotypes that were less obviously related to the vaccine strain were detected. Three cattle were tested on Bergville farm 1, resulting in 10 *msp1aS* sequences. Interestingly, the vaccine genotype was only detected in one of these animals despite the fact that these animals were reported as being vaccinated, while two animals contained the related genotype VV3. Seven additional genotypes were detected on farm 1 that were not closely related to the vaccine genotype.

Interestingly, the vaccine genotype and one of the vaccine variant genotypes were also detected in unvaccinated animals, including buffalo (HIP_6, AEP_1003, and KNP_586) and cattle (WC_108). Genotype Ac33 Ac3 Ac6 was detected in a buffalo from Hluhluwe National Park and in a cow from Bergville farm 1. Several truncated repeats were detected (i.e., Ac36T), and although these predominated in the buffalo samples, a genotype containing a truncated repeat was also detected on Bergville farm 1.

RepeatAnalyzer is a program we developed recently to house, curate, and provide metrics for repeat sequences used to characterize bacteria (29). In the present study, we applied it to the analysis of *msp1aS* repeats. The most common genotype structure we detected contained four repeats, with genotypes having from one to five repeats (Fig. 5B). Most repeats occurred only once with two repeats being detected in six different genotypes (Ac1 and Ac6) (Fig. 5C). The Ac1 repeat is not only detected in the vaccine strain but also in several vaccine variant genotypes that were detected on Bergville farm 2. The Ac6 repeat was prevalent in genotypes detected in wildlife and, interestingly, was also detected in genotypes found on Bergville farm 1. In general, we found that the average number of amino acid changes (edit distance) between any two *A. marginale* subsp. *centrale* repeats was high (13.7) and was normally distributed, with 97.8% of data falling within 2 standard deviations. There was a mean of 0.9 and 1.4 repeats at an edit distance of 1 and 2, respectively, from any given repeat. Despite the high level of variation between repeats, we found five repeats within an edit distance of two from Ac1 (Ac2, Ac26, Ac12, Ac20, and Ac48) and seven repeats within two edits of Ac2 (Ac1, Ac14, Ac28, Ac15, Ac16, Ac22, and Ac26).

Diversity analysis and repeat distribution. Using RepeatAnalyzer, we see that South Africa has a large number of unique *A. marginale* subsp. *centrale* repeats (Table 3, GDM1-L), while having an intermediate amount of repeat diversity in general (Table 3, GDM1-G). There is a higher diversity of repeats among the samples isolated from buffalo hosts than among those from cattle hosts, although this would be expected as many of the cattle were vaccinated and would be expected to exhibit the same repeat structure as the vaccine strain. GDM2 measures how uniformly the repeat occurrences in the strains in a region (local) or the region as a whole (global) are distributed. For both GDM2 metrics, the South African values are low, indicating that the repeats are dispersed; i.e., there is not a preponderance of a single repeat type in individual strains or for the country as a whole. The GDM2 values are higher for cattle than for buffalo-derived samples, reflecting more uniformity in the repeats detected in samples from cattle than from buffalo. When examining whether repeats and strains occur in multiple provinces, we have *msp1aS* data from seven of South Africa's nine provinces (Fig. 6). The repeats and strains are mapped according to GPS coordinates, so multiple locations within a province can be visualized and distinguished. Several repeats were detected in multiple locations (Fig. 6A). Repeats Ac1 and Ac2 were found in Mpumalanga, Gauteng, KwaZulu-Natal, Eastern Cape, and Western Cape provinces. The vaccine strain is detected in cattle from KwaZulu-Natal, the Eastern Cape, and the Western Cape (Fig. 6B), which is interesting as we tested vaccinated animals only in KwaZulu-Natal. Gauteng also shows positive for the vaccine strain, but this is due to the purchased vaccine itself.

DISCUSSION

We tested animals from several different parks and farms and showed that *A. marginale* subsp. *centrale* infection is prevalent in black and blue wildebeest, eland, buffalo, waterbuck, and cattle. *A. marginale* subsp. *centrale* has rarely been examined on its own, as typically researchers/ranchers are interested in *A. marginale* infection, and the competitive enzyme-linked immunosorbent assay (cELISA) often used for detection does not discriminate between *A. marginale* and *A. marginale* subsp. *centrale* infection. One study using the cELISA showed high seroprevalence of *Anaplasma* spp. in wildlife from Kenya with eland and blue wildebeest testing at 100% and 96%, respectively. With a reverse line blot assay, it was shown that *Anaplasma* spp. are prevalent in buffalo in northern Botswana with *A. marginale* subsp. *centrale* being the most preva-

TABLE 2 *A. marginale* subsp. *centrale* genotypes detected from South African bovine hosts (cattle, buffalo, and black wildebeest)

Genotype	Country code[a]	Province code[a]	Yr	Animal no.	Sample clone ID	Host species	Origin, park, farm	Vaccine status	Size (bp)	No. of repeats	Short name
Ac1 Ac1 Ac1 Ac2	IL	M	2010	Genome sequence	CP001759	Cattle	Israel 2010	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	GP	2014		OBP vaccine	Cattle	OBP 2014[b]	+	814	4	Vaccine
SANParks Biobanked samples[c]											
Ac11 Ac8	ZA	EC	2007	CNP_986	G	Buffalo	Cambedoo	−	525	2	
Ac9 Ac8	ZA	EC	2007	CNP_986	C	Buffalo	Cambedoo	−	526	2	
Ac11 Ac11 Ac11 Ac11 Ac8	ZA	EC	2007	CNP_986	C2	Buffalo	Cambedoo	−	940	5	
Ac3 Ac4 Ac5 Ac6	ZA	EC	2007	CNP_987	J2	Buffalo	Cambedoo	−	823	3	
Ac7 Ac8	ZA	EC	2007	CNP_979	D	Buffalo	Cambedoo	−	526	2	
Ac6 Ac35 Ac36T Ac37T Ac6	ZA	NC	2013	MNP_999	L	Buffalo	Mokala	−	889	5	
Ac38 Ac39T Ac34 Ac40T	ZA	NC	2013	MNP_999	N	Buffalo	Mokala	−	759	4	
Ac38 Ac41T Ac42 Ac40T	ZA	NC	2013	MNP_1000	A	Buffalo	Mokala	−	733	4	
Ac6Ac6	ZA	NC	2013	MNP_1000	G	Buffalo	Mokala	−	790	2	
Ac1 Ac1 Ac1 Ac2	ZA	EC	2013	AEP_1003	D	Buffalo	Addo	−	814	4	Vaccine
Ac7 Ac8	ZA	EC	2013	AEP_1006	D	Buffalo	Addo	−	525	2	
Ac38 Ac44T Ac43	ZA	EC	2013	AEP_1006	N	Buffalo	Addo	−	628	3	
Ac31 Ac8	ZA	EC	2013	AEP_1006	S	Buffalo	Addo	−	526	2	
Ac1 Ac1 Ac1 Ac1	ZA	MP	2008	KNP_586	A	Buffalo	Kruger	−	814	4	VV1
Ac26 Ac26 Ac26 Ac2	ZA	NC	2011	MNP_958	F_w	Black wildebeest	Mokala	−	862	4	
Hluhluwe iMfolozi Park											
Ac1 Ac1 Ac1 Ac2	ZA	NL	2008	HiP_6	1	Buffalo	Hluhluwe	−	815	4	Vaccine
Ac30 Ac24 Ac25	ZA	NL	2008	HiP_6	A	Buffalo	Hluhluwe	−	940	3	
Ac29 Ac29 Ac29	ZA	NL	2008	HiP_6	B	Buffalo	Hluhluwe	−	703	3	
Ac33 Ac3 Ac6	ZA	NL	2008	HiP_6	L	Buffalo	Hluhluwe	−	691	3	
NZG Biobanked samples[c]											
Ac20 Ac32 Ac21 Ac10	ZA	WC	2011	WC_107	E	Cattle	WC	−	700	4	
Ac1 Ac1 Ac1 Ac2	ZA	WC	2011	WC _108	A	Cattle	WC	−	799	4	Vaccine
Ac12 Ac12 Ac13 Ac13 Ac14	ZA	NL	2011	KZN_138	B	Cattle	NL	−	919	5	
Ac12 Ac12 Ac13 Ac13Ac14	ZA	NL	2011	KZN_132	A	Cattle	NL	−	941	4	
Ac12 Ac12 Ac13 Ac13 Ac14	ZA	NL	2011	KZN_130	B	Cattle	NL	−	980	5	
Ac15 Ac16 Ac16 Ac16	ZA	FS	2011	FS_56	B	Cattle	FS	−	821	4	
Ac16 Ac16 Ac16	ZA	FS	2011	FS_383	B	Cattle	FS	−	637	3	
Farm 1						Cattle					
Ac33 Ac3 Ac6	ZA	NL	2015	Berg 10	A	Cattle	Bergville	+	691	3	
Ac19 Ac19 Ac3 Ac6	ZA	NL	2015	Berg 10	G	Cattle	Bergville	+	814	4	
Ac17 Ac18 Ac45 Ac46T Ac47	ZA	NL	2015	Berg 10	J	Cattle	Bergville	+	922	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 12	B	Cattle	Bergville	+	811	4	VV3
Ac20 Ac21 Ac21 Ac20	ZA	NL	2015	Berg 12	E	Cattle	Bergville	+	937	5	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 12	N	Cattle	Bergville	+	814	4	Vaccine
Ac23 Ac24 Ac25 Ac34	ZA	NL	2015	Berg 19	A	Cattle	Bergville	+	940	5	
Ac26 Ac12 Ac12 Ac27 Ac14	ZA	NL	2015	Berg 19	A_2	Cattle	Bergville	+	946	5	
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 19	B	Cattle	Bergville	+	811	4	VV3
Ac19 Ac3 Ac6 Ac6	ZA	NL	2015	Berg 19	I	Cattle	Bergville	+	826	4	
Farm 2											
Ac1 Ac1 Ac1 Ac22	ZA	NL	2015	Berg 25	A	Cattle	Bergville	+	814	4	VV3
Ac1	ZA	NL	2015	Berg 25	E	Cattle	Bergville	+	391	1	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 25	B	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 25	E_2	Cattle	Bergville	+	814	4	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 25	X	Cattle	Bergville	+	914	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 27	D	Cattle	Bergville	+	814	4	Vaccine
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 27	E	Cattle	Bergville	+	956	5	VV1
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 27	B	Cattle	Bergville	+	955	5	VV2
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 17	A	Cattle	Bergville	+	943	5	Vaccine
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 24	A	Cattle	Bergville	+	814	5	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 24	C	Cattle	Bergville	+	955	5	VV2
Ac1 Ac28 Ac2 Ac28	ZA	NL	2015	Berg 24	V	Cattle	Bergville	+	814	4	
Ac1 Ac1 Ac1 Ac2	ZA	NL	2015	Berg 30	G	Cattle	Bergville	+	811	4	Vaccine
Ac1 Ac1 Ac1 Ac2 Ac2	ZA	NL	2015	Berg 30	I	Cattle	Bergville	+	954	5	VV2
Ac1 Ac1 Ac1 Ac1	ZA	NL	2015	Berg 20	H3	Cattle	Bergville	+	814	4	VV1

[a] Country and province abbreviations follow ISO 3166-2.
[b] OBP, Onderstepoort Biological Products (Pretoria, South Africa), which produces *A. marginale* subsp. *centrale* vaccine for sale.
[c] SANParks, South African National Parks; NZG, National Zoological Gardens of South Africa Biobanks.

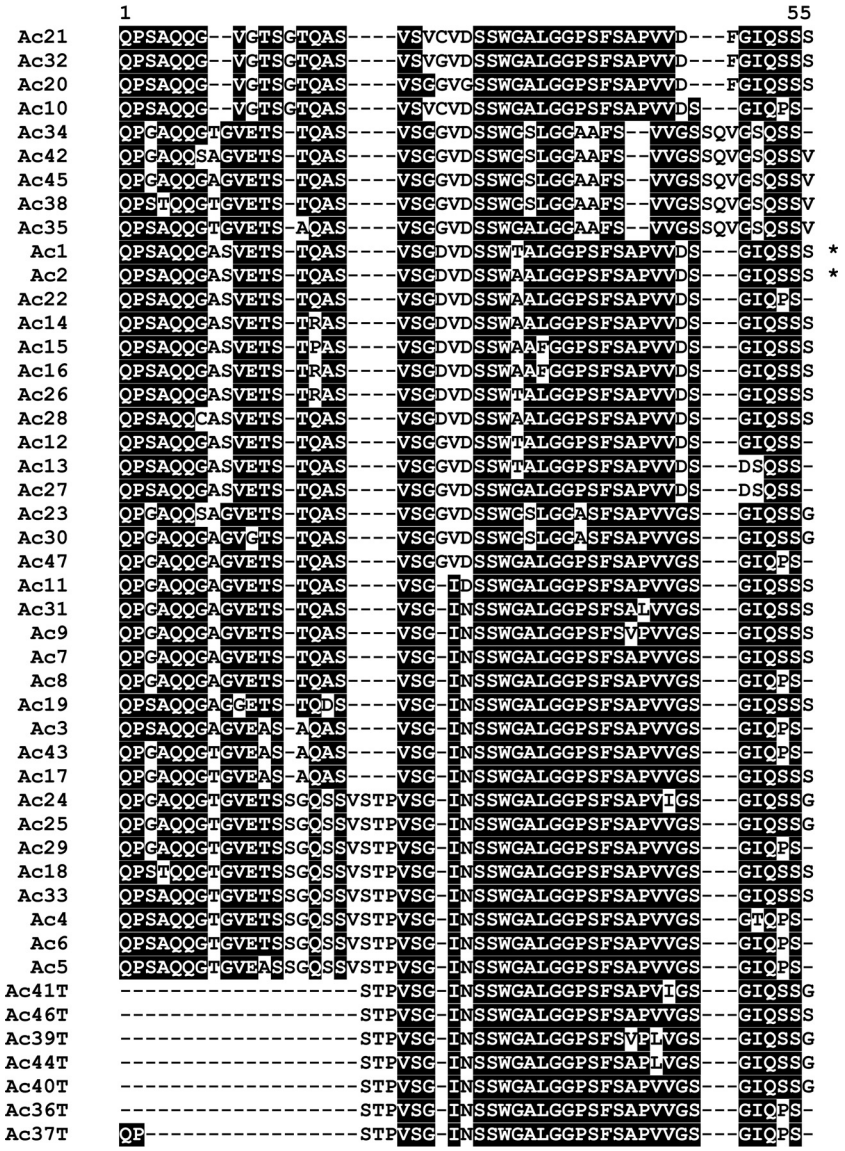

FIG 4 Alignment of *A. marginale* subsp. *centrale* Msp1aS tandem repeats detected from South African cattle, buffalo, and black wildebeest. The 47 repeat types were aligned using the AlignX module of Vector NTI, and groups of identical amino acids are highlighted on a black background. Ac1 and Ac2, the repeats present in the vaccine strain, are indicated with an asterisk.

lent (22). This suggests that wildlife species are reservoirs of *A. marginale* subsp. *centrale*.

We examined positive samples for *msp1aS* genotype, a genotyping scheme that has not previously been employed for *A. marginale* subsp. *centrale*. We identified 47 Msp1aS repeats which corresponded to 32 *A. marginale* subsp. *centrale* genotypes detected in cattle, buffalo, and wildebeest. The most common *A. marginale* subsp. *centrale* genotype among cattle samples was the vaccine genotype. This is not surprising as both farms that we sampled previously vaccinated with *A. marginale* subsp. *centrale* vaccine purchased from OBP. It is worth noting that cattle from farm 1 graze together with goats, sheep, and reedbuck, which might explain the diversity of *A. marginale* subsp. *centrale* strains detected on farm 1. We speculate that there is circulation of *A. marginale* subsp. *centrale* strains among different hosts, which led to the variety of genotypes detected on this farm. Cattle from farm 2 are confined within a grazing area with no interaction with other ruminants. The vaccine genotype was detected in all but one of the animals tested on this farm. In addition to the vaccine genotype, several closely related genotypes were detected, which suggests that the vaccine genotype is changing under selection pressure. This is interesting as we do not see these types of changes in the *msp1α* genotype in *A. marginale*-infected cattle. All repeats detected on farm 2 had an edit distance of two or less from one of the vaccine strain repeats, indicating that these repeats were closely related to the vaccine strain repeats. However, we cannot be sure that the vaccine strain is changing rather than there being an introduction of these new, related genotypes.

The unvaccinated cattle samples from Western Cape and Free State had different *A. marginale* subsp. *centrale* genotypes, while unvaccinated cattle samples from KwaZulu-Natal all had the same *A. marginale* subsp. *centrale* genotype. The vaccine strain was de-

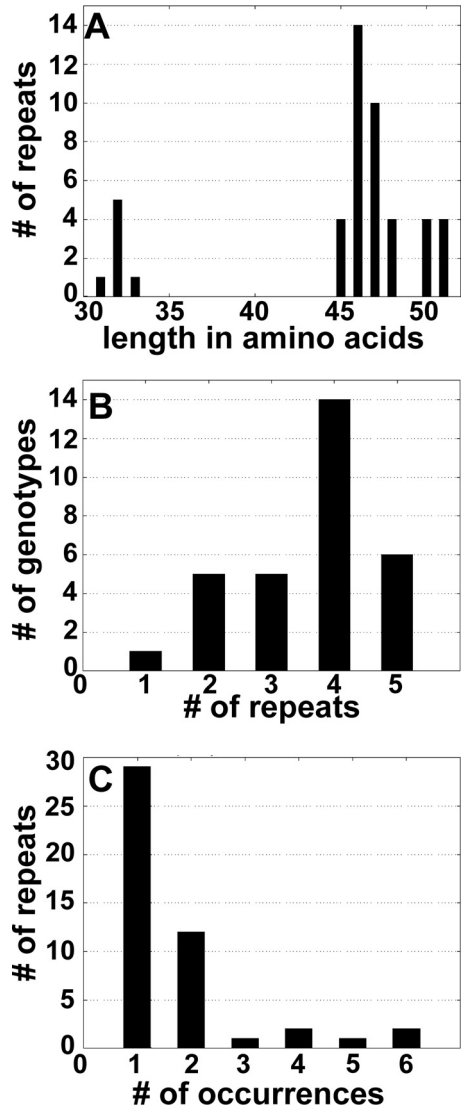

FIG 5 Metrics for *A. marginale* subsp. *centrale* Msp1aS repeats. (A) Number of repeats with a given number of amino acids; i.e., there are four repeats with a length of 45 amino acids. (B) Number of genotypes having a given number of repeats; i.e., 14 genotypes contain four repeats. (C) Number of times a given repeat occurs in our genotype data set; i.e., two repeats occur in six different genotypes.

tected in one of the unvaccinated cattle in the Western Cape. The *A. marginale* subsp. *centrale* genotypes obtained from wild ruminants were diverse, demonstrating geographic segregation of national parks. The repeat Ac8 was common in the *msp1aS* genotypes found in buffalo, even though the buffalo were sourced from parks distributed around South Africa. Ac8 has an edit distance of nine to both repeats Ac1 and Ac2, indicating that it is not closely related to the vaccine strain repeats.

While we have presented diversity metrics broken down by province, we think that the sample size is too small for this to be really meaningful in most cases, i.e., in Mpumalanga and Gauteng, there is an $n = 1$. More importantly, these metrics show us that for South Africa, as a whole, there is a high degree of repeat diversity within genotypes (Table 3, GDM1-L) and a moderate degree of novel genotypes across the country (Table 3, GDM1-G). The low

TABLE 3 Diversity scores for cattle and wildlife hosts by province and host

Location	GDM1-L	GDM1-G	GDM2-L	GDM2-G
All	0.747	0.420	0.065	0.022
Eastern Cape	0.863	0.583	0.069	0.060
Gauteng	0.500	0.500	0.250	0.250
KwaZulu-Natal	0.696	0.419	0.071	0.044
Mpumalanga	0.250	0.250	0	0
Northern Cape	0.760	0.632	0.067	0.050
Free State	0.417	0.286	0.125	0.357
Western Cape	0.750	0.750	0.125	0.093
Buffalo	0.781	0.500	0.051	0.030
Cattle	0.684	0.418	0.081	0.041

GDM2 values indicate that the repeats are dispersed, which is what is expected when the numbers of unique repeats and genotypes are high. This high degree of novel repeats indicates that the repeats have likely been circulating in nature and undergoing selection

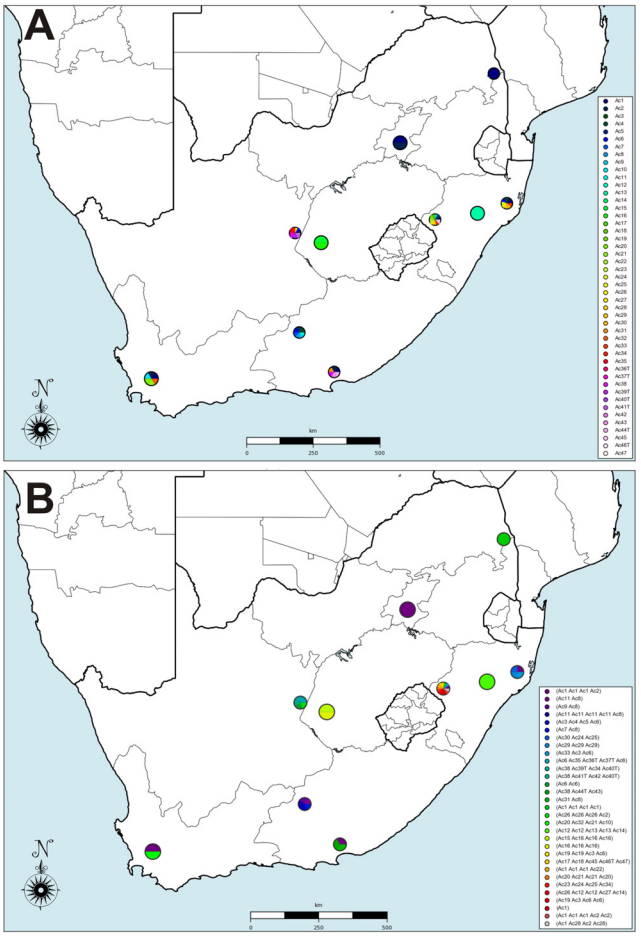

FIG 6 Maps of repeat and strain distribution. (A) Repeats mapped to the provinces of South Africa by GPS coordinates. (B) Strain genotypes mapped to the provinces of South Africa by GPS coordinates. The size of the circle indicates the precision of the location report, with three sizes being possible, corresponding to country, province, and precise GPS location. In these maps, there are no reports that are simply to the country level; i.e., all locations are at the provincial level or more specific. Therefore, there are only two sizes of circles shown. The samples collected from the Free State and Western Cape are marked at the provincial level and, thus, have larger markers.

and change separate from the vaccine strain. As more data are collected, it will be interesting to see if these metrics shift and how these metrics compare with those collected in other countries.

While the *A. marginale* subsp. *centrale* vaccine strain was thought for a long time not to be transmitted by most ticks, it was shown that, in fact, it colonized the tick well but was not secreted into the tick saliva in sufficient quantities for robust transmission (23, 24). Dramatically increasing tick numbers in transmission experiments overcame the transmission barrier (25). Is the reduced ability of the *A. marginale* subsp. *centrale* vaccine strain to be tick transmitted due to long serial needle passage through cattle? Or is there, perhaps, a specific vector-pathogen adaptation? There is a report of apparently efficient tick transmission of *A. marginale* subsp. *centrale* vaccine strain from *Rhipicephalus simus* ticks (26). Although *R. simus* is a proven vector in laboratory conditions, this tick is not found on cattle in large numbers, and the immature stages do not normally infest cattle (27). It would appear that the strains that we have detected circulating in wild animals today are maintained in nature via a natural tick-transmission cycle; however, this remains a speculation at this stage, as we have not tested ticks or performed transmission studies due to the complexities of working with the ecosystem of infections present in South Africa. If, in fact, *A. marginale* subsp. *centrale* is being spread through natural transmission to cattle, it is likely mitigating some of the disease burden of anaplasmosis caused by *A. marginale*.

In conclusion, this paper presents a novel genetic test based on *msp1aS* to discriminate strains of *A. marginale* subsp. *centrale* and shows that the vaccine strain is found widely distributed across South Africa and in animals that do not have a history of vaccination. Further, we present metrics indicating a high degree of Msp1aS repeat diversity in South Africa. Our results indicate the significance of wildlife as reservoir hosts for *A. marginale* subsp. *centrale*.

ACKNOWLEDGMENTS

We thank B. Christoff from Plaas Hongerspoort and A. Shepherd from Tugela Veterinary Clinic, Bergville, KwaZulu-Natal, who helped us collect blood samples from vaccinated cattle and M.S. Mtshali and A. Mutshembele for providing blood samples from unvaccinated cattle from KwaZulu-Natal, Western Cape, and Free State. Wildlife samples were obtained from the South African National Parks SANParks Biobank under reference number LARBJ1118 Conservation Genetics and from the Wildlife Biological Resource Center (WBRC) and Biobank SA under the auspices of the National Zoological Gardens (NZG) of South Africa and Johannesburg Zoo. We thank Dave Cooper for providing buffalo blood samples from Hluhluwe-iMfolozi Park. We thank Erich Zweygarth for *Anaplasma* sp. Omatjenne.

FUNDING INFORMATION

This work, including the efforts of Nicola E. Collins, Marinda C. Oosthuizen, and Kelly A. Brayton, was funded by Technology Innovation Agency, Tshwane Animal Health Cluster (TAHC12-00037). This work, including the efforts of Nicola E. Collins, Marinda C. Oosthuizen, and Kelly A. Brayton, was funded by National Research Foundation (NRF) (81840). This work, including the efforts of Marinda C. Oosthuizen, was funded by University of Pretoria (UP).

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. Any opinion, finding, conclusion, or recommendation expressed in this material is that of the authors, and the NRF does not accept any liability in this regard.

REFERENCES

1. **Theiler A.** 1910. *Anaplasma marginale*: the marginal points in the blood of cattle suffering from a specific disease. Report of the government veterinary bacteriologist, 1908-1909. Transvaal, South Africa.
2. **Losos GJ.** 1986. Anaplasmosis, p 743–795. *In* Infectious tropical diseases of domestic animals. Longman House, Essex, United Kingdom.
3. **Theiler A.** 1911. Further investigations into anaplasmosis of South African cattle, p 7–46. First Report of the Director of Veterinary Research, Union of South Africa. Johannesburg, South Africa.
4. **Aubry P, Geale DW.** 2011. A review of bovine anaplasmosis. Transbound Emerg Dis **58:**1–30. http://dx.doi.org/10.1111/j.1865-1682.2010.01173.x.
5. **Herndon DR, Palmer GH, Shkap V, Knowles DP, Jr, Brayton KA.** 2010. Complete genome sequence of *Anaplasma marginale* subsp. *centrale*. J Bacteriol **192:**379–380. http://dx.doi.org/10.1128/JB.01330-09.
6. **Kuttler KL.** 1984. *Anaplasma* infections in wild and domestic ruminants: a review. J Wildl Dis **20:**12–20. http://dx.doi.org/10.7589/0090-3558-20.1.12.
7. **Anziani OS, Tarabla HD, Ford CA, Galleto C.** 1987. Vaccination with *Anaplasma centrale*: Response after an experimental challenge with *Anaplasma marginale*. Trop Anim Health Prod **19:**83–87. http://dx.doi.org/10.1007/BF02297324.
8. **Neitz WO.** 1935. Bovine anaplasmosis: the transmission of *Anaplasma marginale* to a black wildebeest (*Connochaetes gnou*). Onderstepoort J Vet Sci Anim Ind **5:**9–11.
9. **Potgieter FT.** 1979. Epizootiology and control of anaplasmosis in South Africa. J S Afr Vet Assoc **50:**367–372.
10. **Smith RD, Woolf A, Hungerford LL, Sundberg JP.** 1982. Serologic evidence of *Anaplasma marginale* infection in Illinois white-tailed deer. J Am Vet Med Assoc **181:**1254–1256.
11. **Potgieter FT, Stoltsz W.** 2004. Bovine anaplasmosis, p 598–600. *In* Coetzer JAW, Tustin RC (ed), Infectious diseases of livestock, 2nd ed, vol 1. Oxford University Press, Cape Town, South Africa.
12. **Georges K, Loria GR, Riili S, Greco A, Caracappa S, Jongejan F, Sparagano O.** 2001. Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Vet Parasitol **99:**273–286. http://dx.doi.org/10.1016/S0304-4017(01)00488-5.
13. **Carelli G, Decaro N, Lorusso E, Paradies P, Elia G, Martella V, Buonavoglia C, Ceci L.** 2008. First report of bovine anaplasmosis caused by *Anaplasma centrale* in Europe. Ann N Y Acad Sci **1149:**107–110. http://dx.doi.org/10.1196/annals.1428.069.
14. **Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, McElwain TF, Barbet AF.** 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc Natl Acad Sci U S A **87:**3220–3224. http://dx.doi.org/10.1073/pnas.87.8.3220.
15. **Bowie MV, de la Fuente J, Kocan KM, Blouin EF, Barbet AF.** 2002. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene **282:**95–102. http://dx.doi.org/10.1016/S0378-1119(01)00845-9.
16. **de la Fuente J, Ruybal P, Mtshali MS, Naranjo V, Shuqing L, Mangold AJ, Rodríguez SD, Jiménez R, Vicente J, Moretta R, Torina A, Almazán C, Mbati PM, de Echaide ST, Farber M, Rosario-Cruz R, Gortazar C, Kocan KM.** 2007. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences. Vet Microbiol **119:**382–390. http://dx.doi.org/10.1016/j.vetmic.2006.09.015.
17. **Mutshembele AM, Cabezas-Cruz A, Mtshali MS, Thekisoe OMM, Galindo RC, de la Fuente J.** 2014. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. Ticks Tick Borne Dis **5:**624–631. http://dx.doi.org/10.1016/j.ttbdis.2014.04.011.
18. **Mtshali MS, de la Fuente J, Ruybal P, Kocan KM, Vicente J, Mbati PA, Shkap V, Blouin EF, Mohale NE, Moloi TP, Spickett AM, Latif AA.** 2007. Prevalence and genetic diversity of *Anaplasma marginale* strains in cattle in South Africa. Zoonoses Public Health **54:**23–30. http://dx.doi.org/10.1111/j.1863-2378.2007.00998.x.
19. **Decaro N, Carelli G, Lorusso E, Lucente MS, Greco G, Lorusso A, Radogna A, Ceci L, Buonavoglia C.** 2008. Duplex real-time polymerase chain reaction for simultaneous detection and quantification of *Anaplasma marginale* and *Anaplasma centrale*. J Vet Diagn Invest **20:**606–611. http://dx.doi.org/10.1177/104063870802000511.
20. **Carelli G, Decaro N, Lorusso A, Elia G, Lorusso E, Mari V, Ceci L,**

Buonavoglia C. 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. Vet Microbiol **124:**107–114. http://dx.doi.org/10.1016/j.vetmic.2007.03.022.

21. **Benson G.** 1999. Tandem repeats finder: a program to analyze DNA sequences. Nucleic Acids Res **27:**573–580. http://dx.doi.org/10.1093/nar/27.2.573.
22. **Eygelaar D, Jori F, Mokopasetso M, Sibeko KP, Collins NE, Vorster I, Troskie M, Oosthuizen MC.** 2015. Tick-borne haemoparasites in African buffalo (*Syncerus caffer*) from two wildlife areas in Northern Botswana. Parasit Vectors **8:**26. http://dx.doi.org/10.1186/s13071-014-0627-y.
23. **Ueti MW, Reagan JO, Jr, Knowles DP, Jr, Scoles GA, Shkap V, Palmer GH.** 2007. Identification of midgut and salivary glands as specific and distinct barriers to efficient tick-borne transmission of *Anaplasma marginale*. Infect Immun **75:**2959–2964. http://dx.doi.org/10.1128/IAI.00284-07.
24. **Shkap V, Kocan K, Molad T, Mazuz M, Leibovich B, Krigel Y, Michoytchenko A, Blouin E, De la Fuente J, Samish M.** 2009. Experimental transmission of field *Anaplasma marginale* and the *A. centrale* vaccine strain by *Hyalomma excavatum*, *Rhipicephalus sanguineus and Rhipicephalus* (*Boophilus*) *annulatus* ticks. Vet Microbiol **134:**254–260. http://dx.doi.org/10.1016/j.vetmic.2008.08.004.
25. **Ueti MW, Knowles DP, Davitt CM, Scoles GA, Baszler TV, Palmer GH.** 2009. Quantitative differences in salivary pathogen load during tick transmission underlie strain-specific variation in transmission efficiency of *Anaplasma marginale*. Infect Immun **77:**70–75. http://dx.doi.org/10.1128/IAI.01164-08.
26. **Potgieter FT, van Rensburg L.** 1987. Tick transmission of *Anaplasma centrale*. Onderstepoort J Vet Res **54:**5–7.
27. **Potgieter FT.** 1981. Tick transmission of anaplasmosis in South Africa, p 222. *In* Proceedings of the International Conference on Tick Biology and Control, Grahamstown, South Africa.
28. **Chaisi ME, Baxter JR, Hove P, Choopa CN, Oosthuizen MC, Brayton KA, Khumalo ZTH, Mutshembele A, Mtshali S, Collins NE.** Comparison of molecular tests for the detection of *Anaplasma marginale* and *A. centrale* in South Africa. Onderstepoort J Vet Res, in press.
29. **Catanese HN, Brayton KA, Gebremedhin AH.** 2016. RepeatAnalyzer: a tool for analysing and managing short-sequence repeat data. BMC Genomics **17:**422. http://dx.doi.org/10.1186/s12864-016-2686-2.