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Cytauxzoon felis cytochrome *b* gene mutation associated with atovaquone and azithromycin treatment

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Abstract

Background: Atovaquone and azithromycin (A&A) with supportive care improve survival rates in cats with cytauxzoonosis. Resistance to atovaquone via parasite cytochrome *b* gene (*cytb*) mutations occurs in other Apicomplexan protozoans but is not described in *Cytauxzoon felis*.

Objective: To serially characterize the *C. felis* cytb sequences from a cat that remained persistently infected after A&A treatment.

Animal: A cat with naturally occurring *C. felis* infection.

Methods: Case report of the anemic cat persistently infected with *C. felis* before, during and after A&A treatment. *Cytauxzoon felis* cytb genes were amplified and sequenced before, during and after A&A treatment.

Results: *Cytauxzoon felis* was detected before, during and after A&A treatment including samples collected 570 days after treatment. After A&A treatment, the cat's anemia improved slightly. *Cytb* sequencing revealed only wild-type cytb methionine (M128) in samples collected before treatment. In samples collected after treatment, the cytb coded for isoleucine (M128I) and valine (M128I) at 2- and 4-months after treatment. These M128I and M128V mutations persisted even after a repeat treatment course with a higher dose atovaquone combined with the standard dose of azithromycin.

Conclusions and Clinical Importance: This report documents *C. felis* atovaquone resistance associated with M128 *cytb* mutations. This study suggests parasites with mutations of *cytb* M128 can be selected and impart resistance to A&A treatment even with higher atovaquone dosing.

KEYWORDS

anemia, apicomplexan, cytauxzoonosis, feline, mitochondrial, resistance

1 | INTRODUCTION

Cytauxzoonosis is typically an acute-onset disease of felids caused by infection with the apicomplexan parasite *Cytauxzoon felis*. Felids become infected with this parasite through a tick bite. Clinical signs

Abbreviations: A&A, atovaquone and azithromycin combination therapy; AUS, abdominal ultrasound; CBC, complete blood count bloodwork; *cytb*, cytochrome *b*; DNA, deoxyribonucleic acid; HCT, hematocrit; M128, wild-type methionine 128 of *C. felis* CYTB; NCSU-VBDDL, North Carolina State University College of Veterinary Medicine (CVM) Vector Borne Disease Diagnostic Laboratory; NCSU-VHC, NCSU Veterinary Health Complex; PCV/TS, packed cell volume/total solids; RR, reference range.

include fever, lethargy, icterus, and death in severe cases. In combination with azithromycin, atovaquone is the preferred treatment for *C. felis*. In a randomized, prospective study of acute feline cytauxzoonosis, atovaquone and azithromycin (A&A) treatment results in 60% survival to discharge in cats treated for acute cytauxzoonosis; alternatively, significantly fewer (26%) cats survive to discharge with imidocarb diproprionate treatment alone.[1]

Atovaquone is exploited for its antiprotozoal activity as part of multidrug therapy for treatment of cytauxzoonosis and other Apicomplexan infections, including human malaria and mammalian babesiosis. Atovaquone acts as a structural analog of protozoan ubiquinone, a mitochondrial protein also known as coenzyme Q, that is integrally involved in electron flow during aerobic respiration. In particular, ubiquinone passes electrons to cytochrome bc_1 via binding to a specific Qo cytochrome domain; atovaquone specifically inhibits this step.[2]

Apicomplexan parasite recrudescence is associated with atovaquone monotherapy in human *Plasmodium* sp. infections; concurrent treatment with other agents (such as proguanil for *Plasmodium* sp.) is typically instituted to enhance synergistic antiprotozoal activity. Mechanisms of atovaquone resistance are correlated with parasite cytochrome *b* mutations in both *Plasmodium* sp.[3] and *Babesia* sp.[4] parasites. Previous studies of *C. felis cytb* sequences collected before treatment reveal a pre-existing *cytb1* genotype positively associated with survival in atovaquone and azithromycin-treated cats.[5,6] Yet, to date, atovaquone resistance through parasite gene mutation in *cytb* is yet to be documented in *C. felis*.

In this context, we describe the clinical course of a *C. felis*-infected cat that presented to an academic veterinary referral practice for evaluation and A&A treatment. We hypothesized that a *C. felis*-infected cat remaining persistently parasitemic, despite appropriate A&A treatment, would display *cytb* mutations. Specifically, we aimed to serially characterize the *C. felis cytb* genotypes in this cat after treatment with a standard and high-dose of atovaquone regimen combined with azithromycin.

2 | MATERIALS AND METHODS

2.1 | Patient selection

In August 2015, an approximately 9-year-old, female spayed domestic shorthair from Vass, North Carolina (NC), USA presented to a NC veterinarian for evaluation for health and possible adoption assessment. The cat was quiet, alert, responsive, normothermic at 99 °F, and mildly hyporexic. Bilateral yellow, thick nasal discharge and bilateral complete cataracts were noted. Combination retroviral testing for feline leukemia and feline immunodeficiency viruses (FeLV/FIV) and heartworm testing were negative twice. Bloodwork revealed anemia (hematocrit [HCT] of 23%: reference range [RR], 30%-45%). Abnormalities were not detected on biochemistry bloodwork or thoracic radiographs. After 5 days of receiving doxycycline, iron supplementation, and fenbendazole, an improved HCT of 27%, improved appetite, and no nasal discharge were observed.

When the cat's anemia persisted (HCT 23.2%) 11 days after initial presentation, a comprehensive infectious disease panel was submitted to a veterinary diagnostic laboratory (North Carolina State University (NCSU), College of Veterinary Medicine Vector Borne Disease Diagnostic Laboratory (VBDDL), Raleigh, North Carolina). Results included a positive PCR for *C. felis*. Remaining test results were negative for *Babesia*, *Bartonella*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, and hemotropic *Mycoplasma* organisms by PCR as well as antibodies for *Bartonella henselae*, *Bartonella vinsonii*, and *Bartonella koehlerae* by indirect immunofluorescent antibody (IFA) analysis and to *Borrelia*, *Anaplasma*, *Ehrlichia*, and heartworm by ELISA. The cat was subsequently referred to a veterinary teaching hospital (NCSU Veterinary Health Complex [NCSU-VHC]) for further evaluation and A&A treatment.

2.2 | Samples

Blood from the jugular or medial saphenous veins was collected in EDTA tubes (BD Vacutainer) from day 0 of starting A&A treatment and at various times points to 570 days after first A&A treatment. These blood samples were submitted for *C. felis* deoxyribonucleic acid (DNA) isolation and PCR evaluation. Samples previously submitted to a veterinary diagnostic laboratory (NCSU-VBDDL) for this patient up to 30 days before treatment were identified and included in the study. Weekly packed cell volumes and total solids (PCV/TS) were collected weekly initially and then approximately monthly after 3 months of initial treatment.

2.3 | Atovaquone and azithromycin treatment

The cat was initially treated with atovaquone (Mepron, GlaxoSmithKline, Research Triangle Park, North Carolina) at 15 mg/kg PO q8hr and azithromycin (azithromycin suspension, Greenstone LLC, Peapack, New Jersey) at 10 mg/kg PO q24hr for 10 days.[1] A second course of A&A treatment was pursued at 150 days with an increased atovaquone dosing (25 mg/kg PO q8hr) and standard azithromycin (10 mg/kg PO q24hr) dosing.

2.4 | DNA isolation and PCR

Total DNA was extracted from 200 μL of anticoagulated whole blood using a commercial DNA extraction kit (QIAmp DNA blood mini kit [Qiagen, Valencia, California]) according to the manufacturer's protocol. DNA samples were stored at −20°C. Infected samples were confirmed using a previously described *C. felis*-specific 18S PCR assay.[7] A 181 base-pair fragment of the *C. felis cytb* that encodes for the putative atovaquone binding region was amplified using primers designed based on previously reported sequence (GenBank accession no. KC207821).[6] Additional primers targeting cytochrome *c* oxidase subunit III (*cox3*) were used for replicate testing for enhanced PCR sensitivity on the sample taken 1 month after the first A&A

treatment.[8] Thermal cycler conditions consisted of a denaturing phase at 95°C for 3 minutes followed by 55 cycles of 95°C for 10 seconds, 53°C for 20 seconds, and 72°C for 30 seconds with a plate read following each cycle. Individual PCR reactions contained 25 μL of PCR mastermix (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad, Hercules, California), 25 pmol of CFcytbF (5′-CTA CCT TGG TCA TGG TAT TC-3′), 25 pmol of CFcytb R (5′-CTA CCA ACA CTG TAA CCA C-3′), and 5 μL of DNA totaling 50 μL per PCR reaction. Five replicate PCR reactions were completed for each time point to facilitate the amplification and detection of genotypes present in lower concentrations.[8] Controls consisted of *C. felis*-infected blood samples (positive control) and DNA-free water (negative controls). Amplicons from each replicate reaction were sequenced bidirectionally by a commercial laboratory (Genewiz LLC, Research Triangle Park, North Carolina). Chromatograms were scrutinized manually for mutations associated within the M128 position (based on GenBank accession no. KC207821) of the wild-type *C. felis cytb* gene.

Additional PCR assays were performed to determine cytochrome *b* mutations, specifically which nucleotides at the first codon position were linked to each third codon position coding for amino acid 128. Three reverse primers specific to each nucleotide at the third codon position (T REV: 5′-GTT GCT CCC CAA TAG CTA-3), (A REV: 5′-GTT GCT CCC CAA TAG CTT-3′) or (G REV: 5′-GTT GCT CCC CAA TAG CTC-3) were designed. Individual PCR reactions of samples collected 60 days after treatment contained 25 μL of the same PCR mastermix as above with 25 pmol of each reverse primer paired with 25 pmol of forward primer (CF CYT B FWD: 5′-CTA CCT TGG TCA TGG TAT TC-3). Thermal cycler conditions consisted of a denaturing phase at 95°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 following each cycle. These amplicons were sequenced directly using the forward primer and chromatograms were manually inspected for heteroplasmy.

3 | RESULTS

The cat initially presented to the veterinary teaching hospital (NCSU-VHC) in September 2015, bright, alert, responsive, eupneic, and normothermic. Physical exam revealed bilateral complete cataracts, no nasal discharge, and subtle hindlimb paraparesis. Complete blood count (CBC) revealed a normocytic, normochromic nonregenerative anemia (PCV 24%, 19 000 reticulocytes/μL), mild neutrophilia (7229/μL; RR, 2773-6975), moderate eosinophilia (1541/μL; RR, 118-879), and mild thrombocytosis (platelets 438 000/μL; RR, 198 000-434 000). Microscopic examination of a stained blood film revealed 1 intraerythrocytic organism consistent with *C. felis*. Serum biochemistry revealed a mildly increased BUN (41 mg/dL; RR, 15-37) and mild hypernatremia (156 mEq/L; RR, 148-155); the remainder was within normal limits, with a total bilirubin (<0.2 mg/dL; RR, 0-0.2), of note. Coomb's testing was negative at both 25°C and 37°C; no saline agglutination was noted. A urine specific gravity of 1.020 and 1 + protein was appreciated on urinalysis. Abdominal ultrasound revealed diffuse splenic nodules, heterogenous hepatic parenchyma, mild jejunal lymphadenopathy, and bilateral chronic nephropathy; fine needle aspirates of spleen, liver, and mesenteric lymph nodes were obtained. Splenic aspirate cytology revealed evidence of mild lymphoid hyperplasia, with no evidence of *Cytauxzoon* schizonts. Cytology of a hepatic aspirate revealed mild neutrophilic inflammation, with no etiologic agents or evidence of neoplasia appreciated. Mesenteric lymph node aspiration was consistent with a mildly reactive lymph node. Treatment with atovaquone (15 mg/kg PO q8hr) and azithromycin (10 mg/kg PO q24hr) (A&A) was initiated for 10 days. Seven days into *C. felis* treatment with A&A, a repeat CBC bloodwork revealed regenerative anemia (PCV 21%, 79 000 reticulocytes/μL). A mild leukocytosis (15 020/μL; RR, 4280-14 300) with mild neutrophilia (9312/μL; RR, 2773-6975), improving eosinophilia (1202/μL; RR, 118-879), and resolved thrombocytosis (platelets 295 000/μL; RR, 198 000-434 000) was noted.

Serial PCV measurements, *C. felis* PCR results, and *cytb* genotypes are presented in Figure 1. One-month after A&A initiation, the cat was no longer anemic (PCV 34%) and *C. felis* 18S PCR testing was negative. Replicate testing of this sample targeting *cox*3 demonstrated a low-level parasitemia with 1/10 replicates testing positive for the presence of *C. felis*. There was a moderate inflammatory response (leukocytosis (25 920/μL; RR, 4280-14 300), characterized by a neutrophilia (22 550/μL; RR, 2773-6975) with left shift (banded neutrophils of 518/μL)) and improved eosinophilia (1037/μL; RR, 118-879). Chemistry revealed a persistently increased BUN (46 mg/dL; RR, 15-37), increasing creatinine (1.6 mg/dL [previously 1.2 mg/dL 1 month prior]; RR, 0.7-1.9), hypoalbuminemia (2.7 mg/dL; RR, 2.9-4), hypokalemia (3.2 mmol/L; RR, 3.5-5.1), and increased anion gap (25.2; RR, 16-22). Suspicious that the cat could be suffering from occult pyelonephritis, a 4-week course of marbofloxacin (12.5 mg PO q24hr, Zeniquin, Zoetis Inc, Kalamazoo, Michigan) was instituted and completed. Urine culture results which were finalized 3 days after beginning treatment with marbofloxacin were subsequently negative.

At 60 days after A&A treatment, repeat 18S PCR analysis of blood was again positive for *C. felis* DNA; no merozoites would be seen on blood smear. There was resolved anemia (PCV 33%), persistent eosinophilia (1275/μL; RR, 118-879), and neutrophilia. An increased BUN (44 mg/dL; RR, 15-37) and anion gap (25.6; RR, 16-22) continued, with resolution of creatinine (1.1 mg/dL; RR, 0.7-1.9), electrolyte, and albumin abnormalities. Repeat testing at 90- and 120-days after A&A treatment would confirm persistent *C. felis* infection (Figure 1). Ineffective treatment because of atovaquone resistance was suspected.

To identify for cytochrome *b* mutations yielding atovaquone resistance, PCR amplification of the *C. felis* cytochrome *b* gene (*cytb*) from DNA positive samples was completed. Bidirectional sequencing of 5 replicate PCR reactions revealed mutations (Figure 2A) in the codon for the *C. felis* CYTB amino acid 128 position, which is the putative atovaquone binding site. These mutations changed from original wild-type methionine (ATG, M128) to either isoleucine (ATT, M128I) in 4 of 5 sequence reads or valine (GTG, M128V) in 1 of 5 sequence reads at 60 days after A&A treatment. Repeat PCR

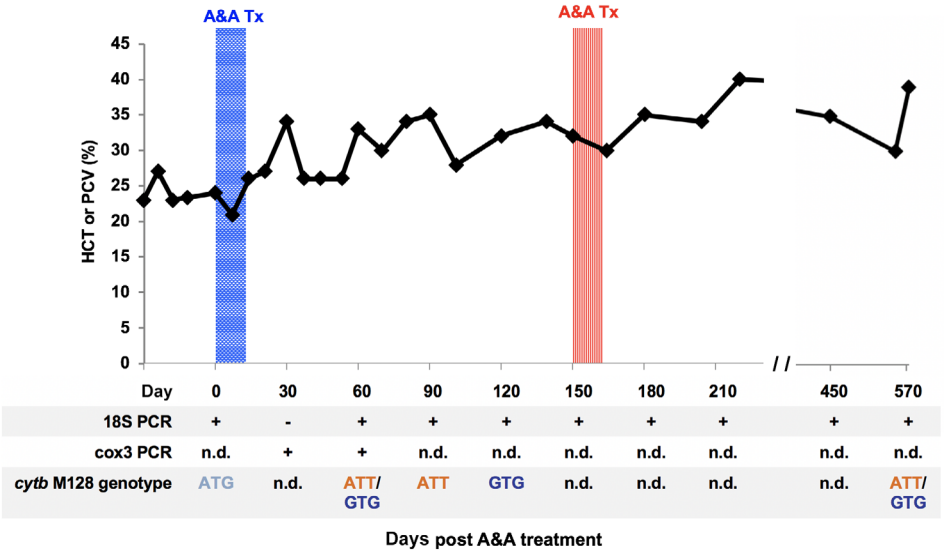

FIGURE 1 Serial PCV or HCT measurements, *Cytauxzoon felis* PCR results, and M128 genotypes during study. Hematocrit (HCT) or packed cell volume (PCV) following initial (blue checkered shade) and repeat increased atovaquone (red striped shade) 10-day A&A treatment (Tx) courses, up to 570 days. Results of *C. felis* DNA detection by 18S and cox3 PCR are indicated by positive ("+") and minus ("−") signs. *C. felis* cytb M128 genotypes are defined as wild type ("ATG") vs mutated M128I ("ATT") or M128V ("GTG"). Samples not determined are indicated by "n.d."

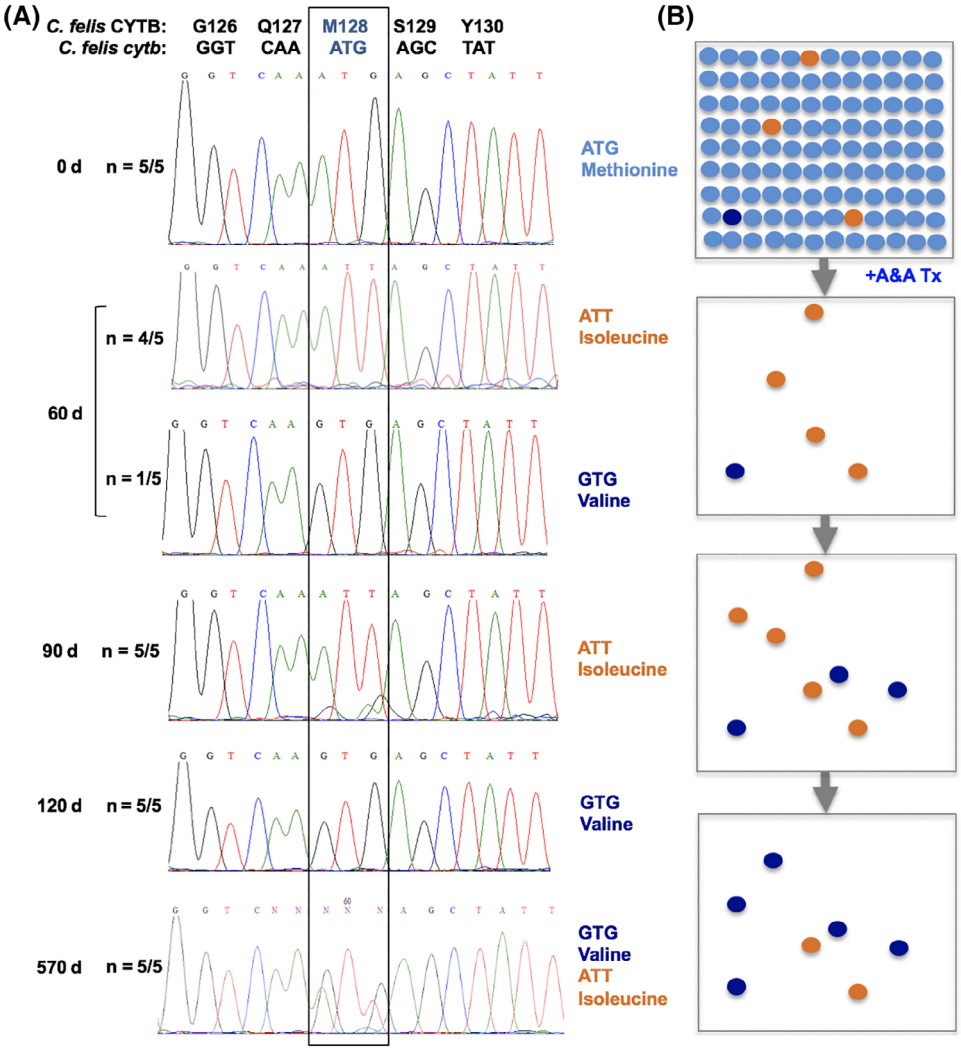

FIGURE 2 *Cytauxzoon felis* cytb sequence reads and proposed parasite populations before and after A&A treatments. A, Sequence reads of the *C. felis* CYTB M128 atovaquone binding region (black box) at time points before (day 0 = 0 d), after first (60 d, 90 d, 120 d) and increased (570 d) A&A treatment courses. Number of individual sequence reads of 5 sequence reads are denoted by "n." B, Hypothesized selection of parasites possessing wild-type methionine (ATG, M128, light blue circles) or mutated isoleucine (ATT, M128I, orange circles) or valine (GTG, M128V, dark blue circles) at CYTB M128 position after treatment

amplification and sequence reads of 90- and 120-days after A&A treatment blood revealed similar mutations of M128I and M128V, respectively. PCR assays and sequencing to assess codons in samples with heteroplasmy at position 128, only detected ATT (isoleucine), ATA (isoleucine), and GTG (valine). There was no detection of the wild-type ATG (methionine).

In an attempt to overcome potential drug resistance, an increased dose of atovaquone (25 mg/kg PO q8hr) with standard dose azithromycin (10 mg/kg PO q24hr) treatment regime was provided for 10 days, 150 days after initial A&A treatment. The cat showed no clinical or hematological adverse effects of this increased atovaquone dose. Fourteen days after the second A&A treatment, *C. felis* DNA was again detected in the cat's blood. *Cytb* sequence analysis revealed persistence of M128V and M128I. There was an increase in red blood cell concentration from start of the second treatment (day 150; PCV/TS 32%/7.7 g/dL) to a maximum at day 220 (PCV/TS 40%/7.2 g/dL). The cat remained PCR positive for *C. felis* for at least 570 days, and the *cytb* mutations (M128V, M128I) remained detectable in all samples collected after A&A treatment (420 days after second A&A treatment). Wild-type M128 was never definitively identified in any samples after day 0.

4 | DISCUSSION

Mutations of *C. felis* cytochrome *b* (*cytb*) M128 position after atovaquone and azithromycin treatment have not been documented previously. This report describes the detection of mutations of the putative atovaquone binding site of CYTB of *C. felis* after A&A treatment. Specifically, the hydrophobic methionine at position 128 of CYTB is replaced by the nonpolar aliphatic amino acid valine or isoleucine in the catalytic Qo site of the cytochrome *b* complex. This finding is striking as M128 is only 1 of over 15 highly conserved amino acid residues responsible for binding of atovaquone to *cyt bc1* in structural models.[9] The M128I and M128V genotypes do not appear to be common as they were not identified in a prior study that characterized *cytb* genotypes from 69 cats with cytauxzoonosis.[5]

We hypothesize these are not *C. felis cytb* mutations that were directly induced by atovaquone but instead represent the selection of pre-existing *cytb* genotypes not previously appreciated in the original sample (Figure 2B). Sequencing 5 amplicons at each time point after treatment allows for increased sensitivity for the detection of genotypes present at lower concentrations. Despite repeated sequencing, the wild-type M128 genotype was never detected in any sample collected after A&A treatment. These results support the theory of selection vs induced mutations.

Documented CYTB methionine residue mutations associated with atovaquone resistance are associated with *Plasmodium falciparum* and *Plasmodium berghei* isolates following antimalarial treatment, and additionally, these parasites can tolerate 1000-fold higher atovaquone concentrations in vitro.[3] Testing of the *C. felis* mutants identified here with atovaquone binding affinity assays as well as in vitro testing of atovaquone concentration tolerance were desired, yet these techniques are currently unfeasible for *C. felis* because of inability to culture or maintain in vitro. In an attempt to investigate if *cytb* mutant parasites could tolerate increased atovaquone concentration, repeat treatment with an almost 2-fold dose of atovaquone was attempted. Results indicate that this cat remained infected with *C. felis* mutant *cytb* after atovaquone treatment. These results additionally suggest the M128V/I mutation may confer this resistance.

Chronic erythroparasitemia with *C. felis* is generally considered to be nonpathogenic in domestic cats. This is supported by a prevalence of *C. felis* averaging 6.2% in healthy domestic cats residing in endemic areas, although clinicopathologic data for the cats in this study were not reported.[10] As the cat had been strictly housed indoors as an individual foster, reinfection was highly unlikely. The apparent clinical response of the cat's anemia in the current report after A&A treatment suggests some cats may have clinical effects secondary to chronic infection with *C. felis*. Clinical improvement despite a failure to eliminate infection after treatment is similarly reported with *Babesia*.[11,12] No definitive cause and effect relationship exists between this cat's persistent anemia and chronic *C. felis* infection. Defining whether or not chronic *C. felis* infection is associated with persistent anemia (ie, anemia of chronic disease or anemia of inflammation) in cats is warranted. PCR assays are imperative to detect chronic infections as blood smear examinations may be negative.

Further studies are indicated to determine the prevalence of atovaquone resistant *C. felis* strains. As our initial characterization of *C. felis cytb* genotypes did not detect any mutations at the M128 position, specific attention should be paid to chronically infected carriers as well as A&A treatment survivors. Housing cats indoors combined with the use of approved acaricides are imperative to prevent transmission.

5 | CONCLUSION

This study documents M128 *cytb* mutations in *C. felis* associated with atovaquone resistance. This case suggests that parasites with mutations of CYTB can be selected with treatment and impart resistance to A&A treatment even in the face of increased atovaquone dosing.

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CONFLICT OF INTEREST DECLARATION

A. Birkenheuer codirects the Vector Borne Disease Diagnostic Laboratory (receives no compensation). H. Marr is paid in part by the Vector Borne Disease Diagnostic Laboratory.

OFF-LABEL ANTIMICROBIAL DECLARATION

Atovaquone and azithromycin are not labeled for the treatment of cytauxzoonosis in cats.

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

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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