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# Development of droplet digital PCR for the detection of *Babesia microti* and *Babesia duncani*

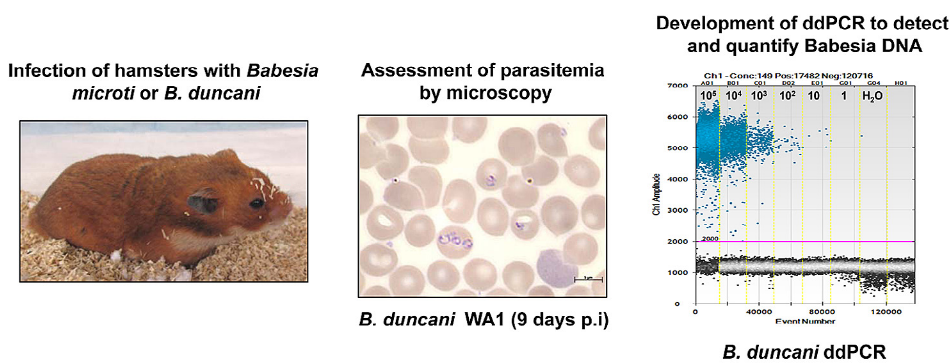
Melisa Wilson, Kathleen C. Glaser, Debra Adams-Fish, Matthew Boley, Maria Mayda, Robert E. Molestina *

*BEI Resources, American Type Culture Collection, Manassas, VA 20110, USA*

## HIGHLIGHTS

- This is the first report of ddPCR assays for *Babesia microti* and *B. duncani*.
- The *B. microti* assay detected parasitemia as early as 3 days of hamster infection.
- The assays were 100% specific when compared with other blood-borne pathogens.
- ddPCR may become a useful tool in the diagnosis of *Babesia* in human blood.

## GRAPHICAL ABSTRACT

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## ABSTRACT

*Babesia* spp. are obligate protozoan parasites of red blood cells. Transmission to humans occurs through bites from infected ticks or blood transfusion. Infections with *B. microti* account for the majority of the reported cases of human babesiosis in the USA. A lower incidence is caused by the more recently described species *B. duncani*. The current gold standard for detection of *Babesia* is microscopic examination of blood smears. Recent PCR-based assays, including real-time PCR, have been developed for *B. microti*. On the other hand, molecular assays that detect and distinguish between *B. microti* and *B. duncani* infections are lacking. Closely related species of *Babesia* can be differentiated due to sequence variation within the internal transcribed spacer (ITS) regions of nuclear ribosomal RNAs. In the present study, we targeted the ITS regions of *B. microti* and *B. duncani* to develop sensitive and species-specific droplet digital PCR (ddPCR) assays. The assays were shown to discriminate *B. microti* from *B. duncani* and resulted in limits of detection of ~10 gene copies. Moreover, ddPCR for these species were useful in DNA extracted from blood of experimentally infected hamsters, detecting infections of low parasitemia that were negative by microscopic examination. In summary, we have developed sensitive and specific quantitative ddPCR assays for the detection of *B. microti* and *B. duncani* in blood. Our methods could be used as sensitive approaches to monitor the progression of parasitemia in rodent models of infection as well as serve as suitable molecular tests in blood screening.



* Corresponding author. Fax: +1 703 365 2730.
*E-mail address:* rmolestina@atcc.org (R.E. Molestina).

At the time of publication, a recent study by Yang R. et al. determined the application of ddPCR for another protozoan parasite, *Cryptosporidium* (Yang R., Paparini A., Monis P. and Ryan U. 2014. Comparison of next-generation droplet digital PCR (ddPCR) with quantitative PCR (qPCR) for enumeration of *Cryptosporidium* oocysts in faecal samples, Int. J. Parasitol. 44, 1105–1113).

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## 1. Introduction

Babesiosis, caused by intraerythrocytic protozoan parasites of the genus *Babesia*, has been recognized in recent years as an emerging infectious disease in humans. Transmission occurs primarily by ixodid ticks (Hunfeld et al., 2008). Although uncommon, other routes of transmission include pregnancy and blood transfusion (Joseph et al., 2012; Lobo et al., 2013). The latter is of particular concern since potential blood donors may harbor asymptomatic infections. A 2008 workshop sponsored by the Food and Drug Administration (FDA) discussed various aspects of transfusion-transmitted babesiosis in the United States, including strategies to identify infected blood donors, epidemiology of the disease, and biology and pathogenesis of *Babesia* spp. (Gubernot et al., 2009). Despite recommendations for the development of tests for babesiosis that met blood donor screening requirements, to this date there is a lack of FDA-approved assays for blood testing against *Babesia* and other vector-transmitted protozoan parasites.

*B. microti* is the causative agent for the majority of human cases of babesiosis in the U.S., with a higher prevalence occurring in the Northeastern region of the country (Johnson et al., 2009; Kogut et al., 2005; Leiby, 2011). Infections by *B. microti* have also been reported in the Upper Midwest, particularly Minnesota and Wisconsin (Centers for Disease Control and Prevention (CDC), 2012; Herwaldt et al., 1995; Setty et al., 2003). A lower incidence of babesiosis is observed in western regions of the U.S. where it is usually caused by infection with *B. duncani* (Conrad et al., 2006; Herwaldt et al., 1997; Persing et al., 1995; Quick et al., 1993).

Infection with *Babesia* is usually asymptomatic or results in mild symptoms that resolve within a few days. Infected individuals may display flu-like symptoms within 1–9 weeks which include high fever, headaches, chills, fatigue, and anemia (Hunfeld et al., 2008; Leiby, 2011). Severe cases featuring acute anemia, thrombocytopenia, organ failure, or even death may occur among the elderly, splenectomized, and immunocompromised individuals (Krause et al., 2008). Limited studies in animal models suggest a higher virulence of *B. duncani* compared with *B. microti* (Wozniak et al., 1996); however, a correlation between the experimental animal data and human clinical cases remains unclear.

Microscopic examination of blood smears stained with Giemsa has been the gold standard test for the detection of *Babesia* infection for many years. This method has limitations since it requires well trained personnel due to the fact that *Babesia* displays morphological similarities with malaria parasites (Leiby, 2011). In addition, microscopic diagnosis is difficult in patients with very low parasitemia during early or chronic stages of infection. Serological methods such as immunofluorescent antibody test and enzyme-linked immunosorbent assay have been developed for detection of *B. microti* infection (Homer et al., 2003; Krause et al., 1994; Luo et al., 2011). As molecular assays have become more commonplace in the diagnosis of parasitic infections, both conventional and real-time polymerase chain reaction (PCR) have been developed for detection of *B. microti* in human samples (Bloch et al., 2013; Chan et al., 2013; Persing et al., 1992; Rollend et al., 2013; Teal et al., 2012). Of note, highly specific and sensitive molecular assays that detect and distinguish *B. microti* from *B. duncani* infections are lacking.

Droplet digital PCR (ddPCR) is a growing technology of nucleic acid detection and quantitation. This method facilitates absolute quantitation of DNA targets without the requirement of standard curves commonly used in real-time PCR (Vogelstein and Kinzler, 1999). In ddPCR, the amplification reaction containing the DNA sample, fluorescently-labeled probe, and components is divided into thousands of microscopic reaction droplets, with each containing one or less copies of the target DNA (Hindson et al., 2011; Nakano et al., 2003; Pinheiro et al., 2012). Following amplification, the measurement of both fluorescent (i.e., positive) and non-fluorescent (i.e., negative) droplets is performed. The number of target DNA molecules present in the sample can be calculated from the fraction of positive reactions using Poisson statistics (Hindson et al., 2011). Uses of ddPCR include measurement of germline DNA copy number variation (Pinheiro et al., 2012), gene expression in single cells (Heredia et al., 2013), detection of genetically modified organisms in food (Morisset et al., 2013), and, more recently, quantitation of bacterial (Roberts et al., 2013) and viral (Hayden et al., 2013; Henrich et al., 2012; White et al., 2012) pathogens in human samples. Applications of the technology in parasitic diseases are lacking to the best of our knowledge.

We report the development of ddPCR assays for the detection and discrimination of *B. microti* and *B. duncani* in experimentally infected hamster blood samples. We targeted the ITS regions of nuclear ribosomal RNA given the divergence in sequence variation among closely related *Babesia* species (Blaschitz et al., 2008; Bostrom et al., 2008; Liu et al., 2008; Niu et al., 2009). Quantitative data of these assays were compared with real-time PCR assays using identical primers and probes. The assays could be used as highly specific and sensitive molecular tests for blood screening of human samples.

## 2. Materials and methods

### 2.1. Babesia *isolates*

*Babesia microti* GI (BEI Resources NR-44070; ATCC® PRA-398™) and *B. duncani* WA-1 (BEI Resources NR-12311; ATCC® PRA-302™) were used in this study. Isolates were propagated in Golden Syrian hamsters (Harlan Laboratories, stock: HsdHan:AURA) according to published protocols (Cullen and Levine, 1987; Oz and Hughes, 1996; Wozniak et al., 1996). Blood was drawn at days 3, 7, and 9 or 10 of infection by peri-orbital route. Parasitemia was determined by microscopic examination of blood films stained with Giemsa. On each sample, a minimum of 500 red blood cells (RBCs) were counted to calculate the percent parasitemia. This included all parasitized RBCs regardless of intraerythrocytic stage or number of parasites per cell. Animals were maintained according to protocols approved by the ATCC® IACUC.

### 2.2. *DNA extraction*

For DNA extraction, 250 µl of blood were collected in heparin and treated with 750 µl of TSE/Triton X-100 buffer (20 mM Tris–HCl pH 7.5, 48 mM EDTA, 100 mM NaCl, 0.2% Triton X-100) for 5 min to lyse the erythrocytes. Subsequent steps were followed according to the Gentra® Puregene® Blood Kit (Qiagen) with the addition of 20 µl of proteinase K to the cell lysis buffer and incubation for 2 h. DNA was dissolved in 100 µl of rehydration buffer in the last step of the protocol and frozen at −20 °C.

### 2.3. *PCR and sequencing*

The entire ITS1, 5.8S rRNA, and ITS2 regions of *B. microti* GI and *B. duncani* WA-1 were amplified as 930–950 bp PCR products using forward primer BABITS-F that targeted the 3′ region of the 18S rRNA gene and reverse primer BABITS-R that targeted the 5′ region of the 28S rRNA (Table 1 and Fig. 1). These primers were also used to sequence the ITS amplicons using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the ABI 3500xL Genetic Analyzer (Applied Biosystems). ITS1, 5.8S rRNA, and ITS2 sequences from *B. microti* GI and *B. duncani* WA-1 were aligned using ClustalW. Sequences in the ITS1 regions that were distinct between each species (Fig. 1B) were selected as targets for real-time PCR and ddPCR assays using the primers and probes listed in Table 1. As positive controls, plasmids were constructed that contained the 930–950 bp ITS1/5.8S