Molecular diagnosis of *Anaplasma marginale* in cattle: quantitative evaluation of a real-time PCR (Polymerase Chain Reaction) based on *msp5* gene[1]

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ABSTRACT.- Bacanelli G.M., Ramos C.A.N. & Araújo F.R. 2014. **Molecular diagnosis of *Anaplasma marginale* in cattle: quantitative evaluation of a real-time PCR (Polymerase Chain Reaction) based on *msp5* gene**. *Pesquisa Veterinária Brasileira 34(1):29-33*. Embrapa Gado de Corte, Avenida Rádio Maia 830, Campo Grande, MS 79106-550, Brazil. E-mail: flabio.araujo@embrapa.br

The rickettsia *Anaplasma marginale* is considered the main agent of bovine anaplasmosis. Due the nonspecific clinical signs of the anaplasmosis, the diagnosis of infection depends of laboratory confirmation. In recent years, molecular diagnostic methods have been used to detect *A. marginale* in cattle. However, the existence of a large number of assays of different sensitivity and cost makes the choice of an appropriate test difficult. In the present study, a real-time Polymerase Chain Reaction (PCR) based on the *msp5* target gene was quantitatively assessed and compared to an end point PCR. Both reactions were subjected to sensitivity and specificity evaluation using plasmid DNA and samples from cattle experimentally infected with *A. marginale*. A comparative field trial of the tests was carried out using samples of cattle from a stable enzootic area for *A. marginale*. The real-time PCR showed a higher sensitivity than the end point PCR. This reaction (i.e. real-time PCR) was able to detect one copy of the *msp5* gene in 100 ηg of plasmidial DNA, and more than 80% of its results were positive among experimentally infected animals seven days after infection. In addition, based on *in silico* analysis, the real-time PCR evaluated in the present study appears to be useful for the detection of *A. ovis*.

INDEX TERMS: Real-time PCR, diagnosis, *Anaplasma marginale*, *msp5,* cattle.

RESUMO.- [**Diagnóstico molecular de *Anaplasma marginale* em bovinos: avaliação quantitativa de uma PCR em tempo real baseada no gene *msp5*.**] A riquétsia *Anaplasma marginale* é considerada o principal agente da anaplasmose bovina. Devido a não especificidade dos sinais clínicos, a confirmação da infecção nos animais depende de testes laboratoriais. Recentemente, métodos de diagnóstico molecular têm sido aplicados para detecção de *A. marginale* em bovinos. No entanto, a grande quantidade de testes com diferentes sensibilidade e custos tem dificultado a escolha do ensaio mais adequado. No presente estudo, uma PCR em tempo real baseada no gene *msp5* foi avaliada quantitativamente e comparada a uma reação de PCR convencional. As reações foram submetidas à avaliação de sensibilidade e especificidade com DNA plasmidial e amostras provenientes de bovinos experimentalmente infectados por *A. marginale*. Uma avaliação comparativa a campo foi realizada entre os testes utilizando amostras provenientes de bovinos criados em uma região de estabilidade enzoótica para *A. marginale*. Embora os testes não tenham apresentado diferença estatisticamente significativa, a PCR em tempo real apresentou valor de sensibilidade maior do que a PCR convencional. A PCR em tempo real foi capaz de detectar uma cópia de *msp5* em 100ng de DNA plasmidial, e mais de 80% de resultados positivos entre bovinos experimentalmente infectados apenas sete dias após infecção. Além disso, baseado em análise *in silico*, a PCR em tempo real avaliada aqui pode ser útil para detecção de *Anaplasma ovis*.

TERMOS DE INDEXAÇÃO: PCR em tempo real, diagnóstico, *Anaplasma marginale*, *msp5,* bovinos.

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INTRODUCTION

Anaplasma marginale is an obligate intracellular pathogen belonging to the genus *Anaplasma* (Order *Rickettsiales*, Family *Anaplasmataceae*) which causes anaplasmosis in bovines (Theiler 1910, Dumler et al. 2001, Kocan et al. 2003). This rickettsia is transmitted biologically by ixodid ticks. The species *Rhipicephalus (Boophilus) microplus* is the only biological vector that has been identified in Brazil (Kessler & Schenk 1998). The clinical presentation of anaplasmosis is nonspecific and can include fever, anemia, jaundice, anorexia, weight loss and death in susceptible cattle (Zaugg 1985).

Because of the difficulty of diagnosing *A. marginale* infection by clinical means, several laboratorial techniques have been used to detect this rickettsia in cattle. In the acute phase of the disease, when rickettsemia is high, initial bodies are easily detected in bovine erythrocytes by microscopy of stained blood smears (Farias 1995). However, the rickettsemia of chronically infected animals is generally low, hindering its diagnosis (Palmer et al. 1986). In this context, molecular diagnostic assays such as PCR have been developed and are both highly sensitive and specific (Palmer et al. 1986, Gale et al. 1996, Corona et al. 2000).

Recently, several types of polymerase chain reactions (PCR) such as nested PCR, multiplex PCR, real-time PCR) have been developed and used for the diagnosis of various pathogens (Courtney et al. 2004, Carelli et al. 2007). Real-time PCR has a number of advantages, including high sensitivity and specificity, usefulness as a quantitative assay, and is able to deliver faster results than end point PCR (Bell & Ranford-Cartwrightet 2002). However, there are few studies of paired comparisons between real-time and end point PCR, making the choice of PCR technique difficult. Therefore, the aim of this study was to quantitatively assess a real-time PCR assay and compare it with an end point PCR based on the same target gene (*msp5*).

MATERIALS AND METHODS

Samples. Blood DNA samples (n=48) from six Aberdeen Angus cattle experimentally infected with a Brazilian isolate of *Anaplasma marginale* were used. These cattle, used in a previous study by Araújo (2005), were kept in an isolation area of the Embrapa Beef Cattle farm, Campo Grande, MS, Brazil. Sampling was performed at 0, 5, 7, 9, 16, 26, 33 and 140 days post-inoculation (DPI). Blood samples (n=99) from a herd kept in an area of enzootic stability for *A. marginale* (Embrapa Gado de Leite, Juiz de Fora, MG, Brazil) were also used.

DNA extraction. DNA extractions were taken from 350μL of bovine blood, using the Easy DNA kit (Invitrogen[5]), following the manufacturer's instructions. The integrity and concentration of DNA samples were assessed by agarose gel electrophoresis 0.8% and spectrophotometry in NanoDrop ND1000 (Thermo Scientific[6]).

Real-time PCR. Real-time PCR was performed with Platinum SYBR Green/ROX (Invitrogen[5]) in a final volume of 12.5μL, using the previously described (Picoloto et al., 2010) primer set *msp5* AMTR F: 5' AAGGCGAGGAGCTGTTTAAG 3' and AMTR R: 5' CTACTGCCTCACAAGGACGA 3'. Amplification was performed with a StepOne Plus thermocycler (Applied Biosystems[7]) as follows: 50°C for 2 minutes, 95°C for 2 minutes, 40 cycles at 95°C for 30 seconds (denaturation) and 54°C for 15 seconds (annealing/extension). A total of 50ηg of each primer and 100ηg of genomic DNA were used in each PCR. Samples were considered positive when the amplification curves exceeded the cutline automatically proposed by the StepOne Software v2.11, and showed a dissociation curve (melt curve) similar to the positive control (±0.5°C).

To check the presence of PCR inhibitors in the extracted DNA samples, a real-time PCR was performed for the bovine constitutive gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primers gadphF 5'GGCGTGAACCACGAGAAGTATAA 3' and gadphR 5'CCCTCCACGATGCCAAAGT 3', and the reaction described by Robinson et al. (2007).

End point PCR. The end point PCR was performed using the primers *msp5*F 5'ATGAGAATTTTCAAGATTGTGTCTAACCTT 3' and *msp5*R 5' AGGAAAGCCCCCAAAGCCCCATACTT 3', which delimit all *msp5* genes of *A. marginale*, and a 3' untranslated region, according to Silva et al. (2006), resulting in an amplicon of 714 bp. Amplification conditions were: 94°C for 4 minutes, followed by 35 cycles at 94°C for 1 minute, 60°C for 30 seconds and 72°C for 45 seconds. A final extension step at 72 °C for 4 minutes was used. The results of PCR were analyzed in 1% agarose gel stained with Sybr Gold (Invitrogen[5]). Samples were considered positive when amplification of a fragment corresponding to the expected amplicon size (714 bp) was visualized under ultraviolet light.

Evaluation of sensitivity. To evaluate the sensitivity of both assays (end point and real-time PCR), the *msp5* gene was cloned into the plasmid p*TrcHis TOPO* (Invitrogen[5]), and the recombinant plasmid (p*TrcHis TOPO/msp5*) was propagated in *Escherichia coli* TOP10. After plasmid purification using Wizard Plus minipreps kit (Promega[8]), plasmidial DNA concentration was quantified by spectrophotometry in Nanodrop ND1000. The number of DNA copies was estimated as described by Ke et al. (2006), and DNA was diluted serially (10^8 to 10^0 copies/ml) in nuclease-free water. The dilutions were subjected to both PCR reactions, as described above.

Clinical sensitivity was evaluated using 48 blood samples from six experimentally infected Aberdeen Angus cattle. The samples collected at 0, 5, 7, 9, 16, 26, 33 and 140 DPI were also subjected to both reactions.

Evaluation of specificity. To evaluate specificity a sample of DNA from *Babesia bovis*, and another from *Babesia bigemina* from blood of experimentally infected cattle were tested. In addition, an *in silico* analysis of specificity was performed with primer sequences used in the real-time PCR using the ClustalW algorithm (www.ebi.ac.uk/Tools/clustalw2/index.html) and the blastn online tool.

Field trial. The field evaluation of PCR reactions was carried out using 99 blood samples from cattle raised in an area of *enzootic stability* for *A. marginale*.

Statistical analysis. The significance of the differences between the frequencies of positive results obtained in the tests was measured by Fisher's exact test. The agreement between the tests was expressed using the kappa index as described by Kramer and Feinstein (1981). Analysis was performed with the aid of Bioestat 5.0 (Ayres et al. 2007) software.

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[5] Invitrogen - Life Technologies Corporations, 3175 Staley Road, Grand Island, New York, USA.

[6] Thermo Fisher Scientific, 81 Wyman Street, Waltham, Massachusetts, USA.

[7] Applied Biosystmes - Life Technologies Corporations, 3175 Staley Road, Grand Island, New York, USA.

[8] Promega Corporation, 2800 Woods Hollow Road, Madison, Wisconsin, USA.

RESULTS

The real-time PCR evaluated in the present study was able to detect one copy of the *msp5* gene in 100ηg of plasmid DNA (Fig.1A), whereas conventional PCR detected up to 1000 copies. The standard curve obtained from the dilutions of the plasmid DNA showed an inversely proportional linear response between the DNA concentration and the number of real-time PCR cycles in the 10^8-10^0 copy range, with values of R^2 =0.998, and amplification efficiency of 107% (Fig.1B). The melt temperature of the amplicon generated in this assay was 81.19±0.26°C.

No unspecific amplification was detected in *msp5* with real-time PCR and end point PCR when DNA from *Babesia bovis* or *B. bigemina* was used as a template. *In silico* analysis using isolate of *Anaplasma marginale* as a reference (Florida, Genbank CP001079), demonstrated that the primers are specific for *A. marginale* and *A. ovis,* showing 100% identity only among isolates of these species (Fig.2).

In the analysis of DNA samples from cattle experimentally infected with *A. marginale* (at 0, 5, 7, 9, 16, 26, 33 and 140 DPI), the real-time PCR was able to detect five positive animals (83.3%) on the seventh day post-inoculation, while the end point PCR detected as positive only one animal (16.7%). At 9 DPI, all six cattle were detected as positive (100%) with real-time PCR, and only three (50%) with end point PCR. From 16 DPI to 140 DPI, all animals were found to be positive using both reactions (Table 1).

The overall frequency of infection by *A. marginale* in cattle from the region of Juiz de Fora, MG, Brazil was 92.92% (92/99) with real-time PCR, and 87.87% (87/99) with PCR (Table 2). No significant difference was observed between tests (p=0.062), with a level of agreement of 94.9% (kappa index =0.87). The feasibility of all DNA samples was confirmed by real-time PCR for the constitutive GAPDH bovine gene.

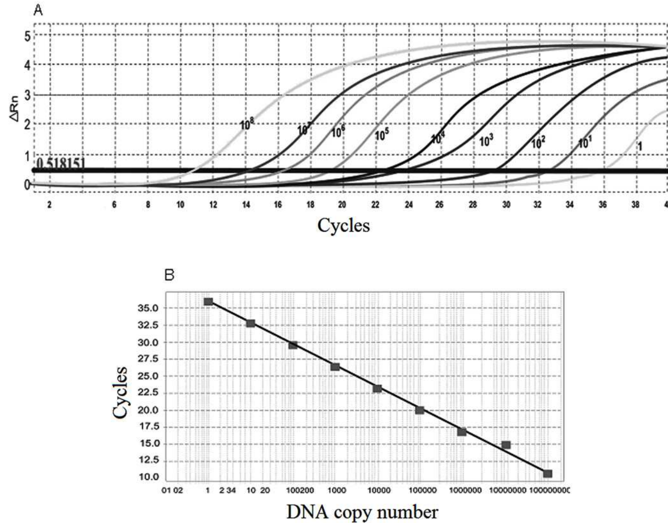
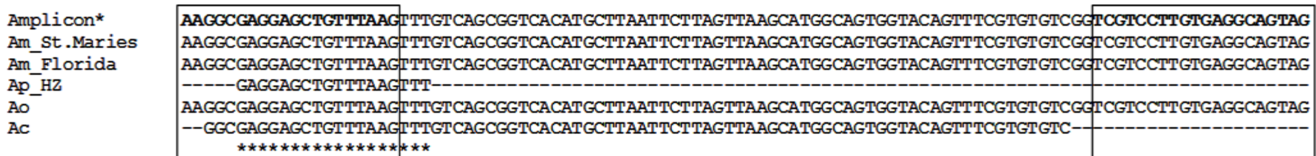


Fig.1. Sensitivity analysis of real-time PCR for the *msp5* gene of *Anaplasma marginale.* (**A**) Amplification plot. (**B**) Standard curve obtained in real-time PCR using serial dilutions of *msp5/pTrcHis*-TOPO (10^8-10^0 copy of the plasmid DNA).



Based on the genome of *Anaplasma marginale* Florida CP001079: Am = *Anaplasma marginale;* Ap = *Anaplasma phagocytophilum;* Ao = *Anaplasma ovis;* Ac = *Anaplasma centrale.

Fig.2. Multiple alignment of the amplicon sequences generated by real-time PCR for *msp5* gene of *Anaplasma marginale* (St. Maries and Florida, Genbank CP000030 and CP001079, respectively) and homologous sequences of *Anaplasma phagocytophilum* (Genbank CP000235), *Anaplasma ovis* (Genbank HM195102) and *Anaplasma centrale* (Genbank CP001759). Annealing sites of primers highlighted.

Table 1. End point PCR and real-time PCR based on msp5 gene of *Anaplasma marginale* using DNA samples of experimentally infected

Animal	PCR	Days post-inoculation (DPI)							
		0	5	7	9	16	26	33	140
1	End point PCR	-	-	-	+	+	+	+	+
	real-time PCR	-	-	-	+	+	+	+	+
2	End point PCR	-	-	-	+	+	+	+	+
	real-time PCR	-	-	+	+	+	+	+	+
967	End point PCR	-	-	-	-	+	+	+	+
	real-time PCR	-	-	+	+	+	+	+	+
970	End point PCR	-	-	-	-	+	+	+	+
	real-time PCR	-	-	+	+	+	+	+	+
975	End point PCR	-	-	-	-	+	+	+	+
	real-time PCR	-	-	+	+	+	+	+	+
982	End point PCR	-	-	+	+	+	+	+	+
	real-time PCR	-	-	+	+	+	+	+	+

+ Positive result, - negative result.

Table 2. Comparison of end point PCR and real-time PCR for the msp5 gene of *Anaplasma marginale* in a herd of cattle from area of enzootic stability

End point PCR	Real-time PCR		Total
	Positive	Negative	
Positive	87	0	87
Negative	5	7	12
TOTAL	92	7	99

DISCUSSION

The real-time PCR evaluated in the present study was 1000 times more sensitive than the end point PCR when plasmidial DNA was used as template. The higher sensitivity of real-time PCR was also evident with the samples of experimentally infected cattle, since, on the seventh DPI, real-time PCR detected a greater number of positive animals (83.3%) than end point PCR (16.7%). However, when the two re-

actions were subjected to a comparative field trial using samples from animals from an enzootic stability region, a significant difference was not observed. Previously described real-time PCR assays for the detection of *Anaplasma marginale* have also shown high levels of sensitivity, as well as those reported by Carelli et al. (2007) and Decaro et al. (2008) (detection limit = 5 copies of DNA). However, in both cases a hydrolysis probe based system was used (TaqMan system), whereas in the present stud the SYBR Green system was used. Despite having some advantages, mainly related to specificity, the TaqMan system is more expensive than the Sybr Green system. Considering only the cost of reagents, the cost of a real-time PCR reaction based on hydrolysis probes is approximately 25% higher in Brazil than a reaction based on Sybr Green. Both real-time PCR systems are at least 50% more expensive than end point PCR.

There are less expensive alternatives for the diagnosis of chronic infection by *A. marginale*, such as serological tests (ELISA, IFAT). However, these are not able to reveal the exact profile of prevalence of infection at a particular point, as detectable levels of antibodies may remain in the animal for long periods, even after elimination of the infectious agent. Molecular techniques, meanwhile, detect the infectious agent but not the byproducts of infection. Recently, discrepancies among *A. marginale* prevalence rates (87.5% with ELISA and 56.9% with real-time PCR) were reported in cattle from Puntarenas Province, Costa Rica (Shebish et al. 2012).

A nested highly sensitive (detection limit = 2 copies) PCR based on *msp5* of *A. marginale* has been described (Ybanez et al. 2013). However, this reaction is carried out in two steps, while real-time PCR is performed in a single step. Furthermore, real-time PCR is widely accepted as a useful quantitative technique for studies of vaccine evaluation, drugs, vector competence, and other studies.

The primers used in real-time PCR were designed to amplify a fragment of 104 *bp* between the end of *msp5* gene and the 5' untranslated region, and between the *msp5* and *pstS* gene of *A. marginale*. Although, other species of Anaplasma were not evaluated in this study, *in silico* analysis using isolate of *A. marginale* (Florida, Genbank CP001079) as a reference, demonstrated that the primers are specific for *A. marginale* and *A. ovis,* showing 100% identity only among isolates of these species. The lack of homology between the sequences analyzed suggests that the test did not amplify DNA from *A. centrale* and *A. phagocytophilum.* However, this reaction has not been tested experimentally. Although the primers evaluated in this study exhibited total complementarity with *A. ovis*, based on *in silico* analysis, this species has not been reported in Brazil and its importance as an infectious agent for cattle is still unknown. However, recent reports provide evidence of the possibility of *A. ovis* infection in cattle (Hornok et al. 2010, Hornok et al. 2012).

The overall frequency of infection by *A. marginale* in cattle from the region of Juiz de Fora, MG, Brazil was 92.92% (92/99) with real-time PCR, and 87.87% (87/99) with PCR. These results suggest that the region is in enzootically stable for *A. marginale*, confirming the results reported by Carvalho et al. (2012). There was no significant difference between the tests (p=0.062), with a level of agreement of 94.9% (kappa index = 0.87). Considering the discordant results, five samples (5.1%) were positive in real-time PCR and negative in the end point PCR (Table 2). False negative results in PCR may occur due to the presence of inhibitors resulting from the process of DNA extraction (Tondella et al. 2002). However, in the present study, the feasibility of all DNA samples was confirmed by real-time PCR for the constitutive GAPDH bovine gene. Therefore, the different frequencies detected by the assays are due to the higher sensitivity of real-time PCR.

Although significant difference between real-time PCR and end point PCR was not observed in field evaluation, the use of real-time PCR is indicated as a quantitative technique when *rickettsial loads* need to be measured (Lohr et al. 2002, Futse et al. 2003). On the other hand, due its relatively lower cost, end point PCR is a more suitable tool for epidemiological studies. However, the high sensitivity of real-time PCR may also be exploited in epidemiological studies, especially in wild ruminants, which usually present chronic infections with low levels of rickettsemia (Picoloto et al. 2010), and are susceptible to infection by both *A. marginale* and *A. ovis*.

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