Babesiosis: Field assessment of protection in cattle immunized with a mixture of *Babesia bovis* recombinant proteins

Babesiosis bovina: Evaluación en campo de la protección de bovinos inmunizados con una mezcla de proteínas recombinantes de Babesia bovis

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ABSTRACT

The aim of the present research was to assess the immunogenicity and protective capability of a combination of the *B. bovis* recombinant proteins MSA-1, MSA-2c and 12D3 in cattle exposed to *Babesia* infection transmitted by ticks under natural conditions. The immunization phase of the study was conducted at CENID-Parasitología Veterinaria in Jiutepec, Morelos, and the challenge phase was carried out in an experimental station in Pichucalco, Chiapas, Mexico. Eighteen steers were randomly distributed into three groups of six animals each. Animals were treated on days 0 and 14 as follows: Group 1 (G1), control, with the adjuvant Montanide 70 (M70) + PBS; Group 2 (G2) with a mixture of the *Babesia bovis* recombinant proteins MSA-1, MSA-2c and 12D3 admixed in M70; Group 3 (G3) with a live attenuated mixed vaccine against bovine babesiosis (day 0 only). Immunization of cattle with a combination of recombinant proteins from *B. bovis* induced specific IgG antibodies that recognized the corresponding antigens of *B. bovis* by three immunological assays: Indirect fluorescent antibody test (IFAT), Enzyme-linked immunosorbent assay (ELISA) and Immunolotting (IB). However, the combination of the *B. bovis* recombinant proteins MSA-1, MSA-2c and 12D3 did not produce an adequate protection. Three animals in GII group were in need of treatment with specific babesiacide, indicating that the specific immune response evoked by these steers, was not necessarily protective (50% protection), as compared to the protection evoked by the attenuated vaccine (only 1 G3 steer required treatment, >80% protection). The immunization of cattle with a combination of recombinant proteins of *B. bovis* induced specific antibodies that recognized the corresponding antigens when analyzed by immunological assays. The combined use of *B. bovis* recombinant proteins MSA-1, MSA-2c, and 12D3 does not produce an adequate immune protection to tick-transmitted *Babesia* parasites as evidenced by the presence of severe signs of babesiosis in the experimental animals. The results, however, are encouraging and support the idea of finding a more suitable combination of recombinant proteins for successful protection of cattle facing a natural challenge with the parasite transmitted in the field by *Rhipicephalus* (*Boophilus*) *microplus* ticks.

Key words: Babesiosis, vaccination, recombinant antigens, MSA-1, MSA-2c, 12D3

RESUMEN

El objetivo de la presente investigación fue evaluar la inmunogenicidad y capacidad protectora de una combinación de las proteínas recombinantes MSA-1, MSA-2c y 12D3 de *B. bovis* en bovinos expuestos a la infección por *Babesia* transmitida por las garrapatas en condiciones naturales. El estudio se llevó a cabo en su fase de inmunización en el CENID-PAVET, en Jiutepec, Estado de Morelos, y la fase de desafío en una estación experimental en el municipio de Pichucalco, Chiapas, México. Dieciocho novillos se distribuyeron al azar en tres grupos de seis animales cada uno que se trataron en los días 0 y 14 de la siguiente manera: Grupo 1 (G1), control, con el adyuvante Montanide 70 (M70) + PBS; Grupo 2 (G2) con una mezcla de las proteínas recombinantes MSA-1, MSA-2c y 12D3 de *Babesia bovis* mezclados en M70; Grupo 3 (G3) con una vacuna viva atenuada mixta contra babesiosis bovina (día 0 solamente). La inmunización de bovinos con una combinación de proteínas recombinantes de *B. bovis* generó anticuerpos IgG específicos que reconocen los antígenos correspondientes de *B. bovis* por medio de tres ensayos inmunológicos: prueba de inmunofluorescencia indirecta (IFAT), ensayo inmunoenzimático ligado a enzima (ELISA) e Inmunoelectrotransferencia (IB). Sin embargo, a la confrontación el uso combinado de las proteínas recombinantes MSA-1, MSA-2c y 12D3 de *B. bovis* no produjo una protección adecuada. Tres de los animales del grupo GII requirieron tratamiento con un babesiacida, indicando que la respuesta inmune específica inducida por estos antígenos no fue protectora (50% de protección), comparada con la protección inducida por la vacuna viva atenuada (donde solo 1 novillo del grupo G3 requirió tratamiento, >80% protección). La inmunización de bovinos con una combinación de proteínas recombinantes de *B. bovis* induce anticuerpos específicos que reconocen los correspondientes antígenos mediante ensayos inmunológicos. El uso de un combinado de las proteínas recombinantes MSA-1, MSA-2c, and 12D3 de *B. bovis* no produce una adecuada respuesta inmune protectora en contra de *Babesia* transmitida por garrapatas, evidenciado por la presencia de signos clínicos de la enfermedad en los animales experimentalmente inmunizados. Los resultados, sin embargo, son alentadores y apoyan la idea de encontrar una combinación más adecuada de proteínas recombinantes para la protección exitosa de ganado frente a una confrontación con el parásito transmitido en el campo por garrapatas *Rhipicephalus* (*Boophilus*) *microplus*.

Palabras clave: Babesiosis, vacunación, antígenos recombinantes, MSA-1, MSA-2c, 12D3

INTRODUCTION

Worldwide, babesiosis is one of the most important parasitic diseases of cattle. It is a tick-transmitted disease caused by protozoa of the *Babesia* genus, characterized by haemolytic anemia and fever, with occasional hemoglobinuria and death (Ristic, 1981; Bock et al., 2004; Hunfeld et al., 2008). The huge costs due to babesiosis have been estimated considering mortality, abortions, loss of cattle production (milk/meat), and control measures. In Mexico, 75% of the 23 316 000 cattle heads (INEGI, 2009) are at risk of acquiring babesiosis (Mosqueda, 2004). Attenuated live vaccines are available (Álvarez y Figueroa, 2007; Shkap et al., 2007; Seitzer y Ahmed, 2008; Florin-Christensen et al., 2014). However, some of these vaccines still have a few drawbacks. For example, the requirement of fresh bovine erythrocytes and serum from specific donors and highly stringent conditions for the maintenance of blood donors for *Babesia* cultures and vaccine production (Álvarez y Figueroa, 2007; Shkap et al., 2007; Florin-Christensen et al., 2014); whereas for vaccine production derived from splenectomized calves, the occasional causation of severe clinical signs after vaccination (Shkap et al., 2005), including death of some of the vaccinated animals (Combrink et al., 2012). In this context, vaccination with recombinant protein antigens has been suggested as an approach for the control of bovine babesiosis (Wright et al., 1992; Hines et al., 1995; Brown et al., 2006; Fish et al., 2008, Willadsen, 2008; Álvarez et al., 2010; Florin-Christensen et al., 2014). *Babesia bovis* MSA-1 and MSA-2c antigens are expressed on the merozoite surface and are involved in the invasion of the parasite into the bovine erythrocyte (Brown et al., 2006; Florin-Christensen et al., 2014). A glycoprotein, 12D3, is localized in the apical complex of the merozoite and is released in soluble form into the cytoplasm of the erythrocyte during parasite division (Harper et al., 1996). The *msa-1* gene locus describes a single-copy gene lacking an intron with an open-reading frame of 961 bp that codes for a 42-kDa membrane glycoprotein (Hines et al., 1995). The *msa-2c* is a single-copy gene lac-

king an intron that contains a 795-bp-long coding sequence that is translated into a 30-kDa protein (Florin-Christensen et al., 2002). The *12d3* gene contains a 1038 bp coding sequence that encodes for a 41-kDa protein (Hope et al., 2005). Previous studies have shown that genes coding for MSA-1, MSA-2c and 12D3 are relatively highly conserved in different *B. bovis* isolates from distinct geographical regions in Mexico (Borgonio et al., 2008; Pérez et al., 2010). The high sequence identity conservation in the Mexican *B. bovis* isolates analyzed suggested that MSA-1, MSA-2c and 12D3 antigens would be good candidates for evaluation in cattle as a recombinant vaccine, so that a controlled trial was carried out. In this, immunization of *Bos taurus* steers with rMSA-1-rMSA-2c-rMSA-r12D3 proteins was not sufficient to prevent clinical signs against challenge; however, the immune response elicited was sufficient to protect steers against a mild virulent strain of *B. bovis* (Álvarez et al., 2010). Based on the previous information, the objective of the present study was to assess the immunogenicity and protective capability of a combination of the *B. bovis* recombinant proteins MSA-1, MSA-2c and 12D3 in cattle exposed to *Babesia* infection transmitted by ticks under natural conditions.

MATERIALS AND METHOD

Use of animals

Animal experiments were conducted in accordance with the Principles for the Care and Use of Animals in Research, and based on the concepts for protection of animal life, health and welfare indicated in the current 'Ley Federal de Sanidad Animal' in Mexico. The protocol was approved by the 'Grupo Colegiado Científico Técnico' (GCCT) at CENID-Parasitología Veterinaria and authorized by INIFAP (A Public Research Center of the Mexican Federal Government) through Project number 16321431988.

Experimental design

The study carried out consisted of a prospective, longitudinal, comparative and experimen-

tal investigation. The experimental design included 18 *Bos taurus* steers (crosses of different breeds), nine to 10 months old, free of *Babesia spp.* as determined by IFAT, ELISA and PCR assays, and free of *Rhipicephalus* (*Boophilus*) *spp.*, which were randomly distributed into three groups of six animals each. They were allocated in pens at CENID-PAVET, in Jiutepec, Morelos State, fed with oat straw and bagasse, supplemented with a commercial feed concentrate, and provided with water *ad libitum* during the phase of immunization. Thirty days post-immunization, the animals were translocated to an experimental station in Pichucalco municipality, Chiapas State, to be challenged with tick-borne *Babesia spp.* in the field, where they fed on native grass supplemented with a commercial concentrate.

Recombinant proteins

Antigens MSA-1, MSA-2c and 12D3 were prepared from *B. bovis*-infected erythrocytes (Sánchez, 2010). Briefly, PCR assays were performed, and primer sets msa-1FN 5'-aatgccgatacttcaatcgtc-3'/msa-1R 5'-aaatgcagagagaacgaagtagc-3'; msa-2cFN 5'-caggtgaatgggagtcatttactgtttg-3'/msa-2cR 5'-aaatgcagagagaacgaagtagcagagagt-3', and 12D3FN 5'-gatcagaat ggtaagctggttcc-3'/12D3R 5'-aagctcctgcctttcgctc-3' were used to amplify *msa-1*, msa-2c and *12D3* truncated coding sequences, respectively, lacking the first 90–96 amino terminal nucleotide residues and the stop codons in each gene. Amplicons were cloned into the pBAD/TOPO thio-expression system (Invitrogen Carlsbad, CA, USA), and expressed as fusion MSA-1-, MSA-2c- and 12D3-thioredoxin proteins, respectively, as described previously (Sánchez, 2010; Álvarez et al., 2010). Plasmid DNA served as template for sequencing by the dideoxy-chain termination method using an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were subjected to a homology search using the BLASTN application (http:// blast.ncbi.nlm.nih.gov/Blast.cgi). The coding sequences obtained for the *msa-1* and *msa 2c* genes were 100% identical with those reported previously for the *B. bovis* RAD

clone (GenBank accession numbers EF640942 and EF640955, respectively) (Borgonio et al., 2008; Genis et al., 2008). The *B. bovis* RAD clone 12D3 sequence obtained was 99% identical to the Texas T2Bo identified previously in the genome of *B. bovis* (NCBI Reference sequence: XM_001610687; Genebank accession number 1233106) (Pérez et al., 2010). The recombinant version of the three *B. bovis* antigens (rMSA-1, rMSA-2c and r12D3 proteins) were purified by affinity chromatography in NI-NTA resin columns and analyzed by SDS-PAGE electrophoresis.

Expression of recombinant proteins from *Babesia bovis*

The genes encoding the recombinant proteins from *B. bovis* merozoite MSA-1 and MSA-2c, as well as protein complex called apical excreted-secreted 12D3 were obtained from cultures of *E. coli* containing the expression vector pBAD/thio-TOPO carrying each of the *B. bovis* genes (Sánchez, 2010; Álvarez et al., 2010). Briefly, the transformed cells were plated on LB agar medium containing ampicillin (50 mg/ml) and were subsequently grown in LB liquid medium by incubation under stirring at 37 °C overnight. After arabinose induction at 20% and centrifugation at 4 000 rpm at 4 °C for 10 minutes, the pellets recovered were added with a lysis solution containing lysozyme (1 mg/ml) to the cultures expressing the recombinant protein MSA-2c (rMSA-2c), and resuspension solution containing sarcosyl (1% v/v) for cultures expressing recombinant MSA-1 protein (rMSA-1) and 12D2 (r12D3). Protease inhibitors, DNases, and RNases were added, incubating for 30 minutes at 4 °C, and pellets were subsequently sonicated three times for 25 seconds with 15 seconds intervals. Next, the lysates were centrifuged at 10 000 rpm for 10 minutes at 4 °C. Supernatants corresponding to purify recombinant proteins were recovered by affinity chromatography on nickel resin column containing Ni- NTA. For this, the supernatant containing rMSA-1 and r12D3 it was added Triton X-100 at a final concentration of 2%. After washing the column with fixing (1x) and washing (2x) solutions, recombinant eluted proteins were

analyzed by separation of components on polyacrylamide gels at 12%, stained with Coomassie blue and were stored at 20 °C until use.

Immunization phase

The immunization phase of the experiment was carried out at CENID-PAVET, INIFAP, at Jiutepec, Morelos State, located in central Mexico. The animals in GI group (Control, n = 6) were inoculated subcutaneously with 500 ml of Montanide 70 + 500 ml PBS to a final volume of 1 ml/animal on days 0 and 14 of the experiment. Steers in GII group (n = 6) were inoculated on days 0 and 14 with 300 μg of recombinant proteins (100 μg each) diluted in PBS + adjuvant in a final volume of 1 ml/animal, whereas the animals in the GIII group (n = 6) were inoculated on day 0 with a live attenuated vaccine derived from in vitro culture and maintained in liquid nitrogen at -196 °C containing 1 x 108 *B. bovis* infected erythrocytes estimated previous to cryopreservation (Álvarez et al., 2004). It has been observed that there is no a 100% effective cross-immunity between *B. bovis* and *B. bigemina* (Vega et al., 1999), so in order to be able to determine and allocate more specifically the protection conferred by the recombinant proteins in calves exposed to a natural challenge by *B. bovis* transmitted by ticks, all animals were premunized with 1 x 108 infected erythrocytes with *B. bigemina* (Figueroa et al., 1998a) prior to their transferring to the experimental station in Pichucalco, Chiapas State, an enzootic area for bovine babesiosis located in Southeast Mexico.

Field challenge

The animals were translocated to the experimental station at Pichucalco at day 30 post-immunization and they were immediately introduced into a pasture paddock naturally infested with common cattle ticks.

Clinical monitoring and sampling after vaccination

Once exposed to the natural infestation with *Rh. microplus* ticks for transmission of *B. bovis*, the steers were monitored from day 30 to 51 post-immunization to determine clinical findings. Blood samples were taken by using heparin as anticoagulant and the presence of specific antibodies against *B. bovis*-infected erythrocytes was determined in blood plasma by the IFAT assay (Goldman et al., 1972) and by ELISA using as antigen each of the recombinant proteins (Castañeda et al., 2013). To prevent death of experimental animals, the babesiacide treatment of any affected animal was based on the following criteria: A drop of more than 25% in packed cell volume (PCV); a rectal temperature (RT) above 40 °C on three consecutive days; and a parasitemia of *B. bovis* as determined by examination of Giemsa stained smears using a 100X objective.

IFAT

This test was carried out in accordance with Goldman et al. (1972) using blood smears of erythrocytes infected with *B. bovis* derived from in vitro-cultured parasites as substrate antigen, the serum from experimental animals as the primary antibody, and sheep anti-bovine IgG conjugated with fluorescein isothyocianate as the secondary antibody.

ELISA

For this test, each of the recombinant proteins were used as antigen and was performed in 96 wells flat bottom plates as described previously (Castañeda et al., 2013) with slight modifications: Recombinant antigens were diluted in coating buffer at a final concentration of 1 μg/ml and 150 μl were placed in each well of the ELISA plate. After overnight incubation at 4 °C, the diluted antigen not adhering to the ELISA plate was discarded, followed by three washes with PBS-solution containing 0,05% (v/v) Tween 20 for 5 min each (100 μl/well). Plates were blocked with 100 μl of PBS-skim milk 5% and were incubated for 1 h at room temperature. Next, plates were washed three times using PBS-Tween 20. Serum samples diluted 1:200 were set up by duplicate (150 μl/well) and incubated for 1 h at room temperature, followed by three washes with PBS-Tween 20. Then, 100 μl of rabbit anti-IgG bovine conjugated with alkaline phosphatase (diluted 1:1 000 in PBS-Tween 20-skim milk) were added to each well. Finally, after the

addition of p-nitrophenyl-phosphate substrate, readings were carried out in an ELISA reader with a filter of 495 nm and optical reads were recorded.

Immunoblotting (IB)

Briefly, purified proteins (1 µg/µl) were mixed with sample buffer and placed in boiling water for 5 minutes followed by 1 minute on ice. Samples were loaded and separated in a 13% polyacrylamide gel at 80 milliampers by using an electrophoresis unit. Next, the electrophoresed proteins were transferred from the gels into nitrocellulose membranes using a commercially available gel transfer system. Subsequently, membranes were blocked by adding PBS solution containing 0,05% (v/v) Tween 20 and 3% (w/v) skimmed milk for 1 hour. After 3 washes with PBS-Tween 20, serum samples diluted 1:100 in PBS were added and incubated overnight at 4 °C. Membranes were washed three times with PBS-Tween 20 and then added with alkaline phosphatase-conjugated anti-bovine IgG diluted 1:5 000 and incubated while stirring for 60 minutes at room temperature. After three washes with PBS-Tween 20 the BCIP/NBT substrate was added as recommended by the manufacturer (Sigma fast BCIP/NBT). Samples analyzed were those collected at day 0, 15, 30 and 52 post-immunization.

Statistical analysis

The significant differences, if any, among the means of variables analyzed (Antibody levels in the ELISA test; Rectal temperature, Packed cell volume and Percent parasitemia values) were examined by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for pairwise comparison of data from the multiple groups (Statgraphics centurion XVI Software, StatPoint Technologies, Inc., Warrenton, VA, USA). Results were considered to be statistically significant when $p < 0,05$.

RESULTS

In order to assess the immunogenicity of a combination of *B. bovis* recombinant proteins in cattle, experimental steers were inoculated with the recombinant version of MSA-1, MSA-2c

and 12D3 antigens. By using the IFAT with *B. bovis*-infected erythrocytes as antigen serum samples collected from each of the immunized Group II steers were screened weekly in search of seroconversion to the *B. bovis* native antigens. Three out of six animals showed a positive, although weak IFAT reactivity at day 14 post-primary immunization with the recombinant proteins. After the second immunization with recombinant proteins, all six GII animals were IFAT positive when tested at day 21 post-immunization showing a relatively strong fluorescence reaction in two of the animals. (Figure 1)

As shown in figure 1A, steers from GII had antibodies in their serum that recognized parasite-derived antigens present in both the trophozoite (single, round shape) and merozoite (double, piriform shape) stages (Figure 1C, 1D), whereas the serum sample from an steer vaccinated with live, attenuated *B. bovis*-infected erythrocytes reacted with both, the *Babesia* parasites and the infected erythrocytes. (Figure 1B)

To more precisely define the specificity of the humoral response to *B. bovis* recombinant antigens the IB assay was performed on the serum samples from GII steers. As expected, on the first date tested (day 0), no immunochemical signal was obtained (Figure 2A, representative of results obtained with serum samples from Group II animals). At day 15 post-immunization, the sera of GII animals showed antibodies recognizing all three *B. bovis* recombinant proteins, although rMSA-2c is weakly detected, yet there is no recognition of the native protein (Figure 2B). At the third date tested (30 days post-immunization) the rMSA-1 and r12D3 proteins are recognized much more intensely than in previous dates and clearly contrasted with the signal obtained with rMSA-2c. A weak signal in the region of 40 kDa is recognized in the native *B. bovis* antigen (Figure 2C). In the fourth sample date tested (52 days post-immunization, 21 days after introduction to tick-infested paddocks), the rMSA-2c protein is no longer detected, while the rMSA-1 and r12D3 proteins continue to have the same signal intensity, and
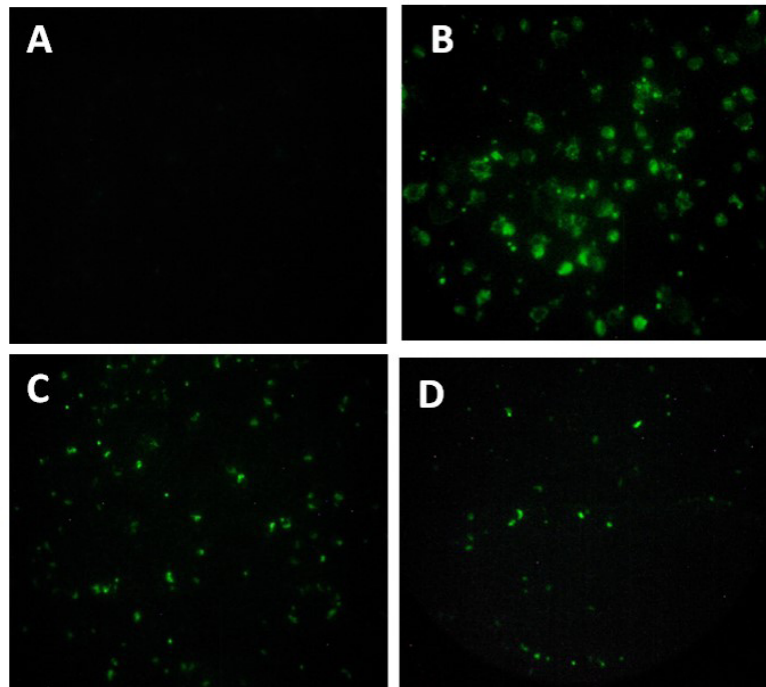
Figure 1. Representative IFAT assay result using bovine sera collected on day 28 post-immunization. A) Negative bovine (GI); B) Positive *B. bovis*-infected bovine (GIII); C) Bovine 13 (GII); D) Bovine 15 (GII).

there is a stronger signal recognition of native antigens consistent with the expected size of approximately 41-42 kDa of native *B. bovis* MSA-1 and 12D3 antigens. (Figure 2D)

The results of serological analysis performed by ELISA, using recombinant proteins as antigens, demonstrated seroconversion at day 10 after first immunization in the GII animals inoculated with the recombinant proteins. The antibody response was apparently faster and of greater magnitude than that determined in the control GI steers and the GIII steers inoculated with the live attenuated vaccine. For the particular case of rMSA-1 protein, seroconversion in the GII steers was significantly higher, as determined by the absorbance values, than that found in the GI and GIII steers (Figure 3).

In GII steers, animals showed significantly higher absorbance levels of specific antibodies against rMSA-1 protein as compared to those of the control group (GI) and those inoculated with the live attenuated vaccine (G III).

The antibody production levels against the rMSA-2c protein showed to be very low, variable, and irregular for all groups, as shown in Figure 4, and mean absorbance value differences were not statistically significant.

With regard to the level of antibodies produced against r12D3 protein these were significantly higher in the GII steers (inoculated with the recombinant proteins) as compared with the level found in the GIII steers, inoculated with the live attenuated vaccine, and the GI group that was inoculated with adjuvant alone. (Figure 5)
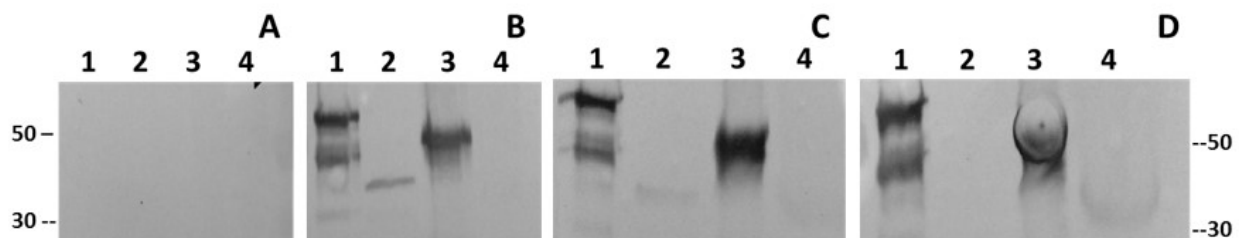

Figure 2. Representative IB assay with serum from GII animals analyzed at day 0 (A), 15 (B), 30 (C) and 52 (D) post-immunization with recombinant proteins. Lane Identification: 1) Recombinant protein MSA-1; 2) MSA-2c; 3) 12D3; 4) protein extract of B. bovis-infected erythrocytes. Left and right outside numbers indicate relative molecular weights (kDa) of commercial markers.
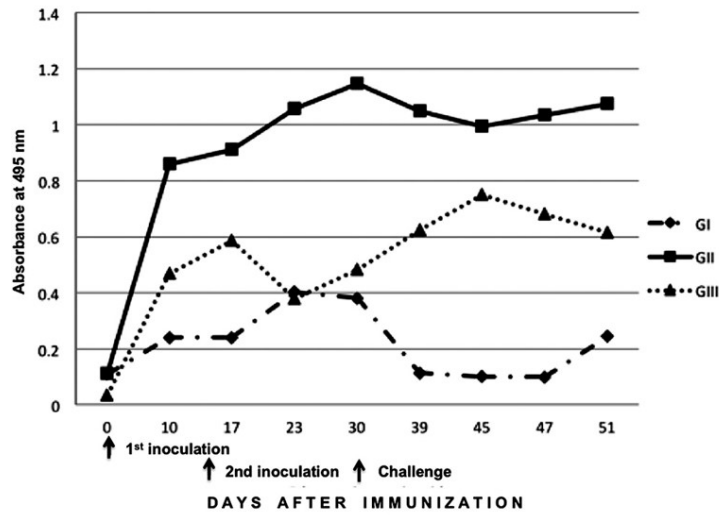
Figure 3. Antibody levels to *Babesia bovis* rMSA-1 protein as determined by ELISA.
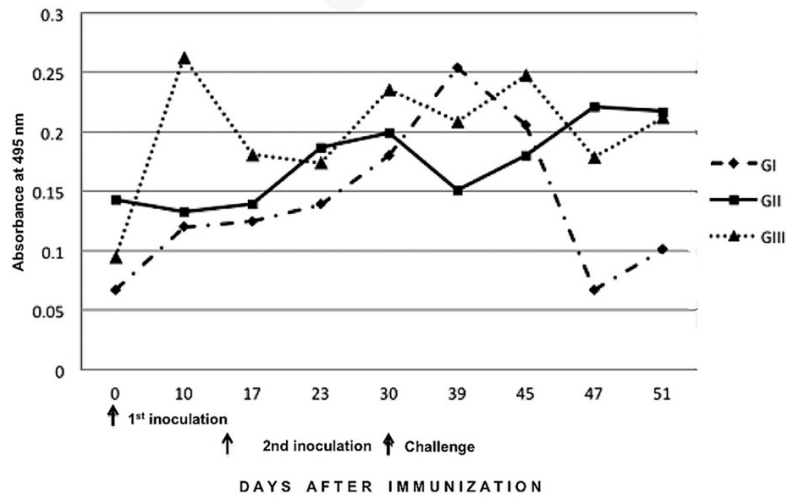

Figure 4. Antibody levels to *Babesia bovis* rMSA2c protein as determined by ELISA.
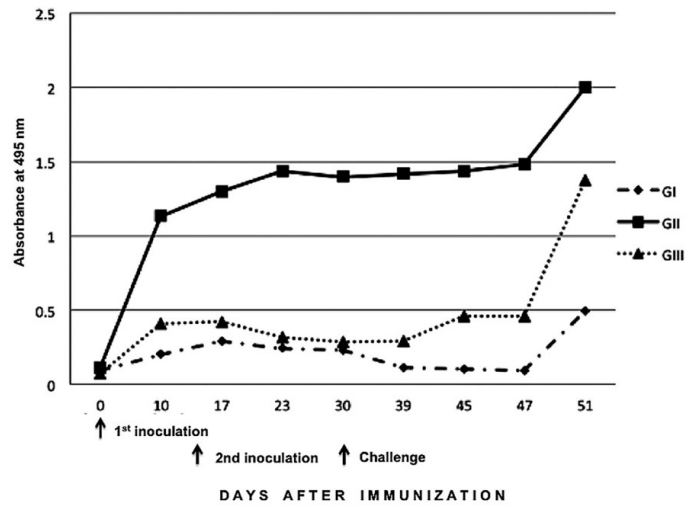

Figure 5. Antibody levels to *Babesia bovis* r12D3 protein as determined by ELISA

Rectal temperature (RT)

A fever exceeding 40 °C was determined around day 9, 10, and 9 post-introduction to the paddock (PIP) for the GI, GII, and GIII steers, respectively. Similarly, a total average of 5,17, 3,83 and 3,0 days with rectal temperatures higher than 40 °C was estimated for GI, GII, and GIII groups, respectively (Table 1). Nonetheless, mean RT value differences were not statistically significant.

Packed cell volume (PCV)

Steers of the three groups showed a decrease in PCV beginning on day 13 PIP, reaching their maximum decrease on days 15, 17 and 16 PIP, with an average (± SD) of 41,77 ± 10,24%; 58,15% ± 8,62 and 42,43 ± 14,33% for the GI, GII and GIII steers, respectively (Table 1). Again, however, mean PCV value differences were not statistically significant.

Percent parasitized erythrocytes (PPE)

B. bovis parasitaemia was observed during 14 days in GI steers reaching a maximum PPE of 0,052; whereas the GII steers had a total of 11 days of parasitaemia with a maximum PPE of 0,185. In contrast, in the GIII steers the parasitaemia lasted nine days, reaching a maximum PPE of 0,218 (Table 1). Again, mean PPE value differences were not statistically significant.

Treatment

Two, three and one animal from each of the GI, GII, and GIII steers, respectively, received one dose of imidocarb (5 mg/kg body weight) intramuscularly to avoid death by bovine babesiosis caused by *B. bovis*. (Table 1)

DISCUSSION AND CONCLUSIONS

The results obtained in this study are comparable to those obtained under controlled conditions, in which a group of experimental animals were immunized with the same recombinant proteins, but challenged by inoculation with blood infected with a *Babesia bovis* isolate of mild virulence (Alvarez et al., 2010); however, it is noteworthy that in this study, several factors such as the change in environment, the stress of transport, as well as the presence of ticks and the daily handling of the experimental animals have a direct influence on the steers that could have rendered the animals more susceptible to infection with *Babesia bovis*, *B. bigemina*, or both, particularly as the virulence of the field microorganisms may also be greater than that one used in a previous study (Álvarez et al., 2010). Additionally, the presence of flies, mosquitoes and the change of feed, may alter the immune response of animals to fight infections, especially those transmitted by ticks.

The effectiveness of recombinant vaccines has been questioned; particularly for those based on fusion proteins. In this particular case, with thioredoxin as the fused protein, the fusion assemble may change the quaternary conformational structure of the recombinant protein, directly affecting exposure of the antigenic determinants and thus its immunogenic or immunoprotective capacity, in such a way that it cannot confer the desired protection. On the other hand, it has been indicated that the use of various recombinant proteins at the same time might produce better results than when a single protein is used as an immunogen (Hope et al., 2005; Willadsen, 2008). However, immunological interference can also be present when using a cocktail of recombinant proteins, thus making it difficult the design of an adequate vaccine cocktail. (Elias et al., 2013)

It has been reported that specific antibodies against surface proteins of the *B. bovis* mero-

Table 1. Immunization trial carried out with 18 steers translocated to bovine babesiosis endemic area for exposure to *R.* (*Boophilus*) *microplus* ticks

Group	Mean (SD)			
	% Decrease in (PCV)	Days with RT higher than 40 ºC	Days of detectable PPE*** (highest PPE)	Animals receiving Chemotherapy after field challenge
GI Control (Adjuvant) (n = 6)	41,77 (10,2)	5,17 (2,6)	14 (0,052)	2/6 (33,33%)
GII (Recombinant proteins) (n = 6)	58,15 (8,6)	3,83 (2,0)	11 (0,185)	3/6 (50%)
GIII (vaccine against *B. bovis*) (n = 6)	42,43 (14,3)	3,0 (2,8)	9 (0,218)	1/6 (18,33%)

*PCV = Packed Cell Volume; **RT = Rectal Temperature; ***PPE = Percent parasitized erythrocytes
Mean PCV, RT and PPE values were not statistically significant.

zoite (MSA) interfere in the process of adhesion for the parasite infective form with the erythrocyte (Hines et al., 1995), having this family of antigens been considered as good candidates for use in the production of vaccines based on recombinant proteins (Brown et al., 2006; Florin-Christensen et al., 2014); however, the MSA-2c recombinant protein was unable to produce an acceptable protective immune response in cattle after challenge (Mosqueda et al., 2002). It has been documented that the recombinant protein 12D3 is an excellent candidate for the same purposes. (Hope et al., 2005; Álvarez et al., 2010; Florin-Christensen et al., 2014)

In this study, the immunological recognition of the *B. bovis* native antigen (by the IFAT assay) and recombinant antigens (by ELISA and IB assays) clearly showed that animals of group II immunized with the recombinant MSA-1, MSA-2c and 12D3 *B. bovis* antigens responded well and elucidated an humoral antibody response. More importantly, the IFAT and the IB assay results using *B. bovis*-infected erythrocyte extract as an antigen demonstrate that these antibodies recognize the authentic *B. bovis* antigens. It should be noticed that the molecular weight of the native protein is 42 kDa for MSA-1; 30 kDa for MSA-2c and 41 kDa for 12D3 (Brown et al., 2006; Borgonio et al., 2008; Pérez et al., 2010). However, since the recombinant proteins are fused to thioredoxin (13,5 kDa), the expected relative sizes would be of approximately 55,5 kDa, 43,5 kDa and 54,5 kDa for MSA-1, MSA-2c and 12D3, respectively, in agreement with the signals obtained in the tests performed. (Sánchez, 2010)

The use of a combination of *B. bovis* recombinant proteins MSA-1, MSA-2c, and 12D3 did not prevent the onset of clinical signs of infection after exposure to tick-borne parasites, but was able to protect 50% of animals vaccinated with these proteins despite having a lower parasitaemia than that determined in the control group. It was shown that the prepatent period of the immunized group was delayed as compared to that of the control group and equal to that of the group immunized with the attenuated *B. bovis* vaccine. This was probably due to the interference of 12D3, involved in the division of the parasite, as well as interference of MSA, involved in the adhesion to the erythrocyte. Nonetheless, only the animals immunized with the live vaccine were capable of preventing the manifestation of severe disease at challenge, as only one animal in this group required babesiacide treatment. It has been previously shown that *in vitro* culture-derived, live attenuated vaccines against bovine babesiosis confer protection rates from 70-100% when vaccinated cattle are exposed to tick-borne babesiosisis under field conditions (Cantó et al., 2002; 2003, Álvarez et al., 2004; Florin-Christensen et al., 2014). Fever in these animals, as in other mammals, is a clinical sign of infection. As already mentioned, in the case of bovine babesiosis it is common to find more than 40 °C rectal temperature values, in addition to severe packed cell volume reduction which is due to the destruction of erythrocytes by the merozoite while exiting from the infected erythrocyte.

Regarding the use of recombinant proteins for inoculation in cattle and subsequent challenge, some studies have been conducted. In this context, Hope et al., (2005) used recombinant proteins 11C5 and 12D3 from *Babesia bovis* for immunizing cattle that were exposed to a highly virulent isolate in a controlled challenge. In such a study, the following findings were reported: rectal temperatures from 41,1 to 41,4 °C in all groups of challenged animals and a PCV decrease from 22 to 19,4%, in addition to a 1,0% of parasitaemia determined in the control group; whereas the parasitaemia was between 0,22% to 0,44% in the vaccinated groups. Moreover, Álvarez et al. (2010), using the same proteins applied in this study but performing a needle-challenge with an isolate of moderate virulence, reported that immunized steers had rectal temperature values between 39,9 and 41,1 °C, decrements in PCV ranging from 26 to 31%, and 80% of the animals were positive to *Babesia* by microscopic analysis of blood smears. The rectal temperature values determined in the present study were significantly lower than those mentioned above. Likewise, the parasitaemia values determined in this experiment were considerably lower than those of the study of Hope et al. (2005). However, the results from the PCV

analysis determined in this study were clearly well above those reported in two previous studies; this was probably due to the handling of livestock under grazing conditions, the mixed infection of babesiosis transmitted by ticks, the high population of infesting ticks, and the virulence of the strains that infected the experimental cattle under field conditions.

The use of a live vaccine in inducing protection against bovine babesiosis showed similar results to previous experiences (Florin-Christensen et al., 2014), ie., as a consequence of prior exposure to the live parasite, both the incubation and the pre-pathogenic period is longer. It is worth noting that at challenge, the prepatent period of the animals inoculated with the recombinant proteins in this study is equal to that from the group inoculated with the live vaccine. However, and even though the PPE value of the recombinant proteins-immunized animals was lower than that determined for GIII animals, the length of time that the parasite could be detected in stained blood smears (up to 11 days), the maximum drop in PCV value (mean of 58,15%), and the number of days having a fever (average 3 days) indicated the babesiacide treatment in 3 of the 6 immunized steers of GII. Rectal temperatures of $39,7 \pm 0,75$ °C in cattle immunized-challenged under controlled conditions have been previously reported (Cantó et al., 1999). On the other hand, rectal temperature values of $40,2 \pm 0,8$ °C and $40,4 \pm 0,8$ °C have been described in animals vaccinated and challenged in the field (Cantó et al., 2002; 2003), this is consistent with the values obtained for GII and GIII steers in this study. As for the decrease in PCV, results found in cattle immunized-challenged varied from $8,82 \pm 5,2\%$ (Cantó et al., 1999), to $37,3 \pm 7,5\%$ (Cantó et al., 2002), and $40,7 \pm 7,5\%$ (Cantó et al., 2003). The first result corresponds to something that is not normally expected, while the others can be considered very similar to those of the present investigation, but not even comparable to those of the GII group inoculated with the recombinant proteins. The observation that only two animals in GI group (control) and three animals in GII group were considered in need of chemotherapy may be explained on the basis that the specific immune response evoked by GII steers, was not necessarily protective. It is worth mentioning that in addition to this fact; most probably a cross-immunity phenomenon occurred. In cattle babesiosis endemic areas of Mexico, it is not unusual that a mixed infection (*B. bovis - B. bigemina*) can be transmitted to cattle, as both parasites share the same tick vector, the common cattle tick *Boophilus microplus* (Figueroa et al., 1998b). It has been shown that cattle immunized with *B. bigemina* can protect up to 50% of the population when challenged with *B. bovis* (Vega et al., 1999). As indicated above, all animals in the experiment were premunized with this parasite species, which would likely result in a partial cross-protection conferred against infection with *B. bovis.*

In conclusion, the immunization of cattle with a combination of recombinant proteins of *B. bovis*: 1) induce specific antibodies that recognized the corresponding antigens when analyzed by IFAT, ELISA, and IB assays; 2) the combined use of *B. bovis* recombinant proteins MSA-1, MSA-2c, and 12D3 does not produce an adequate immune protection to tick-transmitted *Babesia* parasites as evidenced by the presence of severe signs of babesiosis in the experimental animals; however, the results are encouraging to continue looking for the right antigen combination for successful protection against field infection with the parasite transmitted by the natural vector, the common cattle tick.

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