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AN IMPROVED PCR PROTOCOL FOR DETECTION OF *BABESIA DUNCANI* IN WILDLIFE AND VECTOR SAMPLES

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ABSTRACT: Human babesiosis is a tick-borne protozoal disease of increasing clinical significance in North America. Most cases in the eastern and Midwestern regions of the United States are reportedly due to *Babesia microti* infections. By contrast, most human infections reported in California and Washington have been attributed to a new species that was first identified in 1991 and subsequently named *Babesia duncani*. Although the tick vector and mammalian reservoir hosts for *B. microti* are well characterized, the vector and reservoir hosts for *B. duncani* are unknown. As a result, specific risk factors for human infections cannot be characterized. Identification of potential hosts and vector species has been hampered by the lack of specific and sensitive molecular diagnostic tools to amplify parasite DNA. To address this need, a nested PCR assay targeting the *β-tubulin* gene, a well-conserved locus in piroplasm parasites with a highly variable intron region among species, was developed. The assay was evaluated by spiking tick and mammalian DNA extracts with DNA from a *B. duncani* isolate derived from a human patient (WA-1) as well as related *Babesia* spp. from Californian wildlife. This assay was highly specific, with a sensitivity of approximately 1 copy of template DNA in a background of tick DNA. At this level of detection *B. duncani* was detectable in larval tick samples, and the target locus allowed for visual differentiation between species by gel electrophoresis. This assay offers researchers a new tool for elucidating the natural transmission cycle of *B. duncani*.

Babesiosis occurs globally, causing clinical disease in domestic animals and humans (Kjemtrup and Conrad, 2000; Ramos et al., 2010; He et al., 2011; Schnittger et al., 2012). Symptoms and signs in humans include shortness of breath, fatigue, fever, anemia, and thrombocytopenia (Centers for Disease Control [CDC], 2012; Schnittger et al., 2012; Kletsova et al., 2017). The causative agent, a group of intraerythrocytic parasitic eukaryotes belonging to the genus *Babesia*, is widespread and genetically diverse. The pathogens are vectored by a variety of ixodid (hard) tick species but can be transmitted through blood transfusion (Kjemtrup et al., 2002; Herwaldt et al., 2011; Bloch et al., 2012) and vertically from mother to child (Joseph et al., 2012). Human babesiosis in North America is primarily caused by *Babesia microti*, a well-characterized piroplasm with small-mammal reservoirs and a known tick vector, *Ixodes scapularis* (Lack et al., 2012; Hersh et al., 2014). Less commonly reported or well characterized are cases caused by *Babesia duncani*, which to date have only been identified in cases on the west coast of the United States (Quick et al., 1993; Kjemtrup and Conrad, 2000; Conrad et al., 2006). The vector and reservoir for *B. duncani* are unknown, though *B. duncani* has been isolated from bighorn sheep (*Ovis canadensis*), mule deer (*Odocoileus hemionus*), and fallow deer (*Dama dama*) in California (Thomford et al., 1993). The lack of a sufficiently specific and sensitive test for screening tick or vertebrate samples for *B. duncani* DNA has impeded efforts to understand the enzootic cycle of this pathogen.

PCR assays for *Babesia* spp. generally target the *18S rRNA* gene or 1 or both of the internal transcribed spacer (ITS) regions (Persing et al., 1992; He et al., 2011; Kim et al., 2013; Hersh et al., 2014; Wilson et al., 2015). A PCR protocol targeting the *18S rRNA* gene was used previously to sequence WA-1 and other *B. duncani* isolates from various wild ungulate and human samples (Conrad et al., 2006). More recently, *18S rRNA*-based assays have been used to screen *I. scapularis*, the black-legged tick, for *B. microti* (Kim et al., 2013; Hersh et al., 2014). However, primers targeting *18S rRNA* that were developed for screening blood samples may not be sensitive enough to be used for tick screening, and the targeted sequence is of similar length in all species, requiring sequence analysis to determine the specific infectious organism.

Portions of the *β-tubulin* gene that span part of exon1-intron1-exon2 are diagnostic for a variety of *Babesia* and *Theileria* spp. using restriction digest and a nested PCR protocol (Cacciò et al., 2000). However, the nested primers described in the Cacciò protocol provide neither reliable positives nor strain identification. In the present study, the original external primers described by Cacciò et al. (2000) were used to sequence the *β-tubulin* gene partially for 3 strains of *B. duncani* to design new internal primers for a nested PCR protocol that is specific for *B. duncani* and does not amplify host DNA. This new protocol provides a clear molecular weight distinction between *B. duncani* and *Babesia odocoilei*, a parasite common in mule deer, 1 of the suspected reservoir hosts (Yabsley et al., 2005). In addition, the assay allows for the determination of pathogen strains by Sanger sequencing but does not require sequence analysis to differentiate between common *Babesia*/*Theileria* spp. in California.

MATERIALS AND METHODS

Previously characterized parasite-infected whole blood frozen at −80 C was used as the source of *Babesia* DNA as described below for BH3 and FD1 (*Babesia* spp. from bighorn sheep and fallow deer, respectively (Thomford et al., 1993; Kjemtrup et al., 2000). For WA1, procedures for isolation of *B. duncani* from human sources have been previously described (Quick et al., 1993; Kjemtrup et al., 2000, 2002). For this study, merozoite-infected

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TABLE I. *Babesia duncani* sequence accession numbers.

Sample name	Isolation source	GenBank accession numbers
WA1	Quick et al. (1993)	MF978358
BH3	Thomford et al. (1993)	MF978360
FD1	Thomford et al. (1993)	MF978361

hamster red blood cells that were stored frozen at –80 C were used as the *Babesia* DNA source (Kjemtrup et al., 2002).

DNA Extraction

Genomic DNA was extracted from 20 µl of *B. duncani*–infected hamster blood with the use of the DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland) according to manufacturer instructions. DNA was eluted in 100 µl elution buffer and stored at 4 C. Samples were confirmed to be isolates WA1, BH3, and FD1 (see Conrad et al., 2006 for isolate descriptions) with an initial CRYPTO *18S rRNA*-based PCR assay and sequencing.

Nested PCR assay

Nested PCR primers: Each sample was amplified and sequenced with the initial primers F34 and R323 (Cacciò et al, 2000) on an AB3100. The resulting sequences (see Table I) were aligned in Geneious (version 7.1.9; Kearse et al., 2012) and forward (Btub_F_N2) and reverse (Btub_R_N2) internal primers were designed with the use of the Primer3 extension (Table II). These primers specifically target the full first intron of *Babesia* spp. and fall 24 base pairs (bp) before the intron and 116 bp after the intron.

Reaction conditions: The initial PCR reaction was prepared in a total volume of 25 µl, consisting of 4 µl DNA template, 400 nM of each primer, 0.4 nM TaKaRa dNTPs, and 0.625 U TaKaRa taq polymerase (TaKaRa Bio Inc., San Francisco, California). Thermocycling conditions were 2 min at 94 C, then 35 cycles of 30 sec at 94 C, 30 sec at 57 C, and 45 sec at 72 C, with a final extension at 72 C of 5 min. The nested PCR reaction was also prepared in a total volume of 25 µl, consisting of 2 µl PCR product, 400 nM of each primer, 0.4 nM TaKaRa dNTPs, and 0.625 U taq. Nested cycling conditions consisted of 2 min at 94 C, then 40 cycles of 10 sec at 98 C, 30 sec at 63 C, and 45 sec at 72 C, with a final extension at 72 C for 5 min. Negative controls of genomic DNA from uninfected tick, genomic DNA extracted from uninfected hamster whole blood, and PCR H_2O were included.

Sensitivity and specificity: To generate standard concentrations for calculating assay sensitivity, the full *β-tubulin* target sequence was cloned with the use of a TopoClone kit (Invitrogen, Carlsbad, California), and the plasmids were purified with the use of a Miniprep kit (Qiagen, Germantown, Maryland). Purified plasmids were quantified with the use of a Qubit (Invitrogen, Carlsbad, California). Qubit-quantified template DNA at 3.4 ng/µl was subjected to a 10-fold serial dilution and assayed with the nested PCR protocol, with a dilution of the extracted genomic DNA used as a positive control and PCR H_2O as negative control. Final sensitivity tests ranged from 1.02×10^{1} to 1.02×10^{-10} ng of template in the initial PCR reaction. All samples were screened in triplicate.

Purified plasmid was spiked into nymphal tick (*Ixodes pacificus*) and dusky-footed woodrat (*Neotoma fuscipes*) DNA to test for specificity and sensitivity of detection against potential inhibition. Purified plasmid was spiked into tick genomic DNA and serially diluted 10-fold. These dilutions were subject to PCR as described above to determine the detection limit of the assay. The same procedure was followed with woodrat DNA. For both spike-in assays, 10 dilutions were screened in triplicate. The range assayed in both tick and mammal background DNA was 6.8×10^{-1} to 6.8×10^{-10} ng of plasmid target in each PCR reaction.

Samples of *B. microti* and *B. odocoilei* were also assayed to screen for specificity of the nested PCR primers to *B. duncani*.

Sequencing: The sequencing reliability of the nested PCR internal primers was tested by Sanger sequencing on an AB3100. Purified PCR product from positive samples was cycle sequenced with the use of the internal forward (Btub_F_N2) and reverse (Btub_R_N2) primers. Sequences were aligned in Geneious and compared to the sequences generated by the external primers.

RESULTS

Sensitivity and specificity of *Babesia duncani* nested PCR assay

The lower limit of detection was defined for this assay as the last concentration at which all 3 replicates were positive. The nested PCR assay lower limit for amplification of *B. duncani* was as low as 1 template copy. The 3 *B. duncani* isolates used in the creation of this assay differ negligibly in intron length, with a final amplicon size of 175–181 bp (Fig. 1). *Babesia duncani* is distinguishable from other *Babesia* species including *B. odocoilei* and *B. microti* on a gel based on amplicon size of the internal *β-tubulin* target sequence (*B. odocoilei* target sequence length is approximately 450 bp, *B. microti* target sequence length is 160 bp, approximately 15 bp shorter than *B. duncani* and related *Babesia*). The internal primers we designed are not optimized for *B. microti*, and did not amplify the isolates we tested. However, even if amplified, *B. microti* would produce an amplicon of 160 bp and can be resolved and differentiated from *B. duncani* on a sufficiently dense, >2%, agarose gel (data not shown).

TABLE II. *β-tubulin* primers used in this study.

	Primer sequences	Source
Initial forward primer-F34	5′-TGTGGTAACCAGATYGGWGCCAA-3′	Cacció et al. (2000)
Initial reverse primer-R323	5′-TCNGTRTARTGNCCYTTRGCCCA-3′	Cacció et al. (2000)
Nested forward primer-BtubFn	5′-TCWGACGAGCACGGCATYGA-3′	Original
Nested forward primer-BtubRn	5′-CCAGGCTCCAARTCCATYAA-3′	Original

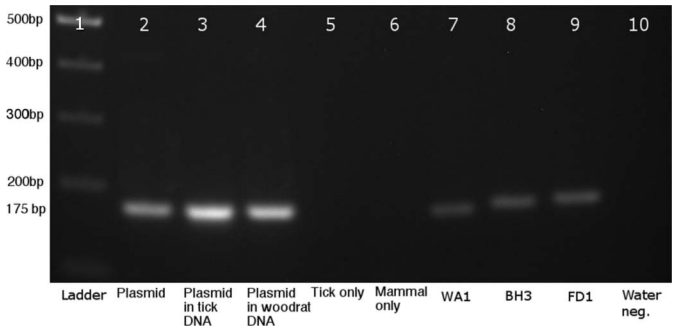

FIGURE 1. Gel electrophoresis of *β-tubulin* nested amplicon from *Babesia duncani* isolates on a 2% agarose gel. Lanes 2–4 show the amplification from 1.02×10^{-9} ng in H_2O, 6.8×10^{-9} ng in tick DNA, and 6.8×10^{-9} ng in woodrat DNA, respectively. Lanes 5 and 6 are negative controls against tick and mammal DNA. Lanes 7–9 are amplified directly from extractions of whole blood infected with *B. duncani* isolates. Lane 10 is the H_2O negative control.

PCR sensitivity: When plasmids containing the target sequence were spiked into tick genomic DNA, approximately 1 plasmid copy was reliably detectable in 100% of replicates (3/3). As few as 1 target plasmid was detected when diluted in woodrat genomic DNA in 100% of replicates (3/3).

Sequence analysis

The region targeted by the internal primers varied in sequence and length (intron length was 35–41 bp) between *B. duncani* isolates WA1, BH3, and FD1. These primers allowed for this region to be easily sequenced and compared to known isolates.

DISCUSSION

Babesiosis is a serious disease, particularly dangerous to the elderly, immunocompromised, and splenectomized. The lack of a sensitive and specific assay has seriously limited research on the enzootic cycle and risk of *B. duncani*. For most cases of human babesiosis in the United States molecular resolution is rarely undertaken. In 2011, only 38% of cases reported to the CDC had species-level data (CDC, 2012). The incidence of babesiosis caused by *B. duncani* relative to *B. microti* could be higher than what is currently recognized. In addition, the development of molecular diagnostic tools is important to identify the tick vector and reservoir species of *B. duncani*, as well as to facilitate investigations into the transmission cycle, spatial distribution, and infection risk of babesiosis.

This is the first report of a functional detection assay that is not only sensitive enough to amplify *B. duncani* from tick vectors, but also specific enough to avoid the amplification of host DNA. A major problem with other commonly used assays for detecting *Babesia* spp. has been the inability to identify species without sequencing or using probe-based protocols, which are costlier than a simple nested PCR. The variable length of the *β-tubulin* intron makes it a diagnostically valuable target but also limits the development of species-specific probes needed for other detection assays such as probe-based real-time qPCR. In order to determine species and strain of the parasite, a probe-based technique would have to be highly multiplexed. Techniques such as droplet digital PCR have been explored for differentiating between *B. duncani* and *B. microti* (Wilson et al., 2015), but have not been tested on tick samples and are not necessarily available to all researchers and laboratories. Conventional nested PCR protocols are potentially time-consuming and labor-intensive in comparison; however, qPCR would also require a secondary sequencing step after screening unless highly multiplexed to determine species with this genetic target. A major strength of this assay is the sensitivity of this assay as well as target specificity. The *β-tubulin* target of the nested PCR protocol we describe avoids co-amplification of host DNA, which may be an issue with universal *18S* primers alone (Shayan and Rahbari, 2005).

The *β-tubulin* gene is highly conserved among apicomplexans but specific enough not to risk false positives from the amplification of host DNA in a nested protocol. The intron sequence is variable and allows for species-level identification on a gel, as well as strain-specific identification by sequencing. Although Cacciò et al. (2000) previously developed a *Babesia* spp. *β-tubulin* protocol and demonstrated its usefulness as a diagnostic tool, their nested primers were not specific enough to detect *B. duncani* reliably, and were unable to produce high-quality sequencing reads. Our novel set of internal primers can detect isolates of *B. duncani* with a functional sensitivity of approximately 1 organism per PCR reaction in mammal and tick background, and can be used for strain identification without necessitating sequencing. This assay will help facilitate much-needed investigation into spatial distribution and prevalence of *B. duncani* in potential tick vectors and reservoir hosts.

Lack of an effective molecular diagnostic tool has hampered research into the ecology of *B. duncani*. The vector and reservoir(s) remain unclear. Although the seasonality of cases in humans with suspected tick-bite history and the large ungulate species from which the pathogen has been isolated present some clues, more information is needed to identify the vector species. Infection prevalence remains unknown, locally and generally, as does the true range of the pathogen. Without this information, disease risk cannot be determined or managed.

Babesia duncani appears to be in a distinct clade from *B. microti* and is more closely related to some *Theileria* species than other *Babesia* spp. (Conrad et al., 2006; Schnittger et al., 2012). This taxonomic relationship may help explain why *B. duncani* and *B. microti* do not appear to share ecological characteristics, and why common human disease vector species have not been found to transmit *B. duncani* (Kjemtrup, 2001). This PCR assay targets a useful gene locus that can be used in phylogenetic analysis, which may provide additional information on the relationship of piroplasmid parasites.

We describe a novel nested PCR assay that builds on 1 developed previously that provides a vital tool for investigation into the natural incidence and transmission of *B. duncani*. With this assay, we can focus epidemiological studies on regions where human cases have been reported or where isolates have been detected in potential pathogen reservoirs like *O. canadensis*, or *N. fuscipes*. In addition, based on the timing of human infections, ticks with phenologies that coincide with known transmissions can also be targeted. These efforts will be essential to understand this important emerging disease.

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