JCM
Journals.ASM.org

Pharmacogenomics of *Cytauxzoon felis* Cytochrome *b*: Implications for Atovaquone and Azithromycin Therapy in Domestic Cats with Cytauxzoonosis

Megan E. Schreeg,[a] Henry S. Marr,[a] Jaime Tarigo,[a] Leah A. Cohn,[b] Michael G. Levy,[a] Adam J. Birkenheuer[a]

North Carolina State University College of Veterinary Medicine, Raleigh, North Carolina, USA[a]; University of Missouri College of Veterinary Medicine, Columbia, Missouri, USA[b]

***Cytauxzoon felis*, an emerging virulent protozoan parasite that infects domestic cats, is treated with atovaquone and azithromycin (A&A). Atovaquone targets parasite cytochrome *b*. We characterized the *C. felis* cytochrome *b* gene (*cytb*) in cats with cytauxzoonosis and found a *cytb* genotype that was associated with survival in A&A-treated cats.**

Cytauxzoonosis is an emerging disease in domestic and wild felines in North and South America caused by the tick-transmitted apicomplexan protozoan parasite *Cytauxzoon felis* (1, 2). Without treatment, cytauxzoonosis is fatal in up to 97% of domestic cats (3). Recent advances in treatment combining atovaquone and azithromycin (A&A) have reduced the mortality rate to 40% (3). Azithromycin targets the mitochondrial ribosomes of the parasite, while atovaquone targets protozoal cytochrome *b* (*cytb*), disrupting electron transport in the parasite mitochondria (4, 5).

In related parasites, including *Babesia* and *Plasmodium* species, resistance to atovaquone treatment has been attributed to mutations in the *cytb* gene (6–11). However, to this point, no similar pharmacogenomic studies characterizing the *C. felis cytb* gene have been performed. The purpose of this study was to determine whether response to A&A treatment is associated with *C. felis cytb* genotype. Therefore, we characterized and compared *cytb* genotypes of *C. felis* isolates from cats with cytauxzoonosis that were treated with A&A or another type of antiprotozoal drug, imidocarb dipropionate, which does not interact with *cytb* (12).

Sixty-nine pretreatment DNA samples from cats with cytauxzoonosis were available from a previous study (3). Total DNA was isolated from 200 μl of infected feline whole blood using a commercial kit according to kit instructions (QIAamp DNA blood minikit; Qiagen Inc., Valencia, CA). All samples were confirmed to be infected by using a *Cytauxzoon felis*-specific PCR assay (13). Cats were treated with A&A ($n = 45$) or imidocarb dipropionate ($n = 24$), and clinical outcome was recorded (3). Full-length *C. felis cytb* was amplified in three overlapping fragments (Fig. 1). Primers for fragment 1 (forward, 5′-CTTAACCCAACTCACGTACC-3′; reverse, 5′-ATCTAGTGACAAGATATGAATCAACAC-3′), fragment 2 (forward, 5′-ACCTTGGTCATGGTATTCAG-3′; reverse, 5′-GATCTAGCTTCAACCAATGC-3′), and fragment 3 (forward, 5′-GCATAGATGTTCAAGTACTAATCC-3′; reverse, 5′-GGTTAATCTTTCCTATTCCTTACG-3′) were designed based on previously reported *C. felis cytb* sequence (GenBank accession no. KC207821) (Fig. 1). Each 50-μl reaction mixture contained 1 μl of DNA template, 50 pmol of each primer, 10 nmol of deoxynucleoside triphosphates (dNTPs), 1.75 U of Expand high-fidelity enzyme mix, and a 1× concentration of Expand high-fidelity buffer with $MgCl_2$ (Roche, Mannheim, Germany). Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 45 amplification cycles (95°C for 20 s, 53 to 56°C for 30 s, and 60°C for 45 s) and a final extension step at 68°C for 7 min (Techne Inc., Burlington, NJ). Annealing temperatures for fragments 1 to 3 were 53.8, 56, and 53°C, respectively (Fig. 1); a 60°C extension temperature was found to be superior to 72°C, presumably due to high AT nucleotide content (14). Positive controls consisted of *C. felis*-infected feline blood samples and negative controls consisted of water (no DNA). Amplicons were visualized on an agarose gel, purified, and sequenced bidirectionally (MCLAB, South San Francisco, CA); chromatograms were carefully inspected for heterogeneity. Any secondary peaks present at 30% or more of the primary nucleotide peaks in both forward and reverse sequence were edited accordingly using IUPAC ambiguity codes (Vector NTI; Invitrogen, Grand Island, NY) (Fig. 2). Contigs were assembled using a commercially available software package (BioEdit Sequence Alignment Editor; North Carolina State University, Raleigh, NC). Se-

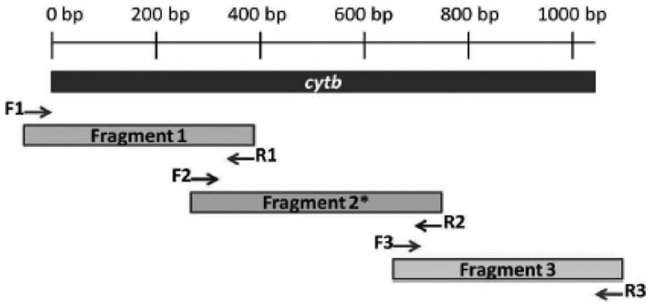

FIG 1 PCR amplification of *C. felis cytb* gene in three overlapping fragments. Full-length *cytb* gene (1,092 bp) was PCR amplified from 69 samples in three overlapping fragments. Primers are indicated with labeled arrows. Fragment 2 includes the putative atovaquone binding sites (*) (6).

Received 31 May 2013 **Accepted** 14 June 2013
Published ahead of print 19 June 2013
Address correspondence to Adam J. Birkenheuer, adam_birkenheuer@ncsu.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.01407-13.

doi:10.1128/JCM.01407-13

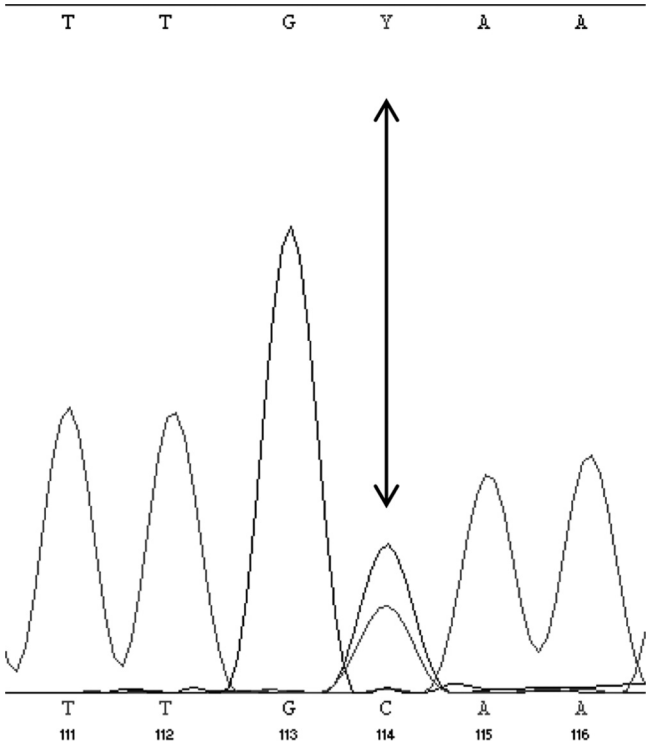

FIG 2 Presence of secondary peaks in *C. felis cytb* sequence as determined with vector NTI. All sequences were analyzed bidirectionally to detect the presence of secondary peaks (for example, the thymine present as a peak secondary to cytosine at position 114 in the chromatogram above). The nucleotide sequence for the sample was edited accordingly using the IUPAC ambiguity code (for example, "Y" in the sequence at the top).

quence data for ribosomal internal transcribed spacer (ITS) regions were characterized for 61 of 69 samples (3) and assessed for any potential associations with *cytb* genotype. Associations between categorical variables were analyzed using two-tailed Fisher exact probability tests, with a P value of <0.05 being considered significant (VassarStats, Poughkeepsie, NY).

A total of 30 *C. felis cytb* genotypes were characterized (Fig. 3). The majority of samples (46/69) showed evidence of infection with *C. felis* possessing a single genotype of *cytb*, while the remaining samples (23/69) had two or more *cytb* genotypes present. In the vertebrate host *C. felis* exists as haploid forms, which replicate asexually. While the majority of genetic material is on chromosomes, some apicomplexan genes, including *cytb*, are on extrachromosomal fragments of DNA within the apicoplast or mitochondria (15). In apicomplexan parasites, up to 20 copies of the mitochondrial genome can exist within one nondividing haploid organism (15). Therefore, samples containing two or more genotypes could represent a single haploid *C. felis* organism with multiple different copies of the *cytb* gene or multiple haploid organisms with different *cytb* genes.

One single *cytb* genotype was found in 13 samples and was designated cytochrome *b* genotype 1 (*cytb1*). Compared to *cytb1*, 35 different locations throughout the *cytb* gene were found to have single nucleotide substitutions. Of the 30 different *cytb* genotypes, only nine genotypes had nonsynonymous substitutions conferring amino acid changes in the *cytb* gene, with three of these being in or near the atovaquone-binding site (Fig. 3 and 4).

Due to a small sample size, only *cytb1* was assessed for association with survival in each treatment group. *cytb1* was found to be positively associated with survival ($P = 0.017$) compared to all other genotypes in cats treated with A&A (Table 1). However, there was no association between *cytb1* and survival ($P = 0.608$) in cats treated with imidocarb dipropionate (Table 1). While no statistical comparisons could be performed, all three cats infected with genotypes conferring amino acid changes in or near the putative atovaquone-binding site died; only one of these cats was treated with A&A (Fig. 3 and 4).

Some genotypes, including *cytb1*, were found only in certain states (see Table S1 in the supplemental material). *cytb1* was found only in Arkansas and Missouri. The association between *cytb1* and survival in the subpopulation of cats treated with A&A from Arkansas and Missouri remained significant ($P = 0.001$) when only these samples were considered, while there was again no association ($P = 0.545$) between *cytb1* and survival in cats from Arkansas and Missouri treated with imidocarb dipropionate. (see Table S2 in the supplemental material). Furthermore, when survival rates of cats from Arkansas and Missouri were compared to those of cats from Oklahoma, North Carolina, and Tennessee, where *cytb1* was not present, there was no significant difference in survival rates (see Table S3 in the supplemental material), indicating that the increased survival benefit of *cytb1*does not merely reflect a decreased virulence of *C. felis* in Arkansas and Missouri. We found no association between the most common ITS genotype (ITSc; GenBank accession no. EU450802/EU450804) and survival or ITS genotype and cytochrome *b* genotype (see Tables S4 to S6 in the supplemental material) (16, 17).

In this study, we found that the *C. felis cytb* gene sequence is highly variable. Despite the high variability of the *cytb* gene and a relatively small sample size, we were able to detect a *cytb* genotype (*cytb1*) that was associated with survival in cats treated with A&A (Table 1). We anticipate that with a larger sample size, the *cytb1* genotype would be detected in other regions, albeit in lower proportions. Additionally, we believe that it is likely that other genotypes, such as *cytb* genotype 3, may confer a survival benefit in cats treated with A&A (Fig. 3). Studies involving a larger sample size across a larger geographic range should be pursued to further assess these hypotheses.

Despite sharing an identical amino acid sequence with nearly all other *cytb* genotypes (Table 1), the *cytb1* genotype was associated with survival in cats treated with A&A. While a synonymous substitution does not cause an amino acid change, "silent" mutations can result in changes in protein amount, structure, or function (18, 19). For instance, differences in *cytb1* nucleotide sequence could have an effect on mRNA structure, stability, and translation kinetics (codon preference) (20). Work with human multidrug resistance 1 gene (*MDR1*) has shown that synonymous substitutions can alter the kinetics of translation, leading to alterations in protein folding and intracellular function (18, 21). Likewise, synonymous substitutions in *cytb* could possibly impact protein folding or structure and alter atovaquone binding. Another possibility is that the *cytb1* genotype is a genetic marker for alterations in promoter regions or neighboring mitochondrial genes. These genes include cytochrome *c* oxidase subunits I and III (*cox1* and *cox3*), which are involved downstream of *cytb* in the electron transport chain, and large subunit (LSU) rRNA fragments believed to be involved in translation of mitochondrial genes (5). Alterations in any of these genes could impact the metabolic effi-

AA	1	13	39	69	80	85	92	108	133	135	145	169	186	193	202	215	226	227	245	250	257	276	279	286	287	288	315	316	318	327	328	344	345	357	361			
NUC	3	38	115	207	240	255	274	324	399	405	435	507	558	578	604	645	676	681	733	750	771	826	835	858	861	862	944	947	952	981	984	1032	1035	1071	1081			
GT																																				n	Tx	%SUR
1	G	T	T	C	T	T	C	T	A	C	G	T	G	A	C	T	A	T	A	A	T	T	C	G	A	A	G	T	T	C	T	T	A	T	G	13	BOTH	0.769
2	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	7	BOTH	0.286
3	G	T	T	C	T	T	C	T	A	C	G	T	G	A	C	T	A	T	A	A	T	T	C	G	A	A	G	C*	T	C	T	T	A	T	G	6	BOTH	0.833
4	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	C	C	T	G	A	A	G	T	T	C	C	C	A	T	G	5	BOTH	0.6
5	G	T	C	C	T	C	C	C	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	4	A&A	0.5
6	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	Y	Y	T	G	A	A	G	T	T	C	Y	C	A	T	G	4	BOTH	0.25
7	G	T	Y	C	T	Y	C	Y	A	C	G	T	G	A	C	T	A	T	A	R	T	T	Y	G	A	A	G	T	T	C	T	Y	A	T	G	3	A&A	0.333
8	G	T	Y	C	T	Y	Y	T	A	C	G	T	G	A	C	T	A	T	A	R	T	T	C	G	A	A	G	T	T	C	T	Y	A	T	G	2	A&A	0
9	G	T	Y	C	T	Y	Y	Y	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	2	BOTH	0
10	G	T	T	C	T	T	C	T	G	C	G	T	G	A	C	T	A	T	A	A	T	T	C	G	A	A	G	T	T	C	T	T	A	C	G	2	A&A	0.5
11	G	T	C	C	T	C	T	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	2	A&A	0.5
12	A	T	C	C	T	C	C	C	A	C	G	T	G	A	T*	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	1	IMID	1
13	G	C*	T	C	G*	T	C	T	A	C	G	T	G	A	C	T	A	T	A	A	T	T	C	G	A	A	G	C*	T	C	T	T	A	T	G	1	IMID	1
14	G	T	C	C	T	C	C	C	A	C	G	C	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	1	A&A	1
15	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	C	T	T	G	A	A	G	T	T	C	C	C	A	T	G	1	IMID	1
16	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	T	T	C	G*	T	G	1	A&A	1
17	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	R*	G	T	T	T	T	C	A	T	G	1	A&A	0
18	G	T	C	C	T	C	C	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	Y	G	A	A	G	T	T	C	Y	C	A	T	G	1	A&A	1
19	G	T	C	C	T	T	C	T	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	1	A&A	0
20	G	T	T	C	T	T	C	T	A	C	G	T	G	A	C	T	A	T	A	A	T	T	C	A	A	A	G	T	T	C	T	T	A	T	G	1	A&A	1
21	G	T	T	C	T	T	Y	T	A	C	G	T	G	A	C	T	A	T	A	A	T	T	C	G	A	A	G	T	T	C	T	T	A	T	G	1	A&A	0
22	G	T	T	C	T	Y	C	T	A	Y	R*	T	G	A	C	T	R*	T	R	R	T	T	C	G	R	A	G	T	T	C	T	T	A	T	R*	1	IMID	0
23	G	T	T	C	T	Y	C	T	A	Y	R*	T	R	A	C	Y	A	T	A	R	T	T	C	G	A	A	R*	T	Y	C	T	T	A	T	R*	1	IMID	0
24	G	T	Y	C	T	Y	C	T	A	C	G	T	G	A	C	T	A	T	A	R	T	T	C	G	A	A	G	T	T	C	T	Y	A	T	G	1	A&A	0
25	G	T	Y	C	T	Y	C	T	A	C	G	T	G	R*	C	T	A	T	A	R	Y	Y	Y	G	A	A	G	T	T	C	Y	Y	A	T	G	1	A&A	1
26	G	T	Y	C	T	Y	C	Y	A	C	G	Y	G	A	C	T	A	T	A	R	T	T	Y	G	A	A	G	T	T	C	T	Y	A	T	G	1	IMID	0
27	G	T	Y	C	T	Y	C	Y	G	C	G	T	G	A	Y*	T	A	T	A	R	T	T	C	G	A	A	G	T	T	C	T	Y	A	Y	G	1	IMID	0
28	G	T	Y	C	T	Y	Y	Y	A	C	G	Y	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	1	A&A	0
29	R	T	C	C	T	C	C	Y	A	C	G	T	G	A	C	T	A	T	A	G	T	T	T	G	A	A	G	T	T	C	T	C	A	T	G	1	IMID	0
30	R	T	Y	Y	T	Y	C	Y	A	C	G	T	G	A	C	T	A	T	A	R	T	T	Y	G	A	A	G	T	T	C	T	Y	A	T	G	1	A&A	1

FIG 3 Characterization of 30 novel *C. felis cytb* genotypes. Thirty different genotypes were characterized from 69 total *C. felis* samples collected from cats with cytauxzoonosis. Thirty-five different point mutations were discovered throughout the gene, 11 of which altered nucleotide sequence in or near the putative atovaquone binding sites (denoted by gray shading and brackets at the bottom). Genotype 1 (*cytb1*) was present alone in 13 samples. Asterisks indicate mutations conferring amino acid changes. Nucleotides differing from the *cytb1* sequence are in black boxes. AA, amino acid; NUC, nucleotide; GT, genotype; Tx, treatment (A&A, atovaquone and azithromycin; IMID, imidocarb dipropionate; BOTH, different cats possessing this genotype were treated with A&A or imidocarb); %SUR, percent survival of cats infected with the indicated *cytb* genotype. Gray shading in the headings indicates the 3 different *cytb* PCR amplicons (fragments 1 to 3).

ciency of the mitochondria and fitness of the organism, rendering the parasite more susceptible to A&A treatment. Further studies are needed to discern the complete pharmacogenomic role of *C. felis cytb* genotypes.

In conclusion, the *cytb* genotype appears promising for predicting survival in cats with cytauxzoonosis treated with A&A. We are evaluating assays such as high-resolution melting curve analysis to rapidly characterize *cytb* genotypes from clinical

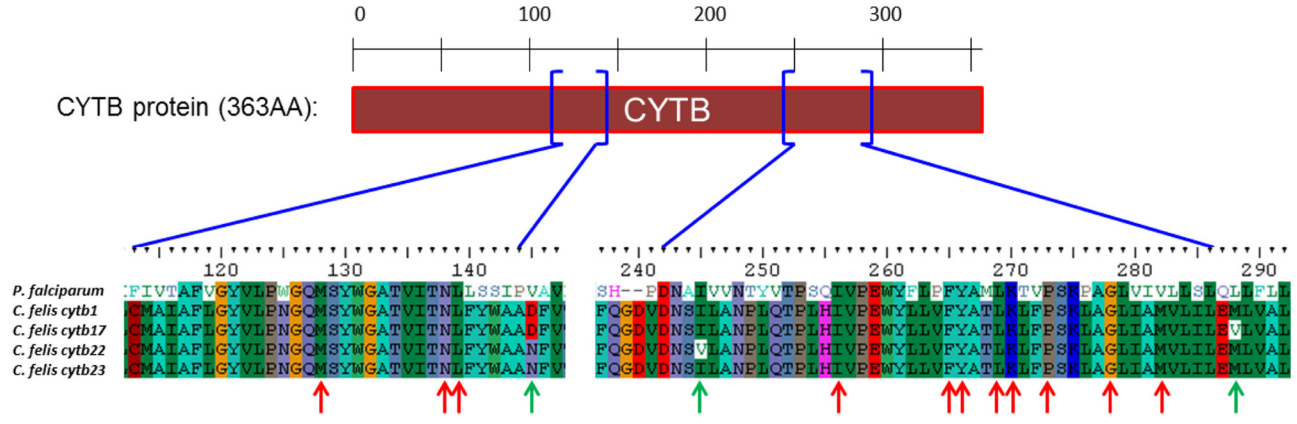

FIG 4 Evidence of missense mutations in or near putative atovaquone-binding sites of *C. felis* CYTB. Putative amino acid sequences of *C. felis* CYTB genotypes with missense mutations in the atovaquone-binding sites were aligned with the *P. falciparum* CYTB sequence. Blue-bracketed regions indicate putative atovaquone-binding sites, predicted by alignment with *P. falciparum* CYTB atovaquone-binding sites (6). Red arrows indicate sites of previously characterized mutations in related *Plasmodium* and *Babesia* species linked to atovaquone resistance. Green arrows indicate sites of missense mutations (compared to the amino acid sequence of *cytb1*) discovered in *C. felis* CYTB in this study.

TABLE 1 Correlation between survival rate of cats treated with A&A and *C. felis cytb* genotype[a]

Treatment and genotype	No. of cats		
	Surviving	Dead	Total
A&A			
cytb1	8	0	8
Non-*cytb1*	20	17	37
Total	28	17	45 ($P = 0.017$)
Imidocarb dipropionate			
cytb1	2	3	5
Non-*cytb1*	5	14	19
Total	7	17	24 ($P = 0.608$)

[a] Survival rate of cats infected with *C. felis cytb1* was compared to survival rate of cats infected with strains of all other genotypes combined ("non-*cytb1*"). Results were analyzed using a two-tailed Fisher exact probability test.

samples to provide prognostic information for cats with cytauxzoonosis.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Karen Gore.

This work was supported by The ALSAM Foundation.

We do not have any conflicts of interest to declare.

REFERENCES

1. **Andre MR, Adania CH, Machado RZ, Allegretti SM, Felippe PA, Silva KF, Nakaghi AC, Dagnone AS.** 2009. Molecular detection of Cytauxzoon spp. in asymptomatic Brazilian wild captive felids. J. Wildl. Dis. **45:**234–237.
2. **Brown HM, Lockhart JM, Latimer KS, Peterson DS.** 2010. Identification and genetic characterization of Cytauxzoon felis in asymptomatic domestic cats and bobcats. Vet. Parasitol. **172:**311–316.
3. **Cohn LA, Birkenheuer AJ, Brunker JD, Ratcliff ER, Craig AW.** 2011. Efficacy of atovaquone and azithromycin or imidocarb dipropionate in cats with acute cytauxzoonosis. J. Vet. Intern. Med. **25:**55–60.
4. **Mather MW, Henry KW, Vaidya AB.** 2007. Mitochondrial drug targets in apicomplexan parasites. Curr. Drug Targets **8:**49–60.
5. **Vaidya AB, Mather MW.** 2009. Mitochondrial evolution and functions in malaria parasites. Annu. Rev. Microbiol. **63:**249–267.
6. **Korsinczky M, Chen N, Kotecka B, Saul A, Rieckmann K, Cheng Q.** 2000. Mutations in Plasmodium falciparum cytochrome b that are associated with atovaquone resistance are located at a putative drug-binding site. Antimicrob. Agents Chemother. **44:**2100–2108.
7. **Sakuma M, Setoguchi A, Endo Y.** 2009. Possible emergence of drug-resistant variants of Babesia gibsoni in clinical cases treated with atovaquone and azithromycin. J. Vet. Intern. Med. **23:**493–498.
8. **Srivastava IK, Morrisey JM, Darrouzet E, Daldal F, Vaidya AB.** 1999. Resistance mutations reveal the atovaquone-binding domain of cytochrome b in malaria parasites. Mol. Microbiol. **33:**704–711.
9. **Syafruddin D, Siregar JE, Marzuki S.** 1999. Mutations in the cytochrome b gene of Plasmodium berghei conferring resistance to atovaquone. Mol. Biochem. Parasitol. **104:**185–194.
10. **Vaidya AB, Mather MW.** 2000. Atovaquone resistance in malaria parasites. Drug Resist. Updat. **3:**283–287.
11. **Wormser GP, Prasad A, Neuhaus E, Joshi S, Nowakowski J, Nelson J, Mittleman A, Aguero-Rosenfeld M, Topal J, Krause PJ.** 2010. Emergence of resistance to azithromycin-atovaquone in immunocompromised patients with Babesia microti infection. Clin. Infect. Dis. **50:**381–386.
12. **Wickramasekara Rajapakshage BK, Yamasaki M, Hwang SJ, Sasaki N, Murakami M, Tamura Y, Lim SY, Nakamura K, Ohta H, Takiguchi M.** 2012. Involvement of mitochondrial genes of Babesia gibsoni in resistance to diminazene aceturate. J. Vet. Med. Sci. **74:**1139–1148.
13. **Birkenheuer AJ, Le JA, Valenzisi AM, Tucker MD, Levy MG, Breitschwerdt EB.** 2006. Cytauxzoon felis infection in cats in the mid-Atlantic states: 34 cases (1998–2004). J. Am. Vet. Med. Assoc. **228:**568–571.
14. **Su XZ, Wu Y, Sifri CD, Wellems TE.** 1996. Reduced extension temperatures required for PCR amplification of extremely A+T-rich DNA. Nucleic Acids Res. **24:**1574–1575.
15. **Wilson RJ, Williamson DH.** 1997. Extrachromosomal DNA in the Apicomplexa. Microbiol. Mol. Biol. Rev. **61:**1–16.
16. **Brown HM, Berghaus RD, Latimer KS, Britt JO, Rakich PM, Peterson DS.** 2009. Genetic variability of Cytauxzoon felis from 88 infected domestic cats in Arkansas and Georgia. J. Vet. Diagn. Invest. **21:**59–63.
17. **Brown HM, Modaresi SM, Cook JL, Latimer KS, Peterson DS.** 2009. Genetic variability of archived Cytauxzoon felis histologic specimens from domestic cats in Georgia, 1995–2007. J. Vet. Diagn. Invest. **21:**493–498.
18. **Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, Gottesman MM.** 2007. Silent polymorphisms speak: how they affect pharmacogenomics and the treatment of cancer. Cancer Res. **67:**9609–9612.
19. **Sauna ZE, Kimchi-Sarfaty C.** 2011. Understanding the contribution of synonymous mutations to human disease. Nat. Rev. Genet. **12:**683–691.
20. **Sauna ZE, Kimchi-Sarfaty C, Ambudkar SV, Gottesman MM.** 2007. The sounds of silence: synonymous mutations affect function. Pharmacogenomics **8:**527–532.
21. **Shabalina SA, Spiridonov NA, Kashina A.** 2013. Sounds of silence: synonymous nucleotides as a key to biological regulation and complexity. Nucleic Acids Res. **41:**2073–2094.