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Multi-locus typing scheme for *Babesia bovis* and *Babesia bigemina* reveals high levels of genetic variability in strains from Northern Argentina

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ABSTRACT

Bovine babesiosis, caused by the protozoa *Babesia bovis* and *Babesia bigemina*, is a tick-borne disease distributed in tropical regions worldwide. Current control measures are based on the use of acaricides and live attenuated vaccines. The major economic impact of babesiosis lies in the cattle industry.

In order to gain insight into the extent of genetic diversity in populations of parasites in the field, we developed two MLST schemes for the molecular genotyping of *B. bigemina* and *B. bovis*. We have also developed a custom-designed bioinformatic pipeline to facilitate the automated processing of raw sequences and further diversity and phylogenetic analysis.

The overall MLST scheme exhibited the maximum discriminatory power (Simpson Index = 1) for *B. bovis* and a high level of discrimination for *B. bigemina* (Simpson Index = 0.9545). Genetic diversity was very high and infections with multiple genotypes were frequently found for both parasites in outbreak samples from the Northeast and Northwest of Argentina. Recombination events, which could have arisen from these multiple infections, were suggested by intra-*loci* linkage disequilibrium analysis and the lack of congruence in phylogenetic trees from individual genes.

The two MLST schemes developed here are a robust, objective and easily adoptable technology to analyze the genetic diversity and population structure of parasites of the genus *Babesia*.

1. Introduction

Bovine babesiosis is caused by the intraerythrocytic protozoan parasites *Babesia bovis* and *Babesia bigemina*. Both parasites are tick-transmitted and distributed in tropical regions worldwide. The major economic impact of babesiosis lies in the cattle industry. In Argentina, this disease is responsible for economic losses of 38.9 million dollars per year (Späth et al., 1994). Costs due to babesiosis are incurred not only from mortality, ill-thrift, abortions, loss of milk and meat production but also for draft power and control measures such as acaricide treatments, purchase of vaccines and therapeutics (Bock et al., 2004).

Live vaccines using attenuated strains are actually the only method to induce long-lasting immunity against babesiosis in cattle.

These vaccines consist of attenuated strains originated after serial passages of pathogenic strains in splenectomised calves (Shkap et al., 2007). The mechanism by which attenuation occurs is not fully understood, but current hypothesis support the selective enrichment of less virulent parasite subpopulations (Shkap et al., 2007) which may have distinct genetic and phenotypic characteristics.

Several methods and markers were developed to characterize the genetic diversity in both *Babesia* species (Hilpertshauser et al., 2007; Wilkowsky et al., 2008; Genis et al., 2009; Wilkowsky et al., 2009; Perez-Llaneza et al., 2010; Simuunza et al., 2011). However these methods are difficult to standardize or provide limited information on the phylogenetic relationships among strains. These limitations demonstrate the need of a more general approach to provide accurate, portable data that are appropriate for the epidemiological investigation of parasite strains and which also reflect their evolutionary and population biology. These studies will allow not only to understand the degree to which parasite populations could be modified by vaccination but also to determine the extent of genetic diversity in populations of parasites in the field. In order to gain insight into these subjects a new panel of polymorphic molecular markers distributed across the genomes of these parasites is essential. When identifying molecular markers for their use in population studies it is important to choose multiple *loci* that are selectively neutral. *Loci* under positive selection

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may give a distorted view of population structure and transmission dynamics, since selection rather than population history may determine the patterns of distribution of alleles within populations for these *loci*.

Multilocus sequence typing (MLST) was proposed in 1998 as a portable, universal, and definitive method for characterizing bacteria, being *Neisseria meningitidis* the species for which the first MLST scheme was developed. In addition to providing a standardized approach to data collection, by examining the nucleotide sequences of multiple *loci* encoding housekeeping genes or fragments of them, the information is compared at the DNA-sequence level. Besides, MLST data are made freely available over the Internet to ensure that a uniform nomenclature is readily available to all those interested in categorizing any given microorganism (Maiden, 2006).

We describe here the development of MLST schemes for *B. bigemina* and *B. bovis*, respectively, using sequences from 6 to 7 genes to type a set of reference and field strains of different geographic origin and phenotypic characteristics. The design of the MLST scheme was assisted by the availability of the completed *B. bovis* genome and the partially completed genome of *B. bigemina*. We have also developed a bioinformatic pipeline for the automated analysis of raw sequences and further diversity and phylogenetic analysis.

2. Materials and methods

2.1. Strains and genomic DNA isolation

Details of the strains and isolates used are described in Tables 1 and 2.

The highly virulent, tick-transmissible T2Bo (Texas) isolate is the published *B. bovis* reference genome. The *B. bigemina* Sanger reference strain is a virulent Australian isolate used for the genome project. *B. bovis* attenuated strain R1A (vaccine), virulent strain S2P and *B. bigemina* attenuated strains S1A (vaccine), S2A and virulent strains Mexico, S2P and S3P were *in vitro* cultured using a microaerophilus stationary phase system (Levi and Ristic, 1980). The *B. bovis* M2P, M3P and S7P virulent isolates, M1A attenuated strain and the *B. bigemina* B38, M1A (attenuated vaccine strain), M2P and M1P virulent strains, were amplified in splenectomized calves.

Blood samples from acute cases of the north region of Argentina (*B. bovis*: 35, 394, Perugorria, Sauce and Tomasito and *B. bigemina*: M30) were obtained from bovines that showed clinical signs of acute infection and were positive for *B. bovis or B. bigemina* by microscopic observation of Giemsa-stained blood smears. *Rhipicephalus microplus* adult ticks that were naturally attached to the bovine that undergone acute infection with *B. bigemina* M30 were removed and incubated at 28° C for oviposition. Resulting egg masses from individual replete females were placed individually into sterile containers (one egg mass per container) under the same incubation conditions and allowed to hatch. Subsequent groups of larvae representing progeny from a single female were kept separate from each other and incubated for 3 weeks at 28° C and 92.5% humidity. One gram of larvae (equivalent to approximately 20,000 organisms) were placed in skin patches over a calf with intact spleen (B32) and determined to be free of *B. bigemina* infection by enzyme-linked immunosorbent assay. When parasites could be detected in B32 calf by Giemsa-stained blood smears, blood was obtained by jugular venipuncture and used to inoculate splenectomized calf B38. When this calf reached the peak of parasitemia (determined by serial Giemsa-stained blood smears), jugular blood was obtained and processed for genomic DNA extraction. Genomic DNA from strains Uruguay and Brasil was kindly provided by Dr. María A. Solari from DILAVE Uruguay and Dr. João Ricardo Souza Martins, Instituto de Pesquisas Veterinárias Desidério Finamor/FEPAGRO, Brazil. The Mexico strain was provided by Dr. Carlos A. Vega y Murguía, CENID-PAVET, INIFAP, Mexico.

Genomic DNA isolation from reference strains from experimentally inoculated bovines or erythrocyte culture was performed from PBS-washed and packed infected erythrocytes. DNA was extracted by SDS lysis/proteinase K digestion, followed by phenol/chloroform extractions, and a standard ethanol precipitation (Sambrook et al., 1989). DNA extraction from field samples was performed by the method previously described by Higuchi (1989) which consists in two lysis steps and an overnight incubation with proteinase K.

All samples were analyzed by the reverse line blot hybridization assay using *Babesia/Theileria* catch-all probes and *B. bovis* or *B. bigemina* specific probes (Petrigh et al., 2008).

Table 1
B. bovis strains used in this study.

Isolate	Geographical location	Phenotype	ST
T2B	USA	Pathogenic	1
M1A	Corrientes, NE Argentina	Attenuated	2
M2P	Corrientes, NE Argentina	Pathogenic	3
S2P	Salta, NW Argentina	Pathogenic	4
M3P	Corrientes, NE Argentina	Pathogenic	5
R1A	Santa Fé, Argentina	Attenuated	6
35	Salta, NW Argentina	Pathogenic	7
394	Salta, NW Argentina	Pathogenic	8
Perugorria	Corrientes, NE Argentina	Pathogenic	9
S7P	Santa Fé, Argentina	Pathogenic	10
Tomasito	Corrientes, NE Argentina	Pathogenic	11
Sauce	Corrientes, NE Argentina	Pathogenic	12
Brasil	Brasil	Pathogenic	13
Uruguay	Uruguay	Pathogenic	14

Table 2
B. bigemina strains used in this study.

Isolate	Geographical location	Phenotype	ST
Sanger	Australia	Pathogenic	1
M1A	Corrientes, NE Argentina	Attenuated	2
M1P	Corrientes, NE Argentina	Pathogenic	3
M2P	Corrientes, NE Argentina	Pathogenic	4
S1A	Salta, NW Argentina	Attenuated	2
S2A	Salta, NW Argentina	Attenuated	2
S2P	Salta, NW Argentina	Pathogenic	5
S3P	Salta, NW Argentina	Pathogenic	6
Brasil	Brasil	Attenuated	7
Mexico	México	Pathogenic	8
M30	Salta, NW Argentina	Pathogenic	9
B38	Salta, NW Argentina	Pathogenic	10

2.2. Target loci

Twenty three *loci* were preselected for *B. bovis* and twenty one for *B. bigemina* using information available from genome strains. All preselected *loci* were single copy and homogeneously distributed through the four *B. bovis* chromosomes. In *B. bigemina*, *loci* were selected using the contigs available at the *B. bigemina* genome project (http://www.sanger.ac.uk/resources/downloads/protozoa/babesia-bigemina.html). The search was performed by Blast (tblastn) using *B. bovis* annotated genes as query. Primers were designed for all genes and amplifications were performed with DNA from 2 reference strains from each parasite. Only those PCR products with high sensitivity and specificity for the corresponding parasite were finally selected. Specificity controls included DNA from uninfected *Bos taurus, Anaplasma marginale* and the heterologous *Babesia* species (i.e. *B. bigemina* for *B. bovis* PCR and viceversa). Amplicons were sequenced in both strands and those *loci* showing a neutral pattern according to the ratio of mean non-synonymous