SEROLOGIC SURVEY AND GENETIC CHARACTERIZATION OF A "LESS-VIRULENT" ISOLATE OF *CYTAUXZOON FELIS* IN DOMESTIC CATS

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CHAPTER I.

INTRODUCTION

THE LIFE CYCLE OF *CYTAUXZOON FELIS*

Since first discovered in Missouri in 1973 (Wagner, 1976) cytauxzoonosis, caused by *Cytauxzoon felis* has been reported to be a rapidly fatal disease in domestic cats, with few known survivors (Hoover et al. 1994, Walker and Cowell 1995). The causative agent is a protozoan blood parasite classified under the order Piroplasmida, family Theileriidae. The life cycle of *Cytauxzoon felis* is initiated when an infected tick vector introduces sporozoites into a susceptible host during blood feeding (Fig. 1). Sporozoites enter endothelial macrophages of blood vessels (Fig. 2), where they multiply by schizogony, producing merozoites. The infected macrophages line the lumen of veins of various organs, including lungs, liver, spleen, and lymph nodes (Wagner, 1976). Schizogony occurs within infected macrophages, which become greatly enlarged, restricting blood supply to the organs. The schizont continues to grow and multiply, filling the entire macrophage until it ruptures releasing numerous merozoites (Fig. 3) into the bloodstream, which subsequently infect red blood cells. While taking a blood meal from an infected host, the tick, a biological vector, picks up the stages in the red blood cells (Fig. 4) where they undergo further development, eventually reaching the tick salivary glands (Kocan et al., 1992). When the infected tick feeds on a domestic cat, the sporozoites are inoculated into the cat. Cytauxzoonosis occurs most commonly in domestic cats during the spring and summer months when tick vectors are active. Cases begin to appear in March, reach

a peak in May and June, and decline towards the end of September (Hoover et al. 1994). Risk factors for cats acquiring *C. felis* includes living in or roaming through rural, wooded environments where reservoir hosts are present during the warm months of the year when infected ticks are active.

THE VERTEBRATE HOST AND BIOLOGIC VECTOR

Numerous studies have shown bobcats (*Lynx rufus*) to be the normal vertebrate host for *C. felis*. In 1980, endemic infections with this parasite were discovered in wild-trapped bobcats in Oklahoma; 50% of the captured animals had detectable infections (Glenn et al. 1982). It was recognized in this study that bobcats are able to survive infections with *C. felis* leaving them with piroplasms in their erythrocytes and making them carriers of the disease.

Transmission studies have demonstrated that inoculations into domestic cats with piroplasms in infected erythrocytes from bobcats does not produce fatal infections, but inoculation of the schizont form in tissues causes rapid onset of clinical signs and death (Kier et al. 1982b, Glenn et al.1983, Blouin et al.1984). Glenn et al. (1983) demonstrated this by parenteral inoculations of peripheral blood from naturally infected bobcats into domestic cats. One cat died and three cats developed parasitemias but suffered no apparent ill effects. One of the parasitemic cats was later challenged with schizont stages from a cat that died of cytauxzoonosis, resulting in its death. The one cat that died was presumably inoculated with blood containing schizonts as well as piroplasms, causing its death. This indicates that blood infection of erythrocytic forms alone did not confer protective immunity. The remaining cats with persistent parasitemias were sacrificed

after two months, necropsied, and no gross lesions were found (Glenn et al. 1983). This study also suggested that bobcats are a natural vertebrate host for *C. felis*. Because *C. felis* produces sporadic fatal infections in domestic cats, they are dead end hosts for parasite. Several other investigations have verified these findings (Blouin et al. 1987, Cowell et al. 1988, Glenn et al. 1982, Glenn et al. 1983).

Kier et al. (1982a) inoculated 4 species of domestic farm animals, 9 species of lab animals, and 17 species of wildlife with tissue homogenates from fatally infected cats. Only bobcats and one sheep showed evidence of infection. Of two bobcats inoculated, one died, and upon necropsy, numerous schizonts were observed in the tissues (Kier et al. 1982b). The second bobcat did not die but developed a persistent parasitemia without clinical signs, and no schizonts were found in the tissues. When domestic cats were inoculated with infected blood from the bobcat that survived, they developed non-fatal parasitemias, whereas cats inoculated with infected blood from the bobcat that died developed clinical signs and died within 2 weeks PI. Cats that developed non-fatal parasitemias were later challenged with a known fatal inoculum of *C. felis* and all died of disease. Terminal animals have schizonts circulating in the blood, and it is assumed that blood inoculations from terminal animals can transfer some schizonts, which can cause death. This showed that only schizont stages cause symptoms of disease and death. It was further shown that blood infections with the erythrocytic stages did not confer protective immunity (Kier et al. 1982b).

In the latter study, a sheep inoculated with a fatal inoculum of *C. felis* developed a non-fatal parasitemia, but no clinical signs were observed. When blood from this sheep was

sub-inoculated a domestic cat, it did not develop either clinical signs or a non-fatal parasitemia suggesting that the sheep are not a reservoir host. No subsequent studies or reports have ever identified *C. felis* organisms from sheep. Evidence of infection was not found in any of the other inoculated animal species and their blood did not produce infections in domestic cats. Bobcats were the only animals in the study, which proved to be potential natural hosts and reservoirs of infection.

Cytauxzoonosis is known to occur in the southcentral and southeastern United States (Fig. 5a), which also would include the distribution area of the only known tick vector, *Dermacentor variabilis*, or the American Dog Tick (Fig. 5b). Studies have shown that *C. felis* infections can be transmitted from bobcats to domestic cats by *D. variabilis*. Blouin et al. (1984) fed laboratory-reared nymphal *D. variabilis* on parasitemic bobcats and allowed them to molt to the adult stage. The adult ticks were then placed on laboratory-reared domestic cats, which became infected and died within 13 to 17 days after attachment. Necropsies revealed numerous schizonts in a variety of tissues, including the liver, spleen, and lymph nodes. They also showed that ticks fed on cats with non-fatal parasitemias also transmitted disease, resulting in typical clinical signs and death (Blouin et al.1984). This suggests that the parasite must pass through a tick to produce fatal infections. It also proved that the parasite was transstadially transmitted by *D. variabilis,* and established the American dog tick as a potential biological vector of *C. felis*.

Clinical Signs Associated with Cytauxzoonosis Infections

Clinical signs of cytauxzoonosis in domestic cats correlate with schizogoneous development of organisms in the mononuclear cells located in the endothelium of veins in the lungs, liver, spleen, and lymph nodes. The clinical signs include anorexia, dehydration, lethargy, depression, icterus, pallor and fever (Cowell et al. 1988, Hauck et al. 1982, Hoover et al. 1994, Kier et al. 1987, Wightman et al. 1977). Anemia, hypothermia, recumbency, and coma are common clinical findings in terminally ill cats (Greene et al. 1999). Death usually occurs in 7 to 10 days following onset of clinical signs (Cowell et al.1988, Greene et al. 1999).

Kier et al. (1982a) characterized pathogenesis of this disease in experimentally induced *C. felis* infections in 55 domestic short hair (DSH) cats, inoculating them with tissue homogenates of a deep-frozen *Cytauxzoon* preparation. Clinical signs first appeared at about 16 days PI. Between days 16 and 19 post-infection (PI), the cats became anorexic, weak, depressed, dehydrated, and icteric. Body temperatures began to rise on day 14 and peaked at 40.6° C on day 19, dropping to a subnormal levels shortly thereafter (Kier et al. 1982a). Piroplasms began to appear in red blood cells approximately 10 days PI and parasitemia levels continued to increased until the cats died or were sacrificed. Necropsies of these animals showed progressive enlargement of spleen, liver, and lymph nodes occurred throughout the course of the disease (Fig. 6). Edema and congestion of the lungs was observed at 15 days PI, and numerous petechial hemorrhages were evident on serosal surfaces throughout the body. Veins, particularly of the mesenteric and renal veins, were noted to be up to 3 times larger in diameter than those of the control cats (Kier et al. 1982a). Clinical signs and necropsy findings were similar in both

experimentally infected and naturally infected cats. Other accounts of naturally and experimentally infected domestic cats have described similar clinical findings (Butt, et al. 1991, Ferris 1979, Franks et al. 1988, Glenn and Stair 1984, Hauck et al. 1982, Hoover et al. 1994, Kier et al. 1982b, Wagner et al. 1980, Wagner 1976, Walker and Cowell 1995, Wightman et al. 1977).

DIAGNOSIS OF *CYTAUXZOON FELIS* INFECTIONS

Antemortem diagnosis of *C. felis* infections is dependent upon microscopic detection of signet ring-shaped piroplasms (Fig. 7) in Wright-Giemsa stained blood films from infected cats (Ferris 1979, Wagner 1976, Wightman et al. 1977). Postmortem diagnosis usually depends on histiologic or cytologic detection of schizont-laden mononuclear cells in vessels of various types of tissues (Ferris 1979, Wagner et al. 1980). However, it can be difficult to find the organisms in erythrocytes, especially when parasitemia is less than 1%. Shindel et al. (1978) showed that only 9 of 14 *C. felis* positive cats had detectable piroplasms on blood smears after initial rise in body temperature. Antibody tests have been developed for diagnosis of cytauxzoonosis in domestic cats (Cowell et al. 1988, Shindel et al. 1978, Uilenberg et al. 1987). A quantitative immunofluorescent assay (IFA) was developed to study the development of IgG antibodies in cats infected with the erythrocytic form of *C. felis* (Cowell et al. 1988). Results showed an initial steady increase (2-4 weeks) in the parasite-specific IgG levels after which titers dropped slightly and remained steady at intermediate levels over a nine-month observation period. It was also found that parasitemia levels in both intact and splenectomized cats fluctuated inversely with the packed cell volume (PCV). Splenectomized cats also exhibited higher antibody levels, higher parasitemias, and lower PCV's than the non-splenectomized cats

(Cowell et al.1988). Although antibody levels were detectable in experimentally infected parasitemic cats for approximately nine months post-infection, IgG antibody levels in clinically ill cats are undetectable in naturally infected cats due to the rapidity of the course of disease (Cowell et al.1988, Shindel et al.1978). Death usually occurs 7-10 days after the onset of clinical signs. Clinically ill cats do not have time to develop measurable antibodies before death occurs, making ante-mortem diagnosis difficult. Recent advancements in molecular biology have seen development of a polymerase chain reaction (PCR) assay, which could be used to detect early *C. felis* infections (Meinkoth et al. 2000). This PCR assay detected 9/9 cats that survived infection and could be a better diagnostic test than blood smears alone for diagnosis of cytauxzoonosis.

RECENT FINDINGS

In 1998, veterinarians in northwestern Arkansas and northeastern Oklahoma, where fatal cases of cytauxzoonosis occurs, reported 18 cats that had survived infections with what appeared to be *Cytauxzoon felis* without any extraordinary treatment or special care provided (Meinkoth et al. 2000). Unlike the usual clinical complications commonly observed in fatal cases, these infected cats did not develop the typical high fevers (<106° F) and their body temperatures never dropped below normal as occurs just prior to death. However, most of these cats had pale mucous membranes, some were icteric, and all were dehydrated. These cats were diagnosed with piroplasm-like organisms in their erythrocytes that were microscopically indistinguishable from the *C. felis* organisms seen in fatal cases (Meinkoth et al. 2000).

Several theories have been postulated to account for the presence of cats with less-virulent infections within the limited area in Arkansas and Oklahoma. Natural immunity, infective dose, treatments, heightened awareness, novel organism, and evolution of a less-virulent strain of *C. felis* are some possible explanations.

Natural Immunity

Natural immunity of cats within the endemic area may alter the severity of disease caused by *C. felis*. However, cats are rarely observed to survive this disease in any of the areas where it is known to occur; only four cases had been documented prior to the discovery of the 18 cats previously mentioned (Meinkoth et al. 2000). Thus, it is unlikely that such a large number of unrelated cats with natural immunity to this parasite would occur within a limited geographic area and within such a small period of time.

Size of the Infective Dose

It is possible that some cats receive a smaller dose of organisms during the transmission process or that the mode of entry limited the infectivity of the organism. Both the number of organisms in the inoculum and/or route of administration has been shown to alter the outcome and severity of other infectious diseases. When cats were given varied doses of inoculum of fatal *C. felis* was given intraperitoneally, parenterally, or intravenously the animals invariably died (Kier et al. 1982a). Thus, the route of infection or size of the infective dose did not alter the course of the disease.

General Use of Therapeutic Agents

Drugs used to treat cytauxzoonosis have not been shown to be effective. Despite aggressive attempts by veterinarians, conventional medications rarely, if at all, alter the course of disease (Hauck et al. 1982, Hoover et al. 1994, Walker and Cowell 1995). It is unlikely 18 survivor cats survived due to effective drug treatments. Most treated cats succumbed to infection regardless of a treatment regimen because the disease progresses so rapidly there is little time for drugs to reverse the pathogenic effects of the parasites. Uilenberg et al. (1987) inoculated two cats with virulent *C. felis* (designated stabilate 153), and treated them with the antimalarial drug parvaquone on days10 and 13 PI when their body temperatures began to rise. One cat became extremely ill showing typical clinical signs, but began to recover 6 days after treatment. Even though the second cat received like treatment, it died 22 days into infection. Because the second cat died, it was not known if the parvaquone was responsible for the successful treatment of the first cat (Uilenberg et al. 1987). In a separate study of 15 experimentally infected cats, neither parvaquone nor buparvaquone were effective treatments, although one animal did survive (Motzel and Wagner 1990).

In cases where cats showing clinical signs are brought to a veterinary clinic before they became critically ill, initial improvement has been observed with supportive therapy (lactated Ringer's solution) but most succumb to the disease (Hoover et al. 1994). Imidocarb, an antiprotozoal compound that is efficacious against similar erythroparasites, has shown some efficacy but does not produce consistent results (Meinkoth et al. 2000).

Greene et al. (1999) successfully treated six of seven experimentally infected cats using imidocarb dipropionate (1 cat) or diminazene aceturate (not available in the United States) along with heparin, blood transfusions, and supportive care. Even though this experimental protocol appears to be effective, it is not routinely used in veterinary practices. In addition, these survivor cats were not treated with these drugs and could not explain the existence of 18 survivor cats found in the endemic area.

Unusual Routes of Infection

Varying the type and location of inoculation of the infectious agent can alter the course of many infectious diseases. Unusual routes of inoculation (i.e. fighting) or some other abnormal method of exposure might alter the course of *C. felis* infections. The life cycle of *C. felis* involves a progression of stages that occur in sequence involving various tissues within both the reservoir host and a biological vector. The infective stages of the parasite transmitted by ticks always produce fatal infections (Blouin et al.1987, Blouin et al.1984, Kier and Greene 1998). The merozoite stages in erythrocytes do not produce fatal infections in domestic cats and do not provide any degree of protective immunity. Infected erythrocytes from the peripheral blood of carrier bobcats most often produce non-pathogenic blood infections when injected intraperitoneally into healthy domestic cats. Conversely, inoculations containing schizonts stages from infected bobcat spleen or lungs consistently produced fatal infections (Blouin et al.1984, Glenn, et al.1983, Kier et al.1982b). Therefore, it seems unlikely that accidental exposures to *C. felis*-infected blood through some unusual route of entry could account for a significant number of survivor cats in a limited period of time (15 months) and confined geographic location (100 mile radius) reported by Meinkoth et al. (2000).

Heightened Awareness of Cytauxzoonosis Survivors

Another explanation for the unusually high number of survivor cats reported in the endemic area may be that local veterinarians are more cognizant of *C. felis* infections, and more apt to find the organisms in the blood of cats that present with usual clinical signs. As a result, they routinely look for these parasites in blood smears from cats with signs of fever, lethargy, and icterus. Thus, survivor cats might be identified more often. However, this is not likely as fatal *C. felis* infections are commonly diagnosed in cats in this area. Local veterinarians routinely examine blood smears from cats exhibiting typical clinical signs of cytauxzoonosis and have not reported any survivor cats until 1998. In addition, veterinarians practicing outside the survivor endemic area have not observed non-fatal infections in cats but where fatal cytauxzoonosis occurs.

Infection with a Novel Organism

It is possible that the survivor cats are infected with an unidentified organism similar to *C. felis*. This is an unlikely explanation as *Babesia* or *Theileria* species have not been reported in cats in the United States (Meinkoth et al. 2000). *Cytauxzoon felis* is the only piroplasm that has been reported in cats in the U. S. Three of the original survivor cats that were tested had detectable antibodies to *C. felis*. Previous studies have shown that there is no serologic cross-reaction between antigens of *C. felis* and those of *Babesia* or *Theileria* minimizing the possibility of another agent (Uilenburg et al. 1987). In addition, DNA analysis has been used to investigate the possible existence of a novel organism in this area. Meinkoth et al. (2000) used a PCR assay to sequence a small fragment of the 18s ribosomal RNA (rRNA) gene of the "less-virulent" isolate and found it to be virtually identical to that of the fatal organism reported by Allsopp et al. (1994). Sequences for

both forms of *C. felis* varied significantly from *Babesia* and *Theileria* species, which are taxonomically similar organisms suggesting that the less-virulent form of *C. felis* is likely to be the same as or a strain of virulent *C. felis*.

Existence of a "Less-Virulent" Form of Cytauxzoon felis

It is possible that a genetic mutation of the fatal *C. felis* organism has given rise to a "less-virulent" strain that does not produce fatal infections. Walker and Cowell (1995) postulated that mutations of such organisms could alter the pathogenicity, changing the severity of the disease. A less-virulent mutant strain could account the non-fatal infections observed in cats in the survivor endemic area. One hypothesis is that a "less-virulent" strain of *C. felis* has emerged and is being perpetuated in the survivor endemic population. If the recently identified cytauxzoonosis survivors represent the emergence of such a strain of *C. felis*, then there should be cats from that area that are seropositive for antibodies against *C. felis*, while such cats would not exist in other areas where fatal cytauxzoonosis occurs.

RESEARCH OBJECTIVES

The specific goal of this study was to look for evidence that cats are surviving infections with *Cytauxzoon felis*. To accomplish this, three objectives were employed (the experimental design in shown in Fig. 8):

I. To optimize a serologic test to determine statistical cutoff values for detecting anti-*C. felis* antibodies in domestic cats.

II. To determine if antibodies to *C. felis* can be detected in domestic cats from cytauxzoonosis endemic areas in the states of Arkansas and Oklahoma.

III. To sequence and compare the 18s rRNA gene of a "less-virulent" isolate of *C. felis* to that of the fatal form to assess their genotypic relationship.

CHAPTER II.
OPTIMIZATION OF AN IFA SEROLOGIC TEST FOR DETECTING *CYTAUXZOON FELIS* ANTIBODIES IN DOMESTIC CATS

INTRODUCTION

Objective one of this study was to optimize a useful Immunofluorescent Assay (IFA) test in which to determine statistical cutoff values, and use that test for detecting antibodies to *C. felis* in domestic cats. The IFA employed for this study is a microfluormetric assay that uses the FIAX® 400 Fluorometer to measure the amount of fluorescien isothiocyanate (FITC)-conjugated antibodies in test sera. The FIAX® IFA system has considerable flexibility for developing different types of diagnostic assays (Fox et al. 1986). Custom tests have been developed and used for detecting antibodies to organisms such as *Sarcocystis cruzi*, *Toxoplasma gondii*, *Dirofilaria immitis*, *Leishmania* spp., *Trypanosoma cruzi*, and *Cytauxzoon felis* among others (Fox et al. 1986). A FIAX® IFA serologic test was previously developed by Cowell et al. (1988) to detect antibodies in *C. felis* experimentally infected cats. The previously described *C. felis* IFA test was optimized and used in the current study to detect antibodies of *C. felis* in cytauxzoonosis endemic populations of domestic cats. Since 18 cats have been found to have survived *C. felis* infections, the optimized IFA test could be used to determine if these previously described 18 cats were unique or if there are other cats in that population carrying antibodies to *C. felis*. Finding other cats in the population with antibodies to *C. felis* would support the hypothesis that some cats in the survivor endemic area are surviving *C.*

felis infections and a possible "less-virulent" strain may have become established in that area.

MATERIALS AND METHODS

Antigen Preparation

Red blood cell lysate antigen was prepared from the blood of splenectomized, *C. felis*-infected cats as previously described by Cowell et al. (1988) (Fig. 9). Three cats were splenectomized and sub-inoculated with peripheral blood with piroplasms of *C. felis* from a parasitemic source cat. When parasitemia levels reached 35%, the cats were exsanguinated by cardiac puncture and blood was collected in EDTA tubes. The whole blood was centrifuged and the plasma removed. The RBC pellet was washed twice by suspending the pellet in PBS and centrifuged for 10 minutes. After the final supernatant was removed, an equal volume of sterile distilled water was added to the erythrocytic pellet and mixed to facilitate complete cell lysis. The resulting crude antigen was then titrated against positive control serum to determine the optimum working concentration for the FIAX IFA test.

IFA Test Components

The IFA test employed a FIAX® 400 fluorometer (BioWhittaker Inc., Walkersville, MD) to measure the amount of fluorescence emitted by bound FITC-conjugated anti-cat IgG (Fig. 10). Parasite specific IgG antibodies were bound to an antigen coated nitrocellulose disc and were quantified by comparing fluorescent signal (FS) values of test sera to those of positive and negative control sera. The StiQ™ samplers used in FIAX IFA tests were specially designed plastic double-sided sticks with a nitrocellulose membrane on each

side (Fig. 11). The topside was used to bind the antigen and the bottom disk served as a control disk to measure non-specific binding of the conjugate.

The following protocol (Fig.12) was used to run the IFA test: StiQ™ samplers were spotted with 25 µl of antigen applied to the topside nitrocellulose membrane and allowed to dry for two hours at 37°C. The antigen-coated StiQs™ were placed in glass 12 X 75 mm tubes containing 50 µl of serum samples diluted 1:100 with 0.1 M phosphate-buffered saline (PBS) containing 0.15% Tween 20. StiQs™ were incubated for 25 minutes on a shaker at room temperature. Next, the StiQs™ were transferred to 500 µl of PBS-Tween 20 buffer wash solution and shaken for 5 minutes. After the wash, StiQs™ were transferred to a third row of tubes containing 500 µl of 1:100 FITC-conjugated rabbit anti-feline IgG (Sigma Chemical Company, Saint Louis, MO) diluted in PBS-Tween 20, and shaken for 15 minutes. Finally, the samplers were again washed and shaken in 500 µl of PBS-Tween 20 for 5 minutes. The samplers were then individually removed from the tubes and inserted into the FIAX® 400 Fluorometer and the fluorescent signal values (FS) were read and recorded. The FS values for the control sera were used to determine the FIAX values for the test sera. The mean of the duplicate StiQ™ samplers' FS values were used to compute the FIAX value of each sample as described below.

Optimization and Statistical Analysis of the IFA Test

Optimization of the serology test was accomplished by using positive control sera derived from archived experimentally infected cats (Cowell et. al 1988) and negative control sera

collected from those same cats prior to exposure to *C. felis*. The positive and negative control sera were processed as previously described and the StiQs™ were read on the FIAX® 400 Fluorometer. The StiQ™ with the highest mean FS values was then re-inserted into the FIAX® 400 Fluorometer and all other StiQs™ were re-read. Once read, the FS values were then converted to FIAX values. To establish FIAX values for the control sera, the duplicated StiQs™ with the highest FS value was arbitrarily given a number of 150. A negative control was chosen and given the value of 30. The mean of the duplicate FS values for the control sera and their arbitrarily-assigned FIAX values were then used to calculate a regression curve from which FIAX values for all other test sera were extrapolated (Logan et al. 1985).

Once FIAX values were established for known positive and negative controls, then 60 serum samples collected from domestic cats from the Pullman, Washington area, where cytauxzoonosis has never been reported and the tick vector does not occur. These samples served as a representative of a *C. felis* negative population. Statistics were run on the extrapolated FIAX values for these samples to establish the baseline response level for a normal range of negative sera and a cutoff point between negative and positive values.

A Kolmogrov-Smirnov (K-S) normality test was run on the results for the 60 representative negative samples. The mean and standard deviation was calculated along with the cutoff point for the negative FIAX values, which were based on a standard normal distribution. The normal range for non-infected cats was defined as adding 3

times the standard deviation of the mean that will include 99.73% of the population and will specify 0.27% of the non-infected population as 'abnormal' for these unusual responses.

To validate the accuracy of antibody detection by the FIAX IFA test, sera from cats with fatal and "less-virulent" *C. felis* infections were tested to determine if antibodies against both forms of *C. felis* would react to the RBC lysate antigen prepared from a fatal isolate. Serum along with normal and hemolyzed plasma collected the same day from two cats, experimentally inoculated with peripheral blood from one of the 18 original survivor cats, were tested by the FIAX IFA test to examine the effects of these parameters on the results. Lipemic plasma samples from one of the two cats at approximately 2-week intervals (5 samples) were also tested (Table 1).

RESULTS

Statistical Analysis of Data

Figure 13 shows the frequency distribution of FIAX values for 60 samples collected from domestic cats from the Pullman, Washington area where *C. felis* is not known to exist. Based on the FIAX values of the 60 negative samples, the range was 19-42 with a mean of 29.8 with a standard deviation of 4.54. Based on the K-S test ($p=.062$), the null hypothesis was not rejected with the conclusion that the negative samples from Washington fit a normal distribution. The cutoff between negative and positive values was set at 3 standard deviations above the mean as previously described. Using this estimate, the cutoff value for negative samples was determined to be 43. Thus, any

sample with a FIAX value of 43 and above was considered positive and samples with values less than 43 were considered negative.

Table 1 shows the FIAX values obtained by testing serum along with plasma that was normal, lysed, or lipemic from two infected cats designated as 3440 and 3467. The fatal form of the RBC lysate antigen was able to detect antibodies in these two cats experimentally infected with the "less-virulent" form of *C. felis* with relatively high FIAX values. The range for cat 3440 was 93-116. The range for cat 3467 was 126-133 with serum having a value of 166. There were slight inconsistencies when comparing normal serum to plasma samples. In cat 3440, the serum FIAX value (93) was lower than the plasma sample (116). In cat 3467, the normal serum sample was significantly higher (166) than the plasma sample (133), indicating that there was some effect on antibody detection when serum or plasma was compared. However, despite those inconsistencies, when normal plasma was compared to hemolytic plasma in either cat, there was little affect on antibody detection by the FIAX IFA test. Also, the degree of hemolysis between all plasma samples did not affect antibody detection. In addition, lipemic sera collected from 3467 between December 1999 and January 2000 ranged from 103-126 again indicating a minimal effect on antibody detection. Because inconsistent results were found between normal serum and plasma samples, and the fact that the degree of hemolysis or lipemia in plasma samples provided consistent FIAX values when compared to normal plasma samples, we requested that veterinarians submit samples in EDTA tubes and plasma samples were used for subsequent testing.

DISCUSSION

Immunofluorescent Assays (IFA) have been developed for detection of cytauxzoonosis in domestic cats (Shindel et al. 1979, Uilenberg et al. 1987). While these tests were effective at detecting *C. felis* organisms, they could only be used on tissues of cats collected post-mortem. They could not be used to detect antibodies in serum samples, or diagnose cats with *C. felis* infections antemortem. A FIAX IFA test was developed by Cowell et al. (1988a) to detect antibodies in experimentally infected cats antemortem. They compared rising antibody levels to PCV and parasitemia levels. The results of that portion of this study indicated that not only could antibodies be detected in the serum of *C. felis*-infected cats, but it also showed a relationship between increasing antibody levels in experimentally infected cats to the increase in parasitemia levels and decrease in PCV levels, making this a useful IFA test in which to detect *C. felis* antibodies (Cowell et al. 1988). The objective of this portion of the current study was to standardize the previously developed FIAX IFA test to screen general domestic cat populations for the presence of antibodies to *C. felis*.

In order to determine cutoff points for the standardized IFA test, 60 domestic cat samples were obtained from the state of Washington where *C. felis* infections have never been reported and the known tick vector, *Dermacentor variabilis*, does not occur. The distribution of the only known tick vector, *Dermacentor variabilis*, includes the areas where fatal cytauxzoonosis occurs but not in the Pullman, Washington area (Fig. 5). The absence of the tick vector in the Pullman, WA region was an important factor for choosing that area. These samples represent an unexposed cat population and were used to obtain a range of IFA FIAX values that would be indicative of negative samples. The

statistical analysis of these values provided the cutoff point (43) between positive and negative sera. Using this cutoff point, other parameters were evaluated and it was found that all sera from experimentally-infected animals had values >43 and that using plasma samples, whether normal, hemolysed, or lipemic did not seem to effect the quality of the results (Table 1). These latter findings provide some level of confidence that the quality of samples used in other future studies would not adversely affect the results.

One advantage of using the FIAX® IFA includes developing tests for detecting antibodies against various organisms. FIAX® IFA individual assays can be adapted to work for several animal species. For example, assays developed to detect *Dirofilaria immitis* infections to dogs were easily adapted to detect infections in cats. Other advantages include ease of conducting the test, elimination of subjective readings, ability to process large numbers of samples in one run. Since the fluorescence is read on a fluorometer, the need for microscopic readings of infected tissues are eliminated (Fox et al. 1986, Logan et al. 1985).

Another advantage of using the FIAX® method of detecting antibodies is that soluble antigens are not necessarily required for the test to work (Fox et al. 1986). Although soluble antigens are ideal for ELISA tests, crude antigens can be used to detect antibodies effectively in serum of infected animals by using FIAX because soluble and particulate material will bind to the cellulose-nitrate surface of the StiQ™ samplers. A particulate antigen derived from RBC of experimentally infected cats, as previously described, was used to detect antibodies in this study. Purified antigen of *C. felis* cannot be cultured in

vitro due to the inability to isolate sporozoites in cell culture. However, Shindel et al. (1979) isolated and maintained *C. felis* schizonts in vero cell lines and used them to develop an IFA test. The schizonts maintained in these cultured vero cells were used to produce antiserum by injecting them into a naïve domestic cat. The antiserum produced by the cat was subsequently used to detect organisms in infected spleens of experimentally infected cats by IFA. Since *C. felis* schizonts has only been maintained and not actually developed in cell culture, a purified antigen of *C. felis* has yet to be prepared and only a crude particulate antigen was available for this study.

Although the FIAX® test can be developed to detect antibodies to just about any organism using soluble or particulate material (Fox et al. 1986), it is not as sensitive as the ELISA assays. In addition, to its dependence on the use of soluble antigens, another disadvantage of ELISA is that it is laborious and time-consuming to run the dilutions associated with the test. FIAX, on the other hand, uses only a single dilution eliminating the serum titrations needed in ELISA to determine antibody levels, and cutting down the time in which it takes to run a test. FIAX can be run in 1 hour whereas it takes most of the day to run one ELISA. However, an advantage of ELISA is that it is a more sensitive assay than FIAX due to using purified antigens. In this and the previously mentioned study (Cowell et al. 1988), it was possible to measure an immune response in *C. felis* infected cats despite the antigen being so crude. The IFA test based on the FIAX® system proved to be a reliable test for detecting *C. felis* infections in domestic cats. Since there is no purified antigen in which to develop an ELISA for *C. felis*, we optimized the FIAX IFA test, and use it as the basis for the serologic survey conducted in this study.

CHAPTER III.

SEROLOGIC SURVEY FOR DETECTING *CYTAUXZOON FELIS* INFECTIONS IN CYTAUXZOONOSIS ENDEMIC POPULATIONS OF DOMESTIC CATS

INTRODUCTION

Objective two of this study was to conduct a serologic survey to determine if *Cytauxzoon felis* antibodies can be detected in cats in the general population where *C. felis* survivors have been reported. Prior to the Meinkoth et al. (2000) discovery of a group of domestic cats that were able to survive cytauxzoonosis, it was thought that all cats, with few exceptions, died of the disease. Therefore, serologic tests to diagnose the diseases did not seem useful since cats died before antibody titers could be developed. With the discovery of survivor cats, population studies became more relevant. Because an unusual number of cats had survived infections with *C. felis* in a limited geographic area over a short period of time, it was hypothesized that a "less-virulent" strain of *C. felis* may have developed. If the hypothesis were true, some seropositive cats would be present in the general population of the endemic area in question. The presence of antibody positive cats in the survivor endemic area would provide evidence that domestic cats do survive infections under natural circumstances. If seropositive animals are restricted only to the geographic area where survivor cats have been previously reported, and no cats outside this area are positive, this would provide further evidence that domestic cats can survive *C. felis* infections. That evidence would also indicate that a "less-virulent" form of *C. felis* exists and may be occurring in the survivor endemic area. However, if this survey

shows survivors in areas other than the survivor endemic area, then that would indicate that cats are surviving *C. felis* infections more often than previously thought and clinicians are simply overlooking the disease, and the geographic range of "survivors" would be extended.

MATERIALS AND METHODS

Collection of Serum Samples

Veterinarians from three geographic areas were asked to collect blood in EDTA tubes from 100 healthy domestic cats that came into the clinics for routine exams greater than six months of age with free access outdoors. Areas selected include: 1) a cytauxzoonosis endemic area where survivors have been reported, 2) a cytauxzoonosis endemic area where no survivors have been reported, and 3) a negative reference area where cytauxzoonosis has never been reported. The first geographic area was located in a 100-mile radius of Northwestern Arkansas and Northeastern Oklahoma (designated the survivor endemic area) where the original 18 survivor cats (Meinkoth et al. 2000) were found, fatal cytauxzoonois is commonly diagnosed in cats, and the tick vector occurs. The second geographic area included the vicinity around the Stillwater, Oklahoma area where fatal cytauxzoonosis is endemic but survivor cats have not been reported, but the tick vector occurs. The third geographic area was located in and around Pullman, Washington where cytauxzoonosis has never been reported, the tick vector does not occur, and served as a source for negative reference samples in the previous chapter. In addition to collecting blood, the participating veterinarians from the survivor endemic area were requested to submit blood smears with each sample. All specimens were forwarded to Oklahoma State University, College of Veterinary Medicine for subsequent

testing. All plasma samples were tested by the IFA protocol to detect antibodies to *C. felis* described in Chapter II.

Blood Smear Examinations

The Diff-Qwik® stained blood smears from seropositive samples from the survivor endemic area were examined microscopically for the presence of the characteristic signet ring-shaped piroplasms in the cytoplasm of erythrocytes (Fig. 7) to further confirm the FIAX IFA results.

Polymerase Chain Reaction of Samples

A Polymerase Chain Reaction (PCR) was performed on the packed blood cell portion of all samples that were positive by serology or blood smears. The PCR served as a "gold standard" in detecting the true infection status of tested cats. All samples from the endemic area were tested, whereas only a representative number of negative samples from the other two areas (14 Stillwater samples and 6 Washington samples) were tested by PCR.

Primer pairs designated 3383 and 3406 described by Meinkoth et al. (2000) were used to amplify genomic DNA extracted from the blood samples (Table 2). DNA was extracted from the samples using a QIAamp Blood Extraction Kit (QIAgen Inc., Santa Clarita, CA) according to the manufacturer's instructions. The PCR was carried out in 30 µL reactions containing 10 µL of sterile H_2O, 10 µL of template DNA, 10 mM 10x buffer A (50 mM KCL, Tris-HCL, 1.5 mM $MgCl_2$, and 0.1% Triton® X-100), 4 mM $MgCl_2$, 200 µM of dNTP, 25 pM each primer, 2.5 U of *Taq* DNA Polymerase in buffer A (Promega

Corp., Madison, WI). The PCR reaction consisted of 30 cylcles with denaturation at 96°C for 1 minute, annealing at 55°C for 1 minute, and extention at 72°C for 2 minutes with an addition extension for 7 minutes. To prevent contamination of samples, DNA purification, master mix assembly and thermal cycling took place in separate rooms to prevent contamination of samples, and aerosol-resistant pipette tips were used in pipetting. Amplified reactions were separated by gel electrophoresis in 1.5% agarose, stained with ethidium bromide, and viewed by ultraviolet light. The positive control DNA was isolated from a cat (3440) experimentally infected with the "less-virulent" isolate of *C. felis*, and was positive by blood smear for piroplasms. The negative controls consisted of DNA isolated from an uninfected cat and a sample with no DNA added to the mix.

RESULTS

Serology Survey

A total of 236 blood samples were collected from domestic cats by veterinarians within the three geographic regions, 75 from Northeastern Oklahoma and Northwestern Arkansas, 98 from the Stillwater, Oklahoma, and 60 from Pullman, Washington (Table 3). Figure 14 shows a frequency of distribution of FIAX values for all 236 samples combined. Eight samples were positive for anti-*C. felis* antibodies, an overall prevalence of 3.4%. Figure 15 shows the frequency distribution of FIAX values for the survivor endemic area. Six of 75 samples from this area were antibody positive, giving an 8% prevalence. However upon further investigation, it was found that two of these samples were from the same cat submitted 6 months apart, so the true prevalence of the survivor endemic area was calculated from 5 of 74 cats with a prevalence of 6.8%. Five

of the 6 positive samples came from the vicinity of Green Forest, Arkansas and one was from Tahlequah, Oklahoma. Two of 98 (2%) samples were positive from the Stillwater, OK region. Figure 16 shows the frequency distribution of FIAX values for samples from that area.

Table 4 contains a summary of FIAX, blood smear, and PCR results for the 8 seropositive cats. Blood smears were available for examination from only 4 of the 6 positive samples in the survivor endemic area. Three of the four blood smears revealed signet ring-shaped *C. felis* organisms in the erythrocytes. A blood smear was not available for the cat named Brown, and the one from Charlie was too poor in quality to be read.

Polymerase chain reaction (PCR) using primers that amplifies DNA from organisms that are members of the Order piroplasmida (Meinkoth et al. 2000) was used as the "gold standard" to determine the true infection status of tested cats. PCR was performed on the 8 seropositive samples, all remaining samples from the survivor endemic area, and 20 seronegative samples from the other two areas with FIAX values near the cutoff value of 43. Figure 17 shows the base pair products that were electrophoresed in ethidium bromide stained gels. The PCR results for the 6 seropositive samples from the survivor endemic area were positive, whereas the two from Stillwater, Oklahoma were negative (Table 4). All other samples from the survivor endemic area were both PCR and seronegative. All seronegative samples selected from Stillwater, OK (14) and Pullman, WA (6) areas were PCR negative.

The sensitivity and specificity of the FIAX-based IFA test as applied to this study was calculated using the FIAX values for the 97 samples for which the true infection status had been confirmed using the "gold standard" PCR test. The sensitivity of the FIAX IFA test was 100%, with a false negative rate of 0%; that is, no false negatives were detected. The specificity was 98%, indicating that 2% of negative samples tested were designated as false positives by the FIAX IFA test. The predictive values show the probability of a positive or negative result of a serologic test reflects the true disease status of the tested samples (Smith 1991). The positive predictive value for the FIAX IFA test was calculated at 75%, meaning that of eight seropositive samples as detected by the FIAX IFA test, 25% of those samples did not reflect their true disease status and were actually non-infected individuals. The negative predictive value was calculated at 100%, meaning that all samples designated as negative by the FIAX IFA test, all were truly non-infected individuals when tested by the "gold standard". The overall accuracy of the FIAX test was 98%, indicating that the test performance in detecting truly infected or non-infected individuals was high.

DISCUSSION

Serologic tests have been developed by Shindel et al. (1978) and Cowell et al. (1988) for detection of *C. felis* infections in domestic cats. The serologic test developed by Shindel et al. (1978) was used to detect *C. felis* organisms in infected tissues of experimentally infected cats but could never be applied to a population study. Although the test proved effective at detecting organisms to *C. felis* in experimentally infected cats, its use was limited to postmortem detection. The IFA test developed by Cowell et al. (1988) was used to compare antibody levels over time to PCV and parasitemias levels in

experimentally infected cats. In addition, 30 random feline samples from a serum bank in the College of Veterinary Medicine at Oklahoma State University were tested against the *C. felis* antigen but no cats were found to be positive (Cowell et al. 1988). The current study is the first application of an IFA test to screen various populations of domestic cats for antibodies to *C. felis*.

Results of the survey reveal that 6.8% of cats in the survivor endemic area (Arkansas-Oklahoma) were seropositive. To confirm these samples were truly positive, they were further tested by blood smear examination and PCR assays. Table 4 shows that of the six samples from the survivor endemic area, only four had blood smears to examine. Of the four blood smears, *C. felis* organisms were found in three of them

All seropositive samples found in the serologic survey were further subjected to PCR tests as a means of confirming the presence of *C. felis* organisms. The PCR primers used to confirm infections were those described by Meinkoth et al. (2000). These primers amplified a 1730 bp product of the 18s rRNA gene from any member of the order Piroplasmida. These investigators used these primers to successfully detect *C. felis* DNA in 9 of 9 domestic cats that had survived infections with *C. felis*. Because the PCR using these primers accurately detected infections (some with very low parasitemias) in the previous study, it was used as the "gold standard" to determine the true infection status of cats in the current study. All six samples from the survivor endemic area were positive, including the cat that was blood smear negative (Table 4). Since the sample negative by blood smear examination was positive by the other two tests, it was considered to be infected with *C. felis* even though it had an undetectable parasitemia. This is a common

occurrence in cats naturally infected with *C. felis* (Wagner 1976). The remainder of the IFA negative samples from the survivor endemic area that were tested by PCR assay to detect if any were carrying piroplasms. All of those samples were PCR negative confirming the effectiveness of the FIAX test.

Two seropositive samples were outside the survivor area, in the Stillwater, OK area. No blood smears were available for examination, but these samples were subjected to a PCR assay and both were negative. Even though these samples had low-positive FIAX values, both were negative by the "gold standard" and considered to be false positives. The Cowell et al. (1988) study also showed that a small population of domestic cats sampled from the Stillwater area was negative for *C. felis*. The current study showed that cats in a significantly larger population in the same area remained negative several years later, indicating there is no evidence of cats from this area surviving infections with *C. felis*. All samples from the Pullman, Washington area were negative by the IFA test. Six of these samples with high-negative FIAX values were subjected to a PCR test to verify the infection status and all were negative. These results substantiate that cytauxzoonosis does not occur in the Pullman, Washington area, which could be due to the lack of a tick vector for that area. *Dermacentor variabilis*, a known biological vector for *C. felis*, is not found in this area, however *Dermacentor andersoni* is present, but not known to be a vector for this parasite. If it is not, the absence of *D. variabilis* may explain why cytauxzoonosis has not been reported in this region.

The IFA test had 100% sensitivity but only 98% specificity. Although the IFA produced two seropositive results for samples from the survivor non-endemic area (Stillwater)

which were PCR negative, the test was 98% accurate making it suitable for a serologic survey. The combination of IFA, blood smears, and PCR tests detected *C. felis*-positive cats in the survivor endemic population (Arkansas), but not in the other two survivor non-endemic areas (Stillwater and Washington). This supports the theory that some cats in the survivor endemic area may be acquiring a "less-virulent" form of cytauxzoonosis and surviving the disease. Cowell et al. (1988) showed that experimentally infected cats developed antibodies approximately 4 weeks post infection. In most cases, cats die within 3 weeks of infection. This is not sufficient time for cats to develop a measurable antibody response prior to death. In such animals, IFA tests would not be useful. Therefore, finding the seropositive cats in the survivor endemic area provided substantial evidence that these animals survived infections with *C. felis* and is consistent with the hypothesis that a "less-virulent" isolate of *C. felis* is being perpetuated only in the area where the original 18 survivor cats were found.

Because survivor cats have persistent parasitemias, they could serve as long-term reservoirs of infection from which tick vectors could ingest the agent and transmit the "less-virulent" isolate from domestic cat to domestic cat instead of bobcat to domestic, thus perpetuating a "less-virulent" form of *C. felis*. One of the first cats (Walker et al.1995) in the survivor endemic area known to have survived an infection with *C. felis* was both parasitemic and PCR positive when examined six years after the initial diagnosis (Meinkoth et al. 2000). In one situation, four housemates of one of the original survivor cats were parasitemic but asymptomatic. All five of these cats were presumably exposed to the same source of infected ticks because prior studies have shown that cat-to-cat transmission does not occur without blood transfer or tick feeding in animals housed

together (Meinkoth et al. 2000). Further evidence to support that theory was shown in an unpublished study, in which a naïve cat was inoculated with peripheral blood from one of the cats that had survived a naturally acquired infection with *C. felis* from the Arkansas-Oklahoma endemic area (J. Meinkoth, personal communication). This cat developed a persistent parasitemia, but did not become clinically ill. Nymphal stages of *Dermacentor variabilis* were fed on this cat and then allowed to molt to adults. The adult ticks were transferred to a second naïve control cat to which *C. felis* was thereby transmitted. Although the blood smears and PCR for this animal were positive, it never became clinically ill. The second cat and a third naïve cat were then challenged with spleen homogenate from a cat with fatal cytauxzoonosis. The second cat developed clinical signs for a few days then recovered. The third (naïve) control cat died of clinical cytauxzoonosis, which was confirmed by histology. It appeared that pre-exposure with this presumed "less-virulent" strain of *C. felis* conferred protective immunity to the fatal form of the organism. That study showed at least in this one cat, survival with a *C. felis* infection can occur when ticks transmit this "less-virulent" form. This further substantiates the possibility of cat-to-cat transmission by ticks in the survivor endemic area. However, more transmission studies are needed to support these findings.

CHAPTER IV.

SEQUENCING AND COMPARISON OF A NON-FATAL FORM OF *CYTAUXZOON FELIS*

INTRODUCTION

The third objective of this study was to sequence and compare the 18s rRNA gene of a "less-virulent" isolate of *C. felis* and compare it to a previously reported sequence of the fatal form to determine their genetic relationship to one another. Highly conserved areas in the ribosomal repeat array of the 18s ribosomal RNA (rRNA) gene can be used to study relationships across phyla (Gerbi 1986, Hillis et al.1991). The 18s rRNA gene has been sequenced and compared in numerous studies, and have shown that the 18s region is nearly identical among different genus/species of organisms (Gerbi 1986). Because the 18s rRNA gene is highly conserved region, it can infer information on the phylogenetic relationship of these clinically distinct forms of *C. felis*. In the initial phylogenetic study, nucleotide sequence of a fatal *C. felis* isolate designated Stabilate 153 (Uilenberg et al. 1987) has previously been compared to the 18s RNA gene sequences of other genera of piroplasms (Allsopp et al.1994). The virulent or fatal isolate of *C. felis* was found to be genetically similar to species of *Theileria* and *Babesia*, with greatest similarity to an isolate of *Babesia equi*. The published genetic sequence of this virulent strain of *C. felis* (GenBank accession number 19080) was used as the basis of comparison for the "less-virulent" isolate under investigation in this study. In addition to a difference in virulence,

identification of variations in the 18s rRNA gene sequences could provide genotypic evidence for separation of the two *C. felis* isolates.

MATERIALS AND METHODS

A cat was experimentally inoculated with peripheral blood from one of the 18 original survivor cats and became parasitemic. When this cat became parasitemic, blood was drawn in EDTA tubes. Genomic DNA was extracted from 200 µL of whole blood using a QIAamp Blood Kit (QIAgen Inc., Santa Clarita, CA). PCR assays were carried out on the extracted DNA according to the protocol described in Chapter III. Primer pairs 3383 and 3409 were used to amplify the 18s rRNA gene of a "less-virulent" isolate of *C. felis* (Fig. 18). Following PCR amplification of DNA and electrophoresis, the resulting 1730 base pair band was excised from the Tris-Acetate-EDTA (TAE) agarose gel, and placed in a Geneclean® II Kit spin column (BIO 101, Carlsbad, CA). The spin column was centrifuged at 10,000 rpm for 30 seconds to extract DNA of the base pair of interest. The extracted DNA was then used as template DNA and subjected to an additional PCR amplification (to ensure that only the single band of interest was sequenced). The final DNA product was purified using the QIAquick Purification Kit (QIAgen, Inc., Santa Clarita, CA) according to the manufacturer's directions. That resultant product was sequenced at the Oklahoma State University Recombinant DNA Core Facility using a 3700 ABI automated sequencer (Applied Biosystems, Foster City, CA). Four additional primer pairs were designed and used to sequence the remaining 18s rRNA gene in both the 5' and 3' directions (Table 5). All primer pairs were synthesized according to specifications by the OSU Recombinant DNA Core Facility (Stillwater, OK). To construct a full sequence of this "less-virulent" isolate of *C. felis*, AssemblyLIGN™ in the

Mac-Vector™ (Oxford Molecular Group Inc., Campbell, CA) software was used to align the overlapping nucleotide fragments obtained from each of the primers (Fig. 19) and a consensus sequence was constructed. The resultant sequence of this "less-virulent" isolate of *C. felis* was deposited into GenBank with the accession number AF399930. The complete nucleotide sequence for the 18s RNA gene from both forms of *C. felis* were then aligned and compared. The DNA sequence of this "less-virulent" isolate of *C. felis* was also aligned with the 18s rRNA gene sequences of other small piroplasms to assess its relationship to that of other piroplasms (Table 6).

RESULTS

A total of 1736 base pairs of the 18s rRNA gene of this "less-virulent" isolate of *C. felis* were successfully sequenced. Attempts to sequence the remaining 38 base pairs were unsuccessful. Comparison of this sequence to 1774 base pair sequence from a *C. felis* fatal isolate (GenBank accession number 19080) revealed that they were 99.8% homologous. Differences were observed at base pairs 99 and 1628 where these bases were designated as "N" by the previous authors in the sequence of the fatal isolate. The sequence obtained in this study and the partial sequence obtained from the same isolate in a previous study by Meinkoth et al. (2000) were also aligned and shown to be 100% identical. Table 6 illustrates base pair and percentage similarities of this "less-virulent" isolate of *C. felis* and that of other piroplasms recovered from various animals in North America. When compared to sequences of members of the *Theileria* group, this "less-virulent" isolate of *C. felis* sequence had 96% identity to *Theileria youngi* with 68 base pair differences, and 95% identity to *Theileria* spp. Type B (*T. cervi*) and *Theileria equi*, differing by 71 and 82 base pairs respectively. When compared to genotypes of the

"true" *Babesia* spp. (*B. gibsoni* and *B. canis*), base pair differences showed a 93% identity with >100 base pairs difference. Comparison of the 18s rRNA gene sequences of our isolate and that of the "Western" isolates of piroplasms (Kjemtrup et al. 2000) showed that the WA1 human piroplasm isolate had 93% identity with 111 base pair differences, and a dog isolate from California (AF158702) had 94% identity with 106 base pair differences. Finally, comparison of two other *Babesia* spp. genotypes revealed that *Babesia felis* (a known piroplasm of felines, not known to occur in the United States) and *Babesia microti* (a small piroplasm found in rodents in the U. S.) both had 93% identity with 105 and 107 base pair differences, respectively, from the gene sequence of this "less-virulent" isolate of *C. felis*.

DISCUSSION

Phylogenetic relationships among species of piroplasms using the 18s rRNA gene have been described by numerous investigators. Allsopp et al. (1994) was first to describe a phylogenetic comparison indicating that members of this group of protozoa can be grouped into three distinct phylogenetic clades. The "true" *Babesia* spp. (those that multiply only in erythrocytes and in which transovarial transmission occurs in the tick vector) were grouped together in the first clade. *Cytauxzoon felis* (fatal isolate) was grouped in a second clade, a sister clade to the true *Babesia,* with the *Theileria* spp. (those that have both an erythrocytic and exo-erythrocytic multiplication in the vertebrate host, and in which only transstadial transmission occurs in the tick vector), and was determined to be most closely related to *Babesia equi*. *Babesia equi* and *B. rodhaini,* for which the phylogenetic placement is controversial, were also placed near *C. felis* in the *Theileria* clade due to *Theileria*-like phenotypic characteristics.

Several new small *Babesia* species were described and phylogenetically grouped in relation to other piroplasms by Kjemtrup et al. (2000). Their study placed the true *Babesia* (*B. bigemina, B. canis, B. gibsoni*) in a separate clade distinct from the *Theileria* group, which includes *Cytauxzoon felis* and *Theileria equi*, formerly known as *Babesia equi* (Fig. 20). In addition, a new group containing Western USA isolates of piroplasms was placed in a third clade similar but distinct from the *Theileria* group. This group differed phenotypically from the true *Babesia* spp., in that they were smaller, pleomorphic, and only transstadial transmission was known to occur in the tick vector. A fourth clade has also been recognized which included *B. felis, B. rodhaini, B. microti*, and a small *Babesia* isolated from a dog in Spain (Kjemtrup et al. 2000).

An 18s rRNA gene base pair comparison of the "less-virulent" and the fatal isolates of *C. felis* indicated that they were virtually identical with 99.8% similarity. With only 2 base pair differences, this result indicates that this "less-virulent" isolate is the same as or could be a variant strain of *C. felis*, but not that of a different genus (Table 6). Because the genotypes were virtually identical, this "less-virulent" isolate would be phylogenetically grouped similar to the fatal isolate of *C. felis* (Fig. 20). Phylogenetic and base pair comparisons of this isolate to other piroplasms indicates that this "less-virulent" isolate of *C. felis* is most closely related to *Theileria* spp. with >65 base pair differences for each *Theileria* isolate compared, again placing it as a sister group to other members of the *Theileria* group. Similar comparisons of this isolate to members of the *Babesia* spp. clade showed differences >100 bp for each *Babesia* isolate compared therefore, supporting placement with the *Theileria* group. This grouping is also supported by morphologic and life cycle differences because *C. felis* shares similar

phenotypic properties with *Theileria* spp. For example, *C. felis* and other members of the Family Theileriidae have life cycles involving both erythrocytic and exo-erythrocytic stages in their cycles, while members of the Family Babesiidae have only erythrocytic stages in their cycle.

Because the 18s rRNA gene is highly conserved, genotypic differences between the two distinct isolates of *C. felis* were not sufficient to make a phylogenetic separation. To do so, sequences from a more variable region within the ribosomal DNA gene family could provide additional evidence that these two isolates are genetically distinct. The 18s rRNA gene has been used to determine phylogenetic relationships among similar genera (Penzhorn et al. 2000) because it is conserved and is one of the slowest evolving sequences found in living organisms (Hillis and Dixon, 1991). However, it is not a reliable region to distinguish between sub-species or strains of organisms. By comparison, the Internal Transcribed Spacer (ITS) regions (Fig. 21) have been shown repeatedly to be useful in distinguishing between taxonomic entities at and below the species level (Zahler et al. 1998). Because the ITS regions do not encode for any product, it evolves at a faster rate than the ribosomal encoding regions resulting in genetic variations that distinguish between genera, species, and within species. Using it sequence comparisons, Zahler et al. (1998) attempted to resolve the controversy associated with the separation of *Babesia canis* into three different subspecies by amplifying and comparing the ITS regions. The three *Babesia canis* sub-species, *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* were historically designated based on differing geographic occurrence, vector specificity, and pathogenicity. When the ITS sequences were compared, the subspecies status of each organism was called into question with an

indication that genotypic differences would support distinct species. Marsh et al. (1999) used the same technology to attempt to differentiate between *Sarcocystis neurona* and *Sarcocystis falcatula*. Results indicated that the 18s rRNA gene showed only 1 base pair difference, and indicated that the two species were synonymous. However, when the ITS regions were amplified and compared, sequential differences were observed and leads to the separation of the two isolates into two distinct species. Amplification and sequence comparison of the ITS-1 and ITS-2 regions of the two *C. felis* isolates could prove useful in determining if these isolates are genetically distinct.

CHAPTER V.

SUMMARY AND CONCLUSIONS

Cytauxzoonosis is a tick-transmitted protozoal disease of domestic cats that poses a health threat to all cats exposed to tick infestation in the southern and southeastern United States. Infections with *Cytauxzoon felis* in domestic cats were thought to be uniformly fatal with only a few animals known to survive natural infections. In 2000, eighteen cats were reported to have survived naturally acquired infections with *C. felis* over a 15-month period in an area where fatal cytauxzoonosis is commonly diagnosed. All cases occurred within a 100-mile radius of one another in Northwestern Arkansas and Northeastern Oklahoma. The objective of the current study was to search for evidence that other cats in the Northwestern Arkansas and Northeastern Oklahoma area are surviving infections with *Cytauxzoon felis*, which could likely be due to a "less-virulent" form of *C. felis*.

An IFA serologic test was optimized to find the ideal cutoff values to be used to detect antibodies to *C. felis* in domestic cats. Once optimized, the serologic test was then used to conduct a survey to determine if domestic cats in cytauxzoonosis endemic areas carried antibodies to *C. felis*. Results showed that antibodies were detected in 5 cats in the "survivor" endemic area with a prevalence of 6.8%. Serologic test results were confirmed by PCR analysis with all 6 seroreactor samples being positive while no PCR reactivity was detected in the non-seroreactors. Although two samples from the

Stillwater, Oklahoma area were positive by FIAX serology, they were negative by PCR assumed to be false positive serologic readings, showing that no samples outside the survivor endemic area were positive for infections with *C. felis*. Results also showed that the "survivor" infections are still confined to the area where the 18 survivors were diagnosed. Lastly, the 18s rRNA gene of a "less-virulent" isolate of *C. felis* was sequenced and compared to the published sequence of fatal *C. felis*. Base pair comparison of these sequences revealed that these two gene sequences were virtually identical with 99.8% homology. When compared to other isolates of small piroplasms that occur in North America, significant base pair differences were noted.

This study shows that there are additional domestic cats in the Northwestern Arkansas and Northeastern Oklahoma "survivor" endemic area are surviving infections with *Cytauxzoon felis*. The reason cats are surviving infections with *C. felis* however, is still unknown, but some possible explanations were postulated previously when the report of the 18 original survivors was published. These included natural immunity, drug therapy, alternate routes of infection, unrecognized carrier-state, infected with a novel organism, or a less-virulent strain. In light of this study, it is still felt that some cats have the ability to survive infections with *C. felis* naturally. But this represents such a small number out of the thousands of cats that have died it is still unlikely to be the reason that cats are surviving infections with *C. felis*. Drug therapy would be eliminated as a possibility because the seropositive cats found in this study were healthy, therefore were not treated with any drugs. Alternate routes of infections, such as exchanging blood from bite wounds inflicted while fighting could still result in a non-fatal parasitemia. However, it would probably not be enough to transmit the organism, so while this has a possibility of

occurring, it is not a likely reason that these cats are surviving infections with *C. felis*. The possibility of an unrecognized carrier state would also be eliminated because PCR's were only positive in the seropositive samples from the survivor endemic area and and negative in all seronegative samples from the same area. This study also showed that veterinarians are not simply overlooking survivor in other cytauxzoonosis areas because no cats outside the survivor endemic area were found to be truly infected with *C. felis* as confirmed by PCR. Finally, the possibility of these cats being infected with a novel organism was eliminated as well because the 18s rRNA gene sequence of this "less-virulent" isolate of *C. felis* was nearly identical to that of a published sequence of the fatal isolate of *C. felis*, but different from that of *Theileria* and *Babesia* spp. So, in light of this research, a "less-virulent" strain of *C. felis* still remain a more viable explanation as to why cats in a limited geographic region are surviving infections with *C. felis*. A "less-virulent" strain of *C. felis* could account for the non-fatal infections observed in the survivor endemic area.

This study provides evidence to support the hypothesis that a "less-virulent" strain of *C. felis* may have become established and is being perpetuated in the survivor endemic area. Such evidence includes finding 6.8% of apparently healthy cats infected with *C. felis* in the survivor endemic area and none were found in the other two study areas. If only the fatal form existed, it would not be likely that seropositive animals could be found in any population of cats. Because infected cats were only found one area in which the original survivors were found, one logical conclusion is that a "less-virulent" form of *C. felis* has become established within that confined geographic area. Both the fatal and "less-virulent" forms of *C. felis* are likely to be of the same genera because antibodies against

both forms of *C. felis* seem to react equally well to the *C. felis* antigen in the IFA test. However, this does not preclude that they are different strains within the species or perhaps subspecies. Secondly, the virulence of each form is clearly different in infected cats even though organisms observed in blood smears are morphologically indistinguishable, further supporting evidence of a "less-virulent" strain of *C. felis*. Finally, the DNA sequence comparison in this study showed that only two base pair differences were noted between the fatal and "less-virulent" forms of *C. felis*, whereas significant base pair differences were noted between those of *Theileria* spp. and *Babesia* spp. This suggests that this "less-virulent" isolate is less likely to belong to either of those genera. Because of this finding, infection of the "survivor" cats with a novel organism would also be eliminated as a possibility.

If domestic cats in the "survivor" endemic area are surviving infections with *C. felis* due to a "less-virulent" form of *C. felis*, they could serve as a reservoir for tick vectors due to the persistent parasitemias that occur in these cats. Persistent parasitemias have been shown to occur in several of the original 18 survivor cats because when examined at 154 days post diagnosis, they were still parasitemic and PCR positive. Also, one cat reported to have survived an infection with *C. felis* was also parasitemic and PCR positive 6 years post diagnosis (Walker and Cowell, 1995).

One theory is that instead of getting bobcat to domestic cat transmission, which usually leads to death, we are getting cat-to-cat transmission due to the persistent parasitemias occurring in these cats. In support of this, a follow-up study to the Meinkoth et al. (2000) report showed that at least one cat could survive challenge with virulent *C. felis* when

first infected with tick transmitted "less-virulent" *C. felis*. Further transmission studies are needed to show that *C. felis* can be transmitted consistently to other experimentally infected cats. Those studies would provide further evidence that it is possible that cats surviving infections with this "less-virulent" strain of *C. felis* could serve as reservoirs, for which ticks could transmit to other cats, establishing it in the local population.

From the clinicians' standpoint in cytauxzoonosis endemic areas, this study gives these veterinarians options when faced with possible cytauxzoonosis victims. Normally when cats are diagnosed with *C. felis* infections, veterinarians advise owners to euthanize their animals because dying of cytauxzoonosis is an excrutiating and agonizing death. However, in light of discovering cats that survive infections with *C. felis*, certain clinical signs can be monitored and if they do not occur, then cats are likely to survive and euthanasia could be avoided. Veterinarians need to monitor body temperatures. Usually, cats with this "less-virulent" form of *C. felis* will not spike as high of a fever as cats with fatal *C. felis*. Also, temperatures in cats with the "less-virulent" form of *C. felis* never drop subnormal as does occur in cats with the fatal form of *C. felis*. Because of this, monitoring cats for subnormal temperatures can give an indication as to whether they are infected with the fatal form of *C. felis* and need to be euthanized if the temperature drops subnormal or whether they are infected with the "less-virulent" form if the temperature never drops subnormal. While these may not be "fool-proof" ways to determine if cytauxzoonosis patients in survivor endemic areas should be euthanized or not, they could be useful alternatives that may give owners options concerning their pets, whereas euthanasia was the only option available before these survivors were known to exist.

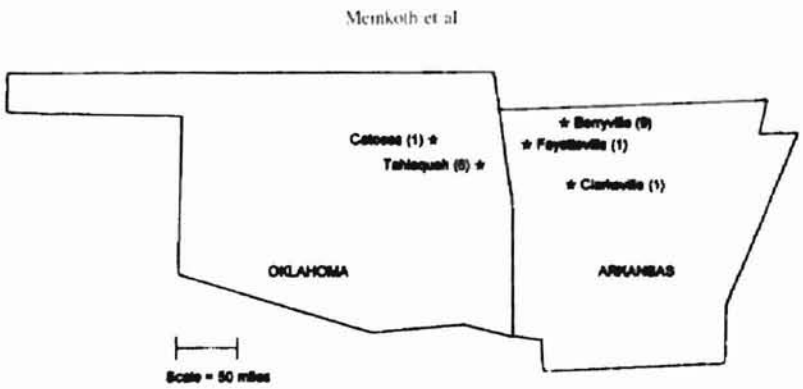
In conclusion, while the serologic study showed that there are cats surviving infections with *C. felis* in the survivor endemic area, it failed to show that the "less-virulent" isolate was different than the fatal isolate of *C. felis* at the 18s rRNA gene level. Therefore it could not be determined if there truly is a "less-virulent" strain of *C. felis*. This study shows overwhelming evidence that a "less-virulent" isolate of *C. felis* exists. However, this study should be followed with further transmission studies and sequencing of the ITS regions of both isolates of *C. felis*, which could possibly differentiate between these two isolates, if two such isolates exist.

Table 1. FIAX values for test validations

Sample	FIAX Values
3440 1/25/00	
Serum	93
Plasma	116
Plasma, slight hemolysis	109
Plasma, moderate hemolysis	108
Plasma, severe hemolysis	109
3467 1/25/00	
Serum	166
Plasma	133
Plasma, slight hemolysis	126
Plasma, moderate hemolysis	129
Plasma, severe hemolysis	130
3467	
Plasma, lipemic, 12/8/99	106
Plasma, lipemic, 12/17/99	103
Plasma, lipemic, 12/27/99	104
Plasma, lipemic, 1/4/00	114
Plasma, lipemic, 1/26/00	126

Table 2. Number of samples and geographic location from which feline blood samples were obtained for the serologic survey

Location	Number of Samples
Survivor Endemic Area	
Tahlequah, OK	5
Berryville, AR	17
Green Forest, AR	53
	75
Survivor Non-endemic Area	
Stillwater, OK	**98**
Non-Endemic Area	
Pullman, WA	**60**



Geographic distribution of the cats surviving *Cytauxzoon felis* infection

Figure 22. Location of the survivor endemic area in Oklahoma and Arkansas.

Table 3. Primer pairs used as a "gold standard" to detect *Cytauxzoon felis* infections in seropositive samples

Primer number	Base pairs, 5'-3'
3383	CCTGGTTGATCCTGCCAG
3406	CGACTTCTCCTTCCTTTAAG

Table 4. Summary of FIAX values, blood smears, and PCR results for all seropositive samples

Cat ID	Location	FIAX	Blood Smear	PCR
Brown	Tahlequah, OK	50	N/A	Positive
Columbus	Green Forest, AR	51	Positive	Positive
Columbus	Green Forest, AR	57	Positive	Positive
Judah	Green Forest, AR	85	Positive	Positive
Charlie	Green Forest, AR	50	N/A	Positive
Tom	Green Forest, AR	45	Negative	Positive
Waldo	Stillwater, OK	47	N/A	Negative
Lobo	Stillwater, OK	44	N/A	Negative

Table 5. Primers used to sequence the 18s rRNA gene of the "less-virulent" form of *Cytauxzoon felis*

Primer number	Primer Sequence, 5' → 3'	Reference
Flanking Primer Pair		
3383	CCTGGTTGATCCTGCCAG	Meinkoth et al. (2000)
3406	CGACTTCTCCTTCCTTTAAG	Meinkoth et al. (2000)
Primer Pair 1		
8632	ATGCGAATCGCATTGC	New
8631	AGACCTGTTATTGCCT	New
Primer Pair 2		
8699	AAAGCAGGCTTTTGC	New
8700	TGGTTAGGACTACGAC	New
Primer Pair 3		
8744	GACAGATTGATAGCTC	New
8745	GCTGCCTTCCTTAGAT	New
Primer Pair 4		
8778	GAGTCATCAGCTCGTG	New
8779	CTAATAAACGCCACC	New

Table 6. Genetic comparison of "less-virulent" *Cytauxzoon felis* to taxonomically similar piroplasms

Organism ID	GenBank #	#BP Differences	% Homology
"Less-virulent" *C. felis*	AF399930	--	--
Fatal *C. felis*	L19080	2	99%
Theileria youngi	AF245279	68	96%
Theileria Type B	U97049	71	95%
Theileria equi	Z15105	82	95%
Babesia gibsoni (Oklahoma isolate)	AF205636	107	93%
Babesia gibsoni (North Carolina isolate)	AF271081	107	93%
Babesia canis	L19079	114	93%
Dog from California	AF158702	106	94%
WA1 Piroplasm (Human, Washington)	AF158700	111	93%
Babesia felis	AF244912	105	93%
Babesia microti	U09833	107	93%

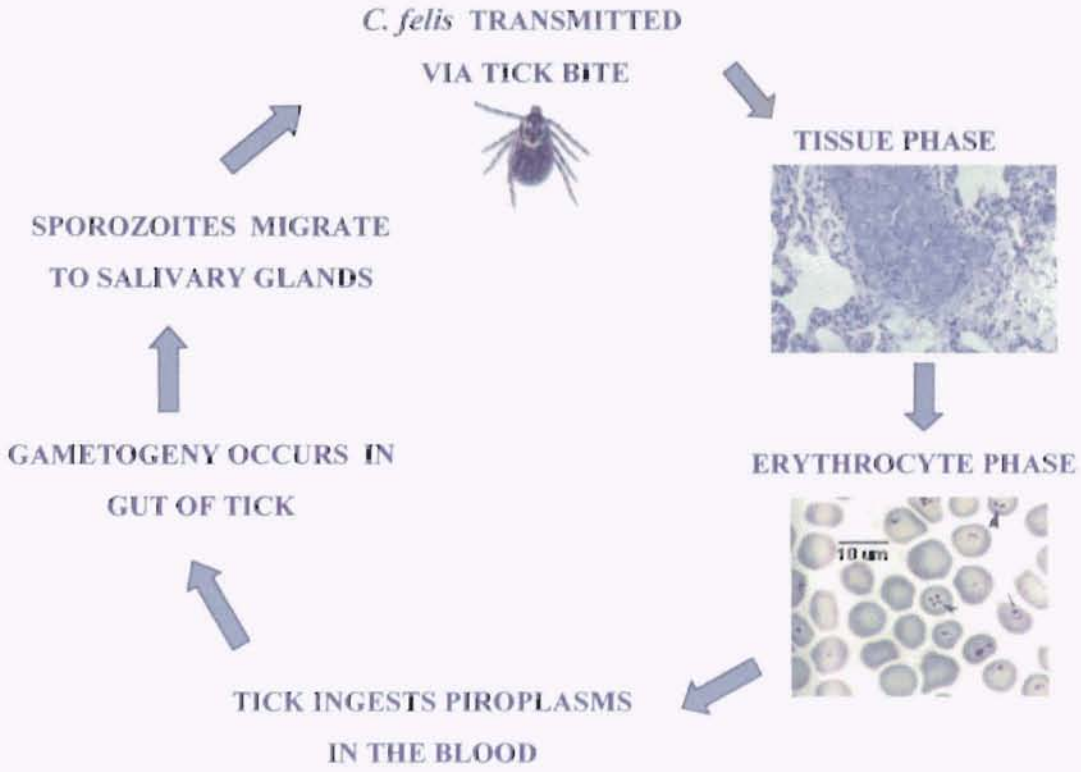


Figure 1. Life cycle of *Cytauxzoon felis*.

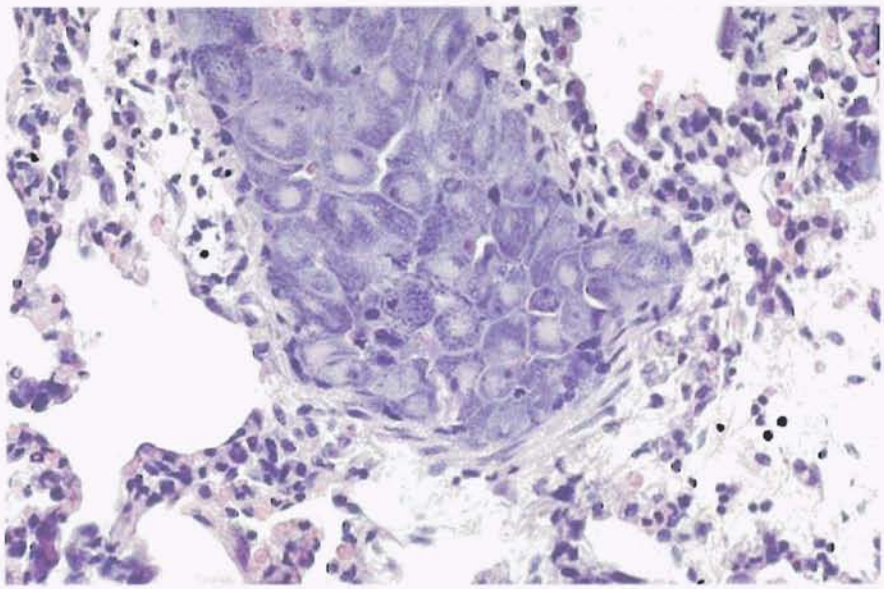


Figure 2. Schizont of *Cytauxzoon felis* within a blood vessel in the lung tissue of an infected cat.

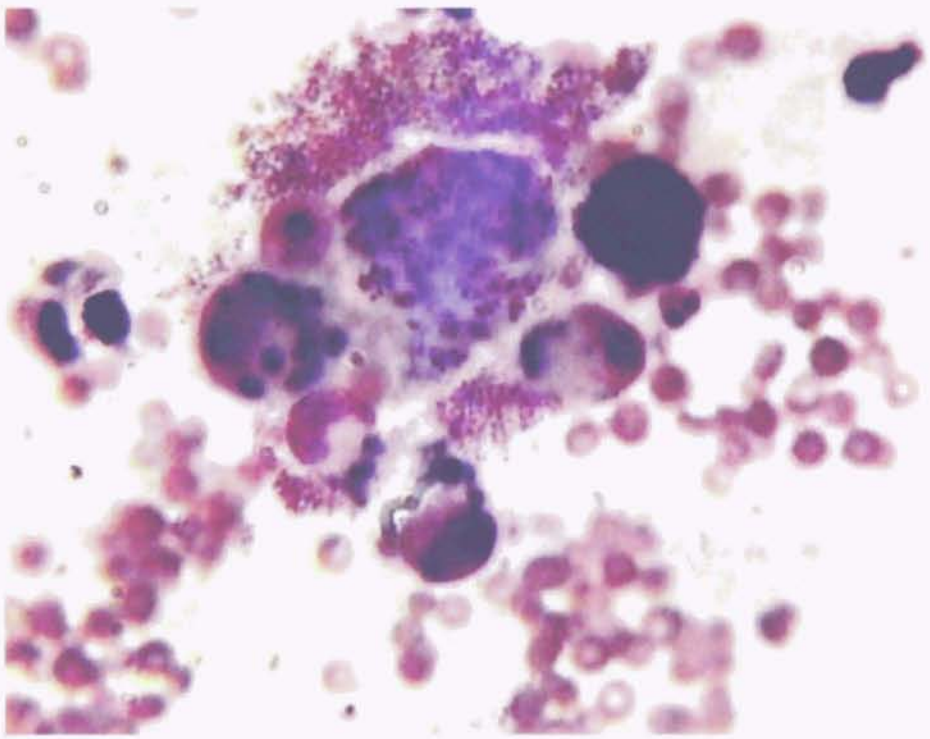


Figure 3. Merozoites and schizonts released from a ruptured macrophage.

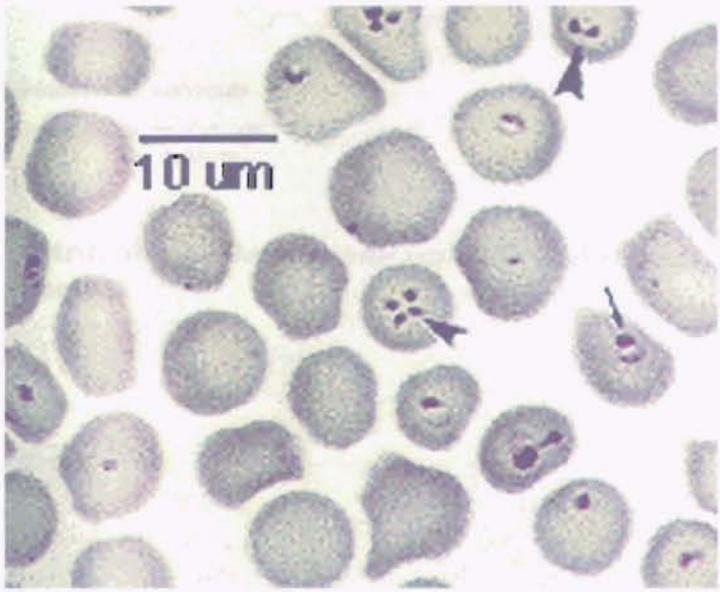


Figure 4. Pleomorphic piroplasms of *Cytauxzoon felis* in erythrocytes of an infected cat.

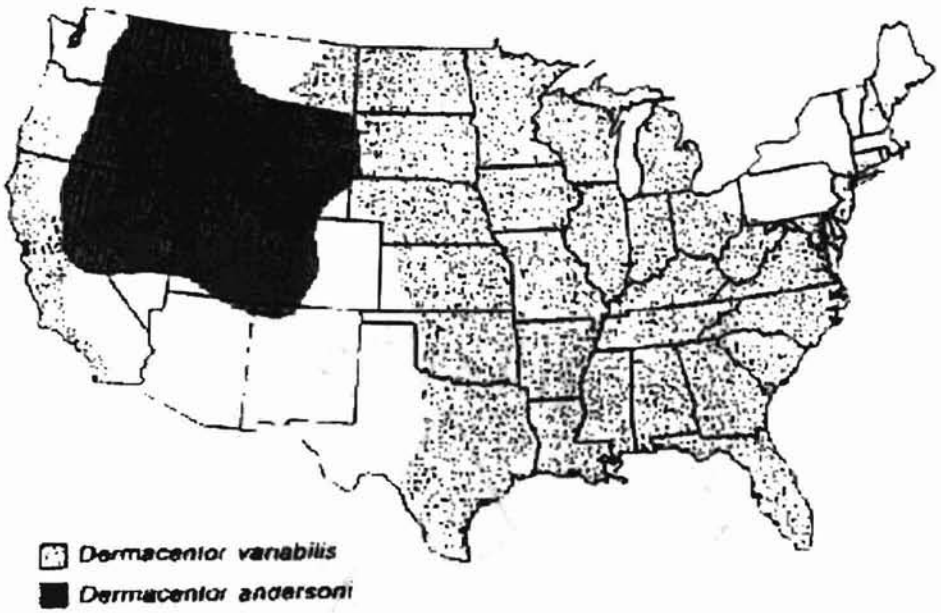


Figure 5a. Distribution of *Dermacentor variabilis* and *D. andersoni* in North America.

Figure 5b. Distribution of *Cytauxzoon felis* based on reported cases in North America.

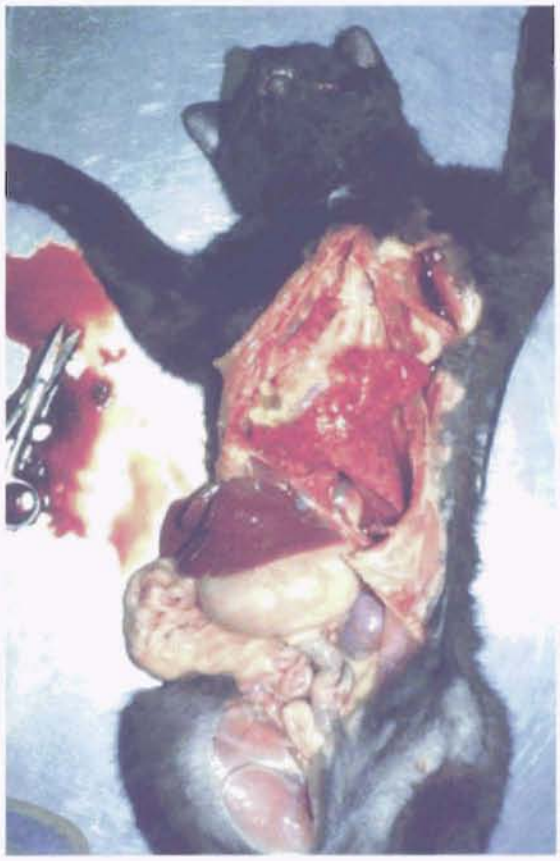


Figure 6. Gross pathology of a domestic cat that died of a *Cytauxzoon felis* infection.

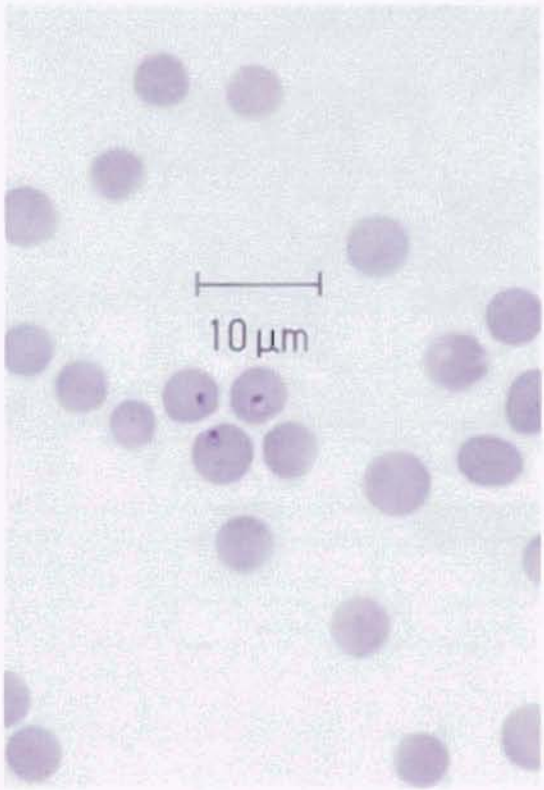


Figure 7. Characteristic signet ring shaped piroplasms in erythrocytes of an infected cat.

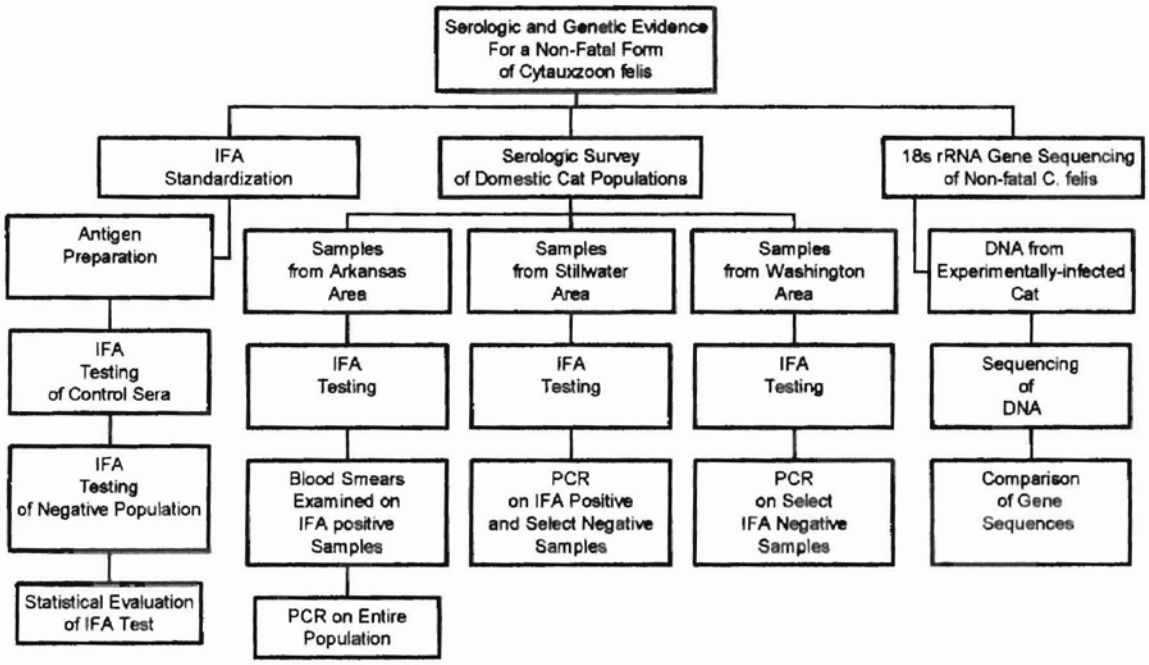


Figure 8. Experimental Design

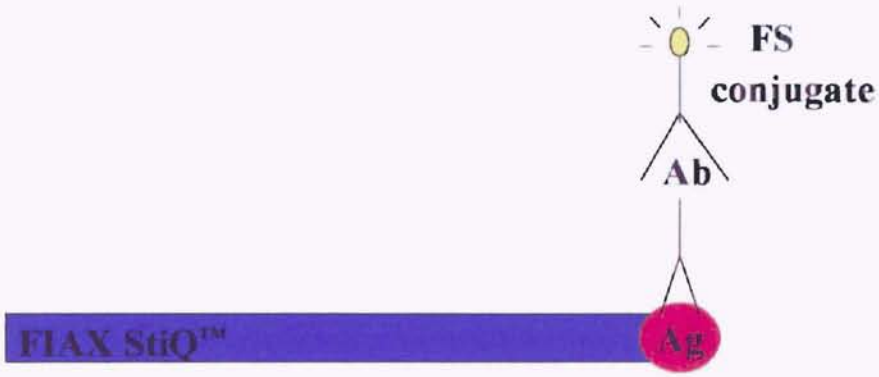
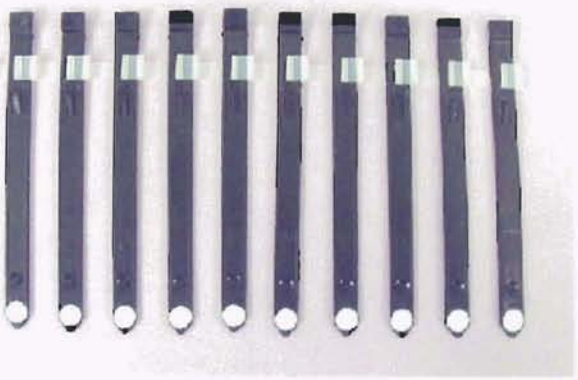


Figure 9. Antibody binding to antigen on a StiQ™ sampler.

A



B

Figure 10. FIAX equipment used for the IFA test. A. FIAX® 400 Fluorometer used to read fluorescent values from StiQ™ samplers; B. Example of StiQ™ samplers.

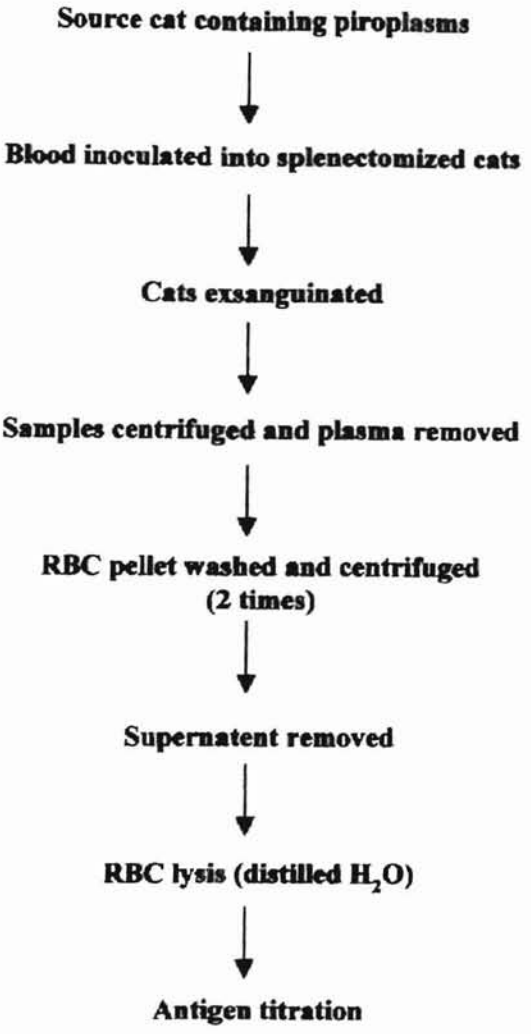


Figure 11. Flow chart of the antigen preparation.

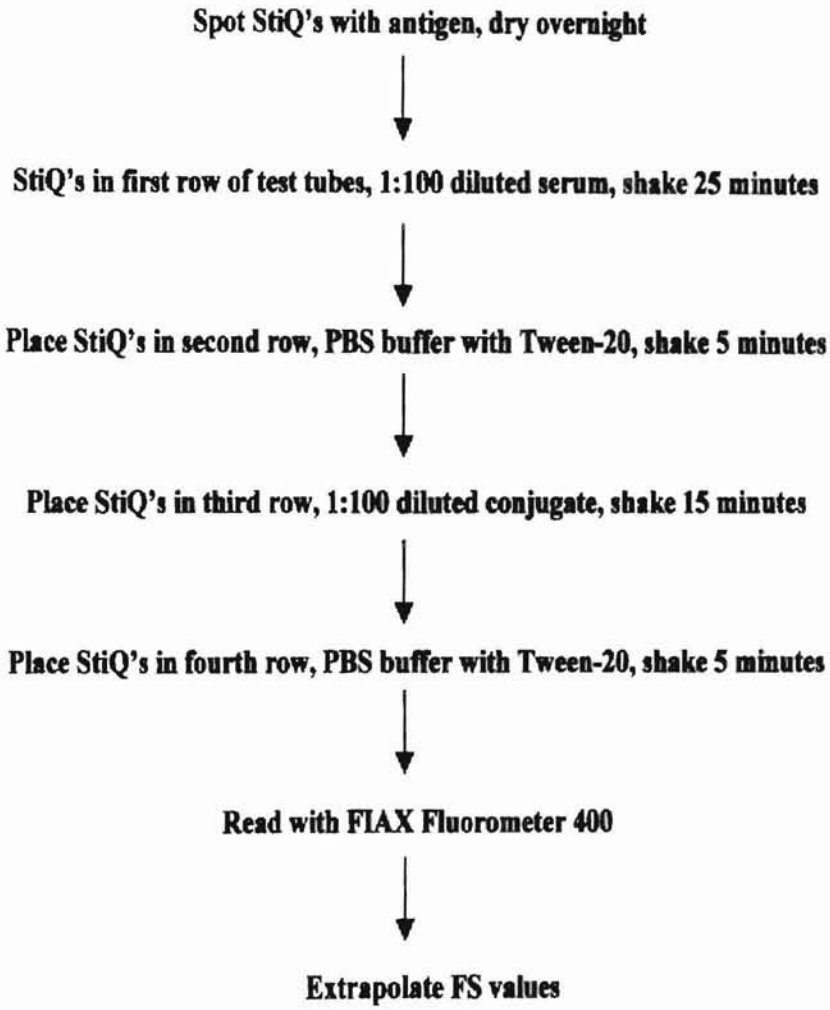


Figure 12. Flow chart of the FIAX® IFA test components.

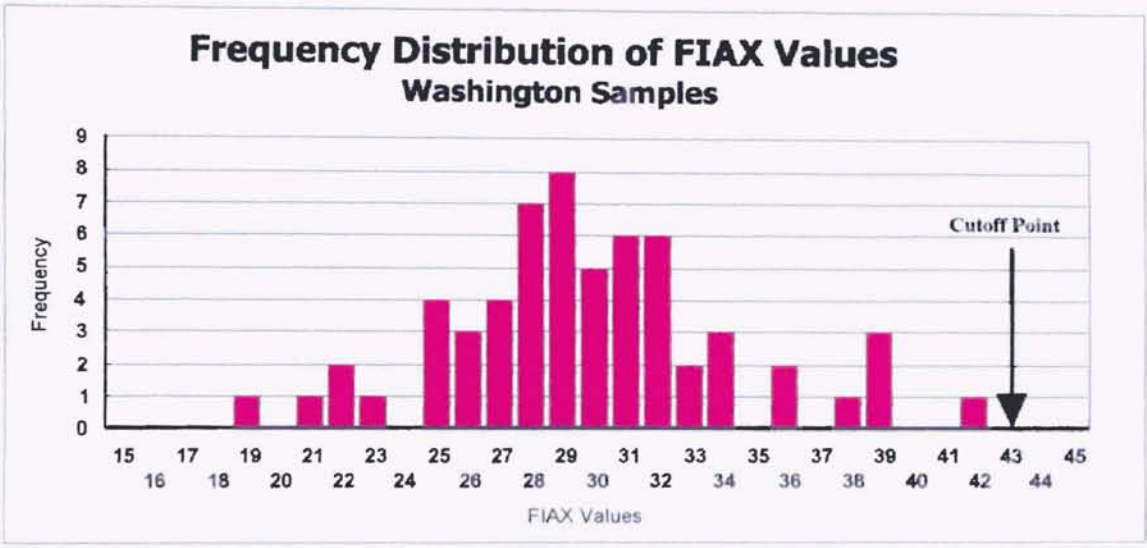


Figure 13. Frequency Distribution of IFA FIAX values (*Cytauzoon felis*-specific IgG) for feline samples obtained from Pullman, Washington. Arrow indicates the statistically calculated cutoff point (43) between positive and negative values.

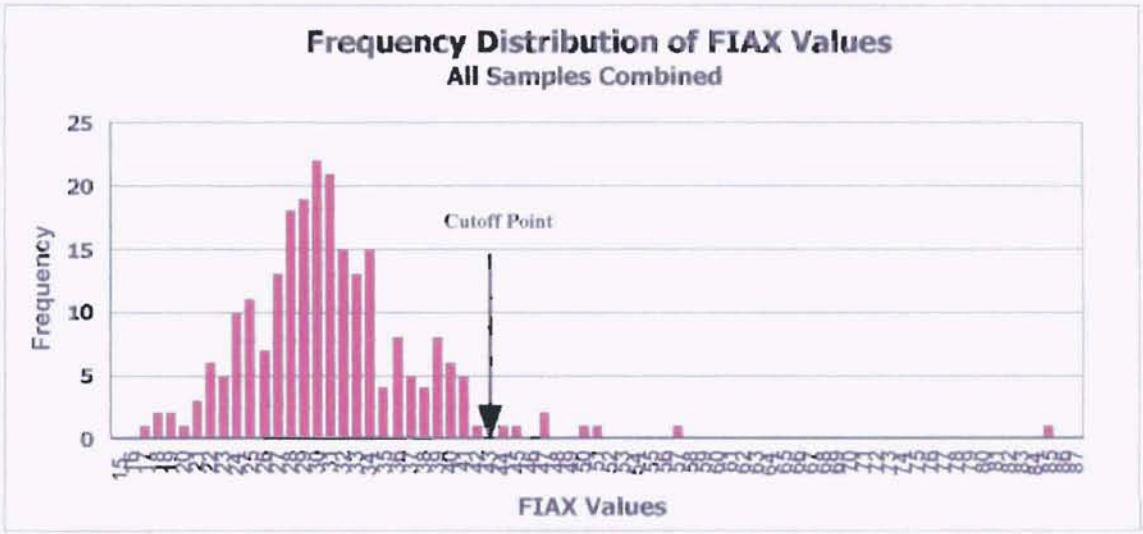


Figure 14. Frequency Distribution of IFA FIAX values (*Cytauzoon felis*-specific IgG) for feline samples obtained from all three areas combined. Arrow indicates the statistically calculated cutoff point (43) between positive and negative values.

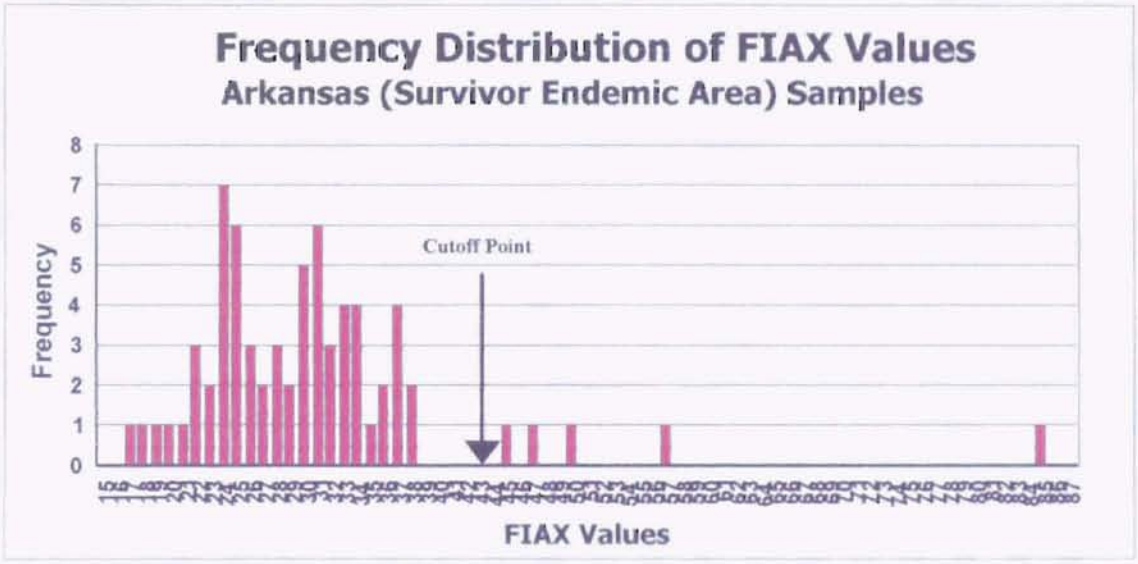


Figure 15. Frequency Distribution of IFA FIAX values (*Cytauzoon felis*-specific IgG) for feline samples obtained from the survivor endemic area (Arkansas/Oklahoma). Arrow indicates the statistically calculated cutoff point (43) between positive and negative values.

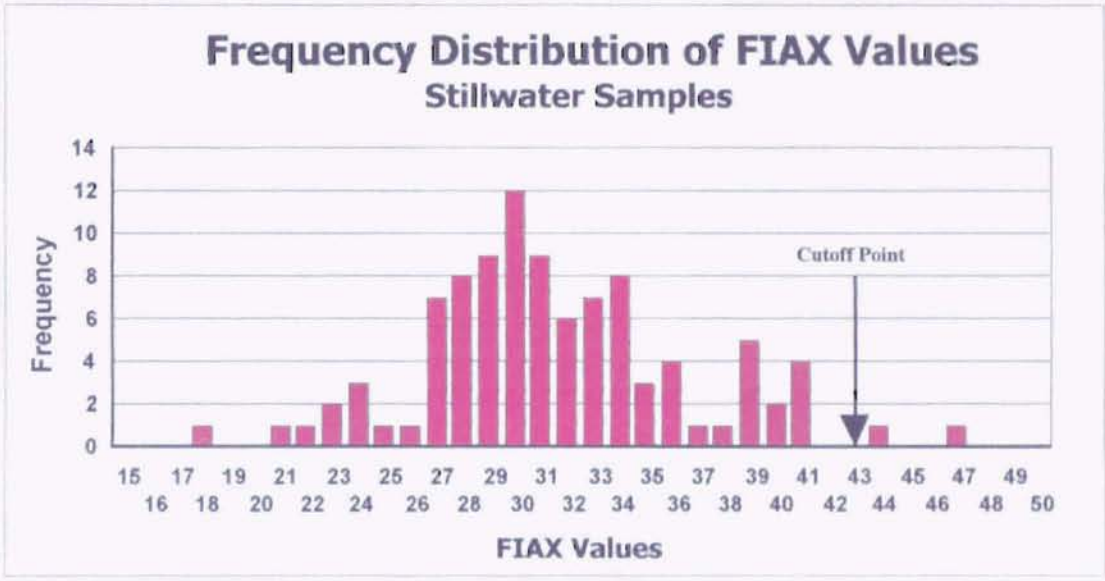
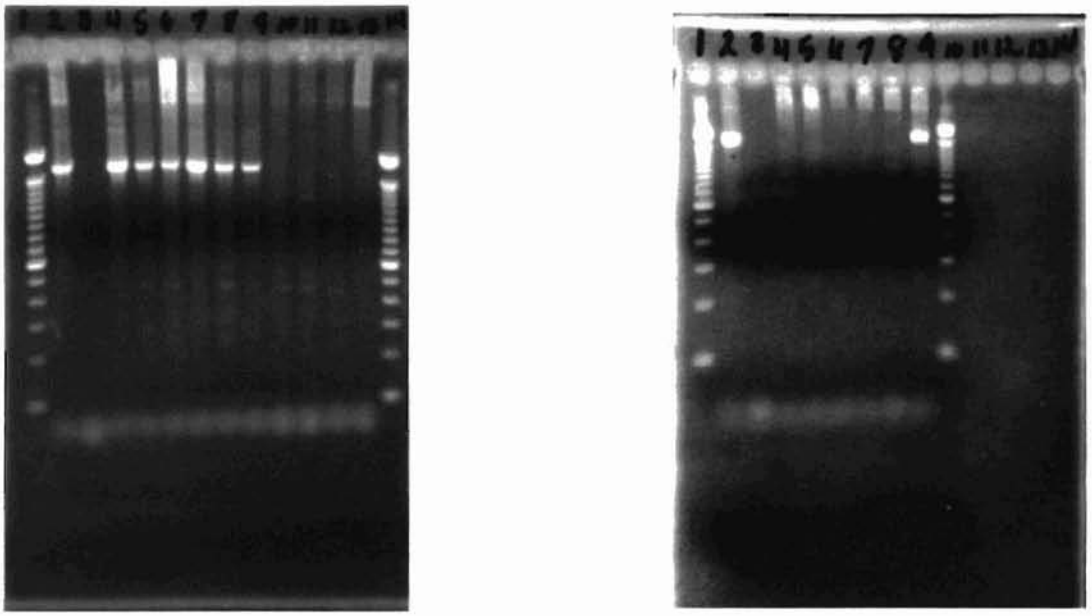


Figure 16. Frequency Distribution of IFA FIAX values (*Cytauzoon felis*-specific IgG) for feline samples obtained from the survivor non-endemic area (Stillwater, Oklahoma). Arrow indicates the statistically calculated cutoff point (43) between positive and negative values.



A B

Figure 17. PCR results for IFA seropositive feline blood samples. A. 100 bp molecular weight (MW) ladder, (1) positive control, (2) negative control, (3) positve control, (4-10) seropositve samples, (11) seronegative sample, (12) seropositive sample, (13) seronegative sample, (14) 100 bp MW ladder. B. 100 bp molecular weight ladder, (1) positve control, (2) negative control, (3) negative control, (4-8) seronegative samples, (9) seropositive sample, (10) 100 bp MW ladder.

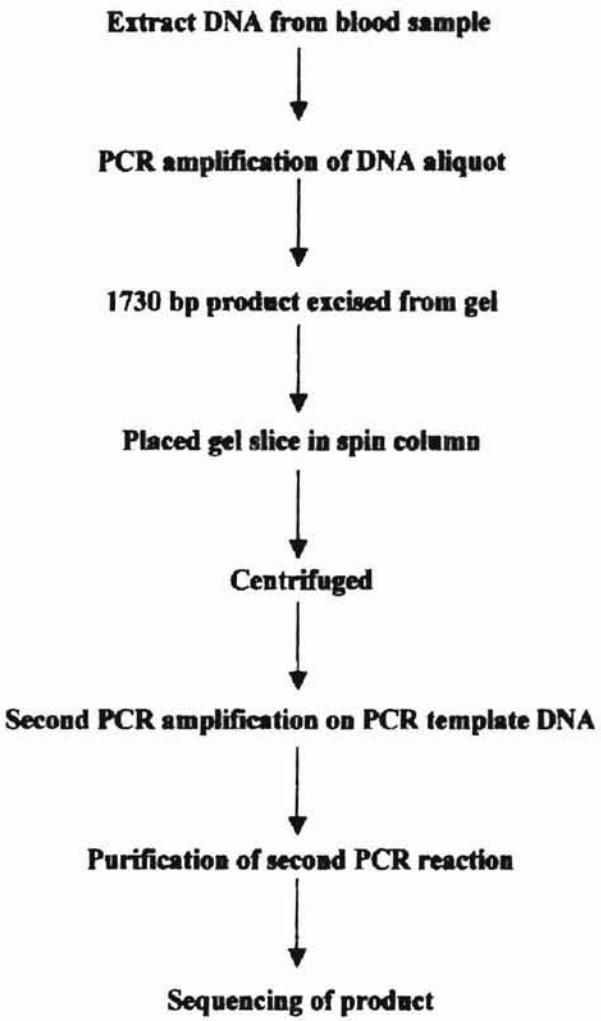


Figure 18. Flow chart of the DNA sequencing protocol.

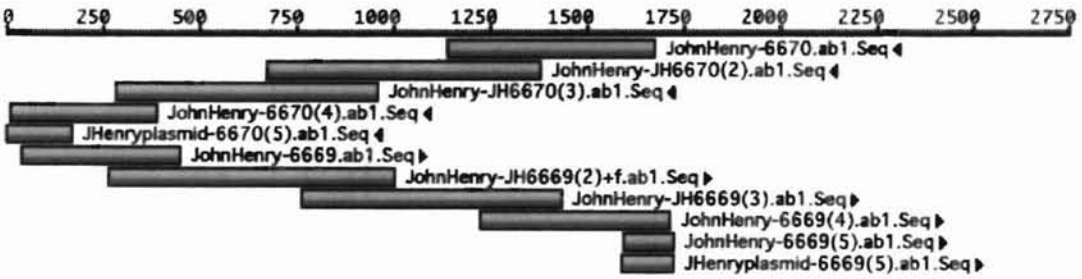


Figure 19. Overlapping sequenced fragments for the 18s rRNA gene of a non-fatal form of *Cytauxzoon felis*.

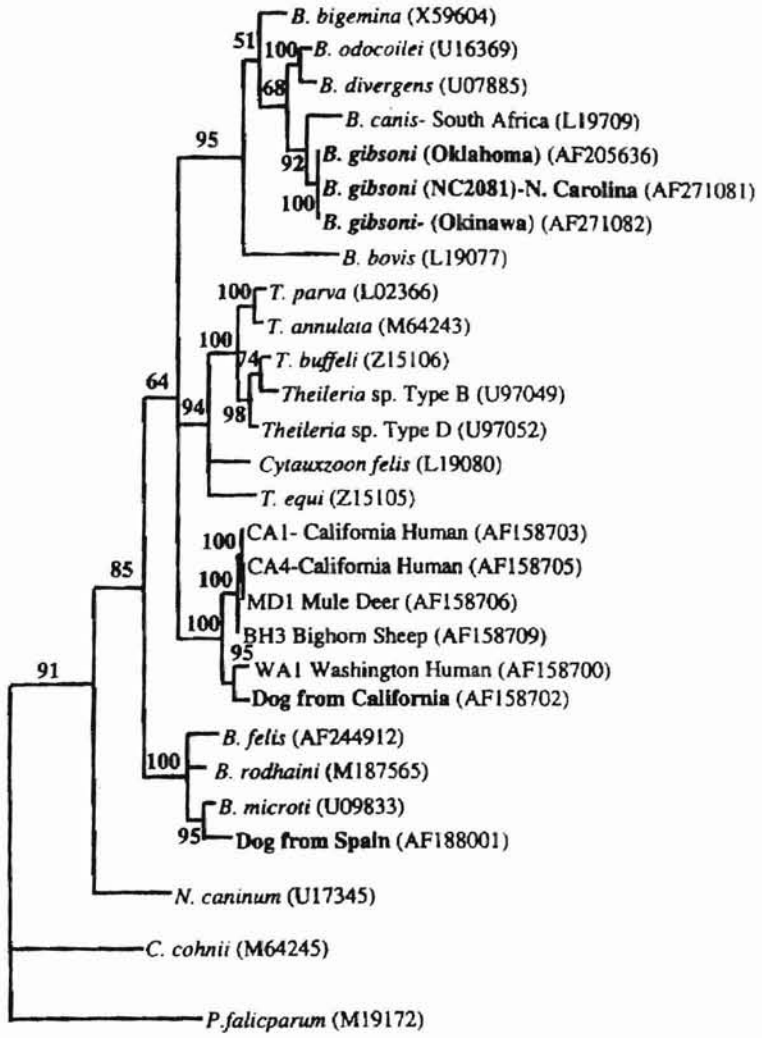


Figure 20. Phylogenetic tree of various members of the Order Piroplasmida (Kjemtrup et al., 2000).

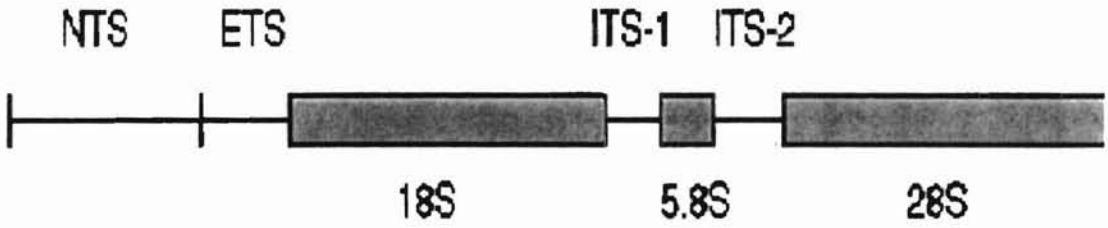


Figure 21. Diagram of the ribosomal DNA gene family in animals (Gerbi 1986).

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APPENDIX

18S rRNA Gene Sequence for a "less-virulent" isolate of *Cytauxzoon felis* "John Henry"

GenBank Accession Number AF399930

CCTGGTTGATCCTGCCAGTAGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAA
GTATAAGCTTTTATATGGTGAAACTGCGAATGGCTCATTAAAACAGTTATAATTTATTTG
ATATTCGTTTCTACATGGATAACCGTGCTAATTGTAGGGCTAATACATGTTCGAGACCTA
TTTTTAATAGGTGGCGTTTATTAGACCTTAAACCATCCCGCTTCGGCGGTATATCGGTGA
TTCATAATAAATATGCGAATCGCATTGCTTTATGCTGGCGATGTATCATTCAAGTTTCTG
ACCTATCAGCTTTGGACGGTAGGGTATTGGCCTACCGGGGCAGCGACGGGTAACGGGGAA
TTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAATGGCTACCACATCTAAGGAAGGCAGC
AGGCGCGTAAATTACCCAATCCTAACACAGGGAGGTAGTGACAAGAAATAACAATACGAG
GCTTAAAGTCTTGTAATTGGAATGACGGAAATTTAAGCTCTTTCCGGAGTATCAATTGGA
GGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAACTTG
TTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTGCATCATTTATATTCCTTAATCGGTT
TATTTATGTTGTGGCTTTTTCTGGTGATTATATTTCGGTATGATTATCCAGATTGTTACT
TTGAGAAAATTAGAGTGCTTAAAGCAGGCTTTTGCCTTGAATACTTTAGCATGGAATAAC
TAAGTAGGACTTTGGTTCTATTTTGTTGGTTTAAGAGCCAAAGTAATGATTAATAGGAAC
AGTTGGGGGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAA
CTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAAGAACGAAAGTTAGGGGATC
GAAGACGATCAGATACCGTCGTAGTCCTAACCATAAACTATGCCGACTAGAGATTGGAGG
TCGTCAGTTTGAACGACTCCTTCAGCACCTTGAGAGAAATCAAAGTCTTTGGGTTCTGGG
GGGAGTATGGTCGCAAGGCTGAAACTTAAAGGAATTGACGGAAGGGCACCACCAGGCGTG
GAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCAGGTCCAGACAGAGGAAGG
ATTGACAGATTGATAGCTCTTTCTTGATTCTTTGGGTGGTGGTGCATGGCCGTTCTTAGT
TGGTGGAGTGATTTGTCTGGTTAATTCCGTTAACGAACGAGACCTTAACCTGCTAAATAG
GATCTGAGAATAAACTTTATGTTGTCTCAGCATCGCTTCTTAGAGGGACTTTGCGGTTAT
AAATCGCAAGGAAGTTTAAGGCAATAACAGGTCTGTGATGCCCTTAGATGTCCTGGGCTG
CACGCGCGCTACACTGATGCATTCATCGAGTATATCCTTGGCCGAGAGGCTTGGGTAATC
TTTAGTATGCATCGTGATGGGGATTGATTATTGCAATTATTAATCATGAACGAGGAATGC
CTAGTAGACGCGAGTCATCAGCTCGTGTCGATTACGTCCCTGCCCTTTGTACACACCGCC
CGTCGCTCCTACCGATCGAGTGATCCGGTGAATTATTCGGACTGTGGTGAATCTAATTCG
TTAGATACGCCATGGAAAGTTTTGTGAACCTTATCACTTAAAGGAAGGAGAAGTCG

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