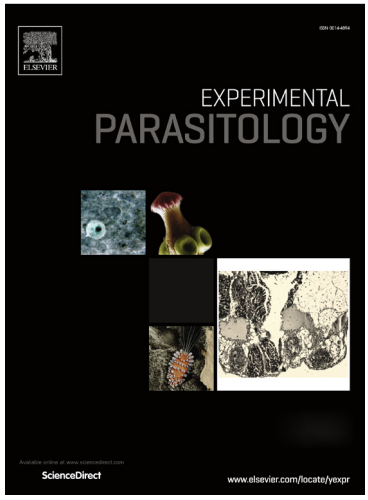


Typification of virulent and low virulence *Babesia bigemina* clones by *18S rRNA* and *rap-1c*

C. Thompson, M.E. Baravalle, B. Valentini, A. Mangold, S. Torioni de Echaide, P. Ruybal, M. Farber, I. Echaide

PII: S0014-4894(14)00059-9
DOI: http://dx.doi.org/10.1016/j.exppara.2014.03.016
Reference: YEXPR 6839

To appear in: *Experimental Parasitology*

Received Date: 6 June 2013
Revised Date: 24 February 2014
Accepted Date: 4 March 2014

Please cite this article as: Thompson, C., Baravalle, M.E., Valentini, B., Mangold, A., Torioni de Echaide, S., Ruybal, P., Farber, M., Echaide, I., Typification of virulent and low virulence *Babesia bigemina* clones by *18S rRNA* and *rap-1c*, *Experimental Parasitology* (2014), doi: http://dx.doi.org/10.1016/j.exppara.2014.03.016

Title: Typification of virulent and low virulence *Babesia bigemina* clones by *18S rRNA* and *rap-1c.*

Thompson, C.[1*]; Baravalle, M.E.[1]; Valentini, B.[1]; Mangold, A.[1]; Torioni de Echaide, S.[1]; Ruybal, P.[2]; Farber, M.[2]; Echaide, I.[1].

[1] *Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela, Ruta 34 km 227, CC 22, CP 2300 Rafaela, Santa Fe, Argentina.*

[2] *Instituto Nacional de Tecnología Agropecuaria, Centro Nacional de Investigaciones Agropecuarias Castelar, Los Reseros y Las Cabañas, CP 1712 Castelar, Buenos Aires, Argentina.*

*Corresponding author

Carolina Thompson

INTA EEA Rafaela

RUTA 34 KM 227

CC 22

2300, Rafaela (Santa Fe)

Argentina

TE: 54 3492 440121

thompson.carolina@inta.gob.ar

Present address of Baravalle M.E.: Laboratorio de Biología Celular y Molecular Aplicada, Facultad de Ciencias Veterinarias, CP. 3080, Esperanza, Santa Fe, Argentina.

Note: Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ216365.2, FJ216366.2, HQ688685.1, HQ688686.1, HQ688687.1, HQ688688.1 and HQ688689.1.

Abstract

The population structure of original *Babesia bigemina* isolates and reference strains with a defined phenotypic profile was assessed using *18S rRNA* and *rap-1c* genes. Two reference strains, BbiS2P-c (virulent) and BbiS1A-c (low virulence), were biologically cloned *in vitro*. The virulence profile of the strains and clones was assessed *in vivo*. One fully virulent and one low-virulence clone were mixed in identical proportions to evaluate their growth efficiency *in vitro*. Each clone was differentiated by two microsatellites and the gene *gp45*. The *18S rRNA* and *rap-1c* genes sequences from *B. bigemina* biological clones and their parental strains, multiplied exclusively *in vivo* or *in vitro*, were compared with strain JG-29. The virulence of clones derived from the BbiS2P-c strain was variable. Virulent clone Bbi9P1 grew more efficiently *in vitro* than did the low-virulence clone Bbi2A1. The haplotypes generated by the nucleotide polymorphism, localized in the V4 region of the *18S rRNA,* allowed the identification of three genotypes. The *rap-1c* haplotypes allowed defining four genotypes. Parental and original strains were defined by multiple haplotypes identified in both genes. The *rap-1c* gene, analyzed by high-resolution melting (HRM), allowed discrimination between two genotypes according to their phenotype, and both were different from JG-29. *B. bigemina* biological clones made it possible to define the population structure of isolates and strains. The polymorphic regions of the *18S rRNA* and *rap-1c* genes allowed the identification of different subpopulations within original *B. bigemina* isolates by the definition of several haplotypes and the differentiation of fully virulent from low virulence clones.

Keywords: *Babesia bigemina*, biological clones, *18S rRNA*, *rap-1c*, HRM

1. Introduction

Babesia bigemina is one of the Apicomplexa hemoparasites responsible for bovine babesiosis, transmitted in Argentina by the bovine tick *Riphicephalus microplus*. The disease is characterized by anemia, hemoglobinurea, and frequently death as a consequence of the hemolysis caused by massive intra-erythrocytic multiplication (Bock 2004). *Babesia* spp. have a biological cycle with two main stages. One is characterized by sexual multiplication in the tick vector, where recombination events frequently occur (Simuunza et al. 2011; Guillemi et al. 2012), and the second is characterized by asexual multiplication inside the bovine erythrocytes, where genetic variations have been observed. These variations have been utilized to characterize populations of other Apicomplexa parasites (Oura et al. 2003; Ajzenberg et al. 2004; Berens et al. 2007; Perez-Llaneza et al. 2010; Simuunza et al. 2011; Guillemi et al. 2012).

Using random amplification of polymorphic DNA (RAPD), repetitive extragenic palindromic elements-polymerase chain reaction (REP-PCR), and enterobacterial repetitive intergenic consensus sequences-polymerase chain reaction (ERIC-PCR), Madruga et al (2001) concluded that Brazilian *B. bigemina* field isolates were genetically distinct and hypothesized that this would result in different subpopulations as a consequence of multiple natural infections. One strategy used to analyze the population structure of hemoparasites has been the isolation of clones from field isolates. Druilhe et al. (1998) established an association between the genetic variations of *Plasmodium falciparum* and anti-malarial drug resistance in genetically different subpopulations using biological clones. The differentiation of subpopulations has been achieved using single-copy genes that carry stable polymorphic sequences. Baravalle et al. (2012) described the *in vitro* selection of virulent and low-virulence subpopulations of *B. bovis* by the higher growth efficiency of the former based on a single copy of the polymorphic *Bv80* gene. Other genes, such as *18S rRNA*, prompted its use for phylogenetic characterizations based on its high conservation (Allsopp et al. 1994; Criado Fornelio et al. 2003). Nevertheless, using reverse line blot hybridization, Bhoora et al. (2008) unexpectedly found intraspecies differences in the *18S rRNA* gene sequences for both *Theileria equi* and *Babesia caballi.* Afterward, they established the sequence heterogeneity among *T. equi* merozoites by analyzing the hypervariable region V4 of *18S rRNA* (Bhoora et al. 2010).

Gene sequences that codify merozoite surface proteins have also been useful for finding intraspecies variability within *Plasmodium vivax* and *Theileria parva* isolates (Beck et al. 2008). The rhoptry protein RAP-1 of *B. bigemina* is encoded by a family of 11 genes located in a single genomic region. This region is characterized by five copies for both *rap-1a* and *rap-1b*, tandemly arranged, and *rap-1c*, which is located downstream of the locus (Suárez et al. 2003). *Rap-1c* is a single polymorphic gene, not transcribed and eligible for characterizing field isolates of *B. bigemina*. Hilpertshauser et al. (2007) used this gene sequence to characterize an outbreak of *B. bigemina* in Switzerland and compared it with the sequences of *B. bigemina* from other regions.

Polymerase chain reaction (PCR) and sequencing are the standard methods to detect point mutations; nevertheless, the recent development of high-resolution DNA melting (HRM) using real-time PCR technology allows the discrimination of nucleic acid sequences based on their melting profiles (Zhou et al. 2005). This technology has been used for several purposes, such as the determination of single-nucleotide polymorphisms (SNPs) associated with *P. falciparum* resistance to anti-malarial drugs (Andriantsoanirina et al. 2009), the detection of predominant species of *Cryptosporidium* spp. (Pangasa et

al. 2009), and the differentiation of *Babesia canis* ssp *canis* (Adaszek and Winiarczyk 2010) as the most relevant applications.

The aim of this work was to characterize the population structure of *B. bigemina* Argentinean reference strains by genotyping low-virulence and fully virulent biological clones multiplied *in vivo* and *in vitro* using *18S rRNA* and *rap-1c* genes.

2. Materials and methods

2.1. Strains

Three virulent *B. bigemina* strains, BbiS2P, BbiM1P, BbiM2P, and two low-virulence strains, BbiS1A and BbiM1A, were evaluated in this work. The pathogenic strains were isolated during babesiosis outbreaks in the Northwest (BbiS2P) and the Northeast (BbiM1P and BbiM2P) of Argentina. The BbiS1A strain showed low virulence after two slow passages of its virulent parental isolate (not available for this work) in splenectomized calves (Aguirre et al. 1989). BbiM1A was derived from the strain BbiM1P and was attenuated in one splenectomized calf after two months of persistent infection (Vanzini, personal communication). Original strains of BbiS1A and BbiS2P were adapted over a 6-month period of continuous *in vitro* culture, denominated BbiS1A-c and BbiS2P-c, and selected for a biological cloning procedure. All strains were stored frozen in liquid nitrogen until use (Palmer, 1982). The three copies of *18S rRNA* described by Reddy et al. (1991) (GenBank accession numbers X59604.1, X596051.1, and X59607.1) and one copy of the sequence rap1-c (GenBank accession number AF026272.1), both from the *B. bigemina* JG-29 clone (Mishra et al. 1991), were used as reference sequences.

2.2. In vitro *cultures*

B. bigemina parental strains were multiplied *in vitro* using the microaerophilous stationary phase method described by Vega et al. (1985). Briefly, the base culture medium (BM) consisted of M199 (Gibco®), buffered with 5 mM HEPES (Sigma®) and 0.5 mM sodium bicarbonate (Sigma®) and enriched with normal bovine serum (40%; V/V). The complete medium was made by the addition of 5-10% (V/V) normal bovine erythrocytes to the BM. Cultures were incubated at 37 ºC under 5% CO_2 in air. BM was replaced every 24 h, and variable proportions of the parasitized complete medium were replaced by normal fresh complete medium after periods of 48-72 h (subculture) to avoid parasite overgrowth.

2.3. Quantification of parasitized erythrocytes

The concentration of parasitized erythrocytes was determined following the method described by Baravalle et al. (2012). Briefly, a suspension of parasitized erythrocytes was diluted 1:50 with BM and mixed with 10 µl of acridine orange working solution. After a 10 min incubation in darkness, parasitized erythrocytes were counted in both grids of the Neubauer chamber using white and UV lights successively under 1,000x magnification. The concentration (iRBC/ml) was calculated by the formula cells/ml = cell No. x 10^4 x dil^{-1} (Archer et al. 1977).

2.4. Biological cloning

Cloning was performed following the limiting dilution of parasitized erythrocytes, which was described by Vega et al. (1986). Briefly, *B. bigemina* suspensions were serially 10-fold diluted to achieve a putative final concentration of one parasitized erythrocyte per ml. Two hundred microliters of this suspension were equally distributed into four wells of a tissue culture plate (96 wells, Corning®), supplemented with 120 µl of complete medium (5% normal erythrocytes [V/V]) to achieve a 5-mm liquid column, and handled as described above over 36 days. Those *B. bigemina* strains that grew in only one of four inoculated wells were considered cloned. These parasites were re-cloned twice following the same procedure. The time that elapsed between the culture medium inoculation and the earliest parasite detection was registered for each clone. All clones were stored and frozen at -196°C until use.

2.5. In vivo *assay.*

To evaluate the virulence status of two biological clones derived from BbiS2P-c (virulent) and two from BbiS1A-c (low virulence), groups of three one-year-old Holstein steers were inoculated subcutaneously with 10^7 erythrocytes that were parasitized by each selected clone (n=12). BbiS2P-c and BbiS1A-c (parental strains) were inoculated in two and one bovines, respectively (n=3). Clinical parameters were evaluated daily between days 6 and 17 post-inoculation. The mean cumulative increase of body temperature above 39.5°C, the mean maximum parasitemia score (Callow and Pepper 1974), the mean maximum hematocrit depression index, and the occurrence of hemoglobinurea were compared among groups. Specific treatment with diminazene aceturate (Ganaseg®, Novartis) was employed when steers had hyperthermia above 41°C over three successive days, hematocrit <15%, parasitemia over 1%, and/or hemoglobinurea (Echaide et al. 1993). All procedures were carried out according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Sciences Societies, 2010) and the protocol was approved by the Ethics and Safety Committee of the Faculty of Veterinary Sciences (Universidad Nacional del Litoral, Argentina).

2.6. Competitive in vitro *growth of virulent and attenuated* B. bigemina *clones*

Two suspensions of 10% erythrocytes that were parasitized by either the virulent *B. bigemina* clone Bbi9P1 (1%) or the low-virulence clone Bbi2A1 (1%) were mixed 1:2 and distributed in 25 cm^2 plastic culture bottles. Three replicas were incubated at 37°C under 5% CO_2 in air and fed daily as described in section 2.2. DNA was extracted from the parasitized erythrocytes every 48 h to PCR-amplify the *gp45* gene and the microsatellites *ms4135* and *ms4179*. The microsatellites were obtained using *Tandem Repeat Finder* software with contigs from an Australian strain sequenced at the Sanger Institute *(http://www.sanger.ac.uk/_resources/downloads/protozoa/babesia-bigemina.html).* Amplified fragments were visualized in agarose gels stained with ethidium bromide.

2.7. Target genes

18S rRNA. The full sequences of *18S rRNA* from the genomic DNA of eight biological clones and seven strains were PCR-amplified using three sets of primers designed *ad hoc* for this experiment. The sequences amplified from this gene were compared with the sequence of clone JG-29 (Fig. 1).

Rap1-c. The 3´ terminal regions of *rap1-c* from the biological clones and strains mentioned above were PCR-amplified using the oligonucleotides defined by Hilperthauser et al. (2007). The sequences were also amplified using real-time PCR (rt-PCR) and were analyzed by HRM. The oligonucleotide sequences, melting temperatures (Tms), and the expected sizes of amplified DNA fragments are displayed in Table 1.

2.8. DNA extraction.

Erythrocytes parasitized by *B. bigemina* clones and strains were lysed and pelleted at 14,000 x*g,* and the hemoglobin was washed off using distilled water. *B. bigemina* DNA was extracted using phenol:chloroform:isoamyl alcohol (Invitrogen®), precipitated using isopropanol (Merck®), and washed in ethanol. The dried pellet was diluted in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and frozen at -20°C.

2.9. PCR.

The PCR mixture prepared to amplify the different gene sequences included 1x reaction buffer (20 mM Tris-HCl ph 8.4 and 50 mM KCl), 2.2 mM $MgCl_2$, 2 mM dNTP mixture, 1 pM of each primer, 3.5 µl of DNA sample, and 1.2 U of Taq DNA polymerase (Invitrogen®) or Proof Reading polymerase (Promega®). The final volume was brought to 50 µl with distilled water. The amplification process was started with 5 min at 94°C to denature all DNA, followed by 35 cycles at 94°C for 10 s, 58-62°C for 20 s, and 72°C for 30 s, and the process was finalized by an extension cycle of 5 min at 72°C. The DNA amplicons were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Finally, the

DNA fragments were purified using a commercial kit (Qiagen®) and sequenced using dye terminator sequencing (Macrogen®, USA).

2.10. High-resolution melting (HRM) analyses

A 287-bp region from the *rap-1c* gene was amplified by rt-PCR using the real-qPCR mix (Biodynamics®) containing Eva Green as a fluorochrome in a final volume of 20 µl. The amplification procedure was performed in a Rotor-Gene-Q6000 (Qiagen®) beginning with a 10 min denaturing step at 94°C, followed by 40 cycles at 94°C for 10 s, 62°C for 20 s, and 72°C for 30 s, and the procedure was finalized by an extension cycle of 5 min at 72°C. The Tms of the rt-PCR products were established by gradually increasing the temperature in 0.2°C intervals within a range of 83°C to 93°C immediately after the amplification.

2.11. Molecular cloning

To analyze the sequences of the V4 polymorphic regions from *18S rRNA*, two fragments were amplified and sequenced from the attenuated clones Bbi2A1, Bbi2A2, Bbi3A1, and Bbi3A2; the virulent clones Bbi8P1, Bbi8P2, Bbi9P1, and Bbi9P2; and strains BbiS1A-c, BbiS2P-c, BbiS2P, BbiS1A, BbiM1A, BbiM2P, and BbiM1P using the primers 18SF2/18SR2 - 18SF4/18SR4.

Similarly, a polymorphic fragment from the 3' terminal regions of *rap-1c* from the biological clones and the strains mentioned above was amplified using the primers Rap-1cF/Rap-1cR (Table 1) and then sequenced.

After sequence comparison, fragments amplified from both genes of biological clones Bbi9P1 and Bbi2A1, their parental strains, and the seven strains mentioned above were cloned into the vector pGem®-T easy Vector System (Promega®) in four different events. Five to fourteen clones from each strain were selected, purified using a commercial kit (Qiagen®), and sequenced using dye terminator sequencing (Macrogen®, USA).

2.12. Sequence analyses

To determine the haplotype profile and to establish the genotypes of the reference strains, the fragment sequences of the a, b, and c units of *18S rRNA* or the *rap-1c* from clones of each strain were compared with the analog sequence of clone JG-29. Sequences were aligned using Bioedit 7.0.5.3 software (Hall 1999). Phylogenetic analyses were performed using the Neighbor-joining method by MEGA® 4.0 (Tamura et al. 2007). The *18S rRNA* secondary structure of *B. bigemina* was obtained from the European Ribosomal Database http://bioinformatics.psb.ugent.be/webtools/rRNA/ssu/ (Wuyts et al. 2004) and was visualized and modified using RNAviz software. Hypervariable region V4 and its E23-n helixes were

adjusted according to Wuyts et al. (2000). To physically localize those regions and to evaluate the mutation probabilities of the nucleotide sites for *B. bigemina*, a SSU rRNA secondary structure variability map was used. This map was developed by computing the nucleotide variability of 500 sequences of species belonging to the eukaryotic 'crown taxa' (Van de Peer et al., 1997).

2.13. Statistical analysis

Biological cloning. The probability of successfully obtaining a biological clone was established using the Poisson distribution (Vega et al. 1985). The differences among the clinical parameters of inoculated cattle were analyzed using ANOVA. Haplotype frequencies from the different cloning sets were compared by using a Chi-Square statistics test ($P<0.05$).

HRM. The Tms from two clones with different phenotypes were arbitrarily selected as references to analyze the genotype differences. For intra-assay variability, the mean and the variation coefficient of the Tms for two reference clones and two parental strains (with five repetitions each) were evaluated. To establish the inter-assay variability, the mean and the standard deviation of the Tms from nine different assays were calculated for the reference clones. The results were analyzed using the software provided by Qiagen®.

3. Results

3.1. B. bigemina *biological clones*

Seven clones were derived from the BbiS2P-c virulent strain (Bbi8P1, Bbi8P2, Bbi8P3, Bbi8P4, Bbi9P1, Bbi9P2, and Bbi9P3) and five from BbiS1A-c low-virulence strain (Bbi2A1, Bbi2A2, Bbi2A3, Bbi3A1, and Bbi3A2). Clones derived from BbiS2P-c required a shorter period for detection during the cloning procedure (a mean of 17 days) than those derived from BbiS1A-c (a mean of 24 days). The difference was not statistically significant. The Poisson probabilities of obtaining a clone after two cloning repetitions were P=0.98 and P=0.97 for BbiS1A-c- and BbiS2P-c-derived clones, respectively.

3.2. In vivo *assay*

The groups of steers inoculated with clone Bbi8P1 or Bbi9P1 had similar hematocrit depressions (35.7%±8.4 and 36.3%±3.8, respectively) and parasitemia scores (10.0%±0.0 and 10.0%±0.0, respectively). These values were significantly higher ($P<0.05$) than those recorded for the groups inoculated with clones Bbi2A2 and Bbi3A1, with respective hematocrit depressions of 27.3%±3.8 and 24.0%±9.5 and respective parasitemia scores of 1.3%±0.3 and 2.0%±0.0. The respective cumulative temperature means were different not only between virulent and low-virulence clones but also between

virulent clones (1.0°C±1.0 and 3.0°C±2.8) ($P<0.05$) and between attenuated clones (1.0°C±1.0 and 0.03°C±1.00) ($P<0.05$).

Two of the three steers inoculated with clone Bbi9P1 and one of two infected with the BbiS2P-c parental strain were treated with diminazene due to hematocrit depression. All steers inoculated with clone Bbi8P1 derived from BbiS2P-c and those inoculated with BbiS1A-c, Bbi2A2, or Bbi3A1 did not require specific chemotherapy.

3.3. Molecular characterization of B. bigemina *clones*

The full sequences of *18S rRNA* genes from four virulent clones (Bbi8P1, Bbi8P2, Bbi9P1, and Bbi9P2), four low-virulence clones (Bbi2A1, Bbi2A2, Bbi3A1, and Bbi3A2), and seven strains (BbiS1A, BbiS1A-c, BbiS2P, BbiS2P-c, BbiM1A, BbiM1P, and BbiM2P) all had a size of 1693 bp, as previously established for the reference clone JG-29. A polymorphic 25 bp fragment from the *18S rRNA* gene allowed for the identification of point mutations and extra nucleotides at sites 201, 215, 618, 619, 621, 623, and 744 in the eight biological clones and the seven above-mentioned strains. Positions 618 to 623, localized in the E23-1 helix of the hypervariable region V4 and visualized in the *18S rRNA* secondary structure (Figure 2), allowed the characterization of six haplotypes (Hs0 to Hs5) (Table 2). In turn, these haplotypes permitted the establishment of three genotypes (Gs): Gs1 (Hs0), Gs2 (Hs1, Hs4), and Gs3 (Hs3, Hs4). Gs1 was restricted to the JG-29 clone, Gs2 was characteristic of all four low-virulence clones and their parental strain, and Gs3 was distinctive from all four virulent clones and their parental strain (Table 2). Virulent (BbiM1P, BbiM2P, and BbiS2P) and low-virulence (BbiS1A and BbiM1A) strains, not adapted to the *in vitro* growth, had multiple genotypes (MGs) defined by four and three haplotypes, respectively (Table 2), including Hs2 and Hs5. Hs0 was also observed in the virulent strains BbiM1P and BbiM2P. Hs4 was shared by all Argentinean strains, and Hs3 was observed only in virulent strains. Haplotype frequencies between the different cloning sets were significantly equal ($P<0.05$), with no new haplotypes found after PCR repetition.

A 287-bp fragment from the 3' region of *rap-1c* gene was amplified from all clones, and the strains were analyzed. The sequences of this fragment allowed for the definition of four genotypes (Gr) based on eight haplotypes (Hr0 to Hr7). Gr1 (Hr0) was characteristic of the JG-29 clone, and Gr2 (Hr5) included the low-virulence clones (represented by Bbi2A1), their parental strain, and the low-virulence strain BbiM1A. Gr3 (Hr4) included the virulent clones (represented by Bbi9P1), their parental strain (BbiS2P-c), and the original (BbiS2P) strains (Table 3). The strains not cultured *in vitro*, BbiS2P (Hr1 to Hr4) and BbiS1a (Hr5 and Hr6), had multiple genotypes (MGr). The virulent strains BbiM1P and BbiM2P had one

haplotype (Hr7) that defined Gr4. The differences between the virulent genotypes Gr3 and Gr4, as compared to the low-virulence clones (Gr2), consisted of two nucleotides. Gr3 and Gr4 had ***G*** nucleotides at positions 164 and 187, while Gr2 had ***T*** and ***A*** nucleotides in the analogous sites, respectively.

3.4. rt-PCR efficiency

The HRM analyses of the rt-PCR products revealed different Tms for the genotypes Gr1, Gr2, and Gr3 (Figure 3). The strains BbiS1A, BbiM1A, and BbiS1A-c were grouped with genotype Gr2, while BbiS2P, BbiS2P-c, BbiM1P, and BiM2P were grouped with Gr3 (data not shown). The levels of confidence obtained for the Tms of clones and strains were >90%; however, the original strain BbiS2P was an exception, with a level of confidence of 68.5%.

Intra-assay variabilities after five repetitions were 84.92ºC±0.06 for BbiS1A-c and Bbi2A1 and 85.17ºC±0.05 for BbiS2P-c and Bbi9P1. The difference between the virulent and low-virulence genotypes was 0.32ºC±0.07, while the difference between the virulent and JG29 genotypes was 0.37ºC±0.06.

3.5. In vitro *growth efficiency of* B. bigemina *Bbi9P1 over clone Bbi2A1*

The biological clones Bbi9P1 and Bbi2A1 multiplied continuously *in vitro* over 18 days and went through nine subcultures. Two fragments, 488 bp (*ms4179*) and 300 bp (*ms4135*) in size, that identify clone Bbi9P1 were amplified from the *B. bigemina* cultures until the end of the experiment. Two other fragments, 404 bp (*ms4179*) and 350 bp (*ms4135*) in size, that are characteristic of clone Bbi2A1 were amplified only during the first seven days of *in vitro* culture (Fig. 4).

A 1044 bp fragment from *gp45* was consistently amplified from clone Bbi2A1 and its parental strain but never from the virulent clone Bbi9P1 or its parental strain. The *gp45* was detected only up to day seven of the *in vitro* cultures. Similar results were obtained in the three replicate experiments.

4. Discussion

The level of virulence exerted by several species of *Babesia in vivo* constitutes a distinctive feature that is useful for the initial classification of strains. Although natural isolates are usually virulent in phenotype, parasites with different levels of virulence (subpopulations) may be included that can eventually be separated *in vivo* or *in vitro* using different protocols. A low-virulence *B. bovis* strain can be selected over several syringe passages in splenectomized calves (Callow and Mellors, 1966) or by *in vitro* cloning (Buening et al. 1986). The virulence phenotype of some hemoparasites is usually established *in vivo* (Lau et al. 2011) or, less frequently, *in vitro* (Nevils et al. 2000; Baravalle et al. 2012; Taylor et al. 2002).

Meanwhile, low-virulence *B. bigemina* parasites induce mild changes in the circulatory systems of infected bovines. The virulent strains also generate high parasitemia, severe anemia, and hemoglobinurea and frequently result in death.

In this work, most of the clones derived from the BbiS2P-c strain were detected earlier during the biological cloning period than those derived from the BbiS1A-c strain, indicating the higher efficiency of the former to invade and multiply in erythrocytes *in vitro*. The experiment designed to challenge this observation confirmed the higher *in vitro* growth efficiency of the virulent clone Bbi9P1 over clone Bbi2A1. Two microsatellites and the gp45 gene were useful for establishing that the parasites of clone Bbi2A1, which putatively has a lower multiplication capacity, were lost and were unable to counter the dilution effect caused by the successive subcultures. We concluded that *in vitro* multiplication of *B. bigemina* isolates might have favored the selection of virulent parasites. However, some steers inoculated with BbiS2P-c or the derived clones Bbi8P1 and Bbi9P1 had similar clinical behavior as those that received low-virulence clones, without requiring chemotherapy. This atypical behavior was also observed in a previous experiment in which two of seven heifers did not require diminazene treatment during the reaction period after inoculation with strain BbiS2P-c (Echaide et al. 1993). Genes sequences used in this work confirmed that biological clones were identical to their parental strains. These results suggest that the phenotypic profile would be driven by the genetic background of the host (Parker et al. 1985) or different levels of gene expression (Cooke et al. 2001). Meanwhile, BbiS2P-c would consist of genetically identical virulent parasites selected after long periods of *in vitro* culture, and the low-virulence strain BbiS1A-c, the product of an *in vivo* attenuation event, maintained the phenotype when cultured in similar *in vitro* conditions. Although the low-virulence phenotypes of only two of the five clones were corroborated *in vivo*, we have assumed that the parental strain was uniformly attenuated because the genotypes of all five derived clones were identical. There are also no reports of the reversion of strain BbiS1A-c, which has been amplified *in vitro* to produce a live vaccine in Argentina since 1993 (OIE, 2012).

The analyses of *B. bigemina 18S rRNA* and *rap-1c* sequences amplified from virulent and low-virulence clones allowed the differentiation of Argentinian *B. bigemina* reference strains. The comparison of *18S rRNA* nucleotide sequences analyzed from local biological clones with the sequence of the Mexican JG-29 clone (Reddy et al. 1991) revealed the presence of more than one alternative nucleotide at specific sites (nucleotide positions 618, 619, 621, and 623) within the gene, generating different haplotypes.

As it was expected, the Bbi2A1 and Bbi9P1 clones showed at least two different gene copies of *18S rRNA* in the *B. bigemina* genome. This observation was attributed to the paralogous copies previously described by Reddy et al., 1991.

The *18S rRNA* profile of the BbiS2P strain, multiplied exclusively *in vivo*, included multiple genotypes, while BbiS2P-c contained one genotype (Gs3). This indicates that the original BbiS2P strain not only had paralogous genes but also included more than one subpopulation, some of which would be lost during the *in vitro* cultivation, as mentioned above. A similar event was observed for the original BbiS1A strain, which included at least two genotypes, one of them likely lost after *in vitro* cultivation (BbiS1A-c). This suggests that strain BbiS1A also underwent modifications in population structure during *in vitro* adaptation. As expected, the persistent genotypes of BbiS2P-c and BbiS1A-c were also identified in their corresponding derived clones.

Regardless of the small percentage of dissimilarity observed with *rap-1c* among the attenuated and pathogenic strains, the point mutations in the sequences were also useful for differentiating the phenotypes. Similarly to *18S rRNA*, by using *rap-1c,* it was possible to establish that the original strains BbiS2P and BbiS1A (attenuated *in vivo*) included at least two genotypes. However, after their *in vitro* multiplication, both strains (BbiS2P-c and BbiS1A-c) had only one genotype. The different *rap-1c* genotypes were also observed by HRM assay, which distinguished virulent from low-virulence reference clones. The lower confidence level observed for BbiS2P (68.5%) with regards to BbiS1A (>90.0%) could be attributed to the instability of the Tm caused by the higher number of haplotypes (n=4) present in BbiS2P compared to BbiS1A (n=2), which should be more stable (Taylor 2009). Therefore, this would not be an advisable method for differentiating field isolates of *B. bigemina*, as they would be composed of multiple genotypes, which is a consequence of multiple tick bites (Madruga et al. 2001).

Based on polymorphic regions of the *18S rRNA* and *rap-1c* genes, we established that: i) original Argentinean isolates of *B. bigemina* consist of subpopulations differentiated by several haplotypes, ii) the sequences of these genes were useful for differentiating the low-virulence vaccinal strain BbiS1A and its derived clones from other virulent reference strains, clones and isolates, and iii) subpopulations of *B. bigemina* isolates are lost during *in vitro* culture.

Acknowledgments

We thank Mariano Ferreira M.V. for control of the *in vivo* assay and Fernando Seguro for technical assistance with *in vivo* experiments and Paula Ramírez for technical assistance with *in vitro* cultures. The study was supported by ANPCyT (PICT0 12920) and TCP INTA EEA Rafaela-Asoc. Coop. 426100.

References

Adaszek, L., Winiarczyk, S., 2010. Application of the SYBR Green real-time HRM PCR technique in the differentiation of the *Babesia canis canis* protozoa isolated in the areas of Eastern Poland. Parasitol. Res. 106, 1253-1256.

Aguirre, D.H., Mangold, A.J., Gaido, A.B., de Ríos, L.G., Guglielmone, A.A., 1989. Evaluación de la infectividad de una vacuna congelada en nitrógeno líquido elaborada en base a *Babesia bigemina* atenuada. Rev. Med. Vet. 70, 142-5 (in Spanish).

Ajzenberg, D., Bañuls, A.L., Su, C., Dumetre, A., Demar, M., Carme, B., Darde, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. Int. J. Parasitol. 34, 1185-1196.

Allsopp, M.T.E.P., Cavalier-Smith, T., De Waal, D.T., Allsopp, B.A., 1994. Phylogeny and evolution of the piroplasms. Parasitology 108, 147-152.

Andriantsoanirina, V., Lascombes, V., Ratsimbasoa, A., Bouchier, C., Hoffman J., Tichit M., Rabarijaona L., Durand R., Ménard D., 2009. Rapid detection of point mutations in *Plasmodium falciparum* genes associated with antimalarial drugs resistance by using High-Resolution Melting analysis. J. Microbiol. Methods 78, 165-170.

Archer R.K., 1977. Technical methods. In: Comparative Clinical Haematology. Archer, R.K. and Jeffcott, L.B. (Eds.), Blacwell Scientific Publications, Victoria, Australia, pp. 537-610.

Baravalle, E., Thompson, C., Valentini, B., Ferreira, M.B., Torioni de Echaide, S., Florin-Christensen, M., Echaide, I.E., 2012. *Babesia bovis* biological clones and the inter-strain allelic diversity of the Bv80 gene support subpopulation selection as a mechanism involved in the attenuation of two virulent isolates. Vet. Parasitol. 190(3-4), 391-400.

Beck, H.-S., Blake, D., Darde, M.-L., Felger, I., Pedraza Díaz, S., Regidor-Cerrillo, J., Gómez-Bautista, M., Ortega-Mora, L.M., Putignani, L., Shiels, B., Tait, A., Weir, W., 2008. Molecular approaches to diversity of populations of apicomplexan parasites. Int. J. Parasitol. 39, 175-189.

Berens, S.J., Brayton, K.A., McElwain, T.F., 2007. Coinfection with antigenically and genetically distinct virulent strains of *Babesia bovis* is maintened through all phases of the parasite life cycle. Infect. Immun. 75(12), 5769-76.

Bock, R., De Vos, A., Jorgensen, W., 2004. Babesiosis of cattle. Parasitology 129, 247-69.

Bhoora, R., Franssen, L., Oosthuizen, M.C., Guthrie, A.J., Zweigarth, E., Penzhorn, B.L., Jongejan, F., Collins, N.E., 2008. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. Vet. Parasitol. 159(2), 112-120.

Bhoora, R., Allsopp, M.T.E.P., Buss, P. Guthrie, A.J., Penzhorn, B.L., Collins, N.E., 2010. Genetic diversity of piroplasms in plains zebra (*Equus quagga burchellii*) and Cape mountain zebra (*Equus zebra zebra*) in South Africa. Vet. Parasitol. 174, 145-149.

Buening, G.M., Kuttler, K.L., Rodríguez, S.D., 1986. Evaluation of a cloned *Babesia bovis* organism as a live inmunogens. Vet. Parasitol. 22, 235-242.

Callow, L.L., Mellors, L.T. (1966) A new vaccine for *Babesia argentina* infection prepared in splenectomised calves. Aust. Vet. J. 42, 464-465.

Callow, L.L., Pepper, P.M., 1974. Measurement of and correlations between fever, changes in the packed cell volume and parasitaemia in the evaluation of the susceptibility of cattle to infection with *Babesia argentina.* Aust. Vet. J. 50(1), 1-5.

Cooke, B.M., Mohandas, N., Coppel, N., 2001. The malaria infecting red blood cells: structural and functional changes. Adv. Parasitol. 50, 1-86.

Criado-Fornelio, A., Martinez-Marco, A., Buling-Saraña, A., Barba-Carretero, J.C., 2003. Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in Southern Europe. Part II. Phylogenetic analysis and evolutionary history. Vet. Parasitol. 114, 173-194.

Druilhe, P., Daubersies, P., Patarapotikul, J., Gentil, C., Chene, L., Chongsuphajaisiddhi, T., Mellok, S., Langsley, G., 1998. A primary malarial infection is composed of a very wide range of genetically diverse but related parasites. J. Clin. Invest. 101, 2008-2016.

Echaide, I.E., De Echaide, S.T., Guglielmone, A.A., 1993. Live and soluble antigens for cattle protection to *Babesia bigemina.* Vet. Parasitol. 51, 35-40.

Federation of Animal Sciences Societies, 2010. Federation of Animal Sciences Societies (FASS) Guide for the care and use of agricultural animals in agricultural research and teaching (Third edition).

Guillemi, E., Ruybal, P., Lia, V., González, S., Farber, M., Wilkowsky, S.E., 2012. Multi-Locus Typing Scheme for *Babesia bovis* and *Babesia bigemina* Reveals High Levels of Genetic Variability in Strains from Northern Argentina. Inf. Genet. Evol. 14, 214-222.

Hall, T.A., 1999. BioEdit, a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95-98.

Hilpertshauser, H., Deplazes, P., Meli, M.L., Hofmann-Lehmann, R., Lutz, H., Mathis, A., 2007. Genotyping of *Babesia bigemina* from cattle from a non-endemic area (Switzerland). Vet. Parasitol. 145, 59-64.

Lau, A.O., Kalyanaraman, A., Echaide, I., Palmer, G, Bock, R., Pedroni, M.J., Rameshkumar, M., Ferreira, M.B., Fletcher, T.I., and McElwain, T.F., 2011. Attenuation of virulence in an apicomplexan hemoparasite results in reduced genome diversity at the population level. BMC Genomics 12(410), 1-13.

Madruga, C.R., Leal, C.R., Ferreira, A.M., Araújo, F.R., Bonato, L.V., Kessler, R.H., Schenk, M.A., Soares, C.O., 2002. Genetic and antigenic analysis of *Babesia bigemina* isolates from five geographical regions of Brazil. Pesq. Vet. Bras. 22, 153-60.

Mishra, V.S., Stephens, E.B., Dame, J.B., Perryman, L.E., McGuire, T.C., McElwain, T.F., 1991. Inmunogenicity and sequence analysis of recombinant p58, a neutralization-sensitive, antigenically conserved *Babesia bigemina* merozoite surface protein. Mol. Biochem. Parasitol. 47, 207-212.

Nevils, M.A., Figueroa, J.V., Turk, J.R., Canto, G.J., Le, V. Ellersieck, M.R., Carson, C.A., 2000. Cloned lines of *Babesia bovis* differ in their ability to induce cerebral babesiosis in cattle. Parasitol. Res. 86, 437-443.

OIE, 2012. Bovine babesiosis. In OIE Terrestrial Manual, seventh edition, Jouve, Mayenne, France, pp. 607-615.

Oura, C.A.L., Odongo, D.O., Lubega, G.W., Spooner, P.R., Tait, A. and Bishop, R.P., 2003. A panel of microsatellite and minisatellite markers for the characterization of field isolates of *Theileria parva*. Int. J. Parasitol. 33, 1641-1653.

Palmer, D.A., Buening, G.M., Carson, C.A., 1982. Cryopreservation of *Babesia bovis* for *in vitro* cultivation. Parasitology. 84, 567-572.

Pangasa, A., Jex, A.R., Campbell, B.E., Bott, N.J., Whipp, M., Hogg, G., Stevens, M.A., Gasser, R.B., 2009. High resolution melting-curve (HRM) analysis for the diagnosis of cryptosporidiosis in humans. Mol. Cell Probes 23, 10-15.

Parker, R.J., Shepherd, R.K., Trueman, K.F., Jones, G.W., Kent, A.S., Polkinghorne, I.G., 1985. Susceptibility of *Bos indicus* and *Bos taurus* to *Anaplasma marginale* and *Babesia bigemina* infections. Vet. Parasitol. 17, 205-213.

Perez-Llaneza, A., Caballero, M., Baravalle, E.; Mesplet, M.; Mosqueda, J., Suarez, C.E., Echaide, I., Katzer, F., Pacheco, G., Florin-Christensen, M., Schnittger, L., 2010. Development of a tandem repeat-based multilocus typing system distinguishing *Babesia bovis* geographic isolates. Vet. Parasitol. 167(2-4), 196-204.

Reddy, G.R., Chakrabarti, D., Yowell, C.A., Dame, J.B., 1991. Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*, expression in erythrocyte culture. Nucleic Acids Res. 19, 3641- 3645.

Simuunza, M., Bilgic, H., Karagenc, T., Syakalima, M., Shiels, B., Tait, A., Weir, W., 2011. Population genetic analysis and sub-structuring in *Babesia bovis*. Mol. Biochem. Parasitol. 177, 106-115.

Suárez, C.E., Palmer, G.H., Florin-Christensen, M., Hines, S.A., Hoetzel, I., McElwain, T.F., 2003. Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina* rap-1 locus. Mol. Biochem. Parasitol. 127, 101-112.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596-1599.

Taylor, L.H., Welburn, S.C., Woolhouse, M.E.J., 2002. *Theileria annulata*, Virulence and transmission from single and mixed clone infections in cattle. Exp. Parasitol. 100, 186-195.

Taylor, C.F., 2009. Mutation scanning using high-resolution melting. Biochem. Soc. Trans. 37, 433-437.

Van de Peer, Y., Jansen, J. de Rijk, P., de Watcher, R., 1997. Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. 25(1), 111-116.

Vega, C.A., Buening, G.M., Green, T.J., Carson, C.A., 1985. *In vitro* cultivation of *Babesia bigemina*. Am. J. Vet. Res. 46(2), 416-20.

Vega, C.A., Buening, G.M., Rodríguez, S.D., Carson, C.A., 1986. Cloning of *in vitro* propagated *Babesia bigemina.* Vet. Parasitol. 22, 223-33.

Wuyts, J., de Rijk, P., Van de Peer, Y., Pison, G., Rousseuw, P., De Watcher, R., 2000. Comparative analysis of more than 3000 sequences reveals the existence of two pseudoknots in area V4 of eukaryotic small subunit ribosomal RNA. Nucleic Acids Res. 28(23), 4698-4708.

Wuyts, J., Perrière, G., Van de Peer, Y., 2004. The European ribosomal RNA database. Nucleic Acids Res. 32, 101-103.

Zhou, L., Wang, L., Palais, P., Pryor, R., Wittwer, C.T., 2005. High-Resolution DNA melting analysis for simultaneous mutation scanning and genotyping in solution. Clin. Chem. 51(10), 1770-1777.

Figure Captions

Figure 1. *18S rRNA* PCR amplification. Scheme of the *Babesia bigemina 18S rRNA* gene and amplified sequences. Arrows indicate the orientations and numbers indicate the location of the oligonucleotides used according to JG-29 *18S rRNA* sequence. The *18S rRNA* gene is represented by an open box, and the PCR-amplified fragments are indicated with patterned boxes.

Figure 2. *18S rRNA* analysis. Hipervariable region V4 from the *18S rRNA* secondary structure of the *Babesia bigemina* JG-29 strain obtained from *http://bioinformatics.psb.ugent.be/webtools/rRNA/ssu/* and adjusted using RNAviz software. Numbers 23-26 are the helices, and E23-n are extra helices, with polymorphic nucleotides denoted in italics.

Figure 3. rt-PCR. Melting temperatures obtained by high-resolution melting analysis for the 287 bp *rap-1c* sequence of *Babesia bigemina* reference clones Bbi2A1 and Bbi9P1 and the JG-29 strain. The dashed line corresponds to Gr2 (attenuated genotype), the dotted line corresponds to Gr1 (pathogenic genotype), and the solid line corresponds to Gr3 (JG29 genotype). The range of analyzed temperatures was 83°C-93°C. dF/dT is the first derivative from Fluorescence/Temperature, and 0.32°C and 0.37°C are the differences observed between the genotypes.

Figure 4. PCR amplification of A) *ms4179,* B) *ms4135,* and C) *gp45* from the *Babesia bigemina* Bbi2A1 and Bbi9P1 clone mixture. Identical numbers correspond to the same days. Columns (Col.) 1 and 2: First culture day; Col. 3 and 4: Seventh culture day; Col. 5 and 6: Tenth culture day; Col. 7 and 8: Eighteenth culture day; Col. 9: BbiS1A-c; Col. 10: BbiS2P-c; Col. 11: water and 100 bp ladder (Promega®). Gel: 1.5% agarose.

Figure A.1. *18S rRNA* analysis. *18S rRNA* secondary structure of *Babesia bigemina* JG-29 strain obtained from *http://bioinformatics.psb.ugent.be/webtools/rRNA/ssu/* and adjusted by RNAviz software. V1-V5;

V7-V9 are known hypervariable regions, numbers from 1-50 are the helices and E10-1 and E23-n are extra helices. The E23-1 helix of the hypervariable region V4 is highlighted in a box. Positions with polymorphic nucleotides are denoted in italic.

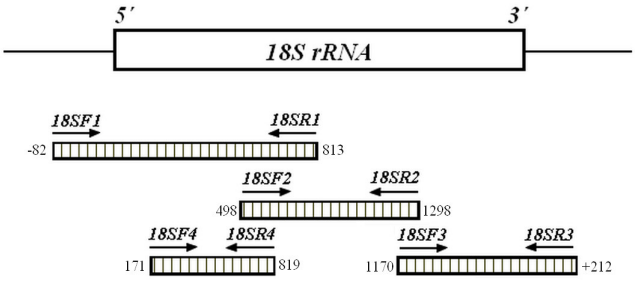
Revised Figure Captions

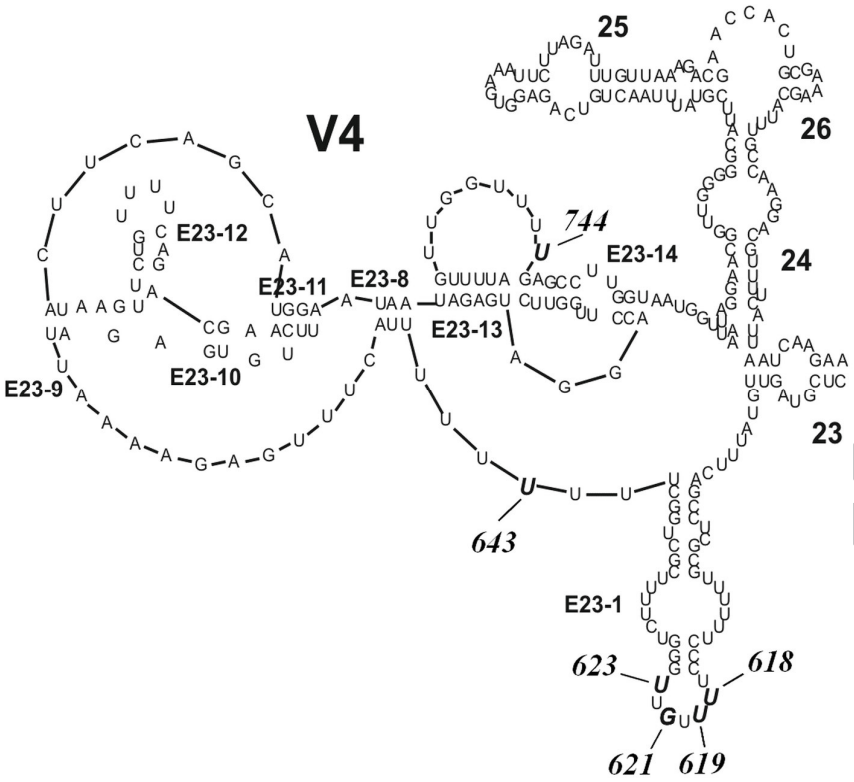
Figure 1. *18S rRNA* PCR amplification. Scheme of the *Babesia bigemina 18S rRNA* gene and amplified sequences. Arrows indicate the orientations and numbers indicate the location of the oligonucleotides used according to JG-29 *18S rRNA* sequence. The *18S rRNA* gene is represented by an open box, and the PCR-amplified fragments are indicated with patterned boxes.

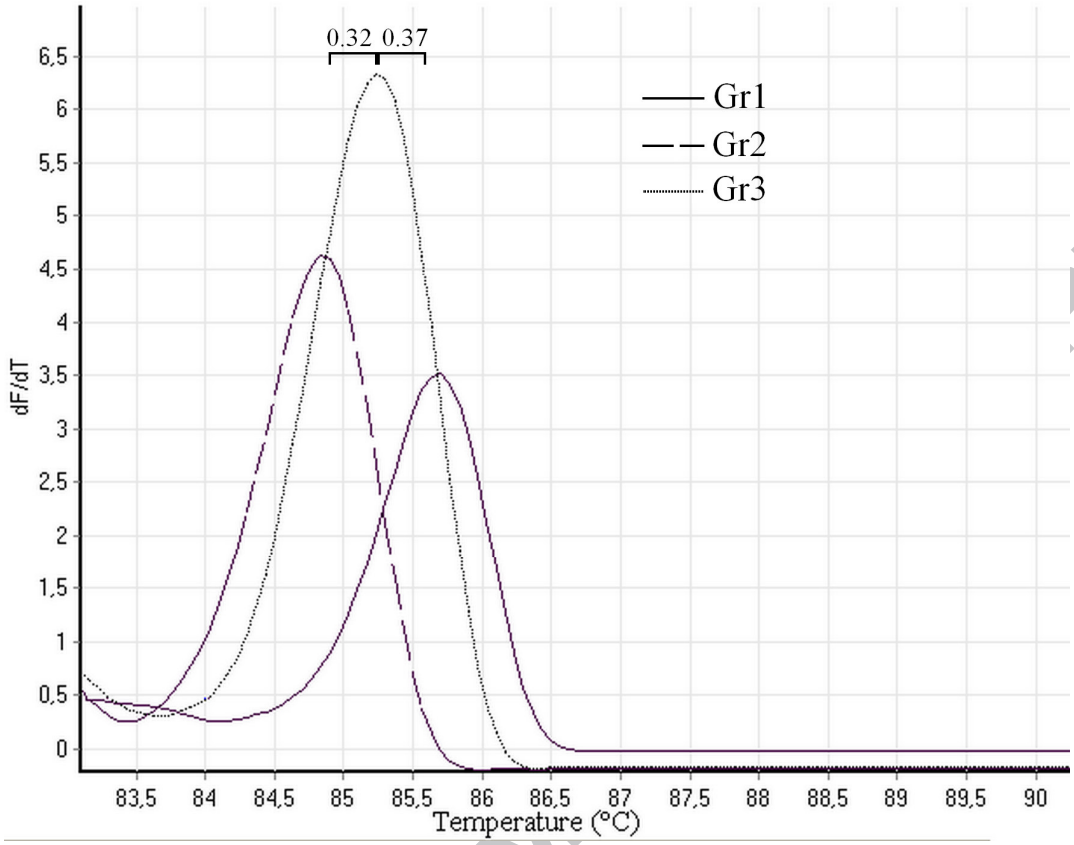
Figure 2. *18S rRNA* analysis. Hipervariable region V4 from the *18S rRNA* secondary structure of the *Babesia bigemina* JG-29 strain obtained from http://bioinformatics.psb.ugent.be/webtools/rRNA/ssu/ and adjusted using RNAviz software. Numbers 23-26 are the helices, and E23-n are extra helices, with polymorphic nucleotides denoted in italics.

Figure 3. rt-PCR. Melting temperatures obtained by high-resolution melting analysis for the 287 bp *rap-1c* sequence of *Babesia bigemina* reference clones Bbi2A1 and Bbi9P1 and the JG-29 strain. The dashed line corresponds to Gr2 (attenuated genotype), the dotted line corresponds to Gr3 (pathogenic genotype), and the solid line corresponds to Gr1 (JG29 genotype). The range of analyzed temperatures was 83°C-93°C. dF/dT is the first derivative from Fluorescence/Temperature, and 0.32°C and 0.37°C are the differences observed between the genotypes.

Figure 4. PCR amplification of A) *ms4179,* B) *ms4135,* and C) *gp45* from the *Babesia bigemina* Bbi2A1 and Bbi9P1 clone mixture. Identical numbers correspond to the same days. Columns (Col.) 1 and 2: First culture day; Col. 3 and 4: Seventh culture day; Col. 5 and 6: Tenth culture day; Col. 7 and 8: Eighteenth culture day; Col. 9: BbiS1A-c; Col. 10: BbiS2P-c; Col. 11: water and 100 bp ladder (Promega®). Gel: 1.5% agarose.







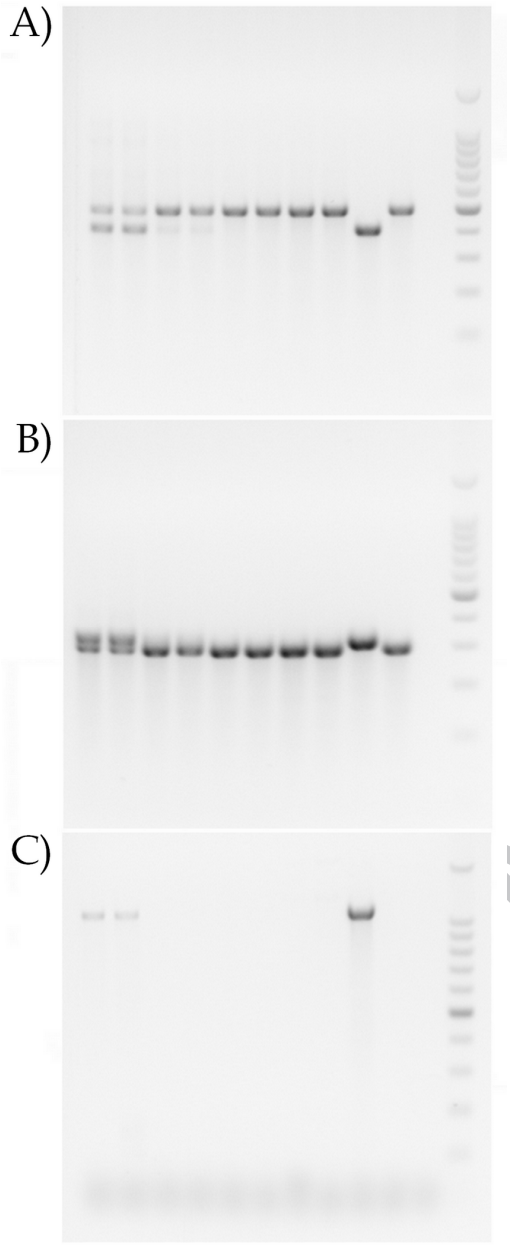


Table 1. Oligonucleotides used to amplify fragments of *18S rRNA* and *rap-1c* genes from *Babesia bigemina*. The table shows the target gene, the melting temperature (Tm) and the expected size of the amplified fragment.

Name	Oligonucleotides 5´-3´	Target genes	Tm (ºC)	Expected size (bp)	Source
18SF1	F-TTTTGCTTTGCGACTCCTGGCGG	*18S rRNA*	58	895	This work
18SR1	R-CTAAGAATTTCACCTCTGACAGT				
18SF2	F-ACCAATTGGAGGGCAAGTCT	*18S rRNA*	56	800	This work
18SR2	R-TTGAAGCACAGGAGTCCCTCTA				
18SF3	F-TGCATGGCCGTTCTTAGTTGGT	*18S rRNA*	58	735	This work
18SR3	R-AGAGCACGCATCACATGACA				
18SF4	F-CGAGGCCTTTTGGCGGCGTTTATTAG	*18S rRNA*	62	643	This work
18SR4	R-ACAAATCTAAGAATTTCACCTCTGAC				
Rap-1cF	F-AGAGTGAAAATGGCGAACTCGC	*rap-1c*	58	287	Hilpertshauser et al., 2007
Rap-1cR	R-TTACGACGATCGTTTGAAGTAC				

F-: Forward primer; R-: Reverse primer

Table 2. Haplotypes and genotypes obtained after the molecular cloning of the E23-1 helix (hypervariable region V4) of the *18S rRNA* gene from *Babesia bigemina* clones (Bbi2A1, Bbi9P1); their parental strains (BbiS1A-c, BbiS2P-c), the original strains (BbiS1A, BbiS2P) and JG29. BbiM1A, BbiM1P and BbiM2P are also included. Nucleotide positions in the *18S rRNA* complete gene (614-624).

Haplotypes	Nucleotide sites											JG-29	BbiS1A	BbiS1A-c	Bbi2A1*	BbiS2P	BbiS2P-c	Bbi9P1*	BbiM1A	BbiM1P	BbiM2P
	614	**615**	**616**	**617**	**618**	**619**	**620**	**621**	**622**	**623**	**624**										
Hs0	C	C	C	T	**T**	**T**	T	**G**	T	**T**	T	x								x	x
Hs1	C	C	C	T	**G**	**G**	T	**T**	T	**T**	T		x	x	x	x			x	x	x
Hs2	C	C	C	T	**T**	**G**	T	**T**	T	**T**	T					x					
Hs3	C	C	C	T	**C**	**T**	T	**T**	T	**C**	T					x	x	x		x	x
Hs4	C	C	C	T	**C**	**G**	T	**T**	T	**T**	T		x	x	x	x	x	x	x	x	x
Hs5	C	C	C	T	**C**	**T**	T	**T**	T	**T**	T		x						x		
	***18S rRNA* gene genotypes**											**Gs1**	MGs	Gs2	**Gs2**	MGs	Gs3	**Gs3**	MGs	MGs	MGs

* Based on their identity, clones Bbi2A1 and Bbi9P1 represent all low virulence a virulent clones respectively.

Table 3. Haplotypes and genotypes obtained after the molecular cloning of the 3´ region of *rap-1c* from *Babesia bigemina* clones (Bbi2A1, Bbi9P1); their parental strains (BbiS1A-c, BbiS2P-c), the original strains (BbiS1A, BbiS2P) and JG-29. BbiM1A, BbiM1P and BbiM2P are also included. Nucleotides positions in the *rap-1c* analyzed fragment.

Haplotypes	**Nucleotide sites**													JG-29	BbiS1A	BbiS1A-c	Bbi2A1*	BbiM1A	BbiS2P-c	Bbi9A1*	BbiS2P	BbiM1P	BbiM2P
	67	**75**	**126**	**136**	**153**	**164**	**183**	**187**	**212**	**222**	**236**	**252**	**265**										
Hr0	G	G	G	C	C	G	A	G	C	G	T	G	A	x									
Hr1	G	G	C	C	C	G	A	G	G	C	T	G	G								x		
Hr2	A	C	G	T	C	G	A	G	G	C	T	G	G								x		
Hr3	G	G	C	C	C	G	A	G	G	G	A	A	G								x		
Hr4	A	C	G	T	C	G	A	G	G	G	A	A	G						x	x	x		
Hr5	A	C	G	T	C	**T**	A	**A**	G	G	T	A	G		x	x	x	x					
Hr6	A	C	G	T	C	T	G	A	G	G	T	A	G		x								
Hr7	A	C	T	T	G	G	A	G	G	G	T	A	G									x	x
Rap-1c gene **Genotypes**														**Gr1**	**MGs**	**Gr2**	**Gr2**	**Gr2**	**Gr3**	**Gr3**	**MGs**	**Gr4**	**Gr4**

* Based on their identity, clones Bbi2A1 and Bbi9P1 represent all low virulence a virulent clones respectively.

Highlights

- Two phenotipically different reference strains were biologically cloned *in vitro*
- The virulence of clones derived from the BbiS2P-c strain was variable.
- The haplotypes obtained by *18S rRNA,* allowed the identification of three genotypes.
- The *rap-1c* gene was analyzed by high-resolution melting.
- Original Argentinean isolates of *B. bigemina* consist of subpopulations.