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A PCR for Differentiate between *Anaplasma marginale* and *A. centrale*

Ana Carolina Joazeiro[1,2], João Martins[3], Aoi Masuda[1], Adriana Seixas[1,4] & Itabajara da Silva Vaz Junior[1,2]

ABSTRACT

***Background*:** *Anaplasma marginale* ssp. *centrale* (*A. centrale*) exhibits low pathogenicity and therefore is used as a live vaccine against bovine anaplasmosis in several countries. During production of the vaccine, accidental contamination with *Anaplasma marginale* (*A. marginale*) is a risk that can jeopardize the entire batch of vaccine. Due to limitation of microscopic examination to detect low levels of parasitaemia, the present study aims to standardize a polymerase chain reaction assay using primers for the msp4 gene of *Anaplasma* sp. for detecting and differentiating with greater sensitivity and specificity the species of *A. centrale* and *A. marginale* in blood samples from experimentally infected cattle.
***Materials, Methods & Results*:** The DNA extraction was performed from frozen blood. Erythrocytes infected with known *A. centrale*, *A. marginale* served as positive control and the erytrocytes infected with *Babesia bovis* and *Babesia bigemina* served as the negative control polymerase chain reaction. PCR was standardized from annealing temperature variations of the primers, magnesium chloride concentration, amounts concentration of primers and DNA concentration of rickettsiae. By PCR method, it was analyzed the DNA from blood samples of 13 cattle positive to A. marginale by microscopic examination from smear stained with Giemsa. The PCR assay was specific for *A. centrale* and *A. marginale*, presented 100% identity without presenting cross-reactivity with other bovine hemoparasites. The detection limits of the PCR were 0.25 pg and 0.125 pg of DNA for detection of *A. centrale* and *A. marginale* DNA respectively.
***Discussion*:** *A. marginale* is an obligate intracellular bacterium that infects bovine erythrocytes causing extravascular hemolysis and anemia being considered the main agent of bovine anaplasmosis.The strain of *A. centrale* by having a low pathogenicity has been used since the years 1900 a live vaccine with the aim of decreasing the clinical signs associated with exposure to field strains of *A. marginale* in some countries. The vaccine production involves exposure of calf via inoculation of erythrocytes infected with the *A. centrale* strain, which need to be free from *A. marginale* and other bovine hemoparasites. Because the vaccine *A. centrale* be produced in splenectomized calves, a rigorous care as accidental contamination mainly by blood-sucking insects should be performed. Since a contamination during the isolation of live *A. centrale* vaccine can compromise the entire batch of vaccine. There are some limitations to the current methods used for the detection of a possible contamination of live vaccine *A. centrale*. The serological tests do not allow differentiation between Anaplasma species, because of the similarity of their antigens and cross-reactions with other hemoparasites can take place. Microscopic examination of blood differentiates *A. centrale* from the *A. marginale* strain by of the characteristics and morphological distribution of the parasite in erythrocytes. However, this procedure does not detect pre-symptomatic and carrier animals, since its sensitivity is in the range of 10^6 infected erythrocytes per mL of blood. In contrast, the molecular biology have potential to detect low levels of blood DNA and differentiate *A. centrale* from *A. marginale* through the presence of major surface protein 4 (MSP4), and thus allows the verification of the purity of *A. centrale* vaccine.

Keywords: *Anaplasma marginale*, *Anaplasma centrale*, diagnosis, control.

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[1]Centro de Biotecnologia do Estado do Rio Grande do Sul (CBiot), Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil. [2]Faculdade de Veterinária (FaVet), UFRGS Porto Alegre, RS. [3]Fepagro Saúde Animal, Instituto de Pesquisas Veterinárias Desidério Finamor, (IPVDF) Eldorado do Sul, RS. [4]Departamento de Farmacociências, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, RS. CORRESPONDENCE: I Da Silva Vaz Jr. [itabajara.vaz@ufrgs.br - Tel.: +55 (51) 3308-6078]. Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul (UFRGS). Av. Bento Gonçalves n. 9500, Bairro Agronomia. CEP 91501-970 Porto Alegre, RS, Brazil.

INTRODUCTION

The species *A. marginale* and the strain *A. marginale* ssp. *centrale* are closely related hemoparasites at antigenic level [5]. However, they differ in their pathogenicity towards cattle [22]. *A. marginale* is highly pathogenic, especially towards cattle over two years of age, while the *A. centrale* strain usually causes mild infection [4].

Anaplasmosis is one of the most important diseases of ruminants worldwide, causing significant economic losses in tropical and subtropical areas [12,13]. Animals that recover from acute anaplasmosis maintain persistent infection for life [7]. In turn, chronic anaplasmosis is characterized by five-week repeated cycles of parasitemia and microscopically undetectable levels of infected erythrocytes (between $10^{2.5}$ and 10^{7}) [7,8,11]. Detection of pre-symptomatic and carrier animals makes it possible to transport cattle to disease-free and enzootically unstable areas, and may be seen as an auxiliary measure in controlling live vaccine production.

The main methods for diagnosing anaplasmosis include serological tests and microscopic examination of Giemsa-stained blood smears [1], although these methods have limitations as the specific detection or as the detection of low levels of parasitaemia. Thereby, a specific and sensitive molecular diagnostic method would improve detection and differentiation between *A. marginale* and *A. centrale* strain in cases of low levels of parasitemia. In the present study, it was describe the standardization of a polymerase chain reaction (PCR) for amplification of specific regions of *Anaplasma* sp. MSP4, allowing a quality control for production of vaccine against bovine anaplasmosis.

MATERIALS AND METHODS

Blood samples from experimentally infected cattle

Blood samples for use as positive controls were provided by the "Desidério Finamor" Veterinary Research Institute (FEPAGRO/IPVDF; Eldorado do Sul, Rio Grande do Sul (RS), Brazil). Samples of the *A. centrale* strain and of *A. marginale*, *Babesia bovis* and *Babesia bigemina* were obtained from experimentally infected cattle in a tick-free area (Santa Vitória do Palmar, RS, Brazil). The strains were thawed and kept on ice until used to infect a susceptible splenectomized calf by means of intravenous inoculation.The rickettsemia level in the calf was monitored daily by means of microscopic examination and blood samples were collected when a parasitemia level of approximately 2% was reached.

The blood samples that were used as negative controls were collected from cattleliving in an *Anaplasma* sp.-free area (Santa Vitória do Palmar, RS, Brazil). Blood samples were collected into tubes containing 3.2% sodium citrate; they were cryopreserved with 10% DMSO and frozen in liquid nitrogen.

Field samples

The blood samples for analysis by means of PCR (n = 13) were collected from Hereford, Devon or crossbreed cattle between 12 and 24 months of age in regions of southern Brazil where anaplasmosis is endemic. Giemsa-stained blood smears wereused for microscopic examination to detect the presence of intraerythrocytic *Anaplasma* sp. Species identification was performed based on parasite morphology [21].

DNA extraction

DNA was extracted from frozen blood containing the infected erythrocytes based on the protocol described by Bashiruddin *et al*. [2]. Briefly, blood was thawed at room temperature, washed three times with Tris-EDTA (pH 7.4), and centrifuged at 14,000 *g* for 2 min. The proteins were dissolved and digested in 10% sarkosyl, 10% SDS and proteinase K (5 mg/mL) (Promega[1]) at 62°C for 1 h. DNA was extracted using phenol:chloroform and was precipitated in an alcoholic solution containing 2M KCl. The DNA samples were resuspended in 20 µL of water and stored at 4°C until use.

DNA quantification and purity analysis

The DNA concentration (in ng/µL) was measured by means of spectrophotometry, in the Ultrospec 10,000 apparatus (Pharmacia Biotech[2]).The absorbance of DNA samples at 260 nm determined the DNA concentration, and the absorbance of DNA samples at 260 nm and 280 nm determined the purity of nucleic acids [18].

Standardization of polymerase chain reaction (PCR) for A. marginale *and* A.centrale *strain detection*

The DNA sequence encoding the major surface protein-4(MSP4) of the *A. centrale* strain was amplified with specific primers[3]: 5´-CATGGGGCATGAATGTGTG-3´ (forward) and 5´-AATTGGTTGCAGTGAGCGCA-3´ (reverse), to amplify a 394-bp

DNA fragment. The primers used for *A. marginale* were 5'-CCCATGAGTCACGAAGTGG-3' (forward) and 5'-GCTGAACAGGAATCTTGCTCC-3´ (reverse), to amplify a 753-bp PCR DNA fragment. PCR was performed in a final reaction volume of 25 µL. To optimize the PCR, the variations of the annealing temperature of the primers, magnesium chloride concentration, amounts of primers and DNA concentration were tested. The PCR parameters used were 4 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s of annealing at 50°C and 1 min of extension at 72°C, with a final extension at 72°C for 5 min. Blood samples obtained from cattle infected with *B. bovis* and *B. bigeminabut* free from hemoparasites were used as negative controls. The products were viewed under UV light after electrophoresis on 0.8% agarose gels stained with 0.5 mg/µL of ethidium bromide. To confirm the PCR product identity, the amplicons obtained from PCR reactions were purified using the Geneclean® II kit (MP Biomedicals LLC[4]; QBiogene[5]), as directed by the manufacturer, and were sequenced using the ABI Prism 3130xl apparatus (Applied Biosystems[6]). The nucleotide sequences for the msp4 gene were compared with the nucleotide sequences deposited in GenBank, using the Blast algorithm.

RESULTS

Blood smear analysis

The microscopic examination was able to identify structures resembling *A. marginale* in four of the 13 samples analyzed that were suspected of having anaplasmosis.

PCR reaction standardization

The average yield of DNA from the blood samples was around 200 ng/µL, and the DNA purity, as calculated using the 260/280 nm ratio, was around 1.7, thus showing that the samples had suitable DNA for PCR amplification.

The standardized PCR reaction contained 1 ng of DNA as template, 1 x PCR buffer (10 mMof Tris-HCl and 500 mM of KCl; pH 8.5), 1.5 mM of $MgCl_2$, 5 pMol of each primer, 0.16 mMol of deoxynucleoside triphosphate and 1.25 U of Taq DNA polymerase. The annealing temperature was 53°C for both species. The amplicons showed 100% identicalness with the nucleotide sequences for the msp4 gene deposited in GenBank (access number: JN564652 for *A. marginale* and CP001759 for the *A. centrale* strain.

Sensitivity and Specificity of PCR

The detection limit of the PCR for the *A. centrale* strain was 0.25 pg and for A. marginaleit was 0.125 pg (Figures 1.A and 1.B).The amplification of the DNA samples detected an expected PCR product of 753 bp for *A. marginale* and of 394 bp for the *A. centrale* strain (Figure 2). The analytical sensitivity of the PCR test was determined using concentrations of 0.1 ng to 0.1 pg of DNA extracted from the erythrocytes of cattle experimentally infected with *A. marginale* and the *A. centrale* strain. The pairs of primers designed for the msp4 gene of the *A. centrale* strain and for *A. marginale* did not amplify the DNA of *B. bovis* and *B. bigemina*, which are commonly found together infecting cattle (Figure 3).

Detection of Anaplasma *sp. within an endemic region*

As shown in Table 1, the results from the PCR analysis on the blood samples from naturally infected cattle were compared with the values obtained from the blood smear analysis. All 13 samples analyzed by means of PCR were positive for *A. marginale*, and two of these samples showed *A. marginale* and the *A. centrale* strain simultaneously.

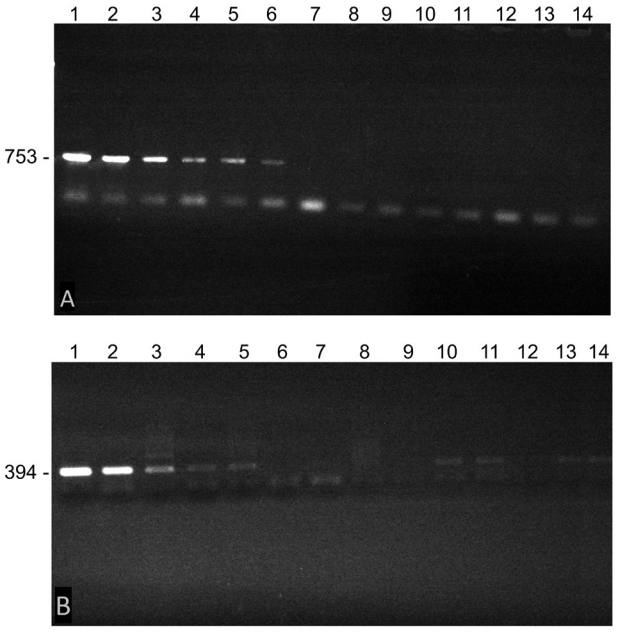

Figure 1. PCR sensitivity with primers for the gene msp4 of *A. marginale* or *A. centrale*. The amount of DNA used as template was 0.1 ng, 0.01 ng, 0.001 ng, 0.5 pg, 0.25 pg, 0.125 pg, 0.1 pg of DNA of *A. marginale* (Figure A, lines 1-7) or *A. centrale* (Figure B, lines 1-7) or bovine (Figures A and B lines 8-14). Electrophoresis on 0.8% agarose gel stained with ethidium bromide.

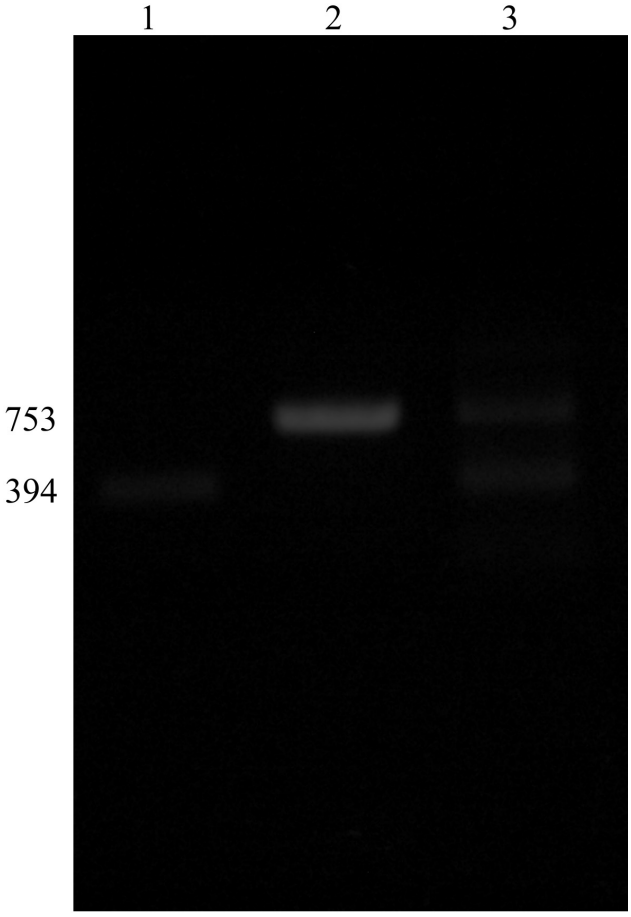

Figure 2. PCR specificity with primers for the gene msp4 of *A. marginale* or *A. centrale*. Sample 1. DNA A. centrale with primers for the gene msp4 for *A. centrale*, sample 2. DNA *A. marginale* with primers for the gene msp4 for *A. marginale* and sample 3. DNA *A. centrale* and *A. marginale* with primers for gene msp4 for *A. centrale* and *A. marginale*. PCR was performed using 1.5 mM magnesium chloride, 0.16 Mm dNTP, 5.0 pmol of each primer, 1 ng DNA, 1.25 U Taq DNA polymerase enzyme buffer and 53° C.

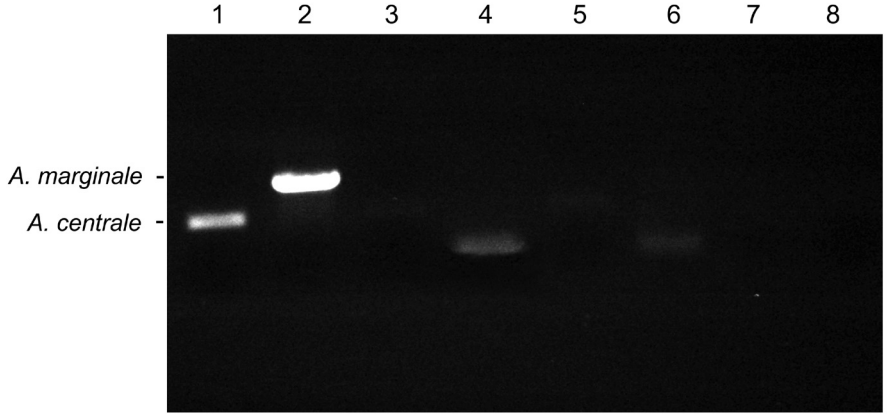

Figure 3. Analysis of the specificity of the PCR standardized. The DNA used as template was of *A. centrale* (line 1), *A. marginale* (line 2), *B. bovis* (lines 3 and 4), *B. bigemina* (lines 5 and 6) and bovine (lines 7 and 8). The primers used were for *A. centrale* (lines 1, 3, 5 and 7) or *A. marginale* (lines 2, 4, 6 and 8). Electrophoresis on 0.8% agarose gel stained with ethidium bromide.

Table 1. Comparison of results of microscopy and PCR analysis of bovine samples from the municipalities of Guaiba, Sao Gabriel and Alegrete naturally infected with *Anaplasma* sp.

Samples	Microscopy	PCR	
	A. marginale	*A. centrale*	*A. marginale*
1	Positive	Negative	Positive
2	Positive	Positive	Positive
3	Positive	Negative	Positive
4	Positive	Negative	Positive
5	Negative	Negative	Positive
6	Negative	Negative	Positive
7	Negative	Negative	Positive
8	Negative	Negative	Positive
9	Negative	Negative	Positive
10	Negative	Negative	Positive
11	Negative	Negative	Positive
12	Negative	Positive	Positive
13	Negative	Negative	Positive

DISCUSSION

The *A. centrale* strain has been widely used in Australia, Israel, South Africa and South American countries for controlling of bovine anaplasmosis [3,16,17].

The vaccine needs to be produced in splenectomized calves that are serologically negative for blood-borne infections, including *Babesia*, *Anaplasma*, *Ehrlichia*, *Theileria* and *Trypanosoma* [23]. During the process of replicating the *A. centrale* strain vaccine in calves, it is important to control for accidental contamination by *A. marginale*, which is transmitted mainly by blood-sucking insects [10]. Thus, an assay that enables accurate detection of *A. marginale* contamination during the process of isolating the *A. centrale* strain is desirable.

Microscopic examination of Giemsa-stained blood smears is routinely used to confirm acute *Anaplasma* sp. infection. However, it does not reach detection levels below 10^7 infected erythrocytes per mL of blood [14] and therefore is not sensitive enough to detect persistently infected animals (or chronically infected animals). The parasitemia levels in chronically infected animals usually range from 10^3 to 10^7 [9], and blood smears detect only one range of infection in these animals [15]. As observed in Table 1, microscopic examination made it possible to identify *A. marginale* in four of the 13 samples analyzed, which were suspected of anaplasmosis. PCR detected *A. marginale* in all the field samples tested and in two other samples with concomitant A. centrale infection, thus agreeing with the findings of Shkap *et al*. [20].

In the present study, a molecular assay was standardized for detection and differentiation of *A. marginale* and the *A. centrale* strain in infected cattle. Selection of an appropriate target for accurate and precise determination of infection is critical for the development of diagnostic methods for *Anaplasma* sp. The nucleotide sequence of the msp4 gene showed identicalness of 83% between A. marginale and the *A. centrale* strain [19]. This makes it possible to design primers that differentiate between these two species of *Anaplasma*. In the present study, specific primers for the msp4 gene of *Anaplasma* sp. were designed for amplification of the DNA obtained from an extraction of frozen blood from cattle experimentally infected with the *A. centrale* strain and/or *A. marginale*. The extraction of DNA from *Anaplasma* sp. from frozen blood was optimized based on *Bashiruddin et al*. [2], and this showed yield and quality suitable for PCR.

The PCR standardized here was specific. It did not amplifying the DNA of other hemoparasitessuch as *B. bovis* and *B. bigemina*. The amplicons of 394 bp and 753 bp were confirmed by sequencing. The detection limits were 0.25 pg of DNA from the *A. centrale* strain and 0.125 pg of DNA from *A. marginale*, which

are consistently low levels of parasitemia, undetectable by means of optical microscopy. PCR made it possible to identify and differentiate the Anaplasma species in infected and persistently infected cattle, from field samples. PCR was more sensitive than analysis of blood smears, even taking into consideration detection of *A. marginale* in asymptomatic animals, which is more difficult.

In conclusion, an important diagnosis tool for detection of persistently infected animals and for differentiation of the closely related species *A. centrale* strain and *A. marginale* was standardized. These findings can help in quality control for live vaccine of the *A. centrale* strain, for use against bovine anaplasmosis.

In this context, standardization of an efficient detection method that clearly differentiates between *A. marginale* and the *A. centrale* strain in blood is of considerable importance for vaccine safety and quality control.

These results indicate that this is a sensitive and specific test that provides an alternative for detecting *Anaplasma* spp. in relation to controlling production standard for the vaccine against bovine anaplasmosis.

MANUFACTURERS

[1]Promega. Madison, WI, USA.

[2]Pharmacia Biotech. Piscataway, NJ, USA.

[3]Integrated DNA Technologies. Coralville, IA, USA

[4] MP Biomedicals. Sant Ana, CA, USA.

[5]QBiogene. Montreal, Quebec, CA.

[6]Applied Biosystems. South Sant Francisco, CA, USA.

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