

Transplacental transmission of *Anaplasma marginale* in beef cattle chronically infected in southern Brazil

Transmissão transplacentária de *Anaplasma marginale* em bovinos de corte cronicamente infectados no sul do Brasil

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Received February 8, 2012

Accepted March 5, 2013

Abstract

In this study, we have investigated the incidence of transplacental transmission of *Anaplasma marginale* in chronically infected cows with no history of acute anaplasmosis during gestation. In addition, we evaluated various techniques for detection of transplacental transmission of *A. marginale*. Blood samples were collected from 30 cows at three different periods: at the time of artificial insemination, at gestational diagnosis, and after calving. Also, blood was collected from the newborn calves, including one sample before colostrum intake, and another three days after birth. *A. marginale*-specific antibodies were detected in 100% of the cows with an indirect fluorescent antibody test (IFAT), and in 97% of them, using an indirect enzyme-linked immunosorbent assay (ELISA). Also, we observed that all of the three-day-old newborn calves were seropositive by IFAT. According to polymerase chain reaction, 63.3% of the cows were carriers of *A. marginale*, as well as 6.7% of the newborn calves. This represented a transplacental transmission rate of 10.5%. Furthermore, a correlation of 93.3% was observed between the two serodiagnostic techniques, demonstrating that both ELISA and IFAT can be used in epidemiological surveys of *A. marginale*. These results confirm the occurrence of transplacental transmission of *A. marginale* in chronically infected cows and suggest the importance of this transmission route in areas of enzootic instability.

Keywords: *Anaplasma marginale*, transplacental transmission, enzootic instability.

Resumo

O objetivo do estudo foi avaliar a transmissão transplacentária de *A. marginale* em bovinos de corte cronicamente infectados utilizando diferentes testes de diagnóstico. Amostras de sangue foram colhidas de trinta matrizes durante o período de inseminação artificial, no diagnóstico de gestação e após o parto. Foi colhido sangue dos bezerros antes da ingestão do colostro e três dias após o nascimento. Anticorpos foram detectados em 100% das matrizes pela Reação de Imunofluorescência Indireta (RIFI) e 97% pelo Ensaio de Imunoabsorção Enzimática Indireta (ELISA). Aos três dias de idade 100% dos bezerros estavam soropositivos na RIFI. Conforme os resultados da PCR, 63.3% das matrizes e 6.7% dos bezerros eram portadores do agente, representando taxa de transmissão transplacentária de 10.5%. Correlação de 93.3% foi observada entre os resultados das técnicas sorológicas, demonstrando que tanto ELISA quanto RIFI podem ser utilizadas em levantamentos epidemiológicos do agente. Os resultados confirmaram a transmissão transplacentária do agente em vacas cronicamente infectadas. Isto demonstra a importância desta via de transmissão do *A. marginale* em áreas de instabilidade enzoótica.

Palavras-chave: *Anaplasma marginale*, transmissão transplacentária, instabilidade enzoótica.

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Introduction

Anaplasma marginale is a pathogen of the Rickettsiales order, family Anaplasmataceae (DUMLER et al., 2001), and is an obligate intraerythrocytic organism in vertebrates (KOCAN et al., 2000) that infects domestic and wild ruminants (KUTTLER, 1984). It causes jaundice, progressive anemia, weakness, and anorexia (REHBUN, 1995).

Anaplasmosis alone, or along with babesiosis, is responsible for significant economic losses in livestock farming worldwide, as it affects the performance of the cattle and limits their production. In fact, anaplasmosis causes death, weight loss, growth delay, low fertility in bulls, interference with the estrous cycle, and abortions (SWIFT et al., 1983; RIBEIRO; LIMA, 1996).

Enzootic stability to bovine anaplasmosis is found in most regions of Brazil (RIBEIRO; REIS, 1981; KESSLER et al., 1983), where at least 75% of nine-month-old cows show antibody seropositivity (MAHONEY; ROSS, 1972; GONÇALVES, 2000). In contrast, enzootic instability has been identified in the southern Rio Grande do Sul region, where 60-64% of nine-month-old cattle are seropositive for *A. marginale* (ARTILES et al., 1995; SILVA, 2003). This instability likely results from climate variations, leading to periods of low temperatures in which vector arthropods cannot reproduce.

The mechanisms of *A. marginale* transmission, as well as the epidemiologic importance of potential vectors, are very controversial (KESSLER, 2001; PYPERS et al., 2011). According to Kuttler (1984), *A. marginale* transmission does not occur transplacentally. However, other reports have suggested that transmission can occur when cows have acute anaplasmosis during gestation (ZAUGG; KUTTLER, 1984) or under conditions of constant inoculation in endemic areas (POTGIETER; VANRENSBURG, 1987; RIBEIRO et al., 1995).

Laboratory diagnosis of chronic anaplasmosis is performed via direct and indirect methods. The direct test involves identification of *A. marginale* DNA by polymerase chain reaction (PCR) and allows detection of parasitemia as low as 0.000025% in asymptomatic carriers (STILLER, 1992).

For indirect diagnosis, serological techniques are employed to detect antibodies against *A. marginale*. Indirect enzyme-linked immunosorbent assay (ELISA) is a widely used technique for detecting and/or quantifying antibodies in serum samples, especially during epidemiological studies (SOARES, 2001), showing a sensitivity of approximately 96.9% (MADRUGA et al., 2000). Also, the indirect fluorescent antibody test (IFAT) continues to be one of the most used serological assays, displaying a sensitivity of approximately 97.2% (MADRUGA et al., 2000). The aim of the present study was to evaluate the occurrence of transplacental transmission of *A. marginale* in chronically infected beef cattle in areas of enzootic instability using various diagnostic tests.

Materials and Methods

Study area and animals

The study was carried out on a beef cattle farm (geographic coordinates: 31° 45' 48" S and 52° 29' 02" W; average altitude: 21 meters). During initial screening, 50 Braford cows were selected

for blood collection, and 60% (30/50) of them were found to be seropositive for *A. marginale*. Thirty seropositive cows were included in the experiment, with deliveries occurring during the spring months (September and October). The cows were clinically followed throughout the gestational period by daily visual observation. Additional serological evaluations were performed at gestational diagnosis (during the third month of pregnancy) and three days after calving. The animals had been naturally exposed to ticks and hematophagous Diptera, as well as other handling procedures (injections and surgical procedures), which could be responsible for mechanical transmission. Moreover, the cows were not subjected to additional stress during the experiment.

Collection of blood

In order to obtain serum and packed red blood cells from animals, blood was collected in vacutainer tubes with and without ethylenediaminetetraacetic acid (EDTA). Samples were collected in a manner that did not interfere with the routine management procedures of the farm. For blood collection, either the coccygeal or jugular vein was punctured while the animals were in the corral of the farm. Blood samples were collected from the jugular veins of newborn calves before colostrum intake and then once again when they were three days old. All experimental animals were handled ethically. The collected material was kept at 4 °C and processed at the Laboratory of Parasitology of the Institute of Biology, Federal University of Pelotas. The serum was separated by centrifugation at 1,800 × g for 10 minutes at room temperature, and then stored at -20 °C. Samples with EDTA were also centrifuged at 1,800 × g for 10 minutes, and the plasma and white cells were removed, leaving only the packed red blood cells. This material was kept in collection tubes to avoid contamination and was stored at -20 °C until analysis at the Animal Health Laboratory of the Centro Nacional de Pesquisa em Gado de Corte (CNPGC) at EMBRAPA (Campo Grande, MS, Brazil).

Serological tests

Indirect fluorescent antibody test (IFAT)

Detection of *A. marginale*-specific IgG antibodies using IFAT was performed in accordance with Madruga et al. (2001). Antigens were prepared in the Protozoology and Animal Health Laboratory of the CNPGC at EMBRAPA. Serum and control samples were diluted at 1:160 in phosphate-buffered saline, pH 7.2 (PBS), and fluorescein isothiocyanate (FITC)-labeled anti-bovine IgG (Sigma Chemical Company) was diluted at 1:160 in PBS. The IFAT was performed with the support of a Nikon Eclipse E400 microscope, and samples were considered positive when yellow-green fluorescence was detected.

Indirect enzyme-linked immunosorbent assay (ELISA)

ELISA-based testing for *A. marginale*-specific antibodies in bovine serum samples was performed by the Protozoology and Animal Health Laboratory of the CNPGC at EMBRAPA. The serum samples were diluted at 1:1,000 in PBS with 0.05% Tween 20. Absorbance was measured at 490 nm using a BIOTEK ELX 800 GIDX. Negative and positive controls were included on each plate. ELISA cut-off values were empirically determined using readings (mean + 3 SD) of serum samples of healthy cattle from tick-free areas in accordance with Madruga et al. (2000).

Polymerase chain reaction (PCR)

Obtaining the DNA of *A. marginale*

DNA extraction was carried out using the Easy DNA Kit according to the manufacturer's instructions (Invitrogen). The *A. marginale msp5* gene (GenBank: M93392) was amplified using forward (5'-ATGAGAATTTTCAAGATTGTGTCTAACCTT-3') and reverse (5'-AGGAAAGCCCCAAAGCCCCATACTT-3') primers. For PCR, Taq DNA Polymerase (Invitrogen) was used, and the thermocycler conditions included 35 cycles (94 °C 1 minute; 55 °C 1 minute; 72 °C 1 minute). PCR products were visualized using a 1% agarose gel, which was stained with ethidium bromide. The 1 Kb Plus DNA Ladder (Invitrogen) was used as the molecular size marker, and the amplified *msp5* fragment was found to be 700 bp. Electrophoresis was performed at 80-90 V, and was stopped when the dye had migrated two-thirds of the gel length. Following electrophoresis, the gel was soaked in a solution of ethidium bromide (0.5 g/mL) for 10-30 minutes, and subsequently unstained in a water bath for 10-20 minutes. Bands were visualized using a UV transilluminator according to Araújo et al. (2002).

Statistical analysis

Sensitivity, specificity, predictive values (both positive and negative), and test accuracy were evaluated according to Coggon et al. (1993), using ELISA results as the gold standard. Comparisons between the prevalence of seropositive results detected by IFAT and ELISA techniques were performed by chi-square and Fischer's

exact tests, with a confidence interval of 95%. Statistical analyses were performed using Statistix 9.0 software (STATISTIX®, 2008).

Results

All experimental cows (100%) continued to test positive for *A. marginale*-specific antibodies by IFAT until the end of the experiment. In addition, three calves (10%) were found to be seropositive at birth (before colostrum intake). Three days after birth, when the test was repeated, 100% of newborn calves were found to be positive.

The results obtained by ELISA (at the dilution tested) indicated that a total of 29 cows (97%) were seropositive. However, no antibodies were detected by ELISA in the samples from the newborn calves. The sera collected three days after birth were not tested by ELISA. Considering all of the test results from the 60 serum samples (30 cows and 30 calves), the rates of seropositivity by IFAT and ELISA were 55% and 48.3%, respectively. Moreover, the difference between these values was not statistically significant ($p > 0.05$) in the chi-square test (Table 1).

The index of co-positivity was 100% (29 samples were both ELISA⁺ and IFAT⁺) and co-negativity was 87.1% (27 samples were ELISA⁻ and IFAT⁻), which according to Coggon et al. (1993), corresponded to a concordance of 93.3% between the two tests (Table 2). A separate analysis of the results from only the adult animals revealed a concordance of 96.6% between the two techniques (ELISA and IFAT), whereas analysis of the data from the calves yielded a concordance of 90%.

Using the Kappa Index according to Smith (1995), we obtained a value of 0.93, which demonstrated almost perfect concordance between the two tests. To determine sensitivity and specificity of IFAT, it was compared to the ELISA test, which was considered to be the gold standard because it presents high specificity and produces few false positive results (MARANA et al., 2009; BRAZ et al., 2000).

A total of 19 of the 30 cows were positive (63.3%) for *A. marginale* by PCR. Also, examination of calves at birth revealed that 2 out of 30 (6.7%) were infected. These results indicated that the transplacental transmission rate detected by PCR was 10.5% (2/19). Notably, antibodies against *A. marginale* were detected by IFAT in 10% (3/30) of newborn calves. Thus, *A. marginale* DNA was detected in 66.7% (2/3) of the seropositive newborn calves (IFAT⁺).

Table 1. Results of IFAT and ELISA tests for *A. marginale* in the serum of 30 cows and 30 calves at birth, in beef cattle chronically infected in southern Brazil.

	IFAT			ELISA		
	Positive	Negative	%Positive	Positive	Negative	%Positive
Total (n = 60)	33	27	55	29	31	48.3
Cows (n = 30)	30	0	100	29	1	96.7
Calves (n = 30)	3	27	10	0	30	0

The difference between the occurrence detected by both tests for each animal category and the two categories together were not statistically significant ($p > 0.05$).

Table 2. Results and validity analysis of IFAT tests for *A. marginale* in the serum of 30 cows and 30 calves at birth in relation to ELISA (gold standard) in beef cattle chronically infected in southern Brazil.

ELISA	IFAT		Total
	Positive	Negative	
Positive	29 ^a	0 ^b	29
Negative	4 ^c	27 ^d	31
Total	33	27	60

According to Coggon et al. (1993): Sensitivity: $a / (a+c) \times 100 = 87.9\%$; Specificity: $d / (b + d) \times 100 = 100\%$; Predictive Value (+): $a / (a + b) \times 100 = 100\%$; Predictive Value (-): $d / (c + d) \times 100 = 87.1\%$; Accuracy: $(a + d) / (a + b + c + d) \times 100 = 93.3\%$.

Discussion

The experimental year (2007) presented favorable weather conditions for the development of hematophagous Diptera and ticks during autumn, spring, and summer. However, unfavorable weather conditions were observed from May to August, with temperatures lower than 15 °C, even though relative humidity (RH) of the air was higher than 80%. During spring, weather conditions once again became favorable for the vectors, as RH ranged from 72.2% to 84.6%, and mean temperature was between 16.2 °C and 21.7 °C.

The results obtained in the initial screening test by IFAT, showed that only 60% of cows had antibodies against *A. marginale*. This prevalence was also found in areas of enzootic instability (MAHONEY; ROSS, 1972), as demonstrated by Artiles et al. (1995) and Silva (2003). Only seropositive cows were selected for this study, as the aim was to evaluate transplacental transmission of *A. marginale* in chronically infected beef cattle. The transplacental transmission rates detected by PCR and IFAT in this study were in agreement with those previously reported by Potgieter and Vanrensburg (1987), who studied animals with chronic infection and reported a transplacental transmission rate of 12.5% using a card agglutination test (CAT). In Brazil, Ribeiro et al. (1995) also reported transplacental transmission of *A. marginale* in fetuses during the second and third of gestation in endemic areas, identifying a rate of 2.1% by ELISA. Therefore, our data might indicate that the incidence of transmission is higher at the end of gestation, which could result from increased time for transmission. However, this finding could also be due to the fact that the older fetuses become immunologically competent allowing seropositivity.

Our observed concordance between the serological tests is similar to that previously determined by Madruga et al. (2000) (96%), in which ELISA was used to detect antibodies against *A. marginale* of initial corpuscles obtained from splenectomized calves (80% displaying rickettsial co-infection).

In the present study, ELISA results did not indicate transplacental transmission of *A. marginale* because no immunoglobulins could be detected in any calves at birth. This finding may have resulted from a low concentration of circulating antibodies in these newborn calves. In this regard, it is possible that the high dilution of sera used in our tests (1:1,000 at ELISA, and 1:160 at IFAT) could have contributed to these negative results.

Here, all newborn calves were positive by IFAT three days after birth. This finding shows the efficacy of immunoglobulin transmission through colostrum. These data are in agreement with a study by Silva (2003), which reported 100% transmission of immunoglobulins via colostrum in newborn calves aged 5 and 15 days in a herd under similar epidemiological conditions to those of our experiment.

The fact that one animal was found negative by PCR, whereas all samples had tested positive by IFAT, probably indicates a false positive serum result. This finding likely stems from the specificity of IFAT, which is not 100% (BRAZ et al., 1997). This hypothesis is confirmed by the fact that techniques based on DNA amplification display high sensitivity and specificity and are capable of identifying animals with extremely low parasitemias, like carriers and asymptomatic young calves (BRITO et al., 2007).

Conclusion

This study demonstrated that even in an area of enzootic instability, cows chronically infected with *A. marginale*, without a history of acute anaplasmosis during gestation, could infect offspring transplacentally. Moreover, all cows serologically positive for *A. marginale* transferred antibodies to their offspring via colostrum at levels detectable by IFAT. Taken together, we have shown that IFAT and ELISA are efficient for indirect diagnosis of *A. marginale* infection in cattle, particularly in epidemiological surveys.

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