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Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood

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Abstract

With the aim of developing more simple diagnostic alternatives, a differential single-round and multiplex polymerase chain reaction (PCR) method was designed for the simultaneous detection of *Babesia caballi* and *Babesia equi*, by targeting 18S ribosomal RNA genes. The multiplex PCR amplified DNA fragments of 540 and 392 bp from *B. caballi* and *B. equi,* respectively, in one reaction. The PCR method evaluated on 39 blood samples collected from domestic horses in Mongolia yielded similar results to those obtained from confirmative PCR methods that had been established earlier. Thus, the single-round and multiplex PCR method offers a simple tool for the differential diagnosis of *B. caballi* and *B. equi* infections in routine diagnostic laboratory settings as well as in epidemiological studies.

Keywords: *Babesia caballi*; *Babesia equi*; Multiplex PCR; Diagnosis

1. Introduction

Babesia parasites are Ixodid tick-transmitted protozoa of many wild and domestic animals; they have a unique life cycle involving asexual and sexual developmental stages within the erythrocytes of vertebrates and the tissue of ticks (Ristic et al., 1988). Two species of *Babesia* parasites, *Babesia caballi* and *Babesia equi*, are known to infect horses, and the disease is endemic in most tropical and subtropical regions of the world (Avarzed et al., 1997a; Schein, 1988). Acute equine babesiosis is usually characterized by fever, anemia, icterus, lethargy, and, in some cases, death (Knowles, 1996). Horses that recover from an acute infection often continue to function as reservoirs for subsequent infecting ticks (Holbrook, 1969).

Babesial infection is usually diagnosed by microscopic examination, which enables the detection of

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parasites from Giemsa or Wright-stained blood films (Saal, 1964). Although this method is simple, it is insufficient for the accurate identification of *B. caballi* and *B. equi* during mixed infections and low parasitemias (Quintao-Silva and Ribeiro, 2003; Krause, 2003). Serological methods have also been developed for the diagnosis of equine babesiosis (Hirata et al., 2003; Ikadai et al., 2000; Kappmeyer et al., 1999; Bruning et al., 1997; Bose et al., 1995). However, these assays are generally restricted by antibody detection limits and cross-reactivity (Allred, 2003; Tenter and Friedhoff, 1986; Weiland, 1986; McGuire et al., 1971).

Recently, the polymerase chain reaction (PCR) method has been applied for the detection of *Babesia* parasites (Bashiruddin et al., 1999; Calder et al., 1996; Figueroa et al., 1993; Conrad et al., 1992; Fahrimal et al., 1992). The sensitivity of these PCR methods for detecting equine babesiosis has been shown to be higher than that of microscopic detection methods (Rampersad et al., 2003; Nicolaiewsky et al., 2001; Bashiruddin et al., 1999). However, these methods are still relatively time-consuming and require complex procedures such as nested PCR and or hybridization to achieve higher sensitivity. Therefore, there is a need to develop simpler PCR-based systems suitable for routine diagnosis. In this study, we developed a single-round and multiplex PCR method for the simultaneous detection of *B. caballi* and *B. equi* with improved rapidity and sensitivity, based on the 18S ribosomal RNA genes, which are present in multiple copies through the genome, and evaluated it on field blood samples.

2. Materials and methods

2.1. In vitro cultures of parasites

United State Department of Agriculture (USDA) strains of *B. caballi* and *B. equi* were grown in equine erythrocytes with a microaerophilous stationary-phase culture system as described previously (Zweygarth et al., 2002; Ikadai et al., 2001; Holman et al., 1998; Avarzed et al., 1997b).

2.2. DNA extraction

The parasites were harvested from the cultures in order to extract the parasitic DNA as described by Battsetseg et al. (2002). Briefly, 50 μl of each *B. caballi*- and *B. equi*-infected erythrocytes were washed three times with cold phosphate-buffered saline (PBS) by centrifuging at 1000 × *g* for 5 min at 4 °C and resuspended in a DNA extraction buffer (0.1 mM Tris–HCl [pH 8.0], 0.1% sodium dodecyl sulfate (SDS), 100 mM NaCl, and 10 mM EDTA). The mixture was digested with 100 μg/ml proteinase K (Invitrogen, Carlsbad, CA, USA) for 2 h at 55 °C. The parasitic DNA was extracted with phenol-chloroform and precipitated with ethanol. A mixed infection study was simulated by simultaneously extracting DNA from a mixture of *B. caballi* and *B. equi*-infected erythrocytes at a ratio of 1:1. The purified DNA was used as a template for subsequent PCR amplifications. Normal horse blood DNA was also purified and used as a negative control.

2.3. Primer design and PCR amplification

The 18S ribosomal RNA gene sequences of *B. caballi* and *B. equi* (Criado-Fornelio et al., 2003; Allsopp et al., 1994) were used to design suitable diagnostic primers.

The accession numbers used in this study are Z15104 for *B. caballi* and Z15105, AY150062, and AY150063 for *B. equi*. By aligning these sequences using a Mac Vector (Oxford Molecular, Ltd., Oxford, UK), a universal screening primer pair common for *B. caballi* and *B. equi*, Bec-UF1 and Bec-UR, was designed to amplify the DNA of both parasites in one reaction. Additionally, a set of primer combinations including Bec-UF2 as a universal forward primer and Cab-R and Equi-R as reverse primers specific for *B. caballi* and *B. equi*, respectively, was also designed for the species-specific detection. Furthermore, species-specific primer pairs were designed based on the genes of *Babesia equi* Merozoite Antigen 1 (EMA-1) and *Babesia caballi* 48-kDa antigen (BC48), and used to confirm the accuracy of the results obtained by the multiplex PCR. The EMA-1 is encoded by a single copy gene of *B. equi* (Knowles et al., 1997; Kappmeyer et al., 1993) and BC48 is present as a multi-copy gene encoding the 48-kDa rhoptry protein of *B. caballi* (Ikadai et al., 1999). The primer pairs designed from these genes have so far been used for the single detection of these parasites in horse blood