Detecting *Giardia*: Clinical and Molecular Identification

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ABSTRACT

The protozoan parasite *Giardia duodenalis* (syn. *G. lamblia, G. intestinalis*) can cause

diarrhea in humans, cats, dogs and other animals. *Giardia duodenalis* consists of eight

assemblages (A-H) that are morphologically identical but genetically distinct. Assemblages C-H

are generally species-specific, while A and B infect people and animals and are considered

potentially zoonotic. Most canine and feline isolates belong to their respective species-specific

assemblages, but isolates of assemblages A and B (predominantly found in humans) have also

been recovered from dogs and cats. Diagnosis of infection has historically been by morphologic

techniques (observing trophozoites on direct fecal smears or cysts on centrifugal zinc sulfate

fecal flotations), and it is currently recommended to use morphologic techniques in conjunction

with a sensitive and specific antigen test. Diagnosis is important for management of clinical

giardiasis in cats and dogs and also to identify the assemblage present to determine its zoonotic

potential.

In my dissertation research I evaluated diagnostic techniques in use for companion

animals, including centrifugal zinc sulfate fecal flotation, antigen tests optimized for use in dogs

and cats, direct immunofluorescent assay (IFA), and Polymerase Chain Reaction (PCR). I

showed that when compared to the reference IFA the veterinary optimized antigen tests

performed similarly and had no statistically significant differences in sensitivity or specificity

when combined with a centrifugal zinc sulfate fecal flotation test. Sensitivity and specificity by

comparison to IFA was ≥ 82% and ≥ 90%, respectively, for all diagnostic tests evaluated in dogs and cats. When analyzed via Bayesian analysis sensitivity and specificity for all diagnostic tests was ≥83% and ≥95%, respectively. The Bayesian analysis also showed that using the direct immunofluorescent assay (IFA) as the reference test was supported. I also evaluated PCR as a molecular diagnostic technique to detect *Giardia* infections in dogs with soft stool or diarrhea (mimicking clinical signs of infection). I utilized both conventional and real time PCR assays and compared the results to the recommended method of diagnosis, the zinc sulfate fecal flotation combined with an immunoassay test. I found that agreement between PCR and microscopy combined with an immunoassay was poor to fair and varied depending on the molecular parameters and size of the DNA target underscoring the complexity of test evaluation and molecular diagnostics for *Giardia*.

I also evaluated cats from a varied population (owned, shelter, feral) in Virginia to determine to what extent (if any) they were infected with potentially zoonotic assemblages of *Giardia.* The species-specific assemblage F was detected in 57% of the samples and assemblage A, which is considered potentially zoonotic, was recovered from 32% of the sampleI. In 11% both assemblages F and A were detected. We showed for the first time that cats in Virginia are infected with potentially zoonotic assemblages of *Giardia.*

GENERAL AUDIENCE ABSTRACT

Giardia duodenalis (syn. *G. lamblia, G. intestinalis*) is a parasite that can cause diarrhea in humans, cats, dogs and other animals. *Giardia* is divided into eight assemblages (A-H) that are identical when viewed under the microscope but when genetic analysis is performed they are actually distinct. Humans are infected with assemblages A and B. Dogs are commonly infected with the species-specific assemblages C and D, and cats are usually infected with their species-specific assemblage F. However dogs and cats can be infected with assemblages A and B and are therefore considered potentially zoonotic.

Giardia exists in two stages: the feeding stage called a trophozoite and the infectious stage the cyst. Diagnosing *Giardia* infections has historically been diagnosed by observing trophozoites on direct fecal smears or cysts on fecal flotation tests with zinc sulfate. There are also fecal antigen tests that detect *Giardia* antigen in the feces. It is recommended to combine antigen testing with the fecal flotation to detect infections. Diagnosis is important for the management of clinical disease in dogs and cats and also so that the assemblage an animal is infected with can be analyzed to determine if there is any zoonotic potential.

In my dissertation research we evaluated diagnostic techniques in use for dogs and cats, including centrifugal zinc sulfate fecal flotation and antigen tests optimized for use in dogs and cats. We showed that when compared to a reference test these veterinary optimized antigen tests performed very similarly to each other. We also evaluated a molecular diagnostic test to detect *Giardia* infections in dogs with soft stool or diarrhea (mimicking clinical signs of infection). We utilized two methods of molecular diagnosis and compared the results to the recommended method of diagnosis, the zinc sulfate fecal flotation combined with an antigen test. We found that

the molecular tests did not agree well with the recommended detection method and that test evaluation and molecular diagnostics for *Giardia* are complex.

We also evaluated cats from a varied population (owned, shelter, feral) in Virginia to determine to what extent (if any) they were infected with potentially zoonotic assemblages of *Giardia*. The species-specific assemblage (F) was detected in the 57% of the samples and assemblage A, which is considered potentially zoonotic, was recovered from 32% of the samples, and in 11% both assemblages F and A were detected. We showed for the first time that cats in Virginia are infected with potentially zoonotic assemblages of *Giardia*.

DEDICATION

This dissertation is dedicated to my family and friends, without their unwavering love and

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Chapters 2 through 4:

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INTRODUCTION

Giardia duodenalis (syn. *Giardia intestinalis*, *Giardia lamblia*) is a protozoan parasite that commonly infects humans and many animals including dogs and cats. *Giardia* consists of eight genetic assemblages (A-H) that are genetically distinct but morphologically identical. The only way to determine the assemblage present is via genetic analysis. Humans are infected with assemblages A and B. Assemblages C-H are found in animals and are usually species-specific. There have been no reports of humans infected with assemblages C-H in the United States. However assemblages A and B have a broad host range and have been recovered from dogs and cats. Therefore dogs and cats can be considered potential sources of zoonotic *Giardia* assemblages. There have been more studies assessing the assemblages of *Giardia* in dogsthan there have been in cats.

Infections can be asymptomatic or may result in acute or chronic diarrhea. The microscopic diagnosis of infections can be difficult because cysts are shed intermittently and trophozoites are only occasionally present in diarrheic feces. Immunoassays that detect soluble cyst antigen have been developed to improve *Giardia* detection. Studies evaluating immunoassays and fecal flotations have shown that no single test detects all infections. It is currently recommended to use morphologic techniques combined with an immunoassay to diagnose infections in companion animals. There is not a perfect diagnostic test for detecting *Giardia*, and it is widely accepted to use a direct immunofluorescent assay (IFA) as the reference test. The IFA has been shown to have high sensitivity and specificity for detecting *Giardia,* and it is used for diagnostic test comparisons as the de facto gold standard. However, false positive and false negative results can and do occur on the IFA.

Molecular techniques are being used more and more frequently to detect infectious organisms, and the same is true in regards to *Giardia* diagnosis in companion animals. Several reference labs now offer PCR panels to detect *Giardia* infections. These commercial tests utilize real time PCR and do not provide any information about the assemblage of *Giardia* present. The increased sensitivity (if any) that these PCR panels offer in detecting *Giardia* infections is unknown. Determining the assemblage of *Giardia* present in a fecal sample is done using PCR; it is widely known that not all cyst positive *Giardia* samples can be amplified by PCR, and as such an assemblage determination cannot be made. This is possibly due to PCR inhibitors in the fecal matrix. Even when attempts to isolate cysts from the feces and remove potential inhibitors are made before DNA extraction PCR can still fail.

Given the overall difficulties associated with diagnosing infections and the uncertainty regarding zoonosis from dogs and cats the work presented in this dissertation sought to address these issues by performing robust diagnostic comparisons and evaluating the assemblages of *Giardia* present in an understudied species, cats. The first study sought to compare immunoassays optimized for use in dogs and cats along with fecal flotation and IFA. The suitability of using IFA as the reference test was also determined by analyzing the data via Bayesian analysis to evaluate the tests without a gold standard reference test. This study showed that all of the tests optimized for use in dogs and cats performed very similarly to each other, and that any statistically significant differences among immunoassay sensitivities and specificities were mitigated when combined with the zinc sulfate fecal flotation. The second study evaluated the assemblages of *Giardia* present in cats from a diverse population (owned, shelter, or feral) in Virginia. This study utilized the recommended multilocus genotyping approach targeting four different loci to assess the *Giardia* assemblages present. The final study was designed to assess

the use of molecular diagnostics to detect *Giardia* infections in clinically affected dogs. The recommendation to detect infections in-house is to use fecal flotation with a sensitive and specific immunoassay for dogs and cats. The in-house tests were compared with two PCR assays to detect *Giardia*. This study found that agreement among the molecular tests and recommended in-house tests were poor, and underscored the complexity of diagnosing *Giardia* infections and detecting the organism via PCR.

Chapter 1: *Giardia duodenalis* – Literature Review

BACKGROUND

General History and Life Cycle

The parasite genus, *Giardia,* was first described by Antony van Leeuwenhoek in 1681 while was examining his own diarrheal stools under the microscope. (Adam, 2001; Wolfe, 1992) Antony van Leeuwenhoek did not name the organism he saw at the time *Giardia*, and it was quite some time before the genus name *Giardia* was coined and widely adopted; the first time *Giardia* was used as the genus name was in 1882 when the parasite was described in tadpoles by Kunstler. (Adam, 2001) In 1902 Stiles changed the name to *Giardia duodenalis,* and in 1915 Kofoid and Christiansen proposed the name *G. lamblia* which was followed by *G. enterica* in 1920. (Adam, 2001) Confusion about the name and number of species of *Giardia* came about because many investigators described new species based on host origin, while others based new species descriptions on morphology. (Adam, 2001) *Giardia duodenalis* (syn. *Giardia intestinalis*, *Giardia lamblia*) is widely used and accepted in the veterinary literature, while *G. lamblia* and *G. intestinalis* are used interchangeably in the human medical literature. (Carranza and Lujan, 2010; Thompson, 2000)

Giardia duodenalis is a flagellated protozoan parasite of the phylum Sarcomastigophora and order Diplomonidida with a worldwide distribution and can infect both humans and animals, and as such is considered a zoonotic parasite. (Adam, 2001; Feng and Xiao, 2011; Ryan and Cacciò, 2013; Thompson, 2000; Wolfe, 1992; Xiao and Fayer, 2008) There are two stages of the parasite in the life cycle: the cyst and trophozoite. Transmission is via the fecal oral route, and cysts are the infective stage. The cysts of *Giardia* are transparent and oval shaped measuring 10-12μm in length and about 7μm in width, while the trophozoite, which is the reproductive stage,

is pear shaped and about 15μm long and 8μm wide. (Bowman, 2014; Tangtrongsup and Scorza, 2010) Upon ingestion the cyst responds to pH changes in the stomach and then undergoes excystation in the small intestine, giving rise to two trophozoites that reproduce via longitudinal binary fission. (Gardner and Hill, 2001) The trophozoite has a ventral sucking disk that attaches to the mucosa of the small intestine. The trophozoite responds to changing environmental conditions in the small intestine and each trophozoite forms a cyst that is then passed in the feces. (Gardner and Hill, 2001) Cysts are shed intermittently and are immediately infective when passed in the feces. (Barr et al., 1998). The cystsare surprisingly hardy in the environment. Olson et al., (1999) showed that at 4°C cysts can survive for 11 weeks in the water, 7 weeks in soil, and up to 1 week in cattle feces, and that at 25°C the cysts do not remain infective for as long. Food and drinking water can easily become contaminated by cysts in the environment. (Carranza and Lujan, 2010; Thompson, 2000) Trophozoites can be passed in diarrheic feces, but do not survive long outside the host. (Bowman, 2014)

Pathology and Pathogenesis

In people *Giardia* infections usually cause a self-limiting illness with diarrhea, abdominal cramps, weight loss, and malabsorption, but asymptomatic infections are not uncommon in developing countries. (Feng and Xiao, 2011; Thompson, 2000) In animals many *Giardia* infections do not cause clinical signs, but acute or chronic diarrhea can occur. (Barr and Bowman, 1994) The pathogenesis of *Giardia* is not clearly understood, but i*n vitro* and *in vivo* studies have indicated that the mechanisms of disease are complex. (Ankarklev et al., 2010; Buret, 2008) Many different disease mechanisms have been proposed, including damage from direct contact with the trophozoite, inflammation of the mucosa after infiltration of lymphocytes

and mast cells, increased bile salt uptake, and the inhibition of brush-border enzymes. (Ankarklev et al., 2010)

A key factor of pathogenesis is the induction of enterocyte apoptosis (Cotton et al., 2011), which then results in disruption of the tight junctions. (Buret, 2008) These tight junctions are a part of the larger apical junctional complexes that create a selective barrier between adjacent enterocytes and separate the host tissue from the lumen of the intestine, and when these apical junction complexes are altered or damaged during *Giardia* infection the result is increased intestinal permeability. (Cotton et al., 2011). One example of tight junction disruption is that *Giardia* infection in humans affects the epithelial proteins that are involved in tight junction sealing capability, which in turn also disrupts the intestinal barrier (Buret, 2008).

It has also been determined that *Giardia* causes malabsorption of water and other nutrients, and that this is due to blunting of the microvilli (Buret, 2008). Studies have shown that people infected with *Giardia* suffer maldigestion due to a lack of enzymes from the microvillous brush border (Cotton et al., 2011). This malabsoprtion and maldigestion results in water being drawn into the lumen of the small intestine leading to intestinal distension and rapid peristalsis increasing intestinal transit (Cotton et al., 2011). Stress has also been shown to play a role in the induction of clinical signs as well as the immune status of the host (Buret, 2006; Buret, 2008). Different strains of *Giardia* also can vary in pathogenicity, which would influence the presence or onset of clinical signs, and several studies have suggested that the acute and chronic forms of giardiasis are caused by different species/assemblages. (Al-Mohammed, 2011; Haque et al., 2005; Homan and Mank, 2001; Read et al., 2002; Sahagun et al., 2008; Thompson and Monis, 2012)

MOLECULAR CHARACTERIZATION

Assemblages

Giardia duodenalis is divided into eight assemblages designated A-H. These assemblages are determined by protein and DNA polymorphisms, and each assemblage is morphologically identical, but genetically distinct (Cacciò and Ryan, 2008; Monis et al., 1999; Ryan and Cacciò, 2013). Detailed genetic studies of *Giardia* in the late 1980s identified specific genotypes that formed distinct groups via clustering analysis, and further studies showed that there were more genetic groupings within these groups, which resulted in the concept of *Giardia* consisting of genetic assemblages. (Thompson and Monis, 2012). Assemblages A and B have a broad host range, can infect both humans and animals, and are considered potentially zoonotic (Ballweber et al., 2010). Generally assemblages C-H have a more narrow host range and are considered to be species specific. Dogs are infected with assemblages C and D, cloven-hoofed livestock (cattle, sheep, pigs, etc.) are infected with assemblage E, cats are infected with assemblage F, rodents are infected with assemblage G, and assemblage H is found in marine mammals (Feng and Xiao, 2011; Ryan and Cacciò, 2013; Xiao and Fayer, 2008). Regarding subtypes, within assemblage A there are four subtypes (AI, AII, AIII, and AIV); human isolates belong to AI and AII, while animal isolates belong to AI, AIII and AIV (Monis et al. 2003). Within assemblage B subtypes BI, BII, BIII, and BIV have been proposed, with humans infected with subtypes BIII and BIV, and animals infected with BI and BII (Monis et al., 2003). However these subtypes are based on allozyme electrophoresis and are not supported when the DNA sequence is analyzed. Because of discrepancies in the literature regarding the validity of these subtypes within assemblage B, they are not considered true subtypes. (Feng and Xiao, 2011)

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Reported Assemblages in Dogs and Cats

Dogs are typically infected with the species-specific assemblages C and D. However assemblages A and B have also been detected in dogs (Ballweber et al., 2010). Researchers examining the assemblages of *Giardia* present in a UK animal shelter found that 1/41 (2%) of the samples with sequencing result was infected with the potentially zoonotic assemblage A and the remaining samples were assemblage C or D. (Upjohn et al., 2010) A similar finding was reported by the authors of a 2012 study evaluating *Giardia* assemblages in 183 dogs from the US; assemblages C and D were most commonly identified and 5 of 183 isolates were genotyped to assemblage A. (Scorza et al., 2012) Studies from Japan and Germany found that most dogs that were group housed were infected with assemblage A. (Itagaki et al., 2005; Leonhard et al., 2007) Covacin et al. (2011) used multilocus genotyping (MLG) to analyze sequences from 128 cyst positive canine fecal samples from owned dogs in the US and found that 41% were assemblage B, 28% assemblage A, 16% assemblage D, and 15% assemblage C.

Cats are also infected with both their species-specific assemblage (F) and the potentially zoonotic assemblages A and B. However, there are also some reports of cats infected with species-specific assemblages C, D, and E in addition to A, B, and F. (Jaros et al., 2011; Read et al., 2004; Scorza et al., 2012) In Mississippi and Alabama, cats were reported to have assemblages F and AI (Vasilopulos et al., 2007), and assemblages A and B were reported in cats from New York (van Keulen et al., 2002) and Ontario, Canada. (McDowall et al., 2011) In a study from Colorado 3 of 13 cats were infected with assemblages C and D, which are generally considered to be dog specific. These findings were not thought to be from contamination and the assemblage of 2 of the samples was confirmed by other gene targets. The remaining samples with interpretable sequence data were assemblage F (Scorza et al., 2012.) The potentially

zoonotic assemblages A and B were recovered in cats from Poland along with the canine specific assemblage D (Jaros et al., 2011), and in Japan Assemblage F was reported from 3 Household cats in Japan (Itagaki et al., 2005.)

Genotyping

The determination of *G. duodenalis* assemblages is based on genetic sequencing of various housekeeping genes. The most frequently used loci are the small subunit ribosomal RNA (ssu-rRNA), (Appelbee et al., 2003; Hopkins et al., 1997) b-giardin (bg), (Lalle et al., 2005) glutamate dehydrogenase (gdh), (Read et al., 2004) and triose phosphate isomerase (tpi). (Sulaiman et al., 2003) The currently recommended practice for genotyping *Giardia* isolates to determine the assemblage present is to utilize multilocus genotyping (MLG) using at least the gdh, bg, and tpi loci (Cacciò and Ryan, 2008; Covacin et al., 2011; Ryan and Cacciò, 2013) The sensitivity of each locus target can vary, (Gomez-Munoz et al., 2012) so utilizing a MLG approach can maximize the chances of identifying the *Giardia* assemblage that is present.

The ssu-rRNA gene is considered to be the most sensitive locus for genotyping, likely due to its highly conserved multicopy nature. (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012; McDowall et al., 2011) The bg, gdh, and tpi loci are much less conserved compared to the ssu-rRNA, but this difference allows them to distinguish among subtypes within assemblages. (Cacciò and Ryan, 2008; Covacin et al., 2011; Scorza et al., 2012; Sprong et al., 2009) Conversely the variability within these other loci results in decreased sensitivity of the PCR assays because they can result in mismatches in the binding region, which can result in conflicting genotyping results. (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012) McDowall et al., (2011) found that the ssu-rRNA locus was much more sensitive than the gdh, bg, and tpi

loci. The ssu-rRNA locus amplified 64% (75/118) of *Giardia* positive dog samples and 87% (13/15) of cat samples, while the other 3 loci were only able to amplify a maximum of 32%of the dog samples and a maximum of 27% of the cat samples. This has also been demonstrated in a study of dogs and humans living in the same community. (Traub et al., 2004) Three loci were evaluated for genotyping (ssu-rRNA, tpi, and elongation factor 1-alpha) and of the 3 loci the ssu-rRNA locus was determined to be the most sensitive, amplifying 83% of samples compared to the tpi and ef1-a loci, which amplified 55% of the samples each.

The ability of the more variable loci (bg, gdh, and tpi) to distinguish among subtypes within assemblages has been documented in the literature. (Cacciò and Ryan, 2008; Covacin et al., 2011; Scorza et al., 2012; Sprong et al., 2009; Traub et al., 2004) A study of human and dog samples found the species-specific canine assemblages C and D in humans using the ssu-rRNA locus, but when the tpi locus was sequenced and analyzed this finding was not sustained. (Traub et al., 2004) Authors utilizing the gdh and ssu-rRNA loci on a variety of animal and human specimens found that when using the ssu-rRNA locus isolates were genotyped to species-specific assemblages C and D, but when using the gdh locus they were determined to be potentially zoonotic isolates of assemblages A and B. (Read et al., 2004) This could be due partially to preferential amplification of assemblages when using different loci targets. (Cacciò and Ryan, 2008; Scorza et al., 2012) *Giardia* isolates from dogs were genotyped to assemblages B, D and C using the ssu-rRNA locus, but when genotyped using the bg locus assemblages A and B were preferentially amplified. (Covacin et al., 2011)

In addition to varying levels of sensitivity and preferential amplification of certain assemblages by each locus, *Giardia* genotyping is further complicated by the presence of mixed assemblages in a single infection. Mixed infections have been demonstrated in humans, dogs,

cats, cattle, goats, sheep, pigs, and wildlife. (Feng and Xiao, 2011) Sprong et al. (2009) characterized the genotype of 908 human and animal samples at 2 or more loci, and they found mixed assemblages in 13% of the samples. In dogs specifically 34% (45 of 134) had infections with mixed assemblages.

Several researchers have advocated considering assemblages A, B, and E as distinct species with separate species names (Ryan and Caccio 2013). These are the only three assemblages with completed whole genome sequences for which comparisons have been made (Franzen et al., 2009; Jerlström-Hultqvist et al., 2010). It has been further suggested that unique species names should be adopted as follows: assemblage A-*Giardia duodenalis*, assemblage B-*Giardia enterica*, assemblages C and D-*Giardia canis*, assemblage E-*Giardia bovis*, assemblage F-*Giardia cati*, and for assemblage G-*Giardia simondi* (Monis et al., 2009; Thompson and Monis, 2004, 2011; Thompson et al., 2008). However, there is still controversy that must be addressed before these names can be accepted; there is no proposed name for assemblage H, which is found in marine mammals, and the fact that assemblages C and D, which are genetically distinct, will be grouped into the same species are causes for concern. (Ryan and Caccio, 2013).

DIAGNOSIS

***Giardia* detection in humans**

Historically *Giardia* has been diagnosed using morphologic techniques; either a direct smear of the feces to observe motile trophozoites or observation of cysts via formol-ether or zinc sulfate concentration (Meyer and Radulescu, 1979). It was recommended to examine three fecal samples on nonconsecutive days, and if samples were negative, to examine intestinal fluid for trophozoites either via duodenal intubation or small intestinal biopsies. In 1970 Beal et al.,

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created the Enterotest capsule, which has a nylon string inside a gelatin capsule that is ingested and after several hours is retrieved and the fluid on the string is examined for trophozoites. Examinations of duodenal fluid were reported to be more reliable than fecal examinations by some, but others reported that fecal examination was more reliable than biopsy or duodenal fluid examinations (Wolfe, 1992). To improve *Giardia* detection immunoassays were developed that detect soluble cyst antigen in the feces (Wolfe, 1992). Now antigen detection assays are widely used for *Giardia* detection, and these include enzyme linked immunosorbent assay, monoclonal antibody, and direct fluorescent-antibody tests. (Fletcher et al., 2012) In 1987 the direct immunofluorescenct assay (IFA) was developed to detect *Giardia* using fluorescein isothyocyanate (FITC) labeled monoclonal antibodies against cell wall antigen in *Giardia* cysts (Garcia et al., 1987). These antibodies bind to the antigens on the *Giardia* cyst wall and show a bright green color under a fluorescent microscope to allow for easier detection. Early studies on human fecal samples showed the test was more sensitive and specific for detecting *Giardia* than conventional morphologic tests (Alles et al., 1995; Garcia et al., 1992).

***Giardia* detection in small animals**

In veterinary medicine several studies have shown that centrifugation using a 33% $ZnSO_4$ solution allows for the best recovery of *Giardia* cysts (Zimmer *et al.,* 1986; Payne *et al.,* 2002; Barr *et al.,* 1992; Zajac *et al.,* 2002). Correct identification of *Giardia* cysts can be difficult because the cysts are so small (10-12um) and transparent, so proper training is required, and when that is coupled with the sporadic nature of cyst shedding identification via zinc sulfate centrifugal fecal flotation can be difficult (Barr and Bowman, 1994; Bowman, 2014).

To improve *Giardia* detection in companion animals immunoassays have been developed that detect soluble cyst antigen in the feces. It has been recommended to use a direct smear and centrifugal fecal flotation in conjunction with a sensitive and specific fecal ELISA test for the diagnosis of *Giardia.* (Payne and Artzer, 2009) There are two USDA approved point of care immunoassays for *Giardia* antigen detection in veterinary medicine: the Idexx SNAP *Giardia* Antigen Test, and the Abaxis VetScan *Giardia* Antigen Test. The IDEXX SNAP Giardia Test has reported sensitivities ranging from 70% to 95% and specificities ranging from 92% to 100%, (Mekaru *et al.*, 2007; Rishniw *et al.*, 2010 and package insert) and the Abaxis VetScan has reported sensitivities and specificities of 98.1% and 99.3%, respectively. It is important to note that samples can be positive on fecal flotation but negative on an antigen test and vice versa. A study by Payne et al. (2002) found that 18 of 57 (31.6%) fecal samples were cyst positive but antigen negative, and that 4 of 94 (4.3%) fecal samples tested antigen positive but were cyst negative. The combination of the fecal flotation and antigen test has been shown to improve the sensitivity of both tests. In one study the SNAP *Giardia* test alone had a sensitivity of 85.3%, but when used with fecal flotation test sensitivity improved to 97.8% (Mekaru *et al.*, 2007). Correct diagnosis of *Giardia* is essential in the management of dogs that present with diarrhea. Moreover, identification and appropriate treatment also limit additional contamination of the environment with cysts (Rishniw *et al.*, 2010). This is particularly important since dogs can be infected with potentially zoonotic assemblages of *Giardia.* In 2007 Rimhanen-Finne et al. used the IFA as the reference test to evaluate human antigen test performance to detect *Giardia* in dogs, and since that time the IFA has been adopted as the reference test for evaluating *Giardia* tests in companion animals (Geurden et al., 2008; Mekaru et al., 2007; Rishniw et al., 2010).

With the advent of PCR, it has been assumed that it is the most sensitive diagnostic test for the detection of *Giardia,* particularly the use of the SSU-rRNA locus. (Bouzid et al., 2015; Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012) In some prevalence studies PCR detected infection more often the immunofluorescent antibody test (IFA), the traditional gold standard for *Giardia* testing. In Spain, Gomez-Munoz et al. (2012) diagnosed more *Giardia* infections with PCR, finding 107 out of 120 (89.2%) samples to be positive by nested PCR at the SSU-rRNA locus; 30 of those positives were negative by IFA. Several large diagnostic laboratories offer diarrhea panels that include *Giardia* PCR but have not published information indicating what improvement in sensitivity is offered by PCR test compared to the method recommended to practitioners of direct smear and centrifugal fecal flotation in conjunction with a sensitive and specific fecal ELISA test. In a recent survey, Tupler et al (2012) found 19 *Giardia* positive pound dog samples with PCR; only 11 of these tested positive by antigen detection and none by fecal flotation, but these investigators used a sodium nitrate flotation solution without centrifugation as opposed to the recommended zinc sulfate centrifugal fecal flotation for the recovery of *Giardia*. However, in other studies PCR did not detect all cyst positive samples, and much like the combination of fecal flotations and antigen testing, it is possible to have cyst positive samples that are PCR negative and vice versa. Tangtrongsup and Scorza (2010) stated that "in experiments in our laboratory, *Giardia* PCR fails to amplify DNA from approximately 20% of samples that are positive for *Giardia* cysts or antigens in other assays." In a genotyping study of 238 dogs that were positive for *Giardia* via fecal flotation only 148 (62%) were positive by PCR (Covacin et al., 2011). Researchers evaluating *Giardia* genotypes in cats found that 13.6% (34/ 250) were cyst positive on IFA, but only 23 of the 34 cyst positive samples resulted in PCR amplicons (Vasilopulos et al., 2007).

Giardia prevalence in dogs and cats

The prevalence of _Giardia_ infection in dogs and cats varies depending on the age, clinical status, housing, and geographic region of the animals surveyed (Rishniw et al., 2010); the reported prevalence is also influenced by the detection method used. (Ballweber et al., 2010) Reported prevalence in dogs can be as little as 0% and as much as 100%, with higher rates in shelters and breeding colonies. (Rishniw et al., 2010) In a 1996 study by Blagburn et al. a _Giardia_ prevalence of 0.62% was found in a national survey of dogs in animal shelters. However the authors believed that their study underestimated the prevalence of _Giardia_ because of the detection method used, which was centrifugal fecal flotation using sugar solution as opposed to the recommended zinc sulfate flotation solution. A study reviewing the results of 1,199,293 canine fecal samples submitted to Antech Diagnostics for zinc sulfate fecal flotations found a _Giardia_ prevalence of 4%, and the authors suggest that the reason the prevalence of parasitism was so low is that the study population was composed of mostly adult well cared for pets who received routine veterinary care. (Little et al., 2009) Tupler et al. (2012) performed a study of 100 dogs entering a Florida animal shelter (50 with diarrhea and 50 with normal stool) utilized sodium nitrate for passive fecal flotations, the SNAP _Giardia_ antigen test, and PCR to detect _Giardia._ Investigators found that 16% (18/50) of dogs with normal stool and 22% (11/50) with diarrhea were infected with _Giardia._ (Tupler et al. 2012) None of these infections were detected by fecal flotation, and of the 19 positive samples from PCR 11 were also positive on the SNAP test. (Tupler et al., 2012)

Prevalence variation is observed in cats as well, with higher prevalence reported in clinically affected cats. (Hill et al., 2000; Vasilopulos et al., 2006) An evaluation of client-owned and shelter cats in Colorado found that owned cats had a higher prevalence of _Giardia_ infection

than the shelter cats, and that cats with diarrhea had higher rates of infection with *Giardia* than healthy cats. (Hill et al., 2000) Prevalence of *Giardia* infection in cats ranges from less than 1% to as much as 44%. (De Santis-Kerr et al., 2006; Fayer et al., 2006; Gookin et al., 2004; Hill et al., 2000; Lucio-Forster and Bowman, 2011; Mekaru et al., 2007; Vasilopulos et al., 2006) A study of 211,105 cats visiting Banfield hospitals found a prevalence of 0.58%, (De Santis-Kerr et al., 2006) while a study consisting of cats from catteries at an international cat show had a much higher prevalence of 31%, (Gookin et al., 2004) and a closed cat colony at the USDA had 44% (8/18) of cats infected with *Giardia.* (Fayer et al., 2006) The increased prevalences are likely due to the group-housing situation of the animals, and in studies of pet cats the reported prevalence is at most 15%. (Ballweber et al., 2010) In a 2006 US study authors utilized a fecal antigen test in symptomatic dogs and cats and reported a national prevalence of 15.6% and 10.3% respectively. (Carlin et al., 2006) A 2010 study by researchers in Canada utilized direct fecal smears and the Idexx SNAP test and found a *Giardia* prevalence of 16.0% and 7.7% in symptomatic dogs and cats respectively. (Olson et al., 2010)

Giardia treatment

There are no FDA approved drugs for the treatment of giardiasis in dogs and cats, but fenbendazole and metronidazole are often used to treat *Giardia* infections in dogs. (Bowman, 2014) Studies have demonstrated that using the anthelmintic dose (50 mg/kg PO for 3 days) of fenbendazole is an effective *Giardia* treatment. In a 1994 study Barr et al. (Barr et al., 1994) found that naturally infected beagles administered fenbendazole, at 50 mg/kg PO for 3 days were negative for *Giardia* cysts 5 days post-treatment. Prior to the start of the study and during treatment feces was normal in all dogs, however during the 10-day observation following treatment dogs in the control group (untreated) and one of the treatment groups did have soft

stool and diarrhea. A study utilizing experimentally infected beagles found that fenbendazole at 50 mg/kg PO for 3 days eliminated *Giardia* cyst shedding in 9 of 10 dogs, with the one positive sample occurring 3 weeks after treatment. (Zajac et al., 1998) Successful treatment has also been demonstrated using a febantel-pyrantel-praziquantel combination product. Febantel is a pro-benzimidazole that is metabolized to fenbendazole and oxfendazole in the liver. (Bowman, 2014) Payne et al. (Payne et al., 2002) demonstrated that the febantel-pyrantel-praziquantel combination product when given for 3 days eliminated cyst shedding in most (7/9) of the dogs. Metronidazole (22mg/kg 2x/day for 5 days) has also been shown to be efficacious in treating *Giardia* infections in dogs. (Zimmer and Burrington, 1986) Ronidazole has also been used in conjunction with intensive hygiene practices to successfully treat *Giardia* infections in group-housed dogs. (Fiechter et al., 2012)

Scorza et al. (2006) used the febantel-pyrantel-praziquantel combination product (56.5 mg/kg, 11.3 mg/kg, 11.3 mg/kg, respectively, PO, q24h, for 5 days) to successfully treat cats. Four of the six kittens that received the combination product tested negative for *Giardia* on IFA after the conclusion of the experiment. (Scorza et al., 2006) Metronidazole has also been used in cats. (Kirkpatrick and Farrell, 1984; Scorza and Lappin, 2004) Cats with diarrhea that were treated with metronidazole (10mg/kg 2x/day for 5 days) ceased *Giardia* cyst shedding during the observation period (5-6 weeks) and clinical signs resolved or were markedly reduced in all animals. (Kirkpatrick and Farrell, 1984)

***Giardia* Prevention and Control**

Complete elimination of *Giardia* is difficult because when the cysts are shed they are immediately infective, making reinfection highly possible (Bowman, 2014; Payne and Artzer,

2009; Tangtrongsup and Scorza, 2010). Preventing fecal contamination of the environment is crucial to preventing reinfection. Feces from infected dogs and cats should be picked up immediately after defecation (Tangtrongsup and Scorza, 2010). Bathing to remove any fecal debris containing cysts in the fur is also recommended (Fiechter et al., 2012; Payne and Artzer, 2009; Tangtrongsup and Scorza, 2010). Disinfection of the kennel or home is also recommended; quaternary ammonium products, boiling water, and chlorine, are described as effective against *Giardia* cysts. (Jarroll et al., 1981; Kahn et al., 2010) There were previously vaccines licensed for *Giardia* prevention in dogs and cats, although there were mixed reports of efficacy (Anderson et al., 2004; Olson et al., 2000; Payne et al., 2002). The vaccine is categorized as not recommended in The 2006 American Animal Hospital Association canine vaccine guidelines (Paul et al., 2006), and it has since been discontinued by the manufacturer (Tangtrongsup and Scorza, 2010).

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Chapter 2: Comparison of Diagnostic Techniques for Detection of *Giardia duodenalis* in Dogs and Cats.

ABSTRACT

Background: A comprehensive evaluation of currently available patient-side diagnostic tests for canine and feline *Giardia* infection has not been performed, and there is discordance among the previously published diagnostic comparisons.

Hypothesis/Objectives: We sought to evaluate two patient-side commercial *Giardia* diagnostic tests, zinc sulfate ($ZnSO_4$) fecal flotation, a direct immunofluorescence assay (IFA), and a newly licensed immunoassay in a population of dogs and cats, and to compare two methods of analysis: comparison to a reference test and Bayesian analysis.

Animals: Fecal samples from 388 animals (127 feline and 261 canine) located in Colorado, Oklahoma, and Virginia.

Methods: Fecal samples were tested for *Giardia* by all five diagnostic tests. The results were analyzed via comparison to a reference test (IFA) and by Bayesian analysis.

Results: Sensitivity and specificity by comparison to IFA was $\geq 82\%$ and $\geq 90\%$, respectively, for all diagnostic tests in dogs and cats. When analyzed via Bayesian analysis sensitivity and specificity was $\geq 83\%$ and $\geq 95\%$, respectively.

Conclusion and Clinical Relevance: Both methods of statistical analysis support using the IFA as the reference test. When combined with centrifugal fecal flotation test there was no

longer a significant difference between the sensitivities of the commercial patient-side immunoassays.

Key words: *Giardia,* diagnostics, coproantigen, Bayesian

INTRODUCTION

Giardia duodenalis is a common enteric parasite of dogs and cats. Most canine and feline

infections are subclinical, but acute or chronic diarrhea can occur [12,124,221]. The prevalence of

Giardia infection varies depending on the age, clinical status, housing, and geographic region of

the animals surveyed [187]; the reported prevalence is also influenced by the detection method used

[12]. Historically, diagnosis of *Giardia duodenalis* in dogs and cats has been via microscopic

examination of feces for trophozoites and/or cysts. [22] However, the microscopic diagnosis of

Giardia duodenalis is difficult because trophozoites are only occasionally present in diarrheic

feces and cysts are passed intermittently and can be difficult to identify. [13,117,247]The direct

immunofluorescence assay (IFA) has been shown to be more sensitive and specific for

diagnosing *Giardia* than conventional flotation tests; [7] as such the IFA has been adopted as the

reference test for evaluating *Giardia* tests in companion animals. [73,138,185,186] Fecal antigen

immunoassays have also been developed that detect a soluble cyst antigen in the feces to

improve *Giardia* detection. The Companion Animal Parasite Council (CAPC), which is widely

cited as a source of guidelines for parasite control, recommends that centrifugal fecal flotation be

used in conjunction with a patient side-immunoassay for diagnosing *Giardia* infections in

veterinary practices. Dryden[53] (2006) showed that the sensitivity of fecal flotations was

improved when used with a commercial immunoassay. [53]

Diagnostic test evaluation is performed by comparison to a gold standard. A gold

standard reference test "…is absolutely accurate. It diagnoses all of the specific disease that

exists and misdiagnoses none." [49] However, there is not always a true gold standard for test

comparison, and this is the case for *Giardia*. The IFA is widely accepted as the most sensitive

and specific test for *Giardia,*[7,73] and is often used as the reference test. However, it is important

to note that it is not a true gold standard. Using an imperfect reference test as the gold standard for diagnostic comparison can lead to miscalculation of test performance for the test(s) being evaluated. Several authors have likened this practice to using an "alloyed" gold standard.[73,168,215] To overcome this problem a Bayesian analysis can be performed. The Bayesian analysis is a statistical method that allows for diagnostic test evaluation in the absence of a gold standard[49] and has previously been used for *Giardia* diagnostic test evaluations.[73,74,168]

Diagnostic test evaluations for small animal infections have been performed using various *Giardia* diagnostic tests, [53,73,138,168,187] but not all have included a reference test. The purpose of this study was to evaluate diagnostic tests that are optimized for *Giardia* in dogs and cats, and are commercially available in the US, with each other by comparison to a reference test (IFA) and to compare the diagnostic tests including the IFA using a Bayesian analysis.

MATERIALS AND METHODS

Fecal Specimen Collection and Screening

Fecal samples (n = 338) were collected from dogs and cats at three distinct study sites. Study sites were parasitology laboratories at veterinary teaching hospitals in Fort Collins, Colorado; Stillwater, Oklahoma and Blacksburg, Virginia. Samples at all 3 sites consisted of hospital submissions (wellness exams and clinical cases), plus collection surveys from animal shelters, and rescue organizations.

At each study site samples were screened for the presence of *Giardia duodenalis* cysts with zinc sulfate ($ZnSO_4$) centrifugal fecal flotation as outlined below. *Giardia* positive samples of sufficient quantity to perform all diagnostic tests were included in the study. Fecal consistency and the presence of other parasite(s) were recorded for each sample. For each

Giardia positive sample, a matching *Giardia* negative sample (by $ZnSO_4$ flotation) of the same

fecal consistency from the same geographic location was included in the study. We used this

procedure to select samples because *Giardia* prevalence is low, and a survey would have resulted

in fewer positive samples for testing. Near the end of the study period all fecal samples from

shelter collections in Virginia regardless of positive or negative status were included in the

sample pool to increase the overall sample size. Samples from Oklahoma and Colorado were

refrigerated and shipped weekly on ice to the main study site (Blacksburg, VA) where the

immunoassays were performed. Samples collected at the Blacksburg study site were refrigerated

until immunoassays were performed.

Fecal Flotation

Zinc sulfate ($ZnSO_4$) centrifugal fecal flotation was performed as described in Zajac and

Conboy (2012). Two to four grams of feces was suspended in 20 mL of 33% $ZnSO_4$ solution,

(s.g.=1.18), mixed and strained through a double layer of cheesecloth. The fecal mixture was

centrifuged in a 15-ml tube with a coverslip in place for 5 minutes at 200 x g. After

centrifugation the coverslip was removed to a glass slide and scanned for *Giardia* cysts with a

compound microscope at 100X and 200X. *Giardia* and any other parasite species present were

recorded as present, but no quantitation was performed.

Immunoassays

The *VETCHEK* ELISA (TECHLAB[®] Inc., Blacksburg, VA) (VetChek) is an enzyme

immunoassay under development by TechLab for the qualitative detection of *Giardia* cyst

antigen in canine and feline fecal specimens. The test was used as a research assay and was

performed following the manufacturer's instructions. This test represents the first well plate ELISA that will be commercially available and is optimized for companion animals.

The SNAP® *Giardia* Antigen Test (IDEXX Laboratories, Westbrooke, ME) (SNAP) is a rapid patient-side enzyme immunoassay for the detection of *Giardia duodenalis* antigen in canine and feline feces. Tests were performed following the manufacturer's instructions.

The Abaxis VetScan® *Giardia* Antigen test (VetScan), is a rapid patient-side enzyme immunoassay for the detection of *Giardia duodenalis* antigen in canine feces only. Tests were performed following the manufacturer's instructions. Although this test is not intended for use in cats it was included in this comparison because practitioners may consider using the VetScan for cats if the test is available in their clinic. The VetScan cat results were excluded from the statistical analysis because the manufacturer does not intend for the test to be used in cats.

The MERIFLUOR® *Cryptosporidium/Giardia* direct immunofluorescence assay (Meridian Bioscience Inc., Cincinnati, Ohio) (IFA) was used as the reference test and performed following the manufacturer's instructions to identify *G. duodenalis* cysts in feces. Samples were run in batches, with positive and negative controls each time the test was run. Slides were examined at 100 and 200X magnification using a fluorescence microscope. A sample was considered positive if any *G. duodenalis* cysts were detected. The presence of *Cryptosporidium* oocysts was also recorded if any were observed, but results are not reported here.

Statistical Analysis

The data was analyzed by multiple methods. The sensitivity, specificity, and positive and negative predictive values (PPV and NPV) were calculated for each diagnostic test by comparison to the IFA [7,73,186]. The sensitivities and specificities of each diagnostic test were then

compared for differences using McNemar's test for significance of changes [2]. These analyses were conducted on data from dogs and cats separately for their respective diagnostic tests. Additionally, the patient side immunoassays (SNAP and VetScan) were analyzed in conjunction with the $ZnSO_4$ fecal flotation in dogs to mimic their use in clinics as recommended by CAPC. The combined results were then compared for differences in sensitivities and specificities using McNemar's test for significance of changes. Predictive values were also calculated at 5, 15, and 50% prevalences to examine diagnostic test performance in a variety of scenarios. A Bayesian analysis was performed to estimate the sensitivities and specificities of each diagnostic test for dogs and cats separately in the absence of a gold standard[73,103,104].

The Bayesian analysis framework expanded the 3-test script BayesDiagnosticTests\src\tt3.txt by Lawrence Joseph and colleagues (available at www.medicine.mcgill.ca/epidemiology/Joseph/Bayesian-Software-Diagnostic-Testing.html) to 5 tests for data from canine samples and to 4 tests for data from feline samples. Theoretical foundations of the actual software (BayesDiagnosticTests version 3.10.2, January 2016) have been reported[103,104]. The new scripts were implemented and executed using WinBugs version 1.4.3. Parameters to be estimated included overall prevalence for either canines or felines, and sensitivity and specificity for each of the diagnostic tests. Pairwise comparisons between diagnostic tests for sensitivity and specificity were included as differences within the scripts while positive and negative predicative values were generated from a combination of prevalence, sensitivity and specificity. Prior information for the model was obtained from published studies[53,73,138,187]and manufacturers' information. The prior information was collated and summarized as mean (± standard deviation) that was subsequently converted into alpha and beta parameters (Table 1) of the beta prior density. Research evidence to update the prior information

is presented in the form of cross-classification for canine and feline samples (Table 2). After initialization, all models were run for 25,000 iterations (5000 for burn-in and 20,000 for parameter estimation). Trace plots were inspected to verify convergence and the Monte Carlo error for each parameter was also verified to be less than 5% of the sample standard deviation. Finally, for each parameter of interest, a median together with the 2.5 and 97.5 percentiles were obtained from the posterior distribution.

RESULTS

Comparison to IFA

Overall 388 samples (127 feline and 261 canine) were included in the study and evaluated for the presence of *G. duodenalis* by each diagnostic test. The sensitivity, specificity, positive and negative predictive values (P/NPV) of each test compared to the IFA reference test for dogs and cats are presented in Tables 3 and 4. Analysis of canine data alone shows that the test with the highest sensitivity when compared to IFA was VetChek (94.1%) followed by SNAP (89.8%), and $ZnSO_4$ flotation (88.1%), with VetScan having the lowest sensitivity in dogs (82.2%). However VetScan was the most specific test (97.2%) in dogs followed by the SNAP test (95.1%), and VetChek and $ZnSO_4$ flotation, both with specificities of 92.3%. The results of the McNemar's test in dogs showed that the $ZnSO_4$ flotation, VetChek, and SNAP all had significantly higher sensitivities than the VetScan. However, the VetScan and the SNAP both had significantly higher specificities than the VetChek. When the patient-side immunoassays in dogs were combined with the $ZnSO_4$ fecal flotations (the CAPC result) there was no longer a significant difference in sensitivities between the SNAP and VetScan immunoassays in dogs. In

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cats all three tests had the same sensitivity (92.5%) when compared to the IFA (Table 4), and the

SNAP and $ZnSO_4$ had a higher specificity (98.9%) than the VetChek (95.4)

There were no statistically significant differences between the sensitivities and

specificities of any of the tests in cats. When the feline patient-side immunoassay (SNAP) was

combined with the $ZnSO_4$ fecal flotations (the CAPC result) sensitivity did improve from 92.5%

to 97.5%, but there was not a statistical significance. With regards to the geographic origin of

each sample there was no difference in sensitivity or specificity of the initial $ZnSO_4$ fecal

flotation when compared to IFA among the different study sites.

The cross-classified results from each diagnostic test are outlined in Table 2. Of the 388

samples tested 108 (24 feline and 84 canine) were positive for *Giardia* on all five diagnostic

tests, while 210 (82 feline and 128 canine) were negative on all diagnostic tests. In total 318/388

samples (82%) had concordant results across all five diagnostic tests. Among the 70 discordant

results (18%) there were 21 (30%) samples negative on the VetScan but positive on all other

tests (11 feline and 10 canine), and 9 (13%) that were negative on the $ZnSO_4$ but positive on all

other tests (0 feline and 9 canine).

When the prevalence of *Giardia* is set to 5% to mimic a low rate of infection in dogs the

PPV of each diagnostic test is lower than what was calculated from the clinical samples, but the

NPV was high and $\geq$ 99% for each test. At 15% prevalence the NPV was only slightly improved,

and PPV remained high. When the prevalence was raised to 50% PPV was $\geq$ 91% for all tests

and NPV was still high, but lower than what was estimated at 5 and 15% (Table 5). When

estimating PPV and NPV for the diagnostic tests in cats with a prevalence of 5% the PPV was

lower than what had been calculated for the clinical samples, but was higher than the PPV at 5%

prevalence in dogs, and similar to dogs NPV was $\geq$ 99% for each test (Table 6). When the prevalence was set to 15% PPV increased while NPV was $\geq$ 98% for each test. At 50% prevalence both PPV and NPV were high (Table 6).

Bayesian Analysis

The estimated sensitivity, specificity, positive and negative predictive values and 95% confidence interval of each diagnostic test for dogs using the Bayesian analysis are shown in Table 7. According to results of the Bayesian analysis the most sensitive diagnostic test was the IFA (99.4%), and it was also the most specific (99.7%) of all the diagnostic tests. While the VetScan had the lowest sensitivity it did have the highest specificity (99.3%) after the IFA.

The analysis of the feline data using the using the Bayesian analysis is presented in Table 8. The most sensitive and specific diagnostic test was the IFA (99.9% and 99.8% respectively). In cats the $ZnSO_4$ flotation (92.9%) was more sensitive than the SNAP (91.1%), and the VetChek was more sensitive than both (94.4%). Similar to its performance in dogs the SNAP (98.8%) followed the IFA in specificity.

DISCUSSION

Importance of Results

To the authors' knowledge this is the first study evaluating diagnostic tests for *Giardia* detection in dogs and cats using both comparison to a reference test and the Bayesian methodology for data analysis. Overall, when the diagnostic tests were compared to the IFA as the reference test in both cats and dogs no single test stood out as an obvious best diagnostic as all of the tests had relatively high sensitivities and specificities. While there were significant

differences in sensitivity between the patient side immunoassays (SNAP and VetScan) when compared to the IFA in dogs, this difference was no longer significant when combined with the $ZnSO_4$ flotation, and was not statistically significant on the Bayesian analysis either. This data strongly supports the recommendation to use centrifugal fecal flotation in conjunction with a patient side immunoassay for diagnosing *Giardia* infections in veterinary practices. Compared to other diagnostic evaluations in dogs our study had a much higher $ZnSO_4$ flotation sensitivity than has been reported previously and contradicts reports that question the accuracy of the $ZnSO_4$ flotation; this is most likely due to variations in the flotation procedure and is explained further below. [73,138,186] Regarding the evaluation of comparison methods, the Bayesian analysis in this study correlated well with the direct comparison to the IFA. Given how similar the results of the direct comparison to the "alloyed" gold standard—the IFA and the Bayesian analysis were, perhaps it is unnecessary to perform the Bayesian analysis. While it does remove the need for a gold standard, in this study the results between the two comparison methods are very similar, and reach the same conclusions, which we would not have known if both analyses had not been performed.

Comparison to IFA

No other studies have compared the same gamut of tests as the current study. [53,73,138,168,186] The VetChek, which is the newly licensed immunoassay being evaluated, performed very well and in this study lands at the top of ranking for sensitivity in dogs when compared to the IFA. When compared to the IFA the sensitivity of the SNAP test was determined to be 92.5% in cats and 89.8% in dogs which is lower than what is reported on the package insert (95%), but is similar to reported ranges in the literature. [73,138,186]

When examining the estimated predictive values for each diagnostic test when compared to the IFA at different prevalence levels the positive predictive values were highest at a higher prevalence of disease, and NPV was also high. At lower prevalences the NPV remained high, but PPV was lower. This association between prevalence and PPV is consistent with the typical correlation between the two factors, where a low prevalence results in a lowered PPV. The reverse is true for NPV and prevalence, where a lower disease prevalence results in higher NPV.

Increased ZnSO$_4$ Sensitivity

Sensitivitiy of the ZnSO$_4$ flotation in this study (92.5% in cats and 88.1% in dogs) when compared to the IFA was higher than others have reported; a study by Rishniw et al. reported a ZnSO$_4$ sensitivity of 49% in dogs while a sensitivity of 85.3% has been reported in cats [138]. The reason for the increased ZnSO$_4$ sensitivity is unclear, but could be due to the different flotation methods utilized. Both studies (and the present study) utilized trained personnel to perform and read the fecal flotations and while each study used ZnSO$_4$, there were differences in the flotation method. Rishniw et al. used 1g of feces with 3 minutes of centrifugation compared to our 2-4 g4g of feces and 5 minute centrifugation. Also rather than centrifuge with the coverslip on, they used a wire loop to transfer 5-6mm from the flotation's surface on to a slide. [187] Mekaru et al. used 2 g of feces for their fecal flotations, but they had a water step to rinse the samples before the flotation with ZnSO$_4$ with 2 ml of 70% disodium chromate added for contrast. They. They also allowedthe centrifuge tubes sit for 10 minutes after centrifugation before removing the coverslips to a slide. While each study did use ZnSO$_4$ as their flotation solution and these differences may seem minor, it is important to recognize these differences likely account for the increased sensitivity seen in the present study. Furthermore, the study design and sampling selection, inclusion based on ZnSO$_4$ result, possibly increased the sensitivity of the ZnSO$_4$ fecal

flotation. We screened samples using $ZnSO_4$ are equal., and included positives with a matching negative, so this could have increased the $ZnSO_4$ sensitivity in this study as compared to others.

Bayesian analysis

Sensitivities and specificities for the IFA, $ZnSO_4$, and SNAP were higher in the Bayesian analysis than the IFA in this study, and were also higher than what has been reported by others [73,138,187]. Sensitivity for the SNAP test was 92.2%, which is much higher than reported in the Bayesian analysis by Guerden et al (52% and 67%). The estimated sensitivity of the $ZnSO_4$ from the Bayesian analysis was also much higher in this study (90.95%) compared to others (34 and 65%). A possible explanation for these differences is that Guerden and colleagues used more non-informative priors, which has minimal impact on the parameters in question when building their model. We were able to utilize prior information from the Guerden et al study and others [73,138,187], which may have resulted in a more rigorous analysis in the present study.

Other studies have also found the IFA to be the most sensitive test when comparing tests using a Bayesian analysis. [73,74] This underscores the high performance of the IFA and provides more evidence to support the use of IFA as the de facto reference test even though it is not a true gold standard. However, the potential for false negatives/positives must still be acknowledged.

Practical Application of Results

When evaluating diagnostic tests it is important to consider the test purpose. The SNAP and VetScan are both rapid patient side tests that require no additional equipment. While the $ZnSO_4$ centrifugal fecal flotation test has a short turnaround time but does require tslow-start, slow-stop centrifuge.While the VetChek performed as well as the currently available diagnostics by direct comparison to the IFA and Bayesian analysis it should be noted that this is not designed

to be a patient side rapid test. However, given its performance in the test comparison it can be considered a sensitive and specific test for *Giardia* detection. The IFA requires the most specialized equipment and training and is not available patient side in the veterinary practice.

In conclusion there are now a number of highly sensitive and specific antigen tests that are optimized for detecting *Giardia* infections in companion animals. These include patient-side immunoassays and the well-plate ELISA presented in this study. None of these immunoassays can detect other parasites, and while the IFA has the added *Cryptosporidium* detection it still will not detect any other parasites. Only the $ZnSO_4$ fecal flotation has the ability to detect other parasites that may be present. Thus, it is important to remember that these immunoassays are only useful if *Giardia* is suspected, or if animals are being intentionally screened for *Giardia*.

TABLES

Table 1: Table 1. Prior information for each of the 11 parameters to be estimated by Bayesian analysis. N/A = Not available. N/A values were replaced with 7.51 and 4.56 for sensitivity and specificity, respectively. These values were approximately mid-way for all standard deviations. *The model would not estimate this parameter. As such non-informative values of (1,1) were used instead.

†To compute alpha and beta, a mean of 99.9% and a standard deviation of 0.1% were used.

Test	Parameter	Mean (%)	Standard deviation (%)	Alpha	Beta
	Prevalence	7.69	6.42	1.25	14.98
ZnSO$_4$	Sensitivity	67.15	25.67	1.58	0.77
	Specificity	96.85	4.04	17.13	0.56
Vet Chek	Sensitivity	93	N/A	9.80	0.74
	Specificity	99.7	N/A	0.44	0.001
SNAP	Sensitivity	84.77	7.51	18.56	3.33
	Specificity	97.27	4.56	11.45	0.32
VetScan	Sensitivity	98.1	N/A	2.26	0.04
	Specificity	99.3	N/A	2.33*	0.016*
IFA	Sensitivity	100†	0†	997.00	1.00
	Specificity	100†	0†	997.00	1.00

Table 2: Results from 5 diagnostic tests for the detection of *Giardia*. The number of samples with each result combination is shown with + indicating a positive and – indicating a negative result on the respective diagnostic test. ZnSO$_4$: Zinc sulfate centrifugal fecal flotation; Vet Chek: TECHLAB *VETCHEK* ELISA; SNAP: IDEXX *Giardia* Antigen Test; VetScan: Abaxis *Giardia* Antigen test; IFA MERIFLUOR® *Cryptosporidium/Giardia* direct immunofluorescent assay

ZnSO$_4$	VetChek	SNAP	VetScan	IFA	Number of Samples		
					Canine	Feline	Total
-	-	-	-	-	128	82	210
+	+	+	+	+	84	24	108
+	+	+	-	+	10	11	21
-	+	+	+	+	9	0	9
+	-	-	-	-	4	1	5
-	+	-	-	-	2	3	5
+	+	-	+	+	4	0	4
+	+	+	+	-	4	0	4
+	-	-	-	+	3	1	4
+	+	-	-	+	2	1	3
-	-	+	-	+	2	1	3
-	+	+	-	-	2	1	3
-	+	-	-	+	2	0	2
+	+	-	-	-	2	0	2
-	-	-	-	+	1	1	2
-	+	+	-	+	0	1	1
+	-	+	-	+	1	0	1
+	+	+	-	-	1	0	1
Total					261	127	388

Table 3: Diagnostic test performance in dogs compared to IFA. The sensitivity, specificity, positive and negative predictive values of each test with 95% confidence intervals (CI) when compared to the IFA reference test in dogs. Within columns different letters are significantly different (McNemar's p < 0.05). Cells without superscripts had no significant differences. $ZnSO_4$: Zinc sulfate centrifugal fecal flotation; Vet Chek: TECHLAB *VETCHEK* ELISA; SNAP: IDEXX *Giardia* Antigen Test; VetScan: Abaxis *Giardia* Antigen test; IFA MERIFLUOR® *Cryptosporidium/Giardia* direct immunofluorescent assay

Diagnostic Test	95% Confidence Interval			
	Sensitivity	Specificity	PPV	NPV
SNAP	89.8[b] (83.06—94.09)	95.1[bc] (90.24—97.61)	93.8 (87.76—96.97)	91.9 (86.36—95.3)
Vet Chek	94.1[b] (88.26—97.1)	92.31[a] (86.75—95.65)	90.9 (84.57—94.89)	94.7 (89.97—97.54)
VetScan	82.2[a] (74.32—88.06)	97.2[b] (93.03—98.91)	96.0 (90.26—98.45)	86.9 (80.77—91.25)
SNAP w/ $ZnSO_4$	97.5[ab] (92.79—99.13)	90.9[abc] (85.07—94.61)	89.8 (83.4—93.97)	97.7 (93.58—99.23)
VetScan w/ $ZnSO_4$	95.8[ab] (90.46—98.18)	92.31[abc] (86.75—95.65)	91.1 (84.81—94.97)	96.4 (91.74—98.43)
$ZnSO_4$	88.1[ab] (81.07—92.8)	92.3[ac] (86.75—95.65)	90.4 (83.68—94.57)	90.4 (84.55—94.2)

Table 4. Diagnostic test performance in cats compared to IFA. The sensitivity, specificity,

positive and negative predictive values of each test with 95% confidence intervals (CI) when

compared to the IFA reference test in cats. Within columns different letters are significantly

different (McNemar's $p < 0.05$). Cells without superscripts had no significant differences.

$ZnSO_4$: Zinc sulfate centrifugal fecal flotation; Vet Chek: TECHLAB *VETCHEK* ELISA;

SNAP: IDEXX *Giardia* Antigen Test; VetScan: Abaxis *Giardia* Antigen test; IFA

MERIFLUOR[®] *Cryptosporidium/Giardia* direct immunofluorescent assay

Diagnostic Test	95% Confidence Interval			
	Sensitivity	Specificity	PPV	NPV
SNAP	92.5 (87.56—95.97)	98.9 (95.89—99.99)	97.4 (86.5—99.53)	96.63 (90.55—98.85)
Vet Chek	92.5 (92.75—99.1)	95.4 (90.2—97.84)	90.24 (77.45-96.14)	96.51 (90.24—98.81)
$ZnSO_4$	92.5 (85.74—95.13)	98.9 (90.74—97.84)	97.4 (86.5—99.53)	96.63 (90.55—98.85)
SNAP w/ $ZnSO_4$	97.5 (87.12—99.56)	97.7 (92—99.37)	95.1 (83.86—98.65)	98.8 (93.7—99.79)

Table 5: Estimated predictive values for diagnostic tests in dogs when compared to IFA at different *Giardia* prevalence rates. PPV: positive predictive value; NPV: negative predictive value

Diagnostic Test	Prevalence					
	5%		15%		50%	
	PPV	NPV	PPV	NPV	PPV	NPV
SNAP	49	99	76	98	94	90
Vet Chek	39.1	99.6	68.3	98.8	96.7	84.5
VetScan	60.7	99	83.8	96.8	96.7	84.5
SNAP w/ $ZnSO_4$	36	99.8	65.4	99.5	91.4	97.3
VetScan w/ $ZnSO_4$	0.39	99.7	68.7	99.2	92.6	95.6
$ZnSO_4$	37.6	99.3	66.9	97.7	91.9	88.5

Table 6: Estimated predictive values for diagnostic tests in cats when compared to IFA at different *Giardia* prevalence rates. PPV: positive predictive value; NPV: negative predictive value

Diagnostic Test	Prevalence					
	5%		15%		50%	
	PPV	NPV	PPV	NPV	PPV	NPV
SNAP	81.5	99.6	93.7	98.7	98.8	92.9
Vet Chek	51.4	99.6	78	98.6	95.2	92.7
$ZnSO_4$	81.5	99.6	93.7	98.7	98.8	92.9
SNAP w/ $ZnSO_4$	69.1	99.9	88.2	99.6	97.7	97.5

Table 7: Bayesian analysis estimates of diagnostic test parameters in dogs. The sensitivity, specificity, positive and negative likelihood ratios of each test with 95% confidence intervals (CI) estimated by Bayesian analysis. Within columns different letters are significantly different (McNemar's $p < 0.05$). Cells without superscripts had no significant differences. $ZnSO_4$: Zinc sulfate centrifugal fecal flotation; Vet Chek: TECHLAB *VETCHEK* ELISA; SNAP: IDEXX *Giardia* Antigen Test; VetScan: Abaxis *Giardia* Antigen test; IFA MERIFLUOR® *Cryptosporidium/Giardia* direct immunofluorescent assay

Diagnostic Test	95% Confidence Interval			
	Sensitivity	Specificity	PPV	NPV
IFA	99.4 (98.86—99.79)	99.8[b] (99.28—99.98)	99.7 (99.04—99.97)	99.5 (99.05—99.84)
SNAP	90.5[ab] (85.03—94.92)	98.7[ab] (95.96—100)	98.2 (94.47—99.99)	92.8 (88.57—96.25)
VetChek	94.5[a] (90.97—98.69)	96.0[a] (91.94—98.83)	95.0 (89.95—98.56)	96.4 (92.76—98.97)
VetScan	83.3[b] (75.86—89.63)	99.3[b] (97.43—99.98)	98.9 (96.23—99.97)	88.1 (82.63—92.81)
$ZnSO_4$	88.7[b] (82.3—93.73)	95.5[a] (91.59—98.26)	93.9 (88.72—97.66)	91.3 (86.35—95.27)

Table 8: Bayesian analysis estimates of diagnostic test parameters in cats. The sensitivity, specificity, positive and negative likelihood ratios of each test with 95% confidence intervals (CI) estimated by Bayesian analysis. Within columns different letters are significantly different (McNemar's $p < 0.05$). Cells without superscripts had no significant differences. $ZnSO_4$: Zinc sulfate centrifugal fecal flotation; Vet Chek: TECHLAB *VETCHEK* ELISA; SNAP: IDEXX *Giardia* Antigen Test; VetScan: Abaxis *Giardia* Antigen test; IFA MERIFLUOR[®] *Cryptosporidium/Giardia* direct immunofluorescent assay

Diagnostic Test	95% Confidence Interval			
	Sensitivity	Specificity	PPV	NPV
IFA	99.9 (99.6—100)	99.8[b] (99.85—99.99)	99.5 (98.6—99.97)	99.9 (99.83—100)
SNAP	91.1[a] (82.67—96.9)	98.8[ab] (95.74—100)	96.8 (88.81—99.99)	96.6 (93.04—98.87)
VetChek	94.4[a] (86.43—99.08)	95.7[a] (90.5—98.89)	89.6 (78.19—97.24)	97.7 (94.41—99.64)
$ZnSO_4$	92.9[a] (83.27—98.64)	98.5[ab] (95.52—99.88)	96.1 (88.33—99.69)	97.2 (93.37—99.48)

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Chapter 3: *Giardia duodenalis* genotypes in cats from Virginia

ABSTRACT

Giardia duodenalis is considered a species complex that is divided into 8 genetically distinct but morphologically identical assemblages (A-H). Assemblages C-H are generally species-specific, while A and B infect people and animals and are considered potentially zoonotic. Assemblages A, B, C, D and F have all been reported from cats. The objective of the present study was to determine the assemblage(s) of *Giardia* present in cats from Virginia using multilocus genotyping and to assess if there were any differences among the assemblage(s) found in the population of cats surveyed (feral, shelter, owned) or their geographic location within Virginia. Samples that were positive for *Giardia* cysts by centrifugal $ZnSO_4$ fecal flotation and/or direct immunofluorescent assay (IFA) were genotyped using PCR and sequencing targeting fragments of the ssu-rRNA, gdh, bg, and tpi genes. In total 54 samples were analyzed by PCR and sequencing, 43 produced amplicons, and 37 samples had interpretable sequence data at one or more loci. Assemblage F was detected in 21/37 samples, AI (or AI like) was detected in 12/37 samples, and in 4/37 samples both assemblages F and AI (or AI like) were detected. The potentially zoonotic assemblage AI (or AI like) was detected in cats from animal shelters in Blacksburg and Richmond and from one feral cat. It is important to know that potentially zoonotic *Giardia* assemblages are present in cats in Virginia, although the number of positive samples did not allow for the determination of assemblage or sub-assemblage differences among cats from different populations,

INTRODUCTION

The zoonotic potential of *Giardia duodenalis* from animals varies, and is dependent on the assemblage(s) the animal.harbors. In North America the potentially zoonotic assemblages A and B have been reported from cats in Ontario, Canada, New York, Mississippi, and Alabama. (McDowall et al., 2011; Vasilopulos et al., 2007; van Keulen et al. 2002) As such, cats can be considered a potential source of zoonotic *Giardia* infection. (Bowman and Lucio-Forster, 2010; Lefebvre et al., 2006) There are over 156 million cats in the United States, of which 50 million are estimated to be feral or stray cats, (http://www.humanesociety.org/issues/feral_cats/). It has been suggested that cats may be a factor in the transmission of *Giardia duodenalis* to humans, especially considering cats' close proximity to people. (Paoletti et al., 2011)

In studies evaluating *Giardia* in cats in North America prevalences as high as 13% and as low as 0.58% have been reported. (De Santis-Kerr et al., 2006; Vasilopulos et al., 2006; Vasilopulos et al., 2007) *Giardia* has been closely scrutinized at the molecular level and is believed to consist of (at least) eight assemblages (A-H) that are genetically distinct but morphologically identical. (Dado et al., 2012; Feng and Xiao, 2011; Sprong et al., 2009) Assemblages A and B have the broadest host specificity and infect both humans and animals, and as such they are considered potentially zoonotic. (Ballweber et al., 2010) Generally, assemblages C and D infect dogs, assemblage E infects cloven-hoofed livestock, assemblage F infects cats, assemblage G infects rats, and assemblage H is found in marine mammals. (Feng and Xiao, 2011; Ryan and Cacciò, 2013) Assemblages C-H are generally considered to be species specific, but there have been some reports of other species of animals infected with these species-specific assemblages. (Ryan and Cacciò, 2013) Assemblages A, B, C, D, and F have all been reported in cats. (Ryan and Cacciò, 2013; Jaros et al., 2011; Read et al., 2004)

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Giardia assemblages are determined via PCR and sequencing gene fragments at specific loci, specifically the small subunit ribosomal RNA (ssu-rRNA), (Appelbee et al., 2003; Hopkins et al., 1997) b-giardin (bg), (Lalle et al., 2005) glutamate dehydrogenase (gdh), (Read et al., 2004) and triose phosphate isomerase (tpi). (Sulaiman et al., 2003) Mixed infections with more than one assemblage are possible, and cannot be differentiated with a single locus PCR. (Ballweber et al., 2010) Currently it is recommended that researchers use multilocus genotyping (MLG) when attempting to determine isolate assemblages. (Cacciò and Ryan, 2008; Covacin et al., 2011; Ryan and Cacciò, 2013)

The use of MLG is particularly important because the ability of each locus to identify *Giardia* assemblages varies. (Gomez-Munoz et al., 2012) The ssu-rRNA gene is a highly conserved multicopy housekeeping gene that has shown to be the most sensitive locus for genotyping. (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012; McDowall et al., 2011) The bg, gdh, and tpi, which are other loci that are commonly used in MLG, are much less conserved compared to the ssu-rRNA, but unlike the ssu-rRNA they are adequately discriminatory to distinguish subtypes within assemblages. (Cacciò and Ryan, 2008; Covacin et al., 2011; Scorza et al., 2012; Sprong et al., 2009) However, it has been stated that the variability found in these other loci (bg, gdh, tpi) may produce mismatches in the binding region, which then results in reduced sensitivity of PCR. (Cacciò and Ryan, 2008; Gomez-Munoz et al., 2012) For example, McDowall et al (2011) found that the ssu-rRNA primer amplified DNA in 64% (75/118) of the *Giardia* positive dog samples and 87% (13/15) of the cat samples and produced the most amplicons compared to the other 3 loci which were able to amplify *Giardia* DNA in at most only 32% the dog samples and 27% of the cat samples. Researchers using the ssu-rRNA, tpi, and elongation factor 1-alpha (ef1-a) loci to determine assemblages in humans and dogs living in the

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same area also found the ssu-rRNA locus to be the most sensitive compared to the tpi and ef1-a loci. (Traub et al., 2004) Scorza et al. (2012) did not use the ssu-rRNA locus and instead used the gdh, bg, and tpi loci, and found that the gdh locus was the most sensitive of the three loci used. There is also the possibility that some primers result in preferential amplification of some assemblages. (Cacciò and Ryan, 2008; Scorza et al., 2012) This has been demonstrated at the bg and ssu-rRNA loci by Covacin et al. (2011) who found that in dogs the ssu-rRNA locus amplified *Giardia* DNA from assemblages B, D and C and the bg locus preferentially amplified *Giardia* DNA from A and B. Read et al. 2004 found that isolates were genotyped to species-specific assemblages using the ssu-rRNA locus, but when genotyped using the gdh they were determined to be potentially zoonotic isolates of assemblages A and B The authors suggested that this was due to the less conserved nature of the gdh locus, which is able to discriminate among assemblage A subtypes. While the ssu-rRNA locus is more conserved and cannot determine subtypes, and also due to the different target fragment sizes of the two loci, with the gdh locus targeting a larger fragment size. (Read et al., 2004)

At this time there are five published studies describing the assemblage(s) of *Giardia* found in cats in the United States. (Fayer et al., 2006; McGlade et al., 2003; Scorza et al., 2012; van Keulen et al., 2002; Vasilopulos et al., 2007). Each of these studies had fewer than 20 cat samples with complete PCR sequences, and in four of the studies only one locus was analyzed. Three used the ssu-rRNA locus, (Fayer et al., 2006; McGlade et al., 2003; van Keulen et al., 2002), one used the gdh locus, (Vasilopulos et al., 2007) and one used the gdh, bg, and tpi loci. (Scorza et al., 2012) The objective of the present study was to determine the assemblage(s) of *Giardia* present in cats from Virginia using multilocus genotyping and to assess if there were any

differences among the assemblage(s) found in the type of cats surveyed (feral, shelter, owned) or their geographic location within Virginia

MATERIALS AND METHODS

Samples— Fecal samples were utilized that had been collected as part of other parasite prevalence surveys. The samples originated from animal shelters (n = 10) in northern Virginia (Prince William, Fauquier, and Stafford counties in Virginia (K. Monti)), from both a shelter (n=5) and trap-neuter-release program (n = 7) in Richmond, VA (S. Taetzsch), and from collections in Blacksburg, VA (n = 15) that included animal shelters and 2 submissions to the diagnostic parasitology laboratory at the Virginia Maryland College of Veterinary Medicine (Blacksburg, VA).

Morphologic testing—Giardia cyst-positive samples were identified by centrifugal $ZnSO_4$ fecal flotation as described by Zajac and Conboy (2011) and/or direct immunofluorescent assay following the manufacturer's instructions (MERIFLUOR® *Cryptosporidium/Giardia* IFA, Meridian Bioscience Inc.).

Cyst Isolation—Cysts were concentrated using sugar gradient density separation to isolate cysts as previously described. (Scorza et al., 2012) Briefly, 2 grams of feces were mixed with phosphate buffered saline solution containing ethylenediaminetetraacetic acid (PBS-EDTA), filtered through cheescloth and then pipetted on to Sheather's sugar solution (s.g. = 1.26) in a 15 mL conical tube and centrifuged at $800 \times g$ for 10 min. The top layer of the filtrate and the interface were then placed in a new 15 mL conical tube and centrifuged for 10 min at $1200 \times g$. The supernatant was discarded and the pellet was washed twice more with PBS-EDTA. After the

final wash the supernatant was discarded and the resulting cyst pellet was suspended in 1mL of PBS-EDTA and stored at 4°C until DNA extraction was performed.

DNA extraction—DNA was extracted from the concentrated fecal samples using the commercial DNeasy Tissue Kit from Qiagen (Venlo, the Netherlands). Reagents from the kit were used, but with modification as outlined below. (Santin et al., 2006) Fifty microliters of the cleaned fecal sample was added to 180 µL of ATL buffer, vortexed, and then 20 µL of proteinase K (20mg/ml) added, and the sample mixed again. Samples were incubated overnight at 55°C, after which 200 µL of AL buffer was added to the sample. For elution of the sample, 100 µL of AE buffer was used, and the sample was stored at −20° C until PCR was performed.

PCR—To determine the assemblage(s) of *G. duodenalis* in each sample multilocus genotyping was performed. Different regions of four genes were utilized for PCR and sequencing using previously described primers and cycling conditions for each locus with the following changes: 24µL/reaction of a commercial master mix (Promega GoTaq® Green Master Mix) and 24µl of water were used in place of the master mix described in the original publications (Appelbee et al., 2003; Hopkins et al., 1997; Lalle et al., 2005; Read et al., 2004; Sulaiman et al., 2003);2µl of DNA was used as the template as outlined by Scorza et al (2012) . The loci targeted were: small subunit ribosomal RNA (ssu-rRNA) (Appelbee et al., 2003; Hopkins et al., 1997), b-giardin (bg) (Lalle et al., 2005), glutamate dehydrogenase (gdh), (Read et al., 2004) and triose phosphate isomerase (tpi). (Sulaiman et al., 2003) PCR products were visualized on a 1% agarose gel stained with ethidium bromide. Negative and positive controls were included for all PCR assays and were also analyzed via electrophoresis. The positive control was a culture derived *Giardia* isolate from ATCC (Manassas, Virginia), and the negative control contained all of the PCR reagents but no DNA.

Purification and sequencing—Amplicons of appropriate size underwent gel extraction using a commercial kit (Qiagen) following the manufacturer's instructions. The resulting product was sequenced at the Virginia Bioinformatics Institute, Virginia Tech utilizing the same PCR primers. The sample was sequenced in both directions and analyzed using a sequence alignment editor. Isolate sequences were compared with sequences in the GenBank database by BLAST analysis and aligned with reference sequences from the literature (Table 2) for identification.

RESULTS

In total 54 samples were analyzed using the ssu-rRNA, gdh, bg, and tpi PCR assays. Of the 54 samples analyzed 43 produced amplicons of which 37 had interpretable sequence data at one or more loci. Combining information from all four loci there were 21 samples that were genotyped as assemblage F, 12 as assemblage A, and 4 that genotyped to both assemblages F and A (Table 1) Twenty-five samples had sequence data at the ssu-rRNA locus, 25 at gdh, 17 at bg, and 13 at the tpi locus. At the ssu-rRNA, bg, and tpi loci, assemblage F was most commonly identified, but for the gdh assay assemblage AI/AI-like were more commonly identified than assemblage F. Most isolates were the same assemblage across all four loci, but four samples had both assemblages A and F present according to the multilocus genotyping. At the gdh locus there were nine samples that had 98% nucleotide sequence identity with the reference strain, but all had the same four single nucleotide polymorphisms (SNPs) in their sequences and did not align perfectly with any reference sequences in GenBank; these are identified to as AI-like. Interestingly all 9 of these samples were from the same rescue organization in Blacksburg, VA.

Twenty-one samples were genotyped as assemblage F only and of those samples 62% (13/21) were from animal shelters, 29% (6/21) were from feral cats, and 9% (2/21) were from

client owned animals. While over half of the assemblage F isolates came from cats in animal shelters, this matches the distribution of sample origin as animal shelter samples made up 76% (28/37) of our isolates. There were 10 samples that were genotyped as assemblage AI; one sample was from the trap-neuter-release program and the other nine were from animal shelters in Blacksburg and Richmond, VA (Table 3). It is not surprising that the majority of our genotyping results would be for samples from animal shelters. There were no potentially zoonotic assemblages of *Giardia* recovered from samples in Prince William, Fauquier, and Stafford counties in Virginia.

DISCUSSION

This report demonstrates that cats in Virginia are infected with potentially zoonotic strains of *Giardia,* specifically assemblage AI., which has been found in both animals and humans. (Ryan and Cacciò, 2013) These findings are consistent with reports from other parts of the United States (Table 4). Although no zoonotic assemblages were recovered from Prince William, Fauquier, and Stafford counties in Virginia, the small number of samples that were genotyped for those locations does not rule out the possibility that potentially zoonotic genotypes are present in those locations.

The AI-like samples with the SNPs were all from an animal shelter in Blacksburg, Virginia; this could indicate that there is a unique isolate of *Giardia* circulating in this animal shelter. However both F and AI were present in samples from that shelter as well. Unfortunately most of the samples in this study that were positive for *Giardia* on fecal flotation or IFA and of sufficient quantity for DNA extraction and molecular analysis were from shelter cats, so the

differences in assemblage(s) among the type of cat (owned, shelter, or feral) are more reflective of our sample pool and cannot be used to definitively determine any differences.

We confirmed that cats in Virginia are infected with both the species-specific *Giardia* assemblage F and the potentially zoonotic assemblage AI, we did not find the diversity of assemblages in cats that others have reported around the world. Cats infected with assemblages F and AI have been reported from genotyping studies using the gdh locus in Mississippi and Alabama (Vasilopulos et al., 2007) and assemblages A and B have been reported in a study utilizing the ssu-rRNA locus in cats from New York (van Keulen et al., 2002). Outside of the United States, assemblage A has been found in addition to the species-specific assemblage F. Studies that also used the gdh locus have identified both Assemblages A and F in Brazil, only Assemblage A in Italy, and only Assemblage F in Japan. (Berrilli et al., 2004; Itagaki et al., 2005; Souza et al., 2007) Other researchers have found Assemblages A and B using the same 4 loci presented in this study, (McDowall et al., 2011). A study that utilized just the gdh locus found Assemblages A, B, and D, (Jaros et al., 2011), and another study that utilized the ssu-rRNA and gdh loci reported Assemblages A, B, C, D, and E in cats. (Read et al., 2004)

Perhaps even more importantly than the identification of potentially zoonotic assemblages from cats in Virginia, this study underscores the importance of utilizing a multilocus genotyping (MLG) approach as opposed to targeting a single locus. This approach to *Giardia* genotyping is widely advocated throughout the literature. (Ballweber et al., 2010; Ryan and Cacciò, 2013; Thompson and Monis, 2012) If genotyping had been undertaken using only one locus the number of cats infected with assemblage AI or AI-like would have been underreported in these samples. The ssu-rRNA locus is considered to be better at detecting *Giardia* infections; this is because of its multicopy and highly conserved nature compared to

other genes. (Gomez-Munoz et al., 2012) However, while the ssu-rRNA locus did differentiate between the two assemblages (A and F), it cannot be used to determine sub assemblages. The more variable loci (bg, gdh, and tpi) are used for subtyping within assemblages, and while we only identified sub assemblage AI and an AI-like assemblage, if these other loci had not been included, the subtypes would not have been detected.

The ssu-rRNA, bg, and tpi loci all identified the same five cats as infected with assemblage AI, but the addition of the gdh locus allowed for six additional samples to be identified as assemblage AI and five more as assemblage AI-like. The distinction between assemblages AI and AI-like at the gdh locus is based on the 4 SNPs that are observed in the AI-like samples. These isolates have 98% sequence homology to the gdh reference strain, but they do not align perfectly, nor do they have 100% homology with any other genotype in GenBank. The ssu-rRNA locus is considered to best for the detection of *Giardia* infections; this is because of its multicopy and highly conserved nature compared to other genes. (Gomez-Munoz et al., 2012) However, while the ssu-rRNA locus did differentiate between the two assemblages (A and F), it cannot be used to determine sub assemblages. The more variable loci (bg, gdh, and tpi) are used for subtyping within assemblages, and while we only identified sub assemblage AI and an AI-like assemblage, if these other loci had not been included, the subtypes would not have been detected.

. In contrast to other studies where the gdh gene has provided limited genotyping information compared to the other loci used (Covacin et al., 2011; McDowall et al., 2011), in this study, as in Scorza et al. (2012) the gdh locus proved to be a valuable and informative target for *Giardia* genotyping, as it identified the most assemblage AI and AI-like infections in samples besides the five that all of the loci identified. Another study found the ssu-rRNA locus to be the

most sensitive for genotyping (n =107), followed by the gdh gene (n = 83), then the bg and tpi genes which were equal (n = 77). (Gomez-Munoz et al., 2012) This variability in genotyping information obtained from each locus could be due to differences among laboratories, but that cannot be definitively determined. Interestingly Covacin et al. (2011) also found that the bg gene seemed to preferentially amplify assemblages A and B as opposed to the species-specific assemblages, but that was not the case in this study. In addition to only identifying five samples with assemblage A the bg locus did not identify any samples with assemblage B even though cats can be infected with assemblage B. (van Keulen et al., 2002)

While the number of positive samples did not allow us to determine if there were assemblage or sub assemblage differences among cats from different populations, it is still valuable to know what *Giardia* assemblages are present in cats in Virginia. This is the first report of a multilocus genotyping study of four loci to determine the assemblage(s) of *Giardia* present in cats. By using the recommended MLG we have identified that cats in Virginia do have potentially zoonotic *Giardia* genotypes in addition to the species-specific assemblage, and these results can provide the basis for further epidemiologic studies on the importance of cats in transmission of human infection *Giardia* infection.

TABLES

Table 1: Multilocus characterization of isolates based on sequencing data from the ssu-rRNA, gdh, bg and tpi genes. Isolates were compared with reference data in GenBank.

− : no sequence data for that locus

Assemblage results for each locus

No. of cats	ssu-rRNA	gdh	bg	tpi
3	F	F	F	F
2	F	F	F	-
6	F	-	-	-
3	F	-	-	F
2	F	-	F	-
5	-	AI	-	-
2	F	F	-	F
2	-	AI-like	-	-
2	F	F	-	-
1	A	AI	AI	AI
4	A	AI-like	AI	AI
1	-	AI	F	-
1	-	-	F	-
3	-	AI-like	F	-

Table 2: GenBank accession numbers for sequences used for genotyping at each locus

GenBank accession numbers for reference sequences

Assemblage	ssu-rRNA	gdh	bg	tpi
F	AF199444	AF069057	AY647264	EU781003
A*/AI	DQ414242	DQ414242	AY655702	L02120

*For the ssu-rRNA locus assemblage subtype cannot be determined

Table 3: Assemblages of *Giardia* determined by multilocus genotyping categorized by population and geographic location.

	Assemblage F			Assemblage A/AI*			Assemblages F and AI*			
Location	Shelter	Feral	Owned	Shelter	Feral	Owned	Shelter	Feral	Owned	Total
Blacksburg	3	-	2	6	-	-	3	-	-	14
Richmond	-	6	-	4	1		1	-	-	12
Northern VA	10	-	-	-	-	-	-	-	-	10

Table 4: *Giardia duodenalis* assemblages identified in cats in the North America.

Location	No. of samples genotyped	Assemblage(s)	Loci tested	Reference
Canada-Ontario	15	A, B, F	ssu-rRNA, gdh, bg, tpi	(McDowall et al., 2011)
USA-Alabama and Mississippi	17	A, F	gdh	(Vasilopulos et al., 2007)
USA-Colorado	13	A, C, D, F	gdh, bg, and tpi	(Scorza et al., 2012)
USA-Maryland	18	F	ssu-rRNA	(Fayer et al., 2006)
Canada and USA-New York	9	A, B	ssu-rRNA	(van Keulen et al., 2002)

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Chapter 4: Diagnosis of Canine Infections with *Giardia duodenalis* using PCR

ABSTRACT

Giardia duodenalis infections in dogs have historically been diagnosed microscopically by observing trophozoites in a direct fecal smear or cysts on a fecal flotation test. These techniques provide limited sensitivity because cysts are shed intermittently, and trophozoites are only found occasionally in diarrheic samples. To combat these difficulties, fecal antigen tests that detect cyst antigen have been developed. Currently, a direct smear and centrifugal fecal flotation with a sensitive and specific fecal antigen test are recommended for diagnosis. However, commercial labs now offer PCR diagnostic tests to detect *Giardia*. There are few direct comparisons of PCR to conventional microscopic techniques with an immunoassay. The objective of this study was to compare diagnostic techniques to determine whether PCR significantly improved *Giardia* detection. Conventional and real time PCR assays targeting the ssu-rRNA gene were evaluated. Diarrheic fecal samples were collected from dogs at local clinics and animal shelters and tested for *Giardia* with centrifugal zinc sulfate fecal flotation, a commercial immunoassay, and both PCR assays. Sensitivity and specificity of each PCR assay was determined by comparison to the recommended method of *Giardia* diagnosis (microscopy and immunoassay) and agreement was measured using Cohen's kappa statistic. The real time PCR assay amplified an approximately 62 base pair gene fragment and was very sensitive (100%), but not very specific (11%) and had poor agreement with the recommended tests. Conventional PCR amplified a larger approximately 292 bp fragment, was moderately sensitive (42%) with high specificity (93%) as well as fair agreement with the recommended diagnostic tests. This data indicates that agreement between PCR compared to microscopy combined with

an immunoassay varies depending on the molecular parameters and size of the DNA target and

underscores the complexity of test evaluation and molecular diagnostics for *Giardia*.

Key words: Giardia duodenalis; diagnostics; PCR; dogs

INTRODUCTION

Giardia duodenalis infections in dogs have historically been diagnosed using morphologic techniques to identify cysts and/or trophozoites, but cysts are shed intermittently, and trophozoites are only found occasionally in diarrheic samples. (Barr et al., 1994; Carlin et al., 2006; Zimmer and Burrington, 1986) Several studies have shown that centrifugation using a 33% $ZnSO_4$ solution allows for the best recovery of *Giardia* cysts. (Barr and Bowman, 1994; Payne et al., 2002; Zajac et al., 2002; Zimmer and Burrington, 1986) However, even with proper technique identification of cysts is difficult for inexperienced individuals. (Carlin et al., 2006) To improve *Giardia* detection, immunoassays that detect a soluble *Giardia* cyst antigen have been developed. IDryden *et al* (2006) found that using a commercial immunoassay with fecal flotation improves *Giardia* detection. In another study the SNAP *Giardia* test alone had a sensitivity of 85.3%, but when used with a fecal flotation test sensitivity improved to 97.8%. (Mekaru et al., 2007) Given that *Giardia* is a common cause of acute and/or chronic diarrhea in dogs, and that the cysts are immediately infective when passed in the feces correct diagnosis of infections is essential in the management of dogs that present with diarrhea. (Ballweber et al., 2010; Barr and Bowman, 1994; Leib and Zajac, 1995; Leib and Zajac, 1999) It is currently recommended that morphologic techniques (the direct smear and centrifugal fecal flotation) be combined with a sensitive and specific fecal antigen test for the diagnosis of *Giardia.* (Tangtrongsup and Scorza, 2010)

With the advent of PCR, it has often been assumed that it is the most sensitive diagnostic test for the detection of *Giardia*. (Bouzid et al., 2015) While PCR is a ubiquitous laboratory technique and is often used for diagnosis of various infectious diseases, it is important to remember that with regards to *Giardia* there is no true gold standard and discordant results

between PCR, IFA, fecal flotation, and antigen testing in the detection of *Giardia* are not uncommon,(Fayer et al., 2006; Hascall et al., 2016; Meireles et al., 2008; Scaramozzino et al., 2009; Traub et al., 2009; Tseng et al.)

In some prevalence studies conventional PCR detected infections more often than microscopy or antigen testing, (McGlade et al., 2003; Scaramozzino et al., 2009) but in other studies the reverse was true.(Hascall et al., 2016; Meireles et al., 2008; Traub et al., 2009; Tseng et al.) In Spain, Gomez-Munoz et al (2012) diagnosed more *Giardia* infections with PCR, finding 107 out of 120 (89.2%) samples to be positive by nested PCR at the SSU-rRNA locus; 30 of those positives were negative by immunofluorescent antibody test (IFA), which is the most common reference test for *Giardia*. In a survey of pound dogs by Tupler et al (2012) 19 *Giardia* positive samples were found with PCR, but 11 of these tested positive by antigen detection and none by fecal flotation, however these investigators did not use the 33% zinc sulfate flotation solution recommended for recovery of *Giardia*. Another study that utilized $ZnSO_4$ fecal flotation and PCR, detected 10 fewer infections by PCR than microscopy. (Meireles et al., 2008) A study by Fayer *et al* (2006) found the same number of positive samples using IFA and PCR, but the positive result differed among four specimens.

Traub et al (2009) tested 139 dogs for *Giardia* using $ZnSO_4$ fecal flotations, IFA, antigen testing, and PCR and found that 95.9% were positive by antigen testing while only 70.7% were positive by PCR and only 7.9% were positive by fecal flotation. Dogs attending California dog parks were tested for *Giardia* using $ZnSO_4$ fecal flotation, IFA, and two types of PCR (a university laboratory assay and the commercial IDEXX Canine Diarrhea RealPCR Panel) and had discordant results among all four diagnostic tests; of 300 dogs tested 27 were positive for *Giardia* using results from all tests. Fecal flotation recovered the most positives 21/27, followed

by IFA 19/23 (four samples were not tested), then the university PCR 14/27, and finally the commercial PCR assay 10/27.(Hascall et al., 2016) In the study by Hascall et al (2016) the university assays (fecal flotation and PCR) detected 13 more positives than the commerical lab PCR, but the commercial lab PCR detected 3 positive samples that the university lab did not, however 11/27 samples that were PCR negative by the commercial PCR assay were cyst positive on fecal flotation and/or IFA.

PCR findings for a particular study only apply to that particular assay, not to every PCR assay that is the same type, such as nested, conventional, semi-nested, or real time; there can also be sizeable disparity between labs for the same DNA target. Bastien et al (2008) emphasize that PCR is a complex process made up of a number of scientific techniques, with many variable outcomes that are dependent on a multitude of variables, and that real time PCR is an advanced PCR technique but has many of the same limitations especially with comparisons between laboratories. This variation could explain some of the variability seen in PCR performance compared to morphologic techniques and antigen detection across different laboratories.

Several large diagnostic laboratories offer diarrhea panels that include a real time PCR assay for *Giardia* but have not published information indicating what improvement in sensitivity/specificity is offered by this PCR test compared to the recommended in-house method of direct smear and centrifugal fecal flotation in conjunction with a sensitive and specific fecal ELISA test. The objective of this study was to compare diagnostic techniques to determine whether either PCR technique significantly improved *Giardia* detection compared to the current testing recommendation (microscopy and immunoassay) in dogs with soft stool or diarrhea..

MATERIALS AND METHODS

Sample Collection and Fecal Scoring—Fecal samples were collected from animal shelters, client owned dogs at local veterinary clinics, and the diagnostic parasitology laboratory at the Virginia Maryland College of Veterinary MedicineTeaching Hospital (VMTH). Samples were scored for consistency using the VMTH teaching hospital fecal scoring system, which utilizes a 1-5 scale, with 5 being formed stool and 1 liquid. Because we wanted to evaluate clinical cases where an owner would be concerned and seek veterinary intervention for diarrhea or soft stool we only included fecal samples scoring 1-3 in the study. Within 24 hours of receiving the sample zinc sulfate ($ZnSO_4$) centrifugal fecal flotations were performed. Because most samples were refrigerated upon collection and during transport to the laboratory the direct smear for trophozoites was not performed. If *Giardia* was detected by fecal flotation, an antigen test was not performed. A sample was considered positive if cysts were identified on the $ZnSO_4$ centrifugal fecal flotation or was it antigen positive on the immunoassay; this was called the in house result. Cyst isolations for DNA extraction were also performed within 24 hours of receiving the sample.

33% Zinc Sulfate Fecal Flotation test—The zinc sulfate centrifugal fecal flotation test was performed as described in Zajac and Conboy (2012). In short, 2 grams of feces was suspended in 20 mL of 33% $ZnSO_4$ solution, mixed and strained through a double layer of cheesecloth. The fecal mixture was poured into a 15 mL plastic centrifuge tube forming a reverse meniscus and covered with a 22 mm^2 glass coverslip. Samples were centrifuged for 5 minutes at 200 x g. After centrifugation the coverslip was removed to a glass slide and scanned for *Giardia* cysts with a compound microscope at 100 and 200X.

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Fecal Antigen Test—The *IDEXX SNAP Giardia Antigen Test* was performed following the manufacturer's directions. The swab device is removed from the tube and the swab is coated with the fecal sample. The bulb on the opposite end of the swab is filled with a reagent containing conjugate-bound antibody. The feces coated swab is placed back in its plastic tube, and the reagent valve is broken by bending the assembly back and forth below the bulb. Then the reagent solution is passed over the swab coated with feces by squeezing the bulb three times. The swab device is then used like a pipette and 5 drops of the fecal solution are deposited into the well of the SNAP test device where it flows across the device's membranes, and is "snapped" into place once the solution moves past the designated spot on the membrane. If conjugated antibody is present a white to blue color change occurs on the sample test spot.

Cyst Isolation—Fecal samples were processed using a sugar gradient density separation to isolate cysts if present as previously described. (Scorza et al., 2012) Briefly, 2-4 grams of feces were filtered through a double layer of cheesecloth using 4 ml of phosphate buffered saline solution containing ethylenediaminetetraacetic acid (PBS-EDTA), and the resulting solution was placed on top of 7 mL of Sheather's sugar solution (s.g.= 1.26) in a 15 mL conical tube and centrifuged at 800 × g for 10 min. The top layer of the filtrate and the interface were then carefully pipetted into a separate 15 mL conical tube and centrifuged for 10 min at 1200 × g. The supernatant was discarded and the washes with PBS-EDTA were repeated two more times for 10 min at 1200 x g. After the final wash step the supernatant was decanted and the cyst pellet was suspended in 1mL of PBS-EDTA and stored at 4°C until DNA extraction was performed.

DNA extraction—DNA was extracted from the concentrated fecal samples using the Qiagen DNeasy Tissue Kit (Venlo, Netherlands). Reagents from the kit were used, but with modification as outlined below. Fifty microliters of the cleaned fecal sample was added to 180

µL of ATL buffer, vortexed, and then 20 µL of proteinase K (20mg/ml) added, and the sample

mixed again. Samples were incubated overnight at 55°C, after which 200 µL of AL buffer was

added to the sample. For elution of the sample, 100 µL of AE buffer was used. (Santin et al.,

2012) The sample was stored at −20°C until PCR was performed.

PCR Assays—For the nested PCR a ~500 base pair (bp) fragment of the small-subunit

ribosomal RNA (ssu-rRNA) was amplified using the external primers Gia 2029 (5'-

AAGTGTGGTGCAGACGGACTC-3') and Gia 2150c (5'-CTGCTGCCGTCCTTGGATGT-3')

and then the internal primers RH11 (5'-CATCCGGTCGATCCTGCC-3') and RH4 (5'-

AGTCGAACCCTGATTCTCCGCCAGG-3') were used to amplify a ~300 bp fragment of the

ssu-rRNA gene. (Appelbee et al., 2003; Hopkins et al., 1997) For the primary and secondary

PCR steps, the PCR mixture contained 1 X GoTaq® Green Master Mix (Promega Corp,

Madison, WI) containing 1.5 mM MgCl^2, 0.2 mM each dNTP, 2.5 mL of dimethyl sulfoxide

(DMSO), and 0.5 mM of each forward and reverse primer in a total of 50uL reaction volume.

The cycling conditions for the primary amplification were as follows: an initial hot start

at 95°C for 2 minutes followed by 35 cycles of 95°C for 45 seconds, 58°C for 30 seconds, and

72°C for 45 seconds, then a final extension at 72°C for 4 minutes. Cycling conditions for the

secondary amplification were an initial hot start at 95°C followed by 35 cycles of 95°C for 45

seconds, 55°C for 30 seconds, and 72°C for 45 seconds, then a final extension at 72°C for 4

minutes. The PCR reaction was visualized by electrophoresis on 1% agarose gels stained with

ethidium bromide.

Because commercial reference labs offer a real time PCR to diagnose *Giardia* infections

we chose to evaluate both a real-time and conventional PCR assay targeting the ssurRNA gene

were evaluated. However the specifics of the commercial laboratory diarrhea panels are proprietary and not available to use. As such, we chose to utilize a real-time protocol that was validated for use in diagnosing human *Giardia* infections (Haque et al., 2007) to mimic the commercial real time assay as closely as possible. For the real time PCR the primers Gia-F (5'-GACGGCTCAGGACAACGGTT-3') and Gia-R (5'- TTGCCAGCGGTGTCCG-3') were used with a Taqman probe (FAM-CCCGCGGCGGTCCCTGCTAG-DDQ1) to amplify a 62 bp fragment of the ssu-rRNA gene. (Verweij et al., 2003) Assays were performed in a 25uL volume using iQ Power Mix (BioRad Laboratories, Hercules, CA) containing dNTPs, 6 mM MgCl^2, and iTaq DNA polymerase [50 units/mL]. The cycling conditions were 3 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 55°C, and then a final step for 30 seconds at 72°C and fluorescence was emitted and measured at 490nm. (Haque et al., 2007) For each set of PCR reactions, positive and negative controls were included.

Statistical Analysis—The prevalence of *Giardia* infection along with sensitivity, specificity, and predictive values were calculated for each PCR assay by comparison to the recommended testing method (combined morphologic techniques with a sensitive and specific fecal antigen test). Agreement between PCR assays was analyzed using an unweighted Cohen's kappa statistic. (Tooth and Ottenbacher, 2004)

RESULTS

In total, 141 samples met the screening criteria, and 38 out of 141 (27%) were positive for *Giardia* using the recommended in house test (morphologic technique combined with a sensitive and specific fecal antigen test). Sixteen of the 38 positive samples were positive on both

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nested and real time PCR, and the remaining 22 were positive on just the real time PCR. Of the 103 samples that were negative on fecal flotation and antigen test 12 were negative on both PCR assays, 84 were positive on real time PCR only, and 7 were positive on both nested and real time PCR (Table 1). Sensitivity, specificity, and predictive values are presented in Table 2. Compared to the in house test result the conventional PCR had a sensitivity of 42% and a specificity of 93%, while the real time PCR had a sensitivity of 100% and a specificity of 12% (Table 2). Both the real time and conventional PCR assays had high negative predictive values, 81% and 100% respectively, while the conventional PCR had a moderate positive predictive value of 70% and the real time PCR had a low positive predictive value of 29%.

Agreement between the tests ranged from poor to fair. The strength of agreement between the real time PCR assay and the fecal flotation and antigen test result was poor, with a Cohen's kappa statistic of 0.066. For the conventional PCR Cohen's kappa statistic was 0.403, and the strength of agreement was fair. The scale for Kappa coefficient strength of agreement is as follows: ≤0=poor, 0.01–0.20=slight, 0.21–0.40=fair, 0.41–0.60=moderate, 0.61–0.80=substantial, and 0.81–1=almost perfect.

DISCUSSION

This data shows that agreement between PCR assays and microscopy and immunoassay varies greatly. For the conventional PCR specificity (93%) was greatly improved by comparison to the real time PCR (11%). This could be explained by the larger gene fragment that was being targeted and amplified, ~292 bp versus only 62 bp, which would allow for less nonspecific binding. The conventional PCR was not as sensitive (42%) as the real time PCR (100%). The conventional PCR identified an additional 7 positive samples that were negative by antigen

detection and fecal flotation, and the real time PCR assay identified 91 positive samples that were negative by antigen detection and fecal flotation. The low specificity of the real time PCR assay is a result of the large number of samples that tested positive, but were cyst and antigen negative. These results were considered false positives, and possible explanations are that the analyte being targeted was too small, only 62 bp in size, that binding was occurring in a non-specific region, or that in diarrheic canine fecal samples something innocuous in the feces was being amplified, It is also possible that a primer dimer was being formed and interpreted as a positive result.

The nested ssu-rRNA PCR had been previously utilized and optimized in our laboratory (data not shown). The real time PCR protocol had been previously validated and optimized for use in humans, (Haque et al., 2007; Verweij et al., 2003) and while the evaluations in humans showed greater specificity that was not the case in this study on a canine population. The real time PCR presented us with several challenges and underwent protocol optimization in an attempt to improve the assay for use with canine samples. The real-time PCR optimization included raising the annealing temperature from 55°C to 60°C to improve the specificity of the primers, utilizing a new PCR buffer and DNA polymerase at both annealing temperatures, and simplifying from a 3 step to a 2 step protocol to try and limit nonspecific amplification. Ultimately even with these attempts at optimization the results of the real time PCR did not change. As such further attempts to determine the nature of these false positive results were undertaken. The PCR products were run on a 2% agarose gel to determine if a primer dimer had formed, unfortunately the resolution required to visualize the 20bp difference between a possible primer dimer and a true positive sample, which would be ~62bp in size could not be obtained.

A melting curve analysis was performed on a subset of 25 samples to determine if the positive results of the real time PCR assay were truly double stranded DNA (dsDNA). A melting curve is used after real time PCR to distinguish if positive results are from real dsDNA or from reaction artifacts such as primer dimer or incomplete binding. (Winder et al., 2011) Melting peak analysis provides a greater level of certainty that fluorescence from the dsDNA dye is from the target as opposed to nonspecific amplification. (Ririe et al., 1997) The melting curve consisted of 4 steps: 95°C for 15 s, 60°C for 1 min, and heating to 95°C for 30s, then 60°C with acquisition at step 2. The melting curve analysis showed numerous nonspecific peaks with lower melting temperatures that did not align with the positive control peak, as such it was determined that nonspecific amplification had occurred in 20 of the 25 samples and the resulting products were not dsDNA and not true positives.. Haque et al. (2007) reported a sensitivity of 91% and specificity of 99% when using human samples. In contrast, antigen testing specificity was 99%, which is much more specific than the real time PCR assay in dogs. It has been shown that antigen tests optimized for use in humans do not always perform as well when used on canine feces, (Geurden et al., 2008; Rishniw et al., 2010a) probably due to differences in the fecal matrix, so perhaps we are seeing a similar trend with this human PCR assay.

Considering the results of the melting curve analysis at least 20 of the 91 additional positives by real time PCR can be considered false positives, and given that 80% of the subset samples tested were determined to be false positives it is likely that there are more false positives in the remaining 66 samples that tested positive. However, the nested PCR identified 7 more positive samples than the in house tests, indicating that the nested PCR did improve *Giardia* detection compared to the fecal flotation and antigen test.

Both the real time PCR assay and conventional PCR had relatively high negative predictive values (NPV), 100% and 81% respectively. Given the high NPV of the real time PCR there is a good chance that a sample that tests negative by real time PCR is indeed truly negative. However the real time PCR in this study had a low positive predictive value (PPV) of only 29%, with such a low PPV there is a low chance that a positive result is truly positive. The conventional PCR had a PPV of 70%, so there is a greater probability that a positive result via conventional PCR is indeed positive.

Agreement between both PCR assays and the in house results was lacking. With such low values both PCR assays (real time PCR Cohen's kappa statistic = 0.066, nested PCR Cohen's kappa statistic = 0.403) would not be considered good diagnostic tests when compared to the in house tests. (Pfeiffer, 2010)

We chose to utilize the same technology platform, real time PCR, in an attempt to mimic the diarrhea panels that are performed in commercial diagnostic labs. This endeavor proved challenging and optimization was difficult. Typically the largest obstacle facing *Giardia* PCR is lack of amplification for certain samples, and several studies have shown that not all of their *Giardia* positive samples are amplified by PCR. (Hascall et al., 2016; Scorza et al., 2012; Scorza and Lappin, 2011) In this study we found the opposite when using the real time assay, future use of this particular real time assay should include a melting curve analysis on all samples tested to determine if positive results are truly dsDNA and therefore real true positives or if they are the result of nonspecific amplification.

These results underscore the complexity of test evaluation and molecular diagnostics for *Giardia* detection. With regard to molecular diagnostic evaluations there is a trend to generalize

all molecular tests have improved sensitivity, even within molecular techniques such as real time versus conventional PCR, with the former being considered more sensitive. (Bastien et al. 2008) We show here that not all PCR assays for *Giardia* are the same, even when both assays target the same gene, and when PCR is compared to the recommended diagnostic tests for *Giardia* that these molecular tests are not inherently better just because they are more sophisticated.

TABLES

Table 1. Comparison of results for the in house test (fecal flotation and antigen test), conventional PCR, and real time PCR.

In House Result	Conventional PCR	Real Time PCR	Number of Samples
+	+	+	16
+	-	+	22
-	-	-	12
-	-	+	84
-	+	+	7
Total			141

Table 2. The sensitivity, specificity, positive and negative predictive values of PCR with 95% confidence intervals (CI) when compared to the recommended fecal flotation and antigen test in dogs.

Test	95% Confidence Interval			
	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Fecal flotation plus antigen test*	100.0%	100.0%	100.0%	100.0%
Conventional PCR	42% (27.85—57.81)	93% (86.63—96.67)	70% (49.13—84.4)	81% (73.38—87.35)
Real time PCR	100.0% (90.82—100)	12% (6.79—19.27)	29% (22.28—37.83)	100% (75.75—100)

*When used as the reference test fecal flotation plus antigen test is assumed to be 100% accurate

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Chapter 5: Conclusions

Giardia duodenalis in companion animals is a concern in veterinary medicine both as a pathogen that causes acute or chronic diarrhea in dogs and cats and from a public health perspective as a potential zoonosis. As a pathogen in dogs and cats this protozoan parasite has proved challenging to diagnose and control, and detection by morphologic techniques can be difficult and reinfection is common as the cysts are immediately infective when shed. The concern with regard to zoonotic disease potential has changed over the years as molecular studies on *Giardia* revealed the occurrence of species-specific assemblages and a few potentially zoonotic assemblages with broader host ranges that can infect both animals and people.

In veterinary medicine diagnosis of infections is largely based on morphologic identification of organisms and antigen testing. This dissertation provided an in depth comparison of currently available antigen tests optimized for use in companion animals, zinc sulfate fecal flotation, and the direct immunofluorescent assay (IFA). Although several diagnostic comparisons have been reported in the literature the work presented here was a more complete comparison as it included both USDA approved antigen tests that are commercially available to veterinarians and a new well-plate ELISA optimized for use with canine and feline fecal samples. Furthermore most studies in the veterinary literature have compared diagnostic tests using the IFA as a reference test; few studies have utilized a Bayesian approach to compare tests without a gold standard, and the practice is becoming more widely used. We demonstrated that these antigen tests perform similarly to each other, and that any differences in sensitivity can be overcome when combined with a centrifugal zinc sulfate fecal flotation. Our work also

provided further evidence for the use of the IFA as the de facto reference test for *Giardia*, and the reliability of utilizing a Bayesian analysis as a means to compare diagnostic tests in the absence of a gold standard.

Further analysis of the methodologies of other diagnostic comparisons allowed for the recognition that not all zinc sulfate fecal flotations performed by parasitologists or trained personnel are the same. While this is not a novel revelation, it is not emphasized in the current literature, and it seems that all zinc sulfate fecal flotations are assumed equally good, when in fact these different methods may be accounting for differences among flotation sensitivities between studies. Thus the importance of a thorough analysis of the methods should be undertaken when comparing studies and perhaps more detail should be included in reports as opposed to the common practice of glossing over a portion of the methods in favor of a simple citation.

Using PCR as a diagnostic tool for *Giardia* in companion animals has been gaining ground in veterinary medicine. PCR was initially (and still is) used to genotype *Giardia* assemblages to determine the zoonotic potential of isolates. However as molecular diagnostics have gained ground in human and veterinary medicine the utility of PCR to detect *Giardia* infections has been explored with mixed results. Human medicine has seen more success with agreement between PCR and traditional diagnostic assays, while some veterinary studies have shown PCR to be more sensitive and others have shown that PCR does not always amplify cyst positive samples, which is a source of great frustration among researchers. Once again when evaluating the literature and currently used molecular assays the importance of thorough evaluation of the methods in the original work cannot be overemphasized. It is widely accepted and many reports have claimed that *Giardia* detection via PCR is more sensitive than

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conventional techniques, however when the original studies are examined more closely it is apparent that the PCR was only performed on cyst or antigen positive samples.

While the public health importance of *Giardia* from dogs and cats has changed over the years, companion animals are capable of and do serve as reservoirs of potentially zoonotic assemblages. However, we have progressed from assuming that all *Giardia* from animals is zoonotic to recognizing that, at least in the United States, most companion animals harbor species specific assemblages, although reports of zoonotic assemblages A and B in dogs and cats are not rare. There continue to be reports of different assemblages in dogs and cats based on geographic location and housing type (group or singly housed) and other risk factors such as park attendance or the presence of other pets in the home. This work showed that in Virginia cats are infected with both their species-specific assemblage, F, and the potentially zoonotic assemblage, A, with subtyping revealing AI which is shared by humans and animals. This finding demonstrates that animals cannot be dismissed as sources of potentially zoonotic *Giardia* assemblages.

In conclusion, we feel that *Giardia* infections in dogs and cats can be reliably detected using currently available antigen tests that are optimized for use in companion animals, and also with centrifugal zinc sulfate fecal flotations, and IFA. Where there are differences in commercial antigen test performance these can be overcome when combined with the zinc sulfate fecal flotation when performed as outlined throughout this write-up. While different studies have reported varying zinc sulfate fecal flotation sensitivities when examining the literature more closely the differences seem to stem from variations in methodologies among researchers, and we feel that it is important to emphasize this point. Regarding molecular diagnostics to detect *Giardia* in companion animals, while attempts were made to determine the

suitability of the commercially available real-time PCR assays for *Giardia* detection we were unable to evaluate them directly, and the results presented in this body of work do not apply to these commercial tests. However, this work does emphasize the difficulty and challenges associated with applying molecular techniques from humans for use in dogs, and molecular methods should face careful scrutiny and validation before being accepted just because they are more sophisticated techniques. Improved *Giardia* diagnostics will allow for more accurate identification of infected animals, which will allow for the determination of what assemblage(s) the animal is infected with. This knowledge can in turn help determine the zoonotic risk that animals may or may not pose and result in better management of *Giardia* infections in companion animals.

Appendix A: Immunologic detection of *Giardia duodenalis* in a specific pathogen–free captive olive baboon (*Papio cynocephalus anubis*) colony

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ABSTRACT

Several commercial *Giardia* immunoassays were evaluated in baboons for sensitivity and specificity as well as ease of use in a large specific pathogen–free (SPF) colony. An additional objective was to identify the assemblage(s) of *Giardia duodenalis* present in this baboon colony. A direct immunofluorescent antibody test (IFAT) was used as the reference test. Tests evaluated were a patient-side rapid test for dogs and cats, a human rapid test, and a well-plate ELISA designed for use with humans. Test sensitivities and specificities were compared using the McNemar paired *t*-test and were further evaluated for agreement using an unweighted Cohen kappa statistic. When compared to the IFAT reference, both human tests were more sensitive than the veterinary test. Based on PCR and sequencing of the *G. duodenalis* small-subunit ribosomal RNA and glutamate dehydrogenase loci, assemblage AI was present in this baboon colony. We found that 10 of the 110 (9%) baboons in this SPF colony were infected with a zoonotic strain of *G. duodenalis*.

Key words: Baboons; *Giardia*; giardiasis; immunoassays; parasitology.

INTRODUCTION

Giardia duodenalis is an enteric protozoan parasite with a broad host range that includes humans and nonhuman primates (NHPs). The parasite is commonly found in NHPs, where it is of both veterinary and public health importance.[8,12] *G. duodenalis* infections in NHPs can result in diarrhea and ill thrift, particularly in young animals.[6] The prevalence of *G. duodenalis* infection in NHPs varies greatly depending on species surveyed, geographic location, and type of housing (free range, zoo, or laboratory animals), with estimates of 2.2–57%.[10,11,19] In captive baboons, prevalences of 10% were reported from Belgium and 17% from China.[12,14] In wild baboons, prevalences of 26% in Saudi Arabia[10] and as high as of 58% in Uganda[11] have been reported.

G. duodenalis has been divided into 8 assemblages (A–H) that are genetically distinct but morphologically identical.[4,8] Assemblages A and B infect both humans and a wide range of mammals; assemblages C and D are typically found in canids, E in cloven-hoofed livestock, F in felids, G in rodents, and H in marine mammals.[4,8] In addition, there are subtypes within assemblages A and B, although there is not complete agreement among researchers as to how many subtypes exist. Assemblage A currently consists of subtypes AI–AIV, with human isolates belonging to AI and AII, and animal isolates belonging to AI, AIII, and AIV.[18] Assemblage B is considered to have many subtypes that do not form genetically authentic sub-assemblages.[18] Both assemblages A and B have been reported in NHPs, with assemblage B being the predominant assemblage reported.[12,24] Specifically in olive baboons (*Papio cynocephalus anubis*), only assemblage B has been reported in the literature.[16] Therefore, NHPs are capable of serving as reservoirs for zoonotic transmission of *G. duodenalis*.

Detection of *G. duodenalis* infections in NHPs by standard morphologic techniques is difficult. Cysts are shed intermittently in feces, and are difficult to recognize without training. In nonhuman primates, endoparasites are often diagnosed using preserved feces and variations of ether sedimentation techniques, direct smears, or fecal flotations.[10,11] If researchers are specifically concerned with finding *G. duodenalis*, an immunofluorescent antibody test (IFAT) is sometimes used,[19] but such tests require specialized equipment and training. There are rapid tests available to detect *Giardia* in humans, dogs, and cats. However, there are no rapid detection tests that are designed and/or optimized for use in NHPs.

MATERIALS AND METHODS

Previously, *G. duodenalis* cysts were detected on routine centrifugal zinc sulfate fecal flotations in our specific pathogen–free (SPF) baboon colony. Fecal samples were then screened with a commercial rapid veterinary fecal antigen test (VFAT; SNAP *Giardia* antigen test, IDEXX Laboratories, Westbrook, ME) validated for use in dogs and cats. There were discrepant results between the fecal flotations and the VFAT, which prompted us to seek a reliable immunoassay that could be used for screening NHPs, and led to the consideration of human detection tests for use in NHPs. We evaluated the VFAT for use in dogs and cats and 2 tests for humans—a rapid test (RT; Giardia/Cryptosporidium Quik Chek, TECHLAB, Blacksburg, VA) and a well-plate ELISA (WPE; Giardia II ELISA, TECHLAB)—and compared each immunoassay to a direct IFAT (Merifluor Cryptosporidium/Giardia, Meridian Bioscience, Cincinnati, OH), which is widely recognized as the most sensitive and specific procedure for detection of *G. duodenalis*.[2,9]

All housing and husbandry procedures for the baboon colony were in compliance with the Guide for the Care and Use of Laboratory Animals[7] and the Association for Assessment and Accreditation of Laboratory Animal Care International. The University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee approved protocols for the maintenance of the baboon colony. The baboon colony was derived and maintained using procedures outlined previously.[23] Baboons were fed a high-protein monkey diet (5045, LabDiet, St. Louis, MO). Their diet was enriched with a forage mix of grains on a daily basis, and they were fed a mix of fruits and vegetables several times per week.

Animal handlers collected fecal samples from 110 clinically normal baboons in the SPF colony within a few minutes after each baboon was observed defecating. Fecal samples were placed in coolers with ice packs during transportation and were stored at 4°C until tested. ELISAs were performed on each sample within 1 wk of collection. At the time of testing, a portion of each sample was fixed in 10% phosphate-buffered formalin for the IFAT, which was performed at a later date.

The WPE is an enzyme immunoassay for the qualitative detection of *Giardia lamblia (duodenalis)* cyst antigen, and the RT is a membrane enzyme immunoassay for the simultaneous qualitative detection of *Giardia* cyst antigen and *Cryptosporidium* oocyst antigen in a single test device; both tests are intended for use with human fecal specimens.[15] The VFAT is a rapid patient-side enzyme immunoassay for the detection of *G. duodenalis* antigen in canine and feline feces.[5] We used the direct IFAT as our reference test to identify *G. duodenalis* cysts in feces. Samples were run in batches, with positive and negative controls each time the test was run. We performed all tests following the manufacturers' instructions.

To determine the assemblage(s) of *G. duodenalis* present, a fragment of the small-subunit ribosomal (r)RNA and glutamate dehydrogenase genes were amplified from a representative cyst-positive sample using primers and protocols previously described in the literature.[3,16] We isolated cysts for DNA extraction from a cyst-positive sample using sugar density gradient separation as described previously,[21] and we extracted DNA from the cysts (DNeasy tissue kit, Qiagen, Valencia, CA). Reagents from the kit were used, but with modification as outlined in the literature.[20] Samples positive by PCR underwent gel extraction (QIAquick gel extraction kit, Qiagen) following the manufacturer's instructions. The resulting product was sequenced at the Biocomplexity Institute of Virginia Tech (Blacksburg, VA) utilizing the same PCR primers. The sample was sequenced in both directions and analyzed using a sequence alignment editor.

We calculated the prevalence of *G. duodenalis* infection along with sensitivity, specificity, and predictive values for each immunologic test by comparison to the IFAT, which was used as the reference standard.[9,17] The McNemar test for significance of changes was used to compare differences in the sensitivities and specificities between the immunologic assays.[1] We analyzed agreement between immunoassays using an unweighted Cohen kappa statistic,[22] with common interpretations of κ from the literature.[13]

RESULTS

The prevalence of *G. duodenalis* infection as determined by the IFAT was 9% (10 of 110). The test with the highest sensitivity when compared to the reference IFAT was the WPE (90%), followed by the RT (80%) and the VFAT (50%; Table 1). The VFAT had the highest specificity (99%) when compared to the reference IFAT followed by the human WPE (97%) and the human RT (95%). The negative predictive value (NPV) for each test was >95% for the immunoassays;

none of the tests demonstrated a positive predictive value (PPV) >84%. There were some

discrepant results among the samples. Of the 10 samples that were positive by the reference

IFAT, 5 were positive on all other immunoassays, 3 were only positive on the human WPE and

the human RT, 1 was only positive on the human WPE, and 1 was negative on all other

immunoassays (Table 2). Of the 100 samples that were negative on the reference IFAT, 94 were

negative on all other immunoassays, 3 were positive on the human WPE and the human RT, 2

were positive on just the human RT, and 1 was positive on the VFAT (Table 2). The only

comparison of the 3 tests that showed a statistically significant difference in sensitivities was

between the human WPE and the VFAT. There was no statistically significant difference in

specificities between any of the test comparisons. Agreement between immunoassays ranged

from moderate to nearly perfect agreement (Table 3). There was nearly perfect agreement

between the human WPE and human RT. There was also substantial agreement between the

IFAT and the human RT, and between the human WPE and the IFAT. Agreement between the

other immunoassays was only moderate: VFAT and IFAT, human WPE and VFAT, and VFAT

and human RT (Table 3).

We sequenced the parasite DNA to assemblage A at the small-subunit rRNA locus and

further subtyped to assemblage AI at the glutamate dehydrogenase locus. The isolate had 100%

nucleotide sequence identity to the assemblage AI sequence referenced in the literature

(GenBank accession DQ414242).

DISCUSSION

The prevalence of *G. duodenalis* infection found in this SPF baboon colony (9%; based

on the reference IFAT) is similar to previous reports in the literature.[12] Although infection with

G. duodenalis can cause diarrhea and ill thrift,[6] and some fecal samples in our study were soft or diarrheic, the baboons in the colony were considered healthy by husbandry staff and caretakers. However, even when all animals are clinically normal, reliable detection of *Giardia* is important in establishing and maintaining SPF status of the colony.

The IFAT is widely recognized as the most sensitive and specific procedure for *G. duodenalis* detection.[9] However, it is time-consuming, requires specialized equipment, and, in large colonies where rapid and simple screening procedures are desired, use of an antigen test may be appropriate even though it may lack the sensitivity and specificity of the IFAT. Because some of the immunoassays only detect cyst antigen and not the presence of cysts, it is possible that some of these discrepant results (Table 2) were actually true positives. The discrepant results seen in our study highlight the difficulty in determining which test is most accurate in the absence of a true gold standard for the detection of *G. duodenalis.*

Based on our results, it appears that the human WPE could be used as a more convenient and yet still reliable test for detection of *Giardia* in baboons than the IFAT. This conclusion is based on its high sensitivity and specificity when compared to the reference test as well as its substantial agreement with the reference IFAT. Although the sensitivity and specificity of the human RT were lower, this test also had substantial agreement with the IFAT and nearly perfect agreement with the human WPE, making the human RT also suitable for the detection of *Giardia* in baboons. Selecting which test to use depends on the purpose of testing, either general screening of an entire colony or testing of individual baboons in which *Giardia* infection is suspected. For large-scale screening, the human WPE might be preferred given that it allows for testing multiple samples at the same time. If only a few baboons need to be tested, then the human RT could be a useful patient-side test. Not surprisingly, the results of this comparison

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also illustrate that the immunoassays developed for humans (RT and WPE) performed better in NHP testing than the VFAT. The human immunoassays also had higher sensitivity and better agreement with the IFAT than the VFAT.

To our knowledge, assemblage AI *Giardia* has not been previously reported in olive baboons. Assemblage B has been reported in olive baboons, and is the more common assemblage reported in NHPs in the literature.[12] Our finding of assemblage AI *Giardia* provides additional evidence that baboons can serve as reservoirs of zoonotic *G. duodenalis* assemblages.

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Table 1. Performance of immunoassays when compared to the direct immunofluorescent antibody test (IFAT) as the reference test.

Immunoassay	No. of positive specimens*	Sensitivity†	Specificity†	PPV†	NPV†
IFAT	10 of 110 (9.1)	100.0%‡	100.0%‡	100.0%‡	100.0%‡
RT	13 of 110 (12)	80% (49–94)	95% (89–98)	62% (36–82)	98% (93–99)
VFAT	6 of 110 (5)	50% (24–76)	99% (94–100)	83% (44–97)	95% (89–98)
WPE	12 of 110 (11)	90% (60–98)	97% (92–99)	75% (47–91)	99% (94–100)

RT = human rapid test; VFAT = veterinary fecal antigen test; WPE = human well-plate ELISA.

* Numbers in parentheses are percentages.

† Numbers in parentheses are 95% confidence intervals.

‡ IFAT assumed to be 100% accurate and used as the gold standard

Table 2. Discrepant results among direct immunofluorescent antibody test (IFAT)-positive and IFAT-negative samples.

IFAT-positive samples (*n* = 10)				IFAT-negative samples (*n* = 100)			
RT+ VFAT+ WPE+	RT– VFAT– WPE+	RT+ VFAT– WPE+	RT– VFAT– WPE–	RT– VFAT– WPE–	RT+ VFAT– WPE–	RT– VFAT+ WPE–	RT+ VFAT– WPE+
5	1	3	1	94	2	1	3

RT = human rapid test; VFAT = veterinary fecal antigen test; WPE = human well-plate ELISA.

Table 3. Agreement between tests as determined by an unweighted Cohen kappa statistic.

Immunoassay compared	Agreement		Interpretation
	Cohen κ statistic	95% CI	
WPE to IFA	0.80	0.60–0.99	Substantial agreement
VFAT to IFA	0.60	0.28–0.91	Moderate agreement
RT to IFA	0.66	0.42–0.90	Substantial agreement
WPE to VFAT	0.52	0.20–0.84	Moderate agreement
WPE to RT	0.86	0.71–1.00	Almost perfect agreement
VFAT to RT	0.49	0.17–0.81	Moderate agreement

CI = confidence interval; IFAT = direct immunofluorescent antibody reference test; RT = human rapid test; VFAT = veterinary fecal antigen test; WPE = human well-plate ELISA.

Appendix B: Development and evaluation of a protocol for control of *Giardia duodenalis* in a colony of group-housed dogs at a veterinary medical college

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Development and evaluation of a protocol for control of *Giardia duodenalis* in a colony of group-housed dogs at a veterinary medical college

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OBJECTIVE
To develop and evaluate a protocol for control of *Giardia duodenalis* in naturally infected group-housed dogs at a veterinary medical college.

DESIGN
Prospective evaluation study.

ANIMALS
34 dogs.

PROCEDURES
All dogs were tested for evidence of *G duodenalis* infection. Dogs were treated with fenbendazole on study days 1 through 10. On day 5, dogs were bathed and moved into clean, disinfected kennels in a different room to allow for disinfection and drying of their assigned kennels at 26.7°C (80°F) for 24 hours on day 6. After treatment, dogs were returned to their original housing; fecal samples were collected weekly from days 8 through 41 and then every 3 weeks until day 209. Samples were fixed in formalin and examined by direct immunofluorescence assay. Additionally, 1 pretreatment sample underwent PCR assay and DNA sequencing to determine the assemblage (genotype) of the organism. Normal handling routines for the dogs and their use in teaching activities were not changed.

RESULTS
Initially, all dogs in the colony shed *G duodenalis* cysts. During and immediately after treatment (days 8 and 13), no cysts were detected in any dogs. On day 20, 1 cyst was observed in the fecal sample from 1 dog; results for all subsequent fecal analyses were negative. The *G duodenalis* cysts collected from the pretreatment sample had an assemblage C genotype.

CONCLUSIONS AND CLINICAL RELEVANCE
The integrated protocol was successful in controlling *G duodenalis* infection in this dog colony, despite exposure of dogs to a variety of environments and frequent handling by multiple individuals. Sequence analysis identified an assemblage typically found in dogs but not in people, indicating that zoonotic transmission would be unlikely. (*J Am Vet Med Assoc* 2016;249:644–649)

The enteric protozoan parasite *Giardia duodenalis* (also termed *Giardia intestinalis*) has a broad range of hosts, including humans and dogs.[1-4] Infections in dogs are usually subclinical, but acute and chronic fatty diarrhea with vomiting and lethargy can occur.[1,5,6] Dogs become infected by ingesting *G duodenalis* cysts, which are immediately infective when passed in the feces. Although pathogenesis resulting from host-parasite interactions at the intestinal villi is poorly understood, stress and the number of cysts ingested may play a role in the severity of disease.[7] Prior *G duodenalis* infection does not induce permanent protective immunity against reinfection.[8] A higher prevalence of *G duodenalis* infection has been reported in dogs housed in kennels and shelters than in household dogs.[9-11]

Eight assemblages (designated A through H) of *G duodenalis* that are genetically distinct but morphologically identical are recognized.[12-14] Isolates of the parasite found in dogs typically belong to assemblage C or D. However, assemblage A and B types, which are predominantly found in people, have been detected in dogs in some studies,[1,2,4,15,16] and the zoonotic status of *G duodenalis* is controversial.[17] Several researchers have found that most dogs that are infected with *G duodenalis* and are housed in kennels or shelters harbor assemblage C or D[3,9,10,18,19]; however, in studies[15,16] performed in Japan and Germany, *G duodenalis* cysts shed in the feces of dogs housed in kennels were primarily assemblage A. As such, there is still cause for concern about zoonotic transmission of assemblage A or B *G duodenalis* from dogs housed in kennels.

Elimination of *G duodenalis* infection from kennel-housed dogs is difficult, and most described protocols have not resulted in the absence of the organism after treatment.[20-25] Although there are no FDA-approved drugs

ABBREVIATIONS
IFA   Immunofluorescence assay

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for treatment of giardiasis in dogs in the United States, metronidazole and fenbendazole are commonly used.[24] Fenbendazole administered at the labeled anthelmintic dosage was effective for treatment of *G duodenalis* infections in dogs in several studies.[21,25] Treatments with a febantel-pyrantel pamoate-praziquantel combination product[20,23] and with metronidazole[26,27] were shown to have some successful results with short-term cessation of cyst shedding. Ronidazole is a nitroimidazole, like metronidazole, and has been reported to have some efficacy against *G duodenalis* in vitro and in mice and dogs.[22,28,29] In addition to pharmacologic treatment, cleaning and disinfection of the environment and bathing of dogs to prevent reinfection via ingestion of cysts in residual fecal material are recommended.[7,22-24] The purpose of the study reported here was to develop and test a protocol to control and possibly eliminate *G duodenalis* from a colony of naturally infected, group-housed dogs kept at a veterinary medical college.

Materials and Methods

Dogs and facility

Dogs in the study were owned by the Virginia-Maryland College of Veterinary Medicine at Virginia Tech and kept as a colony for use in veterinary medical teaching. The study took place between November 4, 2013, and June 2, 2014. Dogs used for teaching were purchased from a commercial supplier in the fall and were adopted by new owners by the following summer.

The kennel facility included 3 rooms (2 with 12 runs each and 1 with 10 runs). Each room was divided in half by a central alley, and dogs were housed in pairs in one-half of the room (in 5 or 6 runs on 1 side of the central alley). The runs had concrete floors, and adjacent runs were separated by concrete block walls. Each pair of dogs was moved daily to an empty run in the room for 24 hours to allow for cleaning of the previously used run. During daily cleaning, waste and debris were washed (by use of hot water from a high-pressure hose) from the front to the back of each run and into a drain covered by a metal screen. The drain was continuous for all runs on a given side of the room. Personnel made an effort to avoid contaminating a run with material from other runs in the cleaning process, but the drain represented a potential source of exposure between runs. All dogs had outdoor access at least 3 times/wk (when they were walked by students in the area around the veterinary college campus or allowed to roam freely in an outdoor enclosure). The dogs were routinely handled by animal care staff, students, and clinicians. There were no clothing or sanitation requirements for individuals handling the animals (other than a requirement for students to wear a laboratory coat), and use of footbaths for entering and exiting animal rooms was not required. The housing and care of animals and all study procedures were in compliance with requirements of the Virginia Tech Institutional Animal Care and Use Committee.

In August 2013, 22 Beagles purchased from a commercial supplier were added to an existing group of 12 dogs (11 mixed-breed dogs and 1 Beagle), bringing the colony population to a total of 34 (13 males and 21 females; age range, 1.5 to 2.5 years). The dogs purchased in August had received broad-spectrum anthelmintic treatment with fenbendazole (50 mg/kg [23 mg/lb], PO, q 24 h for 3 days) at the facility of origin 2 weeks prior to arriving at the study facility. In September, several dogs in the colony developed diarrhea. Animal care staff collected feces from all dogs for which samples were available (32/34) and created several pooled fecal samples that were submitted to the diagnostic parasitology laboratory at the veterinary teaching hospital. The samples were evaluated by means of a centrifugal flotation test with 33% zinc sulfate solution as described elsewhere.[30] After centrifugation, the cover slip was removed from the centrifuge, placed on a glass slide, and scanned for *G duodenalis* cysts with a compound microscope at 100X magnification. *Giardia duodenalis* cysts were detected in all pooled samples. All dogs in the colony were treated with metronidazole (30 mg/kg [14 mg/lb], PO, q 24 h for 7 days). Animal care staff reported that most dogs continued to have diarrhea, although records of fecal consistency for individual dogs were not available because dogs were housed in pairs. The initial treatment was considered unsuccessful by veterinarians in charge of the colony, who were also concerned about zoonotic risk to staff, students, and clinicians handling the dogs on a daily basis for routine husbandry and teaching laboratories.

Subsequently, an integrated *Giardia* control protocol was devised on the basis of information from previous studies[20-23] and the clinical experience of the authors. Before implementation of the program (study day –1), individual fecal samples were collected from each dog in the colony and analyzed following centrifugal zinc sulfate flotation; *G duodenalis* cysts were identified microscopically in all (34/34) samples.

Integrated control protocol

All dogs in the colony were treated with fenbendazole (50 mg/kg, PO, q 24 h for 10 days) on study days 1 through 10, with the day between the previous sample collection and the first treatment considered day 0. On day 5, all dogs were bathed by wetting with a hose in a tub, lathering with a grooming shampoo,[a] and rinsing. After bathing, the dogs were temporarily moved to kennels in rooms located in a separate part of the facility that had not housed dogs for > 1 year, and their original kennels were disinfected with a quaternary ammonium solution.[b] Following disinfection, the temperature in the unoccupied original kennels, typically maintained at 20° to 21°C (68° to 69.8°F), was raised to 26.7°C (80°F) for 24 hours (on day 6) to promote drying. The dogs were returned to their original housing on day 7. During the study, the dogs continued to be handled by students, staff, and faculty with no restrictions. Students handling the dogs were required to wear a laboratory coat, but there were no specific sanitation requirements for individuals handling the animals, and use of footbaths for entering and exiting animal rooms was not required. No new animals were introduced to the colony during the study period.

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Sample collection

Individual fecal samples were collected from each dog once weekly through study day 41 (ie, on days 8, 13, 20, 27, 34, and 41), and then the frequency was reduced to once every 3 weeks through day 209 (ie, days 62, 83, 104, 125, 146, 167, 188, and 209). The night before sample collection, each run was routinely cleaned as previously described to remove any fecal matter or debris. The pair of dogs in each run was separated; 1 dog was returned to the original run and 1 was moved to an assigned secondary run (so that all runs in the room were in use and each dog was housed separately). After sample collection on the following morning, the dogs were housed again in pairs. Samples were fixed in formalin ≤ 24 hours after collection and were examined by means of a commercially available direct IFA.[c]

Direct IFA of fecal samples

Throughout the study period and thereafter, a commercially available IFA kit[c] that was shown to be a highly sensitive and specific means of *G duodenalis* diagnosis[31,32] was used in accordance with the manufacturer's instructions to identify *G duodenalis* cysts in feces. Positive and negative controls were prepared each time the test was run. Slides were allowed to dry for ≥ 30 minutes and were examined at 100X magnification with a fluorescence microscope. A sample was considered to test positive if any *G duodenalis* cysts were detected.

PCR assay and sequencing of parasite DNA

After initial metronidazole treatment and prior to starting the integrated control protocol, parasite DNA was extracted from 1 fecal sample (from 1 dog) that contained high numbers of *Giardia* cysts; PCR assay amplification of the small subunit ribosomal RNA gene and sequencing were performed to determine the assemblage of *G duodenalis*[33] present. Cysts were concentrated and isolated from the fecal sample with PBS containing EDTA and Sheather sugar solution (specific gravity, 1.26).[34] The DNA was extracted from the processed sample with a commercially available kit.[d] Reagents from the kit were used with modifications as described elsewhere.[35] A nested PCR assay protocol was used to amplify a 292–base pair fragment of the small subunit ribosomal RNA gene.[36] The PCR products were visualized following electrophoresis on a 1% agarose gel stained with ethidium bromide with a low-molecular-weight ladder and positive control on the gel to allow for identification of the correct band size. The 292–base pair PCR product was isolated and extracted from the gel by use of a commercial kit[e] following the manufacturer's instructions. The resulting product was sequenced at the Virginia Bioinformatics Institute with the same PCR primers that were used for amplification. The sample was sequenced in 5′ to 3′ and 3′ to 5′ directions, and the resulting chromatograms underwent analysis with a sequence alignment editor.[f]

Environmental temperatures

Monthly climatological summaries were obtained from the national weather database[g] for Blacksburg, Va,

for the period of the study. The data were used to assess ambient environmental temperatures and the number of days with freezing temperatures (≤ 0°C [32°F]).

Statistical analysis

Likelihood of a cure (defined as absence of *G duodenalis* cysts on day 125 [115 days after fenbendazole treatment ended]) was compared with the likelihood of a cure just before treatment (on day 0). Day 125 was selected because it was the last sampling date for which all dogs were available for sample collection. Data were analyzed by means of exact conditional logistic regression analysis with dog identification specified as strata. Because the model could not be run with zeros, a constant was added to the data points.[37] Values of $P < 0.05$ were accepted as significant. The analysis was performed with a statistical software package.[h]

Results

Kennel personnel reported that many of the dogs continued to have diarrhea during the first week of fenbendazole treatment, but the individual dogs affected were not specifically identified at that time. By day 13 (3 days after fenbendazole treatment ended), 29 of 34 (85%) dogs had normal fecal consistency; 4 (12%) dogs had loose stool and 1 (3%) still had diarrhea. The dog with persistent diarrhea was being treated with metronidazole (30 mg/kg, PO, q 24 h for 7 days [days 11 through 17]) because of staff concerns, although no *G duodenalis* cysts had been detected in the dog's feces after day 1. Diarrhea had resolved by day 20 (10 days and 3 days after the end of fenbendazole and metronidazole treatments, respectively).

All fecal samples tested negative for *G duodenalis* cysts by IFA on day 8 (during treatment) and on day 13 (after treatment). All samples on all remaining sample collection days tested negative, except that on day 20, 1 *G duodenalis* cyst was detected in 1 sample from a dog that had been clinically normal since day 8. The IFA was repeated in triplicate for this sample, but no other cysts were detected, and no additional treatment was undertaken. Of 34 dogs in the colony, all were present through day 125; the numbers subsequently decreased as dogs were adopted by new owners, with 27 (79%), 13 (38%), and 9 (26%) dogs remaining through days 167, 188, and 209, respectively. The cure rate on day 125 (34/34), compared with that on day 0 (0/34), was significant ($P < 0.001$). The odds of a cure after treatment were 35 times the odds prior to treatment (OR, 35.0; 95% confidence interval, 5.9 to infinity).

Parasite DNA from the *G duodenalis*-positive pretreatment sample was identified by sequencing of the small subunit ribosomal RNA gene as belonging to assemblage C (a *G duodenalis* group for which dogs are known hosts). The isolate had 99% nucleotide sequence identity to the assemblage C strain referenced in the literature (Genbank accession No. AF199449).[2]

The ambient environmental temperatures for the study time period were reviewed. From November 2013 to April 2014, there were 152 days in which temperatures were ≤ 0°C.

Discussion

Several authors have reported short-term successful control of *G duodenalis* infection and elimination of cyst shedding in dogs with drug treatment and various hygienic measures.[22,23] In the present study, dogs continued to be exposed to a variety of staff members and students during the treatment period and continued to have outdoor access to an area heavily used for exercising college-owned and privately owned dogs. Substantial control of *G duodenalis* infections was achieved in the group-housed dogs of this report by use of an integrated protocol that included hygienic measures and fenbendazole treatment. Even considering the single positive IFA result on day 20, the protocol can be considered effective, as *G duodenalis* cysts were not detected in any of the other dogs and clinical signs resolved in all dogs. The detection of only 1 cyst in 1 sample on day 20 of the 6-month observation period after the completion of the integrated control program was considered to indicate a very low degree of residual infection or to represent a contaminant from the positive control for the test or from another sample being evaluated in the laboratory.

Procedures used in investigations by Payne et al[23] and Fiechter et al[22] also included bathing of dogs and environmental disinfection in addition to medical treatment, but each study had > 1 dog test positive for *G duodenalis* during the monitoring period following treatment (on days 17 and 40, respectively, with the day of treatment considered day 0).[22,23] Our protocol included several features from the aforementioned studies[22,23] but also differed from them in some respects. We used a 10-day course of fenbendazole, whereas a 3- or 5-day course of febantel (probenzimidazole; 27 to 35 mg/kg [12 to 16 mg/lb], PO, q 24 h) or a 7-day course of ronidazole (30 to 50 mg/kg, PO, q 12 h) was used in the other studies.[22,23] The dogs in the present study had been treated with the related drug, metronidazole, after several developed diarrhea, but that treatment did not appear to be effective. This may have been attributable to reinfection because no hygienic measures had accompanied that treatment beyond routine cleaning with hot water from a high-pressure hose to remove feces and debris from runs. Alternatively, the dogs had been given a lower dosage of metronidazole (30 mg/kg, PO, q 24 h for 7 days) than is often recommended (45 to 60 mg/kg [20 to 27 mg/lb], PO, q 24 h for 5 days)[27,38] as treatment for giardiasis, and this may not have been sufficiently effective to eliminate infection from all dogs.

Of particular interest, the kennel hygiene practices, including measures to limit the spread of parasites, used in the present study were less intensive than in the 2 aforementioned studies,[22,23] in which footbaths or shoe changes were used for personnel entering and exiting the dogs' area, and dogs had limited outdoor access. We did not instruct study animal care staff and veterinary students to change shoes, and disinfectant footbaths were not used. Dogs continued to have access to the outdoors multiple times each week and were handled and moved routinely throughout the facility for purposes of veterinary instruction.

Bathing is routinely recommended in conjunction with medical treatment for *G duodenalis* infection to prevent reinfection from cysts in the fur.[7,22,23,25] Bathing was performed on day 5 of treatment in the present study and was followed by another 5 days of fenbendazole administration; in the study by Fiechter et al,[22] dogs were bathed prior to the start of treatment and again on day 6 of the 7-day medical treatment protocol, and Payne et al[23] bathed study dogs on the last day of treatment. It is possible that bathing at the end of treatment allowed for reinfection following cyst ingestion from the fur, whereas bathing in the middle of the treatment period allowed for the potentially ingested cysts to be neutralized by drug treatment, thus preventing reinfection.

The disinfectant used for environmental cleaning could also have influenced the results of these studies. Quaternary ammonium compounds, steam, and boiling water are described as being effective against *G duodenalis* cysts.[39] Fiechter et al[22] used 4-chlorine-M-cresol, which is a chlorinated chemical,[40] for premises disinfection on the day prior to initiating drug administration to dogs and again on the day before treatment ended. Payne et al[23] used a disinfectant that contained 2 quaternary ammonium compounds as active ingredients (didecyl dimethyl ammonium chloride and alkyl dimethylbenzyl ammonium chloride) similar to that used in the present study; however, our daily cleaning practices used pressurized hot water and the disinfectant was used on day 5 only, whereas the earlier investigators applied the disinfectant compound daily throughout the study.

Our protocol also included setting the temperature in the unoccupied kennels after disinfection to 26.7°C for 24 hours to facilitate drying. The survival rate of *G duodenalis* cysts in soil, feces, and water decreases at temperatures > 25°C (77°F),[41,42] and it has been suggested that kennels with concrete or paved floors with grooves can retain cysts after washing.[11] Thus, it is possible that the high temperature allowed for more effective desiccation and death of any remaining cysts during the 24-hour period before dogs were returned to the runs.

The time of year could also have contributed to the successful outcome in the present study. The integrated control program began in November 2013, with weekly fecal collections continuing through December. Fecal samples were subsequently collected and analyzed every 3 weeks from January to June 2014. *Giardia* infection in humans and dogs may be more prevalent in warmer months,[43-46] and after 2 weeks at temperatures below the freezing point (–4°C [24.8°F]), *G duodenalis* cysts were not infective to animals in an experimental study.[42] Intermittent freezing temperatures were recorded in the region where the study was performed from November 2013 through April 2014 and may have reduced the risk of reinfection from exposure to the organism outdoors.

A concerted effort was made to control *G duodenalis* in this colony of teaching dogs because of concerns about possible zoonosis as well as animal health. These dogs were handled on a daily basis by staff, students, and clinicians for routine husbandry and teaching laboratories. It was because of the concern about zoonotic dis-

ease that the *G duodenalis* genotyping was undertaken. The result indicating that the organisms were assemblage C was consistent with other reports[3,9,10,18,19] in which most *G duodenalis* isolates from dogs kept in shelters or kennels were of the assemblage C or D genotype (with some dogs harboring both). Because the dogs in the present study were from the same point of origin, lived in the same environment, and had similar exposures on a day-to-day basis, we deemed it likely that 1 sample would be representative of organisms in the entire colony; however, the identification of assemblage C organisms from 1 sample did not rule out the possibility of other *G duodenalis* assemblages in other dogs. We considered it likely that the dogs were infected with *G duodenalis* before they arrived at our facility, and we did find that replacement dogs acquired from the same supplier the following year were also infected with assemblage C *G duodenalis* on the day that they arrived at the facility. However, it is also possible that the *G duodenalis* infection in the study dogs was acquired from privately owned dogs that were walked in the same outdoor areas as the colony dogs.

No currently available test can determine whether *Giardia* trophozoites are completely absent from an animal, and we believe it is unlikely that complete eradication in a kennel situation is achievable. The control protocol described in this paper had a successful outcome in reducing the *G duodenalis* infection to an undetectable level and substantially reduced its impact, considering that all 34 dogs tested positive for the parasite prior to the treatment and all tested negative on the last day that they were available for sampling (from 115 to 199 days after the treatment ended). This approach could be used in shelters and teaching and research facilities or in other canine group-housing situations as a practical tool to help control *G duodenalis* infections.

Acknowledgments

Footnotes

a. Cleen Sheen Grooming Shampoo, VEDCO, St Joseph, Mo.
b. LabSan256QPC, Sanitation Strategies LLC, Bend, Ore.
c. Merifluor Cryptosporidium-Giardia, Meridian Bioscience Inc, Cincinnati, Ohio.
d. DNeasy Blood & Tissue Kit, Qiagen, Venlo, The Netherlands.
e. QIAquick Gel Extraction Kit, Qiagen, Venlo, The Netherlands.
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h. SAS, version 9.4, SAS Institute Inc, Cary, NC.

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From this month's AJVR

Pharmacokinetics of voriconazole after intravenous and oral administration to healthy cats

Polina Vishkautsan et al

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OBJECTIVE
To determine pharmacokinetics and adverse effects after voriconazole administration to cats and identify an oral dose of voriconazole for cats that maintains plasma drug concentrations within a safe and effective range.

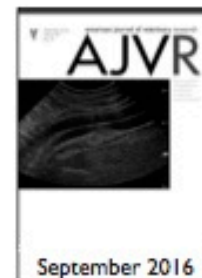### ANIMALS
6 healthy cats.

PROCEDURES
Voriconazole (1 mg/kg, IV) was administered to each cat (phase 1). Serial plasma voriconazole concentrations were measured for 24 hours after administration. Voriconazole suspension or tablets were administered orally at 4, 5, or 6 mg/kg (phase 2). Plasma voriconazole concentrations were measured for 24 hours after administration. Pharmacokinetics of tablet and suspension preparations was compared. Finally, an induction dose of 25 mg/cat (4.1 to 5.4 mg/kg, tablet formulation), PO, was administered followed by 12.5 mg/cat (2.06 to 2.7 mg/kg), PO, every 48 hours for 14 days (phase 3). Plasma voriconazole concentration was measured on days 2, 4, 8, and 15.

RESULTS
Voriconazole half-life after IV administration was approximately 12 hours. Maximal plasma concentration was reached within 60 minutes after oral administration. A dose of 4 mg/kg resulted in plasma concentrations within the target range (1 to 4 µg/mL). Adverse effects included hypersalivation and miosis. During long-term administration, plasma concentrations remained in the target range but increased, which suggested drug accumulation.

CONCLUSIONS AND CLINICAL RELEVANCE
Voriconazole had excellent oral bioavailability and a long half-life in cats. Oral administration of a dose of 12.5 mg/cat every 72 hours should be investigated. Miosis occurred when plasma concentrations reached the high end of the target range. Therefore, therapeutic drug monitoring should be considered to minimize adverse effects. (*Am J Vet Res* 2016;77:931–939)

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