PREVALENCE OF IGG ANTIBODIES TO *ENCEPHALITOZOON CUNICULI*, *TOXOPLASMA GONDII*, AND *SARCOCYSTIS NEURONA* IN DOMESTIC CATS

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(ABSTRACT)

Encephalitozoon cuniculi, *Toxoplasma gondii* and *Sarcocystis neurona* are intracellular parasites that infect a wide range of mammalian host species including domestic cats. The prevalence of antibodies to these parasites in cats was examined using an indirect immunofluorescence antibody assay. *E. cuniculi* targets the kidneys of rabbits but the prevalence of disease in cats is unknown. Chronic kidney disease (CKD) is a common cause of illness in cats. *T. gondii* is a widespread parasite of cats; however, it is not considered a major causative agent of CKD. The first hypothesis was that *E. cuniculi* and *T. gondii* are unrecognized causes of chronic kidney disease in domestic cats. Serum and plasma samples were examined for protozoal antibodies from 232 feline patients at the VMRCVM Teaching Hospital. Thirty-six of the 232 samples met the IRIS criteria for CKD. Antibodies to *E. cuniculi* were found in 15 samples, 4 of which came from cats with CKD. Antibodies to *T. gondii* were found in 63 samples; 10 cats of the 63 had CKD. These were not significantly different from cats with no CKD and the null hypothesis was rejected.

Domestic cats, armadillos, raccoons and skunks are intermediate hosts (IH) for *S. neurona* while opossums are the definitive host (DH). The seroprevalence of *S. neurona* was examined in domestic cats from Virginia and Pennsylvania. The second hypothesis was that domestic cats are important IH for *S. neurona* transmission. A low seroprevalence was found in 32 of the 441 cats and the null hypothesis was rejected.

DEDICATION

I would like to dedicate this thesis to my dear mother and father. Thank you for giving me solace to carry on with my dreams at moments of frustration. Please be the eternal sun in my sky, and let me be the moon that reflects your radiance wholeheartedly. I will love you always.

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INTRODUCTION

Encephalitozoon cuniculi, *Toxoplasma gondii*, and *Sarcocystis neurona* are three intracellular zoonotic parasites that infect multiple mammalian host species including cats. Diagnostic assays including the indirect immunofluorescent antibody assay (IFA) and the direct agglutination test are of considerable value in determining the prevalence of antibodies to these parasites in cats because of their reliable, reproducible, and sensitive nature. Despite the high diagnostic value of these techniques, which make them the methods of choice, they possess a few inherent difficulties. The IFA for instance, requires using a fluorescent compound microscope, and species-specific reagents (Jordan et al., 2006). Furthermore, due to its subjective nature, discrepancies in slide reading might arise. The direct agglutination test requires a special plate reader, and large numbers of spores, tachyzoites, or merozoites harvested as sources of antigens to conduct the assay (Jordan et al., 2006). The direct agglutination test was not employed in the following studies for two reasons. One involves the discontinuation of commercially available bovine serum albumin reagents which were essential for the test. The second reason is that the agglutination test is less sensitive than the IFA when samples were run in tandem. The IFA was conducted with spores of a canine subtype of *E. cuniculi* and tachyzoites of the RH strain of *T. gondii* air dried onto 12-well Teflon-coated slides. Plasma and sera were examined at a 1:10 dilution for *E. cuniculi* antigen and at a 1:25 dilution for *T. gondii*. A secondary fluorescein isothiocyanate (FITC)–coupled goat-anti cat antibody was used to identify the primary antibody at a 1:5

dilution. The bound and labeled slides were mounted in Fluoromount-G for assessment under the epifluorescent microscope.

In manuscript 1, we hypothesized that *E. cuniculi* and *T. gondii* are unrecognized causes of chronic kidney disease (CKD) in domestic cats. *E. cuniculi* is a microsporidian parasite of rabbits, rodents, dogs, humans and other mammals. There are no intermediate hosts and aberrant hosts involved in its life cycle. Unlike *T. gondii,* the term "definitive host" is not commonly applied in the description of life cycle. The hosts acquire the infection upon ingestion or inhalation of urine containing spores or spore-contaminated water. Once inside the hosts, the spores extrude their polar tubules and infect the host cells with sporoplasm containing DNA materials. The sporoplasm undergoes extensive multiplication by merogony within the host cells. Parasitophorous vacuoles are formed to isolate the parasites from their host cells, where the microsporidia develop by sporogony to mature spores. During sporogony, thick walls are formed around the spores. Eventually, the cell membranes are disrupted to release the mature spores via urine into the environment. These free mature spores are capable of infecting new hosts thus continuing the cycle. Clinical signs in rabbits (Jordan et al., 2006) and dogs (Shadduck et al., 1978; Stewart et al., 1988; Snowden et al., 2009) are primarily associated with kidney disease, and to a lesser extent central nervous system disease. Very little is known about the importance or prevalence of microsporidial infections in cats. The presence of the related microsporidian, *Enterocytozoon bieneusi* in the feces of cats has been documented (Mathis et al., 1999; Dengjel et al., 2001; Sadler et al., 2002; Santín et al., 2006), although it does not cause clinical disease in cats. A case report of neurological signs and lesions consistent with

encephalitis and nephritis in a Siamese kitten from South Africa provided the first clear evidence of microsporidial infection in domestic cats (van Rensburg and du Plessis, 1971). Upon microscopic examination, microsporidial parasites were observed in the tissues of this kitten (van Rensburg and du Plessis. 1971). Retrospective examination discovered that 2 of this kitten's littermates had also developed clinical signs of encephalitis. A second case study reported infection of the cornea with *Encephalitozoon* sp. in a 3.5 year old male domestic short-haired cat from the United States (Buyukmihci et al., 1977). Lastly, a prevalence study documented that antibodies to *E. cuniculi* were detected by the IFA in 17 (24%) of 72 cats from Eastern Slovakia (Halánová et al. 2003).

Kidney disease is recognized as a major health problem in cats. The prevalence of renal disease in cats in the United States is estimated to be 1.9% (Lund et al, 1999). Its prevalence increases with age including up to 31% of cats over the age of 15 (Lulich et al, 1992). Numerous infections, congenital and familial diseases, toxicities, neoplasms, and other processes can cause CKD in cats but idiopathic tubulointerstitial nephritis is most commonly identified (DiBartola et al 1987, Minkus et al 1994). Chronic kidney disease in cats is often insidious. It is well accepted that in cats, chronic kidney disease will progress to end-stage renal failure and survival time decreases as International Renal Interest Society stage (IRIS) and urine protein concentration increase (Boyd et al., 2008, Syme et al 2006). CKD is by definition, kidney impairment in the presence or absence of either the occurrence of decreased glomerular filtration rate (GFR), or a > 50% reduction in GRF (Polzin et al., 2005). Feline CKD is commonly diagnosed based on a persistently increased plasma creatinine concentration; although

up to 75% of functional renal mass may be lost before azotemia detection has been reported (Ross, 1981). Different from human medicine, estimated glomerular filtration rate (eGFR) measurements are rarely available to fully evaluate renal function. Besides, longitudinal studies for evaluating increased risks of developing azotemia in cats are lacking (Jepson, 2009). For the studies described herein, feline CKD is diagnosed based on plasma creatinine ≥ 1.6 mg/dl, presence of a concurrent urine specific gravity of < 1.035, and sufficient clinical evidence for staging of CKD by the attending clinician, Dr. Grant. It is characterized by a progressive process involving a loss of functional renal tissue and direct clinical signs become apparent when more than 75% deterioration of nephron function has been reached (Kahn, 2008). The first objective was to determine the prevalence of IgG antibodies to *E. cuniculi* in cats with and without chronic kidney disease.

T. gondii infections are prevalent in all warm-blooded animals globally (Dubey, 2009). Cats as the definitive hosts are crucial in the epidemiology of *T. gondii* by shedding environmentally resistant oocysts in the feces (Dubey, 2009). Oocysts undergo sporulation within 1 to 2 days to become infective. These oocysts persist in the environment and contaminate soil, water or plant material. Upon ingestion of oocyst-contaminated materials, the intermediate hosts, such as small mammals and cats, acquire the infection. Transformation of oocysts into tachyzoites takes place in the lamina propria. These tachyzoites localize in neural and muscle tissue and develop into bradyzoites within tissue cysts. Transmission of infections to cats is achieved by consuming intermediate hosts harboring tissue cysts or sporulated oocysts. However, *T. gondii* is not commonly associated with chronic kidney disease in cats. Clinical and

pathologic data collected from 100 cats with histologically verified clinical toxoplasmosis demonstrated that 11 of the 61 kidneys examined contained stages of *T. gondii* (Dubey and Carpenter, 1993). Renal failure was not listed as a cause of death in any of these cats. Since cats are definitive hosts for *T. gondii* and CKD is common in cats, the second objective was to determine whether *T. gondii* antibody positive cats have a higher prevalence of CKD than other cats in the same population.

In manuscript 2, we hypothesized that domestic cats are important intermediate hosts for *S. neurona*. *S. neurona*, an apicomplexan parasite, is the primary etiologic agent of a frequently diagnosed neurological disease in equids known as equine protozoal myeloencephalitis (EPM) (Dubey, Lindsay, Saville et al., 2001). Its life cycle alternates between the established definitive host, the Virginia opossum (*Didelphis virginiana*), and a wide range of mammalian intermediate hosts. Domestic cats (*Felis domesticus*), along with sea otters (*Enhydra lutris*), raccoons (*Procyon lotor*), nine-banded armadillos (*Dasypus novemcinctus*), striped skunks (*Mephitis mephitis*), and fishers (*Martes pennanti*), have been described as intermediate hosts (Dubey et al., 2000; Cheadle, Tanhauser et al., 2001; Cheadle, Yowell et al., 2001; Dubey, Lindsay, Saville et al., 2001; Dubey, Rosypal et al., 2001; Dubey, Saville et al., 2001; Tanhauser et al., 2001; Butcher et al., 2002; Turay et al., 2002; Gerhold et al., 2005). The bradyzoites undergo sexual replication in the intestines of the definitive hosts that leads to the excretion of sporulated oocysts and environmentally resistant sporocysts in the feces. Upon ingestion of sporocysts by the intermediate hosts, sporocysts excyst and liberate sporozoites into the intestine. Sporozoites penetrate the intestine, enter the vascular system and develop asexually by schizogony in endothelial cells. The

merozoites enter additional endothelial cells and repeat schizogony. Merozoites from the final generation of schizogony enter striated muscle cells. They develop by endodyogeny and produce bradyzoites within sarcocysts. Infection of the definitive host is acquired after ingesting the sarcocysts contained within the muscles of the intermediate hosts. By contrast, the horse (*Equus caballus*) acquires infection by ingestion of the sporocysts that leads to the production of schizonts and merozoites in central nervous system tissue. These stages destroy tissue but do not produce sarcocysts. The horse is unable to transmit *S. neurona* to continue the life cycle and thus is involved as an aberrant host (Dubey et al., 2000). Immunocompetent cats fed *S. neurona* sporocysts seroconverted by day 20 post-inoculation in an agglutination test, while some cats treated with cortisone seroconverted as early as 7 days after inoculation (Dubey, Lindsay, and Saville, 2002). Few studies have examined the seroprevalence of *S. neurona* in cats; therefore, the third objective was to investigate the seroprevalence of antibodies to *S. neurona* in cats from Virginia and Pennsylvania. For this study, a total of 441 feline serum and plasma samples were collected. Of the 441 samples, 232 samples obtained from Virginia were previously used in the study of kidney disease conducted in the Department of Small Animal Clinical Science at the Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia. An additional 209 serum samples were acquired from an animal shelter in Philadelphia, Pennsylvania. These samples were initially used in a study of the seroprevalence of *Toxoplasma gondii* (Dubey et al., 2008).

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MANUSCRIPT 1

PREVALENCE OF IGG ANTIBODIES TO *ENCEPHALITOZOON CUNICULI* AND *TOXOPLASMA GONDII* IN CATS WITH AND WITHOUT CHRONIC KIDNEY DISEASE FROM VIRGINIA

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Key words: *Encephalitozoon cuniculi*, *Toxoplasma gondii*, cat, chronic kidney disease

ABSTRACT

Kidney disease is a common and serious condition in domestic cats. There are numerous causes of kidney disease including parasitic infection. *Encephalitozoon cuniculi* is a microsporidian parasite that develops in the kidneys of rabbits and causes chronic renal disease. Although significant renal impairment is rarely observed, little has been reported concerning *E. cuniculi* in cats and no serological studies on this parasite in cats have been conducted in the United States to date. The present study explored the possibility that *E. cuniculi* is an unrecognized contributor to the high prevalence of kidney disease observed in cats. A serological survey was conducted to determine the prevalence of IgG antibodies to spores of *E. cuniculi* in cats with and without a diagnosis of chronic kidney disease (CKD) according to the International Renal Interest Society (IRIS) staging system. By definition, CKD is the kidney impairment in the presence or absence of either the occurrence of decreased glomerular filtration rate (GFR), or a > 50% reduction in GRF persisting (Polzin et al., 2005). CKD is characterized by a progressive process involving a loss of functional renal tissue. Direct clinical signs become apparent when more than 75% deterioration of nephron function has been reached (Kahn, 2008). By comparison, acute kidney disease is characterized by an abrupt impairment of kidney function, most often accompanied by anorexia, depression, dehydration, oral ulceration, vomiting and/or diarrhea, or oliguria in Stage IV (Kahn, 2008). Impairment of acute injury is potentially reversible by means of resolution of the partial kidney injury, improvement of adaptive compensatory enhancements in kidney function, or a combination of both procedures (Polzin et al., 2005). Therefore, consequences of the chronic and acute kidney disease

are distinguishable (Polzin et al., 2005). Likewise, samples were examined for IgG antibodies to *Toxoplasma gondii*, a common well studied protozoan of cats. Plasma and sera were obtained from 232 feline patients at the Virginia-Maryland Regional College of Veterinary Medicine teaching hospital. With the investigators blinded to the renal status of test subjects, samples were screened via indirect immunofluorescent antibody assay. Thirty-six of the 232 cats met the IRIS staging system criteria for CKD based on plasma creatinine ≥ 1.6 mg/dl, presence of a concurrent urine specific gravity of < 1.035, and sufficient clinical evidence for staging of CKD. Antibodies to *E. cuniculi* were found in 15 of the 232 samples, which included 4 of the 36 cats with CKD. Antibodies to *T. gondii* were found in 63 of the 232 samples, which included 10 of the 36 cats with CKD. The prevalence of antibodies in cats with CKD to either protozoan was not significantly different ($P > 0.05$) from the cats without CKD in the study. Collectively, the results do not support the hypothesis that either *E.* cuniculi or *T. gondii* plays an important etiologic role in the occurrence or progression of CKD in domestic cats.

INTRODUCTION

Kidney disease is recognized as a major health problem in cats. The prevalence of renal disease in cats in the United States is estimated to be 1.9% (Lund et al, 1999). Its prevalence increases with age including up to 31% of cats over the age of 15 (Lulich et al, 1992). Numerous infections, congenital and familial diseases, toxicities, neoplasms, and other processes can cause CKD in cats but idiopathic tubulointerstitial nephritis is most commonly identified (DiBartola et al 1987, Minkus et al 1994). Chronic kidney disease in cats is often insidious. It is well accepted that in cats, chronic kidney disease will progress to end-stage kidney failure and survival time decreases as International Renal Interest Society stage (IRIS) and urine protein concentration increase (Boyd et al., 2008, Syme et al 2006). *Encephalitozoon cuniculi* and *Toxoplasma gondii* are two intracellular zoonotic parasites that infect multiple mammalian host species including cats. *E. cuniculi* is a microsporidial parasite of humans, rabbits, dogs and other mammals. Clinical signs are primarily associated with kidney disease, and to a lesser extent central nervous system disease, in rabbits (Jordan et al., 2006) and dogs (Shadduck et al., 1978; Stewart et al., 1988; Snowden et al., 2009). Very little is known about the importance or prevalence of microsporidial infections in cats. The presence of the related microsporidian, *Enterocytozoon bieneusi* in the feces of cats has been documented (Mathis et al., 1999; Dengjel et al., 2001; Sadler et al., 2002; Santín et al., 2006), although clinically it does not seem to be pathogenic to cats. A case report of neurological signs and lesions consistent with encephalitis and nephritis in a Siamese kitten from South Africa provided the first clear evidence of microsporidial infection in domestic cats (van Rensburg and du Plessis,

1971). Upon microscopic examination, microsporidial parasites were observed in the tissues of this kitten (van Rensburg and du Plessis. 1971). Retrospective examination discovered that 2 of this kitten's littermates had also developed clinical signs of encephalitis. A second case study reported infection of the cornea with *Encephalitozoon* sp. in a 3.5 year old male domestic short-haired cat from the United States (Buyukmihci et al., 1977). Lastly, a prevalence study documented that antibodies to *E. cuniculi* were detected by indirect immunofluorescent antibody assay (IFA) in 17 (24%) of 72 cats from Eastern Slovakia (Halánová et al. 2003).

Toxoplasma gondii infections are prevalent in all warm-blooded animals globally (Dubey, 2009). As the definitive hosts, cats are crucial in the epidemiology of *T. gondii* by producing environmentally resistant oocysts (Dubey, 2009). However, *T. gondii* is not commonly associated with chronic kidney disease in cats. Clinical and pathologic data collected from 100 cats with histologically verified clinical toxoplasmosis demonstrated that only 11 of the 61 kidneys examined contained stages of *T. gondii* (Dubey and Carpenter, 1993). Kidney failure was not listed as a cause of death in any of these cats.

The objectives of the present study were first, to determine the prevalence of IgG antibodies to *E. cuniculi* and *T. gondii* in cats with and without chronic kidney disease. Secondly, to determine whether *E. cuniculi* antibody positive cats have a higher prevalence of CKD than other cats in the same population. Lastly, to determine whether *T. gondii* antibody positive cats have a higher prevalence of CKD than other cats in the same population.

MATERIALS AND METHODS

Sample collection

Serum and plasma samples were collected from any blood submitted for feline patients to the diagnostic laboratory of the Virginia-Maryland Regional College of Veterinary Medicine teaching hospital from September, 2008 to May, 2010. A number code was assigned to each sample by one investigator (DG) and the renal status of the cats was revealed to the investigator performing serology (VH) only after serologic testing was complete. When multiple samples were determined to be from the same animal, only the results from the first received sample were used. This resulted in 232 final samples being included in the present study. All samples were stored frozen at -20^0 C until used.

Retrospective serum and plasma creatinine concentration analysis

Serum and plasma samples were categorized based on their initial creatinine concentrations. Samples with creatinine < 1.6 mg/dl were considered as belonging to cats without kidney disease whereas samples with creatinine ≥ 1.6 mg/dl were considered as belonging to cats that may have kidney disease and their medical records were further reviewed. If a cat with creatinine ≥ 1.6 mg/dl did not have a concurrent urine specific gravity or it could not be placed into one of the two categories (no CKD and with CKD) for any reason, it was eliminated from the study. Cats with chronic kidney disease were assigned to stages 2-4 using the International Renal Interest Society (IRIS, Vienna, Austria) staging system. Cats were considered to have chronic kidney disease of IRIS stage 2 or greater if serum creatinine was > 1.6 mg/dl,

urine specific gravity was < 1.035 and the attending clinician diagnosed the cat with chronic rather than acute disease by the attaining clinician, Dr. Grant, for which the progressive process with the development of clinical signs due to loss of functional tissue (Kahn, 2008).

The IRIS stage 1 includes cats with functional or structural abnormalities of the kidneys in conjunction with creatinine concentrations < 1.6 mg/dl. Since samples were evaluated retrospectively, the inability to distinguish IRIS stage 1 cats from cats with no kidney disease arose. Thus, IRIS stage 1 was not used and all cats with creatinine < 1.6 mg/dl were considered to be without kidney disease and placed under the "no CKD group." As a result, two final groups are formed; cats without kidney disease (no CKD group) and cats with chronic kidney disease (with CKD group).

Parasite culture and antigen production

Techniques employed are according to the modified Cold Spring Harbor laboratory manual (Harlow and Lane, 1988). Spores of a canine subtype of *E. cuniculi* (ATCC 50502, American Type Culture Collection, Manassas, Virginia, USA) and tachyzoites of the RH strain (Sabin 1941) of *T. gondii* were cultivated separately in human foreskin fibroblasts (Hs68, ATCC CRL1635, Manassas, Virginia, USA) which were maintained in 75-mm^2 tissue culture flasks. Growth media used consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin and 100 µg/ml filters (GE Water and Process Technologies, Minnetonka, Minnesota, USA) and pelleted by centrifugation. After 3 washes in phosphate buffered saline (PBS), the cell suspension containing approximately 5 x 10^5 spores or 3 x 10^5

tachyzoites in 25 µl PBS was dispensed onto each well of 12-well Teflon-coated indirect fluorescent antibody test (IFAT) slides (Fisher Scientific, Pittsburgh, Pennsylvania, USA). Antigen containing slides were then left to dry at room temperature for 4 to 12 hours and subsequently stored at -20^0 C until use. *E. intestinalis* (ATCC 50506, American Type Culture Collection, Manassas, Virginia, USA) and *E. hellem* (Dr. Ron Fayer, USDA/ARS Environmental Microbiological Safety Laboratory, Beltsville, Maryland, USA) were cultivated separately in HS68 cells and processed identically for cross reactivity studies, defined as the reaction between *E. cuniculi* antigen and serum or plasma that was generated against similar antigens of *E. intestinalis* or *E.hellem*, resulting in fluorescing on the partial surface of the spores due to conserved antigen.

Indirect immunofluorescent antibody assays

Cat serum or plasma samples were diluted at 1:10 for *Encephalitozoon spp.* or 1:25 for *T. gondii* in phosphate buffered saline solution (PBS), and 25 µl was pipetted into each well of the antigen containing slides. The slides were incubated for 30 minutes at room temperature in a humidified chamber. Subsequent to 3 consecutive washes with PBS in a Copland jar to rinse off unbound antibodies, fluorescence labeled antibody goat anti-cat IgG (Kirkegaard and Perry Labs Inc, Gaithersburg, Maryland, USA) was diluted 1:5 in PBS and 25 µl was added to each well of the slides. The slides were incubated for 30 minutes at room temperature in a humidified chamber. Following 3 consecutive washes in PBS, bound and labeled goat anti-cat antibodies slides were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, Alabama, USA), and assessed using an Olympus BH60 epifluorescent microscope equipped with differential contrast optics (Olympus America Inc., Center Valley,

Pennsylvania, USA). Samples that exhibited fluorescence of the entire surface of the parasite were considered to be positive according to the IFA concentration reagents developed from *T. gondii* positive plasma or serum samples of experimentally infected cats. Positive and negative controls are identified on the basis of the IFA concentration reagents developed from *T. gondii* positive plasma or serum samples of experimentally infected cats tested in conjunction with our initial sample population.

Statistical analysis

To determine the association of *E. cuniculi* or *T. gondii* antibodies in cat populations without kidney disease and with CKD, samples were analyzed by Fisher's exact test. Statistic analyses were performed using SAS® 9.2 Software (Cary, North Carolina, USA). The relationships examined are presented in Tables 1 and 2. The 95% confidence intervals (CI) for prevalence data were calculated for each relationship. P values less than 0.05 were considered statistically significant.

RESULTS

Prevalence of antibodies to *E. cuniculi* and *T. gondii*

Antibodies to *E. cuniculi* were detected by IFA in the sera and plasma of 15 (6%) of the 232 samples, and 4 (11%) of the 36 cats with CKD (Table 1). Antibodies to *T. gondii* were found in 63 (27%) of the 232 cats and 10 (27%) of the 36 cats with CKD (Table 1). If cats with disparate results due to multiple sampling were evaluated using the disparate result for each cat a net gain of 2 *E. cuniculi* positive cats without CKD and a net loss of 1 *E. cuniculi* positive cat with CKD would have been identified. As for *T. gondii* there would be a net gain of 1 *T. gondii* positive cat without CKD and 4 *T. gondii* positive cats with CKD identified.

There was no cross reactivity observed among *E. cuniculi*, *E. intestinalis*, and *E. hellem* when all 15 *E. cuniculi* positive samples were examined by the IFA against spores of these parasites.

Statistical analysis

There were no significant differences ($P > 0.05$) in the prevalence of *E. cuniculi* antibodies in cats with or without CKD. Likewise, there were no significant differences ($P > 0.05$) in the prevalence of *T. gondii* antibodies in cats with or without CKD. Statistical analysis did not demonstrate any statistically significant differences ($P > 0.05$) in any of the other relationships examined (Table 2).

DISCUSSION

The present study examined the prevalence of IgG antibodies to *E. cuniculi* in cats with and without chronic kidney disease from Virginia to investigate the possibility that this parasite plays a role in CKD in cats. There are no previous studies on the prevalence of antibodies to *E. cuniculi* in cats in the USA. Unfortunately, no known positive cat-anti *E. cuniculi* sera from naturally or experimentally infected cats were available to use in developing the IFA and the concentration of reagents was based on experience using *T. gondii* cat sera in the IFA. Due to inherent difficulties in subjective serological tests like the IFA, we chose to limit variability by using results from only the first sample if cats were sampled multiple times. The overall seroprevalence of *E. cuniculi* in the population of 232 cats in the present study was 6%. This is lower than the 17 (24%) of 72 cats from Eastern Slovakia (Halánová et al. 2003) examined using an IFA. There was no cross reactivity observed using sera from *E. cuniculi* positive cats with spores of *E. intestinalis*, and *E. hellem*. Spores of *Ent*. *bieneusi* were not available to examine cross-reactivity to *E. cuniculi* positive cat sera because this parasite cannot be grown in cell culture.

Our finding of 27% seroprevalence of *T. gondii* is lower than previous reports of 34% and 36% in owned cats from assorted locations in the United States (Dubey et al., 2009). A national study of the overall prevalence of *T. gondii* in cats from the United States has not been undertaken. Additionally, the individual profile of cats, which encompasses age, life style of the cat (stray versus domestic), the serologic test employed, and the screening dilution, likely contributes to the variation in percentages reported in the prevalence of *T. gondii* infections in cats (Dubey, 2009).

Statistical analysis of the relationships examined in the present study (Table 2) found no significant differences ($P>0.05$) in antibody positive or antibody negative cats. Thus, our study indicates it is unlikely that either *E. cuniculi* or *T. gondii* plays an important role in CKD in cats in the United States.

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LIST OF TABLES

TABLE 1. PREVALENCE OF POSITIVE SERUM ANTIBODIES TITERS AGAINST *ENCEPHALITOZOON CUNICULI* AND *TOXOPLASMA GONDII* IN A POPULATION OF 232 CATS

Group	Total examined	*E. cuniculi* positive	*T. gondii* positive
Cats without CKD	196	11	53
Cats with CKD	36	4	10
total	232	15	63

TABLE 2. ASSOCIATION OF *ENCEPHALITOZOON CUNICULI* AND *TOXOPLASMA GONDII* SEROPOSITIVITY WITH FELINE CHRONIC KIDNEY DISEASE

Relationships	Two-sided P-value
E. cuniculi antibody and total population	0.2606
E. cuniculi antibody and CKD in *T. gondii* antibody positive population	1.0000
E. cuniculi antibody and CKD in *T. gondii* antibody negative population	0.0773
T. gondii antibody and CKD in total population	1.0000
T. gondii antibody and CKD in *E. cuniculi* antibody positive population	0.5165
T. gondii antibody and CKD in *E. cuniculi* antibody negative population	0.6735
T. gondii antibody and *E. cuniculi* antibody in the total population	1.0000
T. gondii antibody and *E. cuniculi* antibody in the CKD positive population	0.5378
T. gondii antibody and *E. cuniculi* antibody in the CKD negative population	0.4925

MANUSCRIPT 2

PREVALENCE OF ANTIBODIES TO *SARCOCYSTIS NEURONA* IN CATS FROM VIRGINIA AND PENNSYLVANIA

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Key words: *Sarcocystis neurona*, equine protozoal myeloencephalitis, cat

ABSTRACT

Sarcocystis neurona is best known as the causative agent of equine protozoal myeloencephalitis of horses in the Americas. Domestic cats (*Felis domesticus*) were the first animals described as an intermediate host for *S. neurona*. However, *S. neurona*-associated encephalitis has also been reported in naturally infected cats in the United States. Thus, cats can be implicated in the life cycle of *S. neurona* as natural intermediate hosts. Hence, the present study examined the seroprevalence of IgG antibodies to merozoites of *S. neurona* in populations of domestic cats from Virginia and Pennsylvania. Overall, serum or plasma from 441 cats (232 from Virginia and 209 from Pennsylvania) was tested by an indirect immunofluorescent assay at a 1:50 dilution. Antibodies to *S. neurona* were found in 32 (7%) of 441 cats. Of these, 22 (9%) of the 232 cats from Virginia and 10 (5%) of the 209 cats from Pennsylvania were seropositive for *S. neurona*.

INTRODUCTION

Sarcocystis neurona, an apicomplexan parasite, is the primary etiologic agent of a frequently diagnosed neurological disease in equids known as equine protozoal myeloencephalitis (EPM) (Dubey, Lindsay, Saville et al., 2001). Its life cycle alternates between the established definitive host, the Virginia opossum (*Didelphis virginiana*), and a wide range of mammalian intermediate hosts. Domestic cats (*Felis domesticus*), sea otters (*Enhydra lutris*), raccoons (*Procyon lotor*), nine-banded armadillos (*Dasypus novemcinctus*), striped skunks (*Mephitis mephitis*), and fishers (*Martes pennanti*), have been described as intermediate hosts (Dubey et al., 2000; Cheadle, Tanhauser et al., 2001; Cheadle, Yowell et al., 2001; Dubey, Lindsay, Saville et al., 2001; Dubey, Rosypal et al., 2001; Dubey, Saville et al., 2001; Tanhauser et al., 2001; Butcher et al., 2002; Turay et al., 2002; Gerhold et al., 2005). The horse (*Equus caballus*) is involved as an aberrant host (Dubey et al., 2000).

Immunocompetent cats fed *S. neurona* sporocysts seroconvert by day 20 post-inoculation in an agglutination test, while some cats treated with cortisone seroconvert as early as 7 days after inoculation (Dubey, Lindsay, and Saville, 2002). Few studies have examined the seroprevalence of *S. neurona* in cats; therefore, the present study was conducted to investigate the seroprevalence of antibodies to *S. neurona* in cats from Virginia and Pennsylvania.

MATERIALS AND METHODS

For the present study, 441 feline serum or plasma samples were collected. Serum or plasma was obtained from 232 cats from Virginia in an ongoing study of kidney disease conducted in the Department of Small Animal Clinical Science at the Virginia-Maryland Regional College of Veterinary Medicine, Blacksburg, Virginia. An additional 209 serum samples were acquired from an animal shelter in Philadelphia, Pennsylvania. These samples were initially used in a study of the seroprevalence of *Toxoplasma gondii* (Dubey et al., 2008). Serum and plasma samples were stored at 20 °C until used.

Techniques employed are according to the modified Cold Spring Harbor laboratory manual (Harlow and Lane, 1988). Merozoites of the SN-37R isolate (Sofaly et al., 2002) of *S. neurona* were cultivated in African green monkey (*Cercopithecus aethiops*) kidney cells, (CV-1, ATTC CCL-70, American Type Culture Collection, Manassas, Virginia) and maintained in 75-mm^2 tissue culture flasks to confluence. Growth media used consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin and 100 µg/ml streptomycin per ml. When the monolayer reached confluence, the growth medium was removed and replaced by a maintenance medium of 2% (v/v) FBS with an otherwise identical formula. Flasks were incubated at 37 °C in a humidified incubator containing 5% CO_2 and 95% air. For collection of merozoites, the infected CV-1 cells were removed with a cell scraper and the media filtered through 3-µm polycarbonate filters (GE Water and Process Technologies, Minnetonka, Minnesota) and pelleted by centrifugation. After 3 washes in phosphate-buffered saline (PBS), the cell suspension, containing approximately 0.5

to 1 x 10^4 merozoites in 25 µl PBS, was dispensed onto each well of 12-well, Teflon-coated indirect fluorescent antibody test (IFAT) slides (Fisher Scientific, Pittsburgh, Pennsylvania). Antigen-containing slides were then left to dry at room temperature for 4 to 12 hr and stored at 20 °C until use. Cat serum or plasma was diluted at 1:50 in PBS, and 25 µl was pipette into each well of the antigen-containing slides. The slides were incubated for 30 min at room temperature in a humidified chamber. Subsequent to 3 consecutive washes in PBS, a secondary fluorescence-labeled goat anti-cat antibody (Kirkegaard and Perry Labs Inc., Gaithersburg, Maryland) was diluted 1:5 in PBS, and 25 µl were added to each well of the slides. The slides were incubated for 30 min at room temperature in a humidified chamber. Following 3 consecutive washes in PBS, slides were mounted in Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, Alabama) and assessed using an Olympus BX60 epifluorescent microscope equipped with differential contrast optics (Olympus America Inc., Center Valley, Pennsylvania). Samples that exhibited fluorescence of the entire parasite surface were considered to be positive. All positive samples were also examined at dilutions of 1:100 and 1:200. Positive and negative controls are identified on the basis of the IFA concentration reagents developed from *T. gondii* positive plasma or serum samples of experimentally infected cats tested in conjunction with our initial sample population.

RESULTS

Antibodies to *S. neurona* were detected by IFA in the serum and plasma of 32 (7%) of the 441 cats. Twenty-two (9%) were of the 232 cats from Virginia, and the other 10 (5%) were of the 209 cats from Pennsylvania. The 32 positive serum and plasma samples were examined at 3 dilutions. Fourteen were found positive at a 1:50 dilution, 2 were found positive at a 1:100 dilution, and 16 were found positive at a 1:200 based on the IFA concentration reagents developed from *T. gondii* positive plasma or serum samples of experimentally infected cats.

DISCUSSION

Equine protozoal myeloencephalitis is the most-commonly diagnosed neurological disease of horses from the Americas caused by a protozoan parasite (Dubey, Lindsay, Saville et al., 2001). As part of its life cycle, a variety of mammals, namely cats, raccoons, armadillos, skunks, and sea otters, can act as intermediate hosts and develop sarcocysts in their muscle tissues (Dubey et al., 2000; Cheadle, Tanhauser et al., 2001; Cheadle, Yowell et al., 2001; Dubey, Lindsay, Saville et al., 2001; Dubey, Rosypal et al., 2001; Dubey, Saville et al., 2001; Tanhauser et al., 2001). Opossums in the genus *Didelphis* act as the definitive hosts that are capable of excreting sporocysts in the feces as a result of ingesting tissues of intermediate hosts that harbor the sarcocysts (Dubey, Lindsay, Kerber et al., 2001). Horses are identified as aberrant or dead-end hosts in the parasite life cycle (Dubey et al., 2000).

A previous study reported the seroprevalence of antibodies to *S. neurona* to be 27% of 196 domestic pet cats (Rossano et al., 2002) from Michigan evaluated by IFAT. Using the direct agglutination test, 13% of 310 feral cats from Ohio were found to be positive (Stanek et al., 2003). None of 502 cats from Brazil was positive via the direct agglutination test (Dubey, Lindsay, Hill et al., 2002). The present study demonstrated that the seroprevalence of *S. neurona* in cats was relatively low in the 2 geographic locations examined. High seroprevalence has been found in raccoons from Connecticut (100% of 12; Mitchell et al., 2002) and from Fairfax County, Virginia (92% of 469; Hancock et al., 2004) using the SAT. Eleven (46%) of 24 skunks from Connecticut were positive by the SAT (Mitchell et al., 2002). These data suggest that raccoons and skunks are more susceptible to *S. neurona* infection than are cats, or that they have a

higher risk of exposure to sporocysts. The role of domestic cats as intermediate hosts in perpetuating the life cycle of *S. neurona* is probably minimal compared to that of raccoons, skunks, and armadillos.

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CONCLUSION

In domestic cats, kidney impairment can be facilitated by various mechanisms, one involving chronic kidney disease (CKD) and the second, parasitic infections, are discussed in the previous studies. CKD is characterized by cats with plasma creatinine ≥ 1.6 mg/dl, presence of a concurrent urine specific gravity of < 1.035, and sufficient clinical evidence for staging of CKD. It is well accepted that CKD is a commonly diagnosed feline health problem and it is recognized as a major health concern in owned cats. Up to 20% of all cats will develop CKD through their lives and 31% of cats over the age of 15 will be affected (Lulich et al, 1992). The presentation of losing kidney function is invariably irreversible, which elicits severe progression of disease (Boyd et al., 2008).

There are no previous studies on the prevalence of antibodies to *E. cuniculi* in cats in the USA. Manuscript 1 examined the prevalence of IgG antibodies to *E. cuniculi* from cats with and without chronic kidney disease from Virginia to investigate the possibility that this parasite plays a causative role in CKD in cats. The overall seroprevalence of *E. cuniculi* in the population of 232 cats in the present study was 6%. This is lower than the 17 (24%) of 72 cats from Eastern Slovakia (Halánová et al. 2003). There was no cross reactivity observed using sera from *E. cuniculi* positive cats with spores of *E. intestinalis*, and *E. hellem.*

The prevalence of IgG antibodies to *T. gondii* from cats with and without chronic kidney disease from Virginia was also examined to investigate the possibility that this parasite plays a causative role in CKD in cats. Our finding of 27% seroprevalence of *T.*

gondii is lower than previous reports of 34 and 36% in owned cats from assorted locations in the United States (Dubey et al., 2009). Additionally, the individual profile of cats, which encompasses age, life style of the cat (stray versus domestic), the serologic test employed, and the screening dilution, contribute to the variable reports in the prevalence of *T. gondii* infections in cats (Dubey, 2009). Statistical analysis of the relationships examined in the present study (Table 2) found no significant differences ($P>0.05$) in antibody positive or anitbody negative cats. Collectively, these results indicated it is unlikely that either *E. cuniculi* or *T. gondii* plays an important role in CKD in cats in the United States. Hence, the null hypothesis was rejected.

Manuscript 2 examined the prevalence of *S. neurona* in domestic cats. *S. neurona* is an apicomplexan parasite, best described as the agent for the most-commonly diagnosed neurological disease of horses from the Americas equine protozoal myeloencephalitis (Dubey, Lindsay, Saville et al., 2001). Domestic cats (*Felis domesticus*) were the first animals described as a proven intermediate. Once domestic cats were identified as potential natural intermediate hosts for transmuting infection to horses, great concern was generated in the equine community, resulting in removal of cats from farms. Serological studies from Michigan have reported *S. neurona* antibodies in 27% of 196 domestic pet cats by IFA (Rossano et al., 2002). In addition, studies from Ohio and Brazil have reported *S. neurona* antibodies in 13% of 310 feral cats and 0% of 502 cats by the direct agglutination test respectively (Stanek et al., 2003, Dubey, Lindsay, Hill et al., 2002). Our finding of 7% in 441 cats is within the range of previous studies. Finally, the seroprevalence of domestic cats was compared to other intermediate hosts; raccoons, and skunks. High seroprevalence rates of 100% in 12

raccoons from Connecticut (Mitchell et al., 2002) and 92% in 469 raccoons from Fairfax country, Virginia have been found by the direct agglutination test (Hancock et al., 2004). As for skunks, 46% of 24 from Connecticut were found positive by the direct agglutination test (Mitchell et al., 2002). It is convincing that raccoons and skunks are more susceptible to *S. neurona* infection in contrast with domestic cats, or they have a higher risk of exposure to sporocysts. Hence, the role of domestic cats as intermediate hosts in perpetuating the life cycle of *S. neurona* is likely minimal in comparison with that of raccoons and skunks. Thus, the null hypothesis was rejected as well.

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SUMMARY

The present studies were conducted to test our two hypotheses in respect to three porotozoan parasites in a population of domestic cats; *Encephalitozoo. cuniculi*, *Toxoplasm. gondii*, and *Sarcocystis neurona*. We hypothesized that *E. cuniculi* and *T. gondii* are unrecognized causes of chronic kidney disease (CKD) in domestic cats. In addition, domestic cats are important intermediate hosts for *S. neurona* transmission was hypothesized.

In manuscript 1, the prevalence of IgG antibodies to *E. cuniculi* and *T. gondii* in domestic cats with and without chronic kidney disease was examined. *E. cuniculi* antibody positive cats did not have a higher prevalence of CKD than other cats in the same population. Likewise, *T. gondii* antibody positive cats did not have a higher prevalence of CKD than other cats in the same population either. Collectively, the results indicated it is unlikely that either *E. cuniculi* or *T. gondii* plays an important role in CKD in cats in the United States. Thus, the first hypothesis was rejected.

As for manuscript 2, the prevalence of antibodies to *Sarcocystis neurona* in cats from Virginia and Pennsylvania was examined. The role of domestic cats as intermediate hosts in the transmission of *Sarcocystis neurona* infections was determined based on serological prevalence, which was not prevalent in contrast with other intermediate hosts; raccoons, skunks, and armadillos. Thus, the second hypothesis was rejected as well.

Further studies are encouraged to eliminate the possibility that *E. cuniculi* as a causative agent of CKD. One such recommendation would be examining biological samples of CKD cats by PCR, as a genetic analysis to confirm the findings of

serological tests, namely the IFA. In addition, to monitor the urine specific gravity of CKD stating cats to validate the chronic rather than acute kidney disease. Moreover, to develop a more effective method of separating IRIS stage 1 cats from cats without CKD. In regards to the degree of significance the domestic cats play as intermediate host, it is important to investigate the association of the antibody titer with clinical signs. Low prevalence resulted from IFA could either be an indication of infection or the immune system is controlling the infection. Therefore, the role of domestic cats as major intermediate hosts for the transmission of *S. neurona* awaits further investigation.