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CLINICAL AND IMMUNOMOLECULAR MONITORING IN CATTLE INFECTED WITH VIRULENT OR ATTENUATED *Babesia bigemina* MICROORGANISMS

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Abstract: The objective of this work was to carry out comparative clinical, serological and molecular monitoring of animals inoculated with virulent or attenuated microorganisms of *B. bigemina*. Seven cattle were used, one of which was splenectomized to reactivate the virulent strain cryopreserved in liquid nitrogen. The remaining 6 cattle were randomly grouped into Groups I (GI), inoculated with $1x10^8$ erythrocytes infected with *B. bigemina* virulent strain, and GII inoculated with $1x10^8$ erythrocytes infected with *B. bigemina*, attenuated strain, derived from in vitro culture. Daily monitoring included taking rectal temperature (°C) value and collecting blood to determine: Packed Cell Volume (PCV) and Percent of Parasitized Erythrocytes (PPE). In addition, the Indirect Fluorescent Antibody Test (IFAT) and the nested PCR test (nPCR) were used. A PPE >9% was determined in the splenectomized calf, obtaining biological material for GI inoculation. The IG animals showed severe clinical signs, associated with acute babesiosis, with fever >41°C, hemoglobinuria, PPE >5% on day 5-6 Post-Inoculation (PI) and decrease in PCV from day 4 PI, with a minimum PCV of 11.3% at the end of the monitoring. Animals were treated with babesiaside to prevent death. The GII cattle did not present fever or signs of acute babesiosis, presenting only a slight decrease in PCV (HT value of 25%), and PPP <0.1%. No GII cattle required treatment. Seroconversion was identified on day 7 PI, with a maximum antibody titer of 1:1280 on day 14 PI in the GI. In the GII, a maximum antibody titer of 1:2560 was determined. The nPCR test confirmed the presence of *B. bigemina* in the animals, visualizing DNA fragments of 170 bp in agarose gels. It is concluded that the strain kept in cryopreservation maintains its virulence when reactivated in a splenectomized calf, observing severe clinical signs in the inoculated animals. The attenuated

strain, maintained in in vitro culture and used as a live vaccine, has not reverted to virulence, is innocuous, and is still immunogenic.

Keywords: *Babesia bigemina*; virulent strain; attenuated strain; virulence genes.

INTRODUCTION

Currently, it is well known that parasitic and infectious diseases transmitted by ticks and blood-sucking insects are classified as problems of high economic impact in tropical livestock production in the world, and bovine babesiosis is included in this category (Álvarez et al, 2019; Rios et al., 2010). The disease is caused by intraerythrocytic protozoa of the genus *Babesia*, whose vector is the common cattle tick *Rhipicephalus* spp (Martínez et al 2021). The distribution of the disease is mainly in tropical and subtropical areas of the world (Smith, 1978; Bock, 2004). Bovine babesiosis is one of the main diseases that affect the livestock economy. In México, as it is the case for most of the countries in Latin América, the disease is caused by the obligatory intraerythrocytic protozoa *Babesia bovis* and *Babesia bigemina*, which are transmitted by *Rhipichepalus (Boophilus) microplus* and *Rhipichepalus (Boophilus) annulatus*. In addition, the disease is considered one of the main factors that limit health for the development of livestock in the tropical and subtropical regions of Mexico and other countries of the world, its distribution is delimited by the presence of its vector which is related to environmental factors such as temperature, relative humidity and vegetation cover, among other factors (Cantú and García, 2013). The tropical and subtropical regions of Mexico represent 53% of the national territory, where more than 75% of bovine livestock are found in these regions (Bautista and Martínez, 2012). That is, of the national inventory, approximately 33.5 million cattle heads, 25.1 million are found in areas of high endemicity. In 2016, an estimate was made for annual economic losses of $68,878,694 dollars, generated by the decrease in milk production due to the presence of *Rhipicephalus (Boophilus) microplus* (Rodríguez et al., 2017); While losses in fattening animals located in the tropics of Mexico (*Bos indicus* x *Bos taurus*) a loss of $504,729,382 US dollars was estimated due to vector infestations (Rodríguez et al., 2017). Despite large economic losses just due to the presence of the vector, however, today there is no national estimate for losses caused by bovine babesiosis. As control and prevention strategies, different methodologies have been used for many years, such as vector control, through the use of ixodicides, implementation of resistant cattle or breeding of *Bos taurus* with *Bos indicus*, and control of the movement of specialized cattle in areas of high endemicity for bovine babesiosis (Solorio and Rodríguez, 1997). On the other hand, it is known that cattle develop long-lasting immunity after infection with *B. bovis* and/or *B. bigemina*, without developing an adequate cross-immunity between the two species (Vega et al., 1999). Thus, immunization would be the procedure that offers the best prospects for the prevention and control of bovine babesiosis (Cantó et al., 2003 a; 2003b; Figueroa et al., 1998). In addition, different studies of vaccine material have been published, some of them based on the use of parasitic antigens obtained from lysates of infected erythrocytes, soluble antigens present in the supernatant of *Babesia* spp cultures have also been used (Schetters et al., 2001), as well as from *Babesia bovis* and *B. bigemina* (Montenegro-James et al, 1987). The use of live attenuated vaccines by multiple passages in splenectomized calves has been described in Australia (Callow, 1977). With these type of vaccines induction of protection of up to 95% has been achieved. However, adverse reactions, contamination of the vaccine with pathogenic organisms

and sensitization against other blood groups have occurred (Bock et al., 2004). To solve the aforementioned, an alternative has been the use of in vitro culture, from which erythrocytes infected with attenuated parasites can be obtained and inoculated into susceptible cattle, which has been highly effective in preventing bovine babesiosis (Cantó et al., 2003a; 2003b). In Mexico, the in vitro culture of *Babesia* spp. is maintained with parasites that were originally isolated from field outbreaks. Thus, *B. bovis* and *B. bigemina* parasites have been adapted, cloned and maintained in cryopreservation and in vitro culture to date (Rodríguez et al., 1983; Vega et al., 1986a; 1986b). The development of live immunogens, from attenuated strains derived from in vitro culture, has been proposed as a control alternative in bovine babesiosis (Cantó et al., 1999). Currently, the use of attenuated vaccines is one of the best intervention strategies that leads to the need for continuous improvement of this type of live vaccines (Shkap et al., 2007). *B. bigemina* is one of the two species involved in infecting cattle in Mexico, for this reason it is important in Mexican livestock production (Rojas et al., 2004; Alvarez et al, 2019). There are investigations where it is mentioned that up to now the use of attenuated vaccines is one of the best strategies for the control of vector-borne diseases (Shkap et al., 2007). However, in México there is currently no commercially available vaccine. it is important to mention that for more than 30 years studies have been carried out at CENID-PAVET, now CENID-SAI, INIFAP, which have included the development of a methodology for the preparation of a live attenuated mixed vaccine against *B. bigemina* and *Babesia bovis*, derived from strains maintained under laboratory conditions base don in vitro culture (Bautista et al., 2012; Rojas et al, 2018a; 2018b). The objective of this study was to clinically, serologically, and molecularly compare a virulent strain and an attenuated strain (vaccine strain) of *Babesia bigemina* of Mexican origen.

MATERIALS AND METHODS

Location. The study was carried out at Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, located at Cuernavaca-Cuautla highway No. 8534, Colonia Progreso, C.P. 62574 Jiutepec, Morelos, México.

Animals experimental design. Seven native cattle from a *Rhipicephalus* tick-free zone were used. One of the animals, a 2 months old calf, was splenectomized to later be inoculated with a virulent strain of *B. bigemina* in order to reactivate the cryopreserved strain (Figueroa et al, 1998). The six remaining cattle, steers of approximately 1 year old, were randomly divided into two groups. Group I (GI) cattle were inoculated with $1x10^8$ infected erythrocytes (IE) derived from splenectomized cattle inoculated with the virulent strain of *B. bigemina*. The cattle of Group II (GII) were inoculated with $1x10^8$ IE of an attenuated strain (vaccine strain) from in vitro culture (Vega et al, 1985; Sachman et al, 2021). Both groups were inoculated via intramuscular.

Clinical monitoring. Animals were kept under daily monitoring, recording rectal temperature (RT) and packed cell volume (PCV) by the microhematocrit technique. Likewise, blood samples were obtained and the percentage of parasitised erythrocytes PPE was determined by light microscopy examination of blood smears stained with Giemsa dye (Rojas et al., 2011).

Serological monitoring. The kinetics of anti-*Babesia bigemina* antibodies was determined using the Indirect Fluorescent Antibody Test (IFAT) (Bautista et al., 2012). Antigen was obtained by making smears

with infected erythrocytes from bovines previously splenectomized. To determine the antibody titers in the test sera, doubling serial dilutions were made from 1:80 to 1:10240 in PBS. As a second antibody, anti-bovine IgG conjugate prepared in sheep, labeled with Alexa, was used, and the visualization was performed by reading the IFAT smears in an epifluorescence microscope (Rodríguez and Cob, 2005, Bautista et al., 2012). In addition, for immunological monitoring, an Indirect Immunoenzymatic Assay (ELISAi) was also implemented, using the *B. bigemina* recombinant protein RAP-1 (Roptry Associated Protein-1) as antigen (Santamaria et al., 2020). Briefly, polystyrene plates were sensitized using a 100 µl/well volume of rRAP-1 protein, diluted in carbonate buffer (pH 9.6). Blocking was performed with 3% Bovine Serum Albumin (BSA) in 0.1% PBS-Tween and then 50 µL of positive and negative control sera and test sera were added, using a 1:100 dilution on PBS. Plates were incubated at 37 °C for one hour, followed by 3 washes. Subsequently, 100 µL of peroxidase-labeled goat anti-bovine IgG conjugate was added at a 1:10,000 dilution in PBS and incubated at 37 °C for 30 min. Continuing with 3 washes, 50 µL of TMB substrate (3,3',5,5'-tetramethyl-benzidine) was added to each well of the plate. Finally, the reading was carried out in a spectrophotometer, determining the absorbance values at an optical density of 650 nm (Santamaria et al., 2020).

Molecular monitoring. The detection of *Babesia bigemina* DNA was carried out by the Polymerase Chain Reaction assay in a nested format (nPCR), using specific sequences of primers and previously published amplification protocols (Figueroa et al., 1992; 1993; 1996). Finally, electrophoresis was performed to visualize the amplification products of an expected size of 170 bp.

RESULTS

Clinical monitoring. Clinical signs associated with bovine babesiosis were observed in the splenectomized calf on day five post-inoculation (PI), showing fever of 41°C, hemoglobinuria, PCV of 16%, and a PPE ≥ 9.0%. Due to a clinically severe disease in the splenectomized calf, the decision was made to obtain the biological material to inoculate the three cattle of group I, with a dose of 1 x 10^8 IE. Once bled, the calf was immediately treated with diazaminodibenzamidine diaceturate and sodium dipyrone, and physiological saline was administered via intravascular. The three GI cattle inoculated with the virulent strain presented fever (greater than 40°C) on day 4 PI (Figure 1), and decreased PCV from day 5 PI, reaching 11.3% in a bovine at end of monitoring (Figure 2). The presence of the protozoa was determined by microscopy with the reading of the blood smears on day 5-6 PI, obtaining the highest PEP in one of the experimental steers on day 8 PI and on day 9 PI in the other two steers. Subsequently, and to prevent death, the steers were treated with the same active principles used in the splenectomized calf. While the GII steers, inoculated with the attenuated strain, did not present fever throughout the monitoring and in the case of the PCV only a small decrease was presented between days 3-7 PI (Figures 1 and 2). It is important to mention that none of the G I steers required treatment and the PPE values were minimal, <0.1 %.

Serological monitoring. Readings in the epifluorescence microscope of the IFAT slides, showed that, on average, antibody titers in the GI steers, were determined as 1:80 from day 7 PI, increasing to 1:160 on day 8 PI, to subsequently rise to 1:320 from on day 9 PI to day 12 PI, the day on which antibody titers of 1:640 were obtained. Finally, on day 14 PI the highest antibody titer was found, reaching 1:1280. In the GII steers, similar

values were observed, seroconverting on day 7 PI at a 1:80 dilution, reaching a maximum of antibody titers on day 21 PI with 1:2560 (Figure 3). USing the indirect ELISA, the kinetics of specific antibodies against *B. bigemina* are shown in Figure 4. The cut-off point was established as 0.2 absorbance units at an Optical Density (OD) of 650 nm.

Microscopic and molecular monitoring. By microscopic examination of the Giemsa-stained blood smears, a low parasitemia (<0.1%) was determined in steers inoculated with the atenuatted strain (GII). However, at the molecular level the detection and establishment of virulent and attenuated parasites was determined, managing to visualize the 170 bp amplification fragment in the nPCR assay. Using nPCR, it was possible to detect the parasite DNA from day 4 PI in steers of both experimental groups (Figure 5).

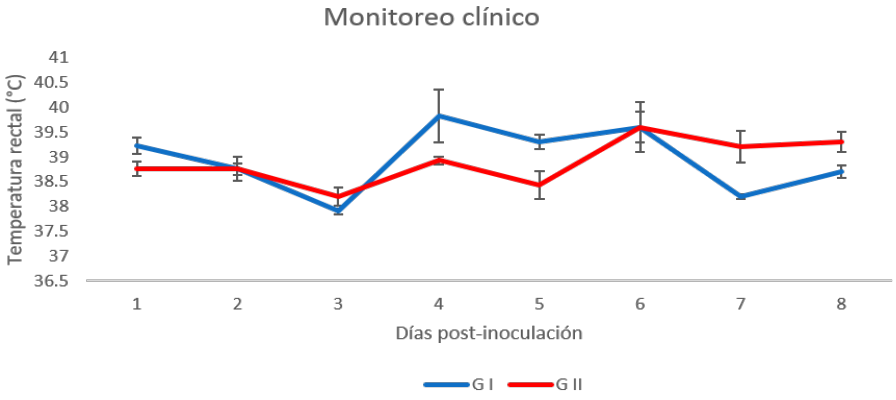

Figure 1. Rectal temperature (RT) monitoring (Mean ± SE) in cattle inoculated with a virulent strain (G I) and with an attenuated strain (G II) of *B. bigemina*. The increased RT that was clinically manifested by the steers coincides with the presentation of parasitemia, occurring from day 4 PI onwards in the G I and with a PCV as high as 3% in one of the steers (not shown). Unlike the G I steers, inoculated with the attenuated strain that had a delay of 2 days in the RT increment and a low PPE (<0.1%).

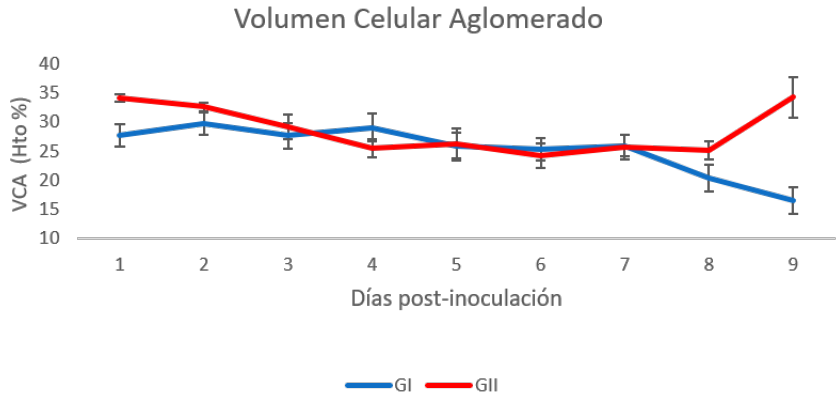

Figure 2. Average PCV values (Mean ± SE) during the experimental infection. The blue line represents G I steers, inoculated with a virulent strain of *B. bigemina*. The red line represents G II steers, inoculated with an attenuated strain of *B. bigemina*. The PCV of the G I steers remained at basal levels from day 5 PI, which is considered the onset of parasitemia, until reaching the lowest value on day 9 PI (11.6 % in one of the steers) requiring babesiacide treatment; while the PCV of G II steers had slight variations, but at the end of the clinical monitoring, the PCV recovered without any babesiacide treatment.

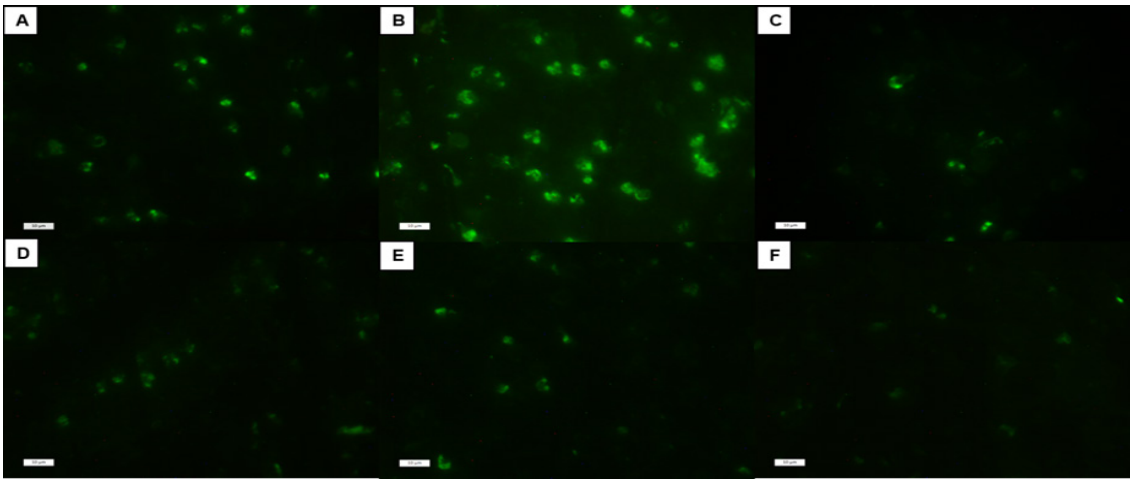

Figure 3. Representative image of the specific antibodies titers to *Babesia bigemina*, using the IFAT. A-F: Serial doubling dilution of 1:80 - 1:2560, respectively. Photographs taken under the epifluorescence microscope, 100 X.

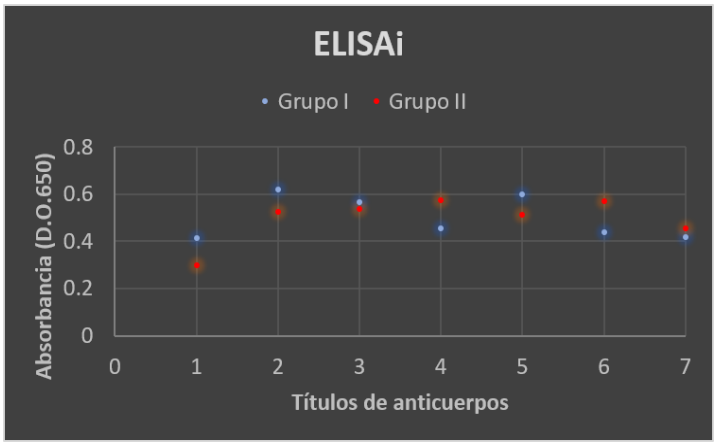

Figure 4. Representative image of the specific antibodies to *Babesia bigemina*, using the indirect ELISA. 1-7: serial doubling dilutions of 1:80 - 1:10240.

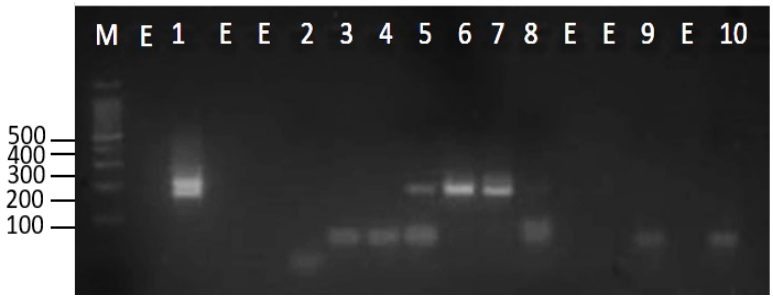

Figura 5. Visualization of the *Babesia bigemina* DNA amplification products by nPCR in a 2.5% agarose gel stained with ethidium bromide. Representative result of a steer from the GI. LAnes: M, 100 bp molecular marker; E, empty lane; 2-8, samples from days 1-7 PI; 9, negative control; 10, H_2O control.

DISCUSSION

Since day 4 PI the rectal temperature monitoring began rising until the day in wich the steers of GI were treated to avoid death, parameters and signs shown were those exclusively pertaining to an infection caused by *B. bigemina*, as it is described in the literature (Alvarez et al, 2019; Bock et al, 2004; Bautista et al, 2012). The experimental work carried out in the two groups of steers was under conditions for a controlled environment, resulting in an expected virulent infection in the GI steers, showing PPE from 1% to 3%, with fever present in all the animals and at least a decrement of 50% in PCV value, unlike the GII steers that was inoculated with an attenuated strain. In addition, similar results to those found in previous studies were obtained both in values of TR, PCV and PPE in blood smears in which it is reported that the establishment of the attenuated vaccine strains in inoculated cattle only reached PPE of 0.01% for *Babesia* spp which makes it more difficult to identify trophozoites and merozoites in groups of animals inoculated with vaccine strains (Bautista et al, 2012; Cantó et al, 1996; 2003). In the molecular monitoring part of the study, the presence of parasites was demonstrated by means of the specific nPCR test for *B. bigemina* in both groups of cattle from day 4-5 PI, demonstrating the high specificity and sensitivity of this test (Figueroa et al 1992; 1996). Finally, throughout the study it was possible to demonstrate that the *B. bigemina* virulent strain maintained in cryopreservation in liquid nitrogen continues being of high virulence, once it is activated in a host, for recovery and subsequent inoculation. Regarding the *B. bigemina* attenuated vaccinal strain, this experiment confirms that the in vitro culture-derived parasites continue to be inocuos, as there is no generation of an anaphylactic reaction or severe clinal signs of bovine babesiosis when administered as a live attenuated vaccine, and there is no reversion to virulence either, managing to effectively stimulate the humoral immune system of GII steers, as well as has been reported in previous studies of the live attenuated vaccine derived from in vitro culture against bovine babesiosis (Cantó et al., 1996, Cantó et al., 2003, Bautista et al., 2015, Rojas et al, 2018a; 2018b). Additionally, its safety margin has been also previously demonstrated, as it is not transmitted by the tick vector *Rhipicephalus microplus*, at least after two successive passages in spleen-intact cattle (Rojas et al., 2011).

CONCLUSION

By using a calf previously splenectomized and inoculated with a virulent strain of *B. bigemina*, it was possible to reactivate the strain that had been kept in cryopreservation. Likewise, it was possible to inoculate it in a group of clinically healthy, naive steers and induced a parasitemia in them; these steers presented clinical signs of a severe infection caused by *B. bigemina*. Through daily monitoring it was possible to determine fever, decrement of PCV value and high parasitemias in blood smears. Despite the fact that the virulent strain had been kept in cryopreservation for several years, it has maintained its virulence, also allowing more biological material to be obtained to continue performing both serological and molecular tests for the benefit of the study of bovine babesiosis in future research. Finally, unlike the severe presentation of clinical signs in highly susceptible steers after inoculation with the virulent strain, steers inoculated with the attenuated vaccinal strain of *B. bigemina*, in addition to still being innocuous, confers adequate induction of a humoral immune response in highly susceptible steers. It is expected that the protection induced immunized cattle that are subsequently challenged under

controlled or field conditions is maintained as reported in previous studies. For this reason, it is of great importance to carry out future research to find out at the genomic and/or transcriptomic level (Sachman et al, 2021), the existing changes that make the attenuated *B. bigemina* population used in this study, phenotypically clinically different from the *B. bigemina* virulent population, and thus be able to elucidate those virulence risk factors, which would allow to obtain new targets at the molecular level, either for diagnosis and/or prophylaxis of bovine babesiosis.

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