International Journal for Parasitology xxx (xxxx) xxx

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

# Evidence for transmission of the zoonotic apicomplexan parasite *Babesia duncani* by the tick *Dermacentor albipictus* ☆

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ARTICLE INFO

*Article history:*
Received 10 January 2018
Received in revised form 23 July 2018
Accepted 27 July 2018
Available online xxxx

*Keywords:*
Babesiosis
*Babesia duncani*
Vector-borne zoonotic disease
*Dermacentor albipictus*
*Odocoileus hemionus*

ABSTRACT

Babesiosis is a potentially fatal tick-borne zoonotic disease caused by a species complex of blood parasites that can infect a variety of vertebrates, particularly dogs, cattle, and humans. In the United States, human babesiosis is caused by two distinct parasites, *Babesia microti* and *Babesia duncani*. The enzootic cycle of *B. microti*, endemic in the northeastern and upper midwestern regions, has been well characterised. In the western United States, however, the natural reservoir host and tick vector have not been identified for *B. duncani*, greatly impeding efforts to understand and manage this zoonotic disease. Two and a half decades after *B. duncani* was first described in a human patient in Washington State, USA, we provide evidence that the enzootic tick vector is the winter tick, *Dermacentor albipictus*, and the reservoir host is likely the mule deer, *Odocoileus hemionus*. The broad, overlapping ranges of these two species covers a large portion of far-western North America, and is consistent with confirmed cases of *B. duncani* in the far-western United States.



## 1. Introduction

Vector-borne diseases are among the most significant emerging infectious diseases globally (Jones et al., 2008). In the United States, one of the most rapidly emerging vector-borne diseases and a leading cause of transfusion-transmitted infections is babesiosis, a malaria-like illness caused by intraerythrocytic *Babesia* spp. parasites. (Kjemtrup and Conrad, 2000; CDC, 2011, https://wwwn.cdc.gov/nndss/conditions/babesiosis/case-definition/2011/). Babesiosis affects domesticated animals, wildlife and humans. Human babesiosis is potentially fatal and its associated symptoms such as fever, chills, anaemia, fatigue and thrombocytopenia (Spielman et al., 1985; Telford et al., 1993; Uilenberg, 1995; Ramos et al., 2010) are especially severe in vulnerable patient groups such as the elderly, asplenic and immunocompromised (Kjemtrup and Conrad, 2000; Herwaldt et al., 2011; Kletsova et al., 2017). Infections in healthy patients may be asymptomatic and subclinical (Krause et al., 1998), therefore the true incidence and distribution of the disease is uncertain. This is especially true in the western United States where the enzootic cycle and ecology of the disease are undescribed.

In the northeastern United States, babesiosis is caused by *Babesia microti* (Spielman et al., 1985), a zoonotic parasite maintained by its rodent reservoir, the white-footed mouse, *Peromyscus leucopus* (Hersh et al., 2014), and transmitted by the blacklegged tick, *Ixodes scapularis* (Spielman et al., 1985; Hersh et al., 2014). Much less is known about babesiosis in the western United States, where the disease is caused by a genetically and pathogenically distinct parasite, *Babesia duncani* (Kjemtrup and Conrad, 2000; Conrad et al., 2006). *Babesia duncani* was first isolated in 1991 from a patient from Washington State, USA, and was referred to as WA1 (Thomford et al., 1994). To date, there have been 12 confirmed human cases and two presumed cases that preceded the description of *B. duncani* (Table 1) (Scholtens et al., 1968; Bredt et al.,

☆ *Note:* Nucleotide sequence data reported in this paper for *Babesia duncani* are available in GenBank under accession numbers MH348898, MH348897, MH333111, submission ID 2164780.

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https://doi.org/10.1016/j.ijpara.2018.07.002
0020-7519/

**Table 1**
Human cases of babesiosis with *Babesia duncani* presumed or identified to be the etiological agent. All localities and counties are from the United States.

| Date[a] | Case name | Location of potential exposure | Patient information | Risk factors | References |
|---|---|---|---|---|---|
| June 1966 | None (pre Bd [b]) | Marin Co., CA | Male | Exposure to natural areas | Scholtens et al.(1968) |
| Sept. 1979 | None (pre Bd [b]) | Central California | 36 yo male | Asplenic, hunter | Bredt et al. (1981) |
| Sept. 1991 | WA1 | South central Washington | 41 yo male | Exposure to natural areas | Quick et al. (1993) |
| Sept. 1991 | CA1 | Fort Ord, Monterey Co., CA | 22 yo male | Asplenic, exposure to natural areas, tick bite history 5 months prior to onset | Jerant and Arline (1993), Persing et al. (1995) |
| Aug. 1992 | CA2 | San Bernardino Mtns, CA or Sierra Nevada Mtns (Fresno Co., CA) | 31 yo male | Asplenic, exposure to natural areas, no tick bite history | Persing et al. (1995) |
| July 1993 | CA3[c] | Lytton Springs, Sonoma Co., CA | 36 yo male, fatal case | Asplenic, tick bite history | Persing et al. (1995) |
| Sept. 1993 | CA4 | Mono Co., CA | 41 yo male | Asplenic, hunter | Persing et al. (1995) |
| 1994 | WA2 | NA-blood transfusion recipient | 76 yo male | Megaloblastic anaemia, myelodysplasia, angina pectoris | Herwaldt et al. (1997) |
| 1994 | WA3 | King Co., WA | 34 yo male, blood donor to WA2 | None | Herwaldt et al. (1997) |
| 2000 | CA5 | NA-blood transfusion recipient | Infant | Premature | Herwaldt et al. (1997), Kjemtrup et al. (2002) |
| 2000 | CA6 | San Francisco Bay Area, CA or central Oregon | Asymptomatic, blood donor to CA5 | None | Herwaldt et al. (1997), Kjemtrup et al. (2002) |
| 2008 | BAB1615 | NA-blood transfusion recipient | 59 yo male | Anemic, chronic blood transfusion recipient | Bloch et al, (2012) |
| 2008 | BAB1615 "Donor A" | Travel throughout far-western U.S. including California and Washington states | 67 yo, blood donor to BAB1615 | Active outdoor enthusiast | Bloch et al. (2012) |
| July 2009 | OR1 | Oregon | 69 yo male | Splenectomised, exposure to natural areas | DeBess et al. (2012) |

Bd, *B. duncani*; yo, years old; NA, not available; Co., County.
[a] Month of initial presentation noted for those clinically affected cases with presumed tick bite exposure.
[b] Presumed *B. duncani* based on exposure, clinical presentation and blood smear analysis.
[c] Fatality.

1981; Quick et al., 1993; Persing et al., 1995; Herwaldt et al., 1997; Kjemtrup et al., 2002; Bloch et al., 2012;). All confirmed cases of *B. duncani* have been restricted to the Pacific coast, namely California, Oregon and Washington States. In rodent inoculation studies using hamsters, *B. duncani* causes significantly higher morbidity and mortality compared with *B. microti* (Dao and Eberhard, 1996; Wozniak et al., 1996). Most described human cases of *B. duncani* infection experienced serious disease and occurred in immunocompromised individuals, although blood donors identified in three blood transfusion cases were asymptomatic (Herwaldt et al., 1997; Kjemtrup et al., 2002; Bloch et al., 2012). In one of those cases, a premature infant became ill with *B. duncani* infection through a blood transfusion from an asymptomatic donor but survived with medical treatment (Kjemtrup et al., 2002; Conrad et al., 2006).

The association of other *Babesia* spp. with tick vectors and patient histories with tick bites suggested that *B. duncani* is transmitted via a tick bite, but the vector and reservoir species of this pathogen have not been identified. Prior xenodiagnostic studies examined the vector competency of *Ixodes pacificus*, *Dermacentor occidentalis* and *Dermacentor variabilis* for *B. duncani*. All three parasitic life stages of these tick species were fed on infected hamsters and then fed on uninfected hamsters to demonstrate their potential for acquiring and transmitting the parasite. These experiments found that these tick species failed to demonstrate parasite transmission and did not have sporozoites in their salivary glands, which would typically be expected for competent tick vectors (Karakashian et al., 1983; Kjemtrup et al., 2002). In search of the tick vector for the WA1-type *Babesia*, 34th Annual Conference of the Society for Vector Ecology, Albuquerque, New Mexico). Another study failed to find evidence of infection in field-collected *I. pacificus*, *D. variabilis*, *Ixodes angustus*, or *Otobius megnini* (Quick et al., 1993). Evidence of piroplasms in small mammals has been documented, however piroplasms were either only morphologically identified or, in the case of woodrats, shown to be a distantly related parasite, *Theileria youngi* (Van Peenen et al., 1968; Kjemtrup et al., 2001). However, *B. duncani* has been isolated from bighorn sheep (*Ovis canadensis*) and mule deer (*Odocoileus hemionus*). In addition, serological data suggests seroconversion in large ungulates (Kjemtrup et al., 1995). Based on the locality and seasonality of human cases of babesiosis having suspected tick bites in the autumn, and evidence suggesting a large ungulate reservoir, we hypothesize that the winter tick (*Dermacentor albipictus*), a large ungulate specialist, is the primary vector of *B. duncani* in the far-western United States. To test this hypothesis, we employed a recently developed molecular assay for *B. duncani* detection (O'Connor et al., 2018) to detect *B. duncani* in *D. albipictus* as well as its primary wildlife host, the Californian mule deer (*O. hemionus*). *Dermacentor albipictus* is a "one-host" tick, typically spending most of its life cycle on a large ungulate such as mule deer, elk, moose, or wild sheep. Only *D. albipictus* larvae quest for hosts on the ground because the nymphal and adult ticks remain and feed on a single ungulate host. Due to this life history, humans are most likely to come into contact with *D. albipictus* larvae which are the only questing life stage, although records exist for humans being bitten by adult *D. albipictus* as well (Howell, D.E., 1939. The ecology of *Dermacentor albipictus* (Packard), 6th Pacific Science Congress of the Pacific Science Association, USA; Merten and Durden, 2000). *Babesia microti* is not transmitted transovarially from adult female ticks to larval offspring, but other *Babesia* spp. including *Babesia canis* and *Babesia bovis* (Howell et al., 2007; Chauvin et al., 2009) do exhibit transovarial transmission. It was

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not previously known whether *B. duncani* is transmitted transovarially because the vector had not been identified until the present study. Human risk of acquiring *B. duncani* from a one-host tick would be influenced by whether larvae could acquire the infection via the eggs of infected female ticks and subsequently infect people and transmit the parasite during their first blood meal (Fig. 1).

Identification of the tick vector and reservoir host of *B. duncani* is crucial to understanding the epidemiology and risk of this emerging and potentially fatal human disease. Identification of *D. albipictis* as the vector of *B. duncani* will influence future studies on the epidemiology of this organism.

## 2. Materials and methods

### 2.1. Experimental design

We sought to test our hypothesis that *D. albipictus* was a competent vector for *B. duncani* and by extension, that the primary blood meal host, mule deer (*O. hemionus*) was a reservoir host. Our study sampled *D. albipictus* ticks that were collected between 1999 and 2014 by the California Department of Public Health (CDPH), R. Lane (University of California, Berkeley), and A. Swei (San Francisco State University) at sites across California, USA (Fig. 1). Samples were selected for testing to represent a range of geographic locations and sought to maximize spatial proximity to known or suspected human cases of babesiosis in California.

### 2.2. Tick sample processing

Larval ticks were collected by dragging or flagging leaf litter or other ground cover (Talleklint-Eisen and Lane, 2000), whereas nymphal and adult ticks were collected directly off mule deer or by flagging deer bedding. All ticks were stored in 70% or 95% ethanol until extraction. Whole nymphal and adult ticks were extracted individually using the DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA) after being surface sterilised with a 3% solution of hydrogen peroxide followed by two washes with 70% ethanol. Ticks were then crushed in lysis buffer using sterile plastic pestles or sterilised scalpels. All nymph and adult ticks were collected off mule deer, *O. hemionus*, in Sacramento County in 2014 and most were partially to fully engorged with deer blood. Initially, larval ticks were extracted in pools of four to five to maximize detection of *B. duncani*. Once positive samples were confirmed, we began to test all ticks individually. As a result, 16 larvae from Yosemite National Park, Mariposa County and all 82 larval ticks from Marin County were individually extracted and PCR tested for infection with *B. duncani*.

### 2.3. Deer and sheep blood collection and extraction

Whole blood samples were collected from mule deer, *O. hemionus*, and bighorn sheep, *O. canadensis*, by scientists at the California Department of Fish and Wildlife in the spring of 2017 as part of ongoing deer and sheep monitoring efforts. Mule deer were sampled from six migratory herds during their winter range in Mono County and Inyo County. Bighorn sheep were sampled from several sites in San Bernardino County. Mule deer and bighorn sheep were captured with a netgun shot from a helicopter that deployed a weighted net over the target animal. Each animal was transported to a sample processing centre where veterinary staff and biologists collected blood samples. Whole blood was drawn by jugular venipuncture from each animal and collected into a 4 ml EDTA vacutainer tube (BD, Franklin Lakes, NJ, USA) and kept on ice, then at 4 °C until sample processing. All animals were released from the processing center after sampling and recovery. However, when the site of the processing center was located outside the normal extent of the herd, animals were flown back to their location of capture for release. All blood samples were extracted with the DNeasy Blood and Tissue kit (Qiagen, Valencia, California, USA).

### 2.4. Pathogen testing and prevalence analysis

All tick and ungulate blood samples were tested for *B. duncani* using a nested PCR protocol targeting the β-tubulin gene, a locus with highly variable, species-specific length based on the size of the intron (O'Connor et al., 2018). The β-tubulin gene sequence for the WA1 strain was sequenced and can be found in Supplementary Data S1 (GenBank Accession number MH348898). Initially, larval ticks were extracted in pools of four to five ticks from Contra Costa, Napa, and Sonoma counties in California. PCR testing was conducted on these pooled samples and a positive pool was conservatively estimated to only contain a single positive tick. Therefore, infection prevalence values for larvae from these counties were calculated as minimum infection prevalence (MIP), however the true prevalence may be higher. Larvae from Marin County, California and Mariposa County, California were extracted and tested individually so that absolute prevalence was calculated for these samples. Each extraction sample was tested in triplicate to reduce the risk of false negatives. Attached nymph and adult *D. albipictus* were collected off mule deer and were partially engorged with blood. It was possible to identify whether a sample was positive for *B. duncani* based on the β-tubulin PCR amplicon size but purification and sequencing of amplicons was difficult from engorged nymph and adult samples, so subsequent PCRs were employed and final infection prevalence was based on sequence confirmed positives. Engorged nymph and adult ticks were tested with three sets of PCR primer pairs targeting the 18S rRNA gene resulting in a 103 bp fragment (F227 + R330), a 353 bp fragment (F227 + R5800), and a 1510 bp fragment (F227 + R1737) (Supplementary Table S1) in the *B. duncani* 18S rRNA gene. The *B. duncani* chloroquine resistance transporter (CRT) gene and β-tubulin gene from the WA1 isolate were also sequenced (GenBank Accession numbers MH348897 and MH348898) and those sequences are provided in Supplementary Data S1).

Bayesian credible intervals (BCI) at the 95% level were calculated for all reported infection prevalence values as previously described (Swei et al., 2011). Briefly, the credible intervals were calculated using the cumulative distribution function of the beta distribution in R using the qbeta function. All analyses were executed in R (R Core Team, 2008; http://www.R-project.org).

### 2.5. Sequencing

Prior to sequencing, DNA was cleaned using Sera-mag SpeedBeads (Rolhland and Reich, 2012) and sequenced on an AB3130 (Applied Biosystems, San Diego, California, USA). Sequences were aligned using Geneious (version 7.1.9) (Kearse et al., 2012) and identified by alignment to previously sequenced β-tubulin genes from three known isolates, WA1, BH3 and FD1 (GenBank Accession numbers MF978358, MF978360, MF978361, respectively; Thomford et al., 1994; Conrad et al., 2006; O'Connor et al., 2018). Sequencing of the 18S rRNA amplicons was performed using primer F227 and the resulting sequences were aligned to the genomic sequence of the 18S rRNA gene of *B. duncani* WA1 strain (GenBank Accession number MH333111).

### 2.6. Phylogenetic analysis

A phylogenetic analysis was conducted on available *B. duncani* isolates as well as other notable apicomplexans based on the 18S rRNA and *cyt b* loci (see Supplementary Tables S2 and S3 for Gen-

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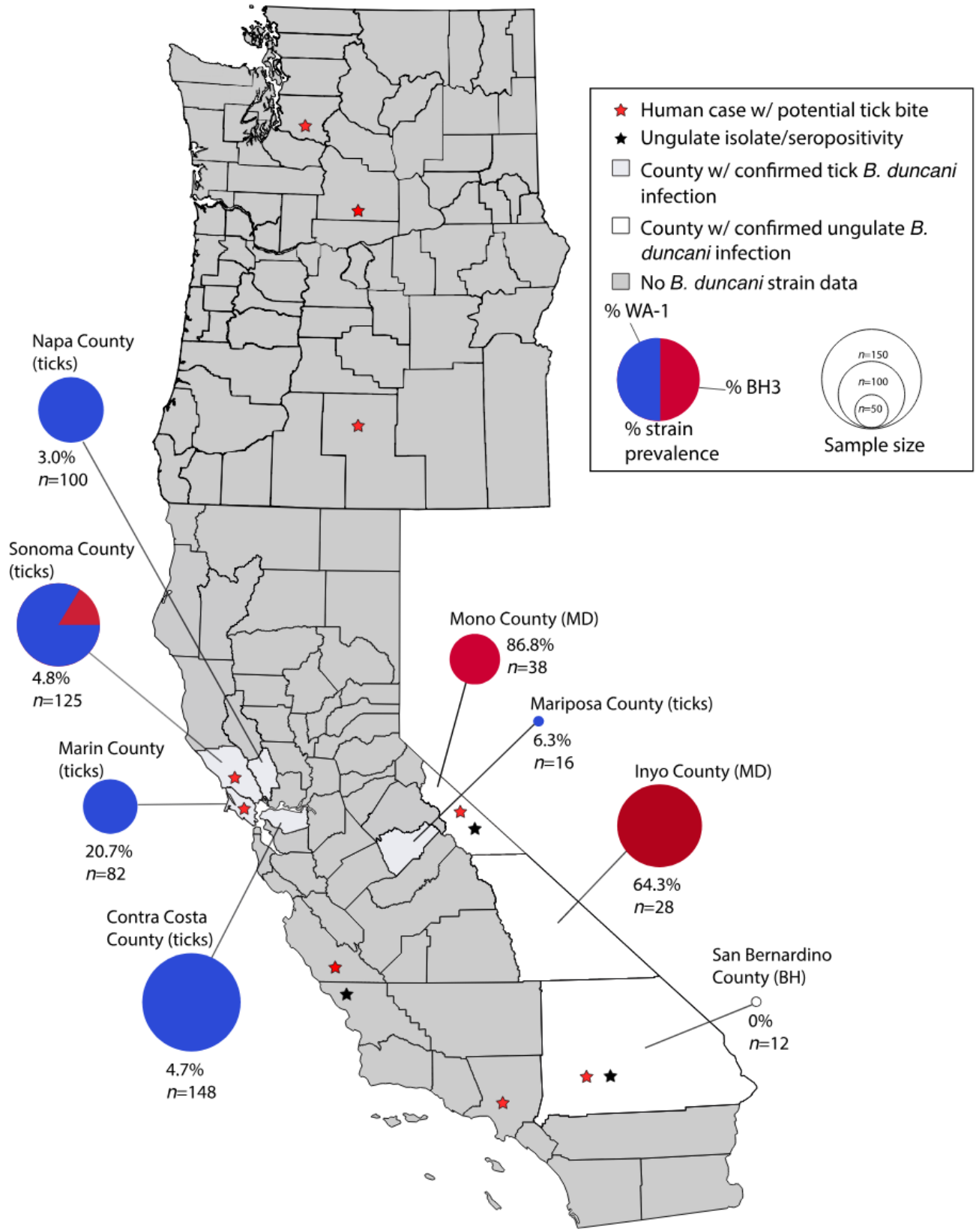


**Fig. 1.** Map of tick-acquired *Babesia duncani* cases at various locations in the USA. The distribution and relative prevalence of *B. duncani* strains detected in *Dermacentor albipictus* larvae (ticks), mule deer (MD), and bighorn sheep (BH). Counties with available *D. albipictus* prevalence data are shown in light grey and counties with only ungulate blood sample data are shown in dark grey. All other counties, which have no available *B. duncani* strain data, are shown in white. Only samples with the *B. duncani* strain type identified are shown. Pie charts indicate the relative sample sizes tested from each county and the relative proportions of different *B. duncani* strains. WA1 was the most prevalent strain, followed by BH3 in ticks.

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Bank Accession numbers). Phylogenetic analyses of both loci used *Toxoplasma gondii* as the outgroup and included key *Babesia and Theileria* spp. with sequences available on GenBank. All available *B. duncani* isolates were used in the analyses. Phylogenetic analyses were conducted in PAUP* (Swofford, 2003) using maximum likelihood model selection and 500 bootstrap iterations.

### 2.7. Data availability

The data generated in this study are all publicly available. All sequence data of *B. duncani* isolates and loci are accessioned in GenBank under accession numbers provided in Supplementary Table S2.

## 3. Results

### 3.1. Tick screening

In total, 585 *D. albipictus* ticks including 471 larvae, 16 nymphs and 98 adults, collected at nine localities in California, were extracted whole and screened for *B. duncani*. The larvae were collected from eight localities in five counties (Table 1) and the nymph and adult ticks were collected from mule deer or by flagging at a ninth locality in Sacramento County. We detected *B. duncani* in larval and adult life stages of *D. albipictus*. Initially, larval tick samples were pooled for molecular testing, thus most prevalence estimates are presented as the MIP by conservatively assuming that a positive pool only had a single infected tick. Overall, larvae had a MIP of 7.21% and a 95% BCI of 5.21–9.92% (Table 2). The highest larval infection prevalence was observed in Marin County, where absolute larval infection prevalence was 20.73% and the 95% was 13.4–30.76%. The lowest county-wide infection prevalence was observed in Napa County where the MIP was 3.0% and the 95% BCI was 1.09–8.43% (Table 2). A limited number of host-attached nymph and adult ticks from Sacramento County were examined for *B. duncani* infection. Of 16 nymphs tested by a β-tubulin PCR protocol, one was positive for *B. duncani* and five of 98 (5.10%, BCI 8.50–11.84%) adult ticks produced a positive PCR. However, we were unable to sequence these β-tubulin PCR products from nymph and adult tick samples so a different PCR assay based on the 18S rRNA gene was employed to confirm the sequence and pathogen strain type. The 18S rRNA PCR produced positive results in two of the five adult tick samples that were positive by β-tubulin PCR but was not able to produce a positive PCR product from three adult samples and one β-tubulin positive nymph sample. The two 18S rRNA positive samples were both from adult male *D. albipictus* and DNA sequencing showed 100% identity to the sequence of the 18S rRNA gene of *B. duncani* WA1 strain (GenBank Accession number MH333111). Based on the 18S rRNA sequence-confirmed positives, the adult infection prevalence with *B. duncani* is conservatively estimated as two positive samples out of 98 samples (2.04%, BCI 0.6–7.10%).

### 3.2. Deer and sheep blood screening

Whole blood samples were collected from 66 mule deer (*O. hemionus*) and 12 bighorn sheep (*O. canadensis*) and were screened by β-tubulin PCR for infection with *B. duncani*. Mule deer samples were obtained from six migratory herds in Mono County and Inyo County. Bighorn sheep samples were collected from San Bernardino County. Mule deer had an overall infection prevalence with *B. duncani* of 75.8% (95% BCI 64.14–84.47%) and all positive deer were infected with the BH3 strain by phylogenetic analysis of the β-tubulin gene. All 12 bighorn sheep samples assayed were negative for *B. duncani* by nested PCR.

### 3.3. Babesia duncani strain identification

Sequencing analysis identified that multiple *B. duncani* strains were circulating in *D. albipictus* larvae (Table 2). Based on β-tubulin sequences, the two most commonly encountered *B. duncani* strains in tick samples were identical to the WA1 isolate and BH3 isolates (Fig. 1). At one site in Sonoma County, *D. albipictus* larvae were infected with two *B. duncani* strains (WA1 and BH3) (Table 2). Sequences were obtained for two adult *D. albipictus* ticks that were both infected with the WA1 strain. Sequence typing of the positive mule deer samples revealed uniform infections with the BH3 strain (Fig. 1).

### 3.4. Phylogenetic analysis

Phylogenetic analysis utilised a maximum likelihood approach. Both the 18S rRNA and *cyt b* analyses show strong support for a clade that includes all known *B. duncani* strains in two well supported subclades (Fig. 2). Based on the 18S rRNA phylogeny, one subclade included strains that were isolated from large ungulates such as bighorn sheep (BH1, BH3) and mule deer (MD1) as well as human patients who were presumably involved in tick-transmitted cases of *B. duncani* (CA1, CA3, CA4). The other subclade

**Table 2**
Minimum infection prevalence of *Babesia duncani* and strain identification in *Dermacentor albipictus* larvae in various locations in California (USA) by county and site. For all sites, the larvae were pooled for extraction and screening with the exception of Marin County, CA (Marin Municipal Water District, China Camp SP) and Mariposa County, CA (Yosemite NP), denoted with [a]where ticks were processed individually and prevalence values are absolute. Bayesian credible intervals (95%) were calculated based on the reported larval prevalence and are provided in parentheses. Strain identity was based on the β-tubulin gene of reference isolates.

| | Larval positives and sample size | Larval prevalence (95% credible interval) | Strain |
|---|---|---|---|
| **Contra Costa Co., CA** | | | |
| Tilden RP | 7/148 | 4.73% MIP (2.35–9.44) | WA1 |
| **Marin Co., CA** | | | |
| China Camp SP | 3/16 | 18.75%[a] (6.81–43.43) | WA1 |
| Marin Municipal Water District | 14/66 | 21.21%[a] (13.11–32.57) | WA1 |
| **Mariposa Co., CA** | | | |
| Yosemite NP | 1/16 | 6.25%[a] (1.46–28.69) | WA1 |
| **Napa Co., CA** | | | |
| Bothe-Napa SP | 3/100 | 3.0% MIP (1.09–8.44) | WA1 |
| **Sonoma Co., CA** | | | |
| Annadel SP | 2/52 | 3.85% MIP (1.18–12.98) | 50% BH3, 50% WA1 |
| Fairfield Osborne Preserve | 1/43 | 2.33% MIP (0.56–12.02) | WA1 |
| Jack London SP | 3/30 | 10.0% MIP (3.63–25.75) | WA1 |
| **Average prevalence** | **34/471** | 7.21% MIP (5.22–9.92) | |

RP, regional park; NP, National Park; SP, State Park.

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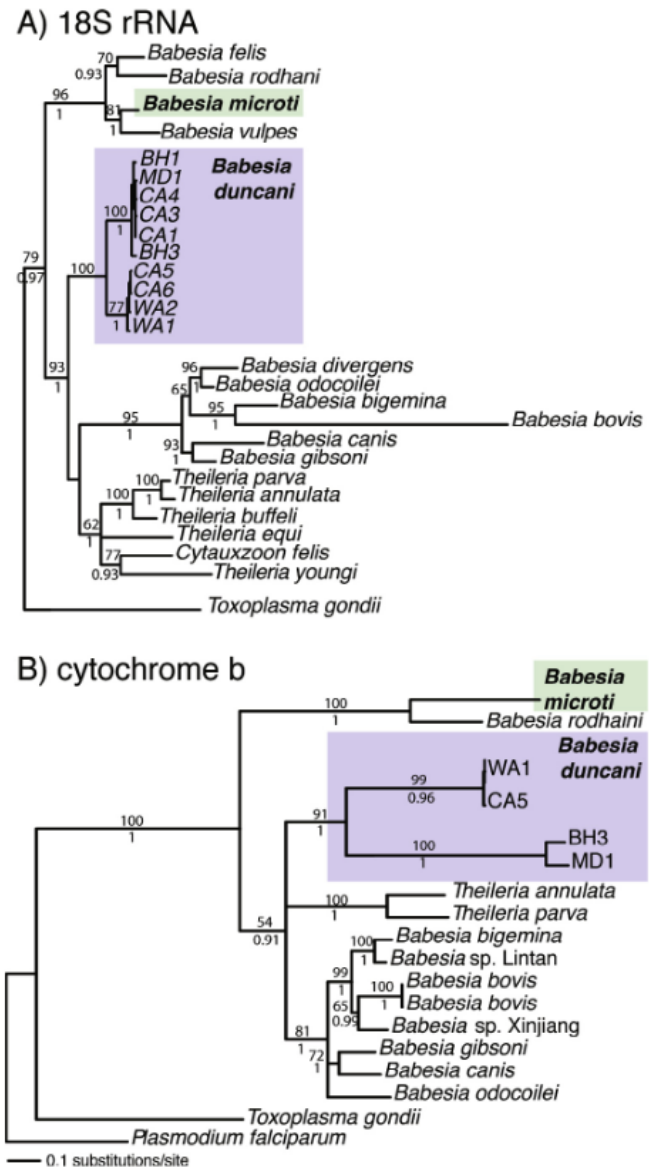


**Fig. 2.** *Babesia duncani* phylogeny. Phylogenetic relationship of *Babesia duncani* relative to other *Babesia* and *Theleria* spp. based on maximum likelihood methods. Bootstrap values are based on 500 model replicates. Maximum likelihood bootstrap values over 60 are displayed above the nodes and Bayesian bootstrap values over 0.9 are displayed below the nodes.

includes several human isolates that were derived from clinical samples including the type strain (WA1), an asymptomatic blood donor (CA6), and two patients who were infected by blood transfusion (CA5, WA2; Table 1). A similar phylogenetic structure of *B. duncani* resulted from the *cyt b* phylogeny with one subclade containing ungulate isolates (BH3 and MD1) and the other subclade comprised of human isolates (CA5 and WA1).

## 4. Discussion

Our study provides evidence that *B. duncani*, a potentially emerging and fatal human pathogen, is likely vectored by the winter tick, *D. albipictus*. As a "one-host" tick, *D. albipictus* larvae quest for a large ungulate blood meal host and then spend the next two life stages on the same animal host, mainly species such as deer, elk, moose and sheep (Fig. 3). Given the life history of *D. albipictus*, larvae are the most likely life stage to be found questing on vegetation and therefore have a higher probability of encountering and biting humans. Records do exist for nymph and adult *D. albipictus* biting humans in California (Merten and Durden, 2000), however it is unclear whether adult *D. albipictus* can transmit *B. duncani* if the parasites are localised in ovaries as has been demonstrated for other transovarially transmitted *Babesia* spp. such as *B. ovis* (Howell et al., 2007). Studies of other transovarially transmitted *Babesia* spp. have shown that adult ticks (e.g. *Boophilus decoloratus*) may transmit the organism to mammalian hosts from the adult stage (Dalgliesh et al., 1978; Büscher, 1988), suggesting that it may be possible for adult *D. albipictus* to transmit *B. duncani*. Our sampling of nymph and adult ticks was much more limited but the confirmation of *B. duncani* WA1 strain in 2.07% of adult *D. albipictus* does suggest that there is transstadial transmission of *B. duncani*. Expanded sampling and transmission experiments will be necessary to more accurately determine the life stage-specific prevalence of *D. albipictus* and transmission potential at each life stage.

Our detection of *B. duncani* from five central and northern Californian counties (Fig. 1) with an overall minimum infection prevalence of 7.2% in questing larval *D. albipictus* strongly suggests that *B. duncani* is transmitted transovarially and that *D. albipictus* is a strong candidate for being a vector. In the United States, human babesiosis is primarily caused by *B. microti*, a *Babesia* sp. that does not exhibit transovarial transmission. However, transovarial transmission is common in several other *Babesia* spp. that are associated with large vertebrate hosts (Chauvin et al., 2009) similar to *B. duncani*, as we demonstrate here. The ability of *Babesia* spp. to be transmitted transovarially is likely an important component in the maintenance of these parasites (Chauvin et al., 2009). Furthermore, the high host infection prevalence in at least one region of California indicates that mule deer (*O. hemionus*) could be a primary reservoir host of *B. duncani* but other species may also be involved.

Half of the tick-transmitted cases of *B. duncani* initially presented in late summer or early autumn months (Persing et al., 1995; Table 1). This is consistent epidemiologically with the seasonality of *D. albipictus* larvae that actively seek a blood meal host from approximately September to January. Interestingly, *D. albipictus* ticks may incidentally drop from their host throughout the year and have the potential to bite humans at that time (Howell, 1939, 6th Pacific Science Congress of the Pacific Science Association, USA) which might explain some reports of later onset months of tick-exposed *B. duncani* patients (Howell, 1939, 6th Pacific Science Congress of the Pacific Science Association, USA; Furman and Loomis, 1984). Additionally, given the high prevalence of infection detected in mule deer, deer hunters could be at an elevated risk of exposure to *B. duncani* from either having direct contact with deer blood while gutting or skinning them or by having increased contact with *D. albipictus* ticks infesting deer (Fig. 3), similar to the exposure circumstances described for the fourth *B. duncani* infection in California (Persing et al., 1995).

Earlier work on *B. duncani* indicates that human cases with no blood transfusion history, and thus transmission of the parasite likely being acquired through a tick bite, are genetically similar to strains isolated from bighorn sheep (*O. canadensis*) and mule deer in California (Conrad et al., 2006; Lack et al., 2012). The mule deer samples tested in this study were uniformly infected with the BH3 strain and had high infection prevalence in Inyo (64.3%) and Mono (86.8%) Counties. These results suggest that mule deer are a potentially important reservoir host. Our molecular testing of bighorn sheep did not detect *B. duncani*, but because our sample size was small and limited to one region this host may be a reservoir in other locations. Previously, *B. duncani* was isolated from bighorn sheep in California (BH3 strain, (Thomford et al., 1993)), thus there is some limited evidence of their reservoir potential and *D. albipictus* may be able to transmit this pathogen between these two ungu-

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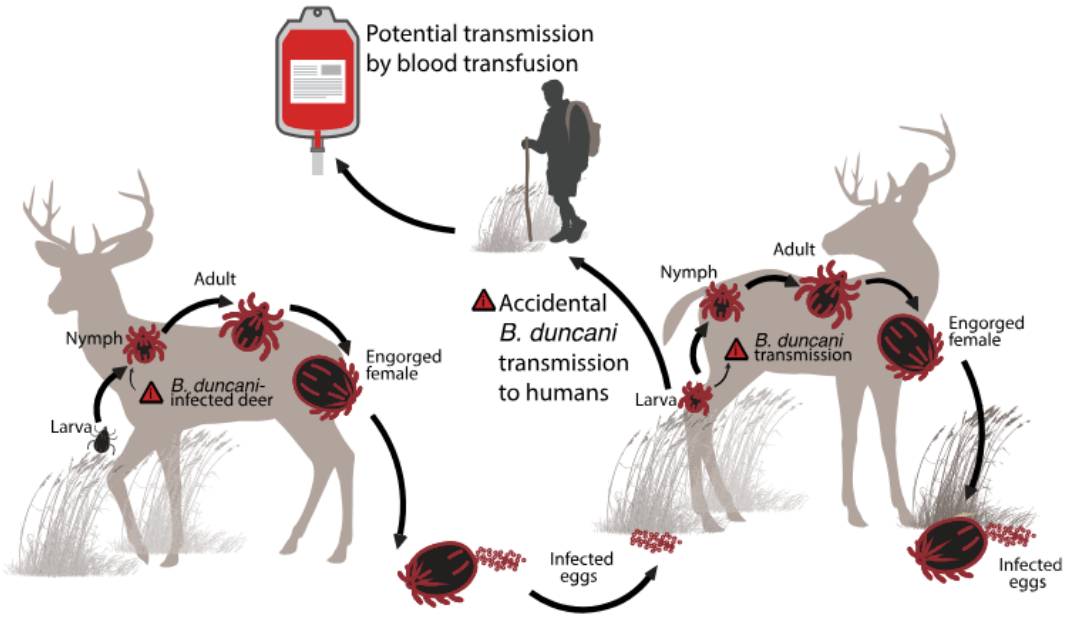


**Fig. 3.** *Dermacentor albipictus* life cycle and proposed pathogen transmission pathways for *Babesia duncani*. The enzootic cycle is maintained by the putative vector, *D. albipictus*, and reservoir host, mule deer, *Odocoileus hemionus*. As a "one-host" tick, *D. albipictus* only quests on the ground as larvae and then remain on the initial blood-meal host until replete females drop off to oviposit on the ground. Larvae then emerge and begin questing for a host. *Babesia duncani* is transmitted vertically from adult female ticks to their offspring; therefore questing larvae can be infected and are the most likely route of pathogen transmission to other ungulates and humans.

late species where they co-occur. However, it is possible that other hosts are also involved as parasite reservoirs. While uncharacterised piroplasms have been documented in Californian rodents (Van Peenan et al., 1968), *D. albipictus* does not typically feed on small rodents, suggesting rodents are unlikely reservoirs. Prior studies in California have also isolated *B. duncani* from fallow deer (*Dama dama*) in Monterey County (Thomford et al., 1993), thus this species or other ungulate species may also be reservoirs. Naturally acquired human cases of babesiosis were contracted in some of the same or adjacent counties where these large ungulate wildlife isolates were obtained (Fig. 1) and further investigations into these and other species as potential reservoirs would be highly valuable.

In the approximately two and a half decades since *B. duncani* was first isolated from a human patient in Washington State (Persing et al., 1995) and subsequently described (Conrad et al., 2006), the enzootic transmission cycle had remained elusive. This gap in knowledge had greatly impaired efforts to understand the risk of this disease both from tick-borne transmission and in the blood supply. Of the 14 documented and presumed cases of *B. duncani* in humans (Table 1), 11 were likely acquired by tick bites and three were contracted through blood transfusions from an asymptomatic donor (Table 1, (Herwaldt et al., 1997; Kjemtrup et al., 2002). Human seroprevalence surveys conducted on human populations in California found elevated antibody titers (≥320) to the WA1 antigen from 0.8% to 20.1% of samples tested from separate human populations (Persing et al., 1995; Fritz et al., 1997). A more recent study (Prince et al., 2010) suggested a high seroreactivity (27% with titers ≥256) to *B. duncani* across a broad geographic area in the United States. However, since the immunoflorescent antibody test (IFAT) used in all these studies has not been validated for appropriate cut-off values for infection due to the low number of known positive samples, the high seroreactivity in some of these studies may be due to a high number of false positives associated with using a low positive cut-off titer or serological cross-reactivity. Thus, improved understanding of the tick vector is critical to assessing the ecology and geographic range of *B. duncani*, human risk for infection, and potential for coinfection with other tick-borne pathogens. Given the evidence that *D. albipictus* is the probable vector of *B. duncani*, it is unlikely that pathogens transmitted by other vector species such as *Borrelia burgdorferi*, which is vectored by *Ixodes* spp., are being co-transmitted with *B. duncani* in the western United States.

Phylogenetic analyses of two informative loci, 18S rRNA and *cyt b*, confirm early work by Persing et al. (1995) that *B. duncani* forms a distinct clade from *B. microti* and may be more closely related to *Babesia* spp. maintained by large ungulates and *Theileria* spp. (Fig. 2). There is strong support for two distinct sub-clades of *B. duncani*; one that contains the original human case isolate from Washington (WA1) and several human isolates from California, and another sub-clade that comprises human isolates as well as wildlife isolates from bighorn sheep and mule deer. Analysis of *D. albipictus* infection with *B. duncani* largely identified the WA1 strain but one tick population in Sonoma County was also infected with the BH3 strain. These two strains can thus circulate sympatrically, although much remains to be learned about the spatial distribution of *B. duncani* strains. Ungulate blood samples collected from wild herds in Inyo and Mono Counties were uniformly infected with the BH3 strains, so further field surveillance is needed to clarify the reservoir host, or hosts, for the WA1 clade in different geographic regions.

Human cases of *B. duncani* have only been confirmed from the western United States thus far, primarily Washington, Oregon and California. As such, this disease has been considered to be restricted to this geographic region. The high prevalence of *B. duncani* infection in mule deer, *O. hemionus*, indicates that this cervid may be a key vertebrate reservoir host. While *D. albipictus* is widely distributed, the geographic range of *O. hemionus* is restricted to western North America (Fig. 4) which is consistent with confirmed *B. duncani* cases that are restricted to the western United States. Currently, there is no evidence that *B. duncani* circulates in the eastern United States. Further investigations are needed to determine whether *B. duncani* is maintained by *D. albipictus* and another reservoir host in regions outside of the western United States.

There remain many facets of the natural enzootic cycle of *B. duncani* that warrant further investigation. The prevalence of *B. duncani* in *D. albipictus* larvae suggests that this parasite is

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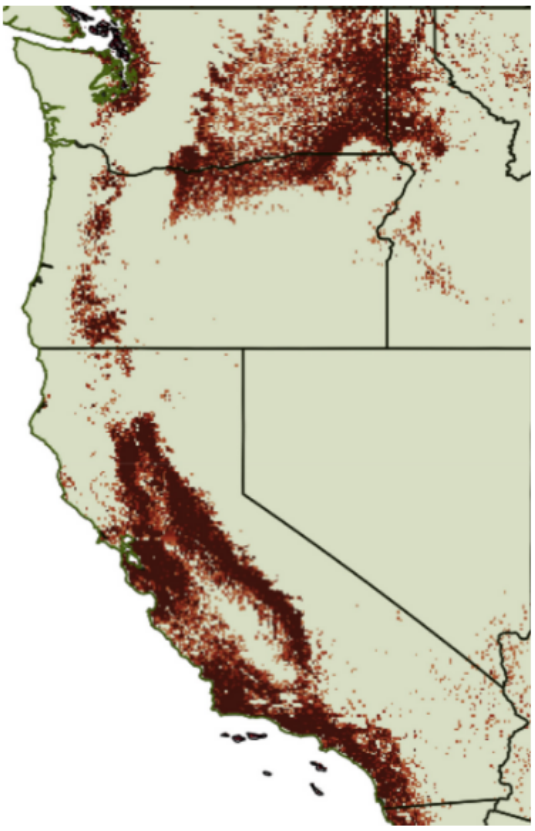

**Fig. 4.** Range of *Dermacentor albipictus* and mule deer (*Odocoileus hemionus*) in western North America. The geographic range of *D. albipictus* and mule deer and shown overlapping. The shaded (green) region is the projected range of mule deer (Sanchez Rojas and Gallina Tessaro, 2016) and the red areas are regions with predicted presence of *D. albipictus* based on environmental niche modelling using Maxent methods (http://vectormap.si.edu/Tick_Metadata.htm#vec147).

transovarially transmitted, which underscores the need to better understand the transmission dynamics of this parasite including the efficiency by which larval ticks can transmit the infection to an animal model, and if transmission efficiency and pathogenicity vary by strain. Transmission experiments are still required to confirm the vector competency of *D. albipictus* and ascertain the preliminary findings suggesting transovarial and transstadial transmission of *B. duncani*. It is also unknown if there are negative health impacts of *B. duncani* infection in mule deer or the critically threatened bighorn sheep. With strong molecular evidence pointing to *D. albipictus* as a vector, it is now possible to better characterize the epidemiology, ecology and pathogenicity of *B. duncani*.

## Acknowledgements

We are grateful for field and laboratory assistance provided by Phillip Galaciano, Jessica Kwan, Betsabel Chicana, Joyce E. Kleinjan, and Dr. Tom Stephenson who led the California Department of Fish and Wildlife Desert Region, USA, project. We are also grateful for graphical assistance provided by Allyx Nicolici and Dr. Marty Castro for initial inspiration for this hypothesis. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.07.002.

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