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

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*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'-CTTAAACCAAATCAGTA CC-3'); reverse, 5'-ATCTAGTGCAAGATATGAATCGC-3'), fragment 2 (forward, 5'-ACCTTGGTCCATGGATTCG-3'; reverse, 5'-GTCTAGCTTCAACCAATGC-3'), and fragment 3 (forward, 5'-GCTCTAGAGTTCTACATTACCC-3'; reverse, 5'-GGTTAATCTTTTCTATTTCCTACG-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 1× concentration of Expand high-fidelity buffer with MgCl₂ (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).

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**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).
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-

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**FIG 2** Presence of secondary peaks in *C. felis* cytb sequence as determined with vector NTL. 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).
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 pre-
dicting 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
samples.

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).

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.
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