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Diagnosis of *Babesia caballi* Infection in Horses by Polymerase Chain Reaction

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ABSTRACT

A set of primers were designed according to the published sequence of the gene encoding a rhoptry protein of *Babesia caballi*, and used to amplify parasite DNA from the blood samples obtained from carrier horses by polymerase chain reaction (PCR) method. The PCR method was sensitive enough to detect parasite DNA from 2.5 μl blood sample with a parasitemia of 0.000001%. The PCR method was compared with fluorescent antibody test (IFAT) in order to evaluate the diagnosis efficiency for *B. caballi* infection in horses. Of 142 field samples from Mongolia, 28 (20%) and 96 (69%) samples were identified positively by PCR and IFAT, respectively. Although the sensitivity of PCR was lower than IFAT, it was noted that the 5 IFAT-negative samples were PCR-positive, suggesting that the combination of PCR method and IFAT for diagnosis of *B. caballi* infection may be effective in detecting the carrier horses.

Babesia caballi is a tick-borne hemoprotozoan parasite that can cause equine piroplasmosis which is one of the most economically important disease of horses. The disease is characterized by fever, anemia, and icterus (Bruning 1996). The disease can be found in most tropical and subtropical areas of the world as well as in temperate climatic zones. Endemic areas include many parts of Europe, Africa, Arabia and Asia. Due to the almost worldwide distribution of the various tick vectors, introduction of carrier horses into non-endemic areas must be

prevented.

To date the complement fixation test (CFT) and indirect fluorescent antibody test (IFAT) are commonly used for detection of *B. caballi* infection. However, these serologic tests are generally restricted by the antibody detection limits and crossreactivity. The advent of PCR has allowed the development of sensitive and specific diagnostic assays for many babesiosis (Brose et al. 1995; Bruning 1996; Calder et al. 1996; Krause et al. 1996; Sahagun-Ruiz et al. 1997).

In this study we established a PCR method for detection of *B. caballi* DNA from blood samples obtained from carrier horses. Our results indicated that the PCR method is sufficiently sensitive, specific, and reproducible for use in the diagnosis of *B. caballi* infection in horses.

B. caballi and *B. equi*, both USDA strains, were cultured in equine erythrocytes by previously described method (Avarzed et al. 1997a). When the parasitemias of *B. caballi* and *B. equi* were reached 10%, the cultured erythrocytes were washed 3 times with phosphate-buffered saline (PBS), and lysed in 0.1 M Tris-HCl (pH 8.0), containing 1% SDS, 0.1 M NaCl and 10 mM EDTA, and then treated with proteinase K (100 μg/ml) for 2 hrs at 55°C. The DNA was extracted with phenol/chloroform and precipitated by ethanol, and then subjected to used as a PCR templates. Two oligonucleotide primers (5'-ACTCTTCAAGGAGAGTGACG-3' and 5'-ATGTACAGGTACCTGGACGC-3'), were designed to the published sequence of the gene of rhoptry protein of *B. caballi* (Dalrymple et al. 1996). PCR was performed in 50 μl of a mixture containing 2.5 μl of template DNA, 50 pmol of each primer, 200 μM of dNTPs, 1.25 U of Taq Gold polymerase in 1×buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.001% gelatin). PCR was performed by 10 min at 95 °C to activate the Taq Gold DNA polymerase, and then the reaction was repeated for 40 cycles under the following conditions: 1 min of denaturation at 94 °C, 1 min of annealing at 58 °C, and 1 min of extension at 72 °C.

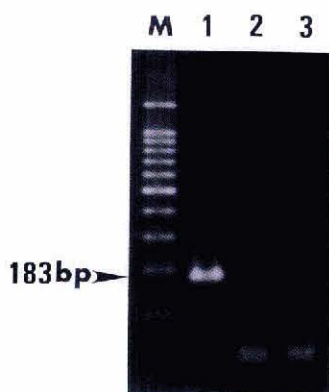

Figure 1. Specificity of the PCR method. Ethidium bromide stained agarose gel of PCR products from *B. caballi* (lane 1), *B. equi* (lane 2), and horse leucocytes (lane 3). Lane M, 100 bp DNA ladder marker.

The specificity of the PCR method was examined with DNA from *B. caballi*, *B. equi* and horse leucocytes. As shown in Fig. 1, the expected 186 bp fragment was amplified only from *B. caballi* DNA, but not from *B. equi* and horse leucocyte DNAs. To further confirm the specificity of the PCR method, the product of PCR was inserted into pBluescript SK, and then subjected to DNA sequencing. The sequence of PCR product was completely identical to the published DNA sequence (Dalrymple et al. 1996) (data not shown).

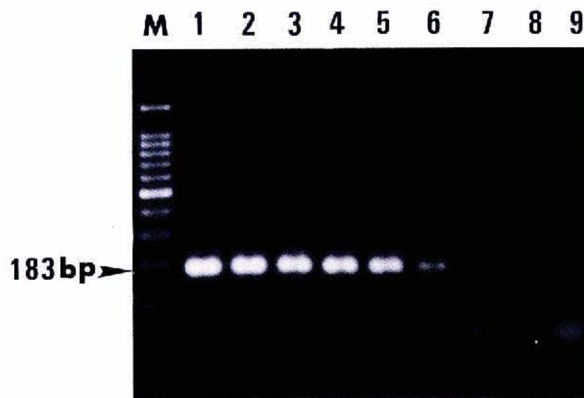

Figure 2. Sensitivity of the PCR method. Ethidium bromide stained agarose gel of PCR products from 10-fold serial diluted samples. Lane 1, dilution of 10^{-1} with 1% parasitemia; lane 2, dilution of 10^{-2} with 0.1% parasitemia; lane 3, dilution of 10^{-3} with 0.01% parasitemia; lane 4, dilution of 10^{-4} with 0.001% parasitemia; lane 5, dilution of 10^{-5} with 0.0001% parasitemia; lane 6, dilution of 10^{-6} with 0.00001% parasitemia; lane 7, dilution of 10^{-7} with 0.000001% parasitemia; lane 8, dilution of 10^{-8} with 0.0000001% parasitemia. Lane M, 100 bp DNA ladder marker.

Table 1 Comparison of PCR and IFAT to detection of *B. caballi* infection.

	IFAT [a] +	IFAT -	Total
PCR +	23 (16%)	5 (4%)	28 (20%)
PCR -	73 (52%)	41 (28%)	114 (80%)
Total	96 (68%)	46 (32%)	142 (100%)

[a] IFAT was done as described previously (Avarzed et al. 1997b).

To determine the sensitivity of the PCR method, *B. caballi*-infected erythrocytes (10% parasitemia) was serially 10-fold diluted with normal horse erythrocytes, and the parasite DNA of each dilution was extracted, and then subjected to PCR amplification. As shown in Fig. 2, the PCR method was sensitive

enough to detect parasite DNA from 2.5 μl blood sample with a parasitemia of 0.000001%.

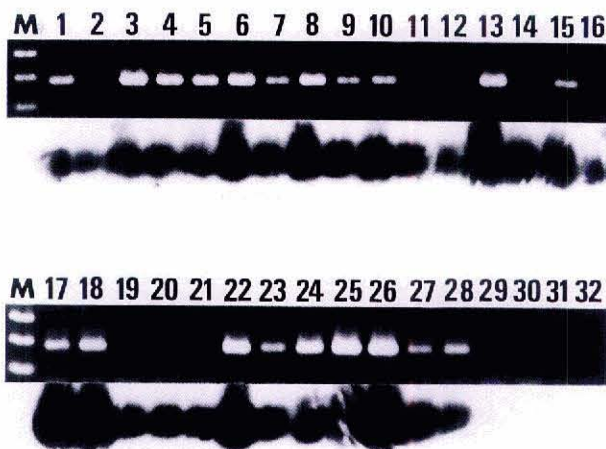

Figure 3. Southern blot hybridization of PCR products with specific DNA probe. Ten microliter of PCR-amplified DNA was separated on 1.5% agarose gel, and then transferred to nylon membranes. The specific DNA probe was labeled with DIG (Boehringer Mannheim). Hybridization was performed in a buffer containing 0.5 M $NaPO_4$ (pH 7.1), 2 mM EDTA, 7% SDS, 0.1% Sodium pyrophosphate, and 100 μg/ml denatured salmon sperm DNA at 65 °C. The membrane was reacted with anti-DIG-AP solution, and then exposed to X-ray film. Lanes 1-28, PCR positive field samples; lanes 29-32 PCR negative field samples. Lane M, 100 bp DNA ladder marker.

The PCR method was compared with IFAT and thin blood smear examination in other to evaluate the diagnosis efficiency for *B. caballi* infection in horses. As shown in Table 1, of 142 field samples from endemic areas of central Mongolia, 28 (20%) and 96 (69%) samples were identified positively by PCR and IFAT, respectively. All of the PCR-amplified products were hybridized with specific DNA probe from *B. caballi* USDA strain (Fig. 3). The parasites were not found microscopically on all of the thin blood smear samples (data not shown). Although that the sensitivity of PCR was lower than IFAT, it was noted that 5 IFAT-negative samples were PCR-positive, indicating that some carrier horses with undetectable antibody titer existed. Our results suggests that the combination of PCR method and IFAT for diagnosis of *B. caballi* infection may be more effective in detecting the carrier houses.

ACKNOWLEDGEMENTS

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