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Babesia bovis biological clones and the inter-strain allelic diversity of the *Bv80* gene support subpopulation selection as a mechanism involved in the attenuation of two virulent isolates☆

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ABSTRACT

The virulence phenotype of *Babesia bovis* subpopulations was evaluated using biological clones derived from the high-virulence BboS2P and the low-virulence BboR1A strain and two original virulent isolates, BboL15 and BboL17, multiplied extensively *in vitro* or attenuated by successive passages in splenectomized calves. The virulence phenotype was assessed both by inoculation of normal Holstein adult steers and by analyses of polymorphic fragments of the single-copy *Bv80* gene as a subpopulation marker. BboS2P and its nine derived clones contained a single 750 bp fragment with identical nucleotide sequences and numbers of repeats. A single fragment of approximately 850 bp was observed in BboR1A and its derived clones (Ca3B1, Ca2B1). Ca3B1 and Ca2B1 were differentiated by a stable deletion of 15 contiguous nucleotides in the *Bv80* allele of Ca3B1. Both alleles were identified in the parental strain. Original isolates BboL15 and BboL17 contained two *Bv80* fragments of different sizes. Interestingly, the heavy and light fragments persisted in the *in vivo*-attenuated strains and the virulent *in vitro*-multiplied strains, respectively. Despite the inter-strain allelic diversity of the *Bv80* gene, the fragments had identical nucleotide sequences and numbers of repeats compared to their respective parental *Bv80* genes. The high-virulence and low-virulence phenotypes remained unchanged after they were multiplied *in vitro*. In conclusion, the polymorphic *B. bovis Bv80* gene, was a useful marker for differentiating subpopulations with different phenotypes. The brevity of the procedure to isolate one parasite from the original isolate or strain before *in vitro* cloning and the fact that the continuous *in vitro* multiplication did not modify the virulence phenotype of *B. bovis* clones strongly suggest that the *in vivo*-attenuated subpopulations existed in the original isolates before they were selected by passages in splenectomized calves.

☆ Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers JX088041; JX088042; JX088043; JX088044; JX088045; JX088046; JX088047; JX088048; JX088049 and JX088050.

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1. Introduction

Bovine babesiosis, caused by *Babesia bovis*, is a deadly disease of cattle in tropical and subtropical regions of the world, where *Rhipicephalus* (*Boophilus*) tick vector species are enzootic. The relevant economic losses attributed to babesiosis are largely the consequence of its high mortality rates in naive cattle populations. The disease is clinically characterized by anemia, hyperthermia, severe depression, respiratory failure and, frequently, death. Vaccination with live attenuated *Babesia* strains is the only method currently available to successfully prevent and control bovine babesiosis (Bock and De Vos, 2001). Virulent isolates of *B. bovis* are attenuated *via* successive syringe passages of parasitemic blood among 20–30 splenectomized calves every 4–5 days (Callow and Mellors, 1966; Callow et al., 1979).

The leading hypotheses for explaining the mechanisms involved in the virulence attenuation of *B. bovis* isolates in splenectomized calves include: first, abrogation of the expression of virulence determinants in attenuated parasites; second, the enrichment of a subpopulation of low-virulence parasites by *in vivo* selection during the process of attenuation; and third, the combination of these two mechanisms. So far, none of these hypotheses has been definitively accepted (Timms et al., 1990; Bock et al., 1995).

Apicomplexa hemoparasites exist as subpopulations that each express distinct virulence phenotypes, which can result from gene mutations or the differential regulation of gene expression (Carter, 1978; Carson et al., 1990; Lew et al., 1997b). These subpopulations can be isolated by *in vivo* (Gill et al., 1987; Timms et al., 1990) or *in vitro* biological cloning (Rosario, 1981; Trager et al., 1981; Rodríguez et al., 1983; Nevils et al., 2000). Changes in the population structure of *B. bovis* at the whole-genome level were recently demonstrated for the first time. No specific genes responsible for *B. bovis* virulence have been identified (Lau et al., 2011), and studies on the presence or absence of such genes or their up- or down-regulation in low-virulence strains have not been possible. Nevertheless, some other genes could be useful to mark and differentiate those parasite subpopulations with stable virulence phenotype.

Bv80, also known as *Bb-1*, is a single-copy gene encoding the *B. bovis* spherical body protein-1, with conserved 5′- and 3′-terminal regions separated by arrays of variable-length tandem repeat sequences, which are responsible for the polymorphism of the corresponding genes (Dalrymple et al., 1992; Lew et al., 1997a; Brayton et al., 2007). PCR amplification of the region that includes the repetitive sequences of *Bv80* has been used to differentiate subpopulations of *B. bovis* and to genotype isolates (Lew et al., 1997b; Bock et al., 2000; Wilkowsky et al., 2009). Recently Mazuz et al. (2011) identified intra- and inter-strain diversity in the *Bv80* gene in both the virulent field isolates and the attenuated strains.

Assuming that each subpopulation that constitutes a natural isolate expresses different virulence phenotypes and using the *Bv80* alleles as a marker, we obtained experimental evidence supporting the hypothesis that the attenuation procedure is based on the selection of less virulent subpopulations of *B. bovis* present in the original isolates. To accomplish this, we: (i) biologically cloned high-virulence and low-virulence *B. bovis* parasites *in vitro*, (ii) attenuated two *B. bovis* isolates by rapid passages in splenectomized calves, and (iii) compared the sizes and sequences of the *Bv80* genes amplified and cloned from *B. bovis* strains and clonal lines used in this study.

2. Materials and methods

2.1. B. bovis strains and isolates

The *B. bovis* pathogenic strain BboS2P, which has been adapted to and maintained in continuous *in vitro* culture (Echaide et al., 1993), was originally isolated from a bovine with severe clinical babesiosis in Salta province, Argentina. The low-virulence *B. bovis* BboR1A strain, derived from a virulent isolate of an acutely infected steer in Santa Fe province, was attenuated by a series of 22 passages of parasitized blood in splenectomized calves (Anziani et al., 1993). BboR1A is a vaccinal strain and is non-transmissible by *R. microplus* (Mangold et al., 1993). BboS2P and BboR1A, which underwent seven extra *in vivo* passages (29) and were adapted to continuous *in vitro* growth for 6 and 2 months, respectively, were elected to generate biological clones. Two *B. bovis* isolates, BboL15 and BboL17, were obtained from bovines with acute babesiosis from two distant endemic regions of Argentina (North Central Córdoba and Salta, respectively), and each was passed between two calves by the bite of uninfected *R. microplus* ticks. Parasitized blood samples referred to as original isolates were frozen in liquid nitrogen until their use for *in vivo* attenuation. Both the original isolates and the derived *in vivo*-attenuated strains were adapted to grow continuously *in vitro*. The *B. bigemina* reference strain BbiS1A was used as the control for *Babesia* gene sequences.

2.2. ***In vitro*** *cultures*

B. bovis strains were multiplied *in vitro* using a microaerophilous stationary-phase culture method (Levy and Ristic, 1980). Briefly, the basic medium (BM) consisted of M199 (Gibco®) supplemented with 0.1 g/l penicillin, 0.16 g/l streptomycin, 2.2 g/l $NaHCO_3$, and 4.25 g/l HEPES and enriched with 40% normal bovine serum (v/v). The complete medium (CM) included 5–10% (v/v) normal bovine erythrocytes. Cultures were incubated at 37 °C under 5% CO_2 in air or under 5% O_2, 5% CO_2 and 90% N_2. BM was replaced every 24 h, and a variable proportion of parasitized CM was replaced by normal CM after 48, 72 or 96 h (subculture).

2.3. Quantification of parasitized erythrocytes

The concentration of parasitized erythrocytes was determined using a Neubauer hematocytometer and fluorochrome acridine orange to stain the DNA. The working solution of fluorophore was prepared daily before use by adding 0.05 ml of 1% stock solution to 5 ml of 0.2 M acetate buffer, pH 4 (Mc Carthy and Senne, 1980). A suspension of parasitized erythrocytes was diluted 1:50 with BM and then mixed with 10 μl of acridine orange working solution. After 10 min incubation in darkness, parasitized

erythrocytes in both grids of the Neubauer chamber were counted using white light followed by UV light at 1000× magnification. The white light permitted visualization of the erythrocytes, and the UV light allowed for the identification of the parasitized erythrocytes. The concentration (iRBC/ml) was calculated by the formula: cells/ml = cell No. $\times 10^4 \times$ dil^{-1} (Schalm et al., 1981).

2.4. *B. bovis* biological cloning

The biological clones were obtained from BboS2P and BboR1A *B. bovis* reference strains, following the technique described by Rodríguez et al. (1983). The presence of parasites was evaluated every 4 days during subculturing, and cultures were continuously maintained for 36 days. Cultures were incubated in 5% O_2, 5% CO_2, 90% N_2. The cloning procedure was repeated twice to obtain tertiary clones, which were stored in liquid nitrogen until use (Palmer et al., 1982).

2.5. Characterization of B. bovis clones

2.5.1. Clone virulence levels

To validate the virulence status of the *B. bovis* biological clones, 12-month-old Holstein steers, free of hemoparasite infections, were subcutaneously inoculated with 10^7 parasitized erythrocytes.

Two groups of three bovines each were inoculated with two respective *B. bovis* clones derived from BboS2P, and two groups of three and four steers were inoculated with two different respective clones derived from BoR1A. The parental strains were inoculated in two and three bovines, respectively, as a control of virulence status.

The clinical reaction was monitored by daily records of body temperature, hematocrit, parasitemia in peripheral blood and sickness behavior (Echaide et al., 1993). Infected steers were treated with 3 mg kg^{-1} of diminazene (Novartis®) if they underwent 3 days with ≥41 °C body temperature, ≤15% hematocrit, or >0.5% parasitemia or exhibited nervous system imbalance together with listlessness, breathlessness and CNS signs (circling, head pressing, and convulsions).

The mean increases in body temperature over 39.5 °C, the minimal hematocrit values and the parasitemia scores (Callow and Pepper, 1974) were compared among the groups by ANOVA.

2.5.2. Stability of the attenuated phenotype

The stability of the attenuated behavior of BboR1A and a derived clone were evaluated by successive inoculation of two groups of ten 17- to 18-month-old normal Holstein steers. Five steers from the first group were subcutaneously inoculated with 10^7 BboR1A, and five were inoculated with 10^7 derived clone-parasitized erythrocytes. Fifteen days after inoculation, 180 ml heparinized blood from each bovine of the first group was subcutaneously inoculated in a corresponding steer of the second group, following the same distribution of five steers per strain. Clinical parameters were obtained and analyzed as described previously (Section 2.5.1).

2.5.3. In vitro growth efficiency of clones

To evaluate the *in vitro* growth efficiency of the biological clones, the capacity to achieve the highest parasitemia without the extinction of the culture by overgrowth was assessed. Two high-virulence and two low-virulence *B. bovis* clones were individually and simultaneously cultivated *in vitro* using the MASP technique. Each clone was suspended 1:9 in M199 + serum and then mixed with a second suspension of normal erythrocytes to achieve a parasitemia of 0.5%. The suspensions were distributed in 24-well plastic culture plates and were incubated at 37 °C under 5% CO_2 in air. Parental strains were used as controls. The percentage of parasitized erythrocytes of each clone was estimated daily, and the dilution factor required to restart the cultures with a parasitemia of 0.5% was estimated every 72 h for 14 days. Three replicates were simultaneously evaluated per clone, and the mean total cumulative dilutions utilized for the replicates were compared among clones.

2.5.4. Competitive in vitro growth of virulent and attenuated B. bovis clones

A 0.5% of erythrocytes parasitized by either a high-virulence *B. bovis* clone or a low-virulence clone were mixed (50:50), suspended in culture medium as described in Section 2.5.3, and distributed in 25 cm^2 plastic culture bottles. The culture and two replicates were incubated continuously at 37 °C under 5% CO_2 in air. DNA from the parasites was extracted from the parasitized blood every 48 h and the *Bv80* gene was amplified by PCR.

2.6. In vivo virulence attenuation of B. bovis BboL15 and BboL17 isolates

Virulent *B. bovis* BboL15 and BboL17 isolates were attenuated by serial passages (P) of parasitized blood in 3- to 6-month-old splenectomized Holstein calves free of hemoparasites, according to the procedure of Callow et al. (1979). Calves were successively infected by jugular inoculation of 10^9 infected erythrocytes every 4–5 days. Calf infection was monitored by daily records of body temperature and the percentage of parasitized erythrocytes in thin smears of jugular blood stained with Giemsa (Bosë et al., 1995). The success of generating low-virulence strains from BboL15 and BboL17 isolates was evaluated in susceptible steers using passages number 26 and 24 respectively. Groups of 16-month-old Holstein steers were inoculated with BboL15 ($n = 5$) or its putatively attenuated derived strain ($n = 5$) and with BboL17 ($n = 6$) or its derived attenuated strain ($n = 6$). The parameters evaluated to compare the clinical reactions and to specifically treat those steers critically affected were described above (Section 2.5.1).

2.7. Bv80 gene analyses

2.7.1. DNA extraction

B. bovis genomic DNA (gDNA) from infected bovine blood and parasitized erythrocytes from *in vitro* cultures were extracted using a standard phenol/chloroform/isoamyl alcohol method. Briefly, erythrocyte suspensions were lysed with erythrocyte

lysis buffer (0.14 M NH_4Cl, 0.17 M Tris–HCl) at room temperature for 30 min and then pelleted. The hemoglobin was washed off using distilled water by centrifugation at 14,000 × *g* for 15 min, and pellets were lysed at 58 °C for 1 h in lysis buffer (0.05 M Tris–HCl pH 8.0, 0.1 M EDTA, 0.1 M NaCl, 2% SDS) with 160 μg of proteinase K (Invitrogen®). gDNA was extracted with 1 vol of phenol/chloroform/isoamyl alcohol (Invitrogen®), precipitated with ice-cold isopropyl alcohol and washed once with 75% ice-cold ethanol. Pellets were suspended in 50 μl distilled water and were kept at −20 °C until use.

2.7.2. PCR

The variable repeat region in the *Bv80* gene of *B. bovis* was amplified by PCR. The PCR mix included 0.2 mM dNTPs, 2.2 mM $MgCl_2$, 1.25 U recombinant *Taq* DNA polymerase (Invitrogen®), 0.8 mM of each primer, and 3 μl (0.05–0.2 μg) of gDNA template. The primers 1Bf (5′TGTGTTAATGTAACTCAGCCCG3′) and 2Br (5′AAAGCCTGTTAGTTGA TGGACC3′) were previously described by Lew et al. (1997a). PCR amplification was performed at 94 °C for 2 min; 40 cycles of 30 s at 94 °C, 30 s at 58 °C, and 60 s at 72 °C; and a final extension period of 7 min at 72 °C. Amplification products were analyzed by electrophoresis in 1.5% UltraPure agarose (Invitrogen®) gels in TAE buffer (40 mM Tris-acetate, 2 mM Na_2EDTA). The amplified products were visualized under UV light after ethidium bromide staining. gDNA from clones, their parental strains and isolates BboL15 and BboL17 multiplied *in vivo* and *in vitro* were used as templates. *B. bigemina* gDNA (BbiS1A strain) and test reagents without DNA template were included as negative controls.

2.7.3. Sequence analysis

The *B. bovis Bv80* gene was amplified by PCR from: (i) strains BboS2P and BboR1A and two derived biological clones from each strain, which had been multiplied *in vitro* first and inoculated *in vivo* later; (ii) original isolates BboL15 and BboL17 multiplied *in vivo* or *in vitro*; and (iii) different passages obtained during the attenuation procedure of BboL15 and BboL17. These PCR products were purified using a QIAquick PCR Purification Kit (Qiagen®). The PCR amplifications were repeated at least 4 times for each strain/clone and at least two PCR products sequenced (Macrogen, USA; Korea) and analyzed.

PCR products from the biological clones and their parental strains, the original BboL15 isolate multiplied *in vitro* and the passages P2/P22 (BboL15) and P2/P20 (BboL17), were cloned into the vector pGEM-T Easy (Promega) following the manufacturer's instructions. *Bv80* fragments amplified by PCR and cloning were sequenced by Macrogen (USA; Korea). Four molecular clones from each biological clone and strain mentioned in the previous paragraph were sequenced using T7 Promoter-F and SP6-R primers. This entire procedure was repeated at least once starting from the DNA extraction. Sequences were analyzed using BioEdit and MEGA software (Tamura et al., 2007). The *Bv80* sequences were compared with those deposited in GenBank (M93125; M93126; M99575) using BLAST. The DNA extracted from the blood samples from the six bovines inoculated with the biological clones and their parental strains were amplified and sequenced. The *Bv80* repetitive sequences from BboL17 P3 and P20 grown *in vitro* were amplified, and the fragments were compared in agarose gels.

3. Results

3.1. *B. bovis* clones

Nine clones were derived from the pathogenic strain BboS2P (Cp5D1, Cp5D2, Cp5D3, Cp5D4, Cp5D5, Cp7A1, Cp7A2, Cp7A3 and Cp7A4), whereas only two clones were obtained from the attenuated strain BboR1A (Ca2B1 and Ca3B1). The cloning procedure was successful using an incubating atmosphere of 5% O_2, 5% CO_2 and 90% N_2, while cultures did not progress when incubated in 5% CO_2 in air.

3.2. In vivo and in vitro characterization of B. bovis clones

The virulence of *B. bovis* clones was evaluated in highly susceptible cattle, as determined by their breed and age. All biological clones tested exhibited virulent phenotypes that were similar to their respective parental strains, without significant differences, except clone Ca3B1. The steers inoculated with clone Cp5D3 or Cp7A4 registered similar average maximum hematocrit depressions (52.0% ± 4.4; 48.7% ± 1.2), cumulative temperatures (4.5 °C ± 0.7; 4.6 °C ± 0.6), and parasitemia scores (10.0 ± 00; 10.0 ± 00). These values were significantly higher ($P < 0.05$) than those measured in the groups inoculated with clones Ca2B1 and Ca3B1 (32.7% ± 4.0 and 20.5% ± 6.0; 0.6 °C ± 0.6 and 0.7 °C ± 0.4; and 1.0 ± 0.0 and 1.0 ± 0.0, respectively). Clinical parameters evaluated in bovines infected with high-virulence and low-virulence parental strains were also significantly different (not shown). However, the hematocrit depression caused by clone Ca3B1 (20.5% ± 6.0) was lower than the Ca2B1 clone and its parental strain ($P < 0.05$) (Table 1).

All bovines exhibited patent parasitemia in thin and thick blood smears. Only bovines inoculated with high-virulence clones or their parental strains had to be treated with diminazene to avoid death.

3.3. In vitro growth efficiency of B. bovis clones

The assessment of the clones' growth efficiencies *in vitro* revealed that high-virulence clones Cp5D3 and Cp7A4 reached a higher mean parasitemia than the low-virulence clones Ca2B1 and Ca3B1, though these differences were not significant. The cumulative dilutions required for the high-virulence clones every 72 h were significantly higher ($P < 0.05$) than the cumulative dilutions needed for low-virulence clones (Fig. 1). Together, these data strongly suggest that the high-virulence clones are more efficient for *in vitro* growth than the low-virulence ones.

3.4. Stability of BboR1A and Ca3B1 low-virulence phenotypes

Most clinical parameters evaluated between normal steers receiving the first *vs.* the second passage of BboR1A

Table 1
Clinical parameters from steers inoculated with *Babesia bovis* strain BboR1A and the derived clone Ca3B1 after two successive passages through normal steers.

Strain/clone	Passage 1		Passage 2	
	BboR1A	Ca3B1	BboR1A	Ca3B1
Steers per group	5	5	5	5
Age (months)	17	17	18	18
Mean pre-patent period (days)	6.6 ± 0.5^{a}	7.0 ± 0.7^{a}	5.0 ± 0.5^{b}	6.0 ± 0.8^{ab}
Days with temp. ≥41 °C*	0.8 ± 0.8^{a}	0.0 ± 0.0^{a}	1.0 ± 1.6^{a}	1.0 ± 1.2^{a}
Days with temp. ≥40 °C*	4.0 ± 0.7^{a}	1.0 ± 0.7^{b}	5.0 ± 2.4^{a}	3.0 ± 2.6^{ab}
Days with temp. ≥39.5 °C*	5.0 ± 1.0^{a}	2.0 ± 1.0^{b}	7.0 ± 1.5^{a}	5.0 ± 1.8^{a}
Cumulative temp. >39.5 °C (°C)*	3.8 ± 1.1^{a}	0.8 ± 0.5^{b}	5.6 ± 3.0^{a}	3.7 ± 2.0^{a}
Maximum PCV depression (%)*	32.4 ± 11.0^{a}	17.6 ± 2.0^{c}	44.8 ± 8.3^{b}	43.7 ± 8.2^{b}
Maximum parasitemia (score)*	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}
Steers requiring treatment	0/5	0/5	0/5	0/5

PCV: packed cell volume. Values with different superscripts in each row are significantly different ($P < 0.05$).
* Values are the mean obtained for each group.

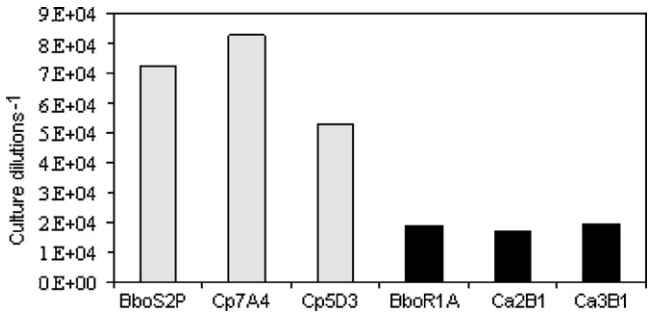

Fig. 1. Mean cumulative dilutions used during the subcultures of three replicates from high-virulence *Babesia bovis* clones (Cp5D3, Cp7A4), low-virulence clones (Ca2B1, Ca3B1), and their respective parental strains (BboS2P, BboR1A), every 72 h (subcultures). The average dilution required by the high-virulence parasites is represented by gray bars and the low-virulence by black bars.

or receiving the first *vs.* the second passage of the derived clone Ca3B1 had no significant differences (Table 1). The exception was hematocrit depression, which was greater in steers receiving a second passage of BboR1A and Ca3B1 compared to the first passage ($P < 0.05$). The clinical reaction caused by the first inoculation of clone Ca3B1 was milder than the reaction caused by the parental strain BboR1A (Table 1). No bovine required specific treatment to control the clinical reaction after the first or second passage. These data demonstrate that both the parental and the clonal strain maintain a stable phenotype after one *in vivo* passage.

3.5. *In vitro* prevalence of *B. bovis* Cp7A4 over the Ca3B1 clone

The PCR amplification of the *Bv80* gene from the original mixture of *B. bovis* clones Cp7A4 and Ca3B1 revealed 670 bp and 870 bp double bands. After 68 days (28 subcultures) of *in vitro* multiplication and during an extra period of 15 days, only a fragment of 670 bp was amplified (not shown). This result was reproduced in the replicates that were evaluated simultaneously.

3.6. Evaluation of virulence attenuation of *B. bovis* BboL15 and BboL17 isolates

The lower levels of virulence of the strains derived from *B. bovis* isolates BboL15 and BboL17 were confirmed after 26 and 24 passages in splenectomized calves, respectively. The comparison between the original isolates and the derived passages showed that, in all cases, all clinical parameters evaluated were milder in the steers inoculated with the attenuated strains ($P < 0.05$) than with the original parental isolates. All inoculated steers exhibited patent parasitemia, at least in the thick blood smears. Only steers inoculated with the high virulence strains had to be treated to avoid death (Table 2).

3.7. *Bv80* gene analyses

3.7.1. PCR

The *B. bovis Bv80* gene fragment was PCR-amplified from all biological clones and their parental BboS2P and BboR1A strains (Fig. 2). No amplification product was obtained from *B. bigemina* BbiS1A gDNA or DNA-free samples (controls).

The *Bv80* fragments amplified by PCR indicated that the parental strain BboS2P and its 9 derived clones contained a unique fragment of approximately 670 bp and identical sequences. BboR1A and its derived clones (Ca2B1 and Ca3B1) contained similar fragments of approximately 870 bp (Fig. 2). The sequences of the fragments from the low-virulence clones were not identical between them and both alleles were contained in the parental strain BboR1A (Section 3.7.2). In addition, similar PCR analysis demonstrated that the *Bv80* gene pattern remained identical after

Table 2
Clinical parameters from steers inoculated with the high-virulence BboL15 or BboL17 *Babesia bovis* isolate, compared with the derived low-virulence strains, attenuated by consecutive passages through splenectomized calves.

Isolate/strain	High-virulence isolates		Low-virulence strains**	
	BboL15	BboL17	BboL15	BboL17
Steers per group	5	6	5	6
Age (months)	14	16	14	16
Mean pre-patent period (days)	8.0 ± 1.0^{a}	8.5 ± 1.9^{a}	8.2 ± 1.3^{a}	8.0 ± 0.9^{a}
Days with temp. ≥41 °C*	2.6 ± 0.5^{b}	3.2 ± 0.8^{b}	0.4 ± 0.5^{a}	0.2 ± 0.4^{a}
Days with temp. ≥40 °C*	4.4 ± 1.3^{b}	5.8 ± 1.5^{c}	2.8 ± 0.8^{a}	2.0 ± 1.3^{a}
Days with temp. ≥39.5 °C*	5.2 ± 1.1^{ac}	6.3 ± 1.5^{a}	5.8 ± 1.1^{a}	3.7 ± 1.9^{bc}
Cumulative temp. >39.5 °C (°C)*	6.7 ± 1.7^{b}	8.7 ± 2.0^{b}	3.1 ± 1.9^{a}	2.0 ± 1.4^{a}
Maximum PCV depression (%)*	60.0 ± 3.0^{c}	48.0 ± 9.3^{b}	32.0 ± 6.0^{a}	25.7 ± 5.2^{a}
Maximum parasitemia (score)*	7.2 ± 4.1^{b}	6.3 ± 4.2^{b}	1.0 ± 0.0^{a}	1.0 ± 0.0^{a}
Steers requiring treatment	5/5	6/6	0/5	0/6

PCV: packed cell volume. Temp.: Temperature. Values with different superscripts in each row are significantly different ($P < 0.05$).
* Values are the mean obtained for each group.
** Twenty four (BboL17) and twenty six (BboL15) passages in splenectomized calves.

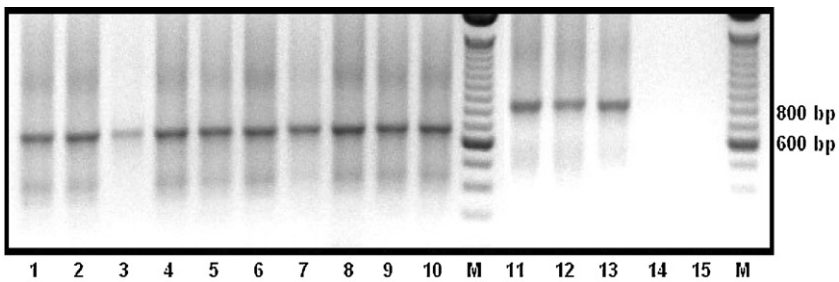

Fig. 2. *Bv80* gene fragments from *Babesia bovis* strains and clones, amplified by PCR, and separated by electrophoresis in 1.5% agarose gel, stained with ethidium bromide. Lanes 1–10: high-virulence parental strain and clones. BboS2P (1); Cp7A1 (2); Cp7A2 (3); Cp7A3 (4); Cp7A4 (5); Cp5D1(6); Cp5D2 (7); Cp5D3 (8); Cp5D4 (9); Cp5D5 (10). Lanes 11–13: low-virulence parental strain and clones: BboR1A (11); Ca2B1 (12); Ca3B1 (13). Lane 14: template-free sample. Lane 15: BbiS1A. M: DNA size marker (100 bp).

the inoculation of normal bovines with clones and their parental strains (data not shown).

Identical PCR amplifications of the *Bv80* genes performed on the original BboL15 isolate produced two fragments of 775 bp and 880 bp. Interestingly, both of these *Bv80* PCR fragments were observed during the attenuation process until P17, but only the 880 bp fragment was consistently amplified afterwards. A single fragment of 775 bp was also amplified after 6 months of *in vitro* multiplication of the virulent BboL15 isolate (Fig. 3A).

Similarly, *Bv80* PCR amplifications of the original isolate BboL17 also produced two fragments of 676 bp and 772 bp until P18, but only the fragment of 772 bp was consistently amplified afterwards and in the resulting final attenuated strain. The original BboL17 isolate, grown *in vitro* for 6 months, produced only a single fragment of 676 bp (Fig. 3.B). The scenario suggested by these experiments is that the smaller 775 and 676 bp PCR fragments are present in the high-virulence subpopulation of the BboL15 and BboL17 isolates, respectively, whereas the larger 880 and 772 bp correspond to their low-virulence versions. Consistent with the observations described in Section 3.2, it is possible to conclude that exclusive amplification of the 775 and 676 bp fragments in long-term cultures of the BboL15 and BboL17 strains, respectively, is due to the selection of the high-virulence strains resulting from their superior fitness for *in vitro* growth.

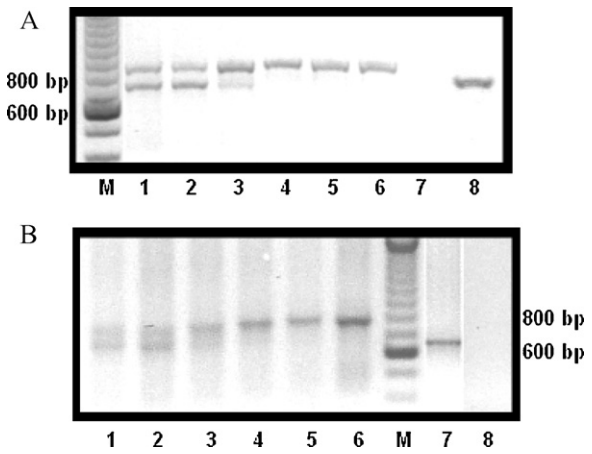

Fig. 3. *Bv80* gene fragments from *Babesia bovis* original isolates (BboL15 and BboL17) and derived strains, amplified by PCR. (A) Blood passages (P) of the BboL15 isolate. Lane 1–6. BboL15-P2 (1); BboL15-P5 (2); BboL15-P10 (3); BboL15-P18 (4); BboL15-P22 (5); BboL15-P26 (6). Lane 7: template-free sample. Lane 8: BboL15 (*in vitro*). (B) Blood passages (P) of the BboL17 isolate. Lane 1-20: BboL17-P3 (1); BboL17-P10 (2); BboL17-P15 (3); BboL17-P18 (4); BboL17-P19 (5); BboL17-P20 (6). Lane 7: BboL17 (*in vitro*). Lane 8: template-free sample. M: DNA size marker (100 bp).

3.7.2. Sequence analysis

BLAST analysis between sequences of *Bv80* gene obtained from strains and isolates used for this work against those deposited in GenBank revealed wide range of genetic polymorphism from 1% to 42%. Regions of the *Bv80* genes encoding the repeat modules corresponding to 4–7 amino acids were variable both in sequence and in number of repeats between high-virulence and low-virulence strains (Fig. 4). All virulent clones had *Bv80* sequences that were identical to the BboS2P parental strain.

Remarkably, the Ca2B1 clone also had a *Bv80* sequence that was identical to its BboR1A parental strain but different from clone Ca3B1, which had a deletion of 15 contiguous nucleotides, coding for one repeat module of 5 amino acids. Cloned *Bv80* fragments amplified from BboR1A confirmed the presence of these two alleles, corresponding to Ca3B1 and Ca2B1, respectively. Sequence analysis of the PCR products obtained from the same strain multiplied *in vivo* revealed absolute sequence conservation of these two distinct alleles amplified from either the clonal lines or the parental strain (not shown). The degree of nucleotide polymorphism between the sequences from high-virulence and low-virulence clones was 18%.

The sequence of the 880 bp fragment identified in the BboL15 original isolate was identical to the sequence of the fragment from its derived low-virulence strain. The sequence of the 775 bp fragment was identical to the sequence found in the original isolate after *in vitro* multiplication. Similarly, the sequence of the 772 bp fragment identified in the BboL17 original isolate was identical to the sequence of the fragment from its derived low-virulence strain. The sequence of the 676 bp fragment was identical to the sequence found in the original isolate after *in vitro* multiplication.

4. Discussion

It has been hypothesized that the mechanism by which attenuation of *B. bovis* occurs involves down-regulation of a virulence gene(s), selective enrichment of a less virulent parasite subpopulation (Carson et al., 1990; Timms et al., 1990), or a combination of these two mechanisms.

Four main conclusions emerge from this study. (1) Consistent with previous observations, the *Bv80* repertoire of the parental strains is diverse, and all of the PCR-amplified *Bv80* sequences in the biological clones are also present in the parental strains. (2) PCR amplification of the variable region of the *Bv80* gene demonstrated that, in contrast with their parental strains, all of the biological clones likely encode a single *Bv80* allele, which also confirms the clonal nature of these parasites. (3) The attenuated phenotype remains essentially stable upon two sequential inoculations in susceptible intact bovines. (4) The BboL15 and BboL17 strains include high-virulence subpopulations containing *Bv80* allelic variants that are distinguishable from their low-virulence subpopulations. Taken together, these results strongly suggest that the process of attenuation of *B. bovis* parasites involves *in vivo* selection of a low-virulence parasite subpopulation.

Changes in the population structure at the whole-genome level were recently demonstrated by the comparison of three virulent *B. bovis* strains (BboL17 among them) and their respective attenuated derivatives (Lau et al., 2011). These authors reported a reduced diversity of the population gene pool in the derived attenuated strains, which might be associated with the loss of parasite populations. Here, we have confirmed not only the reduction of the number of *B. bovis* subpopulations of the parental isolates but also the absolute conservation of the *Bv80* polymorphic sequence in the low-virulence strains selected by *in vivo* passage.

The extensive biological cloning performed in this work consistently demonstrated for the first time that the vaccinal strain BboR1A, currently extensively used for vaccination in Argentina, includes at least two low-virulence clones (Ca2B1 and Ca3B1). A stable deletion of 15 contiguous nucleotides in the *Bv80* gene found only in the Ca3B1 clone distinguishes it from the Ca2B1 clone. The deleted sequence would encode one repeat module of 5 amino acids. Although a single fragment of approximately 870 bp was visualized in the agarose gels, both alleles were identified in the parental strain. These fragments could not be attributed to the existence of paralogous copies because *Bv80* is a single-copy gene in the *B. bovis* genome (Hines et al., 1995; Wilkowsky et al., 2009; Brayton et al., 2007). Dalrymple et al. (1992) and Lew et al. (1997a) noted that the number of *Bv80* alleles found in each isolate indicates the number of genetically distinct subpopulations. Because the parental isolate of BboR1A obtained from a bovine with acute babesiosis was no longer available, we assumed that the high-virulence subpopulations were lost during the *in vivo* attenuation procedure, as only the low-virulence phenotype was found after cloning.

The identical *Bv80* nucleotide sequences and numbers of repeats in BboS2P and its biological clones allowed us to establish that BboS2P constitutes only one subpopulation. The selection could have been the consequence of 92 *in vitro* subcultures (6 month) with a frequency of 48-72 h. A straightforward experiment allowed us to demonstrate that the high-virulence biological clone (Cp7A4) displayed higher growth efficiency *in vitro* and prevailed over the low-virulence clone (Ca3B1), which was lost when they were cultivated together. A similar association between *in vitro* growth efficiency and virulence phenotype has been observed for *B. bovis* by Nevils et al. (2000) and for *Theileria annulata* by Taylor et al. (2002), profiles that were attributed to the major protease components of the rhoptries of virulent *P. falciparum* (Lyer et al., 2007).

We also confirmed that the *Bv80* sequences and the phenotypes of strains BboS2P and BboR1A and their derived clones were stable during long periods of asexual multiplication *in vitro*. An equivalent observation of *Bv80* stability has been described for a virulent Mexican *B. bovis* strain after three passages by normal bovines (Wilkowsky et al., 2009). The phenotypes of strain BboR1A and clone Ca3B1 were essentially stable after the first sub-inoculation in normal steers. Although the mean hematocrit depression was significantly increased compared to that of the first inoculation, the steers did not require specific treatment. Timms et al. (1990) observed that isolated low-virulence clone K-19-47 reverted to a fully virulent phenotype after the first sub-inoculation in normal steers. This phenotype

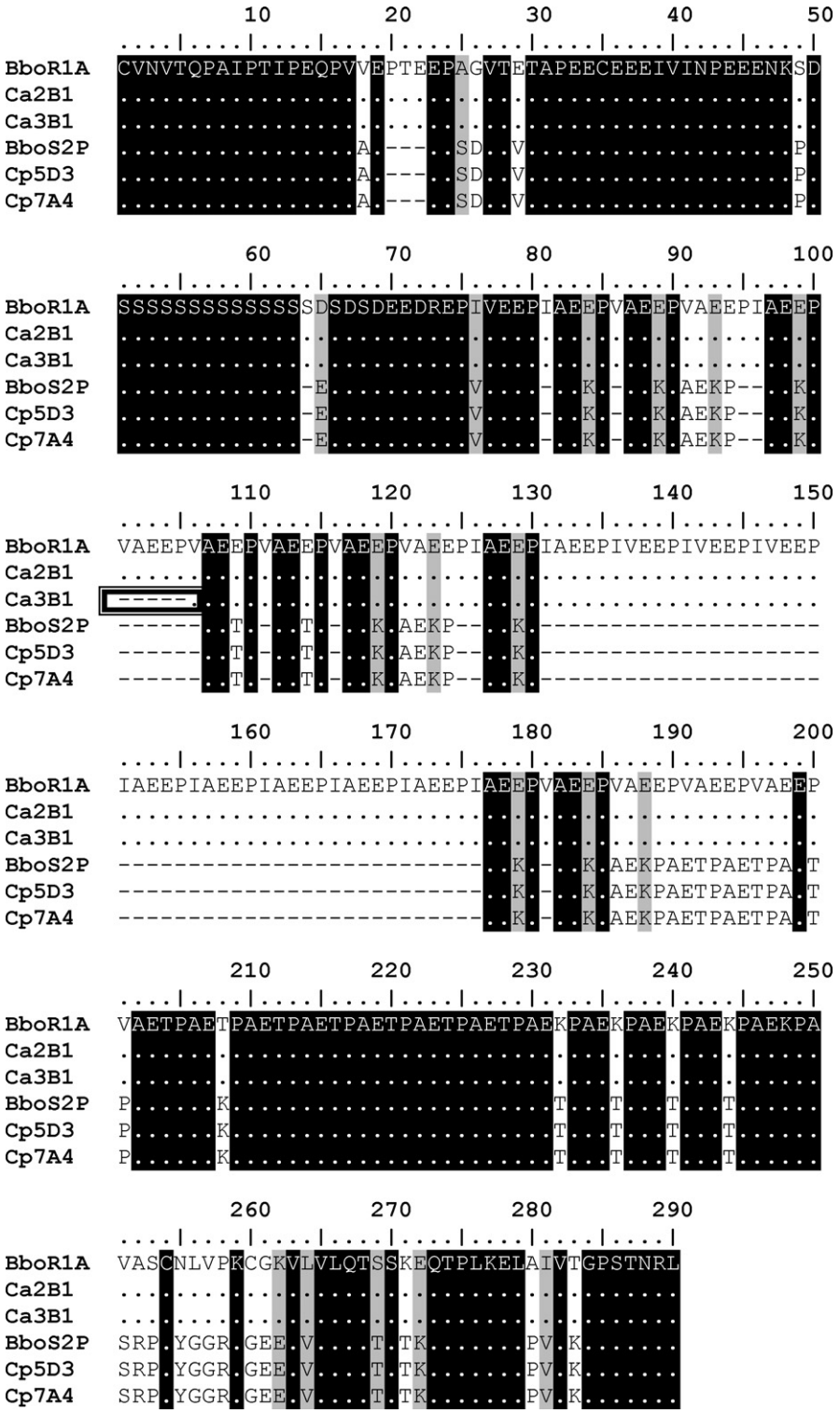

Fig. 4. Comparison of amino acid sequences of *Bv80* from strain BboR1A, clones Ca2B1 and Ca3B1, strain BboS2P, and clones Cp5D3 and Cp7A4. Amino acid numbers are shown on top. Areas of amino acid identity among all strains, conservative amino acid substitutions or variant amino acids have a black, gray or white background, respectively. Deletions are indicated by dashed lines and conservation by dots. Deletion of 5 contiguous amino acids in clone Ca3B1 is shown inside the double-line frame.

variation might correspond to the up-regulation of a virulence gene(s) induced by the immune systems. The genetic structure of a pathogen population would be shaped by the balance between mechanisms that generate diversity, such as recombination and mutation, and the host immune response (Gupta and Anderson, 1999).

A similar selection situation in high-virulence subpopulations was demonstrated *in vitro* for isolates BboL15 and BboL17. Meanwhile, the selection of low-virulence subpopulations occurred during the *in vivo* attenuation process. In both events, the selection was associated with the reduction of allelic *Bv80* diversity. Although both original isolates displayed two fragments of different sizes, only the lighter 775 bp (BboL15) and 676 bp (BboL17) fragments remained conserved after 6 months of continuous *in vitro* multiplication, and only the heavier 880 bp (BboL15) and 772 bp (BboL17) fragments remained conserved after the attenuation process *in vivo*. In contrast to our observations, Lew et al. (1997b) observed that the attenuated Australian Dixie strain (P25) persisted as only a subpopulation with a single light *Bv80* fragment of 700 bp and lost the parasites containing the heavy fragment of 820 bp. These observations suggest that the sizes of *Bv80* fragments are not necessarily associated with virulence status, although fragment sizes are still relevant as subpopulation markers.

We confirmed a wide inter-strain *Bv80* polymorphism, independently of whether the virulence phenotype or geographic origin. Conversely, we observed high conservation of this sequence between each parental strain or isolates and their derived clones or low virulence strains respectively. In contrast to our results, the recently characterized *B. bovis* Israeli strains exhibited extensive inter- and intra-strain *Bv80* polymorphisms, irrespective of whether they were attenuated or virulent field isolates, which hampered their discrimination. Moreover, during the attenuation process of the Gon strain, the *Bv80* gene sequence that was conserved during the first six passages was not detected in the attenuated strains (P17); instead, two new subpopulations were identified (Mazuz et al., 2011). The higher complexity of the population might favor a shift from minor to dominant *B. bovis* subpopulations during the *in vivo* attenuation process (Carson et al., 1990).

The absolute conservation of the *Bv80* sequence between the low virulence strains and their respective parental isolates (BboL15 or BboL17), confirmed that attenuation was the consequence of the selection of pre-existing subpopulations, characterized by *Bv80* allelic variants not shared with other attenuated strains. The biological cloning of the BboL15 and BboL17 original strains would be relevant for analyzing the subpopulation dynamics of *B. bovis* at the gene level during passage in normal bovines. Lau et al. (2011) were unable to identify common genes associated with the virulence phenotype. Although the single-nucleotide polymorphisms in 14 genes distinguished all attenuated parasites from the virulent parental strain, all non-synonymous changes resulted in no deleterious amino acid modifications. The virulence or attenuation mechanisms may not be shared among all populations of parasites at the gene level, but instead may reflect expansion or contraction of the population structure in response to shifting milieus (Lau et al., 2011).

Our results are consistent with the mechanism of selection based on the modulating effect of the spleen on the virulence of *B. bovis* (Schetters and Eling, 1999). They postulated that the knobs-like structures observed on the surface of *B. bovis*-parasitized erythrocytes (known as ridges) appear to be related to the endothelial sequestration of high-virulence strains (knob^{+}) in normal bovines. Repeated passages of a virulent knob^{+} strain of *B. bovis* through splenectomized calves reduced the virulence of the original isolate, event associated with knob^{low+} parasites. When the knob^{low+} isolate was passaged through intact calves, the knob^{+} parasites were re-isolated (Aikawa et al., 1985).

The coexistence of subpopulations with different virulence phenotype is supported by two previous *in vitro* cloning procedures. Buening et al. (1986) obtained a low-virulence clone from a virulent isolate of *B. bovis* using the limiting dilution and Nevils et al. (2000) isolated high- and low-virulence clones from a Mexican isolate using fluorescence-activated cell sorting. The brevity of the procedure for isolating one parasite from the original isolate (by dilution or FACS) before *in vitro* cloning and the fact that the *in vitro* multiplication did not modify the virulence phenotypes of *B. bovis* clones strongly suggest that the *in vivo*-attenuated subpopulations existed in the original isolates before they were selected by passages in splenectomized calves.

Although *Bv80* was not considered a virulence marker here, the simultaneous detection of heavy and light fragments from the Argentinean *B. bovis* strains utilized in this study confirm the coexistence of subpopulations with different virulence statuses. Moreover, the *Bv80* gene has potential epidemiological value for identifying cattle vaccinated with the BboR1A strain.

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