STUDIES ON THE *Anaplasma marginale* THEILER, 1910 INFECTION IN *Boophilus microplus* (CANESTRINI, 1887) USING 'NESTED' PCR

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ABSTRACT. MOURA A.B. DE, VIDOTTO O., YAMAMURA M.H., VIDOTTO M.C.; PEREIRA A.B. DA L. **Studies on the *Anaplasma Marginale* Theiler, 1910 infection in *Booplhilus microplus* (Canestrini, 1887) Using 'Neste' PCR.** [Estudos da infecção por *Anaplasma marginale* Theiler, 1910 no *Boophilus microplus* (Canestrini, 1887) utilizando a técnica de Nested PCR]. *Revista Brasileira de Parasitologia Veterinária*, v. 12, n. 1, p. 27-32, 2003. Universidade Norte do Paraná-UNOPAR, Arapongas, PR 86700-000, Brazil. E-mail: anderbmoura@hotmail.com.br

The nested Polymerase Chain Reaction (nPCR) technique was used to detect the presence of *Anaplasma marginale* DNA fragments in *Boophilus microplus* tick progeny (eggs and larvae) and to determine when they become infected by this rickettsia. Eggs and larvae were obtained by incubation of tick engorged females, harvested from *A. marginale* carrier cattle. The nPCR carried out on the tick donor cattle blood and on the engorged females, eggs and larvae of *B. microplus* identified DNA bands of *A. marginale* with 345 bp, in which specificity was confirmed by cleavage with the Eco R I restriction enzyme. The results showed that animals with a low percent of infected erythrocytes (0-0.2%) detected by Giemsa staining are capable of infecting *B. microplus* females. In the progeny of positive engorged females, fragments of *A. marginale* DNA were only detected in the eggs of the fourth day and in the larvae of the first and seventh days of oviposition (three positive samples in 104 analyzed, or 2.88%) and, in the progeny of negative engorged females, thirty-four of the 152 samples analyzed (22.4%) showed positive larvae and eggs after the first and second days of oviposition, respectively. Specific fragments of *A. marginale* DNA were identified in eggs and larvae from *B. microplus* engorged females that fed on cattle with low parasitemia under field conditions, suggesting a possible migration of *A. marginale* through the general cavity of the tick, infecting the ovaries and then, eggs and larvae.

KEY WORDS**:** *Anaplasma marginale*, *Boophilus microplus*, migration, ovaries, nested-PCR.

RESUMO

A técnica de *nested* PCR (nPCR) foi utilizada para detectar a presença de fragmentos do DNA de *Anaplasma marginale* na progênie (ovos e larvas) de *Boophilus microplus* e determinar quando ela se torna infectada pela rickettsia. Ovos e larvas foram obtidos por incubação de teleóginas colhidas de bovinos portadores de *A. marginale*. A nPCR realizada no sangue dos animais doadores de carrapatos e nas teleóginas, ovos e larvas identificou bandas de DNA de *A. marginale* com 345 pbs, cuja especificidade foi confirmada pela clivagem com a enzima de restrição Eco R I. Os resultados mostraram que animais com baixo percentual de eritrócitos parasitados (0 – 0.2%), detectados na coloração de Giemsa, são capazes de infectar teleóginas de *B. microplus*. Na progênie das teleóginas positivas, fragmentos de DNA de *A. marginale* foram detectados somente nos ovos do quarto dia e em larvas do primeiro e sétimo dias de oviposição (três amostras positivas em 104 analisadas, ou 2,88%) e, na progênie das teleóginas negativas, 34 das 152 amostras analisadas (22,4%) mostraram larvas e ovos positivos a partir do primeiro e segundo dias de oviposição, respectivamente. Fragmentos específicos de DNA de *A. marginale* foram identificados em ovos e larvas de teleóginas de *B. microplus* que se alimentaram em bovinos com baixa parasitemia sob condições de campo, sugerindo

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uma possível migração de *A. marginale* através da cavidade geral do carrapato, infectando os ovários e, então, ovos e larvas.

PALAVRAS-CHAVE: *Anaplasma marginale, Boophilus microplus*, migração, ovários, Nested-PCR.

INTRODUCTION

Ticks and diseases that they transmit to cattle are one of the major problems of livestock in tropical and subtropical regions throughout the world. Among these diseases are the anaplasmosis and babesiosis popularly known in Brazil as "Tristeza Parasitária Bovina (TPB)", caused by three distinct pathogens: the protozoans *Babesia bovis* (Babès, 1888) and *B. bigemina* (Smith; Kilborne, 1893) and the rickettsia *Anaplasma marginale,* Theiler (1910).

Anaplasmosis causes meat and milk production losses, abortion and death during the acute phase of the infection (ALDERINK; DIETRICK, 1983). It is transmitted mainly by the tick, *Boophilus microplus* (THOMPSON; ROA, 1978; ALONSO et al., 1992; MARTINS; CORRÊA, 1995). It can also be transmitted by other ticks, as well by blood sucking insects and surgical materials (ANZIANI, 1979; RICHEY, 1981; EWING et al., 1997) and through the placenta (ZAUGG, 1985; ANDRADE, 1998).

The presence of different evolutionary phases of this rickettsia in *B. microplus* intestine epithelial cells suggests that sequential development stages of *A. marginale* may occur in the invertebrate host, thus characterizing its replication in this arthropod (RIBEIRO; LIMA, 1996) which shows its function as a biological vector.

Although the predominant transmission of rickettsia is by the tick *B. microplus*, its biology in this vector is still under discussion (FARIAS, 1995). Guglielmone (1991), ALONSO et al. (1992) and Vanzini and Ramirez (1994) quoted several authors who reported successful transovarial transmission of *A. marginale* by *B. microplus* and other studies indicating the contrary. Thompson and Roa (1978) and Ribeiro et al. (1996) working in Colombia and Brazil, respectively, did not confirm *A. marginale* transovarial transmission by *B. microplus*. By the other hand, Laranja et al. (1975), in Brazil and, Lopes-Valencia and Vizcaino-Gerdts (1992) in Colômbia, have showed evidences of transovarian transmission of A. marginale by the *B. microplus*. This paper shows the result with *A. marginale* in engorged *B. microplus* females collected from naturally infected cattle evidencing the rickettsia migration to the eggs and larvae during the oviposition period.

MATERIAL AND METHODS

Farm and carrier animal selection

A farm was selected based on the results obtained in a serological survey carried out on dairy farms around Londrina city, (ANDRADE, 1998) where 100% of the animals showed positive serology for *A. marginale* and there was a high incidence of clinical anaplasmosis cases. Londrina city is located in the North of the State of Paraná, Brazil (between 23º 08'47"N and 23º55'46"S), at an altitude of 576 m with an average annual temperature and rainfall of 22°C and 1876 mm, respectively.

EDTA blood samples from 12 animals (heifers and cows) were taken on this farm to select the engorged *B. microplus* females donors, by nPCR. Blood smears, stained with Giemsa, were also made to determine the parasitemia of these animals (IICA, 1984).

Tick collection and *in vitro* cultivation

Six to 16 engorged females were collected from four nPCR identified *A. marginale* infected animals (parasitemia ranging from 0 to 0.2% by Giemsa stained blood smears - Table 1). Ten engorged females weighing ^{3}200 mg, previously checked for viability, were selected and incubated in a chamber at 28°C and 80% relative air humidity for oviposition. The pre-oviposition, oviposition and egg hatching were recorded.

The egg mass eliminated every 24 hours was collected daily from each engorged female, until the end of the oviposition period. Half of the daily egg production was kept at –20°C to run the nPCR trying to detect *A. marginale* DNA fragments eventually present on different laying days. The other part of the eggs was incubated under the same conditions described above until the larvae hatched and submitted to the nPCR.

Each engorged female was examined by nPCR ten days after the end of oviposition to see whether or not they were infected by *A. marginale*. Based on this information, the engorged females and their progeny were divided into two groups: Group 1 had the progeny from five positive females and the Group 2 had the progeny from five negative engorged females.

DNA extractions

Blood. EDTA collected blood was washed three times with PBS buffer to remove the leukocyte layer. The resulting red blood cell mass was processed to extract the DNA according to the manufacturer's recommendations (Purogene, Gentra Systems). The extracted DNA was kept refrigerated (4°C) until the nPCR analysis was carried out.

Boophilus microplus engorged females, larvae and eggs

Boophilus microplus engorged females, eggs and larvae were processed for DNA extraction by the modified silica technique described by Boom et al. (1990). Engorged females, eggs and larvae were squashed in TE solution (10 mM TRIS, 1 mM EDTA, pH 8.0) and, 25 ml (eggs and larvae) or 50 ml (engorged females) of this preparation were added to a 1.5 ml microtube containing 450 ml of "lise" L6 buffer (120 g guanidine "isotiocianide" diluted in 100 ml of 0.1M TRIS, pH 6.4 with 22 ml of 0.2M EDTA solution (0.01%). After a short agitation period, the material was placed at room temperature for 10 minutes and was again agitated and centrifuged at 12,000 x g for 20 seconds. The supernatant was discarded. The DNA

containing pellet was then washed twice in L2 buffer (120 g guanidine "isotiocianide" diluted in 100 ml 0.1M TRIS, pH 6.4), twice in 70% ethanol and once in acetone. After the acetone removal, the microtube was open, incubated at 56°C for 10 minutes and the DNA was eluted in 100 ml of ultra pure water. The tube was incubated at 56°C for 10 minutes for elution and the supernatant was recovered after centrifugation and stored at –20°C for amplification by nPCR.

"Nested"-PCR

A nPCR was carried out according to TORIONI de ECHAIDE et al. (1998) using the nPCR master kit (Boehringer Mannhein) in a MiniCycler™ thermocycler from MJ Research. The *msp*5 sequence from the *A. marginale* Florida strain provide the following primers: external forward 5'-GCATAGCCTCCCCCTCTTTC-3'; external reverse, 5'-TCCTCGCCTTGCCCCTCAGA-3' and internal forward, 5'-TACACGTGCCCTACCGACTTA-3'. The first amplification was made using 12.5 ml of the master kit solution (2.5 U DNA Taq polymerase in Brij35 0.005% (v/v), 0.2 mM of each dATP, dCTP, dGTP, dTTP , 10 mM Tris-HCl, 50 mM KCl and 1.5mM $MgCl_2$), 1 ml of the external primers (20 mM), 5.5 ml of ultra pure water and 5ml of DNA template. The volume of 12.5 ml of the master kit solution, 1 ml of the 'external reverse' primer and 1 ml of the 'internal forward' primer (20mm), 8.5 ml of ultra pure water and 2 ml of the product from the first amplification were used in the second amplification. A drop of mineral oil was added to the amplifications to prevent reagent evaporation. The reactions were processed in the thermocycler programmed for 5 minutes at 95°C, 35 cycles at 95°C for 1 minute, 65°C for 2 minutes and 72°C for 1 minute with a final extension at 72 °C for 10 minutes followed by cooling to 4°C for undetermined period of time in each amplification. nPCR (10 ml) products were visualized in a 1.5% agarose gel following electrophoresis staining with 0.015% ethidium bromide (0,5ml/ml). Negative (cattle blood proven not infected by *A. marginale* and water) and positive (*A. marginale* Florida strain DNA) controls were also subjected to the same treatment. The 345 bp expected DNA fragment was identified by comparison with 100 base pairs weight molecular markers (100 bp Ladder – Gibco BRL).

nPCR reaction specificity

The 345 bp DNA bands were removed from the agarose gel using the Concert Rapid Gel Extraction System (Gibco BRL) commercial kit. This DNA was cleaved with the EcoR I (10 U/ml) (Gibco BRL) restriction enzyme. The cleavage reaction was performed in a water bath at 37 °C for two hours and the product was visualized, as previously described, in SDS polyacrylamide gel (3.5 to 8% gradient concentration) stained with 0.015% ethidium bromide after electrophoresis.

RESULTS AND DISCUSSION

In the Group 1, constituted by the progenies of positive engorged females, fragments of *A. marginale* DNA was only detected in the eggs of the fourth day and in the larvae of the first and seventh days of oviposition (three positive samples in 104 analyzed, or 2.88%) (Table 1). An opposite situation was observed for the progenies of the Group 2 (negative engorged females). Thirty-four of the 152 samples analyzed (22.4%) showed positive larvae and eggs since the first and second days of oviposition, respectively (Table 2). Figure 1 shows the result of the nPCR carried out on the tick donor cattle blood and on the engorged females, eggs and larvae of *B. microplus*. DNA bands with 345 bp can be seen in the positive samples. The nPCR specificity was confirmed by amplified DNA cleavage with the Eco R I restriction enzyme, which cleaves the DNA amplified products in two specific fragments (Figure 2).

These results showed that there was a lower detection of *A. marginale* DNA in the positive than in the negative engorged females. This may be partly explained by the findings

Table 1. Detection of *A. marginale* on eggs and larvae of positive *B. microplus* females, 10 days after the oviposition period, by nested PCR.

Engorged females	Oviposition days												
	1º	2º	3º	4º	5º	6º	7º	8º	9º	10º	11º	12º	13º
	Eggs												
1	-	-	-	-	-	-	-	-	-	-	-	F	
2	-	-	-	-	-	-	-	-	-	-	-	-	F
3	-	-	-	-	-	-	-	-	-	-	F		
4	-	-	-	-	-	-	-	-	-	-	F		
5	-	-	-	+	-	-	-	-	-	F			
	Larvae												
1	+	-	-	-	-	-	-	-	-	-	-	F	
2	-	-	-	-	-	-	+	-	-	-	-	-	F
3	-	-	-	-	-	-	-	-	-	-	F		
4	-	-	-	-	-	-	-	-	-	-	F		
5	-	-	-	-	-	-	-	-	-	F			

+ positive nPCR; - negative nPCR; F final of oviposition.

Table 2. Detection of *A. marginale* on eggs and larvae of negative *B. microplus* females, 10 days after the oviposition period, by nested PCR.

Engorged	Oviposition days																			
females	1º	2º	3º	4º	5º	6º	7º	8º	9º	10º	11º	12º	13º	14º	15º	16º	17º	18º	19º	20º
	Eggs																			
6	-	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	F			
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	F					
8	-	-	-	-	-	-	-	+	-	-	-	-	F							
9	-	+	+	+	+	+	-	+	-	-	+	-	-	+	+	-	-	-	-	F
10	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	F	-	-	-	-
	Larvae																			
6	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	F			
7	-	-	-	+	-	-	-	-	-	+	-	-	-	-	F					
8	+	-	-	+	-	-	-	+	-	-	-	-	F							
9	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	F
10	-	-	+	-	-	-	-	-	+	-	-	+	+	+	+	F				

+ positive nPCR; - negative nPCR; F final of oviposition.

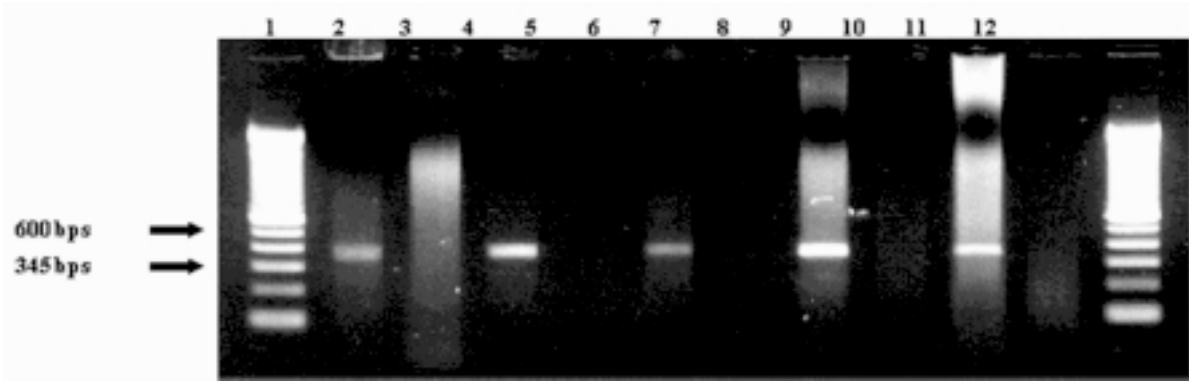

Figure 1. Nested PCR products of tick donor cattle blood, engorged females, eggs and larvae of *Boophilus microplus.* Lines 1 and 12, molecular weight (100bps); line 2, positive control (*A. marginale* Florida strain); line 3, negative control; line 4, blood from positive cattle; line 5, blood from negative cattle; line 6, positive engorged female of *B. microplus*; line 7, negative engorged female of *B. microplus*; line 8, positive eggs of *B. microplus*; line 9, negative eggs of *B. microplus*; line 10, positive larvae of *B. microplus*; line 11, negative larvae of *B. microplus*.

of Ribeiro and Lima (1995), where these authors studied the influence of temperature on *A. marginale* development in *B. microplus* and reported that ticks kept under ideal temperature and humidity conditions may begin the oviposition phase before the rickettsia completes its development cycle in the gut arthropod epithelial cells. This would result in a lower number of positive progeny from these infected vectors. But this does not explain the fact that negative engorged female progeny were sevenfold more positive.

Our data are not in line with Ribeiro and Lima (1995; 1996) for either the progeny from the positive or negative engorged females. They detected the rickettsia presence in engorged *B. microplus* females only after 19 days following oviposition, indicating that transovarial transmission, if occur, would only be possible after that period. This would only occur in the winter period under natural conditions, as in other months more than 90% of the *B. microplus* oviposition occurs between the 10th and 13th days after females detachment (VEGA, 1976; ALVARADO; GONZALES, 1979). Consequently, the possibility of natural transovarial transmission would be limited in our environment conditions. The mean oviposition period was 12 days in the present experiment, with a minimum of nine and a maximum of 19 days. However, *A. marginale* DNA fragments were detected in both eggs and larvae from the 1st to the 16th oviposition day.

A possible hypothesis to explain the observed results and even those of Ribeiro and Lima (1995; 1996) may be related to the moment at which the different tick instars became infected with *A. marginale* while feeding on the host blood. The engorged females whose offspring had a low detection of rickettsia DNA (Group 1) would have had late infection, leaving not enough time for *A. marginale* reaches the ovaries. On the other hand, the engorged females of the Group 2 may have been infected earlier, thus enabling contamination of the ovaries from the beginning of egg production. Connell (1974), investigating the transovarial transmission in *B. microplus* suggested that, the infection did not persist for sufficient time in the adult ticks to be transmitted to their

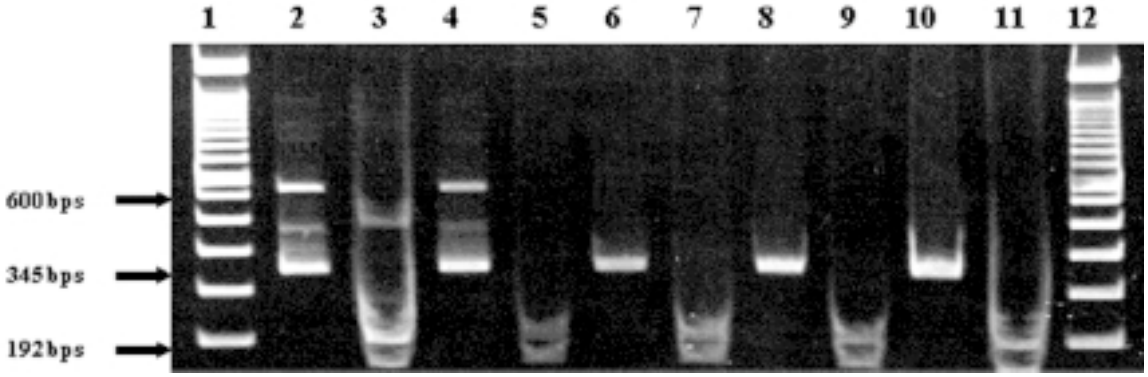

Figure 2. Clivated and intacts Nested PCR products of tick donor cattle blood, engorged females, eggs and larvae of *Boophilus microplus.* Line 1 and 12, molecular weight (100bps); line 2, positive control (*A. marginale* Florida strain); line 3, *Eco*RI cleavage of positive control; line 4, blood from positive cattle; line 5, *Eco*RI cleavage of blood from positive cattle; line 6, positive engorged female of *B. microplus*; line 7, *Eco*RI cleavage of positive engorged female of *B. microplus*; line 8, positive eggs of *B. microplus*; line 9, *Eco*RI cleavage of positive eggs of *B. microplus*; line 10, positive larvae of *B. microplus*; line 11, *Eco*RI cleavage of positive larvae of *B. microplus*.

progenies. The carrier animal parasitemia level may also influence the *A. marginale* transmission by the ticks. KOCAN et al. (1983) reported that *D. andersoni* ticks which were fed on highly infected cattle transmitted the disease to susceptible animals with a lower pre-patent period than those which were fed on cattle with a lower percentage of infected erythrocytes.

Considering that specific fragments of *A. marginale* DNA were identified in eggs and larvae from *B. microplus* engorged females that fed on cattle with low parasitemia under field conditions, this would means that *A. marginale* stages, such as identified by Ribeiro and Lima (1996) in the intestine of engorged female, migrated through the general cavity of the tick, infecting the ovaries and then, eggs and larvae. Whether and when these infected larvae or other stages of the tick will infect cattle, still reminder unclear and, more investigations on *A. marginale* and *B. microplus* biology and, on the potentiality of this vector in the transmission of this rickettsia to cattle are necessary to better understand the epidemiology of anaplasmosis in Brazil.

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