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 el tiempo de prepatencia 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 sus características inmunoprotécticas en todas las series de vacunas producidas. Además, esta descripción del modelo de infección experimental puede ser útil para futuras investigaciones.

**Palabras clave:** ganado; anaplasmosis bovina; *Anaplasma marginale*; rickettsemia.
**Introducción**

Bovine anaplasmosis is an infectious disease caused by *Anaplasma marginale*, 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 (Secoles 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 *Babesia bovis* 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 anaplasmosis 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 and either stored refrigerated or cryopreserved in liquid nitrogen, with the production of *Babesia* 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 male-half-breed Braford Beef bovines (*Bos taurus*), aged 8 to 24 months, weighing 240 to 380 Kg. They were purchased in Gualeguaychú, Entre Ríos, Argentina, and splenectomized (*B. 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 erythrozoosmosis. 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 received an 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 erythrozoosmosis 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 to a water source, for ad libitum access to water. During the infection and post-infection (p.i.) periods, animals were transferred to individual 15 m² boxes and monitored until day 60 p.i. Animals were fed twice a day with 3% body weight balanced diet, consisting in high-fiber pellets containing 16% protein, 10% fiber, and 2850 kcal/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 No. 8574, Resolution 1843/08. Litoral Biológicos SRL, 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 et 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 hemocytometer and Giemsa-stained smears, respectively. A splenectomized calf was subcutaneously inoculated with 4 x 10^5 *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 of infected blood were aseptically withdrawn from 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 bovines were 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 the chronic donor bovine mentioned abovein a single i.v. injection (5 to 15 mL) containing 2x10^8 thechronic donor bovine mentioned above in a single i.v. injection (5 to 15 mL) containing 2x10^8.

Serum samples from 10 animals inoculated with 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 (EIA-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 associations between two variables were studied calculating the Pearson Correlation coefficient (https://www.soceistatistics.com/tests/pearson/).

**Results**

The results of *Anaplasma centrale* infection of bovines are shown in Chart 1. 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, a splenectomized calf was subcutaneously vaccinated with 4 x 10^8 *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 10^5 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 10^5 IE), showed a significantly longer pre-patent period than the other three groups (p < 0.01 for A vs B; and p < 0.05 for 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 C). However, the time lapsed 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 (R^2 = 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 (R^2 = 0.1449, negative correlation) or rickettsemia levels (R^2 = 0.0749, positive correlation).

Rickettsemia levels 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 and 38.78±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 splenectomized calves with *A. centrale* was used in the production of the trivalent vaccine for the prophylaxis of the bovine babesiosis-anaplasmosis syndrome. This vaccine is the result of technology transfer 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 *Anaplasma ovis* 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 of inoculated *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.

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