Estandarización del modelo de infección experimental en bovinos con *Anaplasma centrale* para la producción de inmunógenos

Standardization of the model of experimental infection in cattle with *Anaplasma centrale* for the production of immunogens

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This work was financed by the General Secretary of Science and Technology of the National University of the Northeast (UNNE), Argentina

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Veterinaria (Montevideo) Volumen 54 Nº 210 - 6 (2018) 37-42

DOI: 10.29155/VET.54.210.6

Recibido : 28/02/2018

Aceptado: 14/09/2018

Resumen

La anaplasmosis bovina es una enfermedad infecciosa transmisible causada por la rickettsia *Anaplasma marginale*. La profilaxis se puede lograr mediante la vacunación con eritrocitos infectados con *A. centrale* obtenidos a partir de terneros esplenectomizados. La estandarización de las condiciones para la infección de bovinos con *A. centrale* puede conducir a un sistema de producción eficiente, que es particularmente importante cuando la vacuna se produce como una formulación trivalente que también contiene eritrocitos infectados con formas atenuadas de los parásitos *Babesia bovis* y *B. bigemina.* En este estudio, se inocularon bovinos (n = 26) con diferentes dosis de eritrocitos infectados (EI) con *A. centrale* (2, 3, 4 o 5 x 10^8 EI por animal). El curso de la infección se analizó con respecto al período prepatente, la rickettsemia y la disminución del hematocrito, y se evaluaron las posibles correlaciones entre estos parámetros. El inóculo más bajo dio como resultado períodos de prepatencia más largos con respecto a los otros tres grupos. Además, se observó una moderada correlación negativa entre la duración del período prepatente y la rickettsemia. La sangre de cada uno de estos terneros se usó para la producción de vacunas comerciales. Se confirmó la respuesta humoral contra *Anaplasma sp* sesenta días post-vacunación mediante ELISA competitivo en un grupo representativo de bovinos inoculados con cada lote de vacuna producida. Por lo expuesto, concluimos que el inmunógeno obtenido mantuvo las características inmunoprofilácticas en todas las series de vacunas producidas. Además, esta descripción del modelo de infección experimental puede serútil para futuras investigaciones.

Palabras clave: ganado; anaplasmosis bovina; *Anaplasmamarginale*; rickettsemia.

Summary

Bovine anaplasmosis is an infectious transmissible disease caused by the rickettsia *Anaplasmamarginale.* Prophylaxis can be achieved by vaccination with *A. centrale*-infected erythrocytes (IE) obtained from splenectomized calves. Standardization of conditions for *A. centrale* infection of bovines can lead to a more efficient production system, which is particularly important when the vaccine is produced as a trivalent formulation that also contains erythrocytes infected with attenuated forms of *Babesiabovis* and *B. bigemina* parasites. In this study, bovines (n=26) were inoculated with different doses of *A. centrale*-infected erythrocytes (2, 3, 4 or 5 x 10^8 IE per animal). The course of infection was analyzed with regards to pre-patent period, rickettsemia, and maximal hematocrit decrease, and possible correlations between these parameters were evaluated. The lowest inoculum resulted in significantly lengthier pre-patent periods with respect to the other three groups. Also, a moderate negative correlation was observed between the length of the pre-patent period and rickettsemia. Blood from each of these calves was used to produce commercial vaccines. A humoral response against *Anaplasma* spat sixty days post vaccination was confirmed by competitive ELISA in a representative group of bovines inoculated with each batch of vaccine produced. Therefore, we conclude that the obtained immunogen maintained itsimmunoprophylactic characteristics in all the series produced. Also, this description of the experimental model could be useful for future investigations.

Key words: cattle; bovine anaplasmosis; *Anaplasmamarginale*;rickettsemia

Introducción

Bovine anaplasmosis is an infectious disease caused by *Anaplasmamarginale*, a Gram-negative bacterium, belonging to the order Rickettsiales (Kocan et al., 2010). It is naturally transmitted to cattle by various ticks, including *Rhipicephalus (Boophilus) microplus*, the main cattle tick of South America (Aguirre et al., 1994; Kocan et al., 2004; Mercado-Curiel et al., 2014). Mechanical transmission through blood-contaminated fomites and biting flies is also epidemiologically relevant, especially in tick-free areas (Scoles et al., 2008; Kocan et al., 2004, 2010).Additionally, transplacental transmission has recently been demonstrated (Costa et al., 2016).

*A. marginale*invades the erythrocytes of domestic and wild ruminants, where it forms membrane-bound inclusion bodies containing 4-8 rickettsiae, and can also invade endothelial cells (Carreño et al., 2007). Removal and destruction of infected erythrocytes by the reticuloendothelial system leads to mild to severe anemia and icterus. Other clinical signs include fever, muscular weakness, depression, dehydration, anorexia, increase of heart frequency, respiratory insufficiency, abortion and death. Hemoglobinemia and hemoglobinuria, are not present in acute *A. marginale* infections, which can allow differentiation of this disease from bovine babesiosis, frequently endemic in the same regions. Animals that recover from anaplasmosis remain persistently infected and protected for life (Richey, 1981; Brown and Barber, 2016).

Vaccination of cattle with erythrocytes infected with the benign or mildly pathogenic *A.marginale subsp. centrale (A. centrale)* has been used for over a century to elicit cross-protective persistent immunity against clinical disease (Palmer, 2009). Vaccines frequently have a trivalent formulation and include erythrocytes infected with *Babesiabovis* and *B. bigemina*, which can be amplified either *in vitro* or in splenectomized bovines (Florin-Christensen et al., 2014). On the other hand, *A. centrale* is currently only produced *in vivo* in splenectomized cattle (OIE, 2015). However, recent reports on effective protection against challenge elicited by a vaccine based on cultured *A. marginale*, and the successful propagation of *A. centrale* in a tick-cell line raises the possibility of *in vitro* production of anasplasmosis vaccines in the future (Hammac et al., 2013; Bell-Sakyi et al., 2015).

In the preparation of trivalent vaccines, bovine erythrocytes infected with *B. bovis*, *B. bigemina* and *A. centrale* are mixed and either stored refrigerated or cryopreserved in liquid nitrogen (Mangold et al., 1990; OIE, 2015). Timing is essential to this preparation: since the *A. centrale*-infected splenectomized bovine should reach the peak of rickettsemia at the same time as *B. bovis*and *B. bigemina in vitro* cultures or experimentally infected calves reach an adequate percentage of infected erythrocytes (%IE) and are ready for harvesting. Although most parameters of live vaccine preparation are described in the literature, there is a paucity of information on the conditions for establishing the infection in splenectomized donor bovines by *A. centrale*, including the size of the inoculum, and the influence of these parameters on the pre-patent period, hematocrit decrease and rickettsemia achieved (OIE , 2015, Pipano, 1995; Dagliesh et al., 1990).

The aim of the present study was to find the optimal conditions to synchronize the infection with*Anaplasma centrale* ofsplenectomized calves, through different doses of the inoculum, with the production of *Babesia*sp in cell cultures; during the preparation of trivalent cryopreserved live vaccines. The data obtained is useful for the standardization of this process and for future research.

Materials and Methods

Animals

Experimental infection with *A. centrale* was carried out in 28 healthy malehalf-breed Brafordbeefbovines*(Bostaurus)*, aged 8 to 24 months, weighing 240 to 380 Kg. They were purchased inGualeguaychú, Entre Ríos, Argentina, a*Rhipicephalus (Boophilus) microplus*-free area. All animals gave negative results for serological determination of anaplasmosis and bovine babesiosis, as well as bovine enzootic leucosis, bovine infectious rhinotracheitis, bovine viral diarrhea, brucellosis, tuberculosis, trypanosomiasis and eperythrozoonosis. In addition, after bovines were located in individual pens, bovine enzootic leucosis tests were carried out in sheep, with negative results in all cases. These analyses were performed in the laboratory of immunology of INTA Rafaela (Santa Fe, Argentina) and Laboratory Colon (San Martín, Buenos Aires, Argentina).

Animals were splenectomized following routine procedures and presented no post-surgery complications. Splenectomy was performed under sedation with xylazine (0.2 mg/Kg) and paravertebral anesthesia with lidocaine by the Modified Magda technique (Garnero, 2002) Once the animal was in decubitus, an incision was made in the left flank, and ligated at the level of the ileum to prevent future hemorrhages, followed by spleen removal. Subsequently, animals receivedan intramuscular injection of penicillin-streptomycin (20.000 UI/Kg every 48 h for a maximum of three doses)to prevent postsurgical infections. A few calves presented eperythrozoonosis after the surgery and were treated with Tylosine (10 mg/Kg).

Daily, rectal temperature was recorded and blood samples were collected for hematocrit determination and for preparation of smears that were stained with Giemsa, for rickettsemia determinations (percentages of infected erythrocytes).

During the pre-infection period, animals were weighed and kept in a quarantine area. This is a fenced sector with concrete floor, a galvanized metal roof for shade and a cement drinking container connected toa water source, for ad libitum access to water. During the infection and post-infection (p.i.) periods, animals were transferred to individual 15 m^2boxes and monitored until day 60 p.i. Animals were fed twice a day with 3% body weightof balanced diet, consisting in high-fiber pellets containing 16% protein, 10% fiber, and 2850kcal/ kg metabolic energy

(Beltramino Hnos. S.H., Calchaqui, Santa Fe, Argentina), and had *ad libitum* access to water.

The procedures were carried out in Litoral Biológicos SRL plant, authorized for the manufacture of biological products according to Certificate N° 8574, Resolution 1843/08. Litoral BiológicosSRL, is located in Puerto Tirol, province of Chaco, with a subtropical climate without dry season, which has an annual media temperature of 21±2 °C.The protocols were approved by the Ethics Committee of the School of Veterinary Science- UNNE (certificate 459/2013-CD).

Inoculum preparation

A. centrale M1 strain, isolated in Corrientes, Argentina in 1983 was used (Vanzini el al., 1984). The immunogenicity conferred by this strain was experimentally tested by the inoculation of susceptible calves and subsequent challenge with *A. marginale* (Aguirre et al., 1988; Abdala et al., 1990). Bovine erythrocytes infected with *A. centrale* M1 were preserved in liquid nitrogen in the presence of glycerol as cryoprotectant, according to Mangold et al. (1990). An aliquot was thawed in a water bath at 40°C and the total number of erythrocytes and the percentage of infected erythrocytes (%IE) were microscopically evaluated in a Neubauer hematocytometer and Giemsa-stained smears, respectively. A splenectomized calf was subcutaneously inoculated with 4 x 10^7*A. centrale*-infected erythrocytes (0.5 mL). The pre-patent period lasted 60 days, and at day 70 p.i., a rickettsemia level of 1.5 %IE was reached. Ten milliliters ofblood wereaseptically withdrawnfrom the jugular vein and a second bovine was inoculated intravenously (i.v.) with 7 x 10^7 IE from this suspension. This time, the pre-patent period lasted 40 days and at day 48 p.i., rickettsemia reached 8.5 %. This animal developed chronic infection and was used for all successive inoculations.

Experimental infection

A total number of 26 bovineswere used over a period of two years. Each animal was used for one single time in the production of each cryopreserved vaccine batch. The animals were allocated to four groups and they received blood from thechronic donor bovine mentioned abovein a single i.v. injection (5 to 15 mL) containing 2x10^8 (group A, n=5); 3 x10^8 (group B, n=7); 4 x10^8 (group C, n=8) or 5x10^8 (group D, n=6) *A. centrale* IE. After inoculation, animals were monitored daily for clinical signs (facie and attitude, color of conjunctiva and mucous membrane, appetite, microscopic analysis of urine sediments), rectal temperature, hematocrit, and %IE in Giemsa-stained smears. The length of the pre-patent period, defined as the period of time between the inoculation point and the last day on which rickettsemia cannot be microscopically detected, was recorded.At the end of the experiment, animals were treated with rapid and long-acting formulation of oxytetracycline (10 mg/ Kg and 20 mg/Kg, respectively) and were sent to slaughter (after withdrawal period).

Trivalent vaccine preparation

Volumes of blood withdrawn from each animal depended on the number of vaccine doses required, the hematocrit value and the %IE at the day of blood collection, and varied between 500 and 1500 mL. For example, to obtain 10,000 vaccine doses out of the blood of a bovine that reached 7% rickettsemia and a hematocrit of 25, a blood volume of 555 mL needs to be withdrawn. Erythrocytes infected with *Babesiabovis*, strain R1A, and *B. bigemina*, strain S1A were obtained by *in vitro*cultivationas described by Levy and Ristic (1980) and Vega *et al.* (1985). Parasitized erythrocytes for each *Babesia* sp. were mixed with *A. centrale* infected blood and the suspension was mixed with an equal volume of 3 M glycerol in PBS supplemented with 5 mM glucose, at 37°C (OIE, 2015). The procedure was carried out under sterile conditions. The mixture was incubated at 37 °C for 30 min. Aliquots of 0.5 ml (containing 10^7 erythrocytes infected with each *B. bovis* and *B. bigemina* and 10^7 *A. centrale*IE) were packaged in straws using an automatic fractionator (BlocmachineMoyen IS4 – IMV Technologies) in a laminar flow cabinet, properly labeled, and frozen in liquid nitrogen using a SISTEL freezing equipment (Millennium, 01 model).

Vaccine infectivity and immunogenicity

Serum samples from 10 animalsinoculatedwith each vaccine batch (corresponding to the different *A. centrale* doses) were obtained at days 0 and 60 p.i. The presence of antibodies against *A. centrale Major Surface* Protein-5 (MSP-5) was determined by competitive ELISA at the Animal Diagnostic Service, INTA Experimental Station at Rafaela (EEA-Rafaela), Santa Fe, Argentina, following the procedure described by Torioni de Echaide et al. (1998).

Statistical analysis

The differences in the parameters: %IE, hematocrit reduction, pre-patent period and animal weight for each group were compared by an Analysis of Variance (ANOVA), using the Tukey HSD test (http://astatsa.com/OneWay_Anova_with_Tukey-HSD/). Linear associationsbetween two variables were studied calculating the Pearson Correlation coefficient (https://www.socscistatistics.com/tests/pearson/).

Results

The results of *Anaplasma centrale* infection of bovines are shown in Chart I. In order to set up the production of the trivalent bovine babesiosis-anaplasmosis vaccine, different conditions were analyzed to obtain suitable concentrations of *A. centrale*-infected erythrocytes. First,asplenectomized calf was subcutaneously vaccinated with 4 x 10^7*A. centrale* IE from a liquid nitrogen frozen stock. Given the low rickettsemia reached (1.5% IE at day 70 p.i.), a second splenectomized calf was i.v. inoculated with 7 x 10^7 IE obtained from the first one. The rickettsemia reached in this case was considerably higher and the

pre-patent period shorter (8.5% at day 48 p.i.). However, since these pre-patent periods were too lengthy for an efficient vaccine production system, higher inoculation doses were analyzed. To this aim, splenectomized calves were i.v. inoculated with 2, 3, 4 or 5 x 10^8 IE per animal (groups A to D, respectively).

The length of the pre-patent period, rickettsemia and hematocrit decrease of each animal as well as averages ± SD for each group are shown in Chart1. While no significant differences in the length of the pre-patent period were observed between groups B, C and D, group A, that received the lowest *A. centrale* inoculum (2 x 10^8 IE), showed a significantly longer pre-patent period than the other three groups ($p < 0.01$ for A *vs* D; and $p < 0.05$ for A *vs* B and A *vs* C). This lengthier pre-patent period of group A influenced the day at which blood was collected for immunogen production (Chart1), since significant differences between this and the other groups were also observed in this case ($p < 0.01$ for A *vs* B and A *vs* D; $p < 0.05$ for A *vs* C). However, the time lapses between the end of the pre-patent period and blood collection were similar in all groups, ranging from 7 to 9 days in average.

A moderate negative correlation (R2 = 0.3521) was found between the length of the pre-patent period and % IE, i.e. shorter pre-patent periods correlated with higher rickettsemia levels. In addition, weak correlations were found between hematocrit percent decrease and the length of the pre-patent period (R2 = 0.1449, negative correlation) or rickettsemia levels (R2 = 0.0749, positive correlation).

Rickettsemialevels doubled daily until reaching a suitable %IE for immunogen production, while hematocrit values significantly decreased ($p<0.001$) with respect to the pre-patent period, with averages of 26.1 ± 4.3 and 36.9 ± 3.0, respectively. Rickettsemia and hematocrit levels are two critical parameters for deciding when blood can be collected, since they determine the number of vaccine doses that can be prepared. In this study, the size of the inoculum did not influence the percentages of hematocrit decrease observed at the time of vaccine preparation. Regarding rickettsemia, important individual differences were observed within each group. When group averages were considered, although a tendency to reach higher rickettsemia values with higher inoculum sizes could be observed, the only significant difference ($p < 0.05$) was found between groups A and D that reached % IE values of 4.6 ± 1.1 and 10.8 ± 3.6, respectively (Chart I).

On the other hand, rectal temperature remained around 38.5 ± 0.5 in all cases (39±0.48; 39.12±0.33; 38.94±0.25 and38.78±0.11 for groups A, B, C and D, respectively).No animal became anorexic or prostrated, but increased respiratory rates and jaundice were observed at the peak of rickettsemia.

Blood from each *A. centrale* inoculated bovine was applied to the production of commercial vaccines. Sixty days post vaccination, vaccine efficacy was evaluated by detection of serum antibodies against *A. centrale* MSP-5 by competitive ELISA. All vaccines elicited detectable humoral responses.

A total number of 7.67 x 10^5 vaccine doses were obtained from the 26 bovines of this study, and distributed in small liquid nitrogen containers among farmers of tick-endemic regions in Northwestern Argentina. The use of these vaccines was approved by the Regulatory Agency of Animal Health of Argentina (SENASA).

Discussion

Blood obtained after successive experimental infections of splenectomizedcalveswith *A. centrale* was used in the production of the trivalent vaccine for the prophylaxis of the bovinebabesiosis-anaplasmosis syndrome. This vaccine is the result of atechnologytransfer agreement between the EEA INTA Rafaela and the company LitoralBiologicos SRL and it began to be commercialized in 2010, under the name Biojaja®

For immunogen preparation, *A. centrale*-infected blood was collected at the peak of rickettsemia in each animal,coinciding with considerable hematocrit decreases. It is generally accepted that the decrease in hematocrit is due to the destruction of *A. centrale*-infected erythrocytes triggered by as yet unknown factors (Kocan et al., 2004). Decrease in antioxidant enzyme activities and elevated erythrocyte osmotic fragility were observed in *Anaplasmaovis* infections of sheep, and could indicate a connection between disturbed antioxidant defense mechanisms and anemia during *Anaplasma* spp. infections (Jalali et al., 2016). The lowest individual hematocrit values at the time of blood collection were observed in group A (19%) and D (20%), which received the minimum and maximal *A. centrale* doses, respectively, stressing the notion that hematocrit decrease cannot be predicted by the size of the inoculum.

Taking into account that hematocrit levels in the range of 24-46% are considered normal (Nemi, 1993), the observed hematocrit decreases in these experiments can be considered tolerable.

After blood collection for vaccine formulation, hematocrit values continued to decrease and reached 12% in some of the animals. Blood transfusions from a suitable donor, increase in food rations, and administration of vitamins and minerals were applied until all calves recovered normal hematocrit levels.

The range of the pre-patent period of the animals included in this work was 11 - 26 days, and a moderate negative correlation was found between the length of this period and percentages of IE.

This supports early observations by Lotze (1974), while is not consistent with the suggestions of Ristic et al (1968) and Ajayi et al. (1978), who stated that the length of the pre-patent period in *Anaplasma* spp. infections depends on variable individual characteristics of cattle.

Conclusion

This study shows that it is possible to obtain consistent responses to *A. centrale* infection of bovines. This allows to synchronize the collection of *A. centrale*-infected blood with the production of *in vitro* cultures of *B. bovis* and *B. bigemina*, with the capacity of vaccine generation once a month.The significantly extended pre-patent period observed in the case of the lowest inoculum size used in this study indicates that the number ofinoculated*A. centrale*IE is a critical parameter for the synchronization of the production of immunogens for babesiosis-anaplasmosis vaccines. Importantly, all conditions tested were adequate for the production of infective and immunogenic vaccines against bovine anaplasmosis.

Acknowledgments

This work was financed by grant No. B016/13 from the General Secretariat of Science and Technology of the National University of the Northeast (UNNE), Argentina, for collaborative work between the University and LaboratorioLitoralBiológicos SRL, Argentina. We would like to thank MV. Gustavo Balbin, technical director of the laboratory, for his input in the experimental design and interpretation of results.

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