MOLECULAR ASSESSMENT OF NORTH AMERICAN VECTOR-BORNE HEMOPARASITES AND *IN VITRO* STUDIES

A Dissertation

by

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ABSTRACT

The hemoparasite *Cytauxzoon felis* causes cytauxzoonosis, an emerging infectious disease of cats in the United States. Its life cycle involves sexual reproduction in the tick vector and asexual reproduction (schizogonous and erythrocyctic stages) in the felid. *Cytauxzoon felis* studies involve the use of infected cats, and *in vitro* cultivation would obviate this use as well as provide a continuous parasite source. Modeled after the microaerophilous stationary phase system used in cultures of *Babesia bovis*, a similar hemoparasite, 332 *C. felis* cultures were initiated to optimize the *in vitro* cultivation of the erythrocytic stage. Although no continuous cultivation was achieved, parasites were maintained short-term at a percentage of parasitized erythrocytes ranging from undetectable to 1.1% for two days to 140 days, with an average of 32 days. Five cultures underwent subculture four times, the maximum level achieved.

Cytauxzoonosis can manifest itself as a recovered, chronic, or fatal infection. Historically, nearly all infected cats died, but as years passed, the number of cats that did survive increased, which may be attributed to different strains of *Cytauxzoon felis*. Internal transcribed spacer regions 1 and 2 (ITS1 and ITS2) were assessed for genetic variation and used for determining clinical outcome and spatial correlations. Even though the ITS1 and ITS2 regions are genetically diverse, they do not aid in understanding differences in *C. felis* strains, as there was no association with clinical outcome or geography.

A potential *C. felis* coinfection with a hemoplasma was assessed for difference in clinical outcome. Polymerase chain reaction (PCR) and subsequent cloning showed clones of 11 of 41 *C. felis*-infected cats as positive for *Candidatus* Mycoplasma haemominutum with at least 99% identity and two other cats as positive for *Rhodococcus* spp. with at least a 97% identity. Clones of one of these two cats also matched *Candidatus* Mycoplasma kahanei with 99% identity.

Based on prior molecular evidence of *B. bovis* and the sylvatic component of the *C. felis* life cycle, 161 ticks removed from white-tailed deer in south Texas were molecularly surveyed for hemoparasites. No ticks were positive for species of *Babesia, Cytauxzoon,* or *Theileria.*

DEDICATION

This dissertation is dedicated to my father, Grover Pollard, Jr.✞ He was my biggest supporter and cheerleader. Although he is no longer physically here, I can still feel his presence. He will always be the driving force behind everything I do.

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NOMENCLATURE

DIC	disseminated intravascular coagulation
PCR	polymerase chain reaction
ITS	internal transcribed spacer region
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
18S rRNA	18S ribosomal RNA
rDNA	ribosomal DNA
EDTA	ethylenediamine-tetraacetic acid
TBE	tris/borate/EDTA
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool
FBS	fetal bovine serum
iRBC	infected red blood cells
WTD	white-tailed deer
PPE	percentage of parasitized erythrocytes
MASP	microaerophilous stationary phase

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

CYTAUXZOON FELIS

Cytauxzoonosis is an emerging infectious disease of domestic cats in the United States and is caused by the tick-borne hemoparasite *Cytauxzoon felis*. The *Cytauxzoon* genus is classified as phylum Apicomplexa, class Aconoidasida, order Piroplasmida, and family Theileriidae. This genus was originally used to describe the causative agent of a fatal illness in an African grey duiker (*Sylvicaprae grimmia*) in 1948 (Neitz and Thomas, 1948). Other African ungulates such as antelope (Thomas et al., 1982) and giraffe (Krecek et al., 1990) have shown to be infected with *Cytauxzoon* species.

Cytauxzoon felis was first reported in the United States in 1976 in fatally infected domestic cats in Missouri (Wagner, 1976). Since *Cytauxzoon* species originally were only known to infect African ruminants, the finding of *C. felis* in cats in the U.S. raised concern for domestic food animals (Ferris, 1979; Kier et al., 1982). This warranted the early research conducted on infectivity and host range for this parasite. It was shown that *C. felis* only parasitized domestic and wild felids, especially bobcats. Results also revealed that the disease progression of this parasite varies considerably between domestic and wild felids.

Within less than a decade after the first report of feline cytauxzoonosis in Missouri, this previously unrecognized disease was documented in additional southeastern states including Arkansas, Florida, Georgia, Louisiana, Mississippi,

Oklahoma, and Texas (Glenn et al., 1982). *Cytauxzoon felis* infections were subsequently identified in domestic cats in several other states such as Kansas, Kentucky, North Carolina, South Carolina, Tennessee, and Virginia (Meier and Moore, 2000; Birkenheuer et al., 2006a; Jackson and Fisher, 2006; Haber et al., 2007). *Cytauxzoon felis* is frequently found infecting domestic cats in Arkansas, Missouri, and Oklahoma. The reported high prevalence affirms infected domestic cats as sources of infection for naïve felines and validates the use of acaricides on cats to prevent cytauxzoonosis (Rizzi et al., 2015).

Cytauxzoon felis infection was reported in northern states of the U.S. as well. Cytauxzoonosis was diagnosed in domestic cats in southern Illinois (MacNeill et al., 2015). Although not reported in domestic cats in North Dakota and Pennsylvania, *C. felis* infection has been found in bobcats in these states (Birkenheuer et al., 2008; Shock et al., 2011).

All of the mentioned states lie within specific geographical regions that correlate with the range of the first identified tick vector, *Dermacentor variabilis* (American dog tick) (Birkenheuer et al., 2006a; Jackson and Fisher, 2006).

Transmission

Dermacentor variabilis is mainly found in U.S. states east of the Rocky Mountains. This tick can also be found in certain areas of Mexico, the Pacific Northwest of the U.S., and Canada, east of Saskatchewan (McNemee et al., 2003). Laboratory studies have shown that *C. felis* is capable of being experimentally transmitted by *D. variabilis* as well as *Amblyomma americanum* (lone star tick) (Blouin et al., 1984;

Kocan et al., 1992; Reichard et al., 2009; Reichard et al., 2010). *Dermacentor variabilis* has been experimentally shown as a transstadial vector for *C. felis*, but cytauxzoonosis cases better correlate geographically and epidemiologically with *A. americanum* (Reichard et al., 2009).

An early study concluded that *C. felis* infection was unable to be transmitted via *A. americanum* adults that fed as nymphs on *C. felis* experimentally infected bobcats and domestic cats (Wightman et al., 1977). However, subsequent studies showed *A. americanum* as a more efficient vector for *C. felis* than *D. variabilis* (Bondy et al., 2005; Reichard et al., 2009). Reichard et al., 2009 tested *D. variabilis*, *Ixodes scapularis*, *Rhipicephalus sanguineus*, and *A. americanum* for cat-to-cat tick transmission of *C. felis*, and only the *A. americanum* ticks were able to transmit *C. felis* from a cat that survived a natural *C. felis* infection to a recipient, susceptible cat. In addition, natural *C. felis* infection has been reported in unengorged *A. americanum* nymphs and adults that were caught outdoors (Reichard et al., 2010). Also, the prevalence of *C. felis* infection has been found to be higher in wild felids of states with known *A. americanum* populations (Shock et al., 2011; Shock et al., 2012).

While *C. felis* is the only *Cytauxzoon* species identified in the U.S., infections with other *Cytauxzoon* spp. have been found in felids in other parts of the world. *Cytauxzoon manul* has been found to infect Pallas cats (*Otocolobus manul*) from Mongolia (Reichard et al., 2005). This *Cytauxzoon* sp. can infect domestic cats, but clinical signs are not observed with infection from this species as it does not cause disease. *Cytauxzoon manul* infection does not guarantee protection from a challenge

C. felis infection as well (Joyner et al., 2007). There was also a high prevalence of 62.5% of *Cytauxzoon* sp. in a Eurasian lynx and wild cats. Phylogenetic analysis showed two distinct clades of *Cytauxzoon*, in which all the sequences clustered to *C. manul*/*Cytauxzoon* sp. of Palaearctic felids (Jirsová et al., 2016). In Spain, infections of *Cytauxzoon* spp. have been reported in domestic cats (Criado-Fornelio et al., 2004) and an Iberian lynx (*Lynx pardinus*) (Millan et al., 2007). Domestic cats have been infected with *Cytauxzoon* spp. in France (Criado-Fornelio et al., 2009), northeastern Italy (Carli et al., 2012) , and South Africa (Allsopp et al., 1994). *Cytauxzoon felis* or a morphologically and genetically similar species of *Cytauxzoon* has been implicated in causing cytauxzoonosis in wild felids as well as feral domestic cats in Brazil (Peixoto et al., 2007; Andre et al., 2009). *Cytauxzoon felis* has also been detected in the serum and blood smear of a stray cat in Iran (Rassouli et al., 2015). A retrospective examination of organs from a Bengal tiger at a German zoo revealed a fatal infection resembling cytauxzoonosis after possibly coming in contact with bobcats (*Lynx rufus*) imported from North America (Jakob and Wesemeier, 1996).

Originally, the accepted paradigm of the *C. felis* life cycle was a sylvatic cycle which included the transmission of *C. felis* from an infected bobcat (*L. rufus*) as the reservoir host (Glenn et al., 1983; Blouin et al., 1984) to a domestic, naïve cat as the aberrant or dead-end host (Greene et al., 2006). It was later shown that *C. felis* could be transmitted from a domestic cat to another domestic cat without the necessity of a wild felid host (Reichard et al., 2009). Iatrogenic transmission of *C. felis* was shown possible when a laboratory cat died of cytauxzoonosis 12 days after receiving an intraperitoneal

inoculation of blood mononuclear cells from an infected Florida panther (*Felis concolor coryi* [*Puma concolor coryi*]) (Butt et al., 1991). Ultrastructural and histologic features revealed an acute *C. felis* infection in the domestic cat. This would indicate that the Florida panther can serve as a carrier for *C. felis*.

In addition to the bobcat and the Florida panther, subclinical *C. felis* infection has been demonstrated in Florida cougars (*Puma concolor couguar*) (Yabsley et al., 2006; Harvey et al., 2007) and in Texas cougars (*Puma concolor stanleyana*) (Rotstein et al., 1999). A captive-reared white tiger (*Panthera tigris*) was diagnosed with fatal cytauxzoonosis in an enzootic part of northern Florida (Garner et al., 1996). The findings of *C. felis* infections in these wild felids identify them as hosts for *C. felis* as well.

Life Cycle

The *C. felis* life cycle begins as a naïve tick (usually a nymph) takes in a blood meal of circulating, piroplasm-containing erythrocytes from a felid host. Once engorged, the nymphal tick drops off and molts to the adult stage. At this time, the parasite is released into the tick's gut and undergoes gametogenesis to develop into ray bodies that sexually reproduce by fusing into zygotes (Tarigo et al., 2013). These zygotes differentiate into kinetes that replicate and migrate to the salivary glands of the tick (Tarigo et al., 2013). In the salivary glands, these kinetes asexually reproduce by sporogony to form sporozoites, which infect as the adult tick feeds on a naïve domestic cat (Greene et al., 2006). These sporozoites infect host endothelial associated mononuclear phagocytes. This is followed by a prepatent period of approximately two to three weeks (Snider et al., 2010). During this time, schizonts are developing. The

schizonts in the cytoplasm of host cells start as indistinguishable vesicular structures, but as rounds of nuclear division take place, individual nuclei are more discernible within these enlarged schizonts. Widespread dissemination of occluded vessels with these schizont-infected cells results in formation of parasitic thrombi (Kier et al., 1987; Tarigo et al., 2013). This produces systemic disease leading to multi-organ failure and death (Tarigo et al., 2013). This part of the life cycle is known as the schizogonous or tissue phase (Meinkoth and Kocan, 2005).

Schizonts undergo merogony, giving rise to merozoites (Kocan et al., 1992). Numerous merozoites rupture from schizonts to be released into the bloodstream and enter erythrocytes. Merozoites are identified in host erythrocytes one to three days after a schizont is found in mononuclear cells. Once identified within host erythrocytes, these merozoites are then termed piroplasms (Greene et al., 2006). These piroplasms continue to reproduce asexually. As piroplasms infect new erythrocytes, an erythroparasitemia results, and some feline hosts become carriers of *C. felis*.

Disease Pathogenesis

Cytauxzoonosis in most cats has a rapid onset of severe disease, which is due to the schizogonous phase of *C. felis* (Meinkoth and Kocan, 2005). Schizont-laden macrophages are found in almost any tissue in the body but are the most numerous in the liver, lungs, and spleen (Meinkoth and Kocan, 2005). Schizonts greatly distend mononuclear cells, and this has a profound effect on blood circulation. Schizont-laden monocytes become adherent to the endothelium and impede diapedesis (Susta et al., 2009). The CD18 integrin, which after activation is present on most leukocytes (Altieri

et al., 1992; Lynch et al., 1999; Willeke et al., 2000; Liebig et al., 2009), plays a role in this adherence (Frontera-Acevedo et al., 2013). The CD18 interaction with its ligands also causes an upregulation of proinflammatory cytokines (Frontera-Acevedo et al., 2013). These parasitized mononuclear cells accumulate and totally occlude blood vessels (Wagner, 1976; Kier et al., 1987; Jackson and Fisher, 2006; Peixoto et al., 2007; Brown et al., 2009b; Susta et al., 2009). This vascular obstruction in conjunction with the release of inflammatory mediators causes severe vascular damage and leakage (Ma et al., 1991). This leads to disseminated intravascular coagulation (DIC) and multi-organ failure, which renders the cat in a shock-like state (Peri et al., 1990; Snider et al., 2010).

The manifestations of disease generally vary between bobcats and domestic cats (Meinkoth and Kocan, 2005). The clinical presentation seems to correlate with the degree of the *C. felis* schizogonous phase (Blouin et al., 1987). Most domestic cats that are diagnosed with cytauxzoonosis experience an acute, short lived illness which culminates in death within approximately 19 to 21 days after the first appearance of clinical signs. Along with the massive numbers of schizonts in various tissues, these cats develop an erythroparasitemia as merozoites are released from enlarged monocytes (Meinkoth and Kocan, 2005). An immune-mediated erythrophagocytosis develops (Greene et al., 2006; Peixoto et al., 2007), and this triggers a sudden onset of nonregenerative anemia (Hoover et al., 1994). In bobcats, and in some domestic cats, *C. felis* infection is usually subclinical and leads to a long-lasting erythroparasitemia. In these felids, schizogonous forms are not observed (Glenn et al., 1983), and the erythroparasitemia is not accompanied by a hemolytic anemia (Bondy et al., 2005;

Mendes-de-Almeida et al., 2007; Millan et al., 2007). Occasionally, bobcats succumb to *C. felis* schizont tissue development and acute disease, similarly to acutely-ill domestic cats (Blouin et al., 1987; Nietfeld and Pollock, 2002).

As discussed above, *C. felis* infection can either be subclinical or clinical, with the onset of clinical signs occurring soon after infection by tick transmission (Snider et al., 2010). A cat with cytauxzoonosis may show signs of depression, lethargy, anorexia, anemia, dyspnea, dehydration, icterus, dark urine, and pyrexia above 103°F (Wagner et al., 1980; Greene et al., 2006; Haber et al., 2007; Reichard et al., 2009; Susta et al., 2009; Snider et al., 2010). Laboratory findings correlate with the DIC and inflammation associated with the disease. These findings include abnormalities in complete blood count (CBC), which could be in the form of any combination of cytopenias (Meinkoth and Kocan, 2005). Various combinations of normocytic, normochromic, non-regenerative anemia, neutrophilia (Greene et al., 2006; Reichard et al., 2009), leukopenia, thrombocytopenia (Meinkoth and Kocan, 2005), and neutropenia may be seen. Biochemical findings such as hyperbilirubinemia, hypoalbuminemia (Hoover et al., 1994; Greene et al., 1999), and elevations in liver enzyme activities, serum creatinine, and blood urea nitrogen (BUN) levels (Greene et al., 2006) may be observed as well.

Diagnosis

Since other diseases can produce similar clinical signs to cytauxzoonosis, a definitive diagnosis for *C. felis* infection can be made by direct observation of the organism in erythrocytes on blood smears, visualization of schizonts in affected tissues, and by polymerase chain reaction (PCR) assay. The most common diagnosis typically

was made using stained (Wright-Giemsa or Diff Quik) blood smears (Blouin et al., 1984; Meinkoth and Kocan, 2005) to observe piroplasms or schizonts. Piroplasms are pleomorphic and are morphologically described as tetrad, cocci-like chain, round "signet ring", or bipolar oval "safety pin" (Greene et al., 2006). Although the detection of piroplasms in a blood smear is supportive in diagnosing *C. felis* infection, it has limitations. Depending on the stage of the disease, intraerythrocytic piroplasms may be missed. This is probable at initial presentation of clinical signs when a cat may not have a detectable erythroparasitemia. The schizogonous tissue phase is most responsible for clinical signs, and this point of the disease occurs prior to appearance of piroplasms. Although *Theileria* spp., *C. manul*, and small *Babesia* spp. (*B. felis* and *B. leo*) are difficult to distinguish from *C. felis* with light microscopy, there are no reports of *Theileria* spp. infecting cats in the U.S. Also, there has only been one report of feline babesiosis in Florida panthers (Yabsley et al., 2006) as well as a one report in a bobcat in the U.S. (Shock et al., 2013). Therefore, *Mycoplasma haemofelis*, stain precipitate, Howell-Jolly bodies, and water artifact would more likely lead to false positive identification of *C. felis* infection on blood smears. The presence of schizonts in mononuclear phagocytes in stained aspirates from the spleen, lungs, bone marrow, and lymph nodes can also aid in diagnosis (Greene et al., 2006; Reichard et al., 2009; Susta et al., 2009). Mononuclear phagocytes with tissue phase schizonts may be observed at edges of peripheral blood smears (Haber et al., 2007).

A more thorough diagnosis is made with the detection of these pathognomonic schizonts in macrophages in blood vessels of various affected organs (Peixoto et al.,

2007). Stained impression smears of many tissues can be used to identify the schizogonous phase of *C. felis* at different points of development in mononuclear phagocytes. The tissue stage of the organism can also be observed histopathologically with formalin-fixed tissues, particularly in the liver, lung, lymph node, and spleen (Greene et al., 2006), but this is not routinely conducted because as a confirmable diagnosis it can only be made at necropsy (Meier and Moore, 2000). Gross findings at necropsy usually consist of icterus, enlarged lymph nodes, hepatic and splenic mottling and enlargement, and non-collapsing lungs with petechial hemorrhages (Ferris, 1979; Kier et al., 1987; Hoover et al., 1994; Susta et al., 2009; Snider et al., 2010). In some cats, a gelatinous, iteric fluid may accumulate in the pericardial sac, and petechial and ecchymotic hemorrhages on the epicardium may result (Kocan and Kocan, 1991).

Molecular diagnostics can be used to detect *C. felis.* A PCR based assay was developed in 2006 that amplifies a 284-bp product of the *C. felis* 18S rRNA gene sequence (Birkenheuer et al., 2006b). This test has a sensitivity of 100% at a threshold of 50 gene copies per reaction and is commercially available (North Carolina State University Vector Borne Disease Diagnostic Laboratory, Raleigh, NC; IDEXX Laboratories, Westbrook, ME). This test provides a more specific and sensitive means for detecting *C. felis*, but the rapid disease progression of *C. felis* may preclude its usefulness. Serologic methods have been utilized in order to test for antibody for *C. felis* but have not become commercially available. An indirect fluorescent antibody test was developed in 1978 (Shindel et al., 1978) and a microfluorometric immunoassay in 1988

(Cowell et al., 1988). Serological testing has been used only in prevalence studies and research to date.

Treatments

Different therapies have been evaluated for their effectiveness against cytauxzoonosis. Early on, antibiotics were utilized as therapy for cats with *C. felis* infection. Due to their lack of anti-protozoal activity, the outcome of *C. felis* infection did not solely depend on the use of antibiotics (Hoover et al., 1994; Walker and Cowell, 1995). Two antiprotozoal agents, parvaquone and buparvaquone, are effective against *Theileria* spp., hemoparasites related to *Cytauxzoon* spp., in African cattle (Hawa et al., 1988; Muraguri et al., 2006). However, neither was effective against cytauxzoonosis (Motzel and Wagner, 1990). Imidocarb dipropionate, a drug proven effective at treating *Babesia* spp. infections (Solano-Gallego and Baneth, 2011), and the drug diminazene aceturate have shown minimal success at treating *C. felis* infection (Greene et al., 1999). In a study involving seven cats, five cats survived acute cytauxzoonosis after being given diminazene aceturate and supportive therapy, and one other cat survived after the administration of imidocarb dipropionate and supportive therapy (Greene et al., 1999). Diminazine aceturate is not approved for therapeutic use in the U.S. Imidocarb is available for use in the U.S., but it is labeled for use in dogs only. It has also not consistently proven to be effective at treating *C. felis* infections (Meinkoth and Kocan, 2005). In addition, erythroparasitemia was not eliminated from carrier cats with the administration of imidocarb or diminazene diaceturate (Lewis et al., 2014). In one study, imidocarb dipropionate was evaluated against azithromycin (antibacterial drug) co-

administered with atovaquone (antimalarial drug) (Cohn et al., 2011). This clinical trial yielded the most promising results to date in which 60% of cats suffering from cytauxzoonosis survived to hospital discharge after combination of atovaquone and azithromycin (A&A) along with supportive care was administered versus 26% survival rate of those given imidocarb dipropionate and supportive care (Cohn et al., 2011). Atovaquone targets the *C. felis* cytochrome b (*cytb*). Thirty *cytb* genotypes have been identified, and the *cytb1* genotype has been linked to higher chances of survival in cats administered A&A. Using a high-resolution melt analysis, a PCR panel was predicted to differentiate the *cytb1* genotype from other *cytb* genotypes. With this panel, the *C. felis cytb1* was identified with 100% sensitivity and 98.2% specificity (Schreeg et al., 2015).

Glucosteroids or non-steroidal anti-inflammatory drugs (NSAIDs) can be used for their analgesic effects as well as suppression of pyrexia (Cohn et al., 2011). It has also been beneficial to provide intensive supportive therapy and heparin in order to prevent DIC (Greene et al., 1999; Greene et al., 2006; Cohn et al., 2011). In a recent study, an evaluation of the hemostatic status of five naturally *C. felis*-infected cats revealed that all five cats suffered from overt DIC (Conner et al., 2015). Despite having coagulation abnormalities, the cats had neither an antithrombin deficiency nor clinical signs of hemorrhage. These findings have shed light on conducting further research on the utility of therapies against thrombotic DIC, which could improve the survival of a cat against cytauxzoonosis.

Genetic Variation

Cytauxzoon felis infection was once considered uniformly fatal in domestic cats, but over the years there has been an increasing number of reports of cats surviving infection (Motzel and Wagner, 1990; Greene et al., 1999; Meinkoth et al., 2000; Haber et al., 2007; Brown et al., 2008; Brown et al., 2009a; Brown et al., 2010; Cohn et al., 2011). Some cats' survival could be attributed to treatment (supportive care and/or antiprotozoal therapy), while others received no treatment and survived. Along with the inconsistent findings with therapies against *C. felis* infection, there was speculation that cats surviving infection may be due to a less virulent *C. felis* strain. Furthermore, early reports of *C. felis* infection survival cases in domestic cats appeared to be more limited to certain areas such as western Arkansas (Brown et al., 2009a) and eastern Oklahoma (Meinkoth and Kocan, 2005). This led to the hypothesis that less virulent strains were associated with certain geographical locations, which could further indicate a possible emergence of genetically variable *C. felis* strains.

In order to test this hypothesis, a population of *C. felis* isolates would be assessed for strain variation. This would entail selection of a locus to be used as a genetic marker (Brown et al., 2009a). Polymerase chain reaction amplified DNA sequence analysis of such genetic markers has provided a very sensitive and reproducible means in which to identify and type various microorganisms (Williams et al., 1999). This is also a method for discerning genetic polymorphisms (Williams et al., 1999).

An ideal genetic marker would be one that is a multicopy gene, which would be highly sensitive to a given detection method, and ever-present in the same genus. These

criteria make the ribosomal genes suitable candidates (Ferrer et al., 2001), which led to the 18S ribosomal RNA (18S rRNA) gene being commonly used as a taxonomic marker (Aktas et al., 2007). Initial *C. felis* molecular work involved sequencing the ribosomal genes, with more emphasis on comparing the structural 18S rRNA genes. In an early study, sequencing fragments of the *C. felis* 18S rRNA gene from a cat that survived infection revealed a >99% homology to a previously reported *C. felis* 18S rRNA gene from a fatal infection (Meinkoth et al., 2000). In another study, almost full-length (approximately 1,700 base pairs out of 1,774 base pairs) *C. felis* 18S rRNA gene sequences of four mid-Atlantic cats that either died or were euthanized due to *C. felis* infection had a ≤ 99.9% sequence identity to *C. felis* 18S rRNA sequences reported for cats that either died or survived infection from other U.S. regions (Birkenheuer et al., 2006a). These findings are not surprising since the 18S region is highly conserved. The studying of this area for variation may not lead to distinguishing between very similar species (Aktas et al., 2007), and therefore may not be useful at the strain level.

Genes associated with antigenic variation and pathogenicity also have been used for detection and molecular characterization of closely related apicomplexans, such as *Babesia* spp. (Yamasaki et al., 2007; Jia et al., 2009). The phylogenetic analysis of the heat shock protein 70 (hsp70) gene sequences of the canine *Babesia* spp. revealed the close relation amongst these species, as if evolved from the same ancestor. Using the phylogenetic analysis of hsp70 gene sequences could be useful in classifying *Babesia* and *Theileria* spp. (Yamasaki et al., 2007).

Identification of virulence genes is not an easy feat and can limit molecular studies (Brown et al., 2009a). Genomic studies commonly use the internal transcribed spacer (ITS) regions of the ribosomal RNA operon in order to detect genetic variation between very similar species. The ITS regions are composed of noncoding sequences and do not follow the same functional constraints of the rRNA genes (Brown et al., 2009a). The ITS regions are distinguishing and divergent (Lott et al., 1993; Williams et al., 1995; Lott et al., 1998) as they are usually homogeneous amongst strains but evolve at a higher rate than the structural rRNAs (Windsor et al., 2006). In eukaryotes, the rDNA unit is tandemly organized with the internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2) regions separated by the 5.8S rRNA gene and flanked by the 18S and 28S rRNA genes (Kibe et al., 1994). Genetic variability within the ITS regions has been reported with other similar protozoal parasites, such as *Theileria* spp. (Kibe et al., 1994; Aktas et al., 2007), *Babesia canis* (Zahler et al., 1998), *Cyclospora cayetanensis* (Olivier et al., 2001), and *Eimeria* spp. (Barta et al., 1998). In the study with *B. canis*, ITS variability resulted in division of *B. canis* isolates of varying vector specificity, geographical location, and pathogenicity into three separate groups, approximately at species level, which was reflective of proposed subspecies (*B. canis canis*, *B. canis rossi*, and *B. canis vogeli*) (Zahler et al., 1998).

Genetic analysis of the *C. felis* ITS regions was pursued in order to divulge any distinct parasite populations that could possibly be inferred in the variable outcomes of cytauxzoonosis as well as any differences in geography and hosts (wild and domestic cats). The first study compared *C. felis* ITS1 sequence data for three samples (two

infected ticks and one infected feline blood), in which all the bases were nearly identical except for two ambiguous bases found in one sample (Bondy et al., 2005). In a 2009 study, *C. felis* ITS1 and ITS2 sequences from 88 acutely-ill cats from Arkansas and Georgia were evaluated for genetic variability as well as a positive association with clinical outcome (Brown et al., 2009a).Within the *C. felis* ITS1 sequences, there were eight single nucleotide polymorphisms (SNPs) and one nucleotide insertion, and ITS2 sequences revealed four SNPs and a single 40 bp insertion. The combination of ITS1 and ITS2 sequences yielded 11 different ITS sequences and three unique genotypes. Gentoypes ITSA and ITSB were found only in Georgia samples, and ITSC was found only in Arkansas samples. Fourteen samples revealed the incorporation of two nucleotides at one given position within either the *C. felis* ITS1 or ITS2 sequence. This was understood as either a co-infection in a cat with multiple genotypes or there are multiple copies of the rRNA genes with polymorphic units in the *C. felis* genome, as described with a similar protozoan, *Theileria parva* (Kibe et al., 1994). Brown et al., 2009a also reported survival rates of 79.2% (38/48) for genotype ITSA, 19% (4/21), for genotype ITSB, and 0% (0/5) for genotype ITSC. This led to a conclusion of a strong association of ITS genotype with *C. felis* pathogenicity.

Brown et al., 2009b, analyzed 48 archived tissues of infected domestic cats in GA from 1995-2007 for *C. felis* ITS genetic variation as well as temporal analysis. There were six SNPs in ITS1 sequences and five SNPs in ITS2 sequences. The most common genotype, designated ITSa in this particular study, was found in 27/48 (56.3%) samples, and each of the 27 samples contained two nucleotides at a single position. ITSb was

detected in 8/48 (16.7%) samples, and ITSc was found in 3/48 (6.3%) samples. ITSb and ITSc genotypes only differed from the ITSa genotype at the same polymorphic position (position 180) of ITS2. Each genotype contained one of the two nucleotides of ITSa. The remaining ITS sequences were labeled in this study as ITSe, ITSf, ITSi, ITSj, and ITSk and were all detected in a single sample each. Chronologically, ITSa and ITSb were found in *C. felis*-infected cats over the course of many years. Also, ITSc was only detected in three samples in this study. During the 1995-2007 period, this sequence was not found in samples archived since 2001 (Brown et al., 2009b); whereas, it was the most common genotype (previously designated as ITSA) in samples from 2005-2007 in the earlier study (Brown et al., 2009a).

Brown et al., 2009b suggested that the significant difference in the most common ITS genotypes found may be attributed to changes in the *C. felis* population over the periods of time for this current study and the previous one (Brown et al., 2009a).

In a 2010 study, blood samples of high-risk, subclinical cats and bobcat tissue samples were evaluated for ITS genotype variation within the *C. felis* population (Brown et al., 2010). Out of the 27 *C. felis* positive domestic cats, all ITS1 sequences were identical, with the exception of four SNPs and a single insertion in one sample, and only one SNP within the ITS2 sequence of eight other samples. Together, the sequence data revealed three distinct ITS sequence types. Within the *C. felis* ITS1 sequence from bobcats, five SNPs and one single nucleotide insertion were found as well as three SNPs in the ITS2 region. When combined, 11 ITS sequence types were revealed (three previously reported as infecting domestic cats and eight that were not). *Cytauxzoon felis*

from the bobcats showed some SNPs in the ITS1 and most of SNPs in ITS2 as having two nucleotide bases at a single position. The ITS sequence types of the *C. felis* subclinically infected cats were also detected in clinically infected cats (Brown et al., 2009a). This led to conclusion that ITS sequences may be useful for observing genetic diversity within a given *C. felis* population but not as suitable for distinguishing amongst variably virulent *C. felis* strains.

In 2011, the effectiveness of the co-administration of azithromycin and atovaquone as treatment for *C. felis* was evaluated as well as ITS genotyping and geographic association with outcome (Cohn et al., 2011). Out of 36 genotypes found, 15 were unique. The remaining 21 genotypes matched those previously described (Brown et al., 2009a; Brown et al., 2009b; Brown et al., 2010). Although certain genotypes (ITSa/ITSc) were correlated with less virulence than other genotypes (ITSC) in other studies, this was not found in the 2011 study. And led to the conclusion that ITS genotyping is not ideal for predicting virulence in *C. felis.* Also, the distribution of ITS genotypes of the cats that died versus those that survived was proportional amongst the five states used in the study. This would suggest that survival may not necessarily be based on geographic region (Cohn et al., 2011).

A 2012 study assessed the genetic variability within the *C. felis* ITS1 and ITS2 regions from bobcats and pumas (Shock et al., 2012). For ITS1, 65/144 sequences were identical to those previously reported in domestic cats. The other 79/144 ITS1 sequences were unique. For ITS2, 49/112 sequences were identical to previously reported ITS2 sequences of *C. felis* in domestic cats. The remaining 63 ITS2 sequences were unique.

There were 111 samples in which both ITS1 and ITS2 were obtained. Of those samples, 25 wild felids had five *C. felis* genotypes that had been previously reported. Due to the greater diversity in the ITS1 and ITS2 of *C. felis* from pumas and bobcats found in this study, more *C. felis* strains may possibly circulate amongst wild felids (Shock et al., 2012).

Previous studies analyzing the intraspecific variation of the *C. felis* ITS1 and ITS2 rRNA regions did not utilize cell-based cloning of PCR-amplified DNA (Bondy et al., 2005; Brown et al., 2009a; Brown et al., 2009b; Brown et al., 2010; Cohn et al., 2011; Shock et al., 2012). However, it was reported that there was an incorporation of two nucleotides at a single position in the sequences of *C. felis* ITS1 and/or ITS2. This led to difficulty in sequence analysis as well as some unusable data (Wright, 1984; Brown et al., 2009a; Brown et al., 2009b; Brown et al., 2010; Cohn et al., 2011; Shock et al., 2012). It was speculated that this incorporation was due to either a co-infection with multiple *C. felis* strains or presence of multiple rRNA gene copies which contained polymorphic units. Southern blot analysis could be conducted in order to identify the presence of any heterogeneous copies of the rRNA genes (Brown et al., 2009b). Cloning results in a single sequence of bases, and prior to sequencing, it could be used to alleviate an incorporation of mixed nucleotides at a single position.

Authenticity of a sequence could be compromised as a result of direct sequencing of a highly heterogenic sample (as seen with double peaks in chromatogram) (Winters et al., 2011). Cloning has this drawback too in that a composition of mixed PCR reaction (whether heterogeneity was from adulteration of sample or damage) can be seen when

obtaining sequences (Winters et al., 2011). Carr et al., 2007 stated that with bisulphite genomic sequencing, using direct sequencing could lead to superimposed 'staggered' electropherograms due to dissimilar electrophoretic movement of bisulphite PCR product containing two varying template sequences (two divergent methylation states).

One way to alleviate the presence of more than one nucleotide at a single position would be to clone prior to sequencing. Since a clone contains a single sequence, the differences in movement during bisulfite sequencing do not show, and any spuriously amplified DNA can be quickly recognized and removed (Carr et al., 2007). The majority of strands present during PCR contribute to the bases that are incorporated into strands of the PCR product (Strachan and Read, 1999), and a low-frequency sequence variation is overwhelmed by the sequence in abundance in a given sample (Ruecker et al., 2011). Cell-based cloning is the better way to detect polymorphisms (whether caused by *Taq* DNA polymerase error or a heterogeneous sequence mixture) at a low frequency. Despite cloning being a great candidate for detecting more polymorphisms, it is also more likely to contribute to greater sequence diversity (Ruecker et al., 2011). With any highly heterogenetic pool of molecules, amplification used for both direct sequencing of PCR products and sequencing of clones should be repeated, and their respective resulting sequences should be compared to each other in order to obtain the correct consensus sequence.

FELINE HEMOTROPIC MYCOPLASMA (HEMOPLASMA)

Due to the icterus, anemia, and erythroparasites, some of the first cases of cytauxzoonosis initially suggested *Haemobartonella felis* infection. As a result of DNA

studies, *H. felis* was officially reclassified as a mycoplasma in 2001 (Tasker, 2006), which led to the renaming of the feline hemotropic mycoplasmas (hemoplasmas) as *M. haemofelis*, "*Candidatus* Mycoplasma turicensis," and "*Candidatus* Mycoplasma haemominutum (Sykes, 2010)." The Candidatus distinction is used for newly described prokaryotic species in which more than a sequence is available, but additional evidence needed for classification according to the International Code of Nomenclature of Bacteria is lacking (Murray and Stackebrandt, 1995). Occasionally a fourth species of *Mycoplasma*, a *Candidatus* Mycoplasma haematoparvum-like organism, can be found infecting felines as well (Sykes et al., 2007).

Mycoplasma haemofelis

Feline infectious anemia is caused by *M. haemofelis* (Willi et al., 2005; Sykes, 2010). Pathogenicity ranges from subclinical to severe hemolytic anemia, and this differs between hemoplasma species (Willi et al., 2007b). This also may be due to strain variation within species, in which some strains induce anemia more than others (Sykes, 2010). *Mycoplasma haemofelis* is an epierythrocytic hemoplasma capable of causing hemolytic anemia (Allison et al., 2010) and is difficult to cultivate *in vitro* (Sykes, 2010).

In experimentally infected cats, *M. haemofelis* infection resulted in severe hemolytic anemia. An association with anemia in naturally *M. haemofelis*-infected cats was not always demonstrated (Tasker, 2006). This could be attributed to the differences in population of cats sampled. A population of cats showing clinical signs consistent for hemoplasma infection in a study would have a higher prevalence of *M. haemofelis*

infection with an acute disease than a study including any healthy cats as well as cats with any sign of ill health (Tasker, 2006). Despite this, *M. haemofelis* is still considered the most pathogenic species (Sykes, 2010), and one third of untreated cats with acute *M. haemofelis* infection succumb to the severe anemia (Tasker, 2006).

Five naïve cats and five cats that recovered *Candidatus* M. turicensis infection were exposed to *M. haemofelis*. No cross-protection was witnessed, but differences in infection kinetics were observed (Baumann et al., 2015). In a similar study, five naïve cats (Group A) and five cats that recovered from *M. haemofelis* infection (Group B) were compared. Bacterial loads by quantitative PCR, antibody response to hsp70 by enzyme-linked immunosorbent assay (ELISA), quantitative reverse transcriptase PCR, and blood lymphocyte cell subtypes by flow cytometry were measured. It was observed that all the cats of Group A were parasitized with high bacterial loads and seroconverted, and Group B were protected against reinfection (Hicks et al., 2015).

Candidatus Mycoplasma haemominutum

Candidatus M. haemominutum is also difficult to culture *in vitro,* and infections with *Candidatus* M. haemominutum are chronic and subclinical (Sykes, 2010). Anemia, if present, is often mild, but in some cases, it can be moderate. A co-infection with *Candidatus* M. haemominutum and other diseases, such as feline leukemia virus (FeLV), can lead to significant anemia (Tasker, 2006). Most prevalence studies have not shown an association between natural *Candidatus* M. haemominutum infection and anemia.

Candidatus Mycoplasma turicensis

Candidatus M. turicensis was first identified in Switzerland (Willi et al., 2005) and subsequently has been reported in the U.S., Italy, Japan, United Kingdom, Australia, South Africa, and Canada (Sykes, 2010). This hemoplasma was first discovered by PCR and has not been detected on blood smear or cultivated in the laboratory (Sykes, 2010). The pathogenicity of *Candidatus* M. turicensis can range from moderate to severe anemia. Concurrent infections, such as with FeLV, and immunosuppression are associated with increasing its pathogenic potential (Tasker, 2006).

These mycoplasmas are transmitted by hematophagous ectoparasites, especially fleas (Willi et al., 2007a). The more time cats spend outdoors in temperate and tropical regions, the more they may be exposed to infected ectoparasites, such as fleas and ticks (Loyd et al., 2013). Coincidence of exposure to infected fleas and ticks could potentially lead to a coinfection in cats with a feline hemoplasma and a hemoparasite, such as *C. felis* (Allison et al., 2010).

A coinfection could be implicated in causing a difference in pathogenicity, clinical manifestations, or response to treatment (Shaw et al., 2001). A mycoplasma and *Plasmodium falciparum* coinfection was evaluated in squirrel monkeys (*Saimiri sciureus*) (Contamin and Michel, 1999). It was observed that if a *P. falciparum* infection followed a latent mycoplasma infection, then the mycoplasma infection stayed latent and did not affect the course of the *P. falciparum* infection. If the *P. falciparum* infection was initiated first before the development of patent mycoplasma infection, then the *P. falciparum* infection persisted longer as the host resisted longer or was able to

eliminate *P. falciparum* infection. When intact monkeys with *P. falciparum* infection had a splenectomy performed at different times, then the patent mycoplasma infection was delayed. It was observed that a mycoplasma infection activated after splenectomy may have an impact on the progress of *P. falciparum* parasitemia (Contamin and Michel, 1999). In another *P. falciparum* study using splenectomized squirrel monkeys as hosts, the splenectomy activated a patent infection caused by a wall-less bacterium. The 16S rRNA gene sequence analysis revealed the bacterium as a hemoplasma (Neimark et al., 2002).

With exposure to fleas that harbor a feline hemoplasma and ticks that harbor *C. felis*, cats may naturally acquire these organisms, which could lead to a feline hemoplasma and *C. felis* coinfection. During an evaluation for hemoparasites in a domestic shorthair cat, the diagnosis was *M. haemofelis* and *C. felis* coinfection (Allison et al., 2010). It was suggested that the manifestations of the *C. felis* present might have reactivated a latent *M. haemofelis* infection (Allison et al., 2010). Since *C. felis* undergoes both an erythrocytic and tissue phase, the presence of the tissue phase during postmortem examination helps differentiate between *C. felis* and *M. haemofelis* infections (Meinkoth and Kocan, 2005).

IN VITRO CULTIVATION OF PROTOZOAL HEMOPARASITES

The *in vitro* cultivation of parasitic protozoa has been beneficial at understanding the disease potential and biology of these organisms. This continued supply of viable parasite stock has led to better understanding of the host-pathogen interaction along with producing quantities of parasitic nucleic acids in order to conduct phylogenetic studies,

improve diagnostics (esp. with subclinical infections), produce attenuated strains and parasitic antigen for potential vaccine development (Schuster, 2002a). *In vitro* cultivation of these parasites has also enhanced the assessment in parasite triggered immune factors and drug screening (Visvesvara and Garcia, 2002).

In vitro cultivation of protozoan parasites entails multifaceted techniques that must accommodate numerous factors. Protozoan parasites have complex life cycles, and culture parameters must take the stage of the life cycle into consideration (Visvesvara and Garcia, 2002). The first attempts of cultivating protozoa *in vitro* were with *P. falciparum* and *Plasmodium vivax* (Bass and Johns, 1912). *Plasmodium* spp. undergo sexual reproduction in the invertebrate, mosquito host. As a mosquito feeds, it injects sporozoites into the bloodstream, where these sporozoites travel to the liver and invade the hepatocytes. Here, the sporozoites grow and divide, which results in merozoites. The merozoites exit hepatocytes and re-enter the bloodstream. The asexual reproduction in the vertebrate host (Trager, 1986) occurs as the parasite begins a cycle of invading erythrocytes. In the erythrocytes, they develop into mature schizonts. These schizonts rupture and release numerous merozoites, and the merozoites enter new erythrocytes (Schuster, 2002b). Some merozoite-infected cells continue replicating; while others develop into male and female gametocytes. As a mosquito bites, it ingests the gametocytes, and the cycle repeats.

Due to its involvement in the pathogenesis of malaria as well as potential in vaccine development, the erythrocytic stages of the *Plasmodium* life cycle are the most widely used in cultivation (Schuster, 2002b). Cultivation of these parasites without the

use of animal models or humans dates back as early as 1912 (Bass and Johns, 1912). As the years progressed, more work on improving culture conditions of the erythrocytic stages of malarial parasites of experimental animals as well as human malaria was being conducted (Butcher and Cohen, 1971; Diggs et al., 1971; Trager, 1971; Trigg, 1975). In 1976, Trager and Jensen established the first continuous culture of human malaria parasites (Trager and Jensen, 1976). This entailed maintaining cultures in RPMI-1640 medium with human serum at 38°C under atmospheric conditions of low O_2 (1% or 5%) and 7% CO_2 using a candle jar (Trager and Jensen, 1976). These atmospheric conditions produced by a candle jar were used in cultivating other similar parasites, such as *Babesia* and *Cryptosporidia* spp. (Timms 1980; Upton et al., 1994).

A study to develop an indirect fluorescent antibody (IFA) against *C. felis* was the first attempt at cultivating this parasite *in vitro* (Shindel et al., 1978). The origins of the cultures began with the suspension of spleen and bone marrow cells of a moribund, *C. felis*-infected cat into Medium 199 with Hank's salts supplemented with 20% fetal bovine serum (FBS; v/v), penicillin (200 units/ml), kanamycin (100 µg/ml), mycostatin (60 units/ml), and dihydrostreptomycin (200 µg/ml) in 50 ml Falcon flasks. Five days post initiation, the infected spleen and bone marrow cells were removed and mixed 1:4 with African green monkey kidney (Vero) cells (American Type Culture Collection, Rockville, MD). The co-culture was maintained to a third passage, at which time a cat was inoculated with the cultured material. This successfully immunized the cat against challenges of *C. felis* and serum from this cat was harvested and used as the antiserum in the IFA (Shindel et al., 1978). However, the propagation of the parasite was not verified

through the cultures (Shindel et al., 1978), and there have been no additional reports of *in vitro* cultivation of *C. felis*.

The method developed by Trager and Jensen, 1976 was utilized for short-term culture of *B. bovis* in bovine erythrocytes incubated at 37°C in a candle jar using RPMI-1640 with 10% fetal calf serum (FCS) buffered with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Erp et al., 1978). The cultivation resulted in percentage of parasitized erythrocytes (PPE) that reached 2.85% and did not increase beyond this point (Erp et al., 1978). Erp et al., 1978 also evaluated *B. bovis* cultivation using the spinner flask (SF) method. The SF method entailed using magnetic stirrers to keep the cultures constantly agitated and centrifugation to collect cells each time for subculture. The medium used was 50% Medium 199 supplemented with 50% bovine serum (Erp et al., 1978; Erp et al., 1980). With this exact protocol in addition to lowering the pH to 7, Erp et al. (1980) were able to support the continuous cultivation of *B. bovis.* Short-term cultivation of *B. canis* was also supported using the SF method with HEPES-buffered RPMI-1640 medium and 20% normal dog serum (Molinar et al., 1982). However, this method was not very practical because it required a large quantity of culture that resulted in low numbers of parasites (Levy and Ristic, 1980). The continuous cultivation of *B. bovis* also was made possible at a higher parasitemia in a settled layer of erythrocytes (Levy and Ristic, 1980). This microaerophilous stationary phase (MASP) technique allowed the parasites to proliferate in this settled layer of cells at low oxygen tension similar to the candle jar environment, while being incubated at 37°C to 38°C in an atmosphere of 5% CO_2 and 95% humidified air. The medium consisted of Medium

199 (60%) and bovine serum (40%), which was supplemented with 15 mM HEPES, 100 units/ml of penicillin G, and 100 µg of streptomycin. The cultures were initiated with an inoculum of both normal and infected bovine erythrocytes (Levy and Ristic, 1980). This application is regarded as more suitable for *Babesia* growth (Canning and Winger, 1987; Goff and Yunker, 1988).

Timms (1980) cultivated *B. bovis, B. bigemina*, and *Babesia rodhaini* in their host erythrocytes for up to 96 hours *in vitro* using the candle jar technique (Trager and Jensen, 1976) with some modifications. It was shown that 40% erythrocyte suspension yielded better results than 10-12% suspension of red blood cells (RBC) as shown by Trager and Jensen (1976) for *P. falciparum.* Based on the culture parameters used by Timms (1980) a continuous culture system for *B. bigemina* was developed (Vega et al., 1985). This system included washing erythrocytes used in culture with a special solution. The culture medium consisted 5-10% (v/v) of erythrocytes suspended in Medium 199 supplemented with 20-50% fresh normal bovine serum under atmospheric conditions of 5% CO_2, 2% O_2, and 93% N_2. The inoculation of a susceptible calf also demonstrated that *in vitro* propagation for 36 days and nine subcultures did not negatively affect the pathogenicity or morphology of *B. bigemina* (Vega et al., 1985). Rodriguez et al. (1983) evaluated different culture conditions and subsequently developed a *B. bovis* cloning procedure using *in vitro* cultivation. It was shown that a low-O_2 environment best supports growth of *B. bovis* with a low initial PPE (<0.1%), and a PPE of $\geq$ 0.1% of *B. bovis* grows better in a high-O_2 environment. It was also shown that oxygen consumption in *B. bovis* infected erythrocytes as well as increased

depth of overlaying culture medium restricted O_2 concentration at the settled layer of erythrocytes. A PPE of approximately 30% was the highest parasitemia attained in this study. With the development of this cloning procedure, avirulent *B. bovis* was able to be isolated from carrier animals, and homogeneous *B. bovis* populations were established and cultivated. This cloning procedure resulted in establishing three different *B. bovis* clonal lines (fast-, intermediate-, and slow growing) as indicated by the three different growth rates (Rodriguez et al., 1983).

Goff and Yunker (1986) cultivated *B. bovis* continuously using a modified MASP method with Medium 199 with Earle's salts without $NaHCO_3$ or L-glutamine (60%) and normal adult bovine serum (40%) supplemented with 50 mM TES, 10 µg/ml glucose, 0.25 µg/ml amphotericin B, and 50 µg/ml gentamycin. The complete medium was adjusted to a pH of 7.35. This study evaluated conditions that were essential for obtaining the maximum PPE as well as developed an improved method to assess the growth of *B. bovis in vitro* (Goff and Yunker, 1986). As the PPE was calculated, the observed parasitic forms were also classified as either trophozoites (visible cytoplasm and nucleus within a single parasite), dense forms (no visible cytoplasm), or merozoites (pair of pear-shaped bodies). It was shown that exponential growth varied inversely to the starting PPE and ceased when a threshold was reached (Goff and Yunker, 1986). Even when this peak was reached, individual parasites continued to grow. This culminated in a greater proportion of mature merozoite-infected RBC. Reducing the total number of erythrocytes also elevated the PPE. Besides PPE, the incorporation of [^{3}H] hypoxanthine was used to assess parasite growth *in vitro*. There was a correlation with

uptake of [^{3}H] hypoxanthine and PPE, with trophozoites exhibiting the greatest uptake (Goff and Yunker, 1986).

The variables (pH, buffers, and media storage) were evaluated for their effects on the *in vitro* growth of *B. bovis* (Goff and Yunker, 1988). It was demonstrated that parasitic growth occurred in an alkaline pH range of 7.02-7.79. Optimal growth occurred at a pH range of 7.31 to 7.39. The parasite seemed to be intolerant of acidic pH medium (pH 6.86). The zwitterion buffer 3-(N-tris-[hydroxymethyl] methylamino)-hydroxypropane sulfonic acid (TAPSO) at a concentration of 10 mM served as a better buffer than 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (TES). Exponential growth proceeded through four days in the presence of TAPSO, but for only three days with TES. Although cold medium storage has been reported as leading to less-than-optimal results, refrigeration and/or freezing before use had no profound effect on parasitic growth (Goff and Yunker, 1988).

Babesia divergens is a parasite mainly of cattle but has caused babesiosis in humans throughout Europe (Schuster, 2002a). Adapting the MASP method, a strain isolated from Finland was cultivated continuously. This strain sustained 27 subcultures over the course of 85 days (Vayrynen and Tuomi, 1982). Medium 199 (60%) and bovine serum (40%) were used, and an average of 5-10% PPE was observed (Vayrynen and Tuomi, 1982). A French isolate of *B. divergens* was successfully cultivated in serum-free medium (Grande et al., 1997). The culture was originally initiated with RPMI-1640 (25 mM HEPES) supplemented with 10% human serum (HS) and produced ≥ 40% PPE. Serum was gradually eliminated from the medium and was accompanied with a drop in

PPE. After a period of adaption, the culture remained continuous at a 3% PPE. Neither the addition of vitamins nor reduced glutathione increased the low PPE, but replacement of the serum with either Albumax I or bovine serum albumin (BSA) (Cohn's Factor V) promoted growth with a high PPE (>30%). Neither lipid nor protein fractions alone were enough to promote parasite growth and led to cell lysis. Addition of whole lipid fraction from serum or Albumax I to lipid-free albumin produced a PPE of 7%. This could have indicated that parasitic growth was dependent upon certain lipids with a carrier, such as BSA. These findings showed that Albumax I could replace HS in medium. The replacement of HS could alleviate any issues that could arise from using this serum (Grande et al., 1997).

Konrad et al., 1985 demonstrated that cultures of *B. divergens* and *Babesia major* could be maintained at low temperatures. These lower temperatures could prolong time between subculturing. After giving cultures time to develop, the medium was removed and replaced, and the cultures were switched from a temperature of 38°C to 4°C. This lower temperature delayed the need to subculture for up to 1 week. A lower PPE was observed during this period of lower temperature, but there was an increase in free extra-erythrocytic merozoites. Parasitic growth was restored in cultures stored this way upon returning the temperature to 38°C (Grande et al., 1997).

Novel developments in biotechnology have led to new media for cell culture. Using a chemically defined medium, HL-1 (Ventrex Laboratories, Inc., Portland, Maine) supplemented with 20% normal adult horse serum, 2 mM L-glutamine, 25 µg/ml amphotericin B (Fungizone), 100 U/ml penicillin, and 100 µg/ml streptomycin, *Babesia*

caballi was cultivated *in vitro* (Holman et al., 1993). HL-1 medium contains transferrin, saturated and unsaturated fatty acids, and insulin (Holman et al., 1994a), which are not commonly found in tissue culture media (Schuster, 2002a). This medium was developed for hybridomas and lymphoid cells (Ishikawa et al., 1987). In the first report of an isolate of *Babesia* sp. infective to North American elk (*Cervus elaphus*), the parasite was established in continuous cultures using HL-1 medium supplemented with L-glutamine (2 mM), 20% normal adult bovine serum, 500 U/ml penicillin, 500 µg/ml streptomycin, 125 µg/ml Fungizone, and 100 µg/ml gentamicin (Holman et al., 1994b). Parasitemias ranging from 3-19% were reported (Holman et al., 1994b). Using HL-1 medium supplemented with Albumax I (1 mg/ml), 2% HB101, 20% FBS, 100 µg/ml gentamycin and 2 mM L-glutamine, Holman et al.(1994) were able to establish and maintain the continuous cultivation of the erythrocytic stage of *Babesia equi* (*Theileria equi*). After establishment, normal adult horse serum could replace FBS in culturing later passages of *B. equi* (*T. equi*). Initially, the cultures were maintained in 5% CO_2 and 95% air, but they were later switched to 2% O2, 5% CO_2, and 93% N_2 in order to improve condition of the cells. A PPE in excess of 15% was commonly seen in the established cultures (Holman et al., 1994a).

Zweygarth et al. (1999) cultivated *B. caballi* in serum-free conditions. HEPES-buffered HL-1 medium (80%) supplemented with $NaHCO_3$ (2 g/l), 2 mM L-glutamine, 0.2 mM hypoxanthine, streptomycin (100 µg/ml), penicillin 100 IU, and 20% horse serum was initially used in cultures of two strains of *B. caballi*. In order to replace serum, BSA (5, 10, and 20 mg/ml), Albumax I (5, 10, and 20 mg/ml), and chemically

defined lipids (CDL; 0.25%, 0.5%, 0.75%, and 1%) alone or in combinations were tested. Adaptation to serum-free conditions was reached by gradually reducing serum in media at subculture. *B. caballi* was propagated *in vitro* for over six months in media containing either all concentrations of Albumax I or Albumax I (10 mg/ml) with CDL in various amounts. Bovine serum albumin or CDL alone or in combination could not support *B. caballi in vitro*. Neither RPMI-1640 nor Dulbecco's modified Eagle medium-F-12 in replacement of HL-1 as the basal medium supported growth of the parasite (Zweygarth et al., 1999).

Even with the success of established MASP culture techniques, some attempts at *in vitro* cultivation proved difficult, as seen with the merozoite stage of *Theileria* spp. (Miranda et al., 2006) and with certain isolates of *B. bovis* (Jackson et al., 2001). Therefore, modifications were warranted in order to achieve optimization for *in vitro* cultivation. Miranda et al.(2006) were the first to report the successful cultivation of the erythrocytic stage of *Theileria uilenbergi*. After testing numerous combinations of conditions, HL-1 was shown to be the best culture medium. Best growth was achieved with HL-1 supplemented with 25 mM HEPES, 2 mM L-glutamine, 200 µM hypoxanthine, 0.02 mM bathocuproinedisulfonic acid disodium salt, 1 mM L-cysteine, 24 mM sodium bicarbonate, amphotericin B (2.5 µg/ml), gentamicin (50 µg/ml), 0.75% CDL and 20% sheep serum and incubation at 37°C under a humidified atmosphere of 2% O_2, 5% CO_2, and 93% N_2. The cultivation had a duration for over a year with a PPE of 3%, and with further optimization a higher PPE could possibly be obtained (Miranda et al., 2006).

Field isolates and laboratory-adapted parasite lines of *B. bovis* were cultured *in vitro* using a chemically defined medium (RPMI-1640) and Albumax II (1% w/v) in place of bovine serum. Additional supplements were 25 mM HEPES, 25 mM $NaHCO_3$, penicillin 100 IU/ml, streptomycin 100 µg/ml, and amphotericin B 0.25 µg/ml. With these conditions, some *B. bovis* isolates directly from cattle that were unable to be cultivated in traditional serum-containing medium were able to be propagated. Modifications in culture volume as well as gas composition showed that a 600 µl culture in 24-well plate in atmosphere of 5% O_2, 5% CO_2, and 90% NO_2 resulted in a higher PPE than other tested combinations, and supplementation with hypoxanthine led to improved parasitic growth as well (Jackson et al., 2001).

It was recently observed that a low oxygen environment may not be required to continuously cultivate *T. equi*, a hemoparasite closely related to *C. felis*. Zweygarth and Josemans (2014) speculated that low oxygen tension at culture initiation may cause L-cysteine to be liberated from an erythrocytic source, such as glutathione (GSH). This would suggest that culture initiation and cultivation of *T. equi* may rely more on the availability of L-cysteine than the microaerophilic culture conditions (Zweygarth and Josemans, 2014).

The continuous *in vitro* cultivation of *C. felis* has yet to be achieved. This has slowed the progress with the development of immunological assays (Snider et al., 2010) and vaccines against the parasite. The only way to study *C. felis* is through the use of naturally or experimentally infected cats. The establishment of *C. felis in vitro* cultures

would provide an unlimited source of the parasite for research as well as eliminate the dependence on animal use.

ROLES OF VECTORS IN TRANSMISSION

Ixodid ticks (Acari: Ixodidae) parasitize a broad range of vertebrate hosts worldwide (Bursali et al., 2010) and have been implicated in transmitting pathogens threatening to human and animal health (Sonenshine, 1991). These ticks feed a single time at each active stage of their life cycle. This feeding could occur on any vertebrate hosts from diverse habitats (Sonenshine, 1991).

One common vertebrate host that has been implicated for being a reservoir of certain bacterial and protozoal pathogens is the white-tailed deer (WTD; *Odocoileus virginianus*) (Yabsley et al., 2002; Cantu et al., 2007; Allan et al., 2010; Nair et al., 2014). They may not only potentially harbor certain pathogens, but they can also maintain tick populations (Lane et al., 1991). The most common ticks to parasitize WTD are of the genera *Ixodes, Amblyomma,* and *Dermacentor* (Davidson, 2006). Ticks of these genera are three-host ticks, in which they require a blood-meal from a different host to complete each stage of the life cycle (Allan, 2001). Ticks of *Boophilus,* now *Rhipicephalus,* were also commonly found across the range of white-tailed deer. These one-host ticks, however, have been eradicated from the U.S. but can be found in Texas and California within the quarantine zone along the Mexican border (Allan, 2001). These ticks of WTD also commonly infect other wildlife and livestock.

As with many species of birds, reptiles, and mammals, WTD participate in continuing the wildlife-tick cycle (Davidson and Nettles, 1997). White-tailed deer are the primary reproductive hosts of adult *Ixodes scapularis* (black-legged deer ticks). These ticks spread the Lyme disease bacterium, *Borrelia burgdorferi* (Tillett, 2010). Despite their presence in the *I. scapularis* cycle, WTD seem to have a larger role in the maintenance and transportation of infected ticks than serving as a reservoir for Lyme disease (Lane et al., 1991).

Rhipicephalus (*Boophilus*) spp. ticks primarily feed on and infect cattle with *B. bovis* and *B. bigemina*, the causative agents of Cattle Fever. It has been shown that other ungulates, such as WTD, can be additional hosts of infection (Pound et al., 2010). This could pose a challenge with maintaining the Cattle Tick Fever-free status in the U.S. (Holman et al., 2011).

Data from molecular and serological studies have indicated the presence of *Babesia* spp. in WTD in counties of south Texas (LaSalle and Webb) (Ramos et al., 2010), central Texas (Tom Green) (Holman et al., 2011) and northern states of Mexico that border Texas (Cantu et al., 2007; Cantu et al., 2009). Cantu et al., 2007 studied the presence of *B. bovis* and *B. bigemina* in WTD in the states of Nuevo Leon and Tamaulipas, Mexico by specific nested PCR and indirect fluorescence antibody tests (IFAT) on 20 WTD blood samples. Nested PCR revealed 11 samples as *B. bigemina* positive, and four were positive for *B. bovis.* Subsequent sequences of amplicons were identical to *B. bigemina* and *B. bovis* (*Rap 1*) sequences in Genbank. Results of the IFAT showed antibodies formed against both pathogens in serum samples (Cantu et al., 2007).

In another study, 457 WTD whole blood and serum samples were tested for *B. bigemina* and *B. bovis* using nested PCR and IFAT. A survey was also issued to each WTD ranch used in the study to gather information about management practices. Nested PCR revealed that 19 were *B. bigemina* positive, and six were *B. bovis* positive. The IFAT results were that 274 samples showed antibodies to *B. bovis* and 25 for *B. bigemina.* Statistical analysis of survey information varied with dependent variables, but three management factors (grazing system, habitat, and tick treatment) were associated with positive nested PCR for *B. bigemina.* Findings would suggest that WTD could serve as a reservoir for bovine babesiosis (Cantu et al., 2009).

Ramos et al. (2010) found eight of 49 WTD samples from LaSalle County and two of 47 WTD samples from Webb County, Texas were PCR positive for *B. bovis*. Sequence analysis of subsequent cloning showed 99% identity with *B. bovis* 18S rRNA gene. However, the IFAT showed a poor seroreactivity to *B. bovis* (Ramos et al., 2010).

In a following study, 25 WTD blood samples from Tom Green County, Texas were tested for *B. bigemina* and *B. bovis* (Holman et al., 2011). Three samples were PCR positive for *B. bovis,* as confirmed by ITS sequence analysis. Three samples were PCR positive for *B. bigemina,* but subsequent sequencing of cloned amplicons revealed only one WTD sample as *B. bigemina* positive. The neighbor-joining phylogenetic tree for 18S rDNA sequences revealed a cluster of the WTD *B. bovis* sequences with two bovine *B. bovis* sequences and the WTD *B. bigemina* sequences with bovine *B. bigemina* sequences. The neighbor-joining phylogenetic tree for the rRNA ITS1-5.8S gene-ITS2

genomic region showed no separation between bovine and cervine *B. bovis* sequences (Holman et al., 2011).

It has been shown that *A. americanum* feed on WTD, and these WTD serve as reservoirs of pathogens carried by this tick (Kollars et al., 2000; Childs and Paddock, 2003; Allan et al., 2010). *Ehrlichia chaffeensis* is transmitted by *A. americanum,* and WTD are thought to be the main reservoir responsible for this organism's enzootic cycle (Allan et al., 2010). White-tailed deer naturally infected with *Ehrlichia ewingii* have been documented in several locations that correlate with the distribution of *A. americanum* (Yabsley et al., 2002). In addition, natural infections of *Borrelia lonestari,* vectored by *A. americanum,* has been reported in WTD in the southeastern U.S. (Moore et al., 2003). *Amblyomma americanum* is the primary vector of *C. felis.* With wild felids serving as reservoirs for *C. felis* and *A. americanum* being a 3-host tick, this could possibly lead to WTD as additional hosts in the sylvatic cycle of *C. felis.*

CHAPTER II

EVALUATION OF CULTURE CONDITIONS FOR *IN VITRO* CULTIVATION OF THE ERYTHROCYTIC STAGE OF *CYTAUXZOON FELIS*

INTRODUCTION

Cytauxzoon felis, a tick-borne protozoan hemoparasite, is harbored by wild felids, and its infection in domestic cats causes cytauxzoonosis. High fever, dehydration, anorexia, lethargy, anemia, and dyspnea may be manifestations of cytauxzoonosis (Greene et al., 2006; Meinkoth and Kocan, 2005), which often in the past culminated in death in nearly 100% of *C. felis*-infected cats (Brown et al., 2009). Some cats, however, survive this peracute infection, while others may have a subclinical *C. felis* infection (Reichard et al., 2010).

The pathophysiology of the disease is due to the schizogonous (tissue) phase of the parasite's life cycle (Meinkoth and Kocan, 2005), in which schizonts of *C. felis* distend mononuclear phagocytes and occlude vessels (Snider et al., 2010). The widespread dissemination of vessels occluded by these schizont-infected cells can result in the formation of parasitic thrombi (Kier et al., 1987; Tarigo et al., 2013), which leads to multi-organ failure and death (Tarigo et al., 2013).

The erythrocytic phase occurs when merozoites are released from the schizonts into the bloodstream and enter erythrocytes (Kocan et al., 1992), where they are referred to as piroplasms (Greene et al., 2006). Piroplasms asexually divide and infect new erythrocytes, resulting in erythroparasitemia. This merozoite stage is most exposed to the

host immune system (Miranda et al., 2006), which is advantageous for studies identifying antigenic proteins for diagnostics and vaccine development.

Acquiring parasites for these types of studies involves using either naturally or experimentally infected cats. Ability to cultivate *C. felis in vitro* would eliminate the necessity of infecting cats and lead to a continual source of the parasite. The first reported *C. felis* cultures were used in producing antiserum needed to develop an indirect fluorescent antibody (IFA) test against the parasite (Shindel et al., 1978). Spleen and bone marrow cell suspensions from a moribund *C. felis*-infected cat were used to initiate the cultures. After five days in culture, the infected spleen and bone marrow cells were mixed with African green monkey kidney (Vero) cells and maintained for several passages. The cultures were then used to successfully immunize a cat against challenges of *C. felis*. Serum from this cat was collected and used as the antiserum for developing the IFA test (Shindel et al., 1978). However, Shindel et al. (1978) did not verify propagation of the organism through the subcultures, and there have been no subsequent reports of *in vitro* cultivation of *C. felis*. As a result, the conditions for continuous propagation of the erythrocytic phase of *C. felis* are yet to be described.

However, the erythrocytic stage of a related hemoparasite, *Babesia bovis*, is cultivated *in vitro* using the microaerophilous stationary phase (MASP) system (Levy and Ristic, 1980). In this technique a stationary layer of erythrocytes is overlaid with a buffered cell culture medium containing 25-55% adult bovine serum (Ristic and Levy, 1981). Incubation in a sufficient depth of overlaying medium in an environment of enhanced carbon dioxide content, such as 5% CO_2 and 95% humidified air, results in

reduced oxygen tension in the erythrocyte layer (Levy and Ristic, 1980; Ristic and Levy, 1981). Cultures supporting parasitic growth darken in color due to the oxygen consumption of the parasites as well as the maintenance of the erythrocytic hemoglobin in a deoxygenated state in the reduced oxygen tension (Ristic and Levy, 1981; Schuster, 2002).

A continuous *C. felis* culture system would be advantageous in further developing immunological assays and vaccines, as well as assaying potential therapeutic agents against the parasite (Snider et al., 2010). The objective of this study was to determine the optimal conditions for *in vitro* cultivation of the erythrocytic stage of *C. felis* based on a MASP system.

MATERIALS AND METHODS

Blood Samples

Cytauxzoon felis-infected blood and uninfected donor blood from domestic cats for this study were provided under institutionally approved animal use protocols (Mason Reichard, Department of Veterinary Pathobiology, Oklahoma State University, Stillwater, OK; Animal Care and Use Protocol, "Feline cytauxzoonosis: Establishment of continuous parasites cultures" Protocol Number: VM1121; Leah A. Cohn, Department of Small Animal Internal Medicine, Veterinary Medicine Teaching Hospital, University of Missouri, Columbia, MO; Animal Care and Use Protocol, "*C. felis* source cats" Protocol Number 7301). Normal adult guinea pig, individual male feline, and hamster blood were obtained from a commercial source (BioChemed Services, Inc., Winchester, VA).

Upon receipt, the blood samples were centrifuged at 330 × g for 15 min. The supernatant and buffy coat were removed and discarded. If the resulting packed red blood cells (RBC) volume was ≥ 5 ml, then the RBC were washed 3 times in 5 volumes of Minimum Essential Medium (MEM; Mediatech, Inc., Manassas, VA) or RPMI-1640 medium (Mediatech, Inc.) by centrifugation at 330 × g for 10 minutes. If the volume was < 5 ml, then the cells were similarly washed 3 times by centrifugation at 870 × g for 3 min in a microfuge. After the final wash, the supernatant was removed. Washed RBC (both infected and uninfected) were either used immediately or stored at 4°C in an equal volume of Puck's saline glucose with 10% extra glucose (W/V) (PSG+G) until use for a maximum period of 4 weeks. The percentage of parasitized erythrocytes (PPE) was determined in the washed *C. felis* infected RBC (iRBC) by counting at least 1,000 total RBC on Giemsa-stained smears.

Initiation and Maintenance of Cultures

Cultures were initiated in duplicate wells of 24-, 48-, or 96-well plates in final volumes of 1.1 ml, 800 µl, and 200 µl, respectively, with 10% iRBC (V/V) in various media (Tables 1-4). The initial PPE of the cultures ranged from undetectable to 10%. Some cultures were initiated with a varying initial PPE in 48-well plate (Table 5). The iRBC with approximately 40% PPE were diluted with normal uninfected cat RBC (donor RBC) to yield final PPE of 10%, 5%, and 1% in the cultures.

Table 1. Hl-1 medium and various sera and supplement combinations with inclusion of antibiotic/antimycotic (Ab/Am)[a] tested for *in vitro* cultivation of *Cytauxzoon felis.* The number of cultures initiated (N) are shown. Sera tested were fetal bovine serum (FBS), feline serum (FS), calf serum with iron (CaSI), and others as indicated. Supplements tested include sodium hypoxanthine (Hypo), thymidine (Thym), AlbuMAX I® (Alb), HB 101 supplement (2% V/V prepared according to manufacturer's recommendation) (HB 101), 2 mM L-glutamine (L-glut), bovine serum albumin (BSA), and chemically defined lipids (CDL at 0.375 or 0.75% V/V).

N	Serum	Supplements						
		Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB101 (%)	BSA (mg/ml)	CDL (%)
3	40% FBS	0.2	0.032	2	1	2	-	-
26	20% FBS	0.2	0.032	2	1	2	-	-
6	20% FBS[b]	0.2	0.032	2	1	2	-	-
1	20% FBS	0.2	0.032	2	-	2	-	-
1	10% FS/10% FBS	-	-	2	-	-	-	-
1	30% FS	-	-	2	-	-	-	-
1	40% FS	-	-	2	-	-	-	-
1	10% FS/10% FBS	0.2	0.032	2	1	2	-	-
1	10% FS/10% FBS	-	-	-	-	-	-	-
8	20% FS/20% FBS[c]	0.2	0.032	2	1	2	-	-
1	30% FS	0.2	0.032	2	1	2	-	-
2	20% CaSI	0.2	0.032	2	1	2	-	-
2	40% CaSI	0.2	0.032	2	1	2	-	-
4	40% FS	0.2	0.032	2	1	2	-	-
1	40% FS	0.2	0.032	2	1	2	-	0.75
1	40% FS	0.2	0.032	2	-	2	-	-
3	40% FS	0.2	0.032	2	0.5	2	-	-
1	40% FS	0.2	0.032	2	1	2	12.5	-
1	40% FS	0.2	0.032	2	1	2	-	0.375
4	40% FS	0.2	0.032	2	0.5	2	-	0.375
1	40% FS	0.2	0.032	2	0.5	2	12.5	-
15	20% rabbit serum[d]	0.2	0.032	2	1	2	-	-
1	20% FS	0.2	0.032	2	1	2	-	-
1	20% hamster serum	0.2	0.032	2	1	2	-	-
1	20% rat serum	0.2	0.032	2	1	2	-	-
2	20% mouse serum	0.2	0.032	2	1	2	-	-
1	20% guinea pig serum	0.2	0.032	2	1	2	-	-
3	20% FS	0.2	0.032	2	1	2	-	-
1	10% FS/10% FBS	0.2	0.032	2	-	2	25	-
1	10% FS/10% FBS	0.2	0.032	2	-	2	-	-
1	10% FS/10% FBS	0.2	0.032	2	-	2	25	0.75
2	10% FBS[e]	0.2	0.032	2	1	2	-	-
2	10% FBS[f]	0.2	0.032	2	1	2	-	-
1	20% FBS	0.2	0.032	2	1	2	-	0.75

[a] Final concentration of 200 µg/ml streptomycin, 200 U/ml penicillin, 0.5 µg/ml Fungizone
[b] Histopaque purified.
[c] Two different lots of feline serum were tested.
[d] Four different lots of rabbit serum were tested.
[e] Cultured with a feeder cell layer of baby hamster kidney cells.
[f] Utilized conditioned media from cultured baby hamster kidney cells.

Table 2. HL-1 media combinations tested with no antibiotic/antimycotic added. The number of cultures initiated (N) are shown. Unless stated otherwise, the medium included 2 mM L-glutamine (L-glut), 1 mg/ml AlbuMAX I® (Alb), 0.2 mM sodium hypoxanthine (Hypo) and 0.032 mM thymidine (Thym), and 2% HB101 (V/V) in a total volume of 800 µl in 48-well plates. Sera tested were fetal bovine (FBS), feline (FS), equine (ES), and calf serum with iron (CaSI). Bovine serum albumin (BSA), chemically defined lipids (CDL), Nutridoma-CS (Nutri), and L-glutathione (GSH) were tested as indicated.

N	Serum	Supplements								
		Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)	BSA (mg/ml)	CDL (%)	Nutri (%)	GSH (µg)
2	20% FS	0.2	0.032	2	1	2	-	-	-	-
2	20% CaSI	0.2	0.032	2	1	2	-	-	-	-
21	20% FBS[a]	0.2	0.032	2	1	2	-	-	-	-
2	20% FBS	0.2	0.032	2	-	2	25	-	-	-
1	20% equine serum	0.2	0.032	2	1	2	-	-	-	-
1	40% FBS	0.2	0.032	2	-	2	25	0.75	-	-
2	20% FBS	0.2	0.032	2	-	2	25	0.75	-	-
3	20% FBS [a,b]	0.2	0.032	2	1	2	-	-	-	-
3	20% FBS [a,c]	0.2	0.032	2	1	2	-	-	-	-
3	20% FBS [a,d]	0.2	0.032	2	1	2	-	-	-	-
3	20% FBS	0.2	0.032	-	1	2	-	-	-	-
3	20% FBS	0.3	0.048	-	1	2	-	-	-	-
1	20% FBS [e]	0.3	0.048	2	1	2	-	-	-	-
1	20% FBS	0.2	0.032	2	1	2	-	-	1	-
3	20% FBS	0.2	0.032	2	1	2	-	-	2	-
1	None	0.2	0.032	2	1	2	-	-	2	-
2	20% FBS	0.2	0.032	2	1	2	-	-	-	300
1	20% FBS	0.2	0.032	2	1	2	-	-	-	600
1	20% FBS	0.2	0.032	2	1	2	-	-	-	900
4	20% FBS[f]	-	-	-	-	-	-	-	-	-

[a] Two different lots of fetal bovine serum were tested.
[b] 1.2 ml culture volume in 48-well plate.
[c] 0.9 ml culture volume in 48-well plate.
[d] 0.6 ml culture volume in 48-well plate.
[e] Erythrocytes underwent a hypotonic lysis.
[f] Supplemented with additional 5 mg/ml glucose.

Table 3. Various combinations of medium, sera, and supplements tested for *in vitro* cultivation of *Cytauxzoon felis*. The number of cultures initiated (N) are shown. Media tested were HEPES-buffered Ultradoma (UltraD) without L-glutamine (L-glut) (Lonza, Walkersville, MD), RPMI-1640 (RPMI) with HEPES (Mediatech, Inc.,Manassas, VA) and without HEPES (Lonza), Minimal Essential Medium (MEM) without HEPES (Mediatech), and BSK-II (Millipore, Kankakee, IL). HEPES buffer was added to all media not containing this buffer in the formulation, as indicated. Sera included fetal bovine serum (FBS), feline serum (FS), calf serum with iron (CaSI), or rabbit serum (RS). Supplements included sodium hypoxanthine (Hypo), thymidine (Thym), 1 mg/ml AlbuMAX I® (Alb), HB 101 supplement (HB 101) (2% V/V prepared according to manufacturer's recommendation), 2 mM L-glut, bovine serum albumin (BSA), chemically defined lipids (CDL at 0.375 or 0.75% V/V), 1X MEM Non-essential amino acids (NEAA), Nutridoma-CS (Nutri), and L-glutathione (GSH). BSK-II media consisted of 50 µg/ml of rifampicin, 100 µg/ml of phosphomycin, and 0.25 µg/ml of Amphotericin B (Fungizone). No antibiotic/antimycotic was used in the other media.

N	Medium	Serum	Supplements										
			Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)	BSA (mg/ml)	Ab/Am	CDL (%)	NEAA	Nutri (%)	GSH (µg)
6	UltraD	20% FBS[a]	0.2	0.032	2	1	2	-	-	-	-	-	-
2	RPMI[b]	40% FS	-	-	-	-	-	-	-	-	-	-	-
2	RPMI[b]	40% FBS	-	-	-	-	-	-	-	-	-	-	-
2	RPMI[b]	40% CaSI	-	-	-	-	-	-	-	-	-	-	-
2	RPMI	40% FBS	0.2	0.032	2	1	-	-	-	-	-	-	-
2	RPMI	40% FS	0.2	0.032	2	1	-	-	-	-	-	-	-
2	RPMI	20% CaSI	-	-	-	-	-	-	-	-	-	-	-
4	RPMI	20% FBS	-	-	-	-	-	-	-	-	-	-	-
1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-	-
1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-	300
1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-	600
1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-	900
1	RPMI	20% FBS	0.2	0.032	2	1	-	-	-	-	-	2	-
1	RPMI	10% FBS	0.2	0.032	2	1	-	-	-	-	-	2	-
2	RPMI	20% FBS	0.2	0.032	2	1	-	-	-	-	-	1	-
1	RPMI[c]	None	0.2	-	-	1	-	-	-	-	-	-	-
1	RPMI[c]	20% FBS	0.2	-	-	1	-	-	-	-	-	-	-
1	RPMI[c]	40% FBS	0.2	-	-	1	-	-	-	-	-	-	-
1	RPMI[c]	10% equine serum	0.1	-	-	-	-	-	-	-	-	-	-
3	RPMI[c]	10% FBS[d]	-	-	-	-	-	-	-	-	-	-	-

Table 3. Continued

N	Medium	Serum	Supplements										
			Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)	BSA (mg/ml)	Ab/Am	CDL (%)	NEAA	Nutri (%)	GSH (µg)
2	MEM	None	-	-	-	-	-	-	-	-	-	-	-
2	MEM	None	-	-	-	-	-	-	-	-	1X	-	-
1	MEM	20% FBS	0.2	0.032	-	1	2	-	-	-	1X	-	-
2	MEM[b]	40% FBS	0.2	0.032	-	1	2	-	-	-	1X	-	-
2	MEM	20% FS	-	-	-	-	-	-	-	-	-	-	-
2	MEM	20% FBS	-	-	-	-	-	-	-	-	-	-	-
2	MEM	20% CaSI	-	-	-	-	-	-	-	-	-	-	-
8	BSK-II	20% FBS	-	-	-	-	-	50	+	-	-	-	-
8	BSK-II	20% RS	-	-	-	-	-	50	+	-	-	-	-

[a] Two lots tested.
[b] 20mM HEPES added.
[c] 25mM HEPES added.
[d] Contained 20mg/ml glucose.

Table 4. HL-1 medium supplemented with 2 mM L-glutamine (L-glut), 20% fetal bovine serum (FBS), 1 mg/ml AlbuMAX I® (Alb) or feline serum albumin, 2% HB101 (V/V), and varying concentrations of hypoxanthine (Hypo) and thymidine (Thym) were tested for the *in vitro* cultivation of *Cytauxzoon felis* . A 1:2 or 1:4 initial subculture ratio was employed in some cultures. The media contained no antibiotic/antimycotic. The number of cultures initiated (N) are shown.

N	Serum	Supplements					1:2 Ratio	1:4 Ratio
		Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)		
1	20% FBS	0.1	-	2	1	2	-	+
1	20% FBS	0.2	-	2	1	2	-	+
1	20% FBS	0.3	-	2	1	2	-	+
1	20% FBS	0.4	-	2	1	2	-	+
1	20% FBS	0.2	0.032	2	1	2	-	+
1	20% FBS	-	-	2	1	2	-	+
2	20% FBS	0.1	-	2	1	2	-	-
2	20% FBS	0.2	-	2	1	2	-	-
2	20% FBS	0.3	-	2	1	2	-	-
3	20% FBS	0.4	-	2	1	2	-	-
1	20% FBS	0.1	-	2	1	2	+	-
1	20% FBS	0.2	-	2	1	2	+	-
1	20% FBS	0.3	-	2	1	2	+	-
1	20% FBS	0.4	-	2	1	2	+	-
1	20% FBS	0.2	0.032	2	1	2	+	-
1	20% FBS	-	-	2	1	2	+	-
1	20% FBS	0.5	-	2	1	2	-	-
11	20% FBS[a]	0.3	0.048	2	1	2	-	-
1	20% FBS	0.4	0.064	2	1	2	-	-
1	20% FBS	0.3	0.048	2	1[b]	2	-	-
1	20% FBS	0.3[c]	0.048	2	1	2	-	-

[a] Used in both cat and cat/horse mixed erythrocytes cultures.
[b] 1 mg/ml feline serum albumin replaced Albumax I.
[c] HT increased from 0.2mM, 0.032 mM to 0.3mM, 0.048 mM seven days post initiation.

Table 5. Varying initial percentage of parasitized erythrocytes (PPE) and/or 1:2 weekly subcultures evaluated in *Cytauxzoon felis* cultures. Media used were HL-1, RPMI-1640 (RPMI) with 25 mM HEPES (Mediatech, Inc.,Manassas, VA), and Minimal Essential Medium (MEM) without HEPES (Mediatech), and all were supplemented with 1 mg/ml AlbuMAX I®, 0.2 mM sodium hypoxanthine and 0.032 mM thymidine, and either fetal bovine serum (FBS) or feline serum (FS). Additional supplements consisted of HB 101 supplement (HB 101) (2% V/V prepared according to manufacturer's recommendation), 2 mM L-glut, chemically defined lipids (CDL at 0.75% V/V), and 1X MEM Non-essential amino acids (NEAA). An amount of 20mM HEPES was added to MEM medium. Some media included 200 µg/ml streptomycin, 200 U/ml penicillin, 0.5 µg/ml Fungizone (antibiotic/antimycotic, Ab/Am), and other media contained no Ab/Am. The number of cultures initiated (N) are shown.

N	Medium	Serum	Supplements				Ab/ Am	Weekly Subculture
			HB 101(%)	NEAA	L-glut (mM)	CDL (%)		
4	HL-1	40% FS	2	-	2	-	+	+
2	HL-1	40% FBS	2	-	2	-	+	+
4	HL-1	40% FBS	2	-	2	0.75	+	+
2	HL-1[a]	20% FBS	2	-	2	-	-	+
1	HL-1[b]	20% FBS	2	-	2	-	-	+
1	HL-1[c]	20% FBS	2	-	2	-	-	+
1	HL-1	40% FS	2	-	2	0.75	+	+
1	HL-1[a]	20% FBS	2	-	2	-	-	-
1	HL-1[c]	20% FBS	2	-	2	-	-	-
1	RPMI[a,d]	40% FBS	-	-	2	-	-	-
1	RPMI[c]	40% FBS	-	-	2	-	-	-
1	HL-1[c]	20% FBS	2	-	2	-	-	-
3	HL-1[b,d]	20% FBS	2	-	2	-	-	-
1	RPMI[b,d]	40% FBS	-	-	2	-	-	-
1	MEM[b,d]	40% FBS	2	1X	-	-	-	-
1	MEM[c]	40% FBS	2	1X	-	-	-	-
1	HL-1[a,d]	20% FBS	2	-	2	-	-	-
1	RPMI[a,d]	40% FBS	-	-	2	-	-	-
1	MEM[a,d]	40% FBS	2	1X	-	-	-	-
1	MEM[a]	40% FBS	2	1X	-	-	-	-

[a] Used 1% initial PPE.
[b] Used 5% initial PPE.
[c] Used 10% initial PPE.
[d] Contained no hypoxanthine and thymidine.

The culture medium was replenished daily by removing 900 µl, 700 µl, or 140 µl spent media from wells of 24-, 48-, or 96-well plates, respectively, without disturbing the layer of settled RBC, and then replacing with an equivalent measure of fresh medium. Subcultures were performed at a 1:2 or 1:4 split ratio as needed. The medium was replaced as described above and the cells gently resuspended. The appropriate aliquot was transferred to a new well and fresh medium and donor RBC were added to achieve the final volume (10% RBC). If the PPE was insufficient to subculture, fresh RBC (50 µl, 40 µl, and 10 µl per well in 24-, 48-, or 96-well plates, respectively) were added weekly. Trials of blind passages at a 1:2 split ratio at weekly intervals were performed (Table 5). The cultures were incubated in a humidified gas atmosphere of 5% CO_2, 2% oxygen, and 93% nitrogen at 37°C in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA), except for trials at 39°C or a gas atmosphere of 5% CO_2, 5% O_2, and 90% N_2 (Table 6).

HL-1 medium (Lonza Walkersville, Inc., Walkersville, MD) supplemented with a final concentration of 2 mM L-glutamine, 20% FBS (V/V), 1 mg/ml AlbuMAX I® (Invitrogen, Carlsbad, CA), 0.2 mM sodium hypoxanthine and 0.032 mM thymidine (HT), 2% HB101 (V/V) with or without 200 µg/ml streptomycin, 200 U/ml penicillin, and 50 µg/ml Fungizone (Antibiotic/Antimycotic [Ab/Am]; Gibco BRL, Grand Island, NY), served as the reference medium for comparative studies (Tables 1 and 2).

Table 6. Gas mixtures (5% CO_2, 2% O_2, 93% N_2 and 5% CO_2, 2% O_2, 90% N_2), incubation temperatures (37°C and 39°C), and varying culture volumes (0.6ml, 0.8ml, 0.9ml, and 1.2 ml) in 48-well plates were evaluated in *Cytauxzoon felis* cultures. HL-1 medium and RPMI-1640 without HEPES (Lonza, Walkersville, MD) supplemented with varying concentrations of hypoxanthine (Hypo) and thymidine (Thym), 2 mM L-glutamine (L-glut), fetal bovine serum (FBS), 1 mg/ml AlbuMAX I® (Alb), and 2% HB101 (V/V) were used in the cultures. The media contained no antibiotic/antimycotic. The number of cultures initiated (N) are shown.

N	Medium	Serum	Supplements					O_2 (%)	Temp. (°C)	Volume (ml)
			Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)			
1	HL-1	20% FBS	0.2	0.032	2	1	2	5	37	0.8
1	HL-1	20% FBS	0.3	0.048	2	1	2	5	37	0.8
1	HL-1	20% FBS[a]	-	-	-	-	-	5	37	0.8
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	2	37	1.2
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	2	37	0.9
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	2	37	0.6
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	5	37	1.2
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	5	37	0.9
2	RPMI[b]	40% FBS[c]	0.2	-	-	1	-	5	37	0.6
3	HL-1	20% FBS[c]	0.2	0.032	2	1	2	5	37	1.2
3	HL-1	20% FBS[c]	0.2	0.032	2	1	2	5	37	0.9
3	HL-1	20% FBS[c]	0.2	0.032	2	1	2	5	37	0.6
1	RPMI[b]	None	0.2	-	-	1	-	5	37	0.8
1	RPMI[b]	20% FBS	0.2	-	-	1	-	5	37	0.8
1	RPMI[b]	40% FBS	0.2	-	-	1	-	5	37	0.8
1	HL-1	20% FBS	0.2	0.032	2	1	2	2	39	0.8
1	HL-1	20% FBS	0.3	0.048	2	1	2	2	39	0.8
1	HL-1	20% FBS[d]	0.2	0.032	2	1	2	2	39	0.8
1	HL-1	20% FBS[e]	0.2	0.032	2	1	2	2	39	0.8

[a] Supplemented with additional 5 mg/ml glucose.
[b] 25 mM HEPES added.
[c] Two different lots of fetal bovine serum were tested.
[d] Contained 300µg L-glutathione.
[e] Contained 2% Nutridoma-CS.

Feeder layers of baby hamster kidney (BHK) cells and conditioned medium from the cultures were evaluated for their ability to support the propagation of *C. felis* (Table 1). Cryopreserved BHK cells were thawed at 37°C and transferred to 25-cm^2 culture flasks containing 6 ml of HL-1 medium supplemented with 10% FBS (V/V), 2 mM L-glutamine and Ab/Am (HL10) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. When a confluent monolayer was reached, the medium was removed and the cell layer rinsed twice with 2 ml Dulbecco's phosphate-buffered saline (PBS). The cells layer was trypsinized at 37°C to facilitate detachment. After detachment, 4 ml HL10 was added, and the cells resuspended. The cell suspension (3 ml) was added to duplicate wells of a 6-well plate. The BHK cells were allowed to adhere for 1 h, and the medium was removed and replaced. Cell culture inserts (Falcon Translucent Polyethylene Terephthalate Cell Culture Insert, Becton Dickinson Labware, Franklin Lakes, NJ) were placed in each well and 2 ml of fresh HL10 and 100 µl of packed iRBC were added inside the insert. At 48 h intervals, 4 ml medium was removed and replaced. The cultures were incubated at 37°C in a humidified 5% CO_2 in air atmosphere.

Conditioned HL10 medium was collected daily from monolayer cultures of BHK cells and centrifuged at 870 × g for 3 min to remove any BHK cells. Conditioned medium was tested on *C. felis* cultured in 96-well plates. Each well received 140 µl of conditioned medium daily as described above for culture maintenance.

Density gradient cell separation with Histopaque®-1077 (Sigma Chemical Co., St. Louis, MO) was evaluated in a few cultures as a means of more efficiently eliminating white blood cells from erythrocyte preparations (Table 1). Three milliliters

of *C. felis*-infected whole blood was layered onto 3 ml of 25°C Histopaque-1077 and centrifuged at 330 × g for 30 min. After centrifugation, the resultant layers were removed, and the RBC were washed as described above.

Since iRBC (such as *B. bovis* iRBC) were more resistant to hypotonic saline solution, a differential lysis resulted in a higher concentration of iRBC (Wright, 1990). In order to increase the percentage of *C. felis* iRBC, a hypotonic lysis technique prior to initiation was implemented prior to culture initiation (Table 2). The iRBC were washed as described above. Five milliliters of 0.475% (W/V) NaCl solution was added to 1 ml of iRBC, mixed gently, and allowed to sit at 25°C for 5 min. The mixture was centrifuged at 330 × g for 5 min followed by removal of supernatant. The resultant iRBC were subsequently used in culture.

Erythrocytes from different species (cat, rabbit, or hamster) were tested as donor cells for *C. felis in vitro* as shown in Table 7. The RBC were washed using the wash protocol stated above and introduced to the cultures at the time of subculture and/or RBC replenishment.

Thin smears of cultured RBC were used to monitor parasitic growth and evaluate the culture conditions daily. The smears were stained 1:20 with Giemsa (Accustain, Sigma, St. Louis, MO) and examined by microscopy under oil immersion at 1000X. RBC containing *C. felis* piriforms (tetrads, singles, and multiples) in a total of 1000 RBC were counted to calculate the PPE.

Table 7. Cat, rabbit, or hamster donor normal erythrocytes were tested in *Cytauxzoon felis* cultures (total volume of 800 µl in 48-well plates) as shown. The medium was HL1 with 2 mM L-glutamine, 20% serum (RS, rabbit serum; FS, feline serum; FBS, fetal bovine serum), 1 mg/ml AlbuMAX I® (Alb), and 2% HB101 (V/V)) supplemented with hypoxanthine (Hypo) and thymidine (Thym) as shown, with no antibiotic/antimycotic.

Donor Erythrocytes	Serum	Supplements				
		Hypo (mM)	Thym (mM)	L-glutamine (mM)	Albumax (mg/ml)	HB 101 (%)
Rabbit	20% FBS	0.2	0.032	2	1	2
Hamster	20% FBS	0.2	0.032	2	1	2
Rabbit	20% RS	0.2	0.032	2	1	2
Rabbit	20% FBS	0.3	0.048	2	1	2
Hamster	20% FBS	0.3	0.048	2	1	2
Cat	20% FS	0.3	0.048	2	1	2
Rabbit	20% RS	0.3	0.048	2	1	2

RESULTS

A total of 332 *C. felis* erythrocytic stage cultures were initiated during this study. The minimum and maximum duration of cultures were 2 and 140 days, respectively, with an average of 32 days. Five cultures were subcultured 4 times, the maximum level achieved. The PPE maintained in the cultures ranged from undetectable to 1.1%.

One hundred two cultures were initiated using HL-1 medium with inclusion of antibiotic/antimycotic (Ab/Am) and various sera and supplement combinations. One culture was maintained for 140 days, which was the longest duration achieved. Fifteen cultures underwent a subculture. The PPE maintained in the cultures ranged from undetectable to 0.02% (Table 8).

Table 8. Results of the representative *Cytauxzoon felis* culture of the cultures of HL-1 medium with antibiotic/antimycotic (Ab/Am)[a] and various sera and supplement combinations tested. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown. Sera tested were fetal bovine serum (FBS), feline serum (FS), calf serum with iron (CaSI), and others as indicated. Supplements tested include sodium hypoxanthine (Hypo), thymidine (Thym), AlbuMAX I® (Alb), HB 101 supplement (2% V/V prepared according to manufacturer's recommendation) (HB 101), 2 mM L-glutamine (L-glut), bovine serum albumin (BSA), and chemically defined lipids (CDL at 0.375 or 0.75% V/V).

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Serum	Supplements				
					Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB101 (%)
18	0.2	0.006	-	40% FBS	0.2	0.032	2	1	2
108	0.5	0.01	1	20% FBS	0.2	0.032	2	1	2
94	0.3	0.007	1	20% FBS[b]	0.2	0.032	2	1	2
84	0.2	0.005	-	20% FBS	0.2	0.032	2	-	2
59	0.2	0.003	-	10% FS/10% FBS[c]	-	-	2	-	-
59	0.2	0.005	-	30% FS[c]	-	-	2	-	-
59	0.2	0.003	-	40% FS[c]	-	-	2	-	-
59	0.2	0.005	-	10% FS/10% FBS	0.2	0.032	2	1	2
82	0.3	0.004	-	10% FS/10% FBS[c,d]	-	-	-	-	-
63	0.4	0.008	1	20% FS/20% FBS[e]	0.2	0.032	2	1	2
84	0.2	0.006	-	30% FS	0.2	0.032	2	1	2
32	0.2	0.006	-	20% CaSI	0.2	0.032	2	1	2
11	low	low	-	40% CaSI	0.2	0.032	2	1	2
140	0.2	0.02	1	40% FS	0.2	0.032	2	1	2
18	0.2	0.006	-	40% FS	0.2	0.032	2	1	2
18	low	low	-	40% FS	0.2	0.032	2	-	2
31	0.2	0.006	-	40% FS	0.2	0.032	2	0.5	2
18	0.2	0.006	-	40% FS	0.2	0.032	2	1	2
18	0.2	0.006	-	40% FS	0.2	0.032	2	1	2
28	0.2	0.01	-	40% FS	0.2	0.032	2	0.5	2
18	0.2	0.006	-	40% FS	0.2	0.032	2	0.5	2
84	0.5	0.007	-	20% rabbit serum[f]	0.2	0.032	2	1	2

Table 8. Continued.

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Serum	Supplements				
					Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB101 (%)
113	0.2	0.01	1	20% FS[e]	0.2	0.032	2	1	2
33	0.2	0.006	-	20% hamster serum	0.2	0.032	2	1	2
100	0.6	0.01	1	20% rat serum	0.2	0.032	2	1	2
80	0.2	0.004	-	20% mouse serum	0.2	0.032	2	1	2
33	0.2	0.006	-	20% guinea pig serum	0.2	0.032	2	1	2
82	0.4	0.006	-	10% FS/10% FBS	0.2	0.032	2	1	2
84	0.2	0.006	-	10% FS/10% FBS	0.2	0.032	2	-	2
84	0.2	0.006	-	10% FS/10% FBS	0.2	0.032	2	-	2
14	low	low	-	10% FBS[g]	0.2	0.032	2	-	2
24	0.5	0.04	-	10% FBS[h]	0.2	0.032	2	1	2
73	0.6	0.08	-	20% FBS	0.2	0.032	2	1	2

[a] Final concentration of 200 µg/ml streptomycin, 200 U/ml penicillin, 0.5 µg/ml Fungizone.
[b] Histopaque purified.
[c] Contained no 2% HB101.
[d] Contained no added 2mM L-glut.
[e] Two different lots of feline serum were tested.
[f] Four different lots of rabbit serum were tested.
[g] Cultured with a feeder cell layer of baby hamster kidney cells.
[h] Utilized conditioned media from cultured baby hamster kidney cells.

Sixty cultures were initiated using HL-1 medium with various sera and supplements in the absence of Ab/Am. One culture was maintained for 103 days. Of these cultures, 31 were subcultured, and the maximum subculture level achieved was four. The PPE maintained in the cultures ranged from undetectable to 0.02% (Table 9).

Sixty-six cultures were initiated with various sera, supplement combinations, and various media, which excluded the HL-1 medium. A maximum duration of 50 days was achieved with one culture. Nineteen cultures were subcultured, and the maximum subculture level achieved was two. The PPE maintained in the cultures ranged from undetectable to 0.01% (Table 10).

Thirty-six cultures were initiated using supplemented HL-1 medium without Ab/Am and varying concentrations of hypoxanthine and thymidine. One culture was maintained for 70 days. A subculture was attained in 29 cultures, and the maximum subculture level achieved was four. The PPE maintained in the cultures ranged from undetectable to 0.02% (Table 11).

Using a varying initial PPE and/or 1:2 weekly subculture, 30 cultures were initiated with various media, either FBS or FS, and various other supplements. One culture was maintained for 84 days. Blind, 1:2 weekly subculture were performed on 15 cultures, and passage 10 was the highest attained. The remaining 15 cultures all underwent at least one subculture, in which a 4th subculture passage was the highest reached. The PPE maintained in the cultures ranged from undetectable to 1.1% (Table 12).

Table 9. Results of the representative *Cytauxzoon felis* culture of the cultures of HL-1 medium without antibiotic/antimycotic (Ab/Am) and various sera and supplement combinations tested. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown. Unless stated otherwise, the medium included 2 mM L-glutamine (L-glut), 1 mg/ml AlbuMAX I® (Alb), 0.2 mM sodium hypoxanthine (Hypo) and 0.032 mM thymidine (Thym), and 2% HB101 (V/V) in a total volume of 800 µl in 48-well plates. Sera tested were fetal bovine (FBS), feline (FS), equine (ES), and calf serum with iron (CaSI). Bovine serum albumin (BSA), chemically defined lipids (CDL), Nutridoma-CS (Nutri), and L-glutathione (GSH) were tested as indicated.

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Serum	Supplements						
					Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	CDL (%)	Nutri (%)	GSH (µg)
39	0.2	0.01	1	20% FS	0.2	0.032	2	1	-	-	-
10	low	low	-	20% CaSI	0.2	0.032	2	1	-	-	-
103	0.5	0.02	4	20% FBS[a]	0.2	0.032	2	1	-	-	-
53	0.5	0.02	1	20% FBS[b]	0.2	0.032	2	-	-	-	-
10	low	low	-	20% equine serum	0.2	0.032	2	1	-	-	-
32	0.2	0.01	-	40% FBS[b]	0.2	0.032	2	-	0.75	-	-
36	0.1	0.01	1	20% FBS[b]	0.2	0.032	2	-	0.75	-	-
38	0.2	0.01	2	20% FBS [a,c]	0.2	0.032	2	1	-	-	-
27	0.2	0.01	2	20% FBS [a,d]	0.2	0.032	2	1	-	-	-
35	0.2	0.01	1	20% FBS [a,e]	0.2	0.032	2	1	-	-	-
31	0.1	0.006	-	20% FBS	0.2	0.032	-	1	-	-	-
57	0.2	0.007	-	20% FBS	0.3	0.048	-	1	-	-	-
24	low	low	-	20% FBS[f]	0.3	0.048	2	1	-	-	-
14	low	low	-	20% FBS	0.2	0.032	2	1	-	1	-
49	0.5	0.006	1	20% FBS	0.2	0.032	2	1	-	2	-
13	low	low	-	None	0.2	0.032	2	1	-	2	-
44	0.2	0.01	3	20% FBS	0.2	0.032	2	1	-	-	300
30	0.3	0.01	2	20% FBS	0.2	0.032	2	1	-	-	600
39	0.2	0.005	1	20% FBS	0.2	0.032	2	1	-	-	900
63	0.1	0.008	1	20% FBS[g,h]	-	-	-	-	-	-	-

[a] Two different lots of fetal bovine serum were tested.
[b] Contained 25mg/ml BSA.
[c] 1.2 ml culture volume in 48-well plate.
[d] 0.9 ml culture volume in 48-well plate.
[e] 0.6 ml culture volume in 48-well plate.
[f] Erythrocytes underwent a hypotonic lysis.
[g] Supplemented with additional 5 mg/ml glucose.
[h] Contained no 2% HB101.

Table 10. Results of the representative *Cytauxzoon felis* culture of the cultures of various combinations of medium, sera, and supplements tested. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown. Media tested were HEPES-buffered Ultradoma (UltraD) without L-glutamine (L-glut) (Lonza, Walkersville, MD), RPMI-1640 (RPMI) with HEPES (Mediatech, Inc.,Manassas, VA) and without HEPES (Lonza), Minimal Essential Medium (MEM) without HEPES (Mediatech), and BSK-II (Millipore, Kankakee, IL). HEPES buffer was added to all media not containing this buffer in the formulation, as indicated. Sera included fetal bovine serum (FBS), feline serum (FS), calf serum with iron (CaSI), or rabbit serum (RS). Supplements included sodium hypoxanthine (Hypo), thymidine (Thym), 1 mg/ml AlbuMAXI® (Alb), HB 101 supplement (HB 101) (2% V/V prepared according to manufacturer's recommendation), 2 mM L-glut, bovine serum albumin (BSA), 1X MEM Non-essential amino acids (NEAA), Nutridoma-CS (Nutri), and L-glutathione (GSH). BSK-II media consisted of 50 µg/ml of rifampicin, 100 µg/ml of phosphomycin, and 0.25 µg/ml of Amphotericin B (Fungizone). No antibiotic/antimycotic was used in the other media.

Days	Initial PPE (%)	Maintained PPE (%)	Sub-culture Level	Medium	Serum	Supplements									
						Hypo (mM)	Thym (mM)	L-glut (mM)	Alb (mg/ml)	HB 101 (%)	BSA (mg/ml)	Ab/ Am	CDL (%)	NEAA	Nutri (%)
29	0.1	0.01	2	UltraD	20% FBS[a]	0.2	0.032	2	1	2	-	-	-	-	-
37	0.2	0.005	1	RPMI[b]	40% FS	-	-	-	-	-	-	-	-	-	-
27	0.2	0.007	1	RPMI[b]	40% FBS	-	-	-	-	-	-	-	-	-	-
10	low	low	-	RPMI[b]	40% CaSI	-	-	-	-	-	-	-	-	-	-
25	0.3	0.008	-	RPMI	40% FBS	0.2	0.032	2	1	-	-	-	-	-	-
21	0.2	0.01	-	RPMI	40% FS	0.2	0.032	2	1	-	-	-	-	-	-
30	0.2	0.007	-	RPMI	20% CaSI	-	-	-	-	-	-	-	-	-	-
50	0.2	0.008	2	RPMI	20% FBS	-	-	-	-	-	-	-	-	-	-
33	0.1	0.009	2	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-
28	0.1	0.01	1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-
25	0.2	0.008	1	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-
15	0.2	0.007	-	RPMI[c]	20% FBS	0.28	-	-	-	-	40	-	-	-	-
25	0.2	0.01	1	RPMI	20% FBS	0.2	0.032	2	1	-	-	-	-	-	2
32	0.2	0.009	1	RPMI	10% FBS	0.2	0.032	2	1	-	-	-	-	-	2
49	0.2	0.006	1	RPMI	20% FBS	0.2	0.032	2	1	-	-	-	-	-	1
19	0.1	0.005	1	RPMI[c]	None	0.2	-	-	1	-	-	-	-	-	-
40	0.4	0.008	1	RPMI[c]	20% FBS	0.2	-	-	1	-	-	-	-	-	-
20	0.5	0.01	1	RPMI[c]	40% FBS	0.2	-	-	1	-	-	-	-	-	-
40	0.2	0.008	2	RPMI[c]	10% equine serum	0.1	-	-	-	-	-	-	-	-	-
14	low	low	-	RPMI[c]	10% FBS[d]	-	-	-	-	-	-	-	-	-	-
12	low	low	-	MEM	None	-	-	-	-	-	-	-	-	1X	-
12	low	low	-	MEM	None	-	-	-	-	-	-	-	-	-	-
19	0.1	0.005	-	MEM	20% FBS	0.2	0.032	-	1	2	-	-	-	1X	-
29	0.2	0.01	1	MEM[b]	40% FBS	0.2	0.032	-	1	2	-	-	-	1X	-
17	0.2	0.006	1	MEM	20% FS	-	-	-	-	-	-	-	-	-	-
17	0.2	0.01	1	MEM	20% FBS	-	-	-	-	-	-	-	-	-	-
10	low	low	-	MEM	20% CaSI	-	-	-	-	-	-	-	-	-	-
48	0.3	0.008	-	BSK-II	20% FBS	-	-	-	-	-	50	+	-	-	-
41	0.4	0.01	-	BSK-II	20% RS	-	-	-	-	-	50	+	-	-	-

[a] Two lots tested.
[b] 20mM HEPES added.
[c] 25mM HEPES added.
[d] Contained 20mg/ml glucose.

Table 11. Results of the representative *Cytauxzoon felis* culture of the cultures of HL-1 medium supplemented with 2 mM L-glutamine, 20% fetal bovine serum (FBS), 1 mg/ml AlbuMAX I® or feline serum albumin, 2% HB101 (V/V), and varying concentrations of hypoxanthine (Hypo) and thymidine (Thym) tested. A 1:2 or 1:4 initial subculture ratio was employed in some cultures. The media contained no antibiotic/antimycotic. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown.

Days	Initial PPE (%)	Maintained PPE (%)	Sub-culture Level	Serum	Supplements			1:2 Ratio	1:4 Ratio
					Hypo (mM)	Thym (mM)	L-glut (mM)		
10	low	low	-	20% FBS	0.1	-	2	-	+
10	low	low	-	20% FBS	0.2	-	2	-	+
10	low	low	-	20% FBS	0.3	-	2	-	+
50	0.2	0.01	1	20% FBS	0.4	-	2	-	+
10	low	low	-	20% FBS	0.2	0.032	2	-	+
10	low	low	-	20% FBS	-	-	2	-	+
39	0.1	0.008	1	20% FBS	0.1	-	2	-	-
45	0.2	0.01	3	20% FBS	0.2	-	2	-	-
36	0.2	0.01	2	20% FBS	0.3	-	2	-	-
70	0.5	0.01	4	20% FBS	0.4	-	2	-	-
35	0.2	0.01	2	20% FBS	0.1	-	2	+	-
46	0.2	0.01	2	20% FBS	0.2	-	2	+	-
46	0.2	0.01	2	20% FBS	0.3	-	2	+	-
46	0.2	0.02	3	20% FBS	0.4	-	2	+	-
50	0.5	0.02	2	20% FBS	0.2	0.032	2	+	-
30	0.2	0.01	2	20% FBS	-	-	2	+	-
33	0.2	0.01	2	20% FBS	0.5	-	2	-	-
63	0.2	0.01	4	20% FBS[a]	0.3	0.048	2	-	-
28	0.2	0.01	2	20% FBS	0.4	0.064	2	-	-
11	0.1	0.009	1	20% FBS	0.3	0.048	2	-	-
31	0.2	0.01	2	20% FBS	0.3[c]	0.048	2	-	-

[a] Used in both cat and cat/horse mixed erythrocytes cultures.
[b] 1 mg/ml feline serum albumin replaced Albumax I.
[c] HT increased from 0.2mM, 0.032 mM to 0.3mM, 0.048 mM seven days post initiation.

Table 12. Results of the representative *Cytauxzoon felis* culture of the cultures of varying initial percentage of parasitized erythrocytes (PPE) and/or 1:2 weekly subcultures evaluated. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown. Media used were HL-1, RPMI-1640 (RPMI) with 25 mM HEPES (Mediatech, Inc.,Manassas, VA), and Minimal Essential Medium (MEM) without HEPES (Mediatech), and all were supplemented with 1 mg/ml AlbuMAX I®, 0.2 mM sodium hypoxanthine and 0.032 mM thymidine, and either fetal bovine serum (FBS) or feline serum (FS). Additional supplements consisted of HB 101 supplement (HB 101) (2% V/V prepared according to manufacturer's recommendation), 2 mM L-glut, chemically defined lipids (CDL at 0.75% V/V), and 1X MEM Non-essential amino acids (NEAA). An amount of 20mM HEPES was added to MEM medium. Some media included 200 µg/ml streptomycin, 200 U/ml penicillin, 0.5 µg/ml Fungizone (antibiotic/antimycotic, Ab/Am), and other media contained no Ab/Am.

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Medium	Serum	Supplements				Ab/Am	Weekly Subculture
						HB 101 (%)	NEAA	L-glut (mM)	CDL (%)		
63	0.2	0.01	9	HL-1	40% FS	2	-	2	-	+	+
11	low	low	1	HL-1	40% FBS	2	-	2	-	+	+
42	0.2	0.02	6	HL-1	40% FBS	2	-	2	0.75	+	+
49	1.0	0.06	7	HL-1	20% FBS	2	-	2	-	-	+
61	5.0	0.2	8	HL-1	20% FBS	2	-	2	-	-	+
84	10.0	0.5	10	HL-1	20% FBS	2	-	2	-	-	+
7	low	low	1	HL-1	40% FS	2	-	2	0.75	+	+
52	1.0	0.04	3	HL-1	20% FBS	2	-	2	-	-	-
50	10.0	0.7	3	HL-1	20% FBS	2	-	2	-	-	-
49	1.0	0.004	1	RPMI[a]	40% FBS	-	-	2	-	-	-
14	10.0	1.1	2	RPMI	40% FBS	-	-	2	-	-	-
50	10.0	0.6	4	HL-1	20% FBS	2	-	2	-	-	-
30	5.0	0.05	2	HL-1[a]	20% FBS	2	-	2	-	-	-
49	5.0	0.2	3	RPMI[a]	40% FBS	-	-	2	-	-	-
50	5.0	0.3	3	MEM[a]	40% FBS	2	1X	-	-	-	-
25	10.0	0.9	4	MEM	40% FBS	2	1X	-	-	-	-
30	1.0	0.01	2	HL-1[a]	20% FBS	2	-	2	-	-	-
28	1.0	0.01	3	RPMI[a]	40% FBS	-	-	2	-	-	-
20	1.0	0.01	1	MEM[a]	40% FBS	2	1X	-	-	-	-
39	1.0	0.01	3	MEM	40% FBS	2	1X	-	-	-	-

[a] Contained no hypoxanthine or thymidine.

Thirty-one cultures were initiated using supplemented HL-1 or RPMI media without Ab/Am and incubated in varying gas mixtures (5% CO2, 5% O2, 90% N2 and 5% CO2, 2% O2, 93% N2), incubation temperatures (37°C and 39°C), and varying culture volumes (0.6ml, 0.8ml, 0.9ml, and 1.2 ml). One culture was maintained for 44 days. A subculture was attained in 21 cultures, and the maximum subculture level achieved was three. The PPE maintained in the cultures ranged from undetectable to 0.01% (Table 13).

Seven cultures were initiated using donor erythrocytes from different animal species in HL-1 supplemented medium without Ab/Am. Six of the seven cultures were maintained for 31 days. A subculture was attained in all seven cultures, and the maximum subculture level achieved was two. The PPE maintained in the cultures ranged from undetectable to 0.01% (Table 14).

Table 13. Results of the representative *Cytauxzoon felis* culture of the cultures incubated in varying gas mixtures (5% CO_2, 5% O_2, 90% N_2 and 5% CO_2, 2% O_2, 93% N_2), incubation temperatures (37°C and 39°C), and varying culture volumes (0.6 ml, 0.8 ml, 0.9 ml, and 1.2 ml) in 48-well plates tested. HL-1 medium and RPMI-1640 without HEPES (Lonza, Walkersville, MD) supplemented with varying concentrations of hypoxanthine (Hypo) and thymidine (Thym), 2 mM L-glutamine, fetal bovine serum (FBS), 1 mg/ml AlbuMAX I®, and 2% HB101 (V/V) were used in the cultures. The media contained no antibiotic/antimycotic. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown.

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Medium	Serum	Supplements			O_2 (%)	Temp. (°C)	Volume (ml)
						Hypo (mM)	Thym(mM)	HB 101 (%)			
40	0.3	0.01	3	HL-1	20% FBS[a]	0.2	0.032	2	5	37	0.8
37	0.3	0.005	1	HL-1	20% FBS[a]	0.3	0.048	2	5	37	0.8
43	0.3	0.007	3	HL-1	20% FBS[b,c]	-	-	-	5	37	0.8
42	0.4	0.007	1	RPMI[d]	40% FBS[e]	0.2	-	-	2	37	1.2
16	low	low	1	RPMI[d]	40% FBS[e]	0.2	-	-	2	37	0.9
26	0.3	0.01	2	RPMI[d]	40% FBS[e]	0.2	-	-	2	37	0.6
44	0.3	0.009	1	RPMI[d]	40% FBS[e]	0.2	-	-	5	37	1.2
26	0.3	0.01	1	RPMI[d]	40% FBS[e]	0.2	-	-	5	37	0.9
26	0.3	0.01	1	RPMI[d]	40% FBS[e]	0.2	-	-	5	37	0.6
29	0.3	0.01	2	HL-1	20% FBS[a,e]	0.2	0.032	2	5	37	1.2
29	0.3	0.01	2	HL-1	20% FBS[a,e]	0.2	0.032	2	5	37	0.9
26	0.3	0.01	1	HL-1	20% FBS[a,e]	0.2	0.032	2	5	37	0.6
19	low	low	1	RPMI[d]	None	0.2	-	-	5	37	0.8

Table 13. Continued.

Days	Initial PPE (%)	Maintained PPE (%)	Subculture Level	Medium	Serum	Supplements			O_2 (%)	Temp. (°C)	Volume (ml)
						Hypo (mM)	Thym(mM)	HB 101 (%)			
28	0.4	0.01	1	RPMI[d]	20% FBS	0.2	-	-	5	37	0.8
40	0.3	0.008	1	RPMI[d]	40% FBS	0.2	-	-	5	37	0.8
40	0.3	0.01	2	HL-1	20% FBS[a]	0.2	0.032	2	2	39	0.8
39	0.3	0.008	1	HL-1	20% FBS[a]	0.3	0.048	2	2	39	0.8
34	0.3	0.01	1	HL-1	20% FBS[a,f]	0.2	0.032	2	2	39	0.8
12	low	low	-	HL-1	20% FBS[a,g]	0.2	0.032	2	2	39	0.8

[a] Contained 2 mM L-glutamine.
[b] Supplemented with additional 5 mg/ml glucose.
[c] Contained no 1mg/ml Albumax I.
[d] 25 mM HEPES added.
[e] Two different lots of fetal bovine serum were tested.
[f] Contained 300µg L-glutathione.
[g] Contained 2% Nutridoma-CS.

Table 14. Results of the representative *Cytauxzoon felis* culture of the cultures of cat, rabbit, or hamster donor normal eryrhrocytes tested in total volume of 800 µl in 48-well plates as shown. The medium was HL-1 with 2 mM L-glutamine, 20% serum (RS, rabbit serum; FS, feline serum; FBS, fetal bovine serum), 1 mg/ml AlbuMAX I® (Alb), and 2% HB101 (V/V) supplemented with hypoxanthine (Hypo) and thymidine (Thym) as shown, with no antibiotic/antimycotic. Duration of the culture (Days), the PPE at initiation (Initial PPE), average PPE during maintenance (Maintained PPE) (cultures with undetectable parasitemias for most of culture duration are indicated as low), and subculture level attained are shown.

Days	Initial PPE (%)	Maintained PPE (%)	Sub-culture Level	Donor Erythrocytes	Serum	Supplements		
						Hypo (mM)	Thym (mM)	HB 101 (%)
31	0.2	0.009	1	Rabbit	20% FBS	0.2	0.032	2
17	0.2	0.006	1	Hamster	20% FBS	0.2	0.032	2
31	0.2	0.006	1	Rabbit	20% RS	0.2	0.032	2
31	0.2	0.006	1	Rabbit	20% FBS	0.3	0.048	2
31	0.2	0.006	1	Hamster	20% FBS	0.3	0.048	2
31	0.2	0.01	1	Cat	20% FS	0.3	0.048	2
31	0.2	0.006	2	Rabbit	20% RS	0.3	0.048	2

DISCUSSION

This study evaluated *in vitro* cultivation parameters for the erythrocytic stage of *Cytauxzoon felis* in a microaerophilous stationary phase (MASP) culture system (Levy and Ristic, 1980). Numerous hemoparasites have been cultured using various adaptations of this methodology, including both *Babesia* and *Theileria* species (reviewed by Schuster, 2002). Specifically, the successful establishment of continuous cultures of *Theileria equi* and *Theileria uilenbergi* (Holman et al., 1994; Zweygarth et al., 1995a; Miranda et al., 2006), which are closely related to the genus *Cytauxzoon,* suggested the suitability of MASP for establishing continuous cultures of *C. felis.*

Successful continuous cultivation of *T. uilenbergi* required a PPE greater than 0.1% at culture initiation (Miranda et al., 2006). In the current study, a range of initial

PPE from cats with different clinical presentations of cytauxzoonosis was investigated to determine the effects on *in vitro* propagation of *C. felis*. Those cultures with an initial PPE of approximately 0.2% from subclinical, chronic cytauxzoonosis cases were maintained for the longest periods, while the cultures initiated from acute, recovered cases had the greatest mean length in culture overall. Cultures initiated with these RBC of acute, fatal cases had the highest PPEs maintained in the cultures. However, the parasites in these cultures became undetectable at a fast rate, which likely indicates the high maintained PPEs are attributable to the high initial PPEs. It was also observed that cultures started with an initial PPE greater than 0.2% from acute, fatal cases underwent more subculture passages than others. Inasmuch as continuous cultures were not attained in this study, the impact of a high or low initial PPE is difficult to assess.

Medium and serum selection have long been recognized as critically important factors in continuous *in vitro* cultivation of hemoparasitic protozoa (reviewed by Canning and Winger 1987). In this study, *C. felis* cultures initially propagated in all six media tested, which included HL-1, conditioned HL-1, RPMI-1640, Ultradoma, BSK-II, and MEM. However, only formulations of HL-1 medium were able to support growth for an extended period of time. HL-1 medium is a chemically defined medium that is formulated with known amounts of insulin, transferrin, sodium selenite, testosterone, ethanolamine, proprietary stabilizing proteins, unsaturated, and saturated fatty acids. This medium was developed for of hybridomas and cells of lymphoid origin (Ishikawa et al., 1987). HL-1 medium was first used to culture *Babesia (Theileria) equi* (Holman et al., 1994), and has been shown to support cultures of related hemoparasites (Holman et

al., 1993; Zweygarth et al., 1999; Miranda et al., 2006). In one study comparing HL-1, Medium 199, and RPMI-1640 media for culturing *T. uilenbergi,* only HL-1 supported long term cultivation of the parasite (Miranda et al., 2006). Also, *Babesia caballi* is supported by HL-1 medium in the presence of serum and in the absence of serum (Holman et al., 1993; Zweygarth et al., 1999). Another hybridoma medium, Ultradoma, contains insulin, transferrin and albumin, and *C. felis* cultures with this medium did not last beyond 29 days. Of the different sera tested in this study, fetal bovine serum and feline serum produced the best results. Serum supplementation at less than 20% was not as suitable for cultivating *C. felis in vitro*.

Antibiotics and antimycotics are routinely used in cell cultures to prevent microbial contamination. In this study, the antibiotic and antimycotic combination used was penicillin, streptomycin, and amphotericin B. Penicillin works by targeting the cell wall biosynthesis in bacteria. Streptomycin acts by binding to the 30S subunit of the bacterial ribosome, and this subsequently inhibits the protein synthesis in bacteria (Waksman, 1953). Amphotericin B works by binding to the sterol component in the cell membrane and increases its permeability (Wiehart et al., 2006).

Cytauxzoon felis cultivated in the absence of the antibiotic-antimycotic combination underwent a greater number of subculture levels than those in its presence. This might be due to the amphotericin B component. Amphotericin B more readily binds to the ergosterol in fungal cell membranes, but it can also bind the cholesterol component of mammalian cell membranes, albeit with lower affinity, and lead to cytotoxicity (Wiehart et al., 2006). Erythrocytes parasitized by the trophozoite stage of

Plasmodium falciparum were more susceptible to lysis in the presence of amphotericin B (Wiehart et al., 2006). Amphotericin B also showed effects against *Babesia gibsoni* in normal canine erythrocytes 12 hours after culture initiation (Yamasaki et al., 2014). However, the utilization of the amphotericin B along with penicillin and streptomycin did not the affect the continuous cultivation of *B. caballi* established from blood of horses in a carrier state (Holman et al., 1993). It could be speculated that amphotericin B may play a role in the decreased invasion thus subsequent lowered PPE and number of subculture passages in *C. felis* cultures, but further studies are needed to determine whether this drug impedes cellular invasion by this hemoparasite.

This study demonstrated that hypoxanthine-supplemented cultures maintained a higher PPE for a longer period of time than cultures without this purine derivative. In earlier studies with *Plasmodium* spp., infected erythrocytes incorporated exogenously supplied purines into nucleic acids (Bungener and Nielsen, 1967, 1968; Gutteridge and Trigg, 1970). Just as with *Plasmodium* spp., *C. felis* may require purine from the medium in order to synthesize nucleic acids. When initiated from animals with a very low parasitemia or from liquid nitrogen storage, hypoxanthine seemed to enhance the growth of *T. (B.) equi* and an unknown *Babesia* sp. in cultures (Zweygarth et al., 1995a; Zweygarth et al., 1995b). Hypoxanthine was also vital for *in vitro* growth of *P. falciparum* in serum-free conditions (Asahi et al., 1996). RPMI-1640 medium contains no purine precursors, and when this basal medium was supplemented with hypoxanthine, *Babesia bigemina* was able to grow in every serum condition tested (Neves et al., 2001).

One *C. felis* culture persisted for an extended period without being provided a source of hypoxanthine in the medium. This culture, however, was supplied additional glucose (5 mg/ml). Glucose is metabolized during the pentose phosphate pathway (PPP). During PPP, ribose 5-phosphate is produced from glucose 6-phosphate and used in the synthesis of nucleic acids and nucleotides (Barrett, 1997). Alternatively, glucose is also metabolized for cellular energy to lactate via anaerobic glycolysis. By this means, most, if not all, of the energy of hemoparasites, such as *Babesia* spp., is derived (Rickard, 1969; Yamasaki et al., 2003).

Some cultures were initiated using density gradient cell separation to purify the erythrocytes from lymphocytes and other mononuclear cells. One such culture was maintained for 94 days, but it required a frequent addition of uninfected donor cat erythrocytes due to the loss of RBC integrity occurring at a faster rate compared to washed RBC. Thus, there did not appear to be an advantage to using RBC prepared for culture by this method.

Although continuous cultivation of *C. felis* was not achieved during this study, it has provided insight for further modifications to optimize conditions for parasite growth. In addition to further investigation into culture medium and supplementation, various culture conditions should be evaluated in future studies. Different gas mixtures, particularly with emphasis on the O_2 concentration, as well as the incubation temperature should be investigated.

Based on the work of Rodriguez and others (1983), hemoparasites with a low PPE (generally 0.1%) at culture initiation are often propagated in an oxygen-reduced gas

atmosphere. It was recently shown that *T. equi* cultures initiated from horses with undetectable parasitemias initiation could be propagated in a 5% CO_2-in-air atmosphere with L-cysteine-supplemented culture medium. In comparing an oxygen-reduced environment and 5% CO_2-in-air atmosphere in conjunction with L-cysteine supplementation it was four days before parasites were seen under the oxygen-reduced conditions, whereas six days elapsed before parasites were detected in a 5% CO_2-in-air atmosphere (Zweygarth and Josemans, 2014). Thus, low oxygen tension may not be the requirement for culture initiation. Zweygarth and Josemans (2014) speculated that the low oxygen environment at culture initiation may cause L-cysteine to be released from an erythrocytic source, such as glutathione (GSH). This would suggest that the availability of L-cysteine is the prerequisite for *in vitro* initiation and cultivation of *T. equi* more than the microaerophilic culture conditions (Zweygarth and Josemans, 2014). This is an avenue that certainly might be explored for *C. felis* cultures.

With the successful establishment of continuous cultures of *C. felis,* there would be an unlimited supply of biological materials of this pathogen. This would aid in improving diagnostics and enhance the current state of knowledge regarding the biochemistry behind its nutritional requirements and metabolic pathways. An established culture system would also provide antigens for vaccine development as well as provide a platform for studying drug susceptibility. Perhaps most importantly, *C. felis* cultures would eliminate the need to infect live animals to carry out relevant studies.

CHAPTER III

GENETIC VARIABILITY OF *CYTAUXZOON FELIS* RIBOSOMAL RNA ITS1 AND ITS2 GENOMIC REGIONS FROM DOMESTIC CATS OF VARIED GEOGRAPHIC REGIONS AND CLINICAL OUTCOMES

INTRODUCTION

Cytauxzoon felis, a tick-borne protozoan parasite, is the causative agent of cytauxzoonosis in domestic cats in the United States. The first report described a fatal infection in domestic cats in southwestern Missouri (Wagner, 1976). Since then, *C. felis* infections in domestic cats have been found in south central, southeastern, mid-western, and mid-Atlantic states (Wagner, 1976; Ferris, 1979; Kier et al., 1982; Birkenheuer et al., 2006). Bobcats (*Lynx rufus*) are considered to be the primary reservoir host (Glenn et al., 1983; Blouin et al., 1984), and they typically do not exhibit clinical signs. Subclinical infection is reported in other wild felids in the United States such as Florida panthers (*Felis concolor coryii* [*Puma concolor coryi*]) (Butt et al., 1991), Florida cougars (*Puma concolor couguar*) (Yabsley et al., 2006; Harvey et al., 2007), and Texas cougars (*Puma concolor stanleyana*) (Rotstein et al., 1999).

In contrast to the common subclinical infection of wild felids, *C. felis* infection of domestic cats often leads to rapid disease progression, and most infected cats die within one week of the onset of clinical signs (Hoover et al., 1994; Greene et al., 2006). This is due to the schizogonous phase, in which the lumen of blood vessels in the liver, lungs, lymph nodes, and spleen are occluded by schizont-laden mononuclear phagocytes. Upon maturation the schizonts rupture and release numerous merozoites, which invade

erythrocytes as piroplasms (erythrocytic phase) and cause hemolysis (Kier et al., 1987; Kocan and Kocan, 1991; Kocan et al., 1992, Cohn et al., 2011). The historic high fatality, sporadic incidence, and rapid disease progression of cytauxzoonosis in domestic cats suggest that they may be aberrant hosts (Kier et al., 1987; Greene et al., 2006).

An increasing number of cats that have survived infection are documented (Motzel and Wagner, 1990; Walker and Cowell, 1995; Greene et al., 1999; Meinkoth et al., 2000; Rizzi et al., 2015). Cytauxzoonosis survivors may range from animals that were ill and recovered from the acute infection to cats that never exhibited clinical signs of the infection. Some cats remain detectably parasitemic as long as a year after clinical signs have resolved, and PCR testing has revealed *C. felis* DNA in the blood of clinically healthy cats with microscopically undetectable parasitemias (Meinkoth et al., 2000; Brown et al., 2008; Haber et al., 2007). Piroplasms could persist in erythrocytes of survivors for possibly the life span of the cat, rendering the animal a subclinical chronic carrier (Birkenheuer et al., 2006; Haber et al., 2007; Brown et al., 2008; Lewis et al., 2014). Reichard and others (2009) demonstrated that a natural survivor transmitted *C. felis* via the *Amblyomma americanum* tick vector to a naïve domestic cat resulting in clinical cytauxzoonosis, thus indicating that chronic carriers may serve as reservoirs and increasing the risk of *C. felis* exposure to other domestic cats.

In a previous study examining the intraspecific variation of the internal transcribed spacer (ITS) regions of the *C. felis* ribosomal RNA genes, specific genotypes were associated with clinical outcome and geographic regions (Brown et al., 2009a). However, such an association was not confirmed in subsequent studies (Brown et al.,

2009b; Brown et al., 2010; Cohn et al., 2011; Shock et al., 2012). The previous studies employed direct sequencing of the target regions. Thus, the objectives of this study were to clone and further analyze the *C. felis* 18S rRNA gene, ITS1, and ITS2 regions, and to assess the ITS genotypes for any spatial or clinical outcome associations.

MATERIALS AND METHODS

Fresh or frozen blood samples from 41 *Cytauxzoon felis*-infected blood from domestic cats for this study were provided under institutionally approved animal use protocols (Mason Reichard, Department of Veterinary Pathobiology, Oklahoma State University, Stillwater, OK; Animal Care and Use Protocol, "Feline cytauxzoonosis: Establishment of continuous parasites cultures" Protocol Number: VM1121; Leah A. Cohn, Department of Small Animal Internal Medicine, Veterinary Medicine Teaching Hospital, University of Missouri, Columbia, MO; Animal Care and Use Protocol, "*C. felis* source cats" Protocol Number 7301). The samples originated from Texas, Oklahoma, Arkansas, Kansas, and Missouri from cats with various clinical presentations (Table 15).

Table 15. State of origin and clinical outcomes of *Cytauxzoon felis*-infected cats. The number of isolates for each cytauxzoonosis presentation and the geographic origin are indicated.

Clinical Outcome	No. of isolates	State of Origin				
		OK	KS	AR	MO	TX
acute, fatal	30	26	0	0	3	1
chronic, subclinical	9	2	0	6	1	0
acute, recovered	2	0	1	0	0	1

The *C. felis*-infected cats were categorized for clinical outcome based upon the provided history of the cat. If the history was not known, such as with feral cats and other opportunistic samples, then the provided clinical status of the cat at time of blood draw was used. *Cytauxzoon felis*-infected cats that died were denoted as acute, fatal cases. Ill cats that showed clinical signs but survived were considered acute, recovered. The time period for the acute status for a majority of the cats was not known. Cats that were known to have a persistent *C. felis* infection but never showed clinical signs were considered chronic, subclinical.

The samples were shipped on ice overnight to Texas A&M University and either used upon arrival or stored at -80°C until use. Genomic DNA was extracted (FlexiGene DNA Kit, Qiagen, Valencia, CA) from each blood sample, and the concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). The full-length *C. felis* 18S rRNA gene was amplified in a primary PCR with primers A and B (Sogin, 1990) (Table 16) and 50-100 ng DNA according to manufacturer's instructions (High Fidelity Platinum ® *Taq*, Invitrogen™). Molecular biology grade water served as a negative control and a cloned plasmid with a full-length *C. felis* 18S rRNA gene insert served as a positive control. The reactions underwent a 30 s denaturation at 94°C followed by 45 cycles of 94°C for 15 s, 60°C for 15 s, 68°C for 2 min, with a final extension at 68°C for 7 min, and then were held at 4°C (Labnet MultiGene™ Thermal Cycler, Labnet International, Inc., Woodbridge, NJ). The PCR products were resolved by agarose gel electrophoresis alongside a 100-bp DNA marker (Invitrogen), stained with ethidium bromide, and visualized by ultraviolet light

transillumination (FluorChem 8000, Alpha Innotech Corporation, San Leandro, CA). If a band was not visualized, the primary product was diluted and used in a nested reaction with primers AN50 and BN1700 (Schoelkopf et al., 2005) (Table 16). The cycling parameters were as above, except that the reactions underwent 30 cycles. The PCR products were visualized as above.

Table 16. Oligonucleotide primers used in amplifying the 18S rRNA gene and ITS1-5.8S gene-ITS2 region of *Cytauxzoon felis.*

Primer Name, sense	PCR	Sequence 5'→3'
A, forward	Primary 18S	AACCTGGTTGATCCTGCCAG
B, reverse	Primary 18S	GATCCTTCTGCAGGTTCACCTAC
AN50, forward	Nested 18S	GCTTGTCTTAAAGATTAAGCCATGC
BN1700, reverse	Nested 18S	CGACTTCTCCTTCCTTTAAG
ITSF, forward	Primary and Nested ITS[a]	GAGAAGTCGTAACAAGGTTTCCG
LSU50R, reverse	Primary ITS	GCTTCACTCGCCGTTACTAGG
1055F, forward	Primary ITS[a]	GGTGGTGCATGGCCG
LSURN30, reverse	Nested ITS[a]	AATTCAGCGGGTAGTCTCAC
m13F, forward	Colony 18S and ITS, plasmid (pDNA)	GTAAAACGACGGCCAG
m13R, reverse	Colony 18S and ITS, plasmid (pDNA)	CAGGAAACAGCTATGAC

[a] Designated primer after primer adjustments were made.

The genomic region spanning partial 18S rRNA gene, rRNA internal transcribed spacer 1, 5.8S rRNA gene, ITS2, and partial 28S rRNA gene (ITS) was amplified as above using primers ITSF and LSU50R (Table 16) at an annealing temperature of 55°C and extension time of 2 min. A primary amplification using primers 1055F (Elwood et al., 1985) and LSU50R (Table 16) and a *Pyrococcus*-like polymerase according to

manufacturer's instructions (Phusion High-Fidelity DNA Polymerase, Thermo Scientific, Pittsburgh, PA) was employed for *C. felis* isolates that yielded no amplicons with ITSF and LSUR50. The cycling parameters were a 2 min denaturation at 98°C followed by 45 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 1 min, a final extension of 72°C for 7 min, and a hold at 4°C. The PCR products were resolved and visualized as above. If a band was not visualized, the primary product was diluted and used in a nested reaction with primers ITSF and LSURN30 (Table 16). The cycling parameters were as above, but with a 30 s initial denaturation, 30 s extension time, and 30 cycles for amplification. The PCR products were resolved by agarose gel electrophoresis as above.

Appropriately-sized 18S rDNA and ITS1-5.8S-ITS2 amplicons synthesized by High Fidelity Platinum® *Taq* DNA Polymerase were ligated into pCR®2.1-TOPO® (Invitrogen, Life Technologies, Grand Island, NY) or pPrime (5 PRIME, Gaithersburg, MD) cloning vector. Amplicons synthesized with Phusion High-Fidelity DNA Polymerase were ligated into the pJET1.2/blunt cloning vector (Thermo Fisher Scientific Inc., Waltham, MA). When multiple bands resulted in PCR, the appropriately-sized bands were gel purified (QIAQuick Gel Extraction Kit, Qiagen, Valencia, CA) prior to cloning. TOP10 *E. coli* were transformed according to manufacturer's instructions. Colonies were verified to contain the cloned insert by colony PCR using primers appropriate for the plasmid vector (Table 16). Verified colonies were expanded overnight in broth cultures, and plasmid DNA was prepared (Promega or Bio Basic, Inc.). The plasmid DNA concentration was determined using spectrophotometry. Bidirectional sequencing of the inserts was conducted by Davis Sequencing (Davis, CA).

Sequence and chromatogram data were analyzed, and forward and reverse sequence data were trimmed to exclude primer sequences and unreadable data, aligned, and analyzed for contiguous sequences using Sequencher 4.2 software (Gene Codes Corporation, Inc., Ann Arbor, MI). The obtained sequences were compared to sequences in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The 18S rDNA sequence data served to confirm *C. felis* infection. Using previous *C. felis* ITS1 and ITS2 sequences from GenBank as template, the ITS1 and ITS2 region sequences were identified in the cloned sequences, then aligned and analyzed separately (Clustal Omega, http://www.ebi.ac.uk/Tools/msa/clustalo/). The ITS1 and ITS2 sequences were assigned GenBank accession numbers and combined GenBank accession numbers were designated specific genotypes as previously described (Brown et al., 2009a, 2009b, 2010; Shock et al., 2012). With the chi-square test for independence, genotypes found in this study were evaluated for associations with clinical outcome (fatal, recovered, or chronic) and state of origin (Oklahoma, Kansas, Arkansas, Missouri, or Texas) (JMP®, Version Pro 11. SAS Institute Inc., Cary, NC, 1989-2007). A chi-square test for independence was also employed to evaluate single and multiple variance(s) (SNP[s] and/or single random substitutions) in the delineated ITS1 and ITS2 sequence combinations for a correlation with PCR amplification (primary or nested) and polymerase (High Fidelity Platinum® *Taq* or Phusion) used.

RESULTS

Forty of 41 (98%) samples yielded 18S rRNA cloned amplicons, and subsequent sequencing revealed 1,774-bp inserts sharing 99% identity with C. felis 18S rRNA

gene in GenBank (AY679105). Thirty-five of the 41 (85%) samples yielded C. felis ITS cloned amplicons of approximately 1,000-bp (KR817922-KR818036 and KT783518-KT783524), for a total of 122 cloned sequences (Table 17).

Table 17. State of origin and clinical outcome of the cats from which *Cytauxzoon felis* ITS cloned amplicons were derived.

Clinical Outcome	No. of cloned amplicons (No. of cats)	State of Origin				
		OK	KS	AR	MO	TX
Acute, fatal	89 (30)	80 (26)	0	0	8 (3)	1 (1)
Chronic, subclinical	29 (9)	10 (2)	0	17 (6)	2 (1)	0
Acute, recovered	4 (2)	0	2 (1)	0	0	2 (2)

The partial 18S-ITS1-5.8S-ITS2-partial 28S rDNA sequence for all *C. felis* cloned amplicons from this study were submitted to GenBank. The ITS sequence data were extracted from each of these sequences and used for the analysis of the ITS1 and ITS2 regions, respectively. An alignment of the sequence data from the 122 *C. felis* ITS1 region clones obtained revealed ITS sequences with 21 single nucleotide polymorphisms (SNPs) and three insertions (23-bp insertion and two single nucleotide insertions). The length was generally 458-460 bp, or 481 bp with the 23 bp insertion. Different *C. felis* ITS1 sequences were often found from a single cat. The most common ITS1 sequence matched a *C. felis* ITS1 sequence (GenBank accession number EU450802) from a domestic cat that survived an infection in Georgia. This ITS1 sequence shared 100% identity with 56 total cloned sequences (Table 18) representing 32 cloned amplicons from 15 domestic cats that succumbed to an acute fatal infection (KR817922-KR817930, KR817932, KR817933, KR817959, KR817961, KR817962, KR817968-

KR817970, KR817981, KR817990, KR817991, KR817995, KR818001, KR818007-KR818009, KR818016, KR818017, KR818020, KR818030- KR818032, and KT783524) and 24 clones from 9 cats with subclinical, chronic infections (KR817934-KR817937, KR817971-KR817980, KR818000, KR818002- KR818006, KR818011, KR818012, KR818028, and KR818029). Of the 32 *C. felis* clones from an acute, fatal infection, 27 were from 11 cats in Oklahoma, four were from three Missouri cats, and one was from a Texas cat. Of the 24 *C. felis* cloned amplicons from subclinical, chronic infections, seven clones were from two Oklahoma cats, 15 were from six Arkansas cats, and two were from one Missouri cat.

Table 18. Number and distribution of *Cytauxzoon felis* cloned ITS1 sequences according to the state of origin and clinical outcome that matched sequences previously reported to Genbank GenBank accession no. (GBA) of the previously reported sequence and observed differences in this study are listed. The cloned ITS1 sequences from this study are in the GenBank deposited partial-18S-ITS1-5.8S-ITS2-partial 28S rDNA sequences provided below.

GBA and changes	No. of cloned amplicons (% of total)	Number of clones according to state of origin					Clinical outcome		
		OK	KS	AR	MO	TX	acute, fatal	acute, recovered	chronic, subclinical
EU450802	56 (46)	34	0	15	6	1	32	0	24
1 base change[a]	22 (18)	19	1	2	0	0	18	1	3
23-bp insert[b]	4 (3)	3	0	0	1	0	4	0	0
>1 base change[c]	5 (4)	3	0	1	0	1	3	1	1
GU581166	6 (5)	6	0	0	0	0	6	0	0
1base change[d]	2 (2)	2	0	0	0	0	2	0	0
>1 base change[e]	1 (1)	1	0	0	0	0	1	0	0
HQ383820	4 (3)	2	0	0	2	0	4	0	0
1 base change[f]	4 (3)	4	0	0	0	0	4	0	0
>1 base change[g]	2 (2)	2	0	0	0	0	2	0	0
HQ383856	3 (2)	3	0	0	0	0	3	0	0
1 base change[h]	1 (1)	0	0	0	0	1	0	1	0
>1 base change[i]	1 (1)	1	0	0	0	0	1	0	0
23-bp insert[j]	1 (1)	0	0	0	1	0	1	0	0
JF308498	1 (1)	1	0	0	0	0	1	0	0
1 base change[k]	1 (1)	0	0	0	0	1	0	1	0
>1 base change[l]	3 (2)	3	0	0	0	0	3	0	0
EU450803	1 (1)	1	0	0	0	0	1	0	0
>1 base change[m]	3(2)	3	0	0	0	0	1	0	2
HQ383867	1 (1)	1	0	0	0	0	1	0	0
TOTAL	122 (100)	89	1	18	10	4	88	4	30

Sequences KR817931; KR817940; KR817945-KR817950; KR817955; KR817958; KR817963; KR817965; KR817967; KR817985-KR817989; KR817993; KR817996; KR817997; KR818013 from this study.

[b] Sequences KT783518; KT783523; KR818035; KR818036 from this study

[c] Sequences KR817939; KR817941; KR817942; KR817964; KR817966 from this study.

[d] Sequences KR817960; KR817994 from this study.

Table 18. Continued.
[e] Sequence KT783521 from this study.
[f] Sequences KR817953; KR817954; KR818014; KR818015 from this study.
[g] Sequences KR817951; KR817952 from this study.
[h] Sequence KT783520 from this study.
[i] Sequence KR818022 from this study.
[j] Sequence KT783522 from this study.
[k] Sequence KR817957 from this study.
[l] Sequences KR817944; KR817956; KR818027 from this study.
[m] Sequences KR818025; KR818026; KT783519 from this study.

The second most common ITS1 sequence found in this study matched that of *C. felis* from a Florida bobcat, GenBank accession number GU581166. This sequence was detected in six clones from three fatally infected Oklahoma cats (Table 18) (KR817938, KR817982- KR817984, KR817992, and KR818010). KR818021 was identical to GenBank accession numbers EU450803 (fatal *C. felis* infection of an Arkansas domestic cat), and KR818024 was identical to previously reported and JF308498 (*C. felis* from an Oklahoma bobcat). The ITS1 sequence of KR817943 was identical to the 455-bp submitted for GenBank accession number HQ383867 (Oklahoma bobcat *C. felis*). ITS1 sequences of KR818018, KR818019, KR817998, and KR817999 were a 100% match with the 455-bp of archived HQ383820 (Kansas bobcat *C. felis*); ITS1 sequences of KR818023, KR818033, and KR818034 identically matched the 456-bp submitted for GenBank accession number HQ38385 (*C. felis* from Kansas and North Carolina bobcats) (Table 19). Of the three insertions found in ITS1 in this study (Table 19), the two single nucleotide insertions were previously reported, and the 23-bp insert was novel as it was not reported prior to this study. The 23-bp insert was detected with both polymerases utilized in this study. Five *C. felis* cloned amplicons in this study from Missouri (GenBank accession numbers KR818035 and KT783522) and Oklahoma (GenBank accession numbers KR818036, KT783518, and KT783523) contained the 23-bp insert. These five clones were obtained from three domestic cats with acute, fatal cases. The insert contained a repeat of AACCT within the sequence, and in the absence of the 23-bp insert, three of these five clones (KR818035, KT783518, and KR818036) would be identical to previously submitted GenBank accession number EU450802. Two

of these three clones had identical sequences (KT783518 and KR818036) while the third clone (KR818035) had a SNP inside the 23-bp insert. This exact SNP was shared by one of the two remaining clones with 23-bp insert, KT783522, which also had a single nucleotide insertion and a SNP outside the insert region. Without the 23-bp insert, KT783522 would match the 256-bp of previously submitted GenBank accession number HQ383856 (*C. felis* from Kansas and North Carolina bobcats) with 100%. The other remaining clone had a SNP outside of the insert region (KT78523; Fig. 1).

Table 19. Maximum identity to prior GenBank accession numbers (GenBank match) and nucleotide variation of cloned *Cytauxzoon felis* ITS1 amplicons. Sequence position numbering is based on GenBank accession number EU450803. Insertions, single nucleotide polymorphisms, and miscellaneous mismatches are shown as INS, SNP, and Misc., respectively.

No.	GenBank match	Max. identity	Animal-Clone	GenBank accession number	Polymorphisms											
					Misc.	SNP 80 A>G	SNP 162 A>G	SNP 167 A>C	INS 337 A	SNP 338 G>A	SNP 344 T>C	SNP 397 T>C	SNP 415 T>C	SNP 444 A>G	INS 457 C	23-BP INS
-	-	-	-	EU450802												
1.	EU450802	100%	1 Cl 2	KR817922												
2.	EU450802	100%	1 Cl 6	KR817923												
3.	EU450802	100%	1 Cl 8	KR817924												
4.	EU450802	100%	1 Cl 9	KR817925												
5.	EU450802	100%	1 Cl 11	KR817926												
6.	EU450802	100%	3 Cl 2	KR817961												
7.	EU450802	100%	3 Cl 3	KR817962												
8.	EU450802	100%	4 Cl 1	KR817927												
9.	EU450802	100%	4 Cl 3	KR817928												
10.	EU450802	100%	4 Cl 6	KR817929												
11.	EU450802	100%	4 Cl 9	KR817930												
12.	EU450802	100%	19 Cl 1	KR818011												
13.	EU450802	100%	19 Cl 9	KR818012												
14.	EU450802	100%	23 Cl 1	KR818017												
15.	EU450802	100%	27 Cl 1	KR818016												
16.	EU450802	100%	28 Cl 2	KT783524												
17.	EU450802	100%	28 Cl 3	KR817932												
18.	EU450802	100%	28 Cl 5	KR817933												
19.	EU450802	100%	28 Cl 6	KR817959												
20.	EU450802	100%	32 Cl 4	KR818028												
21.	EU450802	100%	34 Cl 2	KR817934												
22.	EU450802	100%	35 Cl 1	KR817935												
23.	EU450802	100%	35 Cl 2	KR817936												
24.	EU450802	100%	36 Cl 1	KR817937												
25.	EU450802	100%	36 Cl 2	KR818029												
26.	EU450802	100%	39 Cl 3	KR818030												
27.	EU450802	100%	42 Cl 10	KR818020												
28.	EU450802	100%	16 Cl 2	KR817990												
29.	EU450802	100%	12 Cl 3	KR818002												
30.	EU450802	100%	12 Cl 7	KR818003												
31.	EU450802	100%	12 Cl 9	KR818004												
32.	EU450802	100%	12 Cl 10	KR818005												
33.	EU450802	100%	12 Cl 12	KR818006												
34.	EU450802	100%	14 Cl 7	KR817991												
35.	EU450802	100%	18 Cl 1	KR817968												
36.	EU450802	100%	18 Cl 2	KR817969												
37.	EU450802	100%	18 Cl 3	KR818031												
38.	EU450802	100%	18 Cl 4	KR818032												
39.	EU450802	100%	20 Cl 2	KR818007												
40.	EU450802	100%	20 Cl 4	KR818008												
41.	EU450802	100%	20 Cl 5	KR818009												
42.	EU450802	100%	21 Cl 8	KR817970												
43.	EU450802	100%	29 Cl 1	KR817971												

Table 19. Continued.

No.	GenBank match	Max. identity	Animal-Clone	GenBank accession number	Polymorphisms											
					Misc.	SNP 80 A>G	SNP 162 A>G	SNP 167 A>C	INS 337 A	SNP 338 G>A	SNP 344 T>C	SNP 397 T>C	SNP 415 T>C	SNP 444 A>G	INS 457 C	23-BP INS
44.	EU450802	100%	29 Cl 4	KR817972												
45.	EU450802	100%	29 Cl 5	KR817973												
46.	EU450802	100%	29 Cl 6	KR817974												
47.	EU450802	100%	30 Cl 2	KR818000												
48.	EU450802	100%	30 Cl 5	KR817975												
49.	EU450802	100%	31 Cl 6	KR817976												
50.	EU450802	100%	31 Cl 8	KR817977												
51.	EU450802	100%	31 Cl 9	KR817978												
52.	EU450802	100%	31 Cl 10	KR817979												
53.	EU450802	100%	31 Cl 11	KR817980												
54.	EU450802	100%	40 Cl 6	KR817995												
55.	EU450802	100%	41 Cl 2	KR818001												
56.	EU450802	100%	41 Cl 5	KR817981												
57.	EU450802	99%	6 Cl 1	KR817955		X										
58.	EU450802	99%	6 Cl 5	KR817958		X										
59.	EU450802	95%	10 Cl 6	KT783523				X								X
60.	EU450802	99%	15 Cl 7	KR817931	398 A>G											
61.	EU450802	99%	15 Cl 9	KR817940	398 A>G											
62.	EU450802	99%	15 Cl 8	KR817939	350 T>C 398 A>G											
63.	EU450802	99%	15 Cl 12	KR817941	96 T>C 398 A>G		X									
64.	EU450802	99%	17 Cl 12	KR817942	183 T>C 329 T>C											
65.	EU450802	99%	19 Cl 4	KR818013	94 C>T											
66.	EU450802	99%	25 Cl 11	KR817945	93 A>G											
67.	EU450802	99%	26 Cl 1	KR817946	208 A>G											
68.	EU450802	99%	26 Cl 5	KR817948	208 A>G											
69.	EU450802	99%	26 Cl 4	KR817947	221 T>C											
70.	EU450802	99%	32 Cl 5	KR817963	37 A>G											
71.	EU450802	99%	33 Cl 1	KR817965	198 A>G											
72.	EU450802	99%	33 Cl 2	KR817966	217 T>C 323 T>C											
73.	EU450802	99%	34 Cl 1	KR817967	238 T>C											
74.	EU450802	99%	43 Cl 4	KR817964	153 A>G 161 A>G											
75.	EU450802	95%	5 Cl 1	KT783518												X
76.	EU450802	95%	5 Cl 2	KR818036												X
77.	EU450802	99%	16 Cl 4	KR817986	140 G>T											
78.	EU450802	99%	16 Cl 5	KR817987	140 G>T											
79.	EU450802	99%	16 Cl 6	KR817988	140 G>T											
80.	EU450802	99%	18 Cl 5	KR817993	75 A>G											
81.	EU450802	99%	20 Cl 1	KR817996	451 T>C											
82.	EU450802	99%	20 Cl 3	KR817997	451 T>C											

Table 19. Continued.

No.	GenBank match	Max. identity	Animal-Clone	GenBank accession number	Polymorphisms										
					Misc.	SNP 80 A>G	SNP 162 A>G	SNP 167 A>C	INS 337 A	SNP 338 G>A	SNP 344 T>C	SNP 397 T>C	SNP 415 T>C	SNP 444 A>G	INS 457 C	23-BP INS
83.	EU450802	99%	23 Cl 3	KR817950									X			
84.	EU450802	95%	40 Cl 2	KR818035	199 (inside INS) A>C											X
85.	EU450802	99%	27 Cl 4	KR817949									X			
86.	EU450802	99%	21 Cl 12	KR817985									X			
87.	EU450802	99%	16 Cl 1	KR817989	342 A>G											
-	-	-	-	JF308498								X				
88.	JF308498	100%	25 Cl 12	KR818024								X				
89.	JF308498	99%	6 Cl 4	KR817957	61 G>A							X				
90.	JF308498	99%	10 Cl 1	KR818027	37 A>G 406 T>C							X				
91.	JF308498	99%	25 Cl 6	KR817944	84 A>G 262 A>C							X				
92.	JF308498	99%	6 Cl 2	KR817956	284 T>C	X						X				
-	-	-	-	GU581166						X						
93.	GU581166	100%	9 Cl 3	KR817938						X						
94.	GU581166	100%	5 Cl 5	KR818010						X						
95.	GU581166	100%	13 Cl 1	KR817982						X						
96.	GU581166	100%	13 Cl 3	KR817983						X						
97.	GU581166	100%	13 Cl 4	KR817984						X						
98.	GU581166	100%	13 Cl 5	KR817992						X						
99.	GU581166	99%	9 Cl 5	KR817960	126 A>G					X						
100.	GU581166	99%	25 Cl 9	KT783521	93A>G 312 A>G 451 T>C				X							
101.	GU581166	99%	13 Cl 2	KR817994	273 G>A					X						
-	-	-	-	HQ383867		X							X			
102.	HQ383867	100%	25 Cl 4	KR817943		X							X			
-	-	-	-	HQ383856					X		X					
103.	HQ383856	100%	4 Cl 11	KR818023					X		X					
104.	HQ383856	100%	5 Cl 3	KR818033					X		X					
105.	HQ383856	100%	5 Cl 4	KR818034					X		X					
106.	HQ383856	99%	6 Cl 3	KR818022	180 A>G			X	X		X					
107.	HQ383856	99%	43 Cl 1	KT783520	11 A>G				X		X					
108.	HQ383856	95%	40 Cl 5	KT783522					X		X					X
-	-	-	-	HQ383820				X								
109.	HQ383820	100%	23 Cl 4	KR818019				X								
110.	HQ383820	100%	27 Cl 3	KR818018				X								
111.	HQ383820	100%	41 Cl 1	KR817998				X								
112.	HQ383820	100%	41 Cl 3	KR817999				X								
113.	HQ383820	99%	9 Cl 8	KR817951	110 T>C 176 A>C 182T>G 211A>G 283T>C			X								

Table 19. Continued.

No.	GenBank match	Max. identity	Animal-Clone	GenBank accession number	Polymorphisms										
					Misc.	SNP 80 A>G	SNP 162 A>G	SNP 167 A>C	INS 337 A	SNP 338 G>A	SNP 344 T>C	SNP 397 T>C	SNP 415 T>C	SNP 444 A>G	INS 457 C	23-BP INS
114.	HQ383820	99%	9 Cl 9	KR817952	110 T>C 176 A>C 182T>G 211A>G 283T>C			X							
115.	HQ383820	99%	23 Cl 2	KR818015	250 T>C			X							
116.	HQ383820	99%	27 Cl 2	KR818014	250 T>C			X							
117.	HQ383820	99%	23 Cl 5	KR817954				X					X		
118.															
119.															
120.	HQ383820	99%	27 Cl 5	KR817953				X					X		
-	-	-	-	EU450803			X		X		X		X	X		
121.	EU450803	100%	15 Cl 1	KR818021			X		X		X		X	X		
122.	EU450803	99%	19 Cl 3	KR818026					X		X		X	X	X	
123.	EU450803	99%	19 Cl 6	KT783519					X		X		X	X	X	
124.	EU450803	99%	26 Cl 2	KR818025	267 T>C				X		X		X	X	X	

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KT783522      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818026      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818025      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KT783521      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818021      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
EU450803      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818022      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KT783520      ACATGAAATAGTTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818023      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
HQ383856      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818035      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KT783523      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KT783518      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817951      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817964      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817966      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817944      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817942      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817941      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818027      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAGTGACGAAGTCAGTGACGCAGAGC  60
KR817957      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818024      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
JF308498      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817956      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818015      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818019      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
HQ383820      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
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Fig. 1. Clustal Omega alignment of *Cytauxzoon felis* ITS1 sequence representatives of all sequences from this study that identically matched (100% identity) or had 1 or more base differences from the *C. felis* ITS1 sequences previously submitted to GenBank. Alignment also includes the previously reported *C. felis* ITS1 sequences in GenBank (EU450802, EU450803, HQ383820, HQ383856, HQ383867, GU531166, and JF308498), in which the ITS1 sequences of this study were most similar. Nucleotide position number indicated at the right. Matching nucleotides in all sequences are shown by *. Mismatch nucleotides are shown in bold. Gaps (deletions) are indicated by a dash (-).

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KR817996      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817993      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817986      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817989      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817994      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817967      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817965      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817963      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAGTGACGAAGTCAGTGACGCAGAGC  60
KR817947      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817946      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817945      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817943      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817954      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817950      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR818013      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817939      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817931      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817940      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817960      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817938      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
GU581166      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817922      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
EU450802      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
KR817955      ACATGAAATAATTATTTACATGAGTGAGGGAAGTCAATGACGAAGTCAGTGACGCAGAGC  60
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KT783522      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818026      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818025      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KT783521      GAAGCGAAGTTATGAAATCACTGATGTCAGAGGCGTAGTCAATGACTGATGTGATGAATG  120
KR818021      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
EU450803      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818022      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
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Fig. 1. Continued

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KT783520      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818023      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
HQ383856      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818035      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KT783523      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KT783518      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817951      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGACGTGATGAATG  120
KR817964      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817966      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817944      GAAGCGAAGTTATGAAATCACTGGTGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817942      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817941      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGCAGTCAATGACTGATGTGATGAATG  120
KR818027      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817957      AAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818024      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
JF308498      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817956      GAAGCGAAGTTATGAAATCGCTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818015      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818019      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
HQ383820      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817996      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817993      GAAGCGAAGTTATGGAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817986      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817989      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817994      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817967      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817965      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817963      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817947      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817946      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817945      GAAGCGAAGTTATGAAATCACTGATGTCAGAGGCGTAGTCAATGACTGATGTGATGAATG  120
KR817943      GAAGCGAAGTTATGAAATCGCTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817954      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817950      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR818013      GAAGCGAAGTTATGAAATCACTGATGTCAGAGATGTAGTCAATGACTGATGTGATGAATG  120
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Fig. 1. Continued

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KR817939      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817931      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817940      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817960      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817938      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
GU581166      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817922      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
EU450802      GAAGCGAAGTTATGAAATCACTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
KR817955      GAAGCGAAGTTATGAAATCGCTGATGTCAGAGACGTAGTCAATGACTGATGTGATGAATG  120
               ************* **** *** ********  * ************* **********

KT783522      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818026      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818025      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KT783521      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818021      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAGTAACATACTTCAAACTAA  180
EU450803      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAGTAACATACTTCAAACTAA  180
KR818022      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAG  180
KT783520      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818023      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
HQ383856      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818035      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KT783523      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAA  180
KT783518      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817951      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAACCTAA  180
KR817964      AAGTAACGACCGAGGTCGTGATTGTCTTCATAGCTGAGGTGATAACATACTTCAAACTAA  180
KR817966      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817944      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817942      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817941      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAGTAACATACTTCAAACTAA  180
KR818027      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817957      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818024      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
JF308498      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817956      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
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Fig. 1. Continued

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KR818015      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAA  180
KR818019      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAA  180
HQ383820      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAA  180
KR817996      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817993      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817986      AAGTAACGACCGAGGTCGTTATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817989      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817994      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817967      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817965      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817963      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817947      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817946      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817945      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817943      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817954      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACCTACTTCAAACTAA  180
KR817950      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR818013      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817939      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817931      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817940      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817960      AAGTAGCGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817938      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
GU581166      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817922      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
EU450802      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
KR817955      AAGTAACGACCGAGGTCGTGATTGTCTTCATAACTGAGGTAATAACATACTTCAAACTAA  180
              ***** ************ ************ *******  **** ******** ***

KT783522      CTTCATAACCTACTTCAACCTAACTTCATAACCTACTTCAACCTAACGTCATAACTATCT  240
KR818026      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818025      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KT783521      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818021      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
EU450803      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
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Fig. 1. Continued

```
KR818022      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KT783520      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818023      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
HQ383856      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818035      CTTCATAACCTACTTCAACCTAACTTCATAACCTACTTCAACCTAACGTCATAACTATCT  240
KT783523      CTTCATAACCTACTTCAAACTAACTTCATAACCTACTTCAACCTAACGTCATAACTATCT  240
KT783518      CTTCATAACCTACTTCAAACTAACTTCATAACCTACTTCAACCTAACGTCATAACTATCT  240
KR817951      CGTCAT----------------------AACCTACTTCAACCTAACGTCATAGCTATCT  217
KR817964      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817966      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCC  217
KR817944      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817942      CTCCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817941      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818027      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817957      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818024      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
JF308498      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817956      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818015      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR818019      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
HQ383820      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817996      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817993      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817986      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817989      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817994      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817967      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817965      CTTCAT----------------------AACCTACTTCAGCCTAACGTCATAACTATCT  217
KR817963      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817947      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817946      CTTCAT----------------------AACCTACTTCAACCTAACGTCGTAACTATCT  217
KR817945      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817943      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817954      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817950      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
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Fig. 1. Continued

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KR818013      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817939      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817931      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817940      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817960      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817938      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
GU581166      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817922      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
EU450802      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
KR817955      CTTCAT----------------------AACCTACTTCAACCTAACGTCATAACTATCT  217
              *  ***                        *********** ********* ** *****

KT783522      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  300
KR818026      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818025      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCT**C**CTTCGGGAA  277
KT783521      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818021      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
EU450803      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818022      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KT783520      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818023      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
HQ383856      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818035      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  300
KT783523      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  300
KT783518      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  300
KR817951      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817964      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817966      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817944      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAA**C**CGCTTCCTTCGGGAA  277
KR817942      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817941      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818027      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817957      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818024      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
JF308498      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
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Fig. 1. Continued

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KR817956      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818015      CCCTTCATCCTGTAATTTCGTAACGTTGTTTACTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818019      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
HQ383820      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817996      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817993      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817986      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817989      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817994      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCAGGAA  277
KR817967      CCCTTCATCCTGTAATTTCGCAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817965      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817963      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817947      CCCCTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817946      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817945      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817943      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817954      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817950      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR818013      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817939      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817931      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817940      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817960      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817938      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
GU581166      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817922      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
EU450802      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
KR817955      CCCTTCATCCTGTAATTTCGTAACGTTGTTTATTCATAGAGTAAACGCTTCCTTCGGGAA  277
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KT783522      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  360
KR818026      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KR818025      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KT783521      AAACGTTACATTACCACCCCTCCATGTGATTTTAGATCACTTGGGTTATGTTGCAGTGAA  337
KR818021      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
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Fig. 1. Continued

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EU450803      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KR818022      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KT783520      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KR818023      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
HQ383856      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGAA  337
KR818035      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  359
KT783523      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  359
KT783518      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  359
KR817951      AAACGCTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817964      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817966      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGCTATGTTGCAGTGA-  336
KR817944      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817942      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTCGCAGTGA-  336
KR817941      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR818027      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817957      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR818024      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
JF308498      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817956      AAACGTCACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR818015      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR818019      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
HQ383820      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817996      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817993      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817986      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817989      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817994      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817967      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817965      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817963      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817947      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817946      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817945      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817943      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817954      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
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Fig. 1. Continued

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KR817950      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR818013      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817939      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817931      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817940      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817960      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817938      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
GU581166      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817922      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
EU450802      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
KR817955      AAACGTTACATTACCACCCCTCCATGTGATTTTAAATCACTTGGGTTATGTTGCAGTGA-  336
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KT783522      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  420
KR818026      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KR818025      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KT783521      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KR818021      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
EU450803      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KR818022      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KT783520      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KR818023      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
HQ383856      GATTCACTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  397
KR818035      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  419
KT783523      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  419
KT783518      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  419
KR817951      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817964      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817966      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817944      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR817942      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817941      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR818027      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR817957      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR818024      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
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Fig. 1. Continued

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JF308498      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR817956      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
JF308498      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR817956      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATC  396
KR818015      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR818019      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
HQ383820      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817996      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817993      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817986      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817989      GATTCGTTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817994      AATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817967      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817965      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817963      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817947      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817946      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817945      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817943      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817954      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817950      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR818013      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817939      GATTCATTGCAAACGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817931      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817940      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817960      AATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817938      AATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
GU581166      AATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817922      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
EU450802      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
KR817955      GATTCATTGCAAATGTAATTAATAGAGTAATTGCTAATTCTCTATTAGTTGCTGGATATT  396
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KT783522      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  480
KR818026      AATTATCCTTCTTTCTTCCATACAACTACATGAGATGTTTCATCTCGACACATTTATTTC  457
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Fig. 1. Continued

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KR818025      AATTATCCTTCTTTCTTCCATACAACTACATGAGATGTTTCATCTCGACACATTTATTTC  457
EU450803      AATTATCCTTCTTTCTTCCATACAACTACATGAGATGTTTCATCTCGACACATTTATTT-  457
KT783521      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATCTATTT-  457
KR818021      AATTATCCTTCTTTCTTCCATACAACTACATGAGATGTTTCATCTCGACACATTTATTT-  457
KR818022      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  457
KT783520      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  457
KR818023      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  457
HQ383856      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818035      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  479
KT783523      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  479
KT783518      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  479
KR817951      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817964      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817966      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817944      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817942      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817941      AGTTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818027      AATTATCCCTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817957      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818024      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
JF308498      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817956      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818015      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818019      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
HQ383820      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  455
KR817996      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTCATTT-  456
KR817993      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817986      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817989      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817994      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817967      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817965      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817963      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817947      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817946      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
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Fig. 1. Continued

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KR817945      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817950      AATTATCCTTCTTTCTT**C**CATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817943      AATTATCCTTCTTTCTT**C**CATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817954      AATTATCCTTCTTTCTT**C**CATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR818013      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817939      A**G**TTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817931      A**G**TTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817940      A**G**TTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817960      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817938      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
GU581166      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817922      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
EU450802      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
KR817955      AATTATCCTTCTTTCTTTCATACAACTACATGAGATGTTTCATCTCAACACATTTATTT-  456
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KT783522      ATC      482
KR818026      ATC      460
KR818025      ATC      460
KT783521      ATC      459
KR818021      ATC      459
EU450803      ATC      459
KR818022      ATC      459
KT783520      ATC      459
KR818023      ATC      459
HQ383856      ---      456[a]
KR818035      ATC      481
KT783523      ATC      481
KT783518      ATC      481
KR817951      ATC      458
KR817964      ATC      458
KR817966      ATC      458
KR817944      ATC      458
KR817942      ATC      458
KR817941      ATC      458
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Fig. 1. Continued

KR818027	ATC	458
JF308498	ATC	458
KR817957	ATC	458
KR818024	ATC	458
KR817956	ATC	458
KR818015	ATC	458
KR818019	ATC	458
HQ383820	---	455[b]
KR817996	ATC	458
KR817993	ATC	458
KR817986	ATC	458
KR817989	ATC	458
KR817994	ATC	458
KR817967	ATC	458
KR817965	ATC	458
KR817963	ATC	458
KR817947	ATC	458
KR817946	ATC	458
KR817945	ATC	458
KR817943	ATC	458
KR817954	ATC	458
KR817950	ATC	458
KR818013	ATC	458
KR817939	ATC	458
KR817931	ATC	458
KR817940	ATC	458
KR817960	ATC	458
KR817938	ATC	458
GU581166	ATC	458
KR817922	ATC	458
EU450802	ATC	458
KR817955	ATC	458

[a] Only 456-bp submitted to GenBank for HQ383856.
[b] Only 455-bp submitted to GenBank for HQ383820.

Fig. 1. Continued

The remaining 45 ITS1 sequences varied from archived *C. felis* ITS1 sequences by one or more nucleotide substitution(s). Thirty had a single substitution that occurred, and of these, 22 clones (KR817931, KR817940, KR817945-KR817950, KR817955, KR817958, KR817963, KR817965, KR817967, KR817985-KR817989, KR817993, KR817996, KR817997, and KR818013) differed from prior GenBank accession number EU450802; two (KR817960 and KR817994) from deposited GenBank accession number GU581166; one (KR817957) from prior GenBank accession number JF308498; four (KR818015, KR818014, KR817953, and KR817954) from prior GenBank accession number HQ383820; and one (KT783520) from previously submitted GenBank accession number HQ383856 (Table 19). Fifteen *C. felis* ITS1 clones (KR817939; KR817941; KR817942; KR817944; KR817951; KR817952; KR817956; KR817964; KR817966; KR818022; KR818025; KR818026; KR818027; KT783519; KT783521) from 10 cats had two or more bases that varied from GenBank deposited sequences EU450802, HQ383820, GU581166, EU450803, and JF308498 (Table 18).

The alignment of the sequence data from 122 *C. felis* ITS2 region clones revealed nine SNPs, one insertion, and one deletion (Fig. 2; Table 20), which resulted in a length of 264-265 bp, or 305 bp with the insert. Different *C. felis* ITS2 sequences were often found from a single cat. The most common ITS2 sequence (63 clones) matched a *C. felis* infection survived by a domestic cat in Georgia, EU450804, and was found in 47 cloned amplicons from 16 domestic cats with acute, fatal *C. felis* infections (KR817922-KR817933, KR817939-KR817941, KR817943, KR817944, KR817946, KR817953,

KR817954, KR817956, KR817959, KR817968-KR817970, KR817986-KR817990, KR817993, KR817996-KR817999, KR818001, KR818010, KR818021-KR818024, KR818033-KR818035, KT783522, KT783524) and 16 cloned amplicons from seven cats with subclinical, chronic *C. felis* infections (KR817934- KR817937, KR817963, KR817967, KR817971- KR817980, KR818036) (Table 21). Of the 47 cloned *C. felis* amplicons from an acute, fatal infection, 42 clones were from 14 domestic cats from Oklahoma, and the five others were clones from two domestic cats from Missouri. The 16 cloned *C. felis* amplicons from a subclinical, chronic infection were from one domestic cat (one clone) from Missouri and six domestic cats (15 clones) from Arkansas.

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KT783518      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
EU450806      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR818028      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR818027      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR818011      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR818017      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KT783523      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817958      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817957      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTGCAATCCC 60
KR817948      TAAAATCCTAAAAGTACAACTTTTGCTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817947      TAAAGTCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817945      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817950      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR818026      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
JF308506      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817942      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817951      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817960      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGCGAGTAGAGGTGATTTACAATCCC 60
KR817938      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
EU450805      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817955      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817961      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817922      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
EU450804      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
KR817962      TAAAATCCTAAAAGTACAACTTTTGTTGTTCTTTTGTGAGTAGAGGTGATTTACAATCCC 60
              **** ******************** ********** *************** *******

KT783518      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
EU450806      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR818028      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR818027      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR818011      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTT-AA 119
KR818017      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTT-AA 119
KT783523      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
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Fig. 2. Clustal Omega alignment of *Cytauxzoon felis* ITS2 sequence representatives of all sequences from this study that identically matched (100% identity) or had 1 or more base differences from the *C. felis* ITS2 sequences previously submitted to GenBank. Alignment also includes the previously reported *C. felis* ITS2 sequences in GenBank (EU450804, EU450805, EU450806, and JF308506), in which the ITS2 sequences of this study were most similar. Nucleotide position number indicated at the right. Matching nucleotides in all sequences are shown by *. Mismatch nucleotides are shown in bold. Gaps (deletions) are indicated by a dash (-).

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KR817958      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817957      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817948      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817947      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817945      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817950      TCTATATCTACATTCTAGAGACTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR818026      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
JF308506      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817942      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAGCTCTATTGTATTTTATA 120
KR817951      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTACA 120
KR817960      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817938      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
EU450805      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817955      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817961      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817922      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
EU450804      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
KR817962      TCTATATCTACATTCTAGAGATTCACTACCTCGCATCTCGAAACTCTATTGTATTTTATA 120
              ******************** ******************** ************  *

KT783518      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
EU450806      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR818028      TACTATACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR818027      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR818011      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 179
KR818017      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 179
KT783523      TACTCTACGTTTTGAGTGAGGGACATGAATTTCCTTATTGAATGACGTGAGGAAGATCCG 180
KR817958      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817957      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817948      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817947      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817945      TACTCTACGTTTTGAGTGAGGGACATGAACTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817950      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR818026      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
JF308506      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817942      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817951      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCT 180
KR817960      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCT 180
KR817938      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCT 180
EU450805      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCT 180
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Fig. 2. Continued.

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KR817955      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817961      TACTCTACGCTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817922      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
EU450804      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
KR817962      TACTCTACGTTTTGAGTGAGGGACATGAATTTCATTATTGAATGACGTGAGGAAGATCCG 180
              **** **** ****************** *** ************************

KT783518      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
EU450806      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR818028      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR818027      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR818011      AACGGAGTGAGGAAGTGTGTGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 239
KR818017      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 239
KT783523      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCGAT 240
KR817958      AACGGAGTGAGGGAGTGTGGGATGTAACGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817957      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817948      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817947      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817945      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817950      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR818026      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
JF308506      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817942      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817951      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817960      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817938      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
EU450805      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817955      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817961      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817922      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
EU450804      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
KR817962      AACGGAGTGAGGAAGTGTGGGATGTACCGACGTGTGAGGATTATCACAGTATTCCTCAAT 240
              ************ ****** ****** ****************************** **

KT783518      ACATCGATATATCTCAACATCTCCATCAATACATCAACATACGTCGATGTATCGATCTAC 300
EU450806      ACATCGATNTATCTCAACATCTCCATCAATACATCAACATACGTCGATGTATCGATCTTC 300
KR818028      ACATCGATGTATCTCAACATCTCCATCAATACATCAACATACGTCGATGTATCGATCTTC 300
KR818027      ACATCGATGTATCTCAACATCTCCATCAATACATCAACATACGTCGATGTATCGATCTTC 300
KR818011      ACATCGATGTATC----------------------------------------GATCTTC 259
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Fig. 2. Continued.

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KR818017      ACATCGATGTATC----------------------------------------GATCTTC 259
KT783523      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817958      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817957      ACGTTGATGTATC----------------------------------------GATCTTC 260
KR817948      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817947      ACGTCGATGTATC----------------------------------------GATCTTC 260
KR817945      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817950      ACATCGATGTATC----------------------------------------GATCTTC 260
KR818026      ACGTCGATGTATC----------------------------------------GATCTTC 260
JF308506      ACGTCGATGTATC----------------------------------------GATCTTC 260
KR817942      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817951      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817960      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817938      ACATCGATGTATC----------------------------------------GATCTTC 260
EU450805      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817955      ACATTGATGTATC----------------------------------------GATCTTC 260
KR817961      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817922      ACATCGATGTATC----------------------------------------GATCTTC 260
EU450804      ACATCGATGTATC----------------------------------------GATCTTC 260
KR817962      ACATCGATGTATC----------------------------------------GATCTTC 260
              ** * *** ****                                          *******

KT783518      CTTCA     305
EU450806      CTTCA     305
KR818028      CTTCA     305
KR818027      CTTCA     305
KR818011      CTTCA     264
KR818017      CTTCA     264
KT783523      CTTCA     265
KR817958      CTTCA     265
KR817957      CTTCA     265
KR817948      CTTCA     265
KR817947      CTTCA     265
KR817945      CTTCA     265
KR817950      CTTCA     265
KR818026      CTTCA     265
JF308506      CTTCA     265
KR817942      TTTCA     265
KR817951      CTTCA     265
KR817960      CTTCA     265
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Fig. 2. Continued.

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KR817938      CTTCA     265
EU450805      CTTCA     265
KR817955      CTTCA     265
KR817961      TTTCA     265
KR817922      CTTCA     265
EU450804      CTTCA     265
KR817962      TTTCA     265
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Fig. 2. Continued.

Table 20. Maximum identity to prior GenBank accession numbers (GenBank match) and nucleotide variation of cloned *Cytauxzoon felis* ITS2 amplicons. Sequence position numbering is based on GenBank accession number EU450804. Insertions, single nucleotide polymorphisms, miscellaneous mismatches, and deletions are shown as INS, SNP, Misc., and DEL, respectively.

No.	GenBank match	Max identity	Animal-Clone	GenBank accession number	Polymorphisms							
					Misc.	SNP 80 T>C	119 DEL T	SNP 180 G>T	SNP 243 A>G	SNP 249 G>A	254 40-BP INS	SNP 261 C>T
-	-	-	-	EU450804								
1	EU450804	100%	1 Cl 2	KR817922								
2	EU450804	100%	1 Cl 6	KR817923								
3	EU450804	100%	1 Cl 8	KR817924								
4	EU450804	100%	1 Cl 9	KR817925								
5	EU450804	100%	1 Cl 11	KR817926								
6	EU450804	100%	4 Cl 1	KR817927								
7	EU450804	100%	4 Cl 3	KR817928								
8	EU450804	100%	4 Cl 6	KR817929								
9	EU450804	100%	4 Cl 9	KR817930								
10	EU450804	100%	4 Cl 11	KR818023								
11	EU450804	100%	6 Cl 2	KR817956								
12	EU450804	100%	6 Cl 3	KR818022								
13	EU450804	100%	15 Cl 1	KR818021								
14	EU450804	100%	15 Cl 7	KR817931								
15	EU450804	100%	15 Cl 8	KR817939								
16	EU450804	100%	15 Cl 9	KR817940								
17	EU450804	100%	15 Cl 12	KR817941								
18	EU450804	100%	23 Cl 5	KR817954								
19	EU450804	100%	25 Cl 4	KR817943								
20	EU450804	100%	25 Cl 6	KR817944								
21	EU450804	100%	25 Cl 12	KR818024								
22	EU450804	100%	26 Cl 1	KR817946								
23	EU450804	100%	27 Cl 5	KR817953								
24	EU450804	100%	28 Cl 2	KT783524								
25	EU450804	100%	28 Cl 3	KR817932								
26	EU450804	100%	28 Cl 5	KR817933								

Table 20. Continued.

No.	GenBank match	Max identity	Animal-Clone	GenBank accession number	Polymorphisms							
					Misc.	SNP 80 T>C	119 DEL T	SNP 180 G>T	SNP 243 A>G	SNP 249 G>A	254 40-BP INS	SNP 261 C>T
27	EU450804	100%	28 Cl 6	KR817959								
28	EU450804	100%	34 Cl 1	KR817967								
29	EU450804	100%	34 Cl 2	KR817934								
30	EU450804	100%	35 Cl 1	KR817935								
31	EU450804	100%	35 Cl 2	KR817936								
32	EU450804	100%	36 Cl 1	KR817937								
33	EU450804	100%	5 Cl 2	KR818036								
34	EU450804	100%	5 Cl 3	KR818033								
35	EU450804	100%	5 Cl 4	KR818034								
36	EU450804	100%	32 Cl 5	KR817963								
37	EU450804	100%	16 Cl 1	KR817989								
38	EU450804	100%	16 Cl 2	KR817990								
39	EU450804	100%	16 Cl 4	KR817986								
40	EU450804	100%	16 Cl 5	KR817987								
41	EU450804	100%	16 Cl 6	KR817988								
42	EU450804	100%	18 Cl 1	KR817968								
43	EU450804	100%	18 Cl 2	KR817969								
44	EU450804	100%	18 Cl 5	KR817993								
45	EU450804	100%	20 Cl 1	KR817996								
46	EU450804	100%	20 Cl 3	KR817997								
47	EU450804	100%	21 Cl 8	KR817970								
48	EU450804	100%	29 Cl 1	KR817971								
49	EU450804	100%	29 Cl 4	KR817972								
50	EU450804	100%	29 Cl 5	KR817973								
51	EU450804	100%	29 Cl 6	KR817974								
52	EU450804	100%	30 Cl 5	KR817975								
53	EU450804	100%	31 Cl 6	KR817976								
54	EU450804	100%	31 Cl 8	KR817977								
55	EU450804	100%	31 Cl 9	KR817978								
56	EU450804	100%	31 Cl 10	KR817979								

Table 20. Continued.

No.	GenBank match	Max identity	Animal-Clone	GenBank accession number	Polymorphisms							
					Misc.	SNP 80 T>C	119 DEL T	SNP 180 G>T	SNP 243 A>G	SNP 249 G>A	254 40-BP INS	SNP 261 C>T
57	EU450804	100%	31 Cl 11	KR817980								
58	EU450804	100%	40 Cl 2	KR818035								
59	EU450804	100%	40 Cl 5	KT783522								
60	EU450804	100%	41 Cl 1	KR817998								
61	EU450804	100%	41 Cl 3	KR818001								
62	EU450804	100%	41 Cl 5	KR817999								
63	EU450804	100%	5 Cl 5	KR818010								
64	EU450804	99%	27 Cl 1	KR818016			X					
65	EU450804	99%	27 Cl 2	KR818014			X					
66	EU450804	99%	27 Cl 3	KR818018			X					
67	EU450804	99%	42 Cl 10	KR818020			X					
68	EU450804	99%	18 Cl 3	KR818031			X					
69	EU450804	99%	18 Cl 4	KR818032			X					
70	EU450804	99%	23 Cl 1	KR818017			X					
71	EU450804	99%	23 Cl 2	KR818015			X					
72	EU450804	99%	23 Cl 4	KR818019			X					
73	EU450804	99%	33 Cl 1	KR817965								X
74	EU450804	99%	33 Cl 2	KR817966								X
75	EU450804	99%	43 Cl 4	KR817964								X
76	EU450804	99%	40 Cl 6	KR817995								X
77	EU450804	99%	3 Cl 3	KR817962								X
78	EU450804	99%	19 Cl 1	KR818011	200 T>G		X					
79	EU450804	99%	19 Cl 4	KR818026	200 T>G		X					
80	EU450804	99%	19 Cl 6	KR818013	200 T>G		X					
81	EU450804	99%	19 Cl 9	KT783519	200 T>G		X					
82	EU450804	99%	27 Cl 4	KR817949		X						
83	EU450804	99%	21 Cl 12	KR817985		X						
84	EU450804	99%	23 Cl 3	KR817950		X						
85	EU450804	99%	25 Cl 11	KR817945	150 T>C							
86	EU450804	99%	26 Cl 5	KR817948	26 T>C							

Table 20. Continued.

No.	GenBank match	Max identity	Animal-Clone	GenBank accession number	Polymorphisms							
					Misc.	SNP 80 T>C	119 DEL T	SNP 180 G>T	SNP 243 A>G	SNP 249 G>A	254 40-BP INS	SNP 261 C>T
87	EU450804	99%	6 Cl 5	KR817958	193 A>G 207 C>A							
88	EU450804	99%	10 Cl 6	KT783523	154 A>C 238 A>G							
89	EU450804	99%	3 Cl 2	KR817961	130 T>C							X
91	EU450804	99%	17 Cl 12	KR817942	103 A>G						X	
-	-	-	-	EU450805			X					
92	EU450805	100%	9 Cl 3	KR817938			X					
93	EU450805	100%	13 Cl 1	KR817982			X					
94	EU450805	100%	13 Cl 2	KR817994			X					
95	EU450805	100%	13 Cl 3	KR817983			X					
96	EU450805	100%	13 Cl 4	KR817984			X					
97	EU450805	100%	13 Cl 5	KR817992			X					
98	EU450805	99%	9 Cl 5	KR817960	37 T>C		X					
99	EU450805	99%	9 Cl 8	KR817951	119 T>C		X					
100	EU450805	99%	9 Cl 9	KR817952	119 T>C		X					
-	-	-	-	EU450806[a]			X			X		
101	EU450806	100%	12 Cl 3	KR818002			X		X	X		
102	EU450806	100%	12 Cl 7	KR818003			X		X	X		
103	EU450806	100%	12 Cl 9	KR818004			X		X	X		
104	EU450806	100%	12 Cl 10	KR818005			X		X	X		
105	EU450806	100%	12 Cl 12	KR818006			X		X	X		
106	EU450806	100%	14 Cl 7	KR817991			X		X	X		
107	EU450806	100%	20 Cl 4	KR818008			X		X	X		
108	EU450806	100%	20 Cl 5	KR818009			X		X	X		
109	EU450806	100%	30 Cl 2	KR818000			X			X		
110	EU450806	100%	43 Cl 1	KT783520			X			X		
111	EU450806	100%	20 Cl 2	KR818007			X		X	X		
112	EU450806	100%	25 Cl 9	KT783521			X			X		

Table 20. Continued.

No.	GenBank match	Max identity	Animal-Clone	GenBank accession number	Polymorphisms						
					Misc.	SNP 80 T>C	119 DEL T	SNP 180 G>T	SNP 243 A>G	SNP 249 G>A	254 40-BP INS	SNP 261 C>T
113	EU450806	100%	10 Cl 1	KR818027			X			X		
114	EU450806	100%	41 Cl 2	KR818001				X			X	
115	EU450806	99%	36 Cl 2	KR818029	125 C>A			X			X	
116	EU450806	99%	39 Cl 3	KR818030	125 C>A			X			X	
117	EU450806	99%	32 Cl 4	KR818028	125 C>A			X			X	
118	EU450806	99%	5 Cl 1	KT783518	299 T>A			X		X	X	
-	-	-	-	JF308506					X			
119	JF308506	100%	26 Cl 2	KR818025					X			
120	JF308506	100%	19 Cl 3	KR818026					X			
121	JF308506	99%	26 Cl 4	KR817947	5 A>G				X			
122	JF308506	99%	6 Cl 4	KR817957	53 A>G 243 C>T				X			

[a] Sequence contained a polymorphic site depicted as N at position 249.

Table 21. Number and distribution of *Cytauxzoon felis* cloned ITS2 sequences according to the state of origin and clinical outcome that matched sequences previously reported to GenBank. GenBank accession no. (GBA) of the previously reported sequence and observed differences in this study are listed. The cloned ITS2 sequences from this study are in the GenBank deposited partial-18S-ITS1-5.8S-ITS2-partial 28S rDNA sequences provided below.

GBA and changes	No. of cloned amplicons (% of total)	Number of genetic types according to state of origin					Clinical outcome		
		OK	KS	AR	MO	TX	acute, fatal	acute, recovered	chronic, subclinical
EU450804	63 (52)	42	0	15	6	0	47	0	16
1 base change[a]	20 (16)	15	2	0	1	2	17	3	0
>1 base change[b]	8 (7)	8	0	0	0	0	4	0	4
EU450806	14 (11)	11	0	0	2	1	8	1	5
1 base change[c]	4 (3)	1	1	1	1	0	2	1	1
EU450805	6 (5)	6	0	0	0	0	6	0	0
1 base change[d]	3 (2)	3	0	0	0	0	3	0	0
JF308506	2 (2)	2	0	0	0	0	1	0	1
1 base change[e]	1 (1)	1	0	0	0	0	1	0	0
>1 base change[f]	1 (1)	1	0	0	0	0	1	0	0

[a] Sequences KR817945; KR817948-KR817950; KR817955; KR817962; KR817964-KR817966; KR817985; KR817995; KR818014-KR818020; KR818031; KR818032 from this study.
[b] Sequences KR817942; KR817958; KR817961; KR818011; KR818013; KR818026; KT783519; KT783523 from this study.
[c] Sequences KR818028-KR818030; KT783518 from this study.
[d] Sequences KR817951; KR817952; KR817960 from this study.
[e] Sequence KR817947 from this study.
[f] Sequence KR817957 from this study.

The second most common ITS2 sequence matched a *C. felis* ITS2 sequence from a fatally infected cat from Arkansas, EU450806, which had a 40-bp insertion. A similar sequence with a 40-bp insert was found in three ITS2 sequences (HQ383878, HQ383880, HQ383881) of *C. felis* from Florida pumas (Shock et al., 2012). A 41-bp insert in the same location as the *C. felis* ITS2 sequences of the Florida pumas and EU450806 was detected in *C. felis* of a bobcat from Missouri (HQ383897) (Shock et al., 2012). In this study, this 40-bp insert was detected in 14 *C. felis* cloned amplicons from Oklahoma (11 clones), Missouri (two clones), and Texas (one clone) in eight domestic cats that either recovered, survived, or died from cytauxzoonosis (Table 21) (KR817991, KR818000-KR818009, KR818027, KT783520, and KT783521). Of the 11 cloned *C. felis* amplicons from Oklahoma, six clones were from four domestic cats that succumbed to acute, fatal infection, and five clones were from one cat with a subclinical, chronic infection. Two clones from Missouri came from two domestic cats with acute, fatal infections. The one *C. felis* clone from a domestic cat in Texas was from an acute, recovery case.

The ITS2 sequences of three additional clones (KR818028-KR818030) contained this 40-bp insert as well as the same SNP at position 125 of the non-insert region (Table 20). These clones were from three domestic cats (two subclinical, chronic carriers from Arkansas and one acute, fatal infection from Missouri). One other *C. felis* ITS2 clone, KT783518, from a domestic cat from Oklahoma with an acute, fatal infection had the 40-bp insert, which contained a single nucleotide change inside the 40-bp insert region (Fig. 2).

Additionally, two *C. felis* ITS2 clones (KR818025 and KR818026) from two domestic cats (one acute, fatal and one subclinical, chronic) from Oklahoma matched GenBank accession number JF308506 (*C. felis* from a bobcat from Missouri) and six clones (KR817938, KR817982- KR817984, KR817992, and KR817994) from two domestic cats with acute, fatal infections from Oklahoma matched GenBank accession number EU450805 (*C. felis* infected domestic cat from Georgia) (Table 21).

The remaining 33 ITS2 sequences differed from the *C. felis* ITS2 sequences deposited in GenBank by one or more nucleotide substitution(s). Twenty-four had a single substitution, and of these, 20 clones (KR817945, KR817948-KR817950, KR817955, KR817962, KR817964-KR817966, KR817985, KR817995, KR818014-KR818020, KR818031, and KR818032) varied from prior GenBank accession number EU450804 and one (KR817947) from previously submitted GenBank accession number JF308506, and three (KR817951, KR817952, and KR817960) from prior GenBank accession number EU450805 (Table 20). Nine *C. felis* ITS2 clones (KR817942, KR817957, KR817958, KR817961, KR818011, KR818013, KR818026, KT783519, and KT783523) from six cats had two or more bases that varied from GenBank deposited sequences EU450804 and JF308506 (Table 20).

The ITS1 and ITS2 sequences for each clone were joined together for genotyping as previously described (Brown et al., 2009a). When ITS1 and ITS2 sequences were combined, 72 *C. felis* clones from 27 domestic cats had previously described genotypes (Brown et al., 2009a, 2009b, 2010; Shock et al., 2012). This entailed clones that had exact matches and one nucleotide difference with the sequences of the prior genotypes

(Table 22). Due to multiple variances of a previous genotype or not having a genotype that was previously designated, 50 clones did not have prior genotypes. An observation of variances of multiple *C. felis* ITS genotypes infecting the same animal was seen with 25 domestic cats. The other 10 cats produced 42 clones of the same *C. felis* ITS genotypes.

The most common genotype, ITSa (GenBank accession numbers EU450802 and EU450804 for ITS1 and ITS2, respectively) (Brown et al., 2009a), was found in 51 *C. felis* clones of 22 domestic cats. Clones with one random nucleotide substitution in the ITS1 or ITS2 sequence of the ITSa genotype were included, and these clones were represented by GenBank accession number KR817962 (Table 22). There were 14 clones of eight domestic cats with the ITSn genotype (GenBank accession numbers EU450802 and EU450806) (Shock et al., 2012), and this included the clones with a single random base substitution in either one of the ITS sequences of this genotype. These clones were represented by GenBank accession number KR818029 (Table 22). Six clones of two domestic cats were the ITSd genotype (GenBank accession numbers GU581166 and EU450805) (Brown et al., 2010). These six clones consisted of clones with one random nucleotide substitution in their ITS1 or ITS2 sequences in this genotype, and these clones were represented by GenBank accession number KR818029. There was only one clone from one domestic cat with the ITSc genotype (GenBank accession numbers EU450803 and EU450804) (Brown et al., 2009a).

Table 22. Number of *C. felis* isolates (N) assigned a particular ITS genotype in this study. Genotypes include previously reported ITSa, ITSc, ITSd, and ITSn (100% match or one base difference), ITSaa and ITSbb which match ITSa and ITSn except for a 23-bp insert in ITS1, ITScc-ITShh which represent newly identified combinations of previously reported ITS1 and ITS2 sequences, and ITSii-ITStt which show the highest identity to the ITS1 and ITS2 shown but are considered variances. The reference or source and the total number of cloned amplicons for each genotype are shown.

N	Genotype	Previously Reported GBA		Reference/source	GBA this study	Total of cloned amplicons
		ITS1	ITS 2			
22	ITSa	EU450802	EU450804	Brown et al., 2009a	KR817922	51[a]
1	ITSc	EU450803	EU450804	Brown et al., 2009a	KR818021	1[a]
2	ITSd	GU581166	EU450805	Brown et al., 2010	KR817938	6[a]
8	ITSn	EU450802	EU450806	Shock et al., 2012	KR818002	14[a]
3	ITSaa	EU450802	EU450804	This study	KR818036	4[b]
1	ITSbb	EU450802	EU450806	This study	KT783518	1[b]
1	ITScc	JF308498	EU450804	This study	KR818024	1
2	ITSdd	HQ383856	EU450804	This study	KR818023	3
3	ITSee	HQ383820	EU450804	This study	KR818018	6
1	ITSff	GU581166	EU450804	This study	KR818010	1
1	ITSgg	HQ383867	EU450804	This study	KR817943	1
1	ITShh	HQ383856	EU450806	This study	KT783520	1
12	ITSii	EU450802	EU450804	This study	KR818013	17
1	ITSjj	EU450802	JF308506	This study	KR817947	1
2	ITSkk	EU450803	JF308506	This study	KR818025	2
1	ITSll	EU450803	EU450804	This study	KT783519	1
2	ITSmm	HQ383820	EU450804	This study	KR818014	2
1	ITSnn	HQ383856	EU450804	This study	KR818022	1
2	ITSoo	JF308498	EU450804	This study	KR817944	2
1	ITSpp	JF308498	JF308506	This study	KR817957	1
1	ITSqq	JF308498	EU450806	This study	KR818027	1
1	ITSrr	GU581166	EU450806	This study	KT783521	1
1	ITSss	GU581166	EU450805	This study	KR817960	1
1	ITStt	HQ383820	EU450805	This study	KR817951	2

[a] Includes genotypes and sequences with one base difference in ITS1 and/or ITS2 from the previously reported genotype sequence.
[b] Includes clones with the 23-bp insert in ITS1.

The remaining 50 clones did not correspond to these previously described genotypes. These clones either had multiple variances of a prior genotype or had a genotype that was not previously described. Five of these clones contained the novel 23-bp insert in their ITS1 sequence and therefore had novel genotypes (genotypes ITSaa and ITSbb). The ITS1 sequence of three of these clones had a 95% identity to the previously submitted GenBank accession number EU450802. GenBank accession number KR818036 from this study represents these three clones (Table 22). With the previously reported GenBank accession number EU450804 as their ITS2 sequence, these clones were designated ITSaa for their genotype. Another clone from this study, KT783522, had an additional one base insert but was still considered as the ITSaa genotype. One clone contained both the 23-bp insert in the ITS1 sequence and the 40-bp insert in the ITS2 sequence in this study (KT783518) and was designated ITSbb as the genotype.

Thirteen clones either exactly matched or had a single base substitution from exactly matching sequences previously submitted to GenBank, but their combined ITS1 and ITS2 sequences had no prior genotype designations. In the current study, these sequence combinations were designated a genotype. One clone in this study (GenBank accession number KR818024) had previously submitted GenBank accession numbers JF308498 and EU450804 for the ITS1 and ITS2 sequences, respectively. This sequence combination was denoted as genotype ITScc in this study (Table 22). Combined sequences of previously submitted GenBank accession numbers HQ383856 and EU450804 were found in three clones in this study. GenBank accession number

KR818023 from this study represented these clones, and they were referred to as having an ITSdd genotype (Table 22). Six clones in this study had genotype ITSee as they had a combination of previously submitted GenBank accession numbers HQ383820 and EU450804. GenBank accession number of KR817998 from this study represented the two clones with the exact matches, and sequences of four clones, such as GenBank accession number of KR818018, had a one base substitution. One clone (GenBank accession number KR818010) had prior sequences of GU581166 and EU450804 and was referred to as genotype ITSff. Another clone (GenBank accession number KR817943) had prior sequences of HQ383867 and EU450804 and was denoted as ITSgg. The clone with GenBank accession number KT783520 in this study was one nucleotide substitution from being identical to archived sequences HQ383856 and EU450806, and it was referred to ITShh.

Thirty-two clones had more than one nucleotide substitution in the sequence(s) of ITS1, ITS2, or both (Table 22). The previously reported sequences with the highest identities were used to designate the genotypes. Despite these multiple differences, these sequences still shared a 99% identity to the sequences reported from prior studies.

The one clone that had the ITSc genotype was from a cat with a fatal *C. felis* infection, and the ITSd genotype was found in six clones from two cats with a fatal *C. felis* infection. (Table 23). Of the 51 *C. felis* clones with the ITSa genotype, 35 of the 51 clones (69%) were from acute, fatal cases of 15 domestic cats and 16 of 51 clones (31%) from subclinical, chronic cases of seven domestic cats (Table 23). Of the 14 *C. felis* clones of the ITSn genotype, found in this study, eight out of 14 clones (57%)

were from subclinical, chronic cases of four domestic cats, and the other six out of 14 clones (43%) were from acute, fatal cases of four domestic cats (Table 23). The four clones with the ITSaa genotype from three cats and the one clone with the ITSbb genotype from a single cat were all from acute, fatal cases. The 13 clones with ITScc, ITSdd, ITSee, ITSff, and ITSgg were all from cats with a fatal *C. felis* infection, and the clone with the ITShh was found in a cat with an acute, recovered case (Table 23). There was no association found with the previously described genotypes in this study and clinical outcome of *C. felis* infection ($P > 0.05$). The remaining 32 clones were from all clinical outcomes.

Table 23. Clinical outcome and state of origin for cat samples (N) for derivation of 122 *Cytauxzoon felis* cloned amplicons and their matches to previously described genotype (ITSa, ITSc, ITSd, and ITSn) or to new genotype designated in this study (ITSaa-ITStt).

Genotype	N	No. of cloned amplicons for state					No. of cloned amplicons per clinical outcome			Total of cloned amplicons
		OK	KS	AR	MO	TX	fatal	recovered	chronic	
ITSa	22	32	0	15	3	1	35	0	16	51
ITSc	1	1	0	0	0	0	1	0	0	1
ITSd	2	6	0	0	0	0	6	0	0	6
ITSn	8	9	0	2	3	0	6	0	8	14
ITSaa	3	2	0	0	2	0	4	0	0	4
ITSbb	1	1	0	0	0	0	1	0	0	1
ITScc	1	1	0	0	0	0	1	0	0	1
ITSdd	2	3	0	0	0	0	3	0	0	3
ITSee	3	4	0	0	2	0	6	0	0	6
ITSff	1	1	0	0	0	0	1	0	0	1
ITSgg	1	1	0	0	0	0	1	0	0	1
ITShh	1	0	0	0	0	1	0	1	0	1
ITSii	12	14	2	0	0	1	11	3	3	17
ITSjj	1	1	0	0	0	0	1	0	0	1
ITSkk	2	2	0	0	0	0	1	0	1	2
ITSll	1	1	0	0	0	0	0	0	1	1
ITSmm	2	2	0	0	0	0	2	0	0	2
ITSnn	1	1	0	0	0	0	1	0	0	1
ITSoo	2	2	0	0	0	0	2	0	0	2
ITSpp	1	1	0	0	0	0	1	0	0	1
ITSqq	1	1	0	0	0	0	1	0	0	1
ITSrr	1	1	0	0	0	0	1	0	0	1
ITSss	1	1	0	0	0	0	1	0	0	1
ITStt	1	2	0	0	0	0	2	0	0	2

The one *C. felis* clone with the ITSc genotype was from a cat in Oklahoma, and the six clones with the ITSd genotype were from two cats from Oklahoma. The ITSn and ITSa genotypes were detected in clones from this state as well. Of the 14 *C. felis* clones of the ITSn genotype, nine clones (64%) were from three cats from Oklahoma, two clones (14%) from two cats from Arkansas, and three clones (21%) from three cats from Missouri (Table 23). Thirty-two of the 51 total ITSa clones (63%) were from 12 cats from Oklahoma, 15 clones (29%) from six cats from Arkansas, three clones (6%) from three cats from Missouri, and one clone (2%) from one cat from Texas (Table 23). Two of the four clones with the ITSaa genotype were from two cats from Oklahoma, and the other two clones were from a cat in Missouri. The clone with the ITSbb genotype was from a cat from Oklahoma. The six clones with ITScc, ITSdd, ITSff, and ITSgg were from cats from Oklahoma. Four clones with ITSee were from two cats from Oklahoma, and the other two clones were from one cat from Missouri. ITShh was found in a clone from a cat from Texas. There was no association found with the previously described genotypes in this study and geography ($P > 0.05$). The remaining 32 clones were from cats from Oklahoma, Kansas, and Texas.

There were 49 and 73 amplicons from nested and primary amplifications for the ITS region, respectively. Fourteen of 49 nested PCR amplicons contained variance(s), in which nine (64%) contained one variance and five (36%) had multiple variances. Fifty-one of 73 primary amplicons contained variance(s), in which 19 (37%) had one variance and 32 (63%) contained multiple variances. There was no correlation between the single

or multiple variance(s) in the genotypes and PCR amplifications (primary or nested) ($P > 0.05$).

The High Fidelity Platinum® *Taq* DNA Polymerase and the Phusion High-Fidelity DNA Polymerase were used for amplifying the ITS region in 57 and 65 PCR assays, respectively. Of the 28 PCRs with a single variance, 18 (64%) and 10 (36%) utilized the Phusion High-Fidelity DNA Polymerase and High Fidelity Platinum® *Taq* DNA Polymerase, respectively. Of the 37 PCRs with multiple variances, eight (22%) and 29 (78%) were synthesized with Phusion High-Fidelity DNA Polymerase and High Fidelity Platinum® *Taq* DNA Polymerase, respectively. There were more single variances in the PCRs with the Phusion High-Fidelity DNA Polymerase and more multiple variances in PCRs with the High Fidelity Platinum® *Taq* DNA Polymerase, in which there was a correlation between variance(s) and type of polymerase used ($P < 0.05$).

DISCUSSION

In this study, the 18S rRNA gene was the molecular marker used to confirm the identity of *C. felis*. The *C. felis* genotypes were further investigated based on rRNA ITS1 and ITS2 regions amplified and cloned from infected blood samples from domestic cats in Arkansas, Kansas, Oklahoma, Missouri, and Texas. Genotypes previously were assigned based on directly sequenced PCR products (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011; MacNeill et al., 2015). This study was undertaken to genotype *C. felis* through use of cloning and higher fidelity polymerases than previously employed. Additional genotypes were observed in the current study compared to the previous studies. Moreover, this study detected for the first time the same *C. felis* ITS1 and ITS2

sequences in domestic cats previously only found in *C. felis* isolates from wild felids (Shock et al. 2012).

Sequence analysis in previous studies revealed the incorporation of two nucleotides at single, variable positions in the ITS1 and ITS2 of samples (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011; Shock et al., 2012; MacNeill et al., 2015). A coinfection with multiple strains of *C. felis* or the presence of multiple rRNA copies in the genome may have led to these ambiguous sequences. However, since no previous study utilized a high-fidelity DNA polymerase, it is possible that some of the polymorphisms may have been incorporated during amplification. This study was the first to use high-fidelity DNA polymerases in conjunction with cloning amplicons prior to sequencing, and with their utilization, polymorphic sites, as depicted as double peaks in chromatograms in previous studies, were resolved to a single nucleotide. The Phusion High-Fidelity DNA Polymerase and Platinum® *Taq* DNA Polymerase, High Fidelity have 50X and 6X higher fidelity, respectively, than the *Taq* DNA polymerases used in the previous studies. Single and multiple variance(s) in genotypes were found in both nested and primary PCR amplifications, and that variance(s) occurred independently of the type of PCR.

Cloning is a very useful *in vivo* technique that can be used to copy DNA fragments (Strachan and Read, 1999). Cloning does involve the selection for a single DNA molecule, and this could lessen the chance of obtaining ambiguous sequence data as noted with two peaks in chromatogram. This way cloning can be helpful in quickly identifying and dismissing any spuriously amplified DNA (Carr et al., 2007). Cloning

also has the capability of detecting polymorphisms (whether caused by *Taq* DNA polymerase error or a heterogeneous sequence mixture) at a low frequency, which otherwise could be overwhelmed by the sequence in abundance (Ruecker et al., 2011) and missed with direct sequencing. Because of this ability, cloning could lead to an even greater sequence diversity (Ruecker et al., 2011). More costs and time expended can come with cloning as well. Both direct sequencing of PCR products and cloning into a vector prior to sequencing have their benefits and drawbacks. In order to obtain the correct, consensus sequence both approaches should be evaluated and possibly used, especially in the case of any amplification from a highly heterogenetic pool of molecules.

The most common ITS1 sequence found in this study matched Genbank accession number EU450802, an ITS1 sequence from a domestic cat that survived an infection in Georgia. This ITS1 sequence was only found in *C. felis* clones from acute, fatal and subclinical, chronic cases. This ITS1 sequence was found in cloned amplicons from every state included in this study, except Kansas. This could possibly be due to the usage of only one *C. felis* infected cat from Kansas in this study, and it was one of the samples that only yielded two amplicons for analysis. ITS1 sequence EU450802 was also the most commonly found in previous studies in domestic cats (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011; MacNeill et al., 2015), bobcats (Brown et al., 2010), and wild felids (Shock et al., 2012).

In this study, a novel 23-bp insertion was detected within the ITS1 region. This insertion contained a repeated sequence, and repeats are dispersed throughout coding

and non-coding regions of genomes, particularly eukaryotes (Tóth et al., 2000; Viguera et al., 2001a). However, anomalous DNA fragments containing insertions or deletions of repeats may result from polymerase slippage during the extension step of PCR (Litt and Luty, 1989). In previous studies with the *C. felis* ITS regions, an insert was regarded as a finding in the *C. felis* genome, and attributing the inserts to DNA polymerase slippage during PCR was not mentioned (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011; Shock et al., 2012). Slippage is a misalignment of DNA strands during replication of repeated sequences (Castillo-Lizardo et al., 2014). Certain polymerases may undergo slippage more so than others (Viguera et al., 2001b), and the increased processivity polymerases do have a lesser occurrence of slippage (McInerney et al., 2014).

The processivity of a DNA polymerase refers to the number of nucleotides incorporated per binding event (McDougal and Guarino, 1999). A *Taq* DNA polymerase was used in the previous studies (Brown et al., 2009a, 2009b, 2010; Cohn et al., 2011; Shock et al., 2012), and this DNA polymerase has relatively low processivity in PCR (Shinde et al., 2003). In the current study, the High Fidelity Platinum® *Taq* DNA Polymerase and the Phusion High-Fidelity DNA Polymerase were used. The High Fidelity Platinum® *Taq* DNA Polymerase (a blend of *Taq* DNA polymerase and a lesser amount of a proofreading DNA polymerase) is more known for its fidelity (Barnes, 1994) and less for its processivity. However, the fusion of a DNA binding domain to an archaeal polymerase increased the processivity of the Phusion High-Fidelity DNA Polymerase and improved its overall performance (Wang et al., 2004). The 23-bp insert was detected with use of both the Phusion and the Platinum® *Taq* High Fidelity DNA

polymerases, indicating that it was not an artifact introduced by PCR. It could be speculated that the 23-bp insert occurs at a very low frequency, which may be why it was not detected by direct sequencing in previous studies but able to be revealed by cloning in this study.

The most common *C. felis* ITS2 sequence found in this study matched Genbank accession number EU480504, an ITS2 sequence from a *C. felis* infection survived by a domestic cat in Georgia. This sequence was only found in acute, fatal and subclinical, chronic cases from the states of Oklahoma, Missouri, and Arkansas in this study. This ITS2 sequence was also the most common in previous *C. felis* studies with domestic cats (Brown et al., 2009a, 2010; Cohn et al., 2011), bobcats (Brown et al., 2010), and wild felids (Shock et al., 2012). In a previous study, there was an incorporation of two nucleotides, thymine or cytosine, at position 76 in the sequence of two *C. felis* samples. Therefore, the ITS2 sequences of the *C. felis* were either the same as GenBank accession numbers EU450804 (thymine at this position) or HQ383890 (cytosine at this position) (MacNeill et al., 2015). This would mean that the GenBank accession number EU480504 sequence may have been detected in *C. felis* from two domestic cats from southern Illinois, and this further exemplifies the presence of multiple rRNA transcriptional units in an isolate or coinfection with multiple *C. felis* strains.

Genotypes were a compilation of ITS1 and ITS2 sequences (Brown et al., 2009a). The initial study to analyze the intergenic regions of *C. felis* from a large sample size used domestic cats from Georgia and Arkansas (Brown et al., 2009a). This study found an association with *C. felis* ITS genotype and geographic location and clinical

outcome of cytauxzoonosis. The most common genotype found in this previous study was ITSa, and it was associated with survival as well as *C. felis* infection of cats from Arkansas (Brown et al., 2009a). However, the subsequent studies on the ITS regions of *C. felis* did not find an association of ITS genotype with clinical outcome or geographic region (Brown et al., 2009b, 2010; Cohn et al., 2011; Shock et al., 2012). The ITSa genotype was also the most common genotype found in bobcats (Brown et al., 2010), wild felids (Shock et al., 2012) as well as domestic cats (Cohn et al., 2011) over greater locations. In the current study, ITSa was the genotype that was most commonly found, and it was found in both acute, fatal and subclinical, chronic cases. It was found in the states of Oklahoma, Arkansas, and Missouri.

The second most commonly found *C. felis* ITS genotype in this study was ITSn. This genotype was only found in one domestic cat in one previous study (Brown et al., 2009a). This genotype is comprised of Genbank accession numbers EU450802 and EU450806 for ITS1 and ITS2, respectively. The 40-bp insert is found within the ITS2 sequence.

The other previously described genotypes found in this study were ITSc and ITSd. The ITSc genotype was previously found only in domestic cats from Arkansas that either died from acute cytauxzoonosis (Brown et al., 2009a) or had a subclinical *C. felis* infection (Brown et al., 2010). In this study, this genotype was found in one *C. felis* cloned amplicon of a cat from Missouri that had an acute, fatal infection. The ITSd genotype was previously found in a bobcat from Florida (Brown et al., 2010). In the current study, this genotype was found in five *C. felis* cloned amplicons of cats from

Oklahoma that died from acute cytauxzoonosis. This is the first study to report this genotype in domestic cats.

Some genotypes were recategorized since their original designation (Brown et al., 2009b, 2010), and due to discrepancies in these genotypes, they were consolidated (Shock et al., 2012). Shock et al., 2012 obtained all of the respective ITS1 and ITS2 sequences of these genotypes and condensed them into 22 genotypes in order to use them for comparison. Only four of the 22 previously described genotypes were found in the current study. It could be that these *C. felis* genotypes dominated the sampled population of cats. Only a limited number of ITS1 and ITS2 combinations were designated a genotype, and this possibly restricted the detection of more prior genotypes. For instance, one *C. felis* clone from this study matched 100% with HQ383856 and EU450804 for the ITS1 and ITS2 sequences, respectively, and there is no genotype designated for this ITS1 and ITS2 combination before this study. The ITS1 sequence of GenBank HQ383856 was from *C. felis* in bobcats from Kansas and North Carolina (Shock et al., 2012). This sequence was not detected prior to the study conducted by Shock et al., 2012, in which no newly discovered ITS1 and ITS2 sequences were designated genotypes. Also, some of the newly designated genotypes in this study contained only one clone. If the ITS1 and ITS2 sequences of prior studies were all resolved to a single base, then it is possible that the *C. felis* from those cats could have been designated these genotypes as well.

In view of the polymorphism observed in cloned ITS sequences, it would appear that more than one difference in the nucleotide sequence should be considered when

designating genotypes. In prior studies, at base position 180 in ITS2 a guanine substitution differentiated ITSa from ITSb, which had thymine at that position. Similarly, genotypes ITSa and ITSo only differed by a single nucleotide within the ITS2 sequence. The one nucleotide that differed between genotypes of a number of amplicons could have been a true SNP. Since sequencing errors can result in a wrongfully identified SNP (Yu and Sun, 2013), there is a chance the variable base may have been a false positive SNP. A single nucleotide, which was polymorphic, distinguished three additional previously described genotypes from one another. Due to a false-positive SNP or a two nucleotide incorporation at one position, genotyping based on more than one nucleotide difference would have been more practical.

Previously, polymerase errors were taken into account, and the identity of nucleotides was determined for each position from at least two independent PCR reactions in a study of the subspecies of *Babesia canis* (Zahler et al., 1998). Cloning was employed in that study, and ten clones from ten independent PCR amplifications from each of three subspecies were sequenced. Mutations detected in only one clone were considered an error of the *Taq* DNA polymerase (Zahler et al., 1998). A similar interpretation was taken in the current study in which a variable base found in a clone or clones of only a single isolate was regarded as an error. In this study, the sequenced clones were derived from a single PCR amplification. Sequencing each additional clone from an independent amplification might have clarified whether the variation seen in this study was reproducible.

Taq DNA polymerase errors led to false 16S rRNA gene sequence diversity in a complex bacterioplankton sample, and a 99% similarity was proposed as a cutoff in order to accommodate *Taq* DNA polymerase errors (Acinas et al., 2005). Genotype designation should take the possible DNA polymerase errors into account and adjusted accordingly. These observations were taken into consideration for the current study. The approach, therefore, was to accommodate for PCR and/or sequencing induced errors by including the clones with a single base change with the genotype of closest match.

The *C. felis* ITS region, although diverse, did not associate a particular genotype with geography or clinical outcome in this study. However, there seemed to be a trend with the novel 23-bp insert (ITS1) linked with a fatal outcome. Other genetic markers or findings have been associated with outcome and geography in related hemoparasites (*i.e.*, *Babesia* and *Theileria*), and potential homologues of these genes may be beneficial in classification of *C. felis*.

Using the 18S rRNA gene, *Babesia gibsoni* and the three subtypes of *Babesia canis* (*B. canis vogeli*, *B. canis canis*, and *B. canis rossi*) have been classified according to differences in vector specificity and pathogenicity, and in order to enhance the phylogenetic analysis, sequences of additional genes were used (Yamasaki et al., 2007). Heat shock protein 70 (hsp70) is found in virtually all organisms, and it may contribute to the survival of protozoa in the host (Kaufman, 1990). Hsp70 was used to classify piroplasms, and the phylogenetic analysis of the nucleotide and predicted amino acid sequences revealed that when compared to other *Babesia* and *Theileria* spp., *Babesia gibsoni*, *B. canis vogeli*, *B. canis canis,* and *B. canis rossi* were closely related to each

other and formed one cluster (Yamasaki et al., 2007). Also, the nucleotide sequences of this gene were more diverse than the 18S rDNA sequences. A similar study may be useful in classifying *C. felis*, especially with subclinical, chronic infections.

Since *C. felis* has a schizogonous stage in its life cycle, studying a potential homologue of a gene during this phase may be helpful in understanding its pathogenecity as well. The polymorphic immunodominant molecule (PIM) is localized on the schizont surface (Shapiro et al., 1987), and Geysen et al., 2004 found differences (*i.e.*, short repeated sequences and duplicated or deleted long sequence runs) in the PIM gene of *Theileria parva* (a hemoparasite with a schizogonous phase like *C. felis*) from different epidemiological environments. The polymorphisms may indicate that PIM is an important surface antigen in *T. parva*, and establishing the mechanisms responsible for the polymorphisms may be essential for further understanding the host-parasite relationship (Geysen et al., 2004). Use of possible gene homologues like these as well as others may be useful for identifying markers related to pathogenicity in *C. felis.* The annotation of a complete *C. felis* genome (Tarigo et al., 2013) provides additional information on potential genes, which would advance genome wide association research for studying the pathogenicity of *C. felis*.

CHAPTER IV

MOLECULAR DETECTION OF HEMOPLASMAS IN DOMESTIC CATS WITH *CYTAUXZOON FELIS* INFECTION

INTRODUCTION

Cytauxzoon felis is the causative agent of the emerging infectious disease, feline cytauxzoonosis, in the United States. *Cytauxzoon felis* infection was first reported in 1976 in fatally infected domestic cats in Missouri (Wagner, 1976). Originally, cytauxzoonosis had a nearly 100% grave prognosis, but over time there are now numerous reports of survival (Motzel and Wagner, 1990; Greene et al., 1999; Meinkoth et al., 2000; Haber et al., 2007; Brown et al., 2008; Brown et al., 2009; Brown et al., 2010; Cohn et al., 2011). A coinfection with another blood parasite may contribute to a difference in pathogenicity, clinical manifestations, or response to treatment (Shaw et al., 2001).

One concurrent infection with *C. felis* may be with a feline hemotrophic mycoplasma (hemoplasma). The most common feline hemoplasmas are *Mycoplasma haemofelis*, *Candidatus* Mycoplasma turicensis, and *Candidatus* Mycoplasma haemominutum (Sykes, 2010). Occasionally a fourth *Mycoplasma*, a *Candidatus* Mycoplasma haematoparvum-like organism, may be found infecting felines as well (Sykes et al., 2007).

The primary mode of dissemination of feline hemoplasmas is by hematophagous ectoparasites (Willi et al., 2007). The more time cats spend outdoors in temperate and

tropical regions, the more they may be exposed to infected ectoparasites, such as fleas and ticks (Loyd et al., 2013), thus potentially creating a *C. felis* and feline hemoplasma coinfection (Allison et al., 2010). If in a close proximity, these infected domestic cats can pose a threat to endangered felids in the wild, as well as felids in a zoo.

In the U.S., a domestic shorthair cat that went from healthy and appearing normal to markedly lethargic with icterus within a 24-hour period was diagnosed with a *M. haemofelis* and *C. felis* coinfection (Allison et al., 2010). It was purported that the cat had a latent *M. haemofelis* infection, and the presence of the *C. felis* infection reactivated the mycoplasma (Allison et al., 2010). Similarly in Brazil, there was a report of a naturally acquired *C. felis* and *Candidatus* M. haemominutum coinfection in a domestic cat that exhibited neutrophilia, lymphocytopenia, and thrombocytopenia as well as increased blood urea nitrogen, concentration and alanine aminotransferase, and aspartate aminotransferase activities (Maia et al., 2013). It was speculated that the coinfection of these two pathogens may have caused the exacerbation in the hematological and clinical alterations, which led to the cat's demise (Maia et al., 2013).

The objective of this study was to molecularly survey *C. felis* positive cats for *Mycoplasma* spp. using PCR assay targeting the 16S rRNA gene and to determine if an antagonistic or synergistic relationship exists with the coinfection of the two pathogens.

MATERIALS AND METHODS

The genomic DNA from 41 cats previously identified as positive for *C. felis* was provided for this study. A primary PCR assay using primers Hbart250F and Hbart1250R was employed in order to amplify the 16S rRNA gene of mycoplasmas (Table 24). All

samples underwent primary amplification using High Fidelity Platinum® *Taq* DNA Polymerase (Invitrogen™, Carlsbad, CA) following manufacturer's recommendations. A plasmid containing part of the 16S rRNA gene of a mycoplasma served as the positive control and water as the negative. Primary reactions underwent a 2 min denaturation at 94°C followed by 45 cycles of 94°C for 30s, 55.5°C for 30s, 68°C for 2 min, with a final extension at 68°C for 7 min, and followed by a hold at 4°C (Labnet MultiGene™ Thermal Cycler, Labnet International, Inc., Woodbridge, NJ). EconoTaq® Plus Green 2X Master Mix (Lucigen, Middleton, WI) was used in repeated primary amplifications according to the above protocol, except the extension temperature was 72°C. The PCR products were electrophoresed through TBE buffered 1% agarose gel alongside a 100bp DNA marker (Invitrogen), stained with ethidium bromide and visualized by ultraviolet transillumination (FluorChem 8000, Alpha Innotech Corporation, San Leandro, CA).

Table 24. Oligonucleotide primers used in amplifying the 16S rRNA gene of feline hemoplasmas.

Primer Name, sense	PCR	Sequence 5'→3'
Hbart250F, forward	Primary 16S	TGAACAGCCRCAATGGGATTG
Hbart1250R, reverse	Primary 16S	CTGGGAACGKATTCACCC
Mycofe260F, forward	Nested 16S	ACAATGGACGAAAGTCTGATG
Mycofe1220R, reverse	Nested 16S	GGATTACTAGTGATTCCAACTTCA
m13F, forward	Colony 16S plasmid (pDNA)	GTAAAACGACGGCCAG
m13R, reverse	Colony 16S plasmid (pDNA)	CAGGAAACAGCTATGAC

If a band was not visualized, the primary product was diluted 1:5 and used in a nested reaction with primers Mycofe260F and Mycofe1220R (Table 24). The cycling parameters were as above, except that the reactions underwent 30 cycles with an annealing temperature of 50°C and extension temperature of 72°C. The PCR products were gel electrophoresed, stained, and visualized as above.

A PCR using a polymerase with higher fidelity (Phusion DNA Polymerase, Thermo Fisher Scientific Inc., Waltham, MA) was employed according to the manufacturer's recommendations for additional confirmation. Primary PCR utilized primers Hbart250F and Hbart1250R and cycling parameters of one cycle at 98°C for 1 min, 45 cycles at 98°C for 10 s, 55.5°C for 30 s, and 72°C 1 min, and 72°C final extension for 7 min. This was followed by electrophoresis as described above. If no band visualized, then 1:5 diluted products underwent nested PCR for 30 cycles with same parameters as stated, except with a 30 s denaturation, a 50°C annealing temperature, and a 45 s extension step.

Appropriately-sized 16S rDNA amplicons synthesized by High Fidelity Platinum ® *Taq* DNA Polymerase were ligated into pCR®2.1-TOPO® (Invitrogen, Life Technologies, Grand Island, NY) cloning vector. Those amplified using EconoTaq DNA Polymerase or the Phusion DNA Polymerase were cloned into the pJET1.2/blunt cloning vector (Thermo Fisher Scientific Inc., Waltham, MA). TOP10 *E. coli* were transformed according to manufacturer's instructions. Colonies were verified to contain the cloned insert by colony PCR using primers specific for mycoplasma or primers appropriate for the plasmid vector (Table 24). Verified colonies were expanded overnight in broth

cultures, and plasmid DNA was prepared (Bio Basic, Inc.). The plasmid DNA concentration was determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). Bidirectional sequencing of the inserts was conducted by Davis Sequencing (Davis, CA) or Eton Biosciences, Inc. (San Diego, CA). Forward and reverse sequence data were trimmed to exclude primer regions and unreadable data, aligned, and analyzed for contiguous sequences using Sequencher 4.2 software (Gene Codes Corporation, Inc., Ann Arbor, MI). Using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990), the sequences were compared to sequences in GenBank. With the chi-square test for independence, detected *Mycoplasma* spp. were evaluated for associations with the clinical outcomes of cytauxzoonosis (fatal, recovered, or chronic) (JMP®, Version Pro 11. SAS Institute Inc., Cary, NC, 1989-2007).

RESULTS

The primary PCR showed three out of 41 (7%) cats as PCR-positive for the 16S rRNA gene of *Mycoplasma* spp. The three positive samples were from cats representing the three clinical outcomes of cytauxzoonosis - recovered, chronic, or fatal (Table 25). Each positive sample successfully yielded at least one cloned amplicon with five clones in total. Sequencing revealed 1,020-bp inserts in all five. One clone had 100% identity to *Candidatus* M. haemominutum (GenBank accession number AF271154) and four clones shared a 99% identity with *Candidatus* Mycoplasma haemominutum (GenBank accession numbers AF271154, DQ825456, or FJ004275) (Table 25).

When the 38 cat samples that were negative in the primary PCR underwent a nested PCR, 10 (26%) were PCR-positive. Eight when cloned yielded sequences (N=16) 913-bp in length that matched *Candidatus* M. haemominutum (GenBank accession numbers DQ825456, FJ004275, or AF271154) with 99% identity. These cloned samples were from cats that had either a recovered, chronic, or fatal *C. felis* infection. The two clones from the ninth cat sample matched *Candidatus* Mycoplasma kahanei (GenBank accession number AF338269) with 99% identity (Table 25). Six additional clones from that sample were sequenced. One clone matched *Candidatus* M. kahanei (GenBank accession number AF338269) with 99% identity. Four clones matched numerous *Rhodococcus* spp. 16S rRNA sequences reported in the GenBank database with 97-100% identity and the remaining clone matched an uncultured bacterium (GenBank accession number KJ013390) with 93% identity. The two cloned amplicons from the 10th sample also matched numerous *Rhodococcus* spp. with 99% identity.

An association between the *Mycoplasma* sp. and clinical outcome of *C. felis* infection was evaluated. Eleven of the 12 cats positive for *Mycoplasma* spp. were identified as positive for *Candidatus* M. haemominutum. The *C. felis*-infected cats coinfected with *Candidatus* M. haemominutum exhibited all outcomes of cytauxzoonosis, therefore there was no association between degree of cytauxzoonosis and hemoplasma coinfection. The cat sample identified with *Candidatus* Mycoplasma kahanei was from a cat that died, but since it was the only sample matching this particular hemoplasma, no conclusion regarding an association between severity of cytauxzoonosis and mycoplasma coinfection could be determined

Table 25. Identification of cloned samples by animal and clone number, sequence length in base pairs, the *Mycoplasma* spp. and GenBank Accession Number with closest percent identity, and clinical outcome of *C. felis*-infected cats.

Animal-Clone	Size (bp)	NCBI BLAST highest identity (%)		Clinical Outcome
32 Clone 3	1,020	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
32 Clone 4	1,020	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
32 Clone 5	1,020	*Candidatus* Mycoplasma haemominutum	AF271154 (100%)	Chronic
33 Clone 3	1,020	*Candidatus* Mycoplasma haemominutum	DQ825456 (99%)	Recovered
23 Clone 2	1,020	*Candidatus* Mycoplasma haemominutum	FJ004275 (99%)	Fatal
1 Clone 3	913	*Candidatus* Mycoplasma haemominutum	DQ825456 (99%)	Fatal
1 Clone 4	913	*Candidatus* Mycoplasma haemominutum	DQ825456 (99%)	Fatal
13 Clone 1	913	*Candidatus* Mycoplasma haemominutum	FJ004275 (99%)	Fatal
13 Clone 2	913	*Candidatus* Mycoplasma haemominutum	FJ004275 (99%)	Fatal
14 Clone 11	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Fatal
14 Clone 12	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Fatal
19 Clone 7	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
19 Clone 8	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
21 Clone 4	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Fatal
21 Clone 5	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Fatal
34 Clone 14	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
34 Clone 15	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Chronic
43 Clone 4	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Recovered
43 Clone 8	913	*Candidatus* Mycoplasma haemominutum	AF271154 (99%)	Recovered
22 Clone A1	464	*Candidatus* Mycoplasma kahanei	AF338269 (99%)	Fatal
22 Clone A2	911	*Candidatus* Mycoplasma kahanei	AF338269 (99%)	Fatal
22 Clone B1	911	*Candidatus* Mycoplasma kahanei	AF338269 (99%)	Fatal

DISCUSSION

Domestic cats, especially those that are frequently outdoors, are likely to be infected by *Mycoplasma* spp. and/or piroplasms, and they may not exhibit any outward signs. These cats can transport pathogens, which can subsequently infect other naïve felids.

In a 2014 study, blood samples from 260 stray cats in a capture-neuter-release program in northern Italy were screened for *C. felis* or *Babesia microti* infection by PCR (Spada et al., 2014). No samples were positive for *C. felis* (0.0%), but two cats were positive for *B. microti* (0.08%). Both cats were in good clinical condition. One was identified as being positive for *B. microti*, *Candidatus* Myocplasma haemominutum, and *Anaplasma phagocytophilum*. These particular blood-borne pathogens do not seem to be a serious concern to cats in northern Italy (Spada et al., 2014).

Higher infection rates of arthropod-borne pathogens were detected in a smaller sample size of 37 free-roaming cats in a Brazilian zoo (Andre et al., 2014). Blood was screened for the presence of Anaplasmataceae agents, piroplasms, hemoplasmas, *Bartonella* spp., and *Hepatozoon* spp. by PCR. Three (8%) cats were positive for *Anaplasma* spp. (closely related to *Anaplasma phagocytophilum*), 12 (32%) cats for hemoplasmas [two (5%) for *Mycoplasma haemofelis*, five (13.5%) for *Candidatus* M. haemominutum, and five (13.5%) for *Candidatus* Mycoplasma turicensis], 11 (30%) for *Bartonella* spp., six (16%) for *Babesia vogeli*, and one (3%) for *Theileria* sp. (Andre et al., 2014). Some of the sampled cats were positive for multiple vector-borne pathogens, but no cats were positive for *Hepatozoon* spp., *Cytauxzoon* spp., or *Ehrlichia* spp. This

study emphasized the need to control the free-roaming cat population as a way to prevent infections of zoo-housed felids (Andre et al., 2014).

In another study from Brazil, an urban colony of 75 free-roaming cats were molecularly surveyed for the presence of various infectious agents (Mendes-de-Almeida et al., 2007). Cats were shown to be infected with feline immunodeficiency virus, feline leukemia virus (FeLV), *C. felis*, *T. gondii*, and ectoparasites such as lice (*Felicola subrostratus*) and fleas (*Ctenocephalides felis*). There was also a significant association made between cats infected with FeLV and infected with *Mycoplasma* spp.

Since the known vectors of *C. felis* are *AmbylommA. americanum* and *Dermacentor variabilis*, which are not known to occur in South America, the authors suggest there are other competent vectors in Rio de Janeiro, Brazil (Mendes-de-Almeida et al., 2007). A likely candidate is *Rhipicephalus sanguineus* since this was the only tick species found infesting the cats in this study (Mendes-de-Almeida et al., 2007). This study illustrated the need to identify competent vectors outside of the U.S. for non-native pathogens.

In the current study, cats were molecularly assessed for coinfections with *Mycoplasma* spp. and *C. felis* because of the possibility that an underlying coinfection could affect the course of disease. Previously, a coinfection with *Plasmodium falciparum* and a mycoplasma was evaluated in squirrel monkeys (*Saimiri sciureus*) (Contamin and Michel, 1999). It was observed that a latent mycoplasma infection that occurred prior to a *P. falciparum* infection remained latent and did not influence the course of the *P. falciparum* infection. If the *P. falciparum* infection occurred before the patent mycoplasma infection, then the *P. falciparum* infection persisted longer as the host

resisted longer or was able to clear *P. falciparum* infection. When intact *P. falciparum*-infected monkeys were splenectomized at different times, then the patent mycoplasma infection was prolonged. It was demonstrated that a mycoplasma infection activated after splenectomy may have an effect on parasitemia of *P. falciparum* (Contamin and Michel, 1999). In another *P. falciparum* study with splenectomized squirrel monkeys as hosts, it was shown that the splenectomy caused an activation in a patent infection with a hemotrophic mycoplasma (Neimark et al., 2002).

In this study, more than one-fourth of the cats with known *C. felis* infections were coinfected with *Candidatus* M. haemominutum. Determining whether this is a significant finding necessitates screening of another population of clinically normal cats that are *C. felis* negative and are from the same geographic regions.

Interestingly, one cat in the current study was positive for *Candidatus* Mycoplasma kahanei. The cat had a fatal *C. felis* infection and was from Oklahoma, but the history of the cat is unknown. *Candidatus* Mycoplasma kahanei has been found in the blood of colony-reared squirrel monkeys (*S. sciureus*) (Contamin and Michel, 1999; Neimark et al., 2002). To our knowledge, there has been no report of *Candidatus* M. kahanei in domestic cats. The potential host range is unknown, but it may be possible that other animals (including cats) can be suitable hosts (Neimark et al., 2002).

A 16S rRNA gene fragment of *Rhodococcus* spp., was detected in six clones from the blood of two cats in this study. *Rhodococcus* spp. are generally found in the soil and usually are non-pathogenic. However, they have the potential to cause disease in humans, plants, and animals (Baba et al., 2009).

Rhodococcus erythropolis has been shown to be infective to ticks (Tveten and Sjastad, 2011), and this bacterium was identified in the skin lesions of a cat from a case of feline leprosy syndrome in the U.S. (Davies et al., 2006). *Rhodococcus erythropolis* has been found on the surface of a healthy human eye (Graham et al., 2007) and in the bloodstream of a human (Baba et al., 2009), although the exact source of the latter is not known. However, to our knowledge this bacterium has not been reported in the blood of domestic cats. As a skin contaminant, it is possible that the blood samples were exposed to *Rhodococcus* spp. during the blood draw.

It is also not unusual to find *Rhodococcus* spp. DNA contaminating laboratories and reagents, especially DNA extraction kits (Salter et al., 2014). Although this can occur, the DNA of many other organisms has been extracted in this laboratory using the same reagents, then amplified, cloned, and sequenced. To date no sequenced clones from other samples have been found to contain *Rhodococcus* DNA.

There was no association found with the species of *Mycoplasma* and outcome of *C. felis* infection. Using molecular techniques, Maia et al. (2013) described the first detection of a natural *C. felis* infection in a Brazilian cat that was also coinfected with *Candidatus* M. haemominutum in South America. It was suggested that the cat may have succumbed due to the association with these two vector-borne pathogens. More extensive studies employing molecular detection of pathogens in coinfections should be conducted because the presence of both pathogens may cause changes in clinical manifestations, response to treatment, or pathogenicity (Shaw et al., 2001). An

undetected coinfection could ultimately affect the results of studies focusing on a sole pathogen.

CHAPTER V

MOLECULAR SURVEY FOR *CYTAUXZOON FELIS* AND *BABESIA* SPP. IN TICKS INFESTING WHITE-TAILED DEER IN TEXAS

INTRODUCTION

Due to their ability to harbor and transmit pathogens, ticks are the most important vectors of disease in wild mammals (Forrester, 1992)., including wild-tailed deer (WTD; *Odocoileus virginianus*) Ticks that commonly parasitize WTD are hard ticks of the genera *Ixodes*, *Amblyomma, Dermacentor,* and *Rhipicephalus (Boophilus)* (Davidson, 2006). *Boophilus* ticks (now renamed *Rhipicephalus* subgenus *Boophilus*) vector *Babesia bovis* and *Babesia bigemina,* the causative agents of bovine babesiosis. After its eradication from the U.S., this tick can occasionally be seen in California and the buffer quarantine zone along the Mexican border in southern Texas (Allan, 2001).

Data from molecular and serological studies have indicated the presence of *Babesia* spp. in WTD in counties of south Texas (LaSalle and Webb) (Ramos et al., 2010), central Texas (Tom Green) (Holman et al., 2011) and northern states of Mexico that border Texas (Cantu et al., 2007; Cantu et al., 2009) suggesting a role for WTD as reservoirs of bovine babesiosis agents. With WTD potentially serving as additional reservoir hosts, this could pose a challenge in maintaining the Cattle Tick Fever-free status for the U.S. (Holman et al., 2011).

White-tailed deer also have a role in maintaining infected ticks and serving as reservoirs for other protozoal and bacterial pathogens. The Lyme disease bacterium,

Borrelia burgdorferi, is transmitted by *Ixodes scapularis* ticks, for which WTD are the primary reproductive hosts for the adult ticks (Tillett, 2010). The WTD have a greater role in maintaining and transporting the infected ticks than harboring the pathogen (Lane et al., 1991).

Amblyomma americanum adults feed on WTD, and these WTD serve as reservoirs of pathogens carried by this tick (Kollars et al., 2000; Childs and Paddock, 2003; Allan et al., 2010). White-tailed deer naturally infected with *Ehrlichia chaffeensis* (Allan et al., 2010), *Ehrlichia ewingii* (Yabsley et al., 2002), and *Borrelia lonestari* (Moore et al., 2003) have been reported, and all of these bacteria are transmitted by *A. americanum. Amblyomma americanum* is the primary vector of *Cytauxzoon felis*, and wild felids serve as the reservoirs for this pathogen. With *A. americanum* being a 3-host this could possibly lead to WTD as additional hosts in the sylvatic cycle of *C. felis.*

In the current study, actively feeding ticks on WTD were collected in Gus Engeling Wildlife Management Area (Anderson County, East Texas), Aggieland Humane Society (formerly Brazos Animal Shelter) (Brazos County, central Texas), Uvalde and Maverick Counties (south Texas), and Las Palomas Wildlife Management Area (Cameron County, south Texas). Polymerase chain reaction (PCR) targeting the hemoparasite 18S ribosomal RNA gene was utilized to survey ticks for the presence of *B. bovis, B. bigemina, C. felis,* and other closely related piroplasm DNA.

MATERIALS AND METHODS

Extracted DNA from 161 Ixodid ticks were provided for this study by Texas A&M University in College Station, TX (Table 26). To target the full-length 18S rRNA gene of *C. felis* or *Babesia* spp., a primary PCR assay utilized primers A and B (Sogin, 1990) (Table 27). The reactions contained 50-100 ng DNA and primers A and B according to manufacturer's instructions (Accustart™ PCR SuperMix, Quanta BioSciences, Inc., Gaithersburg, MD). Molecular biology grade water served as a negative control and *B. bovis* genomic DNA served as the positive control. The primary reactions underwent a 2 min denaturation at 94°C followed by 45 cycles of 94°C for 30s, 60°C for 30s, 72°C for 2 min, with a final extension at 72°C for 5 min, and then were held at 4°C (Eppendorf® Thermal Cycler, Hauppauge, NY). The PCR products were resolved by electrophoresis through TBE buffered 1% agarose gel alongside a 1-kb DNA marker (New England Biolabs, Ipswich, Massachusetts), stained with ethidium bromide, and visualized by ultraviolet light transillumination (FluorChem 8000, Alpha Innotech Corporation, San Leandro, CA).

Table 26. Species identification and collection locales of ticks (N=161) from infested deer molecularly tested for piroplasm DNA

Tick species	Location	County	Samples
Ixodes scapularis	Aggieland Humane Society	Brazos	BAS-11
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-1 – GEWMA-3
Ixodes scapularis	Gus Engeling Wildlife Management Area	Anderson	GEWMA-4 – GEWMA-9
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-10 – GEWMA-11
Ixodes scapularis	Gus Engeling Wildlife Management Area	Anderson	GEWMA-12 – GEWMA-26
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-27 – GEWMA-30
Amblyomma inornatum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-31
Ixodes scapularis	Gus Engeling Wildlife Management Area	Anderson	GEWMA-32 – GEWMA-33
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-34 – GEWMA-36
Ixodes scapularis	Gus Engeling Wildlife Management Area	Anderson	GEWMA-37
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-38 – GEWMA-50
Amblyomma inornatum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-51
Amblyomma americanum	Gus Engeling Wildlife Management Area	Anderson	GEWMA-52 – GEWMA-64
Amblyomma cajennense	Las Palomas Wildlife Management Area	Cameron	LPWMA-10 – LPWMA-13
Ixodes scapularis	Las Palomas Wildlife Management Area	Cameron	LPWMA-14 – LPWMA-16
Dermacentor albipictus	Uvalde	Uvalde	UMTX-1
Dermacentor albipictus	Maverick	Maverick	UMTX-2 – UMTX-7
Dermacentor albipictus	Uvalde	Uvalde	UMTX-8
Dermacentor albipictus	Maverick	Maverick	UMTX-9 - UMTX-90

Table 27. Oligonucleotide primers used in amplifying the 18S rRNA gene region of *Cytauxzoon felis* and *Babesia* spp.

Primer name, sense	PCR	Sequence 5'→3'	Annealing temperature
A, forward B, reverse	Primary 18S	AACCTGGTTGATCCTGCCAG GATCCTTCTGCAGGTTCACCTAC	60°C
Bbov680F, forward Bbov1270R, reverse	Nested 18S	ACCTTGTATGACCCTGTC TGCCTTAAACCTCACCG	51.5°C
Bbov660F, forward Bbov1330R, reverse	Nested 18S	GCCTGTATAATTGAGCATGG CAAGCATCAGTGTAGCG	50°C
srBoFN, forward srBoRN, reverse	Nested 18S	CACCGTATTTTGACTTTTGTCGACTGTCGG CCCGTAACGGACGAACCTTCTCACGGG	55°C
Piro1F, forward Piro5.5R, reverse	Nested 18S	CCATGCATGTCTWAGTAYAARCTTTT CCTYTAAGTGATAAGGTTCACAAAAC	52°C
Cfelis170F, forward Cfelis1330R, reverse	Nested 18S	GCTAATACATGTTGGAGACC AGAAGCGATGCTGAGAC	50°C
989A, forward 990A, reverse	Nested 18S	GGTAGGGTATTGGCCTACCGT CSCAAAGTCCCTCTAAGAAGC	55°C
m13F, forward m13R, reverse	Colony 18S plasmid (pDNA)	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	50°C

The primary products underwent a 1:5 dilution and were used in nested reactions with primers Bbovis 660F/Bbovis1330R, Bbovis 680F/Bbovis1270R, Piro 1F/Piro 5.5R, srBoFN/srBoRN (Ramos, et al., 2010), 989A/990A (Aktas et al., 2007), or Cfelisspec170F/Cfelisspec1300R (Table 27). The cycling parameters were as above, except a 30s initial denaturation, 30 cycles, and 1-1.5 min extension step were used. In order to improve specificity, higher annealing temperatures (55°C and 56°C) were utilized with the Bbov660F/Bbov1330R primers as well. The PCR products were resolved by agarose gel electrophoresis as above.

Appropriately-sized 18S rDNA amplicons were directly ligated into pPrime (5 Prime, Inc., Gaithersburg, MD) or pCR®2.1-TOPO® (Invitrogen, Life Technologies, Grand Island, NY) cloning vector. TOP10 *E. coli* were transformed according to manufacturer's instructions. Colonies were verified to contain the cloned insert by colony PCR using primers appropriate for the plasmid vector (Table 27). Selected colonies containing the correct size insert were expanded overnight in broth cultures and plasmid DNA was prepared (EZ-10 Spin Column Plasmid DNA Kit, Bio Basic, Inc., Amherst, NY). The plasmid DNA concentration was determined using spectrophotometry (NanoDrop ND-1000 Spectrophotometer, NanoDrop Technologies, Wilmington, DE). Bidirectional DNA sequencing of the inserts was conducted by Davis Sequencing (Davis, CA). Sequence and chromatogram data were analyzed and forward and reverse sequence data were trimmed to exclude primer sequences and unreadable data, aligned, and analyzed for contiguous sequences using Sequencher 4.2 software (Gene Codes Corporation, Inc., Ann Arbor, MI). The sequences were compared to

sequences in GenBank using the NCBI Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). Using obtained tick rDNA sequences, two restriction enzymes (SacI and EcoRI) were selected for their capability to cleave tick rDNA. According to protocol (New England BioLabs, Inc. Ipswich, MA), a dual digestion with these enzymes was used with PCR products that underwent a repeated PCR with an increased annealing temperature in order to discriminate tick from putative parasite rDNA.

RESULTS

Amplification for the 18S rRNA gene showed 105/161 (65%) positive by PCR. Eighty-four PCR-positive samples underwent nested PCR with Bbov660F/Bbov1330R primers, and 62 of these 84 samples (74%) were PCR-positive for *B. bovis*, producing the expected size band of 688 bp. Ten *B. bovis* PCR-positive amplicons were cloned, producing 19 clones. Fourteen clones were sequenced, and results were that 18S rDNA sequences of four clones matched *Ixodes scapularis* GenBank (GU318898) with 99% identity. Four other clones matched *Xiphocephalus ellisi* GenBank (FJ459762) with 97-98% identity, and the six remaining clones matched *Dermacentor nitens* GenBank (KC76962) with 99-100%.

All samples underwent nested PCR using Bbov680F/Bbov 1270R primers. All samples were negative with none producing the expected 600bp *B. bovis* product.

The diluted primary PCR products of the 84 samples underwent nested PCR using Piro1F/Piro5.5R primers. This PCR resulted in 43 positive samples (51%). Three positive amplicons were cloned, and two clones were sequenced. The sequencing revealed that one clone matched the 18S rRNA gene of *Dermacentor andersoni*

GenBank (L76340) with 99% identity, and the other clone matched *D. nitens* GenBank (KC76962) with 98% identity.

Using srBoFN/srBoRN primers (Ramos et al., 2010), all 161 tick samples underwent a nested PCR in order to amplify the 18S rRNA gene of *Babesia odocoilei*. This amplification should result in 688bp of this organism. No samples were positive for *B. odocoilei* by PCR. An additional nested PCR using these same primers was conducted on the 28 *I. scapularis* ticks, in which all failed to yield a positive amplification for *B. odocoilei.*

The 161 tick samples underwent a nested PCR using 989A/990A primers (Aktas et al., 2007). These primers should amplify 1,000 to 1,050bp of *Theileria* spp. as well as closely related hemoparasites. This PCR resulted in 18 (11%) PCR-positive samples. Four positives were cloned with two being sequenced. The results of the sequencing matched the amplification of 18S rRNA gene of an uncultured eukaryote GenBank (GQ462637) with 98% identity and of an uncultured stramenopile (GU823482) with 97% identity.

All 161 tick samples underwent a nested PCR with Cfelisspec170F/Cfelisspec1300R primers. The amplification should result in 1,200bp of the 18S rRNA gene of *C. felis.* Once the PCR assay was complete, no samples were positive.

DISCUSSION

The role of WTD in the expansion of certain hemoparasites, especially bovine *Babesia* spp., raises concern in the southern U.S. Due to the increased rate of vector tick outbreaks in south Texas, especially in areas not in the quarantine buffer zone, WTD are seemingly a crucial factor in the epizootiology of bovine babesiosis (Perez de Leon et al., 2010). Although outbreaks in the Cattle Fever Ticks, *Rhipicephalus (Boophilus)* spp. have not been linked to disease, the resurgence of babesiosis is still a risk to the southern U.S.

It has been shown molecularly and serologically that WTD of central and south Texas and north Mexico may host bovine *Babesia* spp. (Cantu et al., 2007; Cantu et al., 2009; Ramos et al., 2010; Holman et al., 2011). Cantu et al., 2007 was able to detect *B. bovis* in 11 WTD samples and *B. bigemina* in four WTD from the states of Nuevo Leon and Tamaulipas, Mexico by nested PCR assay. In addition, indirect fluorescence antibody test (IFAT) revealed antibodies against both hemoparasites in serum samples (Cantu et al., 2007).

Another study used nested PCR to detect 19 WTD positive for *B. bigemina* and six WTD positive for *B. bovis* out of a total of 457 WTD. Results of IFAT showed 274 samples showed antibodies to *B. bovis* and 25 for *B. bigemina* (Cantu et al., 2009). Ramos et al., 2010 found eight of 49 WTD samples from LaSalle County, Texas and two of 47 WTD samples from Webb County, Texas as *B. bovis*-PCR positive. Sequence analysis of subsequent cloning revealed 99% identity with *B. bovis* 18S rRNA gene, and phylogenetic analysis showed a well-supported group with the bovine *B. bovis* and

B. bovis-like 18S rDNA sequences. There was poor seroreactivity to *B. bovis* with the IFAT (Ramos et al., 2010). In another study, out of 25 WTD blood samples from Tom Green county three were positive for *B. bovis* by amplification and analysis of the internal transcribed spacer (ITS) region. Three samples were PCR-positive for *B. bigemina,* but sequencing revealed only one as positive for *B. bigemina.* There was no difference between the cervine *B. bigemina* or *B. bovis* 18S rDNA or cervine *B. bovis* ITS regions sequences from bovine sequences as depicted in a neighbor-joining phylogenetic tree (Holman et al., 2011).

Despite the numerous accounts of nested PCR detection of hemoparasites such as bovine *Babesia* spp. in WTD, this was not as successful in this study. Unlike the previous studies, this study focused more on the actively feeding ticks rather than the whole blood of the WTD. Despite the source, detection of parasite DNA should have still been feasible.

Increased annealing temperatures and different primer sets were utilized in order to improve specificity. After the initial PCR with the Bbov660F/Bbov1330R primers and subsequent sequencing, it was shown that only tick rDNA was being amplified. To alleviate amplification of tick rDNA, a gradient PCR was employed in order to find the most optimal annealing temperature. The original annealing temperature of 50°C was increased to 55°C and then 56°C to improve specificity. Unfortunately, the increased annealing temperatures still resulted in the amplification of tick rDNA.

Different primer sets were utilized for better specificity. Since the Bbov660F/Bbov1330R primers were able to amplify tick DNA in addition to targeting

B. bovis, another primer set (Bbov680F/Bbov1270R) was used. These primers were more specific for *B. bovis*, and once they were included in the PCR reaction, tick rDNA was no longer amplified. Also, the generic primer sets of Piro1F/Piro5.5R and 989A/990A (Aktas et al., 2007) were used to amplify the DNA of similar hemoparasites. The Piro1F/Piro5.5R primers can be used to amplify part of the 18S rRNA gene of *Babesia microti, B. bigemina, Theileria cervi, C. felis,* and *Theileria equi*. The 989A/990A primers can target part of the 18S rRNA gene of different species of *Theileria* as well as *C. felis, B. microti, and Babesia divergens.*

Even though *Babesia* spp. and other closely related protozoa were not detected in this study, WTD have previously shown to be exposed to *B. bovis* and *B. bigemina.* The exact position of WTD in bovine babesiosis still warrants further clarification. It has been demonstrated that a virulent strain of *B. bovis* failed to establish infection in WTD via larval extract supernatant containing sporozoites from infected *Rhipicephalus microplus* or a *B. bovis* infected blood stabilate (Ueti et al., 2015). More research is still warranted in order to confirm if WTD are affected or not by bovine babesiosis and whether WTD are competent hosts for completion of the transmission cycle of *Babesia* spp. and other similar hemoparasites.

CHAPTER VI

CONCLUSION

The research for this dissertation focused on *Cytauxzoon felis,* feline hemotrophic mycoplasmas, and *Babesia* spp. in the U.S. Historically, it has been believed that bobcats serve as reservoirs of *C. felis* and domestic cats as aberrant hosts, in which the infection was ultimately fatal. However, as years passed, it was observed that cats were surviving infections with and without treatment. It was speculated that survival was due to development of different strains of *C. felis* that possibly varied geographically.

One study used the genotypes of *C. felis* internal transcribed spacer (ITS) regions in order to determine if there was an association with the varying clinical outcomes (fatal, recovery, and chronic) and geographical regions (Arkansas, Missouri, Texas, Oklahoma, and Kansas) of *C. felis*-infected cats. Amplification of the *C. felis* 18S ribosomal RNA (rRNA) gene was used to positively identify cats as infected. The *C. felis* ITS regions showed genetic diversity within the domestic cats, but there was no association with the ITS genotypes and clinical outcome or geography. These ITS genotypes in conjunction with other *C. felis* genes or other associations may provide more insight to them.

These same *C. felis*-infected cats were molecularly surveyed for feline *Mycoplasma* spp. to see if the presence of a mycoplasma might have an effect on the clinical outcome of *C. felis* infection. After amplification of the 16S rRNA gene of *Mycoplasma* spp., it was revealed that nearly a quarter of the cats had a coinfection with

C. felis and *Candidatus* Mycoplasma haemominutum. All of the cats were from different clinical outcomes. Thus it appears the outcome of disease is independent of the presence of this *Mycoplasma* sp. More studies should be conducted in order to confirm if an association of a *C. felis* coinfection with *Candidatus* M. haemominutum and other feline hemotrophic mycoplasmas does exist. Also, *Rhodococcus* spp. were detected in this study. In addition, *Candidatus* Mycoplasma kahanei was identified in one cat that ~~did~~ died. This identification has not previously been reported in domestic cats, and more studies would need to be done in order to confirm this result and understand its implication.

Using a microaerophilous stationary phase system (Levy and Ristic, 1980) the continuous *in vitro* cultivation of *C. felis* was attempted. This would lead to an abundant source of this parasite, which could be used to recognize antigenic proteins for vaccine development and diagnostic improvement. Although the indefinite propagation of *C. felis* was not achieved, the cultures did provide insight. It was demonstrated that HL-1 medium, cat serum or fetal bovine serum, hypoxanthine, and the source of *C. felis*-infected blood had involvement in some of the best culture conditions. These conditions, along with the supplementation of antibiotics-antimycotic should be investigated more and further modified for optimization in order to establish a continuous culture.

In studying transmission of *C. felis* as well as similar hemoparasites, 161 ticks actively feeding on white-tailed deer (WTD) were molecularly surveyed for the 18S rRNA gene of these protozoans. Although there were PCR positive samples, sequencing revealed that the positive amplicons were tick rDNA sequences. Modifications such as

raising the annealing temperature and utilization of more specific primers for hemoparasites also resulted in the amplification of tick rDNA. Despite not detecting hemoparasites in these WTD ticks, there have been other molecular and serological evidence of bovine *Babesia* spp. in WTD. Further studies should be conducted in order detect protozoal parasites and determine the role of these WTD in their transmission cycle.

Since its initial report in 1976 (Wagner, 1976), our knowledge of *C. felis* has progressed with microscopy and molecular research. Without the establishment of a culture system, *C. felis* research relies on either naturally infected cats or infecting healthy cats with a fatal disease. Until a continuous *C. felis* culture is achieved, comparative genomics (Tarigo et al., 2013) and molecular technologies (*i.e*., high resolution melt analysis) (Schreeg et al., 2015) have been able to add to the vast growing knowledge of *C. felis*, especially with finding means of control and aiding in faster diagnoses.

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