Received: 16 March 2018 | Revised: 30 May 2018 | Accepted: 24 July 2018

DOI: 10.1111/jvim.15300

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

STANDARD ARTICLE

Babesia gibsoni cytochrome b mutations in canine blood samples submitted to a US veterinary diagnostic laboratory

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Background: Babesiosis caused by *Babesia gibsoni* is recognized throughout the world and can be difficult to treat. Resistance to atovaquone is associated with mutations in the *B. gibsoni* mitochondrial genome, specifically the M128 position of cytochrome b (*cytb*). The prevalence of *cytb* mutations in North America has not been reported.

Hypothesis/Objectives: The objective of our study was to describe the prevalence of *cytb* M128 mutations in *B. gibsoni* in canine blood samples submitted to a US veterinary diagnostic laboratory. A secondary objective was to determine whether or not some dogs had wild-type *cytb* in our initial samples then had M128 mutations detected in follow-up samples.

Animals: One-Hundred seventy-four dogs that tested positive for the presence of *B. gibsoni* between 2012 and 2017.

Methods: Case series of consecutive samples submitted to a veterinary diagnostic laboratory. Partial *B. gibsoni cytb* genes were amplified by polymerase chain reaction and screened for the presence of mutations at the M128 position.

Results: The overall prevalence of M128 mutants was 3.5% (6/173 dogs) in the initial samples. The incidence of new *cytb* mutants in dogs that tested positive for *B. gibsoni*, which then had follow-up testing, was 12.1% (5/41).

Conclusions and Clinic Importance: Our study reaffirms that *B. gibsoni* infection is widespread and most commonly detected in American Staffordshire Terrier/American Pit Bull Terrier dogs (128/174, 74% of the infected dogs in our study). The prevalence of *cytb* mutations does not warrant pretreatment genotyping.

KEYWORDS

atovaquone, mitochondrial, pharmacogenomics, resistance

1 | INTRODUCTION

Since its emergence in the 1990s,[1] *Babesia gibsoni* has become the most commonly diagnosed species of *Babesia* in dogs in the United States of America and is now widely distributed across the country.[2] A&A has been recommended for *B. gibsoni*, and approximately 80% of treated dogs have clinical "cures" with reductions in parasitemia below the detection limit of sensitive molecular diagnostic assays.[3,4] Mutations in *B. gibsoni cytb* genes confer resistance to atovaquone, resulting in treatment failures.[5,6] These nonsynonymous mutations often result in a M128I or M128V change in the putative atovaquone binding site. Atovaquone resistant mutants have been identified in blood samples collected before and after treatment but are more often documented in dogs in which treatment with A&A failed.[6–8] This suggests selection for rare mutants during treatment, which has been documented *in vitro*.[5] In Asia, *B. gibsoni* strains with M128I mutations

Abbreviations: A&A, atovaquone and azithromycin combination therapy; WT, Wild type; *cytb*, cytochrome *b*; DNA, deoxyribonucleic acid; CDM, clindamycin, doxycycline and metronidazole combination therapy

were detected in 3%–47% of the canine blood samples tested.[6,7,9] To the authors' knowledge, the prevalence of M128 mutations in *B. gibsoni* in the United States has not been reported. Therefore, the primary purpose of our study was to determine the prevalence of M128 mutations in *B. gibsoni*-infected dogs using blood samples submitted to a diagnostic laboratory in the United States of America. The secondary purpose was to describe the number of dogs that had WT *cytb* in initial and M128 mutants in follow-up blood specimens.

2 | MATERIALS AND METHODS

2.1 | Samples

All samples submitted to a veterinary diagnostic laboratory (Vector Borne Disease Diagnostic Laboratory, North Carolina State University, College of Veterinary Medicine, Raleigh, NC) between January 01, 2012 and January 18, 2017 were evaluated to identify dogs that were *B. gibsoni* polymerase chain reaction (PCR) positive and PCR negative for all other *Babesia* spp. Samples that tested positive for *B. gibsoni* were then reviewed to identify the total number of dogs that were tested and whether any dogs that tested positive had multiple samples submitted during the study period. Initial samples were defined as the first *B. gibsoni* positive sample submitted for each dog during the study period. Sample inclusion, exclusion, and subcategorization are outlined in Supporting Information Figure S1.

2.2 | DNA extraction

DNA was extracted from 200 μL of EDTA whole blood using magnetic bead separation via an automated DNA workstation (Qiagen QIAsymphony SP using the DSP DNA Mini Kit 192, version 1 or the Qiagen Biorobot M48 using the Qiagen MagAttract DNA Mini M48 Kit [Qiagen Inc, Valencia, CA]). DNA samples were stored at −20°C.

2.3 | PCR assays

2.3.1 | Quality control PCR

Housekeeping PCR amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to demonstrate the suitability of each sample for PCR with modifications from a previously described protocol.[10] GAPDH PCRs were run using quantitative PCR machines (CFX96 Real-Time System C1000 Touch Thermal Cyclers [Bio-Rad Laboratories, Inc, Hercules, CA]) and amplicon detection and melting curve analyses utilized SYBR green dye (SsoAdvanced Universal SYBR Green Supermix [Applied Biosystems, Life Technologies, Carlsbad, CA]).

2.3.2 | PCR for the detection and confirmation of *B. gibsoni*

All samples were originally screened for the presence *Babesia* spp. DNA using two independent PCR protocols. One targeting 18S rRNA genes[10], and a second targeting mitochondrial DNA.[11] Assays were run using quantitative PCR machines (CFX96 Real-Time System C1000 Touch Thermal Cyclers [Bio-Rad Laboratories, Inc]), and amplicon detection and melting curve analyses utilized SYBR green dye (SsoAdvanced Universal SYBR Green Supermix [Applied Biosystems, Life Technologies]).

2.3.3 | PCR and sequencing for *cytb* gene mutations

cytb PCRs were run using a quantitative PCR machine (MiniOpticon [Bio-Rad, Hercules]) amplicon detection and melting curve analyses utilized SYBR green dye (SsoAdvanced Universal SYBR Green Supermix [Applied Biosystems, Life Technologies]). Thermal cycler conditions consisted of a denaturing phase at 98°C for 3 minutes followed by 50 cycles of 95°C for 10 seconds, 58°C for 20 seconds, and 72°C for 30 seconds with a plate read after each cycle. Individual PCR reactions contained 25 μL of PCR mastermix (SsoAdvanced Universal SYBR Green Supermix [Applied Biosystems, Life Technologies]), 25 pmol of Bgib *cytb* 427F (5′-GCATTCTTAGGTTATGTTTTACCAA-3′), 25 pmol of Bgib *cytb* R (5′-GAACACTAACACTATAACCACC-3′), and 5 μL of DNA totaling 50 μL per PCR reaction. Amplicons were sequenced bidirectionally by a commercial laboratory (Genewiz LLC), and chromatograms were manually inspected for a *cytb* mutation at the M128 position (based on AB499087.1).

3 | STATISTICAL ANALYSIS

Prevalence was calculated using initial samples only as follows (number of *B. gibsoni* PCR+ dogs with M128 mutants / number of *B. gibsoni* PCR+ dogs). A one-sided Fisher's exact test was used to determine whether there was a difference in the proportion of mutant strains of *B. gibsoni* in dogs that only tested positive on follow-up samples that were completed <60 days after the initial positive test compared to those that had positive tests that were completed ≥60 days after the initial positive test (Statcalc, EpiInfo ver. 7.2.2.6). Differences were considered significant at $P < .05$. The incidence of new *cytb* mutants in dogs that tested positive for *B. gibsoni* which then had follow-up testing was calculated as follows: (number of new *cytb* mutants / number of *B. gibsoni* positive dogs with WT *cytb* in the initial sample that had follow-up testing). New *cytb* mutants were defined as *cytb* mutants that were detected in dogs that had only had WT *cytb* detected in our initial sample.

4 | RESULTS

Eight-thousand six-hundred forty-two samples (8642 samples/8174 dogs) were tested for the presence of *Babesia* species DNA, of which 211 were positive for *B. gibsoni* DNA and PCR negative for other *Babesia* spp. Sixteen samples were excluded because they were either not available for mutation testing or *cytb* genes could not be amplified. One additional sample was excluded because *B. canis vogeli* coinfection was detected during repeat testing. One hundred ninety-four *B. gibsoni*-infected samples from 174 dogs were included in the final analyses (Supporting Information Figure S1). These *B. gibsoni*-infected samples were submitted from 29 states and Canada including: Alabama (n = 1), Colorado (n = 1), Connecticut (n = 2), Florida (n = 7), Georgia (n = 1), Illinois (n = 3), Kansas (n = 3), Kentucky (n = 1), Louisiana (n = 3), Massachusetts (n = 3), Maryland (n = 3), Michigan

(n = 3), Minnesota (n = 1), Missouri (n = 3), Mississippi (n = 5), North Carolina (n = 20), New Jersey (n = 1), Nevada (n = 2), New York (n = 65), Ohio (n = 19), Oklahoma (n = 1), Oregon (n = 1), Pennsylvania (n = 4), South Carolina (n = 3), South Dakota (n = 1), Tennessee (n = 2), Texas (n = 6), Virginia (n = 6), Wisconsin (n = 2), and Canada (n = 1). The breeds were reported as: American Bulldog (n = 1), American Staffordshire Terrier/American Pit Bull Terrier (n = 128), Australian Shepherd (n = 1), Beagle (n = 2), Boxer (n = 4), Chihuahua (n = 2), Doberman Pinscher (n = 1), English Bulldog (n = 1), German Shepherd (n = 1), Great Dane (n = 1), Hound (n = 1), Jack Russell Terrier (n = 1), Labrador Retriever (n = 1), mixed breed (n = 19), Plott Hound (n = 1), Redbone Coonhound (n = 1), Rhodesian Ridgeback (n = 1), Shetland Sheepdog (n = 1), Staffordshire Bull Terrier (n = 1), Yorkshire Terrier (n = 1), and no breed listed (n = 4). American Staffordshire Terriers/American Pit Bull Terriers represented 13.7% of the tested dogs, and the prevalence of *B. gibsoni* in these American Staffordshire Terriers/American Pit Bull Terriers was 11.4% (128/1120).

Partial *B. gibsoni cytb* genes were successfully amplified and sequenced from 194 blood samples from 174 dogs. During the study period, repeat testing was requested for 44 of these dogs but was not requested for 130 dogs (Supporting Information Figure S1). One dog had its initial sample excluded because of the lack of availability, so it only has a follow-up sample included. Therefore, there were 173 initial samples with a prevalence of M128 mutations in these samples of 3.5% (6/173 samples).

Of the 44 dogs that were retested, 41 dogs had WT *cytb* on initial samples, 2 had *cytb* mutants, and 1 dog had its initial sample excluded. A total of 76 follow-up tests were performed with an average of 1.73 follow-up tests per dog (range 1-5). Nineteen dogs with follow-up testing (43.2%) were PCR+ on one or more follow-up samples with a total of 21 positive follow-up tests. Thirteen dogs had WT *cytb* in our follow-up samples and six had M128 mutant strains. Five of six dogs with M128 mutants were new *cytb* mutants as they only had a WT *cytb* strain detected in the initial sample. Among dogs that tested positive for *B. gibsoni* which then had follow-up testing, the incidence of new *cytb* mutants in our follow-up tests was 12.1% (5/41). The breeds and states for dogs with M128 mutations (including initial and new *cytb* mutants) were: American Staffordshire Terrier/American Pit Bull Terrier (one each from Michigan, Missouri, North Carolina, New Jersey, New York, South Dakota, and Tennessee), Beagle (Maryland), Mixed breed (two from North Carolina), and one dog of unknown breed (Texas). The specific M128 mutants characterized in our study included ATT (M128I, six dogs), ATW (M128I, two dogs), AYD (Methionine to Isoleucine or Threonine, two dogs), and ACG (M128 T, one dog).

Samples submitted for retesting were also analyzed with respect to timing of subsequent positive testing relative to the initial PCR+ tests. Of the 44 dogs that had repeat testing, 9 dogs only had positive follow-up tests <60 days from our initial positive, and 10 dogs had positive follow-up tests ≥60 days from our initial positive test. The proportion of dogs that had M128 mutations was higher in dogs that tested positive ≥60 days from the initial PCR+ test (6/10) compared to dogs that only tested positive <60 days from the initial PCR+ test (0/9) (P = .008).

5 | DISCUSSION

Our study reaffirms that *B. gibsoni* infections occur in dogs throughout North America, and this infection is detected most commonly in American Staffordshire Terrier/American Pit Bull Terrier type dogs (71% of the infected dogs in our study). Our study also documents the presence of *B. gibsoni* organisms with M128 mutations that have been previously associated with resistance to atovaquone. Nearly half of these M128 mutants were detected in follow-up blood specimens from dogs initially infected with a WT *cytb* strain. Compared to other studies, the prevalence of M128 mutations in our samples (3.5%) were similar to the prevalence found in Japan (5%) and substantially lower than the prevalence found in Taiwan (47%).[7,9,12] There are a number of potential reasons for the differences including study design/sample selection, frequency of atovaquone administration and geographic differences in the epidemiology of disease transmission. Differences could also be because of the decreased genetic variability in the North American isolates of *B. gibsoni*. It is suspected by some that the North American populations are more "clonal" than those in Asia attributable to the apparent lack of tick transmission and sexual reproduction of the parasite. North American isolates may have been introduced from a limited number of individuals, with subsequent propagation via direct dog-to-dog transmission and transplacental transmission.[13] Because of the variations in methodologies among laboratories, direct comparisons among studies documenting M128 mutations should be interpreted with caution. Some studies used methods that screened for any mutations at any location, whereas other studies reported one specific M128 mutation. The clinic and analytic sensitivities of the *cytb* PCR assays used in each study were not determined or compared.

Clinicians should be aware that *B. gibsoni* isolates in the United States of America can have mutations in *ctyb* genes that could confer atovaquone resistance and an incomplete treatment response. However, the prevalence of these mutations in US dogs as the predominant genotype does not appear to be common enough to warrant mutation screening before therapy or the use of first line regimens that do not include atovaquone. In fact, because nearly half of the dogs with M128 mutations only had WT *cytb* detected in our initial samples, they would have been incorrectly identified as susceptible to atovaquone based on our initial samples. Therefore pretreatment screening could actually mislead clinicians regarding the presence or absence of M128 mutations. The "transition" from WT *cytb* to M128 mutants is likely a result of selective pressure induced by treatment with atovaquone, but this is speculative, because treatment information was not available for our study. The higher proportion of WT *cytb* in samples tested <60 days from the initial PCR+ sample, could represent the detection of dead or replication deficient parasites present in red blood cells that have yet to be removed from circulation. Unfortunately, treatment data and sequential additional follow-up samples for mutation screening were not available for the majority of samples, precluding evaluation of treatment efficacy in dogs with *cytb* genotypes. A more sensitive screening method for M128 mutants might be more useful for predicting resistance and treatment failure, but is not commercially available. One such assay using an allele-specific SYBR-

Green PCR was able to detect a rare M128 mutant (<1% of the population) in as many as 66% of the *B. gibsoni* samples tested.[9] The presence of these rare M128 mutants could explain treatment failures rates in Asia (20%–47%) that are substantially higher than apparent prevalence when DNA sequencing is used to define resistance.[14] In addition to *cytb* mutations that confer resistance to atovaquone, mutations to the azithromycin-binding region of ribosomal protein subunit L4 (*rpl4*) have been recently identified in *B. microti*.[15] It is feasible that mutations in *B. gibsoni rpl4* could induce resistance to azithromycin and this is an area that should be the focus of future studies. In *B. microti*, *rpl4* is encoded in the apicoplast genome which has yet to be characterized in *B. gibsoni*. Mutations in *rpl4* are of particular interest in dogs that have persistent *B. gibsoni* infections after A&A that lack *cytb* mutations.

In conclusion, *B. gibsoni* is present across North America and American Staffordshire Terrier/American Pit Bull Terrier type dogs remain the most common breeds associated with this infection. The prevalence of M128 mutations in North America appears similar to or slightly lower than some populations of *B. gibsoni* in Asia. Based on these data, the authors still recommend combination treatment with A&A as the initial treatment of choice. Screening for M128 mutations should primarily be pursued in dogs that remain persistently infected despite treatment. A recent study have shown that that testing <60 days after treatment can yield false positive or false negative results that do not agree with testing performed ≥60 days post-treatment.[4] Based on these results and the higher proportion of WT *cytb* detected in follow-up samples <60 days after the initial positive, PCR testing <60 days from the completion of treatment is not recommended. When M128 mutations are detected, treatment with a combination of CDM with or without artemisin or other antiprotozoal drugs is recommended.[16] Unfortunately, the optimal duration of treatment with the CDM protocol is unknown.

ACKNOWLEDGMENTS

We thank Brittany Sherbert, Penny D'amelio, Kaye Gore, and Kaitlyn Haney for their assistance in the laboratory. All work was performed at North Carolina State University.

CONFLICT OF INTEREST DECLARATION

A. Birkenheuer codirects the Vector Borne Disease Diagnostic Laboratory (receives no compensation). H. Marr and J. Wilson are paid in part by the Vector Borne Disease Diagnostic Laboratory. E. Breitschwerdt codirects the Vector Borne Disease Diagnostic Laboratory (receives no compensation) and the Intracellular Pathogens Research Laboratory at the Institute for Comparative Medicine at North Carolina State University. He also is chief scientific officer at Galaxy Diagnostics and has been a paid consultant and researcher for IDEXX Laboratories. B. Qurollo is a research assistant professor at the Vector Borne Disease Diagnostic Laboratory at North Carolina State University, and part of her salary is funded by IDEXX Laboratories.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Birkenheuer AJ, Marr HS, Wilson JM, Breitschwerdt EB, Qurollo BA. *Babesia gibsoni* cytochrome b mutations in canine blood samples submitted to a US veterinary diagnostic laboratory. *J Vet Intern Med*. 2018; 32:1965–1969. https://doi.org/10.1111/jvim.15300