Supplementary information: Second illness due to *Cytauxzoon felis* infection in a domestic cat

Cohn LA, et al.

DNA extraction methods for Formalin-fixed paraffin embedded (FFPE) liver tissue samples stored on slides

Formalin-fixed paraffin embedded (FFPE) five micrometer sections of liver tissue mounted on slides were submitted to the Vector Borne Disease Diagnostic Laboratory (North Carolina State University, College of Veterinary Medicine, Raleigh, NC) for DNA extraction and PCR testing. FFPE liver tissue slides from a negative cat was submitted for testing and was used as a negative control. The control cat was obtained from a cat without infectious or hepatic disease and submitted for necropsy through the University of Missouri Veterinary Medical Diagnostic Laboratory within days of submission of the tissue from the subject of this report.

Samples were prepared for DNA extraction by scraping off slide contents (three slides each) into 2mL Eppendorf tubes using a standard disposable scalpel with a #10 blade. Sample lysis was performed by adding 400µL of ATE buffer and 40µL of Proteinase K and incubating samples overnight at 56°C (DNeasy Blood & Tissue Kit, Qiagen, Valencia, CA). Being careful to avoid dissolved paraffin along the sides of the tube, a volume of 200µL of lysate from each sample was transferred to a clean 2mL Eppendorf tube. DNA was extracted from the FFPE tissue lysate by magnetic bead separation using an automated DNA workstation (QIAsymphony SP using the DSP DNA Mini Kit 192, Qiagen, Valencia, CA)

Quantitative Polymerase Chain Reaction (qPCR) Assays

qPCRs used for the detection of *Cytauxzoon felis*

Cytauxzoon felis was identified using three gene targets LSU4, COX-3, and 18s rRNA. All three qPCRs were run using the same conditions and with the same qPCR machines, the CFX96 Real-Time System C1000 Touch Thermal Cyclers (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Conditions for each qPCR included a denaturing phase at 98°C for 3 minutes followed by 40 cycles at 98°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. The melt curve analysis ranged from 65°C to 95°C in 0.5°C increments. Each well contained 12.5µL of SsoAdvanced™ Universal SYBR® Green Supermix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), varying concentrations of primer (Supplementary Table 1), 5µL of template DNA, and molecular grade water for a total of 25µL per well.

DNA Quality Control qPCR

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene target to confirm the DNA was amenable to PCR and lacked inhibitors. Conditions for the housekeeping qPCR included a denaturing phase at 98°C for 3 minutes followed by 40 cycles at 98°C for 15 seconds, 57°C for 15 seconds, and 72°C for 15 seconds. The melt curve analysis ranged from 65°C to 95°C in 0.5°C increments. Each well contained 12.5µL of SsoAdvanced™ Universal SYBR® Green Supermix (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), 10pmol concentration of primers (Supplementary Table 1), 5µL of template DNA, and molecular grade water for a total of 25µL per well.

Primers	Primer Sequence 5'->3'	Primer Concentration (pmol)	References
Apicomplexa 18s F3	GCAGTTAAAAAGCTCGTAGTTGAATT	2.5	(1)
Apicomplexa 18s R2	GTT AAA TAC GAA TGC CCC CAA	2.5	(1)
Bab-LSU4 F	ACC TGT CAA RTT CCT TCA CTA AMT T	15	(2)
Bab-LSU4 R2	TCT TAA CCC AAC TCA CGT ACC A	15	(2)
BMIC-LSU4 F	TTG CGA TAG TAA TAG ATT TAC TGC	10	(2)
Cfelis cox3 F	GCA TAT CTT CAA ATT ACA GAT ACA C	10	(3)
Cfelis cox3 R	CCA GTA ACT GTT TAG TGT AGT TAA C	10	(3)
HKA_GAPDH	CCT TCA TTG ACC TCA ACT ACA T	10	(4)
HKB_GAPDH	CCA AAG TTG TCA TGG ATG ACC	10	(4)

Supplementary Table 1

Supplementary References

1. Tyrrell JD, Qurollo BA, Tornquist SJ, et al. Molecular identification of vector-borne organisms in *Ehrlichia* seropositive Nicaraguan horses and first report of *Rickettsia felis* infection in the horse. *Acta Trop*. 2019;200:105170.

2. Qurollo BA, Archer NR, Schreeg ME, et al. Improved molecular detection of *Babesia* infections in animals using a novel quantitative real-time PCR diagnostic assay targeting mitochondrial DNA. *Parasit Vectors*. 2017;10(1):128.

3. Schreeg ME, Marr HS, Griffith EH, et al. PCR amplification of a multi-copy mitochondrial gene (cox3) improves detection of *Cytauxzoon felis* infection as compared to a ribosomal gene (18S). *Vet Parasitol*. 2016;225:123-30.

4. Birkenheuer AJ, Levy MG, Breitschwerdt EB. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *J Clin Microbiol*. 2003;41(9):4172-7.