ASSESSMENT OF PRIMERS DESIGNED FROM THE SMALL RIBOSOMAL SUBUNIT RNA FOR SPECIFIC DISCRIMINATION BETWEEN *BABESIA BIGEMINA* AND *BABESIA BOVIS* BY PCR

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SUMMARY

Six pairs of species-specific primers were designed from the alignment of the sequences of the SS rRNA gene obtained from the Genbank database for *Babesia bigemina* (accession number X59604) and for *B. bovis* (accession number U06105). Three pairs of primers were designed specifically for *B. bigemina* and another 3 sets of primers for *B. bovis*. All oligonucleotide sequences selected as primers were examined for similarities to other organisms through the Genbank Blast procedure and these 6 sets of primers demonstrated high level of specificity. The synthetic oligonucleotides were also tested for specificity by PCR assay using genomic DNA extracted from 40 isolates of *B. bigemina* and 30 from *B. bovis*, obtained in six different States of Brazil. All 6 sets of primers were validated as 100% specific for the respective parasite. The PCR amplified the expected fragments for each set of primers, as follows: a) *B. bigemina*: primers GAU5 forward/GAU6 reverse with amplicon of 1,124 bp; primers GAU5 forward/GAU8 reverse with amplicon of 458 bp; primers GAU7 forward/GAU6 reverse with amplicon of 685 bp; b) *B. bovis*: primers GAU9 forward/GAU10 reverse with amplicon of 541 bp; primers GAU9 forward/GAU 13 reverse with amplicon of 883 bp; primers SOGIN forward/GAU 10 with amplicon of 1211 bp.

KEYWORDS: *Babesia bovis*, *Babesia bigemina*, SS rRNA gene, PCR, primers, bovine babesiosis.

RESUMO

CONTRIBUIÇÃO DOS *PRIMERS* OBTIDOS DE SUBUNIDADES DO RNA RIBOSSÔMICO PARA DISCRIMINAÇÃO ENTRE *BABESIA BIGEMINA* E *BABESIA BOVIS* POR PCR

Seis pares de *primers* espécie-específicos foram construídos a partir do alinhamento do gene SS rRNA de *Babesia bigemina* (número de acesso no Genbank: X59604) e *Babesia bovis* (número de acesso U06105). Três pares de primers foram construídos especificamente para *B. bigemina* e outros três para *B. bovis*. As seqüências de oligonucleotídeos, selecionadas como primers, apresentaram elevada especificidade na avaliação quanto à similaridades a outros microrganismos, através do procedimento designado "basic local alignment search tool (BLAST)" do Genbank. Os oligonucleotídeos sintéticos foram também avaliados quanto à especificidade através da reação de PCR, utilizando-se DNA genômico extraído de 40 isolados de *B. bigemina* e 30 de *B. bovis* procedentes de seis estados do Brasil. Os resultados da amplificação foram 100% específicos para todos os pares de primers testados, obtendo-se, como produtos de PCR, fragmentos de tamanhos esperados para cada combinação de primers e DNA utilizados, como segue: a) *B. bigemina*: primers GAU5/GAU6 = amplicon de 1124 pares de base (pb); primers GAU5/GAU8 = amplicon de 458 pb; primers GAU7/GAU6 = amplicon de 685 pb; b) *B. bovis*: primers GAU9/GAU10 = amplicon de 541 pb; primers GAU9/GAU13 = amplicon de 883; primers SOGIN/GAU10 = amplicon de 1211 pb.

PALAVRAS-CHAVE: *Babesia bovis*, *Babesia bigemina*, SS rRNA, PCR, primers, babesiose bovina.

INTRODUCTION

The microscopic examination is the conventional and widely used method to diagnose acute babesiosis. Thick or thin blood films are usually stained with Giemsa and show a sensitivity to detect parasitemias ranging from 10^{-5} to 10^{-7} and 10^{-5} to 10^{-6}, respectively (Mahoney & Saal, 1961). The quantitative buffy coat (QBC) system is reportedly more sensitive than thick blood films (10^{-7} to 10^{-8}),

but the disadvantages are lower specificity and a higher cost (Levine at al., 1989). The most specific and sensitive method known to detect *Babesia* spp. from carrier animals in the laboratory is the *in vitro* culture (MASP culture) with ~10^{-8} to 10^{-10} of sensitivity (Holman et al., 1993), but it is laborious, much more expensive, time consuming and requires special equipment.

DNA probes have been developed with mainly application in differentiating morphologically similar species or in post-mortem diagnosis in decomposed tissues. In spite of the high specificity its sensitivity is in the range of the thin film examination (10^{-5} to 10^{-6}) and for this reason and also because of the higher cost, time consuming, and requirement of special equipment, it is not applied for routine diagnosis or for carrier identification.

DNA probes have been developed as a highly specific method (Mc Laughlin et al., 1986; Holman et al., 1989; Jasmer et al., 1990; Buening & Figueroa, 1996; Figueroa et al., 1992a; Petchpoo et al., 1992; Reddy & Dame, 1992), although its sensitivity remains in the range of the thin film examination (10^{-5} to 10^{-6}) and for this reason and also because of the higher cost, time consuming, and requirement of special equipment it is not applied as a routine diagnostic procedure.

The polymerase chain reaction (PCR) has been tested to detect *Babesia* spp. by specific DNA sequences. This method has shown to be 100 times more sensitive than any microscopic examination, detecting parasitemia of ~10^{-6} to 10^{-9}, with high specificity and less time-consuming than DNA probes (Fahrimal et al., 1992; Persing et al. 1992; Figueroa et al., 1992b, 1993; Calder et al., 1996), which makes it more suitable for diagnosis.

This study was conducted with the objective to select different sets of primers targeting the SS rRNA genes that could be used in a species-specific detection of *B. bovis* and *B. bigemina* by a single PCR technique or other PCR-based procedures.

MATERIAL AND METHODS

Fourty field isolates of *B. bigemina* (35 from the State of Goiás and 1 from each State: Mato Grosso do Sul, Rondônia, São Paulo, Rio Grande do Sul and Bahia) and 30 field isolates of *B. bovis* (27 from Goiás, 2 from Mato Grosso do Sul, 1 from São Paulo and 1 from Bahia) were used to evaluate primers and protocols for PCR reaction. *A. marginale* DNA was extracted from a field isolate to be used as a control for PCR reaction. Genomic DNA was extracted from 300 µl blood samples by a commercial kit (GFX Genomic Blood DNA Purification Kit – Amersham Pharmacia Biotech).

Partial sequences of the SS rRNA of *B. bovis* and *B. bigemina* gene "A" SS rRNA as registered on the Genbank database (accession numbers L31922 and X59604, respectively) were aligned by the Clustal method using a computer program (DNASTAR Inc., Madison, Wis.). Primers were then selected from 3 variable regions of both *B. bovis* and *B. bigemina* as shown in Table 1. Primers were designed with attention to ensure similar annealing temperatures (AT) in the PCR reaction for each species (*B. bovis* = 57 and *B. bigemina* = 55). The pairs of primers were evaluated for specific amplification by PCR on the following combinations: a) for *B. bigemina*-specific PCR reaction: GAU5/GAU6, predicted amplicon size (PAS) = 1124 bp; GAU5/GAU8, PAS = 458 bp; GAU7/GAU6, PAS = 685 pb; and b) for *B. bovis*-specific DNA amplification: GAU9/GAU10, PAS = 541 bp; GAU9/GAU13, PAS = 883 bp and GAU3/GAU10, PAS = 1212 bp. All oligonucleotide sequences selected as primers were examined for similarities to other organisms through the Genbank Blast procedure and these 6 sets of primers demonstrated a high level of specificity. The primer GAU3 used in this experiments a forward general primer complementary to the coding strand of the SS rRNA gene previously described by Sogin & Gunderson (1987).

The PCR master mix was calculated on the basis of a 50 µl each reaction, prepared as follows: 40.75 µl H_2O ultra pure; 5 µl 10X PCR reaction buffer (Amersham Pharmacia Biotech) containing 500 mM KCl, 15 mM $MgCL_2$ and 100 mM Tris-HCl; 1.0 µl dNTP (Life Technologies) 10 mM; 0.5 µl forward primer 20 µM; 0.5 µl reverse primer 20 µM; 0.25 µl Taq polymerase (Amersham Pharmacia Biotech) 5 U/µl; 2 µl genomic DNA extracted as above. Separate control tubes were included in each PCR run consisting of DNA of *B. bigemina*, *B. bovis*,

Anaplasma marginale, uninfected bovine whole blood DNA and a negative mix control. The PCR was processed in thermal cycler (Research Inc., mod. PTC-100/MJ) under the following conditions: 1 cycle at 94°C for 2 min; 40 cycles at 94°C for 30 sec; 55°C for *B. bigemina* PCR or 57°C for *B. bovis* PCR for 30 sec; 72°C for 1 min; and 1 final extension cycle at 72°C for 5 min. The amplicons were submitted to electrophoresis in a 1% agarose gel in TBE buffer. A 100 bp DNA ladder was used as a size marker (Life Technologies). The amplified DNA products were stained with ethidium bromide (0.4 µg/ml) (Life Technologies) and visualized under UV transillumination.

The sensitivity of the PCR assays were determined by using 10-fold dilutions of *B. bovis* and *B. bigemina*-infected erythrocytes with known concentrations of 3×10^6 and 3×10^7 parasites per ml of blood sample, respectively, as previously described by Torioni et al. (1998).

TABLE 1. Nucleotide seguences of primers used to amplify specific fragments of the SS rRNA gene of *B. bovis* and *B. bigemina* by polymerase chain reaction.

Primer	Sequence	Position	Organism
GAU5 (F)	5'-TGGCGGCGTTTATTAGTTCG-3'	409-428	*B. bigemina*
GAU6 (R)	5'-CCACGCTTGAAGCACAGGA-3'	1532-1515	*B. bigemina*
GAU7 (F)	5'-GTTGGGTCTTTTCGCTGGC-3'	848-866	*B. bigemina*
GAU8 (R)	5'-GCCAGCGAAAAGACCCAAC-3'	866-848	*B. bigemina*
GAU9 (F)	5'-CTGTCGTACCGTTGGTTGAC-3'	675-694	*B. bovis*
GAU10 (R)	5'-CGCACGGACGGAGACCGA-3'	1215-1198	*B. bovis*
GAU13 (R)	5'-CTGGCCGCGAGCGGCGA-3'	1557-1541	*B. bovis*
GAU3 (F)	5'-CTGGTTGATCCTGCCAGTAG-3'	4-20	*Babesia* spp.

F = forward primer; R = reverse primer.

RESULTS AND DISCUSSION

The alignment of the sequence of *B. bigemina* gene "A" SS rRNA with *B. bovis* SS rRNA gene allowed the selection of three variable regions to each protozoan that could specifically discriminate between the two species by polymerase chain reaction and did not cross react with *A. marginale* or uninfected bovine genomic DNA. After checking all primer sequences selected for this study through the Genbank Blast procedure it was concluded that, on the basis of the genomic data available in that Database Center, there were no similarities to other organisms that could allow cross reactions.

PCR amplification of *B. bigemina* specific fragments of the SS rRNA gene were successfully achieved by the following primer sets with respective amplicon size: GAU5/GAU6 (1,124 bp amplicon); GAU5/GAU8 (458 bp amplicon) and GAU7/GAU6 (685 bp amplicon) (Table 2; Figures 1A, 1B and 1C). *B. bovis* SS rRNA gene was also specifically amplified by the following pair of primers and respective amplicon size: GAU9/GAU10 (541 bp amplicon); GAU9/GAU13 (883 bp amplicon) and GAU3/GAU10 (1,212 bp amplicon) (Table 2; Figures 1D, 1E and 1F). *Anaplasma marginale* DNA as well as uninfected bovine genomic DNA were not amplified by any of the sets of primers applied in the experiment (Figures 1A, 1B, 1C, 1D, 1E and 1D).

The results presented in Table 2, demonstrated a 100% specificity for the detection of *B. bigemina* genomic DNA from 40 field isolates by PCR using the primer sets GAU5/GAU6, GAU5/GAU8 and GAU7/GAU6, as well as for the detection of *B. bovis* from 35 field isolates with primer sets GAU9/GAU10, GAU9/GAU13 and GAU3/GAU10.

The PCR assays for *B. bovis* and *B. bigemina* were demonstrated to be sensitive to detect parasitemia as low as 10^{-7}% for both protozoans, which corresponds to 3 parasites per ml of blood (data not shown). Sensitivity of the method verified by this study is in the range of those published by other researchers (Fahrimal et al., 1992, Figueiroa et al., 1992, Persing et al., 1992, Figueiroa et al., 1993, Calder et al., 1996).

The six pair of primers evaluated in this experiment appeared to be highly specific for the detection of the *B. bigemina* and *B. bovis* DNAs. The diversity of amplicon sizes obtained by different combinations of primer sets (1,124 bp, 685 bp and 458 bp for *B. bigemina* and 541 bp, 883 bp and 1,212 bp for *B. bovis*), which were constructed with a very close annealing temperate allows the construction of other PCR-based techniques such as the multiplex-PCR and the nested-PCR.

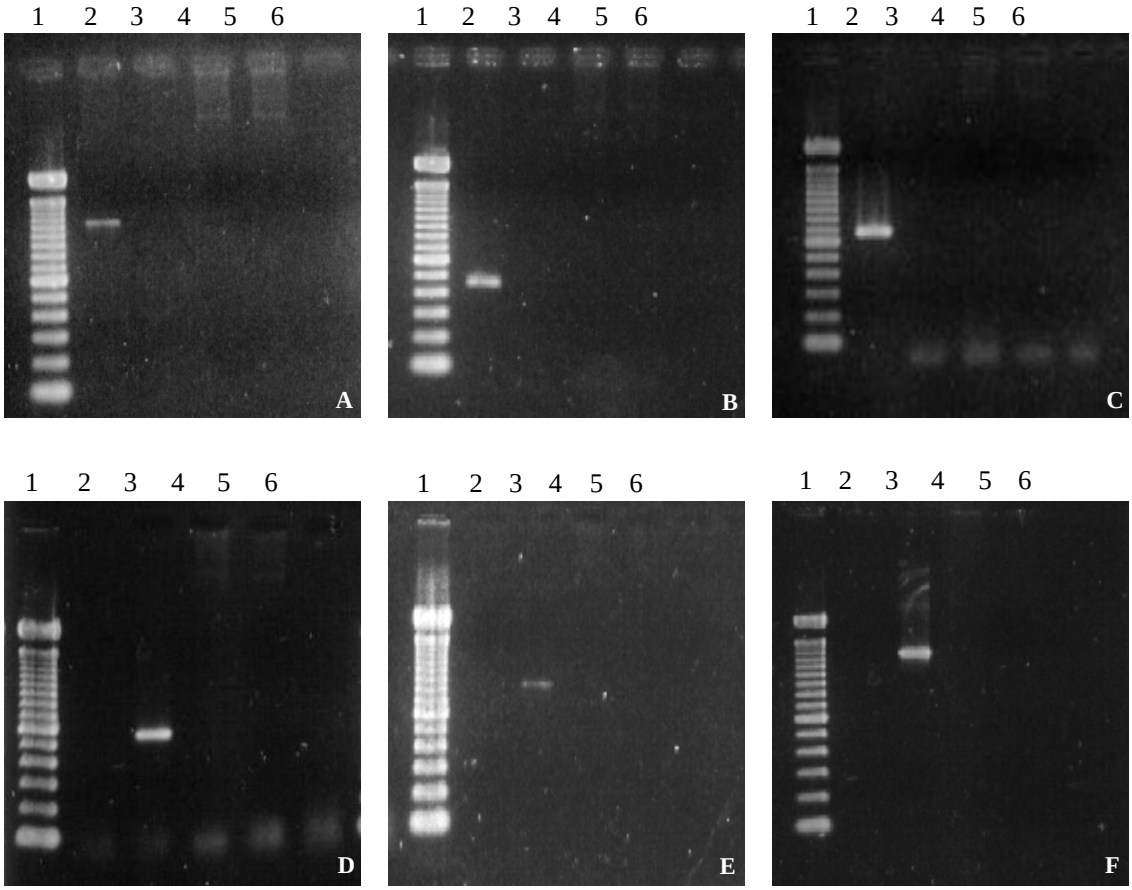

FIGURE 1. A). Agarose gel electro-phoresis of 10 µl ampli-fication of SS rRNA gene of *B. bigemina* with primer set GAU5/GAU6. Lane 1 = 100 bp DNA ladder; lane 2 = 1,124 bp *B. bigemina* amplicon; lane 3 = *B. bovis* DNA; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control. **B)** Agarose gel electrophoresis of 10 µl amplification of SS rRNA gene of *B. bigemina* with primer set GAU5/GAU8. Lane 1 = 100 bp DNA ladder; lane 2 = 458 bp *B. bigemina* amplicon; lane 3 = *B. bovis* DNA; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control. **C)** Agarose gel electrophoresis of 10 µl amplification of SS rRNA gene of *B. bigemina* with primer set GAU7/GAU6. Lane 1 = 100 bp DNA ladder; lane 2 = 685 bp *B. bigemina* amplicon; lane 3 = *B. bovis* DNA; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control. **D)** Agarose gel electrophoresis of 10 µl amplification of SS rRNA gene of *B. bovis* with primer set GAU9/GAU10. Lane 1 = 100 bp DNA ladder; lane 2 = *B. bigemina* DNA; lane 3 = 541 bp *B. bovis* amplicon; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control. **E)** Agarose gel electrophoresis of 10 µl amplification of SS rRNA gene of *B. bovis* with primer set GAU9/GAU13. Lane 1 = 100 bp DNA ladder; lane 2 = *B. bigemina* DNA; lane 3 = 883 bp *B. bovis* amplicon; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control. **F)** Agarose gel electrophoresis of 10 µl amplification of SS rRNA gene of *B. bovis* with primer set GAU3/GAU10. Lane 1 = 100 bp DNA ladder; lane 2 = *B. bigemina* DNA; lane 3 = 1,212 bp *B. bovis* amplicon; lane 4 = *A. marginale* DNA; lane 5 = bovine leucocyte DNA; lane 6 = negative control.

TABLE 2. Results of PCR reaction using six different combination of primer sets for specific amplification of the *B. bigemina* and *B. bovis* SS rRNA genes, with respective amplicon sizes obtained.

Primer Sets	DNA tested	Samples tested	PCR-positive	Amplicon size
GAU5/GAU6	*B. bigemina*	40	40	1,124 bp
GAU5/GAU6	*B. bovis*	35	00	-
GAU5/GAU6	*A. marginale*	40	00	-
GAU5/GAU8	*B. bigemina*	40	40	458 bp
GAU5/GAU8	*B. bovis*	35	00	-
GAU5/GAU8	*A. marginale*	40	00	-
GAU7/GAU6	*B. bigemina*	40	40	685 bp
GAU7/GAU6	*B. bovis*	35	00	-
GAU7/GAU6	*A. marginale*	40	00	-
GAU9/GAU10	*B. bigemina*	40	00	-
GAU9/GUA10	*B. bovis*	35	35	541 bp
GAU9/GUA10	*A. marginale*	40	00	-
GAU9/GUA13	*B. bigemina*	40	00	-
GAU9/GUA13	*B. bovis*	35	35	883 bp
GAU9/GUA13	*A. marginale*	40	00	-
GAU3/GAU10	*B. bigemina*	40	00	-
GAU3/GAU10	*B. bovis*	35	35	1,212 bp
GAU3/GAU10	*A. marginale*	40	00	-

Primers selected from the *B. bigemina* gene "A" SS rRNA gene shared 100% similarities with types "B" and "C", as they were submitted to the Genbank under the accession numbers X59604, X59605 and X59607, respectively (Reddy et al., 1991). Therefore it was accepted that these primers would recognize *B. bigemina* DNA despite minor divergences among types.

CONCLUSIONS

The three primer sets - GAU5/GAU6, GAU5/GAU8 and GAU7/GAU6 - selected from the *B. bigemina* SS rRNA gene and the other three sets - GAU9/GAU10, GAU9/GAU13 and GAU3/GAU10 – from the *B. bovis* SS rRNA gene were considered to be highly specific for the detection of the respective protozoans from infected blood samples by the polymerase chain reaction for diagnostic purpose.

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