Seroprevalence fo *Anaplasma marginale* in dairy cattle and, studies on the dynamics of natural infection of Holstein calves in Southern Brazil

Soroprevalência de *Anaplasma marginale* em bovinos leiteiros e estudos sobre a dinâmica da infecção natural em bezerros holandeses no Sul do Brasil

Gisele Maria de Andrade[1]; Odilon Vidotto[2]*; Marilda Carlos Vidotto[3]; Eidi Yoshihara[4]; Flora S. Kano[5]; Carlos Henrique S. Amaral[6]

Abstract: Sera of 708 animals (cows, heifers and calves) from 13 dairy herds in the Londrina region of Paraná, Brazil, were tested for antibodies to *Anaplasma marginale* by a competitive ELISA assay (cELISA). Ten 2 to 20 days old Holstein calves, from one of the 13 herds studied, were monitored during one year. Blood samples from each calf were collected monthly and tick burden counting was performed every fortnight. Percentage of infected erythrocytes was established by Giemsa-stained smears, and sera samples were examined by cELISA to detect antibodies against *A. marginale.* In the 13 herds, 92.94% of the animals were seropositive to *A. marginale,* which indicates that Londrina is an area of enzootic stability. Among the three animal categories (cows, heifers and calves), the rates were 98.29%, 96.64% and 81.25%, respectively. Passive transfer of maternal antibodies to calves was demonstrated by cELISA. From ten calves, nine (90%) were seropositive at the first sampling, revealing colostral antibodies anti-A. *marginale*. These antibodies remained in calves for 2 to 3 months. After this period the calves were infected with ticks, and then all of them were seropositive to *Anaplasma*. Five 4 to 7 months old calves showed rickettsemia ranging from 0.1% to 3.8%. Two of them were treated with tetracycline. The rickettsemia and clinical signs of anaplasmosis of these calves were coincident with tick burden increase.
Key words: *Anaplasma marginale*, seroprevalence, dairy cattle, cELISA, tick burden.

Resumo: Soros de 708 animais (vacas, novilhas e bezerros) oriundos de 13 propriedades leiteiras da região de Londrina, Estado do Paraná, Brasil, foram testados para a presença de anticorpos contra *Anaplasma marginale* pelo teste ELISA competitivo (cELISA). Dez bezerros recém nascidos, pertencentes a um dos 13 rebanhos utilizados no levantamento sorológico, foram monitorados durante um ano. De cada bezerro foram colhidas amostras de sangue mensalmente e a cada quinze dias foi realizada a contagem de carrapatos. A porcentagem de eritrócitos parasitados foi determinada pela coloração de Giemsa em esfregaços sanguíneos e as amostras de soros foram submetidas ao cELISA para a detecção de anticorpos contra *A. marginale*. Dos animais dos rebanhos, 92,94% foram soropositivos para *A. marginale* indicando que a região de Londrina é uma área de estabilidade enzoótica. Nas três categorias de animais estudadas (vacas, novilhas e bezerros) as taxas de soropositivos foram de 98,29%, 96,64% e 81,25%, respectivamente. Nove dos 10 bezerros (90%) foram positivos pelo cELISA logo após o nascimento, o que demonstra a transferência de anticorpos colostrais. Os níveis de anticorpos colostrais foram detectados até o segundo e o terceiro mês de idade com um decréscimo neste período. Os níveis de anticorpos contra *A. marginale* voltaram a crescer nos meses seguintes, após os animais se infestarem com o *Boophilus microplus*, quando todos eram positivos. Cinco bezerros com idades entre quatro e sete meses apresentaram rickettsemias detectáveis pela coloração de Giemsa, variando de 0,1% a 3,8%, sendo que dois destes animais foram tratados com tetraciclina. Estas rickettsemias e os sinais clínicos apresentados por estes bezerros foram coincidentes com o aumento do número de carrapatos sobre os animais.
Palavras-chave: *Anaplasma marginale*, soroprevalência, bovinos leiteiros, cELISA, infestação por carrapato.

Introduction

Bovine anaplasmosis is an economically important disease caused by the Anaplasmataceae *Anaplasma marginale*, and has a widespread distribution in areas with tropical-to-temperate climates (RISTIC, 1968). *A. marginale* is transmitted to cattle biologically by ticks and mechanically by biting flies and fomites (HAWKINS *et al.*, 1982; ZAUGG *et al.*, 1986; AGUIRRE *et al.*, 1988). The infection becomes patent microscopically 2 to 6 weeks post-transmission, depending on the number of organisms transmitted and the virulence of the isolate. At peak infection the rickettsemia levels exceed 10^9 infected erythrocytes per ml and the clinical signals developed are severe anemia, fever, weight loss and death. Following acute anaplasmosis, repetitive cycles

[1] Curso de Pós-graduação em Med. Vet., UNESP- Jaboticabal, Doutorado (Patologia Animal).
[2]*Universidade Estadual de Londrina (UEL), Depto de Med. Vet. Preventiva, Campus Universitário, Caixa Postal 6001, Londrina, Paraná, 86051-990. E-mail: <vidottto@uel.br>.
[3] Depto de Microbiologia, UEL.
[4] Fundação Faculdades *Luiz Meneguel* (FFALM), Depto de Zootecnia, Bandeirantes, Pr.
[5] Fundação Educacional de Guarapuava (FEG) – Escola Superior de Ciências Agrárias.
[6] Secretaria de Agricultura e de Abastecimento do Estado do Paraná.

of rickettsemia ranging from approximately $10^{2.5}$ to 10^{7} infected erythrocytes per ml characterize the persistent infection (ERIKS *et al.*, 1993; KIESER *et al.*, 1990; FRENCH *et al.*, 1998). Persistently infected cattle remain carriers with low levels of rickettsemia, which can not be detected microscopically, serving as a permanent source of infection for susceptible cattle (ZAUGG *et al.*, 1986; KUTTLER, 1984; SWIFT; THOMAS, 1983).

The identification method commonly used to confirm acute anaplasmosis is the microscopic examination by Giemsa stained blood smears, by which can be only detected levels over 10^{6} infected erythrocytes per ml (GALE *et al.*, 1996). Nested-PCR (nPCR) can reveal carrier cattle by amplification of *A. marginale* DNA, and serological reactions detect *A. marginale*- specific antibodies in serum.

The performance of four serological tests for the detection of antibodies against *A. marginale* (Rapid Conglutination Test - RCT, Immunofluoerescence antibody test (IFA), enzyme linked Immunosorbent assay) (Araújo *et al.*, 1998) and a competitive ELISA (cELISA) based on the *A. marginale* recombinant protein MSP5 and its monoclonal antibody (mAb) ANAF16C1 (Knowles *et al.*, 1996) was evaluated by Marana *et al.* (1998) with 237 bovine sera. When the tests were analyzed individually, the cELISA showed the lowest percentage of discrepancy (4.9%) as compared to indirect ELISA (10.4%), IFA (23.0%) and RCT (28.2%), showing that cELISA test was the most specific and sensitive of them.

A previous study about prevalence of anaplasmosis infections in dairy cattle in Londrina, Paraná, by cELISA test, showed that the epitope on MSP-5 defined by mAb ANAF16C1 is conserved within *A. marginale* in this region (VIDOTTO *et al.*, 1998).

The present study, using a larger number of sera from three animal categories, shows a more accurate prevalence for *A. marginale,* by the cELISA test and, evaluation of the *A. marginale* natural infection in a dairy herd from the Londrina region.

Material and Methods

Sera samples and animals

This study was carried out in Londrina county situated in northern Paraná, in Southern Brazil (23º08' 47 W; 23º 55' 46 S, 576m) with an average temperature of 22ºC and an average annual pluviometric rainfall of 1876mm (Instituto Agronômico do Paraná – IAPAR, 1998). A total of 708 sera from 13 Holstein pure and crossbred herds (208 calves, 149 heifers and 351 cows) were randomly selected for testing, during January-April 1998. Blood samples were collected, from the jugular vein, using disposable needles for each animal. Sera were separated, then removed from the clotted blood samples and stored at -20ºC until to be used.

One of the 13 herds studied, showed 100% of cows seropositive for *A. marginale* and was chosen to study the dynamics of the natural infection, by monitoring of ten Holstein calves, age ranging from 2 to 20 days old, at fortnight intervals, during one year from April 1997 to March 1998. Calves received the colostrums immediately after birth and then they were separated from their dams at one to two days of age and fed artificially in individual places, until being turned out to pasture, when they were between two-three months old.

Blood samples were collected monthly from each calf into two tubes, one containing ethylenediaminetetraacetic acid (EDTA) anticoagulant and the other without it. EDTA-blood samples were used to make thin blood smears. Non-EDTA blood samples were token to perform cELISA. The rickettsemia observed by microscopic examination of Giemsa-stained blood smears was calculated according to the Instituto Interamericano de Cooperation para la Agricultura (IICA) (1984). Every two weeks half body counts of standard female *Boophilus microplus* ticks (>4.5mm) were carried out according to Wharton & Roulston, 1970. Calves received acaricide treatments according to the farm management practices schedule considering tick infestation. Clinical signs and treatments that had occurred to calves during the experimental study were recorded.

rMSP5-cELISA

The test was performed as previously described (Knowles *et al.*, 1996), based on the recombinant major surface protein 5 (rMSP5) conserved among *Anaplasma* sp. Two negative male Holstein calves raised at an isolation area of Londrina State University Veterinary Hospital, persistently negative by IFA and nPCR, were included as negative controls and a sera pool of *A. marginale* infected animals from Londrina region was used as positive controls. Reactions were stopped with 25 mL of 2N NH_2SO_4, and optical density at 492 nm (OD_{492}) was determined with microplate reader. The percentage of inhibition (PI) for the test sera was calculate relative to the negative control serum using the formula: PI = 100 – (100 x test serum absorbance/ negative control absorbance). The cutoff point selected to discriminate between negative and positive sera was 25% inhibition.

Statistical analysis

The Chi-square test (FLEISS, 1981) was used in the prevalence survey, to compare the positive sera percentages among the animal categories studied with a $p<0.01$ level of significance.

Results and Discussion

Of 708 serum samples tested, from three animal categories, 658 (92.9%) were positive to *A. marginale* antibodies by the rMSP5-cELISA assay (Table 1). The sera tested presented PI between 5% and 100%, with the most part over 70% (Fig. 1). These values show

that the mojority of animals (74.0%) had high levels of antibodies against *A. marginale*. Considering the three animal categories in separete, the statistical analysis showed association between animal ages and reactivity to *A. marginale.* Calves exhibited a lower frequency of seropositive (81.2%) than heifers (96.6%) and cows (98.3%). These results suggest that calves should be more exposed to *A. marginale* infection than other animal categories.

In a previous study with 410 sera samples collected in 1991-1992 from Londrina, the prevalence for cows was 87.5%, varying from 78.2% to 93.9% among 12 herds (VIDOTTO *et al.* 1998). The seroprevalence rate for *A. marginale* found in Londrina, in both studies, is in agreement with other rates (86.5% to 98.0%) found throughout the country: Minas Gerais (RIBEIRO; REIS, 1981), Mato Grosso do Sul (MADRUGA *et al.*, 1985), Bahia (ARAUJO *et al.*, 1995; ARAUJO *et al.*, 1998) and Rio Grande do Sul (DALAGNOL *et al.*, 1995). Considering the criteria defined by Mahoney and Ross (1972), all these regions studied are areas of enzootic stability. In such conditions (high prevalence rates) the herds maintain high levels of antibodies against *A. marginale* all over the year and outbreaks of clinical disease rarely occur. However, there are some particular situations, such as low tick load, lack of rain and arid regions, where the prevalence rates are lower, characterizing areas of enzootic instability (OLIVEIRA *et al.*, 1992; ARTILES *et al.*, 1995).

In this work, passive transfer of maternal antibodies to calves was demonstrated by the rMSP5-cELISA. From ten calves, nine were seropositive on the first month with ages varying from 2 to 20 days old, showing colostral antibodies anti-A*. marginale* rMSP5 transfers (Table 2). Only one 15 days old calf (number 527) was negative and remained serologically negative until 135 days old. In the following month, this calf became infected, showing 0.1% of rickettsemia by Giemsa stain. At this time it was counted 24 adult ticks fixed on it and one month later the rickettsemia was increased to 3.8% and the animal presented clinical signs of anaplasmosis.

Table 1 – Seroprevalence rates for *Anaplasma marginale* in dairy herds from the Londrina region of Paraná State, Brazil, by cELISA assay.

(p< 0.01)

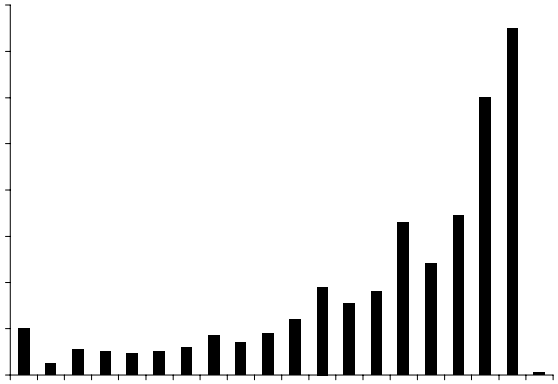

Figure 1 – Frequency distribution of rMSP-cELISA percent inhibition values for 708 dairy cattle from Londrina, Paraná State, Brazil.

Table 2 – Results of monthly samples collections during 12 months, from April 1996 to March 1997, analyzed by Giemsa stain and cELISA, from Holstein calves in Londrina County, Paraná State, Brazil.

The transferred colostral antibodies level decreased at 2nd sampling, when the animals had about two months until the 4th sampling and, after the 6th month all calves had high *A. marginale* antibody levels, corresponding to the ticks' presence (Fig. 2 and 3). Similar data were found by Herrero *et al.* (1998) that found 79% of the calves with post colostral antibodies at birth, by cELISA assay, decreasing to 13% at first month. Madruga *et al.* (1987), monitoring beef calves from birth until 210 days old, detected decreasing of colostral antibodies against *A. marginale* on 47th day. The passive transfer of colostral antibodies from the dams was also demonstrated experimentally in heifers infected with *A. marginale* during gestation period (SWIFT *et al.*, 1978).

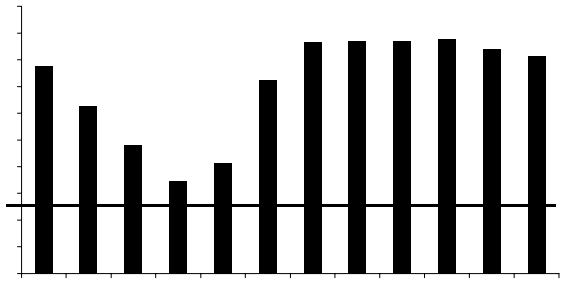

Figure 2 – Frequency distribution of rMSP5-cELISA percent inhibition by sera of ten calves during a period of 12 months, in an *A. marginale*-infected herd, in Londrina, Paraná State, Brazil.

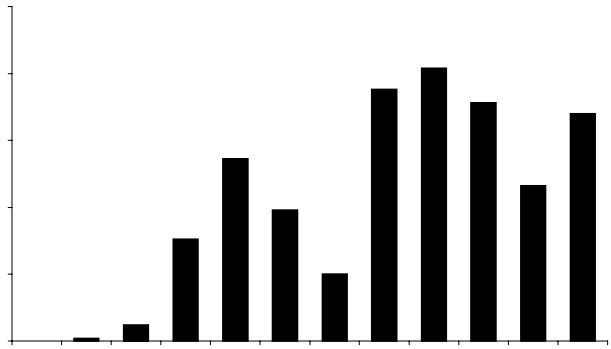

Figure 3 – Mean frequency of tick burden on Holstein calves during a period of 12 months, beginning in April 1997, in Londrina, Paraná State, Brazil.

Detection of the *B. microplus* on the calves occurred for the first time at the 2nd month (calf 534 showed two ticks) and next, at the 3rd month (calves 529, 533 and 998, had six, two and two ticks, respectively). All the calves had tick burden counting recorded and increased around the 4th and 5th month (Table 2, Fig. 3).

None of the calves presented clinical disease until the fifth month after birth, indicating that colostral antibodies may have an important role on protecting them against *A. marginale* infection during the first four months after birth. Additionally, the ticks started to appear on the calves around the 2nd and 3rd months, but the tick burden increased significantly only in the 5th month after birth, when rickettsemia was detected for the first time. Rickettsemia detectable by Giemsa stained slide smear occurred in four calves (526, 527, 529 and 530). Two calves (527 and 530) showed clinical signs of anaplasmosis and they had treatment with tetracycline during rickettsemia. All cases of anaplasmosis with recorded rickettsemia occurred from the fifth to eighth months after birth, coinciding with the higher tick burden coutings. *A. marginale* infection usually becomes patent microscopically 20 to 40 days post rickettsia inoculation (RISTIC, 1981). In this experiment the tick burden increased between 15 and 30 days before the detection of rickettsemia and the appearance of clinical signals of anaplasmosis (Table 2). These facts together, strongly suggest an horizontal transmission of *A. marginale* by *B. microplus*, after the calves are turned out to pasture. Furthermore, the calves showed low levels of antibodies from the third to the fifth months and then increased significantly remaining high until the end of the experiment.

References

AGUIRRE, D.H.; BERMUDEZ, A.C.; MANGOLD, A.J.; GUGLIELMONE, A. A.. Natural infection with *Anaplasma marginale* in cattle of the Hereford, Criolla and Nelore breeds in Tucumán, Argentina. *Ver. Latinoam. Microbiol.* v.30, p.37-41, 1988.

ARAUJO, F.R.; LEAL, C.R.B.; MADRUGA, C.R.; MIGUITA, M.;CARVALHO, E.L.L. Levantamento sorológico para *Anaplasma marginale* em duas microregiões do Estado da Bahia. *Rev. Bras. Parasitol. Vet.* v. 4, p.187 (suplemento 1), 1995.

ARAÚJO, F.R.; MADRUGA, C.R.; LEAL, C.R.B.; BASTOS, P.A.S.; MARQUES, A.P.C. Frequência de anticorpos anti-*Anaplasma marginale* em rebanhos leiteiros da Bahia. *Arq. Bras. Med. Vet. Zootec.*, v. 50, n. 3, p. 243-246, 1998.

ARTILES, J.; ALVES-BRANCO, F. de P.J.; MARTINS, J.R.; CORREA, L.B.; SAPPER, M.F. de M., 1995. Prevalência de *Babesia bovis*, *B. bigemina* e *Anaplasma marginale* no Município de Bagé, RS. *Rev. Bras. Parasitol. Vet.*, v. 4, p. 179 (suplemento 1), 1995.

DALAGNOL, C.A.; MARTINS, C.R.; MADRUGA, C.R. Prevalência de anticorpos contra *B. bovis*, *B. bigemina* e *Anaplasma marginale* em bovinos de corte na região de clima Cfb. *Rev. Bras. Parasitol. Vet.*, v. 4, p. 220 (suplemento 1), 1995.

ERIKS, I.S.; PALMER, G.H.; MCGUIRE, T.C.; ALLRED, D.R.; BARBET, A.F. Detection and quantification of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. *J. Clin. Microbiol.,* v. 27, p. 279-284, 1989.

ERIKS, I.S.; STITLER, D.; PALMER, G.H. Impact of persistent *Anaplasma marginale* on tick infection and transmission. *J. Clin. Microbiol.*, v. 31, p. 2091-2096, 1993.

FLEISS, J. L. *Statisticals Methods for Rates and Proportions*. 2nd ed. [S.l.]: John Wiley& Sons, 1981. p. 14-15.

FRENCH, D.M.; MCELWAIN, T.F.; MCGUIRE, T.C.; PALMER, G. H.. Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect. Immun.* v. 66, p. 1200-1207, 1998.

GALE, K.R.; DIMMOCK, C. M.; GARTSIDE, M.; LEATCH, G. *Anaplasma marginale*: detection of carrier cattle by PCR. *Int. J. Parasitol.*, v. 26, p.103-1109, 1996.

HAWKINS, J.A.; LOVE, J.N.; HIDALGO, R.J. Mechanical transmission of anaplasmosis by tabanids (Diptera: Tabanidae). *Am. J. Vet. Res.,* v. 43, p. 732-734, 1982.

IAPAR. *Cartas climáticas do Estado do Paraná*. Londrina, 1999.

INSTITUTO INTERAMERICANO DE COOPERATION PARA LA AGRICULTURA (IICA). *Tecnicas para el diagnostico de babesiosis y anaplasmosis.* Costa Rica: IICA, 1984. (Serie Salud Animal. Publication Cientifica, n. 8).

KIESER, S.T.; ERIKS, I.S.; PALMER, G.H. Cyclic ricketsemia during persistent *Anaplasma marginale* infection in cattle. *Infect. Immun.*, v. 58, p. 1117-1119, 1990.

KNOWLES, D. P.; TORIONI DE ECHAIDE, S.; PALMER, G. H.; MCGUIRE, T.; STILLER, D.; MCELWAIN, T. Antibody against *Anaplasma marginale* MSP-5 epitope common to tick and erytrocytes stages identifies persistently infected cattle. *J. Clin. Microbiol.,* v. 34, p. 2225-2230, 1996.

KUTTLER, L.K. Anaplasma infections in wild and domestic ruminants: a review. *J. Wildl. Dis.*, v. 20, p.12-20, 1984.

MADRUGA, C. R.; HONER, M. R.; SCHENK, M.A.M.; CURVO, J. B. E. Avaliação preliminar de parâmetros epidemiológicos da tristeza parasitária bovina no Mato Grosso do Sul. Centro Nacional de Pesquisa de Gado de Corte (CNPGC). *EMBRAPA, Boletim Técnico*, n. 38, p.1-7, 1987.

MADRUGA, C. R.; KESSLER, R. H.; GOMES, R. H.; SCHENK, M. A. M.; ANDRADE, D. F. Níveis de anticorpos e rickettsemia em área enzoótica, nos bezerros da raça Nelore, Ibage e cruzamentos de Nelore. *Pesq. Agropec. Bras.*, v. 20, p. 135-147, 1985.

MAHONEY, D. F.; ROSS, D. R. Epizootiological factors in the control of bovine babesiosis. *Australian Veterinarian Journal*, v. 48, p. 292-298, 1972.

MARANA, E. R. M.; VIDOTTO, O.; MADRUGA, C. R.; ANDRADE, G. M.; ALFIERI, A. A. Evaluation of agglutination card test (CA), IFA, ELISA and cELISA for the detection of antibodies against *Anaplasma marginale*. In: ANAIS XVI Congreso Panamericano de Ciencias Veterinarias, Santa Cruz de la Sierra. Bolivia, 1998. p. 83. (Resumo)

OLIVEIRA, A.A. de; PEDREIRA, P.A.S.; ALMEIDA, M.P.R.S de *et al.* Doenças de bezerros. II. Epidemiologia da Anaplasmose no estado de Sergipe. *Arq Bras Med Vet Zoot.,* v. 44, n. 5, p. 377-386, 1992.

RIBEIRO, M.F.B.; REIS, R. Prevalência de Anaplasmose em Quatro Regiões do Estado de Minas Gerais. *Arq. Esc. Vet.* UFMG, Belo Horizonte., v. 33, n. 1, p. 57-62, 1981.

RISTIC, M. Anaplasmosis. In: WEINMAN, D.; RISTICK, M. (Ed.). *Infectious blood diseases of man and animals*. New York: Academic Press, 1968. v. 2, p. 478-572.

SWIFT, B.L.; THOMAS, G.M.. Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. *J. Am. Vet. Med. Assoc.*, v. 183, p. 63-65, 1983.

SWIFT, B. L.; SETTLEMIREAND, J.; THOMAS, G. M.. Inoculation of pregnant beef heifers at midgestation with *Anaplasma marginale*. *Theriogenology*, v. 10, p. 481-485, 1978.

TORIONI DE ECHAIDE, S.; KNOWLES, D.P.; MCGUIRE, T.C.; PALMER, G.H.; SUAREZ, C.E.; MCELWAIN, T.F. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. *J. Clin. Microbiol.*, v. 36, p. 777-782, 1998.

VIDOTTO, M.C.; VIDOTTO, O.; ANDRADE, G.M.; PALMER, G.; MCELWAIN, T.; KNOWLES, D P. Seroprevalence of *Anaplasma marginale* in cattle in Parana state, Brazil, by MSP-5 competitive ELISA. *Annals of the N. Y. Acad. of sci.* v.849, p.424-426, 1998.

VISSER, E.S.; MCGUIRE, T.C.; PALMER, G.H.; DAVIS, W.C.; SHKAP, V.; PIPANO, E.; KNOWLES, D.P. The *Anaplasma marginale* msp5 gene encodes a 19-kilodaldonprotein conserved in all recognized *Anaplasma* species. *Infec. Immun.* v. 60, p. 5139-5144, 1992.

ZAUGG, J.L.; STILER, D.; CROAN, M.E.; LINCOLN, S.D. Transmission of *Anaplasma marginale* Theiler by males of *Dermacentor andersoni* Stiles fed on an Idaho field infected, chronic carrier cow. *Am. J. Vet. Res.* v.47, p.2269-2271,1986.

WHARTON, R.H.; ROULSTON, W.J. Resistence of tick to chemicais. *An. Rev. Entomol.,* v. 15, p. 381-404, 1970.