Development of Taqman-Based Real-Time PCR Assays for Diagnostic Detection of *Babesia bovis* and *Babesia bigemina*

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**Abstract.** Two TaqMan-based real-time polymerase chain reaction (PCR) assays devised for the detection of two bovine *Babesia* parasites, *Babesia bovis* and *B. bigemina*, were evaluated for their diagnostic utility using cultured parasites and 92 field bovine blood samples collected from cattle living in Brazil. The real-time PCR assays were compared with previously established nested-PCR assays. The detection limits of both *B. bovis* and *B. bigemina*-real-time PCR assays were identical at the value of 2.5 parasites/μL of the infected blood. When 92 field bovine blood samples were tested using the real-time assays, *B. bovis*-positive signals were observed in 30 samples among 31 *B. bovis*-positive blood samples in the nested-PCR assay (96.9% sensitivity and 100% specificity), whereas the *B. bigemina*-real-time PCR assay could detect the parasite from all of 45 *B. bigemina*-positive blood samples in the nested-PCR assay (100% sensitivity and specificity). The real-time assays using the TaqMan-system can therefore be practically implemented in the epidemiologic survey for bovine babesiosis. Further studies will be necessary to investigate the clinical value of this technique, especially for the quantitative detections of the parasites.

**INTRODUCTION**

Bovine babesiosis is a tick-borne disease in cattle that is caused by intra-erythrocytic protozoan parasites of the genus *Babesia*. Two species, *Babesia bovis* and *Babesia bigemina*, are the most frequent and important tick-borne protozoa among various bovine *Babesia* parasites.1 Bovine babesiosis is often found in most of Africa, Southern Europe, Asia, Central and South America, and many of the islands in the Caribbean and South Pacific.2 It is estimated that half a billion cattle throughout the world may be endangered by bovine babesiosis.3

Routine clinical diagnosis for babesiosis is usually based on the microscopic detection of parasites from collected blood smears. The detection had been considered to be the “gold standard” for the diagnosis of babesiosis.4 However, the technique is relatively laborious when large numbers of blood smear samples must be simultaneously quantified. Furthermore, it is extremely difficult to detect parasites in blood smears during low parasitemia.5 Alternative techniques have been developed for the laboratory diagnosis of babesiosis. For example, many serological diagnostic tests have been developed for the detection of specific antibodies to bovine *Babesia* parasites, such as the complement fixation test (CFT), the indirect hemagglutination (IHA) test, the latex agglutination test (LAT), the indirect fluorescent antibody test (IFAT), and the enzyme-linked immunosorbent assay (ELISA).6 However, the antibodies cannot always be detected in long-term carriers despite the presence of the parasite.7 Furthermore, cross-reactivity of the antibodies against other *Babesia* species has limited the specificity of serological tests.8,9

Polymerase chain reaction (PCR) assays for the diagnostic detection of *Babesia* parasites have the potential to provide rapidly qualitative results with high sensitivity, in particular, nested-PCR assay.10,11 These PCR assays have various advantages over the microscopic and serological diagnostic test. For example, PCR diagnosis is possible in animals as young as 1 month of age, and the data obtained by PCR assays refer to the current prevalence, in contrast to the data obtained by serological assays.7 In addition, the sensitivity of these assays for detecting bovine babesiosis has been shown to be higher than that of microscopic detection methods.5,10,12 However, current PCR-based assays often require multiple processes to be performed on each sample and, moreover, do not provide quantification of the parasitemia.

Real-time PCR is a new methodology that employs fluorescent labels to enable the continuous monitoring of amplification (PCR product) formation throughout the reaction13 and has proven to be useful in various applications, including pathogen detection,14 gene expression and regulation,15 and allelic discrimination.16 In this study, two TaqMan-based real-time PCR qualitative assays were devised for the rapid detection and identification of two bovine *Babesia* parasites (*B. bovis* and *B. bigemina*) and evaluated for their diagnostic utility using cultured parasites and field blood samples collected from cattle in Brazil that were infected by *B. bovis* and *B. bigemina* and from non-infected control cattle. The results of the real-time PCR assays were compared with those of previously established nested-PCR assays.10,12

**MATERIALS AND METHODS**

**Cultured Babesia.** The Texas T2B strain of *B. bovis*17 and the Argentine strain of *B. bigemina*18 were grown in purified bovine red blood cells (RBC) using a previously established continuous microaerophilic stationary-phase culture system,19 Medium M199 (Sigma-Aldrich, Tokyo, Japan) supplemented with 40% bovine or horse serum, 60 U/mL of penicillin G, 60 μg/mL of streptomycin, and 0.15 μg/mL of amphotericin B (Sigma-Aldrich) was used for the cultivation.

**Collection of blood samples from cattle and DNA extraction.** Ninety-two field blood samples were collected from cattle living in Mato Grosso do Sul, Brazil, and then stored at −20°C until use. Genomic DNAs were extracted from 200 μL of the blood samples using a QIAamp DNA Blood Mini Kit
(QIAGEN, Tokyo, Japan) according to the manufacturer’s instructions.

**Real-time PCR assays.** Primers (forward and reverse) and TaqMan fluorescence-labeled probes for real-time PCR assays were designed using Primer Express Software (Applied Biosystems, Foster City, CA) to specifically amplify the 18S rRNA genes of *B. bovis* (GenBank accession numbers, AY150059, AY603398, L19077, L31922, L19078, and M87566) and *B. bigemina* (GenBank accession numbers, AY603402, X59604, X59605, and X59607). The pairs of forward and reverse primers (BoF-BoR and BiF-BiR) and probes (BoP and BiP) are shown in Table 1. For real-time quantitative assays, 1 μL of the extracted genomic DNA template was combined with 450 nM of each oligonucleotide primer and 250 nM of the FAM- (*B. bovis*) or VIC- (*B. bigemina*) and TAMRA-labeled probe in 20 μL of a total reaction volume using TaqMan Universal PCR Master Mix (Applied Biosystems) per reaction. TaqMan PCR reactions were mixed in 96-well optical plates (Applied Biosystems). The PCR samples were subjected to 45 cycles of amplification in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) under the following conditions: 50°C for 2 min (uracil N-deglycosylase digest), 95°C for 10 min (AmpliTaq Gold pre-activation), and then 45 cycles of 95°C for 20 sec and 55°C for 1 min. The ABI 7900 cycler provided a cycle-by-cycle measurement of the fluorescence emission from each reaction. The fluorescence data was analyzed using PE 7900 Sequence Detection System Software (Version 2.1; Applied Biosystems).

**Nested-PCR assays.** Nested-PCR assays for the detection of *B. bovis* or *B. bigemina* were carried out with four oligonucleotides as described previously.10,12 For each of the nested-PCR assays, 1 μL of extracted genomic DNA template and 0.5 μL of the first PCR product were used for the first and second PCR assays, respectively. The nested-PCR products were visualized by 2% agarose gel electrophoresis and then stained with ethidium bromide.

**Sensitivity and specificity.** **Analytical sensitivity test.** Each 200 μL of *B. bovis* and *B. bigemina*-infected RBC with 1% parasitemia (2.5 × 10^5 parasites/μL of the infected blood) was subjected to 10-fold dilutions in non-infected bovine RBC, and DNA extracted from each diluted RBC sample was then used for an analytical sensitivity test. All dilutions of infected RBC were prepared in duplicate for the real-time PCR assay in parallel to the nested-PCR assay.

**Analytical specificity test.** To evaluate the analytical specificity, DNAs from other protozoan parasites including *Theileria parva*, *Trypanosoma evansi*, and *Neospora caninum*, which infect cattle, or from non-infected bovine blood were subjected then to the real-time PCR assays for bovine *Babesia* parasites.

**Clinical evaluations of the sensitivity and specificity.** Clinical sensitivity and specificity of the real-time PCR assays were evaluated on the basis of data of 92 field blood samples identified by the nested-PCR assays and sequence analysis. The sensitivity was calculated as the percentage of the number of true positives (real-time and nested-PCR-double positives) in the total number of the true positives and false negatives (real-time PCR negatives but nested-PCR-positives), and the specificity was calculated as the percentage of the number of true negatives (real-time and nested-PCR-double negatives) in the total number of the true negatives and false positives (real-time PCR positives but nested-PCR negatives).

**DNA sequence analysis.** Amplification products of the discordant samples between the real-time and nested-PCR assays were extracted from 2% agarose gel using a QIAquick Gel Extraction Kit (QIAGEN, Tokyo, Japan) and sequenced using a Big Dye Terminator Sequencing Kit (Applied Biosystems Japan Ltd., Tokyo, Japan) with the ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA) following the manufacturer’s instructions.

**RESULTS**

**Analytical sensitivity and specificity of real-time PCR assays.** To evaluate the sensitivity of real-time PCR assays devised in this study, *in vitro* cultured *B. bovis* or *B. bigemina*-infected RBC with 1% parasitemia (2.5 × 10^5 parasites/μL of the infected blood) were serially 10-fold diluted with non-infected bovine RBC up to 0.000000001% parasitemia (2.5 × 10^-8 parasites/μL of the infected blood). The amplification plots of the real-time PCR assays for *B. bovis* and *B. bigemina* and the products of nested-PCR are shown in Figures 1 and 2, respectively. The real-time (Figure 1A) and nested-PCR (Figure 1B) assays for *B. bovis* were detectable from 2.5 × 10^5 to 2.5 × 10^4 parasites/μL of the infected blood. The detection limits of *B. bigemina* real-time (Figure 2A) and nested-PCR (Figure 2B) were also identical (2.5 parasites/μL of the infected blood). The mean cycle threshold (Ct) value, which is the value that indicates the target gene amount at which the fluorescence exceeds a preset threshold,13 ranged from 21.3–35.2 and 23.1–37.1 for *B. bovis* and *B. bigemina*, respectively. A linear standard curve was routinely generated in each real-time PCR run from 2.5 × 10^5 to 2.5 × 10^4 parasites/μL of the infected blood (*B. bovis*, R^2 = 0.9911; *B. bigemina*, R^2 = 0.9868). The Ct values increased linearly as the target quantity decreased (Figure 3). No positive signal was observed in the real-time PCR assays with DNAs of non-*Babesia* species (*Theileria parva*, *Trypanosoma evansi*, and *Neospora caninum*) and non-infected bovine blood (data not shown).

**Clinical evaluations of real-time PCR assays.** To evaluate the field utilities of the two real-time PCR assays, we tested 92 field bovine blood samples collected from cattle living in

<p>| Table 1 | Primers and probes selected for TaqMan PCR of 18S rRNA gene of <em>Babesia</em> parasites |
|---------|--------------------------------|----------------------------------------------------------------|</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Primer or probe</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bovis</em></td>
<td>BoF</td>
<td>AGCGGTTTCCGCTTGATATAATG</td>
</tr>
<tr>
<td></td>
<td>BoR</td>
<td>AGCTGGTCGTCTACGACAA</td>
</tr>
<tr>
<td></td>
<td>BoP probe (FAM)</td>
<td>CCTTGATGACCCCGTGTTACGTTGG</td>
</tr>
<tr>
<td><em>B. bigemina</em></td>
<td>BiF</td>
<td>AATAACAATACAGGGCTTTCGTCT</td>
</tr>
<tr>
<td></td>
<td>BiR</td>
<td>AACGGGAGCCTGAAAATACAAC</td>
</tr>
<tr>
<td></td>
<td>BiP probe (VIC)</td>
<td>TTTGGAATGATGGTGATGTACAACCTCA</td>
</tr>
</tbody>
</table>

* The nucleotide positions are those reported in GenBank (accession numbers, AY603398 for *B. bovis* and AY603402 for *B. bigemina*).
Mato Grosso do Sul, Brazil. The results of real-time PCR assays were compared with those of nested-PCR as described earlier and the discordant PCR samples were analyzed by DNA sequencing for the final identification. Among 92 field blood samples, 30 and 31 samples were positive in the B. bovis real-time and nested-PCR assays, respectively, whereas 45 and 48 samples were positive in the B. bigemina real-time and nested-PCR assays, respectively (Table 2). DNA sequence analysis for the case of B. bovis revealed that the only discordant sample of the nested-PCR assay had point variations within the complimentary sequence of the BoP probe (5’-H11032-CCTTGTATGACCTTGTCCTACCGTTGG-3’; the underlines refer to the variation sites within the complimentary sequence of the BoP probe). In the case of B. bigemina, three discordant blood samples that were positively diagnosed by the nested-PCR assay but not by the real-time PCR assay revealed non-related sequences to any protozoa in DNA sequence analysis followed by a sequence homology search (NCBI, National Institutes of Health, http://www.ncbi.nlm.nih.gov/BLAST/).

The quantities of positive field blood samples in the real-time PCR assays were calculated on the basis of the standard curve that had been generated in the positive-cultured RBC as described previously. Among the 30 positive blood samples in the B. bovis real-time PCR assay, the most frequent quantity ranged within $2.5 \times 10^3$ to $2.5 \times 10^2$ parasites/$\mu$L (12 head...
of cattle), whereas among the 45 positive blood samples in the B. bigemina real-time PCR assay, the most frequent quantity ranged within 2.5 $\times$ 10^2 to 2.5 $\times$ 10^3 parasites/$\mu$L (15 head of cattle) (Table 3).

The clinical sensitivity and specificity of the B. bovis real-time PCR assay showed 96.9% (30 of 31) and 100% (61 of 61) relative to the results of nested-PCR assay and the subsequent sequence analysis, whereas the B. bigemina real-time PCR assay was able to detect B. bigemina only in all 45 positive blood samples (100% sensitivity), without a positive signal in the remaining 47 negative blood samples (100% specificity).

**DISCUSSION**

In the present study, we describe the development and standardization of two real-time PCR assays for the quantitative detection of B. bovis and B. bigemina from bovine blood. Until now, several diagnostic PCR assays have been developed to assess the frequencies of B. bovis and B. bigemina infections in cattle and transmitted ticks such as Boophilus microplus in endemic areas of babesiosis. The DNA amplification assay, which can detect Babesia parasites from the infected animals at the early phases of infection and the carrier animals, is a more powerful tool for epidemiologic research than the microscopic method because the latter method cannot detect the positive animals with low parasitemia.

Quantification of parasite levels by the PCR assay would be extremely valuable for the investigation of cattle infection with Babesia parasites. The use of the real-time PCR assay has several advantages for the detection and quantification of the parasite over the use of the conventional PCR assay because the detection and quantification of a real-time PCR product can take place in a single tube, obviating the need for post-PCR manipulation and reducing the risk of contamination. Although several real-time PCR assays have proved their availability for the detection of various protozoan diseases, to date, there is no rapid, specific, and sensitive assay using the TaqMan-system available for diagnosing and quantifying bovine Babesia parasites. The real-time PCR assay is characterized by the point of time during cycling (Ct or threshold cycle) at which the amplification of the PCR product, rather than the amount of PCR product accumulated, can be first detected. This Ct parameter is defined as the number of cycles at which the fluorescence of reporter generated by cleavage of the probe (ΔRn) is greater than a fixed threshold above the baseline. The quantification of blood samples is performed by determining the Ct value and the use of a standard curve to determine the starting copy number. The quantification of an infectious load has been used to assess the disease severity and treatment outcome in various protozoan diseases. Thus, the quantitative analyses developed in this study might be useful for comparing different drug regimens and determine the prognostic value of the treatment of bovine babesiosis.

This study demonstrated that two real-time PCR assays are highly specific and sensitive for the detection of B. bovis and B. bigemina. To evaluate the field utilities of two real-time PCR assays, field blood samples obtained from cattle in Brazil were tested. Although both the analytical specificity and sensitivity were identical between real-time and nested-PCR assays, the results with field blood samples showed slight discordances between two PCR assays. One discordant blood sample of B. bovis was caused by variation of two nucleotide sequences within the 18S rRNA gene targeted for the BoP probe. Even though the sequence analyses of the 18S rRNA gene have been widely used for the identification of many pathogens, the sequence data of Babesia species have not been well established, and the species distribution is not well known throughout the world. Further studies based on the 18S rRNA genes regarding the molecular epidemiology of Babesia parasites are required. On the other hand, in the case of B. bigemina, three field blood samples were determined to be positive by the established nested-PCR assay but found negative by the subsequent DNA sequence analysis. Essentially, when compared with the results of nested-PCR assays, the B. bigemina real-time PCR assay developed in this study showed a higher efficacy for parasite detection.

In conclusion, the real-time PCR assays using the TaqMan-system described in this study provided a rapid, sensitive, and quantitative way using continuous fluorescence monitoring of each B. bovis and B. bigemina DNA-specific dye from clinical blood samples. The real-time assays developed in this study can therefore be practically implemented in epidemiologic surveys for bovine babesiosis, even though evaluations on a larger scale are necessary.

Received May 7, 2007. Accepted for publication July 5, 2007.

Acknowledgments: This research was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), the Program of Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), and The 21st Century COE Program (A-1), Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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**REFERENCES**


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**TABLE 3**

Quantities of B. bovis and B. bigemina in field bovine blood collected from Mato Grosso do Sul, Brazil

<table>
<thead>
<tr>
<th>Quantity*</th>
<th>No. of infected cattle</th>
</tr>
</thead>
</table>
| $>1.25 \times 10^5$ | B. bovis 1  
| 1.25 $\times 10^2$ – 2.5 $\times 10^5$ | 2 2
| 2.5 $\times 10^2$ – 2.5 $\times 10^4$ | 3 3
| 2.5 $\times 10^3$ – 2.5 $\times 10^6$ | 8 8
| 2.5 $\times 10^4$ – 2.5 $\times 10^9$ | 12 12
| 2.5 $\times 10^5$ – 2.5 $\times 10^9$ | 15 15
| Total | 30 45

* The quantities are expressed as the number of Babesia parasites per microliter of RBC.
REAL-TIME PCR OF BABESIA BOVIS AND BABESIA BIGEMINA


