Research Note

Rev. Bras. Parasitol. Vet., Jaboticabal, v. 19, n. 3, p. 186-188, jul.-set. 2010
ISSN 0103-846X (impresso) / ISSN 1984-2961 (eletrônico)

Real time polymerase chain reaction to diagnose *Anaplasma marginale* in cattle and deer (*Ozotoceros bezoarticus leucogaster*) of the Brazilian Pantanal

Reação da polimerase em cadeia tempo real para diagnóstico de *Anaplasma marginale* em bovino e veado campeiro do Pantanal brasileiro *Ozotoceros bezoarticus leucogaster*

Graziela Picoloto[1]*; Renileide Ferreira de Lima[2]; Lílian Andressa Oliveira Olegário[2]; Cristiano Miranda Espínola Carvalho[3]; Ana Crystina Reis Lacerda[4]; Walfrido Moraes Tomás[5]; Paulo André Lima Borges[5]; Aiesca Oliveira Pellegrin[5]; Cláudio Roberto Madruga[6]

[1]Programa de Mestrado em Ciência Animal, Universidade Federal de Mato Grosso do Sul – UFMS, bolsista Fundect

[2]Programa de Pós-Graduação em Biotecnologia, Universidade Federal de Lavras – UFLA

[3]Programa de Mestrado em Biotecnologia, Universidade Católica Dom Bosco – UCDB

[4]Programa de Pós-Graduação em Biologia Animal, Universidade de Brasília – UnB

[5]Centro de Pesquisa Agropecuária do Pantanal – Embrapa Pantanal

[6]Programa de Mestrado em Ciência Animal, Universidade Federal de Mato Grosso do Sul – UFMS

Received October 30, 2009
Accepted June 15, 2010

Abstract

Epizootiological study of *Anaplasma marginale* in regions that contain various reservoir hosts, co-existence of rickettsia pathogens, and common vectors is a complicated task. To achieve diagnosis of this rickettsia in cattle and campeiro deer of Brazilian Pantanal, a comparison was made between a real time polymerase chain reaction (RT-PCR) with intercalating Sybr Green fluorochrome and primers based on *msp5* gene of *A. marginale*; a conventional PCR (C-PCR); and parasitological examination using thin blood smear stained with Giemsa-MayGrunwald. Both PCRs showed good performance in the diagnosis of *A. marginale* in cattle, and were superior to the parasitological exam. The RT-PCR detected seven positive campeiro deer (16.3%). This rate was significantly higher compared to C-PCR, which identified one animal as positive (2.3%), and also compared to parasitological diagnosis, which did not find any positive animals. The dissociation temperature average of positive reactions in cattle (81.72 °C ± 0.20) was identical to dissociation temperature found in the cervids (81.72 °C ± 0.12), suggesting that both animal species were infected with *A. marginale*. We concluded that RT-PCR can be used for *A. marginale* diagnosis and in epizootiological studies of cattle and cervids; in spite of the small number of campeiro deer samples, the results indicated that this wildlife species has importance in the *Anaplasma* epizootiology in the Brazilian Pantanal.

Keywords: *Anaplasma marginale*, real time PCR, cattle, campeiro deer, Pantanal.

Resumo

O estudo epizootiológico de *Anaplasma marginale* em regiões que existem vários reservatórios, co-existência de espécies de riquétsias patógenas e vetores comuns é uma tarefa complicada. Com o objetivo de obter o diagnóstico dessa riquétsia em bovinos e veado campeiro do Pantanal brasileiro foi avaliada uma reação da polimerase em cadeia em tempo real (PCR-TR) com o fluoróforo intercalante de fita dupla de DNA Sybr Green e iniciadores baseados na seqüência do gene *msp5* de *A. marginale* comparando-a a uma PCR convencional (PCR-C) e ao exame parasitológico de esfregaço fino de sangue corado com Giemsa-MayGrunwald. Ambas PCRs apresentaram bom desempenho no diagnóstico de *A. marginale* nos bovinos, o qual foi superior ao exame parasitológico. O PCR-TR detectou sete veados campeiros positivos (16,3%), o que foi significativamente maior comparado ao PCR-C identificando um animal como positivo (2,3%), e ao exame parasitológico não encontrou nenhum animal positivo. A média da temperatura de dissociação das reações positivas para amostras de bovinos (81,72 °C ± 0,20) foi idêntica àquelas dos cervídeos (81,72 °C ± 0,12), o que sugere que ambas espécies animais foram infectadas por *A. marginale*. Concluímos que PCR-TR pode ser utilizada para diagnóstico e estudos epizootiológicos de *A. marginale* em bovinos e cervídeos. Apesar da pequena amostragem de veado campeiro os resultados indicam que essa espécie de animal selvagem tem importância na epizootiologia do *Anaplasma* no Pantanal brasileiro.

Palavras-chave: *Anaplasma marginale*, PCR tempo real, bovino, veado campeiro, Pantanal.

***Corresponding author:** Graziela Picoloto
Programa de Mestrado em Ciência Animal,
Universidade Federal de Mato Grosso do Sul – UFMS, bolsista Fundect,
Av. Felinto Muller, 2443, CP 549, CEP 79070-900, Bairro Ipiranga,
Campo Grande - MS, Brazil;
e-mail: graziela.picoloto@gmail.com

The intraerythrocytic *Anaplasma* sp microorganisms of the *Rickettsiales* order and *Anaplasmataceae* family (DUMLER et al., 2001) cause in susceptible animals fever, anemia, icteric, anorexia, weight loss, and abortion (KOCAN et al., 2003). *Anaplasma marginale* produces considerable economic losses to the cattle industry of tropical and subtropical regions of the world due to significant productivity reduction and mortality (KOCAN et al., 2003). In regions where wild ruminant populations are abundant, particularly cervids, which have been implicated as important reservoirs and infection sources of *A. marginale* (DE LA FUENTE et al., 2008), epizootiological studies of this rickettsia are more complicated due to various reservoir hosts, co-existence of rickettsia pathogens, and common vectors. As well as being infected by *A. marginale*, the cervids are also infected with *Anaplasma phagocitophylum* (DUMLER et al., 2001) and *Anaplasma ovis* (HAIGH et al., 2008), which makes it difficult to implement a surveillance by serology. The current serological tests display crossed reactions, even when antigenic sub-units such as MSP5 recombinant protein are employed as antigens, because *Anaplasma* species have similar B epitopes (STRIK et al., 2007).

Several molecular diagnostic methods with high levels of sensitivity and specificity are used, such as reverse line blotting (GUBBELS et al., 1999), conventional polymerase chain reaction (C-PCR) (FRENCH et al., 1998), semi-nested, nested PCR (MOLAD et al., 2006) and real time PCR (RT-PCR) (DECARO et al., 2008). This last technique presents some advantages, such as speed of obtaining results, quantification of the infection level, and a minor probability of contamination. So far, the RT-PCR that has been developed to detect anaplasma in bovines uses the Taqman system as a reaction indicator (DECARO et al., 2008). This system requires a specific probe with a fluorescent reagent and a fluorescence emission quencher, making it more expensive than the technique that utilizes double strand DNA intercalating fluophore, the Syber Green. Because previously RT-PCR with primers based on genes *msp*5 and *msp*2 and Syber Green were used only to detect *A. marginale* gene transcription in the tick vector (FUTSE et al., 2003), this work evaluated a RT-PCR using primers with sequences of the *msp*5 gene with Sybr Green for diagnostic and epizootiological study of *A. marginale* in bovines and cervids.

To assess RT-PCR in the diagnosis of *A. marginale* in bovines, three experimental groups were established according to the following descriptions.Group 1 consisted of ten *Brangus* calves raised in the isolation area at the Embrapa Gado de Corte, Campo Grande, Mato Grosso do Sul State, seven to nine months of age, serologically negative in enzyme linked immunosorbent assay with recombinant proteins of *A. marginale*, MSP2 (ARAÚJO et al., 2005), and MSP5 (SILVA et al., 2006) and in conventional PCR (C-PCR) (ARAÚJO et al., 2005). Group 2 consisted of ten *Brangus* calves raised in an endemic area for *A. marginale*, ratified as positive by the above diagnostic tests. Group 3 consisted of eight *Brangus* calves of the same age and raised in the same isolation area, confirmed as negative through the above diagnostic tests and immunized with attenuated *Babesia bovis* and *B. bigemina* to avoid babesiosis sintomatology. These calves subsequently were exposed to *A. marginale* infection by ticks (20 engorged female *Rhipichephalus* (*B.*) *microplus* that grew on *A. marginale* bovine carriers) or mechanical transmission by insects. To evaluate RT-PCR in the diagnosis of *A. marginale* in campeiro deer (*Ozotoceros bezoarticus leucogaster*) of the Pantanal of the Mato Grosso do Sul State, fourty three campeiro deer were captured (IBAMA authorization number: (005/2007) 02014.000382/2007-22). The capture was done using anesthetic darts according to PIOVEZAN et al. (2006). The *A. marginale* direct diagnosis was performed by thin blood smears from capillaries of calves' ears and stained with May-Grunwald Giemsa, C-PCR according to ARAÚJO et al. (2005), and by RT-PCR. For the RT-PCR procedure, the reaction was obtained in a 25 µL volume containing PlatinunSYBR Green qPCR SuperMix-UDG (Invitrogen), primers forward 5'-AAGGCGAGGAGCTGTTTAAG-3' and reverse 5'-CTACTGCCTCACAAGGACGA-3' based on the sequence of the gene *msp5* of *A. marginale*, at concentration of 20 ηg.µL^{-1}, and DNA at concentration of 5 ηg.µL^{-1}. Three stages were programmed in the thermocycler (Cepheid Smart Cycler): 1) one cycle at 95 °C for 600 seconds; 2) 35 cycles at 95 °C for 15 seconds and 52 °C for 45 seconds; and 3) 60 cycles at 95 °C, for two hundredths of a second, to create the dissociation curve or melting curve. The statistical analysis was done by McNemar test.

The RT-PCR standardization with DNA samples obtained from blood of *A. marginale* infected cattle, showed positive reactions, while blood samples from animals free of this rickettsia infection did not show amplicons. The same results were verified in the C-PCR. In the calves of group 3, RT-PCR and C-PCR showed similar results, with the first *A. marginale* detection by both PCRs at Day 21 after exposure of the calves to tick or insect transmission, while in the peripheral blood smears *A. marginale* was observed at the 26th day post challenge. The only disagreement between the PCRs occurred with the sample from calf number 6, at 59 days post challenge, which displayed positive reaction only in the C-PCR.

The campeiro deer thin blood smear examination did not detect *A. marginale*. Using C-PCR one animal was detected as positive (2.3%), while RT-PCR found seven positive reactions (16.3%), which resulted in a significant statistical difference between the molecular diagnostics. Therefore, RT-PCR using Sybr Green demonstrated superior performance as a diagnostic tool.

The RT-PCR described here has advantages over the recently developed RT-PCRs (DECARO et al., 2008), since they employ a Taqman system, which increases the tests costs, compared to method that uses the intercalating fluorochrome of DNA double strand. Another advantage is the use of the dissociation temperature, which confers a diagnostic specificity that is important for epizootiological studies.

There is serological evidence that cattle infection with *A. phagocitophylum* occurs in areas endemic for *A. marginale* (DE LA FUENTE et al., 2008), as well as deer with infections of *A. phagocitophylum* and *A. ovis* (DUGAN et al., 2006). However, crossed reactions in serological tests (even when using recombinant protein MSP5; STRIK et al., 2007), due to common antigens between the species of *Anaplasma* (DREHER et al., 2005), do not allow a conclusive diagnosis of *Anaplasma* species infecting cattle and cervids. Also, the gene *msp5* is considered highly conserved between *Anaplasma* species (KNOWLES et al., 1996), consequently there is possibility of amplicon production in the PCR with primers based on this gene sequence for different *Anaplasma* species.

However, *msp5* gene blast of different *Anaplasma* species displays different degrees of identity in the gene sequence; for instance

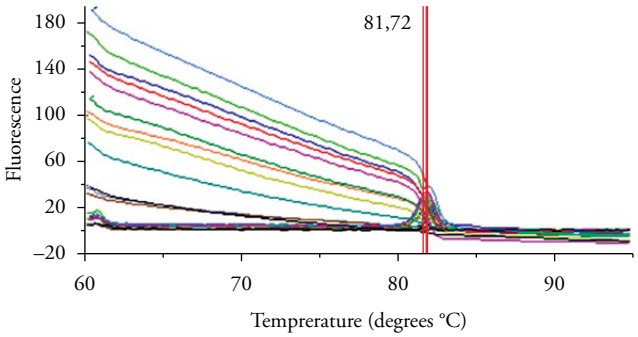

Figure 1. Melting temperature in the Real Time PCR (RT-PCR) performed with DNA extracted from calves infected with *Anaplasma marginale*.

A. marginale msp5 has 89, 85, and 65% of identity, respectively, with ortologous genes of *A. ovis*, *A. centrale*, and *A. phagocitophylum*. It is likely that the RT-PCR melting curve would show distinct temperature dissociation for each *Anaplasma* species. Since the average temperature of dissociation in positive reactions in cattle (81.72 ± 0.20) (Figure 1) and in campeiro deer (81.72 ± 0.12) were identical, there is the suggestion that both animal species were infected with *A. marginale*. This finding agrees with the fact that only *A. marginale* was found infecting deer in Brazil by a direct diagnosis with PCR (MACHADO et al., 2006).

In conclusion, this RT-PCR showed good performance as a diagnostic test for *A. marginale* in cattle and in campeiro deer, as suggested by dissociation temperature. Despite the small number of campeiro cervid samples, the results pointed to the importance of these animals as *Anaplasma* reservoirs in the Brazilian Pantanal. A complementary study on dissociation temperature of *A. ovis*, *A. centrale*, and *A. phagocitophylum* in this RT-PCR would be relevant for its use in epizootiological studies of *Anaplasma* sp., in regions or farms where various animals species co-exist.

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