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A new set of molecular markers for the genotyping of *Babesia bovis* isolates

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ABSTRACT

Babesia bovis is a tick-borne apicomplexan pathogen that remains an important constraint for the development of cattle industries worldwide. The existence of different strains and subpopulations has long been described in this hemoparasite. However, few molecular markers have been reported for strain genotyping and characterization. Minisatellite sequences show high levels of variation and therefore provide excellent tools for both the genotyping and population genetic analysis. In this work we report a set of five molecular markers containing minisatellites that showed a variable degree of polymorphism in several American strains. We have used a bioinformatics approach to search for marker sequences contained in open reading frames. Five genes were chosen and primers were designed in conserved regions flanking the repeat region. Two of the genes were the previously described Bv80/Bb-1 and TRAP. The other three genes were named p200, Antigen 3 and Desmoyokin. Amplification by PCR, sequencing and comparative analysis of 11 strains from Argentina, Brazil, Uruguay, Mexico and USA determined that the tandem repeats varied in number and sequence among the isolates. Genome analysis of the five markers revealed that they were single copy and distributed across the four *B. bovis* chromosomes. When the new markers were analyzed in an experimental infection, absolute sequence conservation was found, indicating the stability of these markers during the course of infection. These markers were also stable during three syringe passages through calves. The application of this panel of molecular markers could provide new molecular tools for the genotyping of *B. bovis* isolates and analysis of changes in parasite populations following vaccination.

1. Introduction

The apicomplexan protozoan *Babesia bovis* is one of the etiological agents of bovine babesiosis, a disease that remains a major constrain to livestock production in tropical and subtropical regions of the world. This tick-transmitted parasite has an intraerythrocytic stage in the mammalian host that ensures both, survival through asexual reproduction and effective transmission to its arthropod vector. In cattle, *B. bovis* causes a disease that is characterized by fever, anemia, hemoglobinuria and jaundice, resulting in high mortality rates among susceptible animals (Bock et al., 2004). Recovered bovines remain infected for years and experimental evidence showed that a single animal can be co-infected with genetically and antigenically distinct strains (Berens et al., 2007).

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The existence of different strains and subpopulations within *B. bovis* parasites displaying distinct biological features has been reported (Timms et al., 1990). Parasite clones with different degrees of pathogenicity and tick-transmissibility have been isolated from the Australian vaccine strain. It was also demonstrated the full reversion of an avirulent clone to a virulent phenotype after one passage through intact cattle (Timms et al., 1990). These findings support the hypothesis that in addition to subpopulation selection mechanisms, recombination or differential gene expression might also be occurring in this parasite (O'Connor et al., 1997).

Several outbreaks in vaccinated herds imposed the need to differentiate field strains from vaccine strains. DNA probes have been used to demonstrate heterogeneity within natural isolates or vaccine strains of *B. bovis* (Cowman et al., 1984; Dalrymple et al., 1992; Jasmer et al., 1990). Its use was later replaced by a PCR-fragment size polymorphism assay based on two previously described genes, namely *Bv80* and *BvVA1* assays (Lew et al., 1997a). These genes contain conserved 5′- and 3′-terminal regions separated by arrays of tandem repeat (TR) sequences with variable length which are responsible for the size polymorphism of the corresponding genes (Dalrymple et al., 1993).

Besides these reports, only few molecular markers have been described for the genotyping of *B. bovis* isolates. Previous results of our laboratory (unpublished data) have shown that the use of highly conserved genes such as the ssRNA could not differentiate among American *B. bovis* isolates. On the other hand, the *msa-2* locus of the variable merozoite surface antigen family has proved useful for the typification of American isolates (Wilkowsky et al., 2008) and to distinguish an American from an Australian isolate (Berens et al., 2007). However, the simultaneous analysis of multiple polymorphic genes is required to assess the extent of allelic diversity among strains (Maiden, 2006; Mallon et al., 2003). TR sequences, particularly minisatellites within coding regions, have been successfully used for the genotyping of diverse microorganisms (Yousef Mohamad et al., 2008; Yanga et al., 2003; Li et al., 2004; Felger et al., 1999).

In Argentina, where live vaccines are routinely used and a large extension of its territory is enzootic for ticks, the degree of genetic diversity among isolates is unknown. For this reason, our purpose was to identify minisatellite sequences that could be applied as molecular markers for the characterization of *B. bovis* isolates. In this work, we have defined a panel of five minisatellite markers and analyzed their degree of polymorphism in different pathogenic and attenuated strains of Argentina, Mexico and USA. We have also evaluated the stability of the genetic markers during the time-course of an experimental infection and during passages through bovines. Finally, a phylogenetic analysis of minisatellite conserved flanking sequences was performed to assess genetic relationships among the American strains under study.

2. Materials and methods

2.1. B. bovis strains and genomic DNA isolation

The Mo7, T2Bo, R1A and S2P strains were *in vitro* cultured using a microaerophilus stationary phase system (Levi and Ristic, 1980). The M2P and M3P virulent isolates and the M1A vaccine strain were amplified in splenectomized calves. DNA from the Mexico isolate was kindly provided by Juan Mosqueda. Details of the strains and isolates used are referenced in Wilkowsky et al. (2008).

Blood samples from field cases of the NW region of Argentina were obtained from bovines that showed clinical signs of acute infection and were confirmed to be positive to *B. bovis* by microscopic observation of Giemsa-stained blood smears.

Genomic DNA isolation from PBS-washed and packed *B. bovis*-infected erythrocytes was performed by SDS lysis/proteinase K digestion, followed by phenol/chloroform extractions, and a standard ethanol precipitation (Sambrook et al., 1989).

2.2. Identification of B. bovis minisatellite sequences

In order to identify minisatellites present in *B. bovis*-coding sequences, the tandem repeat finder (TRF) program (Benson, 1999) was used. This program allows locating and displaying TR in DNA sequences. The search was performed against the available sequences from the *B. bovis* ESTs Sequencing Project (http://www.sanger.ac.uk/Projects/B_bovis/). A subset of sequences was selected from the TRF output according to the period size of the repeat (>9 bp) and its copy number (>4). Sequences were translated with the Translate program (http://www.expasy.ch/tools/#translate) and those containing an open reading frame were further analyzed to identify the repeat region within the amino acid sequence.

A BLAST search against the *B. bovis* genome (www.vetmed.wsu.edu/research_vmp/Babesia-bovis/) was performed to confirm the absence of introns and that the selected genes were single copy. Finally, genes which contained repeat regions up to 2000 bp were selected for PCR amplification.

2.3. PCR analyses

Primers corresponding to the conserved 3′- and 5′-regions flanking the repeat region of each of the candidate genes were designed using Primer 3 software (Rozen and Skaletsky, 2000) and are described in Table 1. Amplifications were carried out from *B. bovis* genomic DNA. To determine the species specificity of all the sets of primers, DNA from *B. bigemina* S1A strain was used as a template negative control. Amplifications were performed using GoTaq polymerase (Promega), Go Taq PCR buffer, dATP, dCTP, dGTP, dTTP (each at 1 mM), and primers (0.4 μM). The total reaction volume was 50 μl. The cycling conditions were: denaturation at 94 °C for 60 s, annealing at 53 °C for 30 s for TRAP, 55 °C for 60 s for p200, 52 °C for 30 s for Desmoyokin and 51 °C for 30 s for Antigen 3. Extension at 72 °C for 60 s was set for all cases with the exception of p200 in which a 2 min extension time was used. The final extension period was set at 72 °C for 10 min for all amplifications. PCR products (7 μl) were separated on 2% agarose gels (except of p200 in which a 0.8% gel was used) in Tris–acetate–EDTA buffer. Gels were stained with ethidium bromide and the DNA bands were visualized

Table 1
Minisatellite markers identified from the *Babesia bovis* EST database.

Satellite marker	Size range of repeat region (bp)	PCR primers 5′–3	Number of alleles identified/number of strains analyzed
p200	1194–2040	Forw CTGGTCACCATGGACTATCTGT Rev ACATAACAGCAGCGAGACCAAC	6/6
TRAP	273–615	Forw AATCATGAGCCTGTGCCAAG Rev ATCACATCAGAAGGCGATGG	9/11
Desmoyokin	144–264	Forw TCTCGAAAAGGCGAAACTTG Rev TGCTTCATCACGTTGGAGTT	4/5
Bv80/Bb-1	285–507	Forw ATAGCCTGTTAGTTGATGGACC Rev TGTGTTAATGTAACTCAGCCCG	10/11
Antigen 3	192–300	Forw GCGCTTCTCCTTTCAACAAC Rev CTCGCATCAGTAGCACTTGG	4/11

on a UV image analyzer (BioRad) and documented. PCR products of intermediate size (400–700 bp) were directly sequenced using the same PCR primers (TRAP, Bv80/Bb-1, Desmoyokin, Antigen 3). In the case of p200, the PCR product was cloned into pCR-TOPO 2.1 vector (Invitrogen) and sequenced using M13 forward and M13 reverse primers. An additional internal primer was designed (5′-TGCTTGGCGTCTCAT-3′) to obtain the complete sequence of this fragment. In all cases sequencing was performed at the sequencing facility of the Instituto de Biotecnología-INTA using the Big Dye Terminator v3.1 kit from Applied Biosystems and analyzed on an ABI 3130XL genetic analyzer from the same supplier. Contigs were assembled using NTI Vector (Invitrogen) and sequences were translated using Translate.

2.4. Nucleotide sequence accession numbers

GeneBank accession numbers of the new genomic DNA sequences from which protein sequences were derived are as follows:

(a) *TRAP*: Bor, FJ423465; Uruguay, FJ423466; Brazil, FJ423467; M2P, FJ423468; M3P, FJ423469, Mo7, FJ423470; S2P, FJ423471; M1A, FJ423472; Mexico, FJ423473; R1A, FJ423474; (b) *Antigen 3*: R1A, FJ423475; M1A, FJ423476; M2P, FJ423477; M3P, FJ423478; Uruguay, FJ423479; Bor, FJ423480; Brazil, FJ423481; S2P, FJ423482; (c) *Bv80/Bb-1*: Bor, FJ423483; R1A, FJ423484; Brazil, FJ423485; M2P, FJ423486; Uruguay, FJ423487; S2P, FJ423488; M3P, FJ423489; Mexico, FJ423490; (d) *p200:* R1A, FJ423491; M1A, FJ423492; Bor, FJ423493; M2P, FJ423494; (e) *Desmoyokin*: Mexico, FJ603468; M1A, FJ603469; S2P, FJ603470; M2P, FJ603471.

2.5. Experimental infection of calves

To determine the stability of the molecular markers used in this study in a time-course experimental infection, one Holstein calf was subcutaneously inoculated with 1×10^7 merozoites of the *B. bovis* pathogenic strain S2P. The trial was carried out in the Experimental Station of INTA, near Rafaela city, Santa Fe province, a tick-free region of Argentina. The clinical parameters were followed daily by assessing the hematocrit value, body temperature and parasitemia. From day 10 on, this animal presented severe clinical signs of babesiosis (3 days with $\geq$41 °C body temperature; hematocrit value $\leq$15% and >1% of parasitized erythrocytes). On the 10th day post inoculation (pi), maximal parasitemia (2%) was detected and the bovine was specifically treated with diaminazene diaceturate (3.0 mg/kg of body weight) to avoid death. Blood samples were taken at days 7, 102, 135 and 206 post infection (pi) and genomic DNA was extracted.

2.6. Blood passages of B. bovis parasites

In order to evaluate whether the markers used in this work were stable after blood passages in susceptible cattle, an experiment was carried out with the Mexico virulent strain of *B. bovis*. Three 12-month-old splenectomized Holstein steers free of antibodies against *Babesia* spp., assessed by the indirect immunofluorescence antibody test (IFAT), were used in this experiment. The first bovine was inoculated intravenously with a frozen stabilate of the *B. bovis* Mexico strain and when clinical babesiosis appeared, blood was taken from the jugular vein, defibrinated and separated from white blood cells and serum. Infected erythrocytes were resuspended in Vega y Martinez solution (VYM) and immediately inoculated into a second steer. This solution is used for washing and storage of infected and uninfected erythrocytes (Vega et al., 1985). The same procedure was followed for the third steer. Unused infected erythrocytes from each passage were maintained at −20 °C until used for DNA isolation. For this experiment, genomic DNA was extracted using the Puregene commercial Kit (Gentra Systems) following the instructions of the manufacturer.

2.7. Phylogenetic analysis

Conserved flanking sequences were identified for each minisatellite and the corresponding 5′- and 3′-regions analyzed in a single datamatrix concatenated using WinClada (Nixon, 1999). Multiple alignments of DNA sequences were accomplished with CLUSTAL X (Thompson et al., 1997). Parsimony analysis was performed using the exact search command implemented in NONA 2.0 (Golob-

off, 1993). Branch support was measured using 1000 bootstrap replicates. Phylogenetic inferences had to be restricted to network analysis without outgroup rooting due to the lack of suitable outgroups.

3. Results

3.1. Identification and PCR amplification of minisatellite sequences

The TRF software was used to screen the *B. bovis* ESTs database for genes containing TR arrays. Among the many sequences identified by the programme, we selected five which fulfilled the specified criteria of length and repeat period sequence. BLAST analysis revealed that the five genes are distributed across the four *B. bovis* chromosomes. TRAP and Bv80/Bb-1 are in chromosome 2 and Desmoyokin, Antigen 3 and p200 in chromosomes 4, 3 and 1, respectively.

PCR amplifications with the five sets of primers were carried out with *B. bovis* DNA from a total of 11 strains from Argentina, Mexico and USA. Positive amplifications were obtained in all strains for Bv80/Bb-1, TRAP and Antigen 3. In the case of Desmoyokin, only three Argentine strains (M1A, M2P and S2P) and the T2B and Mexico strains yielded positive amplifications. In this case, we designed and tested a new set of primers upstream to the previously selected region; however, no amplification products were obtained for the rest of the strains (data not shown). In the case of p200, amplification was successful only for the M1A, M2P, R1A and Bor strains, although low yields were obtained. For this reason, we decided to clone the PCR product and obtain the p200 sequence using vector and internal primers.

A size polymorphism of PCR products in agarose gel electrophoresis was clear for Bv80/Bb-1 and p200 (Fig. 1A and B) and less evident for the rest of the markers (data not shown). No amplification was obtained in any case with *B. bigemina* DNA (data not shown), confirming the species specificity of the primers. Single band PCR products were obtained for all markers and strains.

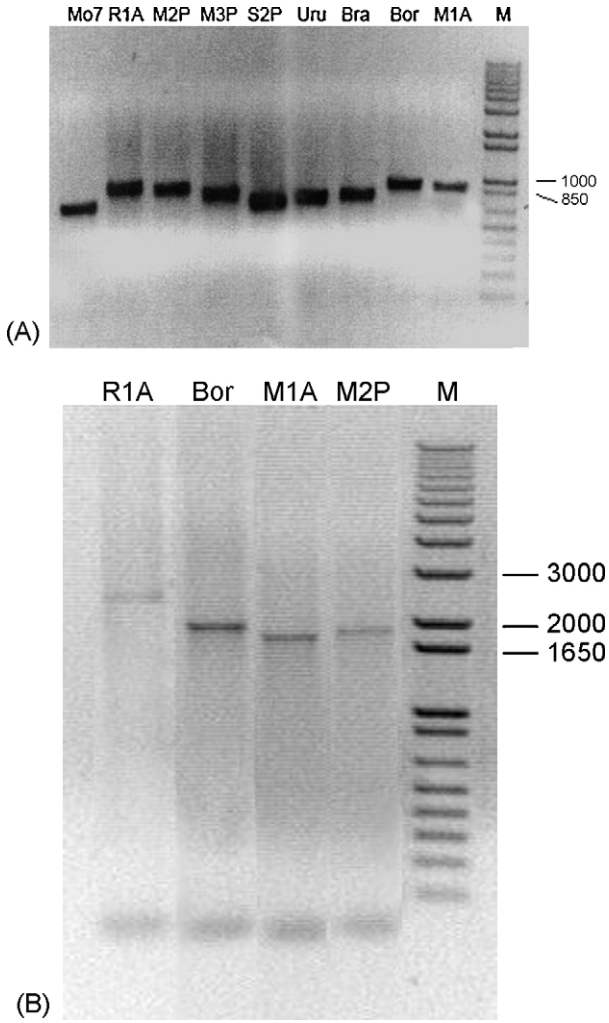

Fig. 1. Size polymorphism of two minisatellite markers in different *B. bovis* strains. Agarose gel electrophoresis of PCR products of (A) Bv80/Bb-1; (B) p200. The 1Kb Plus molecular marker (M) was used and size relevant bands are indicated in base pairs (bp). Uru: Uruguay strain. Bra: Brazil strain.

3.2. Allelic variation in isolates from different geographical regions

DNA sequences were obtained from all positive amplifications and contigs were assembled and analyzed for each marker. In the case of p200, nucleotide sequences for T2B and Mo7 were obtained from the *B. bovis* genome and GeneBank, respectively.

Nucleotide sequence polymorphism was mostly restricted to the repeat region and the repeat modules were variable in sequence and number among all isolates. Size ranges of these modules are shown in Table 1. For Bv80/Bb-1 the variation also extended to a non-repetitive 5′-region.

In order to facilitate the analysis of variation of the repeat modules, sequences were translated and a symbol code was assigned to each repeat (numbers for Bv80/Bb-1, capital letters for TRAP, Greek letters for p200, small letters for Antigen 3 and roman letters for Desmoyokin) (Table 2).

Using the five minisatellite markers, all strains analyzed could be differentiated, with the exception of Mexico and T2B strains that had identical aminoacid sequences with the four markers analyzed (Bv80/Bb-1, TRAP, Antigen 3 and Desmoyokin). Sequence polymorphism was observed for Bv80/Bb-1 in all strains and the same was found for the six strains analyzed for p200. In the case of TRAP, this marker allowed to differentiate all strains with the exception of the pairs Mexico/T2B and S2P/Uruguay, which had identical sequences, respectively. Antigen 3 resulted to be the least discriminatory marker since only 4 alleles were identified out of the 11 analyzed strains. Although Desmoyokin sequences could not be determined for all strains, the five sequences obtained showed a unique pattern of amino acid repeats.

A common feature of the five molecular markers described in this work is that the genotypes were not obviously related to geographical regions or the virulence phenotype of the strain.

3.3. Analysis of protein sequences of the repeat region

To examine the polymorphism of the identified minisatellites, the amino acid sequence of each marker was analyzed for presence, number and type of repeat. Physicochemical parameters were determined using ProtParam (Gasteiger et al., 2005). Results will be described for each marker.

3.3.1. Bv80/Bb-1

The translated region of Bv80/Bb-1 analyzed here comprises residues 273–434 of a total of 596 amino acids in the Mo7 strain (Hines et al., 1995). A high diversity of repeats was found in this region, characterized by degeneration within a repeat segment of 4, 5 or 6 amino acids. In each of these repeats, there is a proline at the first position, with five different consensus sequences: PA(E, D, K)T, PAEK, PV(A, V)E(E, T), PI(A, V)EE and PAETET.

The distribution of repeats in the analyzed strains is quite variable. Some of them, like PAET and PAEK are

Table 2
Variability of the protein sequence of the *B. bovis* minisatellite markers. The aminoacid sequences corresponding to the repeat region of the five markers in different strains are schematized. For each marker a symbol code was assigned. Each letter or number represents a block of repeats. Subscripts brackets represent the number of times one repeat appears sequentially. ND, not determined.

	Antigen 3	TRAP	Bv80/Bb-1	p200	Desmoyokin
Strain					
M1A	abcdefedcgcfcbeh	ABCDBC EEBCDFCG	12332 $(3)_5$221$(2)_6$ $(3)_2$ 6$(4)_6$ $(5)_5$	$\alpha\alpha(\gamma)_5$	v i xi $(ii)_2$ i $(ii)_4$ xii
R1A	abcdefedcgefcbeh	ABCABD G ABG AB	12332 $(3)_5$22111$(2)_6$ $(3)_4$ 6$(4)_6$ $(5)_5$	$\alpha\alpha\beta\alpha\alpha\beta\alpha\beta\alpha\alpha\alpha$ $\beta\alpha\alpha\beta\alpha(\gamma)_{28}$	ND
MO7	abcdefedcgcfcbeh	HEEHDDIGIJDGDJDGIIGIG	7$(5)_4$ $(4)_2$ $(5)_7$ $(4)_3$ 5 $(4)_9$ $(4)_2$	$\alpha(\beta)_6(\gamma)_{17}$	ND
Bor	abcdefedcgcfcbeh	HEEHDDIGIJIGDJDGIDGIG	$(5)_4$ 44$(5)_7(4)_3$5$(4)_9$55	$\alpha(\beta)_{23}(\gamma)_{13}\xi\gamma\gamma$	ND
Pullman	abcdefedcgcfcbeh	EDBCGGCGCBG	177 $(4)_{15}$ $(8)_5$	ND	i vi ii iii ii i iv v ii
T2B	abcdefedcgcfcbeh	EDBCGGCGCBG	177 $(4)_{15}$ $(8)_5$	$\alpha\alpha\beta\alpha\alpha\delta\alpha\alpha\beta\alpha\alpha\delta$ $\alpha\alpha\delta\alpha\alpha\beta\alpha\alpha\delta\alpha\alpha$ $\beta\alpha\delta\alpha\alpha\delta\alpha\alpha\delta\alpha\alpha$ $\delta\alpha\alpha\delta\alpha\alpha(\delta)_3(\gamma)_3(\varepsilon)_8$	i vi ii iii ii i iv v ii
Uruguay	abcdefedcgcfcbeh	HIGIGIGIJDGDGIGIG	$(5)_3$ 44 $(5)_6$ $(4)_4$ 5 $(4)_{13}$ 584455	ND	ND
S2P	ibcdeibcdcfchjfchjfcdjbeh	HIGIGIGIJDGDGIGIG	$(5)_4$ 44 $(5)_6$ $(4)_4$5 $(4)_9$ 5	ND	i i iv v ii ii x ii vii i ii
M2P	ibcdeibcdcfchjfchjfcdjbeh	EJBCBCGEBCDBCG	12332 $(3)_6$22122$(1)_3(2)_4$336 $(4)_3$ $(5)_5$	$\alpha(\beta)_4\xi(\beta)_{18}(\gamma)_{10}$	xi $(i)_2$ vii ii
M3P	ibcdkfchjfchjfcdjbeh	HEEHDDIGIJFJDGDGIGIG	$(5)_7$ 4 5$(4)_3$ 5 447$(4)_6$5 $(4)_{11}$5	ND	ND
Brasil	ibcdeibcdlfchjfchjfcdjbeh	HEEHGIHJDGCGIJIG	12332 $(3)_5$ 2221 $(2)_5$ $(3)_5$6 $(4)_6(5)_5$	ND	ND
Clinical cases					
La Angela 841	abcdefedcgcfcbeh	ND	ND	ND	ND
Salta 394	abcdefkcdcfchjfcdjbeh	HDEODDIGIJDGDDJG	1$(7)_3$ $(5)_{11}$ $(4)_4$ $(9)_2$ $(4)_4$ 5	ND	ND
Salta 35	ibcdeibcdcfchcgcdjbeh	ND	ND	ND	ND
Salta 249	abcdefedcgcfcbeh	ND	ND	ND	ND

(a) Bv80/Bb-1: (1) PIVEE; (2) PIAEE; (3) PVAEE; (4) PAET; (5) PAEK; (6) PVAET; (7) PAKT; (8) PVVEE; (9) PAETET; (10) PADT. (b) p200: (α) QEALEAERKR; (β) QEAEAERKR; (γ) QEEAEAERKR; (δ) QEKRQEKR; (ε) QEEKRQEKR; (ζ) QEEAEAEGKR; (η) QEAEAVRKR; (μ)QEEEEAR; (σ) QAAERKR; (π) QEEEAAR. (c) TRAP: (A) MSSSFTD; (B) MPSSPTD; (C) MSSSTTD; (D) MPSSSSD; (E) MSSSSTD; (F) MPSSPSD; (G)MPSSTTD; (H) MSTGSTD; (I) MSSSSSD; (J) MPSSSTD. (d) Antigen 3: (a) RPER; (b) KHDV; (c) KHEI; (d) KPEI; (e) KHEV; (f) RPEV; (g) RPEI; (h) KEDI; (i) RPEL; (j) RHEI; (k) KHGV; (l) KHEF. (e) Desmoyokin: (i) KEPAPTSP; (ii) KEPAPTTP; (iii) KQPAPAEP; (iv) KEPAPTDP; (v) KEPAPAEP; (vi) KEPEPTT; (vii) KEPAPSTP; (ix) KEPAPISP; (x) KEPAPTAP; (xi) KEPAPTTT; (xii) KQPAPTTT; (xii) KQPVPTSP.

present in all strains. Conversely, repeats like PAETET and PADT are only present in the Uruguay and Salta 394 isolates, respectively. Details are shown in Table 2.

According to the intrinsic characteristics of the variable TR found in this marker, sequence alignment of all alleles could not be performed using the usual matrixes. However, strain association can be done manually by looking at the distribution and order of appearance of the repeat segments. By doing this, at least three clear groups can be distinguished. One formed by the M1A, R1A, M2P and Brazil strains (Table 2); the second composed by S2P, Bor, Uruguay and M3P and the latter includes Mo7, Mexico and T2B strains. The Salta 394 case could not be clearly included in any of the previous groups, although the first three to four repeats of its sequence show similarity with the Mexico and T2B strains (see Table 2).

On the other hand, the total number of Proline repeats is highly variable and fluctuates from 23 to 37 residues (Table 3). No clear association can be made between the number of proline repeats and the three groups of strains already described.

Another striking characteristic of the Bv80/Bb-1 gene fragment amplified in this study is the presence of a stretch of 12–15 serine (S) residues immediately preceding the repeat segment. The S2P, Bor, Uruguay, Mexico, T2B, Mo7 and M3P isolates display 13 S; R1A, M2P, Salta 394 and Brazil had 14 S while M1A was the only strain with 15 S.

The amino acid composition of the Bv80/Bb-1 translated repeat regions was analyzed with the ProtParam software. Glutamic acid is the most frequent residue (25.6–35.6%), followed by proline (21.3–24.8%) and alanine (19–24.4%) in all the strains analyzed. The amino acids lysine, threonine, valine and isoleucine appear with less than 17% frequency.

3.3.2. TRAP

The analyzed TRAP region containing TR comprises residues 324–588, of a total of 660 amino acids of the predicted protein in the T2B strain. The repeat motif is conserved in length and composed by seven residues being the first always a methionine (M) and the last an aspartic acid (D). The consensus motif is M (S/P) (S/T) (S/G) (F/P/T/S) (S/T) D. The fifth position of the seven residues-motif seems to be the most variable within any of the four amino acids (F, P, T or S) present in that position. With the exception of the first and the last positions, the rest of the residues only vary between S and other amino acid (F, P, T or G).

The amino acid composition of the repeat region for this marker is less variable than for Bv80/Bb-1. Only five residues are present, being S the most abundant (37.7–48.6%) followed by threonine (12.9–22.1%), aspartic acid (14.3%), methionine (14.3%) and proline (8.6–11.7%).

3.3.3. Antigen 3

For this predicted protein, we have analyzed a region of 188 amino acids from a total of 795 present in the Mo7 strain. The repeat region comprises residues 195–266 and includes 2 types of motifs with 4 amino acids each. The consensus sequence of each group of repeats is R (P/H) E (R, V, I, L) and K (P, H, E), (D, E, G) (V, I, F). A total of 12 different repeat motifs were found in the 11 strains analyzed. Glutamic acid is the most frequent amino acid (around 21% of the total), followed by K (16–18%), V (8.3–12.5%), I (11.1–14.8%) and to a lesser extent R (6.9–7.4%).

3.3.4. p200

The p200 protein is the longest protein analyzed in this study. In the T2B strain this protein has 718 aa, according to the predicted amino acid sequence. The repeat region is 297 aa in length and comprises residues 197–490. The repeat module varies in length between 8 and 10 amino acids. Seven different repeat motifs were found in the six strains analyzed. The first and last two residues are conserved (QE and KR, respectively). The rest of the amino acids in the middle of the repeat module are either A, E, K, L, R, Q, V or G. Glutamic acid is the most abundant residue in the repeat motif (30–39%), followed by alanine (20–21%) in R1A, M1A, Mo7 M2P and Bor. T2B strain has the longest repeat domain with 54 modules compared with the rest of the strains that only have between 27 and 44 repeat modules.

3.3.5. Desmoyokin

This marker displayed the highest variation in repeat modules with 12 different motifs in the 6 strains that could be amplified and sequenced. The repeat module is composed of eight amino acids in all strains with the exception of the T2B strain that is the only one that contains an additional repeat module of seven amino acids. The consensus sequence is K(E, Q)P(A, E, V)P(T, A, S, I)(S, T, E, D, A)(P, T). Amino acid composition of the repeat region shows a high abundance of P (35.4–37.5%), followed by T (13.9–18.8%), A (12.5–15.3%) and G (10.4–15.5%).

3.4. Assessment of genetic stability of markers

3.4.1. In a time-course infection

In order to determine the molecular stability of the newly described markers for *B. bovis*, an experimental infection of an intact bovine with the S2P pathogenic strain was performed. Agarose gel electrophoresis of PCR products of Bv80/Bb-1, TRAP and Antigen 3 showed positive bands of the same size at days 7, 102, 135 and 206 pi. Sequence analysis of these PCR products revealed total sequence conservation. Stability was not evaluated for Desmoyokin and p200 because, as described earlier, it was not possible to amplify these two markers from the S2P strain.

3.4.2. In passages through bovines

The nucleotide sequences of Bv80/Bb-1, TRAP, Desmoyokin and Antigen 3 were analyzed in three passages through splenectomized calves infected with the pathogenic Mexico strain. An absolute conservation of nucleotide sequence was obtained for Bv80/Bb-1, Desmoyokin and TRAP for the three passages. For Antigen 3, two PCR bands were obtained. Amplicons were cloned in pCR-TOPO 2.1 and at least 4 clones of each passage were sequenced. Two different genotypes of similar size (540 bp and 432 bp) were found and called "L" and "S", being L identical to the Mexico strain genotype. In the first passage,

one of five of the clones were L, while this genotype rose in the second passage to two out of four clones. In the third passage, all four clones were of the L genotype (Fig. 2).

3.5. Phylogenetic analysis

To determine the relationships among different strains and isolates, a phylogenetic analysis of the conserved flanking regions was performed for each marker. The Antigen 3 alignment yielded a 365-character matrix. Of these characters, 357 were invariant and 8 were parsimony informative. The 3′-region exhibited only one variable character, whereas the 5′-region harboured seven variable positions. Parsimony analysis yielded a single most parsimonious tree of 15 steps and revealed the presence of two main lineages with 100% bootstrap support (M2P,

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First passage

  1  NETHSNIKVEERRGMRSDPPPIPKTPITLESKNEVRPERKHDVKHEIKPEIKHEVR----
  2  NETHSNIKVEERRGMGSDPPPIPKTPITLESKNEVRPERKHDVKHEIKPEIKHEVR----
  3  NETHSNIKVEERRGMRSDPPPIPKTPITLESKNEVRPERKHDVKHEIKPEIKHEVR----
  4  NETHNNIKVEERRGMRSDPPPIPKAPITLESKNEVRPELKHDVKHEIKPEIKHEVR----
  5  NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEVRPELK

  1 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  2 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  3 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  4 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  5 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR

  1 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  2 HEIKHDVKHEVKEDIKAEHVSDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  3 HEIKHDVKHEVKEDIKAEHVSDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  4 HEIKHDVKHEVKEDIKAEHVSDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  5 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN

Second passage

  6  NETHSNIKVEERRGMRSDPPPIPKTPITLESKNEVRPERKHDVKHEIKPEIKHEVR----
  7  NETHSNIKVEERRGMRSDPPPIPKTPITLESKNEVRPERKHDVKHGIKPEIKHEVR----
  8  NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEVRPELK
  9  NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEIRPELK

  6 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  7 --------------------------------PEVKHEVKPEIKHEIRPEIKHEIRPEVK
  8 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR
  9 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR

  6 HEIKHDVKHEVKEDIKAEHVSDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  7 HEIKHDVKHEVKEDIKAEHVSDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  8 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
  9 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN

Third passage

 10 NETHSNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEVRPELK
 11 NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEVRPELK
 12 NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELRHDVKHEIKPEIKHEVRPELK
 13 NETHNNIKVEERRGMRSDPPPIPKTPITLESKNEVRPELKHDVKHEIKPEIKHEVRPELK


 10 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR
 11 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR
 12 HDVKHEIKPEIKHEIRPEVKHEIKEDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR
 13 HDVKHEIKPEIKHEIRPEVKHEIKGDIRHEIRPEVKHEIKEDIRHEIRPEVKHEIKPEIR


 10 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
 11 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
 12 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
 13 HEIKHDVKHEVKEDIKAEHVGDIKAETKSESKSSLKNHGRNPYLGRSASAEAHLNVKHMN
```

Fig. 2. Selection of a larger repeat-containing genotype of Antigen 3 in three passages through bovines. ClustalW alignment of the aminoacid sequence of Antigen 3 is shown. Bacterial clones in which the PCR products were cloned are indicated with numbers for the three syringe passages. The larger genotype "L" is underlined.

M3P, Brazil, S2P and La Angela 841, Mo7, Bor, T2B, Uruguay, M1A, R1A and Salta 394). However, no resolution was achieved within either of them. In agreement with these groupings, the same two lineages are apparent from the analysis of repeat units, with the latter showing markedly higher sequence diversity than flanking sequences.

Of the 157 aligned characters included in the TRAP data matrix, 12 were variable, but only 4 were parsimony informative. In addition, these four characters provided contradictory information, thus resulting in no resolution in the consensus of the five most parsimonious trees.

A data matrix of 164 characters was obtained from the alignment of the Bv80/Bb-1 flanking sequences. Of these, 29 were variable and 25 parsimony informative. The two most parsimonious trees of 32 steps were found by exact search. The strict consensus is divided into two main groups with 78% bootstrap support: (i) Mo7, Uruguay, S2P, Bor and M3P and (ii) T2B, Mexico, Salta 394, M1A, R1A, M2P and Brazil. A further subclade including M1A, R1A, M2P and Brazil is also recognized with high bootstrap value (100%). As observed for Antigen 3, the clustering obtained for Bv80/Bb-1 on the basis of the flanking regions is highly consistent with the groupings defined by the number and composition of repeats (Table 2).

4. Discussion

In this work, we have tested the hypothesis that genes containing tandem repeat units, which are known to have a high ratio of variation, could be applied for high resolution molecular fingerprinting of *B. bovis* isolates.

Using the TRF programme and the *B. bovis* EST database we selected five genes containing minisatellites. This software has been successfully used for satellite detection in the genome sequence data from *Cryptosporidium*, *Theileria Trypanosoma* and *Leishmania* species (Mallon et al., 2003; Oura et al., 2003; Goto et al., 2007; Yasuyuki et al., 2007). We here report the characterization of the identified minisatellites with respect to chromosomal location, sequence and copy number in geographically distant isolates, as well as the identification of conserved flanking regions for their PCR amplification.

Of the five genes that were investigated, two of them, TRAP and Bv80/Bb-1 were previously characterized at the genetic and immunological level in *B. bovis* (Gaffar et al., 2004; Hines et al., 1995; Dalrymple et al., 1993). In the case of Antigen 3, a homologous protein, named BC134, was used as antigen in an ELISA for the diagnosis of *B. caballi* (Tamaki et al., 2004). Similarly the *B. bigemina* homologous p200 antigen was used in an ELISA by Tebele et al. (2000). Both proteins have a central region of tandem repeats.

Regarding their use as molecular markers, only Bv80/Bb-1 was already reported to be size polymorphic in Australian strains (Lew et al., 1997a,b; Bock et al., 2000) but no sequence analysis of the variable repeat region in different isolates was described. No publications were found for Antigen 3, Desmoyokin and p200 in *B. bovis* although their nucleotide sequences are deposited in the GeneBank (accession numbers DQ_319896.1, XM_001609362 and DQ_319895 respectively). Sequence analysis using the *B. bovis* EST database (C61411 strain) and the *B. bovis* genome (T2B strain) allowed us to confirm that the genes are transcribed, single copy and distributed across all four chromosomes. This condition was previously defined to avoid amplification of different paralogues and to assure that each marker was independent (i.e., not in linkage disequilibrium on the same chromosome). Additionally, by aligning sequences of these two strains, we were able to identify potentially conserved regions for primer design. Conservation of primer annealing sequence for Bv80/Bb-1, TRAP and Antigen 3 was further confirmed by PCR amplification. Conversely, Desmoyokin and p200 could only be amplified from a subset of isolates, indicating the existence of null alleles or sequence polymorphism in the flanking regions. DNA from the related organism *B. bigemina* that co-exists in cattle of endemic regions was used to assert the specificity of the primers.

Size polymorphism was found in all the genotypes in direct correlation to the variation in tandem repeat number but was insufficient to unambiguously identify each of the strains. To this regard, sequence analysis of each marker provided a much more powerful tool. As it was expected, a high rate of variation in the repeat region was detected among strains, even though each marker exhibited a different level of polymorphism. Specifically, TRAP and Bv80/Bb-1 showed 10 alleles out of 11 strains examined, whereas Antigen 3 only presented 4 in the same strains. Although some degree of similarity could be observed among isolates from the same region, regarding type and repeat order, no strong correlation with geographic location could be found. However, it should be noted that no precise data that could track the origin of the animals from which these strains came from, is available. Finally, in the T2B and Mexico strains, all the markers in this study had identical sequences suggesting a common origin of these strains (J. Mosqueda, personal communication).

Tandemly repeated regions within proteins are widespread in many parasitic protozoa including *Plasmodium*, *Trypanosoma*, *Leishmania* and *Toxoplasma*, and several functions have been assigned to these regions, including ligands for host proteins and immune evasion (Schofield, 1991). Nussenzweig and Nussenzweig (1986) have reported that there appears to be a noticeable bias in the amino acids in the repeat regions with residues such as N, A, D, V, P, E, G, Q and S highly represented. Amino acid composition analysis in the five markers described in this work has confirmed these findings although the structural meaning of this bias is still uncertain.

The fact that 10 unique multilocus genotypes were identified in 11 strains, points to a high level of genetic diversity among *B. bovis* parasites. Genetic differences could arise from mutations during asexual reproduction or by genetic recombination and random assortment of alleles during sexual reproduction in the tick host. However, even though genetic recombination has been demonstrated in other apicomplexan parasites, none of these events has been elucidated for *B. bovis*. In this aspect, the molecular markers described in the present work represent a highly needed tool that will facilitate the study

of genetically mixed populations along with the potential role of genetic exchange in generating diversity.

Mutations in protein-coding regions that arise from asexual reproduction in the mammalian host can be selected by means of the immune system. This is the case of the variable erythrocyte surface antigens family of proteins of *B. bovis*, which generate surface exposed variants in the course of infection (O'Connor et al., 1997). On the contrary, molecular markers should not vary in response to immune pressure. In this work we demonstrated that Bv80/Bb-1 and TRAP sequences were stable during a long-term experimental infection and all markers were also conserved after three passages through naive bovines. In this last experiment, the results obtained for Antigen 3 were surprising. In spite of being the less polymorphic marker, two alleles were identified in the first two passages, meaning that initially there were at least two populations. During repeated blood passaging, the large "L" genotype gradually displaced the "S" genotype, so that by the third passage, "L" was the only sequence observed. This finding suggests the existence of a selective pressure in the bovine for one genotype that in this case favored the longer sequence. The hypothesis of whether the repeat region length would have immunological implications as reported by Schofield (1991) remains to be further evaluated. Finally, the fact that Antigen 3 had two genotypes while only one variant of TRAP, Bv80/Bb-1 and Desmoyokin was observed shows that, at least for these three markers, the subpopulations present in the Mexico isolate have the same sequence.

To our knowledge, the present work is the first to describe polymorphic minisatellites of *B. bovis*. In addition, some new genes of as yet unknown function were revealed. Further assessment of phylogenetic relationships among the *B. bovis* strains analyzed here is still needed to gain additional insights into the evolution of repeat units. The tools we have developed here will enable the investigation of the population genetic structure and the frequencies of multiple and clonal *B. bovis* infections in cattle. This set of markers will also allow determining if sexual recombination is a frequent event in field parasite populations.

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