Comparison of *Babesia microti* Real-Time Polymerase Chain Reaction Assays for Confirmatory Diagnosis of Babesiosis

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Abstract. Babesiosis is an emerging tick-borne disease caused by apicomplexan parasites of the genus *Babesia*. Most human infections in the United States are caused by *Babesia microti,* but other infection-causing *Babesia* parasites have been documented as well.¹,² *Babesia* infection may be asymptomatic in otherwise healthy people, but can be severe in immunocompromised or asplenic individuals. Common clinical manifestations include nonspecific flu-like symptoms (e.g., fever) and hemolytic anemia. The Centers for Disease Control and Prevention (CDC) has supported health departments in their investigations of cases of babesiosis in the United States since the first documented case was identified in 1966.³ Babesiosis is considered an emerging infectious disease, with a majority of cases reported in the northeast and upper midwest. Babesiosis became a nationally notifiable disease in January 2011; that year, CDC was notified of 1,124 cases that fulfilled the established case definition.⁴

The current gold standard for the laboratory diagnosis of babesiosis is microscopic examination of Giemsa-stained blood smears.⁵ However, microscopy can identify *Babesia* only to the genus level, and morphological features alone are not always sufficient to distinguish *Babesia* from *Plasmodium.* Molecular methods or serology can be used to confirm the microscopic findings and in addition provide a species-specific diagnosis.⁶,⁷

In the United States, babesiosis is most often caused by *Babesia microti,* but sporadic human cases caused by other *Babesia* parasites have been documented.⁸,⁹ For many years, CDC relied on a nested polymerase chain reaction (PCR) assay for the species-specific identification of *B. microti* in human clinical specimens¹⁰; however, this assay is time consuming and requires handling of amplified DNA, which is a risk factor for false-positive results due to contamination. To facilitate a faster diagnosis in a more practical format, we evaluated different real-time PCR assays specific for *B. microti,* aiming to identify an alternative for the detection of this species in human blood specimens.

INTRODUCTION

Babesiosis is a zoonosis caused by intraerythrocytic apicomplexan parasites of the genus *Babesia.* These parasites are generally transmitted by tick bites, but blood transfusion and congenital transmissions have been reported as well.² Babesia infection may be asymptomatic in otherwise healthy people, but can be severe in immunocompromised or asplenic individuals. Common clinical manifestations include nonspecific flu-like symptoms (e.g., fever) and hemolytic anemia. The Centers for Disease Control and Prevention (CDC) has supported health departments in their investigations of cases of babesiosis in the United States since the first documented case was identified in 1966.³ Babesiosis is considered an emerging infectious disease, with a majority of cases reported in the northeast and upper midwest. Babesiosis became a nationally notifiable disease in January 2011; that year, CDC was notified of 1,124 cases that fulfilled the established case definition.⁴

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MATERIALS AND METHODS

Specimens. Seventy-eight ethylenediaminetetraacetic acid–whole blood human specimens from different areas of the United States received at CDC between 2001 and 2016 were included in this study. The specimens had originally been submitted for confirmatory diagnosis of parasitic infections and were used in accordance with the CDC Human Subjects Research Protocol titled “Use of residual diagnostic specimens from humans for laboratory methods research.” As part of CDCs reference diagnostic services, these specimens were examined by microscopy of Giemsa-stained blood smears upon reception at CDC. Following the diagnostic algorithm for babesiosis at CDC, specimens that were microscopy positive for *Babesia spp.* (N = 44) were subjected to a *B. microti*–specific nested PCR.¹⁰ Thirty-six specimens were positive for *B. microti* by this PCR assay. Specimens positive for *Babesia* by microscopy but negative for *B. microti* by PCR (N = 8) were subjected to a generic PCR assay followed by DNA sequencing analysis of the amplicon to determine the infecting species¹¹; two were positive for *Babesia duncanii* (of which one case was described previously),¹² one for a *Babesia CA1*-type parasite, four for *Babesia MO1*-type parasites, and one for another *Babesia divergens*–like parasite.¹³ Specimens negative for *Babesia spp.* by microscopy but positive for other blood-borne pathogens were confirmed with species-specific PCR and included as specificity controls.¹⁴,¹⁵ Fifteen specimens were included as parasite-free negative controls. In addition to the specificity controls described above, the specificity of the real-time PCR assay was evaluated with four laboratory isolates of *B. divergens* s.s. (of which one was the Purnell strain),¹⁶ originally cultured from cattle in Europe. Genomic DNA was extracted from 200 μL of each blood specimen using QIAamp DNA Blood Mini Kit using the QIAcube automated system (QIAGEN, Valencia, CA) according to the manufacturer’s instructions.

Nested PCR assay. All specimens positive for *Babesia spp.* by microscopy were subjected to a nested PCR specific for *B. microti* using primers and thermal cycling structure described elsewhere.¹⁰ The PCR reactions contained AmpliTaq Gold
RESULTS AND DISCUSSION

Selection of real-time PCR assays for evaluation. Upon literature review, nine published real-time PCR assays were identified and included in this evaluation. Seven of them target the 18S ribosomal RNA (rRNA) gene, one targets the surface antigen 1 gene (sal), and one targets the thiamine pyrophosphokinase gene (pkp). Five assays targeting the 18S rRNA gene had at least one oligonucleotide with one or more mismatches when aligned to the target gene (Supplemental Figure 1); these assays were eliminated from further evaluation to avoid potential problems with false-negative results. Four assays had correct oligonucleotide designs according to this in silico test and were therefore selected to be evaluated in the laboratory: the SA1 TaqMan assay, the TPK molecular beacon assay, the 18S TaqMan assay, and the 18S SYBR Green assay. 

Comparison of four real-time PCR assays. The detection limits, amplification efficiencies, and dynamic ranges of the four real-time PCR assays were determined using serial dilutions of three samples with known parasite densities. Results are summarized in Table 1 and Figure 1. All real-time PCR assays had comparable dynamic range and amplification efficiency, but the analytical sensitivity (limit of detection) varied. The most sensitive assay was the SYBR Green assay with 2.4 parasites/μL, which is the same level of sensitivity as for the nested PCR. The 18S TaqMan assay had a detection limit of 12 parasites/μL, but it also produced positive results on some samples with fewer parasites, although inconsistently (positive results for two of three replicates of 3 parasites/μL and one of three replicates of 0.5 parasites/μL). The SA1 and TPK assays were both less sensitive, with a detection limit of 14 parasites/μL. The multiplicity nature of the 18S rRNA gene probably contributed to the superior detection limit for the nested, SYBR Green, and 18S TaqMan assays.

The analytical specificity of the assays was determined with a panel of 27 clinical specimens positive for parasites other than B. microti, plus four B. divergens isolates (see Materials and Methods section for details). Only one test, the SYBR Green assay, displayed evidence of nonspecific amplification with nine false-positive results. The Ct values for the false positives were all 33 or above (mean = 36), but the melting curve analysis did not separate the false positives from the weak B. microti specimens that were detected with similar Ct values. A BLAST similarity search of the SYBR Green primers in the GenBank database revealed that these primers have the capability to bind efficiently to other parasites besides B. microti (data not shown). Thus, the SYBR Green assay was considered not specific for B. microti. Taken together, these data indicated that the 18S TaqMan assay was the most accurate real-time PCR assay for B. microti.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Comparison of analytical performance of five PCR-based assays for Babesia microti detection</th>
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</thead>
<tbody>
<tr>
<td><strong>Assay</strong></td>
<td><strong>Target gene</strong></td>
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<tr>
<td>Nested</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>18S TaqMan</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>SYBR Green</td>
<td>18S rRNA</td>
</tr>
<tr>
<td>SA1 TaqMan</td>
<td>sal</td>
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<td>TPK</td>
<td>tbb</td>
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CI = confidence interval; NA = not applicable; PCR = polymerase chain reaction; rRNA = ribosomal RNA.
Diagnostic validation of the 18S TaqMan assay. The 18S TaqMan assay was validated for sensitivity, specificity, accuracy, and reproducibility as defined by the Clinical Laboratory Improvement Amendments of 1988. The assay was performed with 78 clinical specimens (of which 36 were positive for \textit{B. microti}, 27 were positive for other parasites, and 15 were parasite free; see Materials and Methods section for details). We found the sensitivity and specificity for \textit{B. microti} to be 100\% (Table 2). To assess reproducibility, the 78 specimens were retested in six separate runs on four different days, totaling 468 individual result points. All positive specimens remained positive in all runs, and among the negative specimens, only one borderline positive result (Ct = 39) was obtained with one malaria specimen in one single run. These findings led to a reproducibility of almost 100\% (Table 2). Since the false-positive result was so weak and not repeatable, it was most likely due to a random contamination event. Positive and negative predictive values were not calculated since the real prevalence of babesiosis in the test population is unknown and the numbers of positives and negatives included in this validation most likely did not reflect the real prevalence.

Concluding remarks. Real-time PCR assays can provide a faster diagnosis compared with conventional PCR assays, especially nested assays. This study compared the performance of published real-time PCR assays against a conventional nested PCR assay for the specific detection of \textit{B. microti} in human blood specimens and identified a TaqMan assay targeting the 18S rRNA gene to be the best performing assay. The 18S rRNA gene is highly conserved within each \textit{Babesia} species and is present in high copy number in the genome. PCR-based assays targeting this gene therefore have the potential to be very sensitive and species specific. Although not quite as analytically sensitive as the nested PCR assay, the 18S TaqMan showed a 100\% diagnostic sensitivity and did not yield any false-negative results for the actual clinical specimens analyzed in this study.

This study only evaluated the use of real-time PCR as a qualitative method to detect \textit{B. microti}. The possibility to use this as a quantitative assay to estimate parasitemia remains to be explored. Although parasitemia calculations are not part of the diagnostic algorithm at CDC, the parasitologists sometimes noted an estimate of the number of infected

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Calculations</th>
<th>Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>((\text{true positives})/(\text{true positives} + \text{false negatives}))</td>
<td>(36/(36 + 0) \times 100 = 100%)</td>
<td>90.3–100.0%</td>
</tr>
<tr>
<td>Specificity</td>
<td>((\text{true negatives})/(\text{true negatives} + \text{false positives}))</td>
<td>(42/(42 + 0) \times 100 = 100%)</td>
<td>91.6–100.0%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>((\text{true positives} + \text{true negatives})/(\text{true positives} + \text{true negatives} + \text{false positives} + \text{false negatives}))</td>
<td>((36 + 42)/(36 + 42 + 0 + 0) \times 100 = 100%)</td>
<td>95.3–100.0%</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>((\text{results in agreement})/(\text{total no. of results}))</td>
<td>(467/468 \times 100 = 99.8%)</td>
<td>98.8–100.0%</td>
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CI = confidence interval; PCR = polymerase chain reaction.
blood cells when reading the blood smears. Based on these estimates, 18 of the \textit{B. microti}-positive specimens were classified as having few parasites; the mean Ct value for these specimens in the 18S TaqMan assay was 26 (range, 17–37). Four specimens were classified as having high parasite density, with a mean Ct value of 22 (range, 20–23). More studies are needed to clarify the usefulness of this real-time PCR assay for quantification of parasitemia in babesiosis cases.

In conclusion, the 18S TaqMan real-time PCR assay is a rapid, sensitive, and specific method for detection of \textit{B. microti} in human blood specimens. This assay has now replaced the nested PCR as the method of choice for the identification of \textit{B. microti} among cases of babesiosis at the CDC.

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Note: Supplemental figure appears at www.ajtmh.org.

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Disclaimer: The findings and conclusions in this manuscript are those of the author(s) and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

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