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Sensitivity of *Anaplasma marginale* genotypes to oxytetracycline assessed by analyzing the *msp1α* gene in experimentally infected cattle.

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**Highlights**

* The strain S1P of *A. marginale* has several *msp1α* genotypes in different abundance
* Variants with different *msp1α* genotypes have a variable sensitivity to OTC
* The *msp1α* genotype is one of the variables that affect the effectiveness of sterilization treatment

**Abstract**

The aim of this study was to evaluate the influence of the long-acting oxytetracycline (OTC) treatment on *A. marginale* genotypes of the isolate S1P, by analyzing the *msp1α* genotype based on a microsatellite (*ms*) and tandem repeat sequences (TRS) located at the 5´ end of the gene. DNA samples were obtained from a longitudinal study of chemosterilization; 10 2-year-old steers were experimentally infected with blood from a splenectomized calf inoculated with the *A. marginale* isolate S1P. All the steers had received a first dose of 20 mg kg\(^{-1}\) OTC to treat acute disease, and once recovered all steers received a sterilizing treatment based on three doses of 20 mg kg\(^{-1}\) OTC 7 days apart. Blood from two steers not sterilized by the treatment was inoculated into two splenectomized calves (receptors) 104 days after treatment. DNA samples (S) used for
msp1α amplification were obtained from i) the donor calf (S0), ii) 10 steers during acute disease (S1), after the first antibiotic treatment (S2), and after the chemosterilization procedure (S3 and S4), and iii) two receptor calves (S5). Thirty clones from the donor calf and at least 5 clones from the other DNA samples were analyzed. The genotype E/αββββ msp1α identified in the donor calf and steers, before OTC treatment, was not detected either in steers that continued infected after the sterilizing treatment or in the receptor calves, in which only genotype C/EϕFF msp1α was observed. These results highlight the existence of A. marginale genotypes with different sensitivity to OTC and the importance of other variables to successfully sterilize the carriers.

Keywords
Anaplasma marginale; oxytetracycline; msp1α; cattle; strain; genotype

1. Introduction

Bovine anaplasmosis is an infectious disease caused by the obligate intraerythrocytic bacterium Anaplasma marginale (order Rickettsiales; family Anaplasmataceae) (Theiler, 1910); it is biologically transmitted to susceptible cattle by ticks or mechanically by biting flies and fomites. Anaplasma marginale is one of the most prevalent tick-borne pathogens of cattle, being endemic to tropical and subtropical areas of the world. Acute anaplasmosis affects mostly adult bovines and is characterized by severe anemia, weight loss, reduction of milk production, abortion and frequently death (Aubry and Geale, 2011; Kocan et al., 2010). Cattle that recover from acute disease remain carriers and serve as reservoirs for transmission to other animals (Eriks et al., 1993).
Control measures for anaplasmosis vary among herds according to the epidemiological status and include arthropod control, vaccination and antibiotic treatment (Kocan et al., 2010). The long-acting oxytetracycline (OTC) is currently used for acute anaplasmosis treatment and chemosterilization of carriers (Kocan et al., 2010). OTC is a bacteriostatic antibiotic that inhibits the protein synthesis principally through reversible binding with the 30S ribosomal subunit (Scholar and Pratt, 2000). Degenerated forms of Anaplasma inclusion bodies, associated with the inhibitory action of the protein synthesis, were observed by light and electron microscopy, 4 days after the administration of tetracycline (Simpson, 1975). Differences in OTC susceptibility between Virginia and Oklahoma A. marginale isolates were observed in in vitro studies (Coetzee et al., 2006a). Three general class-specific mechanisms of resistance to tetracycline have been well described: efflux, ribosomal protection, and enzymatic inactivation (Ian and Marilyn, 2001). Two multidrug resistance efflux pumps were identified in the genome of A. marginale (Brayton et al., 2005).

The single gene *msp1α* encodes the major surface proteins 1α (MSP1α), an adhesin necessary to invade bovine erythrocytes and tick cells (de la Fuente et al., 2001a; McGarey and Allred, 1994). *Anaplasma marginale* strains were identified using a microsatellite (*ms*) located at the 5′-untranslated region (5′-UTR) and tandem repeat sequences (TRSs, variable in number and sequence) at the 5′ end of the *msp1α* gene (Cabezas-Cruz et al., 2013). Twelve *ms* structures of *msp1α* (A to L) were described (Cabezas-Cruz et al., 2013; Estrada-Peña et al., 2009; Fedorina et al., 2019) and the *ms* size was found to affect the MSP1α expression levels (Estrada-Peña et al., 2009). From the TRS deduced amino acid sequence, more than 300 MSP1α versions were identified in cattle and given alphanumeric names (Cabezas-Cruz et al., 2013; Castañeda-Ortiz et al., 2015; da Silva et al., 2015; de la Fuente et al., 2007; Fedorina et al., 2019; Hove et
al., 2018; Yang et al., 2017; Ybañez et al., 2014). These TRSs, which are known to be stable genetic markers to characterize strains, are conserved throughout the developmental cycle of rickettsia in cattle and ticks (Bowie et al., 2002; Kocan and de la Fuente, 2003). MSP1α varies in molecular weight among geographically distant isolates due to a varying number of TRSs located in the N-terminal region of the protein (Cabezas-Cruz et al., 2013; de la Fuente et al., 2001b). Many epidemiological studies found an association between A. marginale MSP1α sequence lineages and environmental factors, tick population, and livestock movement (Cabezas-Cruz and de la Fuente, 2015; Estrada-Peña et al., 2009; Guarnizo et al., 2020; Hove et al., 2018; Silva et al., 2015). However, the effect of antibiotic treatment has been not considered in terms of genotype selection. Several studies that attempted to sterilize A. marginale infections in cattle using antibiotics showed variable results (Coetzee et al., 2005; Coetzee et al., 2006b; Magonigle and Newby, 1982; Roby et al., 1978). The aim of this study was to evaluate the effect of the OTC treatment on the A. marginale isolate S1P by analyzing the msp1α gene sequence.

2. Materials and Methods

2.1 Isolate

The A. marginale isolate S1P (previously A2), was obtained from a 3-month-old calf with acute anaplasmosis (13 % packed cell volume and 6 % of parasitized erythrocytes) before antibiotic treatment, in northwestern Argentina, where the tick R. microplus is endemic (de Ríos et al., 1988). This isolate has long been characterized mainly by its pathogenicity traits and the ability to be transmitted by ticks (de Ríos et al., 1988; Aguirre et al., 1994; Gaido et al., 1995). The isolate was free of Ehrlichia, Babesia, Theileria and other Anaplasma species defined by reverse line blot hybridization assay
using TBD-RLB kit (Isogen life science). The isolate has been maintained in the hemoparasite collection of our laboratory at -196°C.

2.2 Experimental design

Blood samples stored at -20°C, previously obtained from steers born and raised in an anaplasmosis-free Holstein dairy herd located in a tick-free area, involved in a chemosterilization protocol using OTC, were selected (Sarli et al., 2020; Sarli et al., 2021). Briefly, the experiment consisted of i) multiplication of the *A. marginale* S1P in a splenectomized calf (donor); ii) inoculation of 10 normal steers (2-year-old) with 2-mL of the donor blood contained 10⁷ parasitized erythrocytes by subcutaneous injection (day 0); iii) treatment of steers with acute anaplasmosis using OTC, between 21 and 30 days post-infection (dpi); iv) *A. marginale* OTC sterilizing treatment at 78, 85, and 92 dpi, and v) subinoculation of 50 ml of blood from 2 steers refractory to *A. marginale* sterilization procedure into 2 splenectomized calves (receptors) to confirm the presence of the genotypes in steers at 182 dpi (Fig. 1) (Sarli et al., 2020).

Before the vaccination-challenge experiment, every bovine was confirmed to be free of *Anaplasma* spp. infection by cELISA and nested PCR (nPCR). Steers were maintained in different isolation pens and sprayed weekly with flumethrin (Bayticol1 Pour-On, Bayer) to protect them from biting flies and rule out the possibility of *A. marginale* cross-infection during the experiment (Sarli et al., 2020). All procedures were approved by the Animal Care Committee of the Faculty of Veterinary Sciences, National University of Litoral (Protocol number 243/15).

2.3 Sample collection

The selected samples were obtained from the donor calf during the clinical reaction at 30 dpi (sample 0, S0), from 10 experimentally infected steers at 14, 78, 119 and 182 dpi (S1, S2, S3, and S4 respectively), and from the two receptors bled 19 days after

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1 Bayticol
subinoculation (S5) (Fig. 1). For the characterization of the *msp1α* gene, genomic DNA was purified from blood samples using the phenol/chloroform method (Baravalle et al., 2012).

### 2.4 *Anaplasma marginale msp1α* gene amplification, cloning and sequencing

The *msp1α* gene sequence of a Florida isolate (GenBank accession No. M32871; Allred et al., 1990), was used to design the primers for amplification of a sequence containing *ms* and TRS by PCR. Platinum® Pfx DNA polymerase high-fidelity (Invitrogen, Thermofisher scientific, Waltham, MA) and the forward primer MSP1α1-1603F (5′-acctccgagtctacctctcgtt-3′) and the reverse primer MSP1α2-2926R (5′-tgctaaacgctgtcgtgggt-3′) were used. PCR conditions were (in a mixture of 1X Pfx amplification buffer, 0.3 mM (each) dNTPs, 0.3 µM (each) primer and 1 mM MgSO₄) 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 45 s, 68°C for 90 s, and a final extension of 68°C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis, purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI), cloned into pGEM®-T easy vector (Promega) and transformed into One Shot TOP10F’ *Escherichia coli* competent cells (Invitrogen). Recombinant plasmids from white colonies were purified using Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced using the SP6 and T7 promoter vector primers (Instituto de Biotecnología, INTA CICVya, Argentina and Macrogen, Seoul). Thirty clones of the S0 and between 5-10 clones from S1 to S5 were sequenced.

### 2.5 Sequence analysis

Sequence analysis was performed using the BLAST search (http://blast.ncbi.nlm.nih.gov/). Sequences were aligned, edited, and analyzed using BioEdit (Hall, 1999). Tandem repeat analysis was performed according to the proposed nomenclature (Cabezas-Cruz et al., 2013; de la Fuente et al., 2007). A 5′-UTR *ms*
located between the putative Shine-Dalgarno (SD) sequence (GTAGG) and the translation initiation codon (ATG) were used to assign a genotype (Estrada-Peña et al., 2009). Its structure is \((G/A\ TTT)m\ (GT)n\ T\ ATG\). The SD-ATG distance \((d)\) was calculated in nucleotides as \((4 \times m) + (2 \times n) + 1\) (Table 1). The RepeatAnalyzer software (Catanese et al., 2016) was used to identify new \(msp1\alpha\) repeats. Names X1 to X7 were given to novel repeats that were not recognized by this software.

3. Results

\(Anaplasma\ marginale\ msp1\alpha\) amplified by PCR from the donor calf (S0) showed a strong band of \(\approx 1,200\) bp and two very weak bands of \(\approx 1,000-1,100\) bp (Fig. 2). The most frequent genotype found in the 30 clones analyzed was \(E/\alpha\beta\beta\beta\beta\Gamma\) (73%) (Table 2). A previously undescribed TRS (X1) was identified (Table 2, Fig. 3).

A similar \(msp1\alpha\) pattern observed in the donor calf (S0) was amplified in all steers from the S1 (Fig. 2). Of the five genotypes observed at S0 (Table 2), three (\(D/\alpha\beta\beta\beta\beta\Gamma\), \(E/\alpha\beta\beta\beta\beta\Gamma\) and \(E/\alpha\beta\Gamma\)) were found at S1. \(E/\alpha\beta\beta\beta\beta\Gamma\) was the most frequently found genotype and was identified in all steers of S1, in three of S2 (after the acute infection treatment) and in five steers of S3 (41 days post-sterilizing treatment). Among the new genotypes found in the steers, the \(C/E\phi FF\) showed the highest frequency in S2, S3 and S4 (Table 3) and it was the only genotype identified in S4. After the subinoculation of blood from two steers refractory to OTC sterilization treatment into two receptor calves, a DNA sample was obtained and a 1,000-bp fragment was amplified (Fig. 2); the only genotype identified in that sample was \(C/E\phi FF\). The sequences obtained from S2, S3 and S4 were those expected according to the size of the main bands observed in the agarose gel. The 1,200-bp and 1,000-bp fragments corresponded to TRS \(\alpha\beta\beta\beta\beta\Gamma\) and TRS \(E\phi FF\), respectively (Fig. 2). Seven previously undescribed TRS (X1 to X7) were found in this study, five in repeat (R) 3, and one in R2 and R4 positions (Fig. 3).
In S0 (donor calf) and S1 (steers still not treated with OTC) only \( ms \) D (d = 21 bp) and E (d = 23 bp) were detected, and in S4 (refractory steers) and S5 (receptor calves) only \( ms \) C (d = 19 bp) was identified. The \( ms \) E-D/C ratio decreased in samples after antibiotic treatments (S1 > S2 > S3 > S4) (Table 3).

4. Discussion

In this study, the isolate S1P of \( A. \ marginale \), genotyped using two molecular markers (\( ms/\)TRS) of the single copy gene \( msp1\alpha \), showed different genotypes, with the E/\( \alpha\beta\beta\beta\Gamma \) being the most frequently identified in the donor calf (S0) and steers during acute illness (S1). After the restraint of acute infection with OTC, a different \( A. \ marginale \) genotype, C/\( \phi\FF \), was identified. This variant was the only one found in the five steers refractory to the treatment with OTC and in the two receptor calves subinoculated with the blood from two of those steers. These results show that the isolate S1P of \( A. \ marginale \) has several \( msp1\alpha \) genotypes, consequence of the superinfections expected in areas of high endemicity as described by Hove et al. (2018). This event would determine the differences in the abundance of genotypes and the variable sensitivity to OTC. The \( A. \ marginale \ msp1\alpha \) gene has been used as a marker of genetic diversity in epidemiological studies (Fedorina et al., 2019; Guarnizo et al., 2020; Silva et al., 2015), as a marker of strain identity in transmission studies (dos Santos et al., 2019), and to establish associations between superinfections and transmission by ticks (da Silva et al., 2015; Hove et al., 2018). In those studies, purified PCR products of the \( msp1\alpha \) gene or 2-7 clones were usually sequenced. In our study, the analysis of 30 clones obtained from the donor calf (S0) and 5-10 clones from each of the 10 steers before the OTC treatment (S1) was not enough to detect all genotypes of S1P in all cattle. Although the PCR product used for cloning included both strong and weak bands in the agarose gel (Fig. 2), only the main bands could be sequenced.
Similar electrophoretic patterns (main band and weak bands) were observed in different works, where only one genotype was identified (dos Santos et al., 2019; Lew et al., 2002; Palmer et al., 2001). This finding reflects the limitation of the technique to detect all msp1α genotypes present in a bovine when they vary in abundance.

As mentioned above, the genotypes found in S0 and S1 were not detected in all bovines, except for E/αββββГ, suggesting that all those genotypes were present in the S1P stabilate (-196°C), even though some of them were not identified. The variable detection of genotypes would depend on the relative abundance in the samples, strongly related to the phase of the rickettsemia cycle at the sampling time. Using a competitive PCR to amplify msp5 in persistently infected cattle, French et al. (1998) established cycles of bacteremia between $10^3$ and $10^7$ parasitized erythrocytes per ml of blood. Therefore, the presence of genotypes below the lowest rickettsemia level (approximately $10^{-5}$ % parasitized erythrocytes) may remain undetected by PCR.

The 93% and 7% of the clones from the donor calf genotyped by ms, were E and D, respectively, meanwhile in the receptor calves, 100% of the sequenced clones were genotype C. The SD-ATG distance (nucleotides) of the msp1α genotypes E, D and C are 23, 21 and 19, respectively (Estrada Peña et al., 2009). Dalbøge et al. (1988) established that the distance between the ribosome binding site (SD sequence) and the ATG initiation codon would affect gene expression in prokaryotes. Estrada Peña et al. (2009) showed that msp1α expression in E. coli was lower in the construct containing msp1α sequence with the lowest SD-ATG distance of 19 nucleotides, whereas no differences were observed between constructs containing the msp1α sequences with SD-ATG distances of 23 and 29 nucleotides. These observations could correlate with a higher expression of the MSP1α protein by the genotype E detected in higher abundance relative to D and C (not detected) in the S0 (donor calf) and the S1 (10 steers).
Garcia-Garcia et al. (2004) showed that the expression of the msp1α gene was greater in *A. marginale* derived from bovine erythrocytes than in *A. marginale* from cultured tick cells and tick salivary glands. The authors suggested that this difference in the gene expression might influence the infectivity of *A. marginale* for host cells, since the MSP1α protein is an adhesin required for the invasion. Although the putative pathogenicity differences among genotypes of S1P isolate was not evaluated in this work, higher invasion efficiency would explain the dominance of the genotype E during acute stages (stabilate, S0 and S1). Since the steers were subsequently treated with OTC, the pattern of the S1P genotypes could not be evaluated during the chronic stage. However, different authors reported that msp1α genotypes did not vary during the acute and chronic stages of *A. marginale* infection (Bowie et al., 2002; dos Santos et al., 2019; Palmer et al., 2001); consequently, the OTC would be responsible for the changes in the relative abundance among genotypes observed along the present study. The OTC treatment reduced or eliminated the genotype E and favor the expression of C, suggesting an efficient action of the antibiotic against the most prevalent genotype. At the usual recommended therapeutic dose, OTC interrupts the geometric multiplication of *A. marginale* without clarifying the infection, despite the subsequent immune response (Stewart et al., 1979). In this study, after the OTC treatment, the different genotypes (*ms*/TRS) detected at the acute stage, including the dominant E/αββββГ, were gradually lost and the genotype C/EϕFF (1,000 bp) was the only one detected at the end of the study. We hypothesized that the faint band of about 1,000-bp identified in S0 and S1 may correspond to the C/EϕFF genotype, which becomes detectable after the OTC treatment. These results are the first evidence of a varying susceptibility of *A. marginale* msp1α genotypes to OTC.
The C/EϕFF genotype was detected not only in the five steers refractory to OTC treatment and in the two receptor calves, but also in four of the five remaining animals before the A. marginale infection would have been eliminated (Table 3). This result indicates that C/EϕFF had a lower sensitivity to OTC than the other genotypes; suggesting that the outcome of the sterilization treatment would depend on other factors. Many protocols using OTC have been evaluated to eliminate A. marginale infection of carriers, with different results. Some treatments were successful (Magonigle and Newby, 1982; Roby et al., 1978; Swift and Thomas, 1983) and others failed (Coetzee et al., 2005; Coetzee et al., 2006b; Goff et al., 1990; Hollis et al., 2004). These discordant results could be due to differences in the design of the experiment related to the treatment protocol (dose, route, number and interval of inoculations, time elapsed between infection and treatment), selected cattle (breed, age), and susceptibility of the A. marginale isolate to the antimicrobial (Kocan et al., 2015). Different responses to antibiotic treatment were observed between Jaboticabal and Palmeira A. marginale strains (Alonso et al., 2020) and between Virginia and Oklahoma isolates (Coetzee et al., 2006a). The two multidrug resistance efflux pumps identified in the genome of A. marginale could explain the different susceptibility of the isolate to the antibiotic (Brayton et al., 2005). In those cattle whose infection was chemosterilized, it still was frequently to detect specific DNA after the disappearance of antibodies (Coetzee et al., 2006a; Goff et al., 1990; Sarli et al., 2020). Therefore, the genotypes identified in S3 (41 days after sterilization treatment) could belong to dead Anaplasma bodies and the results obtained in S4 (104 days after the sterilization treatment) when the clearance was achieved, would be more trustworthy. Future studies should consider the use of tests with a higher sensitivity and specificity to identify the relative abundance of each of the genotypes present in an isolate. To the best of our knowledge, this is the first report
where the treatment with OTC altered the pattern of the msp1α genotypes of an isolate of *A. marginale*.

5. Conclusions

OTC affected the relative abundance of the genotypes present in the isolate S1P of *A. marginale*. After the sterilizing treatment, some genotypes were eliminated, showing differences in sensitivity to the OTC among them; this result would explain the partial success of the antibiotic to clear the infection in the steers. Treatment failure would not depend solely on the variant sensitivity but on other, still not established factors.

Declaration of interest

None

Author contributions

**María Evangelina Primso:** Conceptualization, Methodology, Formal analysis, Visualization, Writing- Original draft preparation, Funding acquisition

**Macarena Sarli:** Methodology

**Carolina Soledad Thompson:** Writing- Reviewing and Editing

**Susana Marta Torioni:** Writing- Reviewing and Editing

**Ignacio Eduardo Echaide:** Visualization, Writing- Reviewing and Editing

Acknowledgements

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Reference


Theiler, A., 1910. *Anaplasma marginale* (Gen. and spec. nov.) The marginal points in


**Table 1.** Structure of the *Anaplasma marginale* msp1α microsatellite (ms).

<table>
<thead>
<tr>
<th>Microsatellite genotype</th>
<th>m</th>
<th>N</th>
<th>SD-ATG distance (nucleotides)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>7</td>
<td>19</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>5</td>
<td>19</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>6</td>
<td>21</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>7</td>
<td>23</td>
</tr>
<tr>
<td>F</td>
<td>3</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>G</td>
<td>3</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>H</td>
<td>3</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>4</td>
<td>6</td>
<td>29</td>
</tr>
<tr>
<td>J</td>
<td>1</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>K</td>
<td>2</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>L</td>
<td>1</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

The microsatellite was located between the Shine-Dalgarno (SD) sequence and the translation initiation codon (ATG) with the structure: GTAGG (G/A TTT)m (GT)n T ATG. The SD-ATG distance (d) was calculated in nucleotides as $(4 \times m) + (2 \times n) + 1$ (Cabezas-Cruz et al., 2013; Estrada-Peña et al., 2009; Fedorina et al., 2019).

**Table 2.** *Anaplasma marginale* S1P msp1α genotype, defined by microsatellite (ms) and tandem repeat sequence (TRS) in the infected donor calf (sample 0).

<table>
<thead>
<tr>
<th>Number of clones</th>
<th>Genotype</th>
<th>ms</th>
<th>TRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>D</td>
<td>αββββГ</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>E</td>
<td>αββββГ</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. *Anaplasma marginale* msp1α genotype defined by microsatellite (*ms*) and tandem repeat sequences (TRS) detected in steers different days after experimental infection.

<table>
<thead>
<tr>
<th>Steer (N°)</th>
<th>Sample 1 (S1) day 14</th>
<th>Sample 2 (S2) day 78</th>
<th>Sample 3 (S3) day 119</th>
<th>Sample 4 (S4) day 182</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N  (ms)  TRS</td>
<td>N  (ms)  TRS</td>
<td>N  (ms)  TRS</td>
<td>N  (ms)  TRS</td>
</tr>
<tr>
<td>1</td>
<td>4 E  (\alpha\beta\beta\beta\Gamma)</td>
<td>5 C  E(\phi)FF</td>
<td>5 C  E(\phi)FF</td>
<td>6 C  E(\phi)FF</td>
</tr>
<tr>
<td></td>
<td>1 E  (\alpha\beta\alpha\beta\Xi\Gamma)</td>
<td>1 E  (\alpha\beta\beta\beta\beta\Gamma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5 E  (\alpha\beta\beta\beta\Gamma)</td>
<td>10 C  E(\phi)FF</td>
<td>10 C  E(\phi)FF</td>
<td>5 C  E(\phi)FF</td>
</tr>
<tr>
<td></td>
<td>1 D  (\alpha\beta\beta\beta\beta\Gamma)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5 E  (\alpha\beta\beta\beta\Gamma)</td>
<td>3 C  E(\phi)FF</td>
<td>7 C  E(\phi)FF</td>
<td>PCR negative</td>
</tr>
<tr>
<td></td>
<td>1 D  (\alpha\beta\beta\beta\beta\Gamma)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>4 E  (\alpha\beta\beta\beta\Gamma)</td>
<td>5 C  E(\phi)FF</td>
<td>4 C  E(\phi)FF</td>
<td>5 C  E(\phi)FF</td>
</tr>
<tr>
<td></td>
<td>1 E  (\tau\alpha\beta\beta\beta\Xi\Gamma)</td>
<td></td>
<td></td>
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N: number of clones.
Figure 1. Chemosterilization assay protocol. DNA samples (S) of the donor calf (S0), from 10 steers at 14, 78, 119 and 182 days post-infection (S1, S2, S3 and S4, respectively), and from the 2 receptor calves (S5) were analyzed. OTC; oxytetracycline; dpi: days post-infection; dpt: days post-sterilizing treatment.

Figure 2. *Anaplasma marginale* msp1α PCR products separated in 1% TAE-agarose gel. MW: 100-bp DNA ladder (PB-L Productos Bio-Logicos®, Argentina); S0: sample obtained from the donor calf on the day of infection; S1, S2, S3, and S4: samples obtained from experimentally infected steers at 14, 78, 119 and 182 days post-infection (dpi), respectively; and S5: samples from two calves subinoculated with blood from steer 4 (S5₄) or from steer 8 (S5₈) at 19 dpi. The band pattern observed for steers 4 and 6 was identical to that of steer 2; the band pattern observed for steer 9 and 10 was identical to that of steer 5; and the band pattern observed for steer 7 was identical to that of steer 3.

Figure 3. New tandem repeat sequences of *A. marginale* MSP1α identified in this study. The one-letter amino acid code was used to depict the differences found in
MSP1α repeats. Dots indicate identical amino acid sequences. Tandem repeat A was used as a model for comparison (de la Fuente et al., 2007).