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

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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.

Keywords

Babesia; Babesiosis; Digital PCR; Real time PCR; Blood infection; Molecular method; Detection; Quantitation

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 (Hunfeldt 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 enzymelinked 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, 48mMEDTA, 100mMNaCl, 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 ITS1/5.8SrRNA/ITS2 amplicons of *B. microti* GI and *B. duncani* WA-1 inserted in pUC19. Plasmid concentrations were determined by the Quant-iT[™] PicoGreen[®] assay (Life Technologies) and their equivalences to copies per μ l were calculated assuming a molar mass of dsDNA per base pair equal to 660 g per mole per base pair.

2.4. Real-time PCR

Real-time PCR assays were performed in a Bio-Rad CFX96[™] System using 25 μ l reaction mixtures containing 1 μ M of each primer, 250 nM of probe, 1 $\times$ Bio-Rad SsoFast[™] Probe Supermix, and 5 μ l of DNA. Cycling conditions for *B. microti* were 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s and 60 °C for 10 s. Cycling conditions for *B. duncani* were 95 °C for 30 s followed by 40 cycles of 95 °C for 10 s and 68 °C for 10 s. Standard curves were generated by using serial dilutions of linearized plasmids containing the ITS1/5.8S rRNA/ITS2 regions of *B. microti* and *B. duncani*. DNA samples and standards were tested in triplicate. The relative fluorescence unit (RFU) baseline threshold was auto-calculated using the Bio-Rad CFX Manager[™]3.0 Software. Gene copy numbers of *Babesia* DNA samples were determined by extrapolating the quantification cycle (C_q) values with those from the standard curves.

2.5. ddPCR

Detection and quantitation of *Babesia* DNA by ddPCR was performed using the same primers and probes used in real-time PCR assays. ddPCR assays were carried out in 20 μ l reaction mixtures containing Bio-Rad 1 $\times$ ddPCR Master Mix, 1.25 μ M of each primer, 312 nM of probe, and 5 μ l of DNA. Reactions were loaded into eight-well cartridges and droplets were generated using the Bio-Rad QX100[™] Droplet Generator. Droplets containing the PCR mixtures were transferred to a single PCR plate for amplification according to recommendations from Bio-Rad but using annealing temperatures of 60 °C for *B. microti* DNA and 63 °C for *B. duncani* DNA. Samples were subsequently analyzed with the Bio-Rad QX100[™] Droplet Reader. The absolute limit of quantification and absolute limit of detection were determined for each molecular standard on five replicates. DNA samples from *Babesia*-infected hamsters were tested in triplicate. Samples measuring above the upper limit of detection by ddPCR ($>10^5$ copies per reaction) were diluted and retested. Specificities of real-time and ddPCR assays were examined using template DNA from other vector-borne pathogens (Table 4).

2.6. Statistical analysis

Linear regression analyses of standard curves from real-time PCR were performed with the GraphPad Prism 6.0 software. For ddPCR, positive droplets were discriminated from

negative droplets by applying a fluorescence amplitude threshold with the QuantaSoft™ analysis software 1.3.2.0 (Bio-Rad). After being exported, the ddPCR data were further analyzed in Microsoft Excel to calculate means and standard deviation measurements.

3. Results

3.1. Sensitivities of *Babesia* real-time PCR and ddPCR assays

Sensitivities of real-time PCR and ddPCR assays were determined by performing dilution series of positive control plasmids, hereafter referred to as standards, which contained the 930–950 bp ITS1/5.8S rRNA/ITS2 amplicons from *B. microti* and *B. duncani*. For real-time PCR, cycle thresholds were calculated for each dilution in triplicate. As shown in Table 2, the sensitivities of detection of *Babesia* ITS1 DNA by real-time PCR were approximately 10 copies for both *B. microti* and *B. duncani*. Plotting the calibration curves based on dilution series of the standards and C_q values resulted in slopes 3.44 and R^2 values of 0.997 for both assays (Fig. 2). Calculated efficiencies were 91.2% and 95.1% for *B. microti* and *B. duncani*, respectively.

Copy numbers detected by ddPCR of serial dilutions of standards are shown in Table 3. ddPCR detected fewer *B. microti* and *B. duncani* ITS1 DNA copies than expected by the calculated standard copy number. However, the sensitivity of ddPCR to detect *B. microti* or *B. duncani* ITS1 DNA was similar to that of real time-PCR as both assays obtained positive signals down to 10 copies of diluted standard. A visual representation of positive and negative ddPCR droplet reactions for diluted *Babesia* DNA standards with minimum threshold for droplet positivity is shown in Fig. 3.

3.2. Comparison of real-time PCR and ddPCR using experimentally-infected hamsters

We expanded the utility of these assays to estimate gene copy number in blood samples from experimentally-infected hamsters. Figures 4 and 5, and Appendix: Supplementary Table S1 show the correlations between copy numbers determined by real-time PCR and ddPCR from two hamsters infected with *B. microti* (Fig. 4A) and two hamsters infected with *B. duncani* (Fig. 5A). As shown in Fig. 4A, ddPCR was found to be more sensitive than real-time PCR in the detection of *B. microti* ITS1 DNA at 3 days p.i. in hamsters 1 and 2. Both hamsters were negative for parasitemia by microscopy at this time point (Fig. 4B, 3 days p.i.). At 7 days p.i., increases in copy numbers of *B. microti* ITS1 DNA were detected by both assays in hamsters 1 and 2 (Fig. 4A) as parasitemia became detectable by microscopy (Fig. 4B). Levels of *B. microti* ITS1 DNA at 10 days p.i. were only examined in hamster 2 and showed a continued increase to approximately 9×10^3 and 1.9×10^4 copies per reaction as measured by real-time PCR and ddPCR, respectively (Fig. 4A and Appendix: Supplementary Table S1). In general, measurements of copy numbers at the later time points were two- to fourfold higher by ddPCR compared with real-time PCR (Appendix: Supplementary Table S1). Notably, a correlation between parasite burden determined by microscopy (Fig. 4B) and elevated DNA copy numbers measured by the PCR assays (Fig. 4A) was not observed.

Infection of hamsters with *B. duncani* also resulted in undetectable parasitemia by microscopy at 3 days p.i. (Fig. 5B and Appendix: Supplementary Table S1). However, *B. duncani* ITS1 DNA at this time point was detected by both real-time PCR and ddPCR in hamster 4 (Fig. 5A, 3 days p.i.). As parasitemia became detectable in both hamsters at 7 days p.i. (Fig. 5B), an increase in *B. duncani* ITS1 DNA levels measured by real-time PCR and ddPCR was also observed (Fig. 5A). Subsequent blood samples were examined at day 10 p.i. for hamster 3 and day 9 p.i. for hamster 4. As parasitemia by microscopy reached 3% in hamster 3 (Fig. 5B), levels of *B. duncani* ITS1 DNA measured by real-time PCR and ddPCR continued to increase to approximately 2.2×10^5 and 4.8×10^5 copies per reaction, respectively (Fig. 5A, 10 days p.i. and Appendix: Supplementary Table S1). At 9 days p.i. and a parasitemia of 5%, *B. duncani* DNA levels in hamster 4 were approximately 1×10^6 copies per reaction by real-time PCR and 4.8×10^6 copies per reaction by ddPCR (Fig. 5A and Appendix: Supplementary Table S1). Similar to the *B. microti* assays, ddPCR detected two- to fourfold higher copy numbers of *B. duncani* ITS1 DNA compared with real-time PCR in infected animals. In addition, the extent of parasitemia of *B. duncani* determined by microscopy correlated minimally with DNA copy number measurements obtained with the PCR assays.

3.3. Specificities of Babesia real-time PCR and ddPCR assays

Specificities of the real-time and ddPCR assays were tested with DNA from five additional vector-borne pathogens: *Plasmodium knowlesi*, *Plasmodium falciparum*, *Rickettsia prowazekii*, *Borrelia burgdorferi*, and *Trypanosoma cruzi* (Table 4). Fifteen nanograms of DNA from each organism were used for specificity testing. None of the organisms examined showed cross-reactivity with the Babesia assays. In addition, DNA from *B. microti* (1×10^7 copies of standard) did not cross-react with the *B. duncani* assays and vice versa. DNA samples from *B. microti* strains Peabody (ATCC® PRA-99™) and Nan-Hs-2011 (BEI NR-44071; ATCC® PRA-399™) tested positive in our *B. microti* ITS1 real-time and ddPCR assays (data not shown). Samples from other *Babesia* spp. were not available to us at the time of this study.

4. Discussion

Babesiosis was first recognized as a cattle disease of significant economic impact with initial reports of human infections originating in the 1950s. Human babesiosis has been considered an emerging disease in the U.S. in the last 20 years (Hunfeld et al., 2008). A number of factors, such as increased medical awareness, changes in the parasite's vector ecology, housing development in the countryside, and a growing population of immunocompromised individuals have likely contributed to this emergence. Of note, there has been an increase in Babesia infections transmitted by blood transfusion concurrent with the emergence of human babesiosis in recent years (Bloch et al., 2012; Johnson et al., 2009; Leiby, 2011; Lobo et al., 2013). Current practices used in the prevention of transfusion-transmitted babesiosis include completing a donor questionnaire to identify potential infected individuals (Leiby, 2011; Lobo et al., 2013). Such an approach has many disadvantages as infected blood donors may be asymptomatic or harbor the parasite after symptoms have resolved. The practice may also inadvertently exclude suitable blood donors. Thus, an assay

based on serology and/or nucleic acid testing and approved by the FDA will be of great use in blood screening of *Babesia*.

In the present study, we developed ddPCR assays based on the ITS regions of *B. microti* and *B. duncani* that were both sensitive and species-specific. Sensitivities of the assays were comparable with real-time PCR when using the same primers and probes in the amplification of plasmid controls harboring the *Babesia* ITS sequences. Of note, ddPCR detected fewer DNA copies than the expected standards (Table 3). This may be explained by the fact that the input copy numbers of the standards were low overall. Copy numbers of the standards are calculated prior to amplification and are derived from measuring the number of grams per molecule of the plasmid DNA with a known molecular mass. Discrepancies between the calculated copy numbers of standards and actual detection by ddPCR could be dependent on variations in the measurement of DNA concentration and dilutions of the standards. 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. A limitation of ddPCR as compared with RTPCR is the need to dilute samples with more than 100,000 copies of the target DNA. Overloading the assay may affect the efficient partitioning of standards into reaction droplets which may in turn result in a loss of linearity at high copy numbers.

The ddPCR assays were useful in the detection of parasitemia in hamsters infected with *B. microti* and *B. duncani*. Contrary to real-time PCR, the ddPCR assay for *B. microti* detected parasite infection in 3 day-infected hamsters which were negative by microscopy. Both assays improved detection of parasitemia after 3 days of infection in one of the hamsters infected with *B. duncani* (Hamster 4 in Fig. 5 and Appendix: Supplementary Table S1). As levels of parasitemia observed by microscopy increased over time in response to *Babesia* infection, a concomitant increase in copy numbers was detected by the real-time and ddPCR assays. Overall, ddPCR detected two- to fourfold more copy numbers of *B. microti* and *B. duncani* ITS DNA compared with real-time PCR. In accordance to previous reports (Wozniak et al., 1996), infection of hamsters with *B. duncani* resulted in higher levels of parasitemia compared with *B. microti*, suggestive of a higher virulence potential of the former compared with the latter in animal models. Lastly, the real-time and ddPCR assays were highly specific to *B. microti* and *B. duncani* when compared with five additional vector-borne pathogens. Specificity testing against other members of the genus *Babesia* is pending availability of DNA from these strains.

Importantly, a clear correlation between parasite burden determined by microscopy and DNA copy numbers measured by both PCR assays was not observed in this report. A limitation of our study is that microscopic counts do not take into account RBCs infected with multiple parasites or cells harboring dividing merozoites which may affect the overall DNA copy number detection by the PCR assays. In addition, real-time PCR and ddPCR may detect DNA from extracellular parasites or even free DNA circulating in the blood. These scenarios and the possibility of nuclear-encoded ribosomal RNA genes occurring as multiple copies in the genomes of *B. microti* and *B. duncani* could explain the absence of correlation with the microscopy data and even the differences in DNA copy numbers observed between animals. Future research using a larger number of animal subjects accompanied by a more

accurate determination of absolute parasitemia expressed as parasites/ μ l of blood may help determine better trends between parasite burden and copy numbers detected by the molecular PCR assays. Moreover, the addition of an internal amplification control to the reaction mixture targeted by primers unrelated to *Babesia* may be required to rule out the presence of PCR inhibitors in the DNA samples.

Recently developed quantitative PCR-based methods have shown promise in the diagnosis of *Babesia* infections in humans (Bloch et al., 2013; Chan et al., 2013; Rollend et al., 2013; Teal et al., 2012). However, many of these methods require further validation prior to becoming standardized assays for blood screening. As opposed to microscopy which can be time consuming and requires proficiency in the detection of parasite morphology, quantitative PCR assays can be useful in high throughput screening, particularly in acute cases of infection when levels of parasitemia are high (Leiby, 2011). On the other hand, such assays including ddPCR may be limited in detecting babesiosis during chronic stages of infection as levels of parasitemia become negligible over time (Krause et al., 1998). The addition of serological tests at this stage to detect increased IgG antibody titers may be necessary to rule out false negative PCR results in chronically infected individuals.

In summary, droplet digital PCR is a novel technology that offers increased sensitivity in the detection and quantitation of nucleic acids from different infectious agents (Hayden et al., 2013; Henrich et al., 2012; Roberts et al., 2013; White et al., 2012). As opposed to other quantitative nucleic acid detection tests, this method does not require the use of molecular standards and provides the absolute quantitation of DNA targets (Hindson et al., 2011; Vogelstein and Kinzler, 1999). We determined the application of ddPCR for protozoan parasites by developing specific tests that are applicable for monitoring parasitemia of *B. microti* and *B. duncani* in rodent models of infection. The assays were generally comparable with traditional real-time PCR methods for the detection of *Babesia* ITS DNA. As digital PCR is adaptable to multiplexing (Zhong et al., 2011), a duplex ddPCR assay is foreseeable to simultaneously detect *B. microti* and *B. duncani* using different fluorophores combined with the probes and primers described in this study. The ability to differentiate between both *Babesia* species may be useful in epidemiological studies in the USA, particularly for *B. duncani*, to determine the extent of the parasite's geographic distribution outside the Pacific Coast region (Prince et al., 2010). Pending further validation, the ddPCR methods described herein could be readily adapted for use in the diagnosis of *Babesia* infections in human specimens and screening of blood bank supplies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.exppara.2014.12.003.

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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.

Fig. 1. Diagnostic regions targeted by real-time and ddPCR assays for *B. microti* and *B. duncani*. (A) Representation of eukaryotic nuclear ribosomal RNA. The entire ITS1, 5.8S rRNA, and ITS2 regions of *B. microti* GI and *B. duncani* WA-1 were amplified using primers BABITS-F and BABITS-R. (B) Amplicons were sequenced and aligned using ClustalW. Regions in ITS1 that were unique for each species were selected as targets for real-time PCR and ddPCR assays. Regions shown in red in the *B. microti* sequence indicate binding sites for forward primer BmITS1-F, reverse primer BmITS1-R2_mb, and FAM-labeled probe BmITS1-P. Regions shown in green in the *B. duncani* sequence indicate binding sites for forward primer BdITS1-F, reverse primer BdITS1-R, and FAM-labeled probe BdITS1-P. Asterisks indicate identical nucleotides between sequences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Calibration curves of real-time PCR assays run with standards harboring ITS1/5.8S rRNA/ITS2 regions of *B. microti* (left panel) and *B. duncani* (right panel). The data correspond to a representative experiment of three performed.

Fig. 3.

A visual representation of positive and negative ddPCR droplet reactions for diluted *Babesia* DNA standards of *B. microti* (left panel) and *B. duncani* (right panel). Minimum threshold for droplet positivity is shown with a pink line. Positive droplets in blue and negative droplets in black are shown for the six diluted linear DNA standards (10⁵ to 1) and water as a negative control (H₂O).

Fig. 4.

Quantification of *Babesia microti* ITS1 DNA in infected hamsters by real-time PCR (RT-PCR) and ddPCR. (A) DNA copy numbers measured by RT-PCR (black bars) and ddPCR (gray bars) were transformed to log₁₀ values. Non-transformed copy numbers are shown in Appendix: Supplementary Table S1. Data represent the means $\pm$ standard deviations of each sample tested in triplicate. Samples that were undetectable by RT-PCR at 3 days p.i. are not shown. (B) Parasitemia of *B. microti* infected hamsters determined by microscopy.

Fig. 5.

Quantification of *Babesia duncani* ITS1 DNA in infected hamsters by real-time PCR (RT-PCR) and ddPCR. (A) DNA copy numbers measured by RT-PCR (black bars) and ddPCR (gray bars) were transformed to $\log_{10}$ values. Non-transformed copy numbers are shown in Appendix: Supplementary Table S1. Data represent the means $\pm$ standard deviations of each sample tested in triplicate. Samples that were undetectable by both assays at 3 days p.i. in

hamster 3 are not shown. (B) Parasitemia of *B. duncani* -infected hamsters determined by microscopy.

Table 1

Primers and probes used in this study.

Designation	Target	Sequence (5'–3')
Primer ^a		
BABITS-F	3' region of the <i>B. microti</i> or <i>B. duncani</i> 18S rRNA gene	GGTGAACCTGCRGAAGGATC
BABITS-R	5' region of the <i>B. microti</i> or <i>B. duncani</i> 28S rRNA gene	TCTKCCGCTTARTTATATGC
BmITS1-F	<i>B. microti</i> ITS1	TCCCATTGCGGTACGCTGG
BmITS1-R2_mb	<i>B. microti</i> ITS1	CGTGCAGACAAACCCGCCTT
BdITS1-F	<i>B. duncani</i> ITS1	GCTTCCTAACCCGAGACCAA
BdITS1-R	<i>B. duncani</i> ITS1	CACTGGCGGGGTGAAAAGTA
Probe ^b		
BmITS1-P	<i>B. microti</i> ITS1	AGTGGCCTTGACGTAGCGGC
BdITS1-P	<i>B. duncani</i> ITS1	TACGGCCCTGATGTCGGGG

^aExpected amplicon sizes are as follows: BABITS-F/BABITS-R: 930–950 bp; BmITS1-F/BmITS1-R2_mb: 142 bp; BdITS1-F/BdITS1-R: 125 bp.

^bProbes were dye-labeled with FAMTM. PCR Primers were purchased from IDT. Probes were purchased from Biosearch Technologies.

Table 2

Real-time PCR detection and quantitation of Babesia DNA standards.

<i>B. microti</i> standard ^a	<i>C_q</i>		<i>B. duncani</i> standard ^a	<i>C_q</i>	
	Mean	SD		Mean	SD
1 × 10 ⁸	12.85	0.155	1 × 10 ⁸	10.90	0.194
1 × 10 ⁷	16.36	0.059	1 × 10 ⁷	14.15	0.257
1 × 10 ⁶	19.82	0.075	1 × 10 ⁶	17.39	0.058
1 × 10 ⁵	23.44	0.042	1 × 10 ⁵	21.06	0.202
1 × 10 ⁴	27.03	0.049	1 × 10 ⁴	24.78	0.112
1 × 10 ³	30.54	0.184	1 × 10 ³	28.53	0.414
100	34.24	0.529	100	31.71	0.127
10	37.60	0.408	10	34.13	1.057
1	0.0	0.0	1	0.0	0.0
H ₂ O	0.0	0.0	H ₂ O	0.0	0.0

^aValues reflect copy numbers per reaction based on serial dilutions of plasmid DNA standard by DNA concentration and known molecular mass. Data represent the means ± standard deviations of each dilution tested in triplicate from a representative experiment of three performed.

Table 3

ddPCR detection and quantitation of Babesia DNA standards.

<i>B. microti</i> standard ^a	ddPCR value ^b		<i>B. duncani</i> standard ^a	ddPCR value ^b	
	Mean	SD		Mean	SD
1 × 10 ⁵	38,200	721	1 × 10 ⁵	51,733	1,858
1 × 10 ⁴	3,393	170	1 × 10 ⁴	4,827	50
1 × 10 ³	344	26.5	1 × 10 ³	414	31.4
100	38.5	3.8	100	44.3	3.4
10	1.2	0	10	7.5	1.1
1	0	0	1	0	0
H ₂ O	0	0	H ₂ O	0	0

^aValues reflect copy numbers added per reaction based on serial dilutions of plasmid DNA standard by DNA concentration and known molecular mass.

^bCopies per reaction detected. Data represent the means ± standard deviations of each dilution tested as five replicates from a representative experiment of three performed.

Table 4
Exclusivity panel organisms tested against Babesia real-time PCR and ddPCR assays.

Organism/strain ^a	Catalog no.	<i>B. microti</i>		<i>B. duncani</i>	
		RT-PCR	ddPCR	RT-PCR	ddPCR
<i>Plasmodium knowlesi</i> H strain	BEI MRA-456G	Neg	Neg	Neg	Neg
<i>Plasmodium falciparum</i> 7C46	BEI MRA-172G	Neg	Neg	Neg	Neg
<i>Rickettsia prowazekii</i> BREINL	BEI DD-248	Neg	Neg	Neg	Neg
<i>Borrelia burgdorferi</i> B31	ATCC 35210D-5	Neg	Neg	Neg	Neg
<i>Trypanosoma cruzi</i> SYLVIO X-10	ATCC 50823D	Neg	Neg	Neg	Neg
<i>Babesia microti</i> GI	BEI NR-44070	ND	ND	Neg	Neg
<i>Babesia duncani</i> WA-1	BEI NR-12311	Neg	Neg	ND	ND

^a *B. microti* and *B. duncani* were used at 1×10^7 copies of standard per reaction. The remaining DNA samples were used at 15 ng per reaction. Neg, negative; ND, not done.