GIARDIA DUODENALIS AND *CRYPTOSPORIDIUM* SPP. INFECTION IN THE DOG (*CANIS LUPUS FAMILIARIS):* A ONE HEALTH APPROACH TO TESTING STRATEGIES AND EPIDEMIOLOGIC ANALYSIS

A Dissertation

by

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ABSTRACT

Giardia duodenalis and *Cryptosporidium* spp. are zoonotic protozoal pathogens, spread by a fecal-oral route, which can infect a wide range of hosts including but not limited to dogs and humans. *Giardia* infections were reported to be present in 15.2%, and *Cryptosporidium* infections in 5%, of dogs globally. The main goal of this project was to define factors associated with *Giardia* and *Cryptosporidium* in dogs in Texas. Fecal samples were collected from laboratory dogs from an academic research facility and a local laboratory dog supplier. We first assessed a human *Giardia* and *Cryptosporidium* point-of-care test in dogs. We then determined factors associated with subclinical infections in kennel housed dogs. Multilocus PCR of three gene targets was implemented, followed by sequencing and typing to determine the assemblages of *G. duodenalis*. SSU rRNA PCR sequencing was used to determine the species of *Cryptosporidium.* Finally, we assessed *Giardia* infection, in the State of Texas at the county level, by analyzing publicly available data on canine test positivity and potential social determinants of animal health (SDOAH). We showed that the human point of care test could successfully be used to identify *Giardia* and *Cryptosporidium* in the dog with high specificity (%) but low sensitivity (%). *Giardia duodenalis* assemblages A, C, and D were detected in our dogs, while *C. canis* was the sole species detected. We found the presence of hard feces to be associated with lower odds (0.3 [0.1, 1.0]) of subclinical *Giardia* infection. Dogs that were ≤18 months old had 3 times the odds of subclinical *Giardia* infection compared to older dogs. We found that SDOAH may be indicators of canine *Giardia* infection risk and that counties with low veterinary coverage (represented by the veterinary care accessibility score [VCAS] <25) were also those with more *Giardia* infections (PR 11 [7, 18]) when college graduation rates were low.

With these findings, we demonstrated the potential utility of an available point-of-care test in dogs to detect *Giardia* and *Cryptosporidium* infections and the value of understanding clinical, social, and epidemiologic risk factors of *Giardia, Cryptosporidium,* and other important zoonotic pathogens of public health concern.

DEDICATION

I dedicate this dissertation to my family and friends. Especially my husband, Chris, my children, Adeline and Asher, my parents, Lonny and Tammy, and my mother-in-law Sandy, who provided support, patience, and made sacrifices for the pursuit of this research. Thank you all for the encouragement, faith, and prayers throughout this journey, without which this would not have been achieved.

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Contributors

This work was supervised by a dissertation committee consisting of Dr. Christine Budke [chair] of the Department of Veterinary Integrative Biosciences, Dr. Rebecca Fischer [co-chair] of the Department of Epidemiology and Biostatistics, and Drs. Meriam Saleh and Guilherme Verocai [members] of the Department of Veterinary Pathobiology.

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NOMENCLATURE

BG	Beta-giardin gene
CDC	Centers for Disease Control and Prevention
CI	Confidence Interval
CAPC	Companion Animal Parasite Council
DFA	Direct Immunofluorescent Assy
EDTA	Ethylenediaminetetraacetic acid
ERS	Economic Research Service
GDH	Glutamate Dehydrogenase gene
IQ	Interquartile Range
MUA	Medically Underserved Area
OR	Odds Ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PR	Prevalence Ratio
QC	QuikChek Patient Side Coproantigen Test
ROC	Receiver Operating Characteristic
RUC	Rural Urban Continuum Codes
SDOAH	Social Determinants of Animal Health
SDOH	Social Determinants of Health
SSU RNA	Small Subunit Ribosomal Ribonucleic Acid
TPI	Triosephosphate Isomerase gene
TVMDL	Texas Veterinary Medical Diagnostic Laboratory
USDA	United States Department of Agriculture
VCAS	Veterinary Care Accessibility Score

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CHAPTER I

INTRODUCTION

Giardia duodenalis and *Cryptosporidium* spp. are two important gastrointestinal pathogens in humans and canids alike [1,2]. Co-infections with these two pathogens in dogs have not been well documented, with disagreement in the literature regarding the clinical significance [3,4]. In the fall of 2020, clinical *Giardia* and *Cryptosporidium* co-infections were identified in a litter of puppies born at our institution's research facility, followed by a litter of puppies with clinical infections of only *Cryptosporidium* in the winter of 2021. After this cluster of cases, we realized the need to further evaluate both organisms in dogs. The dogs in this population have different conditions that can be affected by these organisms and can make some of them more susceptible to clinical disease. They also have an increased exposure to more diverse groups of people than most pet dogs due to their contact with many students, faculty, and care staff.

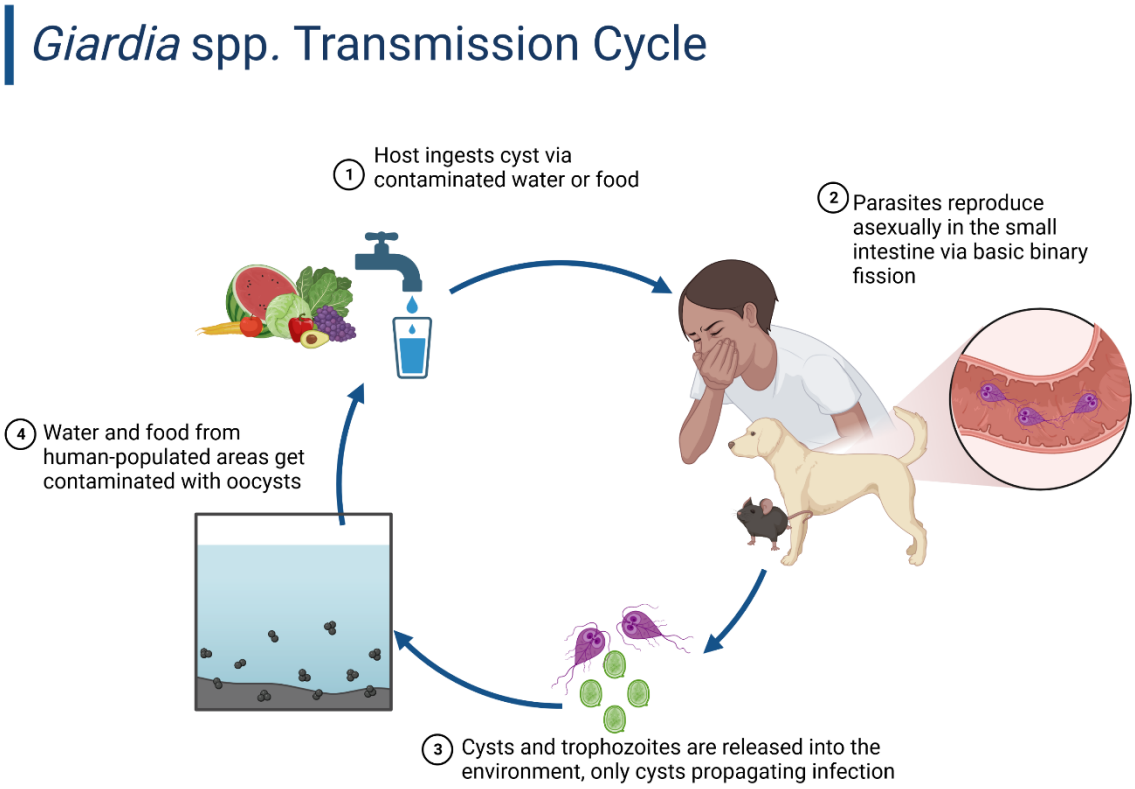
Giardia duodenalis

Overview

Giardia duodenalis is a protozoal pathogen affecting many species of domestic and wild animals and is also potentially zoonotic to humans [5]. It was first identified by van Leeuwenhoek nearly 350 years ago [6]. In 1859 Lambl further described the organism and named it *Cercomonas intestinalis*, it was renamed *Giardia lamblia* in 1915, and is currently synonymous with *Giardia intestinalis* [5,7]. *Giardia duodenalis* belongs to Kingdom Protozoa (syn. Protista), Phylum Sarcomastigophora, Subphylum Mastigophora, Class Zoomastigophora,

Order Diplomonadida, and Family Hexamitidae Subphylum Mastigophora boast flagella [8]. Order Diplomonadida is known for having two karyomastigonts, each with four flagella, no mitochondria, no Golgi apparatus, binucleated, cyst producing, and possess the ability to live freely or parasitic [5,7].

Figure I.1. *Giardia* spp. transmission cycle



Life Cycle

Giardia has a direct life cycle, which has two distinct phases, a vegetative trophozoite and cyst phase (Figure I.1). It undergoes asexual reproduction via binary fission. Both nuclei are transcriptionally active, and divide left to right so that the daughter cells receive one copy of each nucleus from the parent cell [9]. This process produces trophozoites to propagate individual infection and cysts that are passed in the feces. Cysts are immediately infective when shed and remain viable in water in the environment at 4ºC for up to three months, until they are consumed by an animal or human host, commonly via contamination of water or raw food [5,10]. After ingestion, the cysts will immediately undergo excystation in the small intestine and the trophozoites will feed off the nutrients in the host's digestive tract as they pass through the tract with encystation occurring in either the small or large intestine [11]. The prepatent period of *Giardia* is dependent on the host species but is usually around 3-10 days [11].

Assemblages

Giardia duodenalis is currently classified into 8 different genetic assemblages, designated with letters A-H, with variable clinical significance in humans or dogs [12]. The assemblage types, and sub-assemblage types, have been noted to have a potential connection with the resulting clinical disease, but further research is needed to clearly define these relationships [9].

Natural hosts for assemblage A include humans, many domestic animals including dogs, cats, cattle, sheep, pigs, horses, and goats, and many wild animals including fallow deer, white-tailed deer, reindeer, coyotes, foxes, Australian house mice, moose, muskoxen, howler monkeys, alpaca, water buffalo, wild boar, grey kangaroos, opossum, wallabies, koalas, ferrets, red deer,

roe deer, marmosets, planigales, and quendas [9,13]. Assemblage A has the largest array of susceptible host species and is split into assemblages A-I, A-II, and A-III [9,14]. Current studies have shown that assemblage A-I is typically isolated from domestic animals and livestock while assemblage A-II is typically isolated from humans and assemblage A-III is seen in wild ruminants [15]. Assemblage B has been isolated from humans frequently and has been associated with higher rates of clinical illness [9]. Assemblage B has also been isolated in dogs, guinea pigs, rabbits, cattle, sheep, horses, beavers, mandrills, macaques, chimpanzees, howler monkeys, coyotes, kangaroos, red foxes, and chinchillas with variable host level clinical significance [9,13]. Assemblages C and D are most commonly isolated in both clinical and asymptomatic dogs and usually cause little to no disease in humans although rare cases of infection with assemblage C in children and immunocompromised adults have been reported [12,16]. Assemblage E has been isolated from sheep, cattle, deer, and cats [13]. Assemblage E has also been noted to sporadically cause clinical illness in humans [9]. Assemblage F is typically found in cats, assemblage G in rodents, and the newly isolated assemblage H has been found in pinnipeds [9]. With the diversity in clinical illness, natural hosts, and genetics between these assemblages, the following recommendations have been suggested by Thompson and Monis for renaming the assemblages of *Giardia*: assemblage A as *G. duodenalis*, assemblage B as *G. enterica*, assemblages C/D as *G. canis*, assemblage E as *G. bovis*, assemblage F as *G. cati*, and assemblage G as *G. simondi*. This would accompany the eight already named *Giardia* species, including but not limited to, *G. muris* that typically infects mice and *G. microti* which typically infects voles and muskrats [17].

Differences in mechanisms in which *Giardia* induces illness have been noted between the assemblages, but more research is needed to fully classify the mechanisms of each of the

different assemblages and sub-assemblages [9]. Current knowledge of the infection cycle within the host states that the trophozoites of these different assemblages stimulate differences in gene expression of the intestinal epithelial cell (IEC) [9]. Host proteins are secreted upon interaction between the trophozoite and IECs, apoptosis can be induced, and an increase in intestinal permeability occurs [9]. Arginine levels decrease, effectively decreasing enzymes at the IEC brush border, resulting in hypermotility [9]. Furthermore, reactive oxygen species and nitric oxide are both released by the IEC as innate antimicrobial peptides yet certain trophozoites show resistance to these, such as those of assemblage B [9].

Disease Syndrome

Human Illness

Human illness due to *Giardia* occurs typically six to fifteen days post infection [10]. The disease syndrome is classically characterized by diarrhea and can be either subclinical or associated with diarrhea due to malabsorption, nausea, vomiting, and, with prolonged illness, significant weight loss [6]. Illness with *Giardia* has also been associated with non-gastrointestinal signs and symptoms such as pruritis, urticaria, uveitis, food antigen sensitivity, and synovitis [6]. Children who have had giardiasis many times have a more significant illness course including stunted growth and development, poor cognitive function, and malnutrition [6]. Specific nutrients that have been noted to not be well absorbed during infection include electrolytes, fats, D-xylose, lactose, vitamin A, and vitamin B12 [9].

Giardiasis is not limited to an acute disease process and chronic giardiasis is a long-regarded condition. In the 1950s, Rendtorff studied *Giardia* in prisoners where 14 individuals volunteered to be infected with the organism [6,18]. In this study, within 41 days he saw that 12

had successfully stopped shedding the organism. At 146- and 163-days post exposure he noted two participants were still shedding the organism. Another study on chronic malabsorption conducted in India noted that out of adults diagnosed with malabsorption, 24% were infected with *Giardia*, compared to only 8% of controls sampled [19]. In another study, a prospective cohort of individuals from a large (n=124) waterborne outbreak of *Giardia*, that had been previously treated, were evaluated [20]. In this study they found persistent infections in 32.3% of patients and of those patients 87.2% had microscopic duodenal inflammation compared to 28.0% of the *Giardia* negative controls despite treatment with metronidazole. An additional study looking at the chronic conditions of chronic fatigue and irritable bowel syndromes (IBS), 3 years after a history of acute giardiasis, found that 46.1% of individuals in the infected group reported having IBS compared to 14.0% of controls [21]. Chronic fatigue syndrome was reported by 46.1% of individuals compared to 12.0% of controls.

Induction of gastrointestinal (GI) inflammation by *Giardia* is a current topic of interest. *Giardia* has been a well-established cause of disruption of the GI mucosal barrier and disruption of villi function [22]. It is also well known that disruption of the mucus layer of the GI tract can induce pro-inflammatory states [22]. Ample research has shown increased expression of inflammatory cytokines including IL-1β, IL-4, IL-6, CSCL8, IL13, and TNF-α with *Giardia* infection [22]. The organism also has been noted to induce accumulations of neutrophils, which are also touted as a contributing factor to the diarrheal disease process in addition to the role of the organisms' competition for L-arginine within the lumen of the intestine [22]. Alternatively, the organism can also induce inflammation secondary to direct disruption to the intestinal epithelium. A recent report demonstrated *Giardia* trophozoites within the duodenal epithelium of a human patient [23]. This is contradictory to other studies that have failed to identify epithelium

invasion, leading to the thought that this is an assemblage, or sub-assemblage, feature not shared throughout the species [23].

The mechanism that drives chronic giardiasis is still under consideration. Recent work has shown the importance of Immunoglobulin (Ig) A, and immunoglobulin transport proteins, in host defense against the organism [6]. This mechanism is supported by data showing children living in endemic regions having higher levels of infection than adults, and all residents in these areas exhibiting a decreased incidence of giardiasis compared to non-native visitors [6]. Other studies have shown significantly lower IgG and IgA levels in children with acute or chronic giardiasis compared to patients with no clinical signs [6]. IgA is dependent on IL-17A, and if this interleukin is deficient in the host, then disease could be more likely [9].

Canine Illness

Dogs with giardiasis will show similar incubation periods, disease durations, and classic signs of disease as humans with giardiasis. The incubation period in dogs has been cited to be approximately 5 days in dogs [24]. The signs of illness due to *Giardia* include abdominal discomfort, abdominal pain, cramping, watery and mucoid diarrhea, and steatorrhea [25]. Canine GI inflammation is not as well characterized as what has been described in humans [25]. One feature gaining momentum is the disruption in the microbiome associated with *Giardia*. While it is not well understood, currently the thought is that dysbiosis, or disruption on the GI microbiome, is what can predispose dogs to exhibit signs and symptoms of disease [25]. Dysbiosis can be the result of antimicrobial administration leading clinicians to question the use of medications such as metronidazole for the treatment of disease [26]. Furthermore, evidence supporting the theory of dysbiosis resulting in giardiasis has been noted in small animal

laboratory studies where mice given probiotics prior to infection with *Giardia* have reduced severity and duration of disease [27].

***Cryptosporidium* spp.**

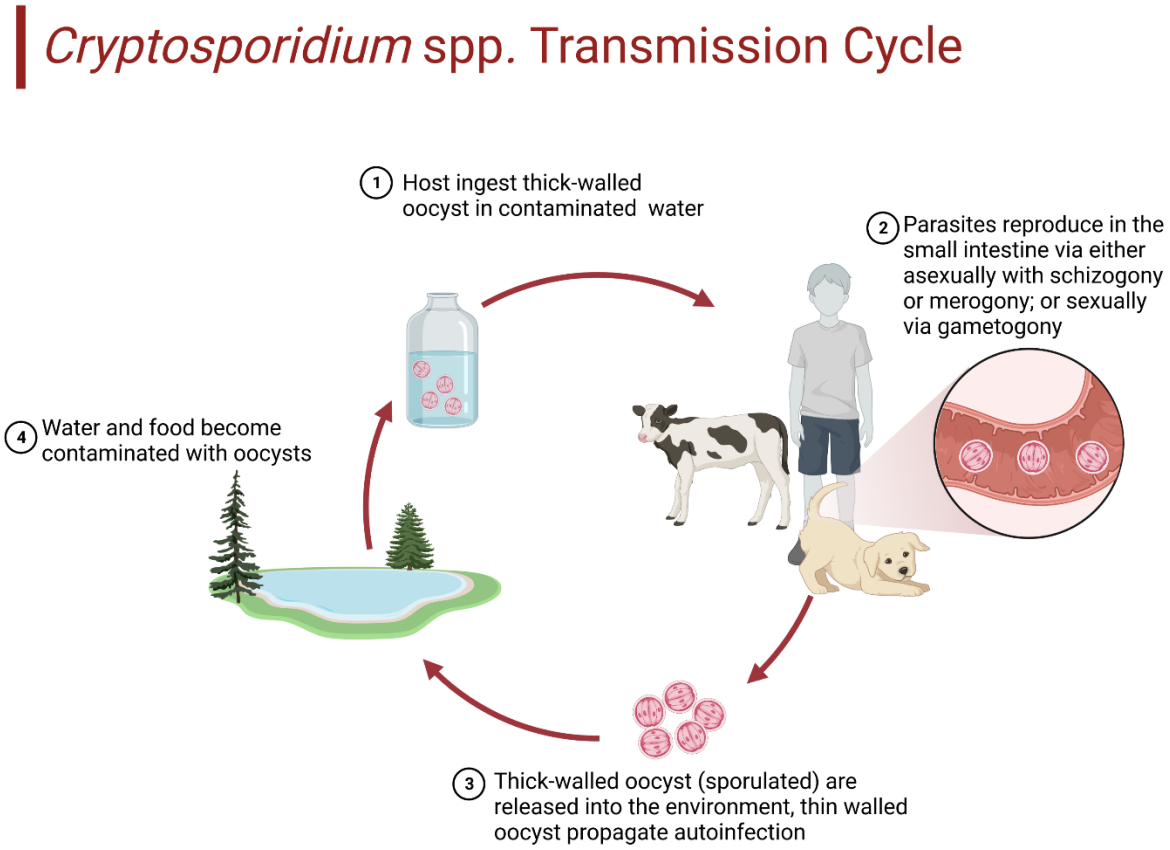
Overview

Cryptosporidium is a genus of protozoal pathogens made up of nearly 30 different species [28]. *Cryptosporidium* was first identified in 1907 when E.E. Tyzzer noted an organism frequently occurring in the gastric glands of laboratory mice, but not of wild mice [29]. It was not until 1976 that *Cryptosporidium parvum* was identified as a human pathogen when it was isolated out of a 3-year-old child what had acute enterocolitis [30]. Shortly after this, in 1981, researchers showed presence of the organism in dogs when they isolated antibodies against *Cryptosporidium* in 16 of 20 dogs sampled [31]. It was suspected that the disease was only of concern in immunocompromised individuals originally. This theory was propagated with the acquired immunodeficiency syndrome (AIDS) epidemic in the 1980s. It has been cited that 14-24% of AIDS patients with life-threatening chronic diarrhea were infected with *Cryptosporidium* compared to 0-4% of AIDS patients without diarrhea [32]. More recently the importance of this pathogen has been reinforced with the occurrence of two large scale outbreaks due to a contaminated drinking water supplies in Swindone and Oxforshire UK and Milwaukee, WI [33].

Cryptosporidium spp. belong to the Kingdom Protozoa, Phylum Apicomplexa, Class Conoidasida, Order Eucoccidiorida, Suborder Eimeriorina, and Family Cryptosporidiidae [34]. The Phylum Apicomplexa is characterized by an apical grouping of organelles and a complex life cycle [35]. *Cryptosporidium* has been classified as a coccidian due to its similarities in life cycle,

yet with many differences from typical coccidia it is often placed at the base of the phylum as it is evolutionarily unique [36].

Figure I.2. *Cryptosporidium* spp. transmission cycle



Life Cycle

Cryptosporidium, like *Giardia*, is spread in a fecal oral route via ingestion of oocysts that are fully sporulated when passed in the feces [35]. Infected animals or humans will shed oocysts into the environment, which are the infective stage (Figure I.2). Upon consumption of the oocyst, it will excyst releasing a sporozoite that enters the epithelial cell and gives rise to a merozoites or schizozoites [35,37]. The parasite exists in a parasitophorous vacuole membrane, extra-cytoplasmically as a Type I meront [36,38]. Asexual reproduction occurs with schizogony or merogony giving rise to merozoites [35]. These merozoites are released and infect surrounding cells and can either give rise to Type I meronts and repeat the cycle or Type II meronts [36]. A Type II meront enters sexual reproduction, through gametogony, producing the gametocytes, microgamonts, males, and macrogamonts, females [35]. The microgamont releases microgametes that fertilize the macrogamont giving rise to the zygote [35]. The zygote can then form an oocyst while in the parasitophorous vacuole, which is then released, with the end results of re-infection, via excystation within the host gastrointestinal tract with a thin-walled cyst (approximately 20%), or excretion into the environment with a thick wall cyst (approximately 80%) [35–37]. The prepatent period is variable among host species but in general is 2-7 days [39]. The organisms can be cultured in vitro, indicating it is not an obligatory intracellular pathogen, and it has been noted to have unequal pairing of ages and plasticity of the life cycle, skipping stages [36]. Recent work has shown that regarding life cycle and genotyping, *Cryptosporidium* is much closer related to the gregarines opposed to coccidia [36].

Table I.1. Currently recognized species of *Cryptosporidium* [40]

***Cryptosporidium* spp.**	**Mammalian Primary Host Spp.**	***Cryptosporidium* spp.**	**Avian/Reptile/Fish Primary Host Spp.**
*C. andersoni**	Cattle	*C. baileyi**	Birds
*C. bovis**	Cattle	*C. fragile*	Toads
*C. canis**	Dogs	*C. galli*	Birds
C. cuniculus	Rabbits	*C. meleagridids**	Birds
*C. cuniculus**	Rabbits	*C. molnari*	Fish
*C. ditrichi**	Rodents	*C. serpentis*	Snakes and Lizards
*C. erinaceid**	Hedgehogs and Horses	*C. varanii*	Lizards
C. fayeri	Marsupials		
*C. fayeri**	Marsupials		
*C. felis**	Cats		
C. hominis	Humans		
C. macropodum	Marsupials		
*C. muris**	Rodents		
*C. occultus**	Rodents		
*C. parvum**	Ruminants		
C. ryanae	Cattle		
*C. scrofarum**	Pigs		
*C. suis**	Pigs		
*C. tyzzeri**	Rodents		
*C. ubiquitu**	Ruminants, Rodents,		
*C. viatorum**	Humans		
*C. wrairi**	Guinea pigs		
*C. xianoi**	Sheep and Goats		
*denotes evidence of zoonosis			

Species

Of the near 30 species currently recognized, *Cryptosporidium canis* is the species most commonly found in dogs, yet *Cryptosporidium parvum, Cryptosporidium hominis,* and *Cryptosporidium muris* have also been identified in dogs and are the species most associated with disease in humans [12,28,35,40,41]. In a recent study of children in developing countries, *C. canis* is the cause of 4.4% of cases of cryptosporidiosis [42]. Of the recognized species, almost 20 have been noted in infections in humans [28]. See Table I.1 for a list of all currently recognized species of *Cryptosporidium* with the host species most affected. In addition to the recognized species of *Cryptosporidium* there are also 29 suggested genotypes of *Cryptosporidium* including ferret, mouse, skunk, marsupial (4), horse, rabbit, monkey, pig (2), cervid (2), fox, muskrat (2), deer mice, squirrel (2), bear, goose (2), duck, bovine, snake, tortoise, lizard, and woodcock that have not been classified into specific species [28,43].

Disease Syndrome

Human Illness

Cryptosporidium in humans normally affects the small and large intestine, but also has been found in the stomach, bile duct, gall bladder, and respiratory tract [36]. Clinical onset of disease occurs two to ten days after infection [32,44]. Classical signs and symptoms include large volumes of watery diarrhea, 1-14 days in duration, cramps, fatigue, vomiting, fever, and malaise [44]. It is most common in children and in Africa and Asia, *Cryptosporidium* is one of the top four causes of severe diarrhea and is considered the second greatest cause of diarrhea and death in children following rotavirus [28].

This is a disease of specific concern in immunocompromised individuals. People suffering with asthma, severe allergies, autoimmune diseases, or with organ transplants who are receiving immunosuppressive therapy are of specific risk as well as individuals with AIDS caused by the Human Immunodeficiency Virus (HIV) [44]. In immunocompromised individuals' case fatality rates have been documented to reach 52-68% [45]. The CDC published a study using the MarketScan Commercial Claims and Medicare data from 2012 to 2017 and found that 6.2% of people between 18-64 years of age and 2.6% of children ≤ 18 years of age had immunosuppressive conditions [46]. Of these individuals, 27% had HIV or some other hematologic condition, 36% were being treated with immunosuppressive drugs due to organ transplant or autoimmunity, and 37% were being treated with immunosuppressives without indication of an immunosuppressive disease process [46]. Globally this is even more of a concern with health inequities still at the forefront of the global health crisis. It is estimated that 1.7 billion individuals, 22% of the world population, are suffering from one or more conditions that could compromise the body's immune response [47].

The relationship between immunosuppression and disease severity with *Cryptosporidium* is evidence based. The body's ability to combat cryptosporidiosis is dependent on the adaptive immune response including IgA, CD4+ T lymphocytes, and cytokines that support a Th1 response such as IFN-γ, IL-18, and potentially TNF-α and IL-12 [41]. Innate immune system mechanisms essential to the response include natural killer (NK) cells, nitric oxide (NO) production from phagocytes, and activation of the mannan-binding lectin pathway of complement [48].

Chronic manifestations of cryptosporidiosis are common in children and one study noted that *Cryptosporidium* was the most common pathogenic parasite (14%) associated with the presence of pediatric patients with malabsorption syndrome [19].

In recent literature, *Cryptosporidium* has been found more commonly in colon cancer patients though the pathogenesis of this relationship has yet to be firmly defined [49]. In a recent immunocompromised mouse model of persistent infection, researchers established that chronic infection with chronic inflammation and an upregulation of inflammatory cytokines plays a key role in the development of parasite induced GI neoplasia [49]. They attributed the chronic inflammation to an upregulation of IFN-γ and downregulation of α-defensins.

Canine Illness

The disease course of *Cryptosporidium* in dogs follows that of humans and other animals. Young dogs are at higher risk of disease, and in those who are immunocompetent, the disease usually lasts one to two weeks and is self-limiting including signs of diarrhea, fever, and inappetence [12,50]. Dogs with diarrhea are more likely to shed oocysts, but it has been documented that asymptomatic dogs can shed oocysts for 21-88 days post infection [51]. Like in humans, puppies that are also experiencing another immunosuppressive disease such as canine distemper, parvovirus enteritis, or other immunosuppressive condition are more likely to have clinical signs [51]. While most infections with *C. canis*, the host specific species, are limited in clinical significance, severe diarrhea, malabsorption, and weight loss have all been reported [51]. Studies into the immunologic response, inflammation, and other components of chronic illness are limited in dogs with no dog specific literature addressing this to the author's knowledge.

One Health

Epidemiology

Giardiasis remains the most prevalent human gastrointestinal parasitic infection the United States, at a consistent incidence of 5.8 per 100,000 population each year for the last decade [52]. The Companion Animal Parasite Council (CAPC) currently estimates test positivity of *Giardia* in dogs to be 7.49% in 2023 [53]. Cryptosporidiosis is a disease on the rise in humans at an incidence of 4.3 people per 100,000 in the United States [54]. While this number is less than estimates of giardiasis, the startling fact is that incidence in humans has increased 47.2% in the past decade and 241% since 2004 [54]. The companion animal parasite council does not currently monitor for positivity of *Cryptosporidium*. To the authors knowledge there are minimal large scale studies assessing presence of the organism in dogs in the United States.

These two pathogens can cause subclinical infections in animals leading to potential zoonotic transmission, especially a risk in immunocompromised people [25,43,55]. In 2004 giardiasis and cryptosporidiosis were included in the WHO Neglected Diseases Initiative, due to an established link between these diseases and poverty [56]. Prevalence, or positive population proportion, values for canine giardiasis are reported to be 15.6% in the United States in a pet population and 15.2% in dogs globally [1,57]. A recent global meta-analysis incorporated studies that based *G. duodenalis* prevalence on microscopy, ELISA, DFA, and polymerase chain reaction (PCR) and found that prevalence varied with testing modality, with microscopy performing poorly when compared to the other testing methods [57]. Giardiasis in humans remains a concern [52]. Between the years of 2012 and 2017 there were 111 giardiasis outbreaks reported in humans from 26 different states with 760 primary cases documented [58]. A recent

Cryptosporidium infection meta-analysis, using data from descriptive, cross-sectional, and case-control studies, with microscopic, molecular, and coproantigenic methods, cited prevalence values of cryptosporidiosis in dogs to be 5% in North America and 8% globally [41]. This study did not find a similar link between testing modality and likelihood of identifying the organism as the *Giardia* study did, yet further research should be conducted in this area as these organisms are difficult to identify microscopically [41]. A recent retrospective study was conducted in the United States that evaluated laboratory test findings (n=4692) from 10 veterinary diagnostic labs. This study found that *Giardia* was the most detected parasite, 8.33%, and *Cryptosporidium* was rarely identified, 0.28% [59].

The underlying mechanism of how dogs, or humans, are often subclinical carriers of these organisms, and if subclinical infection predisposes hosts for other gastrointestinal diseases, is still a mystery [25]. It is plausible that variables such as housing type and geographic locale can increase or decrease likelihood of becoming infected, with either *Giardia* or *Cryptosporidium* [25,42,57,60–65]. Equally so, there is biological plausibility that either subclinical or symptomatic infection with either of these organisms would lead to decreased stool consistency due to how they interact with the gastrointestinal tract mucosa [25]. This lack of consistency in reports calls for further investigation into risk factors that increase the odds of presence of these organisms in dog colonies with proper husbandry standards.

Socioeconomic factors, such as the environment or the economic status of an individual, have been linked recently to parasitism and transmission of parasites, including *G. duodenalis* [66]. Social Determinants of Health (SDOH) are these components of our communities and lives used to evaluate health inequities and health divides in human populations [67]. SDOH are defined as the circumstances in which humans develop, mature, live, work, and die in [68].

These influences are usually broken down into 5 categories: physical environment, social factors, economic factors, medical care, and health behaviors [68]. SDOH have been well studied in human medicine for a variety of infectious diseases of viral, bacterial, and parasitic concern including *Giardia* and *Cryptosporidium* infections [66,69–72]. A One Health perspective, defined as the impact that humans, animals, and the environment have of each other's health, suggests that SDOH will be able to indicate risk areas or factors underlying the epidemiology of disease in not only human but also animal populations. Recently there has been a push to evaluate Social Determinants of Animal Health and arguments have been made that these human focused SDOH can equally impact animal health [73].

Coinfections

Canine

At our institution, we have experienced coinfections with *Giardia* and *Cryptosporidium* in dogs. Coinfections between these two organisms and other GI parasites have been well established. This could be due to the method of infection with the organisms being similar, consumption of contaminated water, food, fomites, or directly through the feces via coprophagy [22]. A recent study conducted in the US demonstrated a 3.82% prevalence of coinfections between two or more organisms in animals [59]. Another study cited that coinfections were detected in 1.6% of dogs sampled with *Giardia* with hookworms being the most common coinfecting organism identified [74]. There is great variability in the research on this topic, with other studies sampling symptomatic, diarrheic, dogs demonstrating a 45.1% prevalence of coinfections most commonly with *Clostridium perfringens* and either *Giardia* or *Cryptosporidium* [3].

Human

In humans, *Giardia* has been commonly isolated with other organisms associated with inflammation. These organisms include *Ascaris* sp., *Cryptosporidium* sp., *Clostridium difficile*, *Helicobacter pylori*, *Salmonella* sp., *Vibrio cholera*, enteropathogenic *Escherichia coli*, norovirus, and rotavirus [22]. Despite the well documented cases of coinfection, the role it plays in the disease course and inflammatory response is not well understood and is an area for growth in research [22].

Research Objectives

Specific Aims

Due to the unexpected emergence of clinical *Giardia* and *Cryptosporidium* co-infections at the institution's research facility, we recognized that strategies to rapidly detect and mitigate these specific pathogens were an important need. A dependable, quick screening test for our canine colonies that would be able to simultaneously detect both pathogens across their genetically diverse backgrounds was needed. We also found a need to identify specific genetic assemblages and species of *Giardia* and *Cryptosporidium* to mitigate transmission sources, and to determine factors associated with subclinical organism carriage for rapid risk-assessment within our canine colonies. We finally identified a need to study canine parasites in the State of Texas, where our facilities are located, with a novel approach at the county and state level to find the potential associations with common SDOH. To address these needs, we proposed the following Specific Aims.

Specific Aim 1

Aim 1 was to evaluate the human Quik Chek (QC), *Giardia* and *Cryptosporidium*, rapid membrane enzyme coproantigen test in dogs using both direct immunofluorescent assay (DFA) and PCR. We hypothesized that the QC test would perform with a sensitivity of at least 50% and specificity of at least 90% in detection of *Giardia* and *Cryptosporidium* in dogs.

Specific Aim 2

Aim 2 was to identify and characterize the assemblages and species of *Giardia* and *Cryptosporidium*, respectively, in laboratory canines utilizing multilocus genotyping. We hypothesized that most species found would be those host-adapted to dogs and of limited human zoonotic risk.

Specific Aim 3

Aim 3 was to identify the risk factors of subclinical infection of either *Giardia* or *Cryptosporidium* in laboratory canines.

Subaim 3.1: We determined if fecal score predicted a positive fecal test result for *Giardia* or *Cryptosporidium*. We expected that a soft fecal score would be associated with a positive *Giardia* test.

Subaim 3.2: We described the association between *Giardia* and *Cryptosporidium* subclinical infection and demographic risk factors. We expected that immature age would be associated with subclinical infection with *Giardia* and *Cryptosporidium*.

Specific Aim 4

Aim 4 was to identify social determinates of health associated with county-level changes in positivity of canine *Giardia* infection in Texas. We hypothesized that rural status, poverty, and poor access to veterinary care would be associated with an increase in county level positive test proportions of *Giardia*.

Impact

Given the zoonotic potential of *Giardia* and *Cryptosporidium*, screening for these parasites, followed by identification of specific assemblage and species respectively, will ensure the health of the dogs in our facility, and also our animal caretakers if there are zoonotic subtypes of organism present [75]. Completion of the aforementioned aims provides clinicians and colony managers with a rapid point of care testing choice for two parasitic organisms of concern. It also adds to the knowledge available on assemblages and species, of *Giardia* and *Cryptosporidium* respectively, found in dogs supplying vital information for public health officials who are conducting risk assessments for infection with these two zoonotic pathogens. It also provides veterinarians and colony managers with individual factors associated with increased likelihood of infection in the dog. Finally, this gives clinicians, colony managers, and public health officials key associations with specific non-medical factors of concern that can help identify increased *Giardia* test positivity risk in the Texas canine population at the county level.

CHAPTER II

COMPARISON OF 3 DIAGNOSTIC TESTS FOR THE DETECTION OF *GIARDIA* AND *CRYPTOSPORIDIUM* SPP. IN ASYMPTOMATIC DOGS (*CANIS LUPIS FAMILIARIS*)[1]

Introduction

Giardia duodenalis and *Cryptosporidium* spp. are important gastrointestinal pathogens in humans and canids [1,2]. Coinfections with these 2 pathogens in canids have not been well documented, with disagreement in the literature regarding its significance [3,4]. *G. duodenalis* has multiple genetic assemblages, most of which are host adapted and have little to no clinical significance in humans [12]. Assemblages C and D are the most isolated in both clinical and asymptomatic dogs and cause little to no disease in humans, yet assemblages A and B occur in dogs and can cause clinical disease in humans and canids [12,16]. Similarly, the genus *Cryptosporidium* comprises more than 25 host-associated species [28], of which *C. canis* is the most commonly found species in dogs; however, *C. parvum* and *C. hominis* have also been identified in dogs and are the most common species associated with disease in humans [12,28,35,41].

A litter of puppies born at our institution in 2020 was diagnosed, via fecal PCR by a veterinary diagnostic laboratory, as having clinical coinfections of *Giardia* and *Cryptosporidium* spp. This diagnosis led us to realize the need for a dependable, quick screening test that could

[1] L.A. Taylor, M.N. Saleh, E.C. Kneese, T.H. Vemulapalli, G.G. Verocai, Comparison of 3 Diagnostic Tests for the Detection of *Giardia* and *Cryptosporidium* spp. in Asymptomatic Dogs (*Canis lupis familiaris*), Journal of the American Association for Laboratory Animal Science. 62 (2023) 139–146. https://doi.org/10.30802/AALAS-JAALAS-22-000108.

identify multiple assemblages and species of both organisms. Inhouse methods for the detection of *Giardia* and *Cryptosporidium* include direct fecal smear and centrifugal fecal flotation with either Sheather's sucrose for *Cryptosporidium* or zinc sulfate for *Giardia* [76]. These techniques, although cost effective, require technicians with specialized training in the recognition of cysts and oocysts as well as the necessary laboratory equipment, such as centrifuges and microscopes, depending on the procedure [77]. For optimal accuracy, *Cryptosporidium* should be stained and viewed at 400× magnification, thus adding another layer of technical complexity [78,79]. Wide ranges of sensitivity and specificity have been reported for these methods, with 34% to 88% sensitivity and 92% to 96% specificity of zinc sulfate fecal flotation for the detection of *Giardia* [55,80,81]. Fecal flotation methods to detect *Cryptosporidium* in dogs have not been well studied, but in other species reported sensitivity ranges from 21% to 68% with a specificity of 93% to 98% [78,79,82,83].

These 2 pathogens can cause subclinical infections in animals, leading to potential zoonotic transmission, particularly in immunocompromised people [25,43,55]. Reported prevalence values for canine giardiasis are as high as 16% in the United States and 15% in dogs globally [1,57]. A recent global meta-analysis incorporated studies that determined *Giardia* prevalence based on microscopy, ELISA, direct immunofluorescent assay (DFA), and PCR analysis and found that prevalence varied with testing modality; microscopy performed poorly compared with the other testing methods [57]. Giardiasis in humans remains a concern, with an incidence rate of 6 per 100,000 population in the United States in 2019 [52]. Between 2012 and 2017, 111 giardiasis outbreaks were reported in humans from 26 different states, with 760 primary cases documented [58]. *Giardia duodenalis* is the most common intestinal parasitic infection of humans in the United States [58]. A recent *Cryptosporidium* meta-analysis, using

data from descriptive, cross-sectional, and case-control studies, with microscopic, molecular, and coproantigenic methods, cited 5% prevalence of cryptosporidiosis in dogs in North America and 8% globally but did not report a link between testing modality and likelihood of identifying the organism [41,57]. *Cryptosporidium* remains a prominent gastrointestinal parasite in humans, with an overall incidence rate of 4 per 100,000 people in the United States in 2019; this value represents an increase in incidence of 47% over the last decade [54].

Several tests are currently approved for *Giardia* detection in dogs, and a few are approved for the diagnosis of cryptosporidiosis. However, no approved tests are available for concurrent rapid diagnosis of both pathogens in dogs [57,81,84]. This lack of dependable and uncomplicated testing modalities led our team to search for an economic, sensitive, and specific test that our technicians could use to screen incoming dogs and suspected clinical cases for *Cryptosporidium* and *Giardia*. Given their zoonotic potential, screening for these parasites would promote the safety of both our dogs and their caretakers. To this end, we compared a commercial point-of-care test (QC test) for use in people with both DFA and PCR analysis to determine whether this test would be useful for screening both healthy incoming and symptomatic dogs for the presence of *Giardia* and *Cryptosporidium*. We hypothesized that the QC test would perform with a sensitivity of at least 50% and specificity of at least 90% for the detection of *Giardia* and *Cryptosporidium* in dogs.

Materials and Methods

Ethics Statement

This study was designed and conducted in accordance with the Texas A&M University IACUC, and in accordance with the regulations of the Animal Welfare Act [85]. An animal use

protocol was deemed unnecessary by the IACUC because none of the experiments directly affected the day-to-day activities of the dogs; all samples were voluntarily voided into the environment and were collected during routine cleaning of the dogs' standard enclosures.

Animals and Sample Collection

Fecal samples (n = 170) were collected from dogs housed at our research facility (population 1; n = 96; age, 3mo to 10y) and from a Texas supplier of dogs for research (population 2; n = 74; age 3 mo to 13 y) during March through October 2021. Samples were collected from all dogs in population 1 and from a convenience sample of dogs in population 2 during daily cleaning activities and routine yearly physical exams.

Experimental Design

This study evaluated a lateral flow assay that is approved to detect coproantigens of both *Giardia* and *Cryptosporidium* in humans (Quik Chek [QC], TechLab, Blacksburg, VA). The test takes approximately 30min to run and requires no specialized equipment.

All fecal samples were individually labeled and stored in sealed plastic bags at 4 to 8°C for 24 to 48h prior to analysis. The first analysis used the QC test according to the manufacturer's recommendations. All reagents and samples were brought to room temperature and a 1.5 µL microcentrifuge tube was prepared for each sample by adding 500 µL of manufacturer-provided diluent and one drop of conjugate. A small, approximately 2 mm diameter, portion of feces was added to this tube. This was emulsified using an applicator stick and vortexer. A 500 µL portion of the diluted-conjugate prepared sample was deposited in the sample well of the test and was incubated at room temperature for 15min. Then, 300µL of the

provided wash buffer was added to the reaction window and allowed to fully absorb. Lastly, 2 drops of the provided substrate were applied to the reaction window and the test was incubated at room temperature for 10min. The result was considered positive for an organism when a blue line appeared next to the corresponding indicator (Figure II.1).

Figure II.1. A QC test that was positive for *Giardia* and negative for *Cryptosporidium* (no blue line), with 3 control dots in the middle.

The samples were then divided, with approximately 0.1g placed in a 1.5-mL microcentrifuge tube containing formalin and stored at room temperature for DFA. The remaining sample was stored in plastic specimen containers at −80 °C for PCR analysis. Formalin-preserved samples were tested using DFA (Merifluor *Cryptosporidium/Giardia*, Meridian Bioscience, Cincinnati, OH) according to the manufacturer's instructions. Samples were examined at 200× and 400× by a single trained reviewer using a fluorescence microscope.

Prior to PCR analysis, all frozen fecal specimens were thawed, and cysts and oocysts were isolated by using a modification of the gradient centrifugation protocol [86,87]. An emulsion was created by mixing 2 to 3 g of feces with approximately 12 mL of a 0.01-M PBS–EDTA, 0.01M, solution and straining through a double layer of cheesecloth. A disposable plastic pipette was used to transfer the eluate into sucrose solution (specific gravity, 1.26) in a 15-mL conical tube. This mixture was centrifuged at 800 × g for 10 min at room temperature. The top layer and emulsion interface were then pipetted into a new tube and centrifuged for another 10 min at 1,200 × g, at room temperature. The supernatant was discarded, the pellet was washed twice with PBS-EDTA, and the final pellet, which contained oocytes and cysts, was resuspended in 1 mL of PBS-EDTA and stored at −80°C until DNA extraction [81,88]. DNA was extracted from stored pellets by using a commercial kit (DNeasy Blood and Tissue Kit, Qiagen, Germantown, MD) [81,89]. DNA was eluted in 100µL of elution buffer and stored at −20 °C until PCR analysis.

Purified and extracted DNA samples were analyzed for Giardia by using a 2-step nested PCR assay (MyCycler, Bio-Rad, Hercules, CA), in which a 292-bp fragment of the 16S rRNA gene was amplified by using the primers 5′ AAG TGT GGT GCA GAC GGA CTC 3′ and 5′ CTG CTG CCG TCC TTG GAT GT 3′ for the primary reaction and 5′ CAT CCG GTC GAT CCT GCC 3′ and 5′ AGT CGA ACC CTG ATT CTC CGC CAG G 3′ for the secondary reaction [90,91]. The primary and secondary PCR master mixes each included 8.75µL of molecular-grade water, 0.625µL of 10-µM forward primer, 0.625µL of 10-µM reverse primer, and 12.5µL of GoTaq Green (Promega, Madison, WI) to which 2.5µL of sample was added for a total of 25µL. After a 2-min initiation at 95 °C for the primary reaction, 40 cycles were run at 95°C for 30 s, 54.5°C for 45 s, and 72°C for 90 s, followed by a final extension at 72 °C for 90 s and storage at

4 °C. The secondary reaction differed in that the 40 cycles were run at 95 °C for 30 s, 56.5°C for 45 s, and 72 °C for 90 s.

Samples were similarly analyzed for *Cryptosporidium* DNA by using a 2-step nested PCR assay. An 800-bp fragment of the SSU rRNA gene was amplified by using primers 5′ TTC TAG AGC TAA TAC ATG CG 3′ and 5′ CCC ATT TCC TTC GAA ACA GGA 3′ for the primary reaction and 5′ GGA AGG GTT GTA TTT ATT AGA TAA 3′ and 5′ CTC ATA AGG TGC TGA AGG AGT A 3′ for the secondary reaction [92]. The primary and secondary reaction master mixes were prepared as described above to a total of 25μL. After a 2-min initiation at 95°C for the primary reaction, 40 cycles were run at 95 °C for 30 s, 48°C for 45 s, and 72 °C for 90 s, followed by a final extension at 72°C for 90 s and storage at 4 °C. For the secondary reaction, 40 cycles were run at 95°C for 30 s, 50 °C for 45 s, and 72°C for 90 s. Negative and positive controls for both *Giardia* and *Cryptosporidium* were included in each batch run. All secondary reaction PCR products underwent gel electrophoresis through a 1% agarose gel stained with GelRed (Biotium, Fremont, CA), with a 100-mV procedure for 45 to 75min, DNA marker ladder (Quick-Load DNA Ladder, New England BioLabs, Ipswich, MA), and gel imaging system (GelDoc Go, Bio-Rad, Hercules, CA). Samples were considered conditionally positive when they had a band at approximately 800bp for *Cryptosporidium* and approximately 300bp for *Giardia*.

All conditionally positive PCR samples were purified (Omega EZNA Cycle Pure Kit, Norcross, GA, or Wizard Gel and PCR Clean-Up System, Promega) according to the manufacturer's recommendations, and the resulting product was submitted for confirmatory sequencing (Eurofins Genomics, Louisville, KY). All genetic sequences were queried in the Nucleotide collection database by using MegaBLAST (National Center for Biotechnology

Information, National Library of Medicine). The sequences for *Giardia* were matched to accession numbers AF310725.1, KY783324.1, LC437354.1, LC437356.1, LC437360.1, LC437361.1, LC437365.1, MG972765.1, MN263895.1, MN593002.1, MT129490.1, and MT484087.1. The sequences for *Cryptosporidium* were matched to accession numbers KT749817.1 and MT329018.1 (Table II.1).

Table II.1. NCBI Megablast results

Sample ID*	% identity	Accession number	Sample ID*	% identity	Accession number
Giardia			*Giardia*		
2	99.06	LC437365.1	84	99.60	LC437356.1
3	87.50	MT129490.1	85	99.58	LC437361.1
4	98.33	MN263895.1	86	92.24	LC437361.1
6	96.61	MN263895.1	89	97.69	LC437361.1
7	91.95	LC437365.1	91	94.61	MT129478.1
10	98.37	MN263895.1	92	100.0	LC437360.1
15	81.15	MT129490.1	94	100.0	MN263895.1
16	98.72	LC437365.1	98	97.56	MN263895.1
18	93.88	LC437365.1	101	97.56	MN263895.1
19	96.49	MT484087.1	108	87.03	LC437360.1
29	96.67	MN263895.1	110	85.19	MT129478.1
31	88.27	MN593002.1	116	86.13	LC437365.1
32	90.43	MN263895.1	117	97.71	MN263895.1
34	98.63	LC437354.1	121	90.16	LC437365.1
35	96.61	MT484087.1	126	90.38	LC437360.1
36	94.74	MT484087.1	130	90.70	LC437365.1
39	95.87	MN263895.1	132	88.05	MN593002.1
41	100.0	LC437354.1	136	94.37	LC437360.1
43	98.73	LC437354.1	138	100.0	LC437365.1
44	98.73	LC437365.1	151	100.0	MG972765.1
72	92.31	LC437365.1	161	91.57	KY783324.1
75	92.70	AF310725.1	164	89.26	LC437365.1
79	84.30	LC437365.1	165	95.38	LC437365.1
81	97.50	MN263895.1	167	97.39	LC437365.1
83	92.99	LC437365.1	168	98.33	MN263895.1
Cryptosporidium					
103	99.62	KT749817.1			
125	98.55	MT329018.1			
151	100.0	KT749817.1			
152	97.84	KT749817.1			
163	95.57	KT749817.1			

*missing sample numbers were negative on PCR for both organisms

Statistical Analysis

All data were analyzed initially by using STATA SE 17.0 (STATA Corp, College Station, TX). The apparent prevalence of each organism was calculated for both test populations for each of the 3 testing modalities. Our testing methods of direct visualization of the organism on DFA and sequencing the products of PCR-positive samples with nucleotide bank verification allowed us to assume that the specificity of both tests closely approached 100%. Therefore, we created a reference standard for the QC test by using both the DFA and PCR results in order to improve overall sensitivity. Apparent prevalence values for each organism in each population were calculated by using this reference standard.

Differences in prevalence of *Giardia* and *Cryptosporidium* between and within populations were calculated by using a z-test statistic, with significance defined as $P < 0.05$. Using the diagt command in STATA, we calculated sensitivity and specificity values for the QC, DFA, and PCR tests by using the reference standard. Likelihood ratio analysis was also performed; this analysis provides the probability that a dog that tests positive truly has disease, whereas one that tests negative truly does not have the disease. Receiver operating characteristic (ROC) curves were calculated for each test compared with the defined reference test for both organisms. Bayesian analysis with the Markov Chain Monte Carlo process was then performed by using WinBugs (version 1.4.3, University of Cambridge, Cambridge, United Kingdom), with the assumption of complete independence and adaption of the code (Figure II.2) from the Center for Animal Disease Modeling and Surveillance (University of California Davis, Davis, CA) [93,94]. BetaBuster (version 1.0, Chun-Lung Su, Informer Technologies, Los Angeles, CA) was used to calculate all α and β priors from previously reported specificities from the literature [55,78,79,81,84,95–97]. Informed priors from the literature differed largely from the sensitivities

obtained for DFA and PCR in the current study. Therefore, for Bayesian analysis, we used the sensitivities for DFA and PCR as compared with the reference standard in the current study in order to avoid overestimating the sensitivities of each of the tests during Bayesian analysis.

Figure II.2. WinBugs code for comparing 3 independent tests

```
model {
y1[1:Q, 1:Q, 1:Q] approximately dmulti(p1[1:Q, 1:Q, 1:Q], n1)
y2[1:Q, 1:Q, 1:Q] approximately dmulti(p2[1:Q, 1:Q, 1:Q], n2)
p1[1,1,1] <- Prev1*(SeT1*SeT2*SeT3) + (1-Prev1)*((1-SpT1)*(1-SpT2))*(1-SpT3)
p1[1,1,2] <- Prev1*(SeT1*SeT2)*(1-SeT3) + (1-Prev1)*((1-SpT1)*(1-SpT2))*(SpT3)
p1[1,2,1] <- Prev1*(SeT1*(1-SeT2))*SeT3 + (1-Prev1)*((1-SpT1)*SpT2)*(1-SpT3)
p1[1,2,2] <- Prev1*(SeT1*(1-SeT2))*(1-SeT3) + (1-Prev1)*((1-SpT1)*SpT2)*(SpT3)
p1[2,1,1] <- Prev1*((1-SeT1)*SeT2)*SeT3 + (1-Prev1)*(SpT1*(1-SpT2))*(1-SpT3)
p1[2,1,2] <- Prev1*((1-SeT1)*SeT2)*(1-SeT3) + (1-Prev1)*(SpT1*(1-SpT2)*SpT3)
p1[2,2,1] <- Prev1*((1-SeT1)*(1-SeT2)*SeT3) + (1-Prev1)*(SpT1*SpT2)*(1-SpT3)
p1[2,2,2] <- Prev1*((1-SeT1)*(1-SeT2)*(1-SeT3))+ (1-Prev1)*(SpT1*SpT2*SpT3)
p2[1,1,1] <- Prev2*(SeT1*SeT2*SeT3) + (1-Prev2)*((1-SpT1)*(1-SpT2))*(1-SpT3)
p2[1,1,2] <- Prev2*(SeT1*SeT2)*(1-SeT3) + (1-Prev2)*((1-SpT1)*(1-SpT2))*(SpT3)
p2[1,2,1] <- Prev2*(SeT1*(1-SeT2))*SeT3 + (1-Prev2)*((1-SpT1)*SpT2)*(1-SpT3)
p2[1,2,2] <- Prev2*(SeT1*(1-SeT2))*(1-SeT3) + (1-Prev2)*((1-SpT1)*SpT2)*(SpT3)
p2[2,1,1] <- Prev2*((1-SeT1)*SeT2)*SeT3 + (1-Prev2)*(SpT1*(1-SpT2))*(1-SpT3)
p2[2,1,2] <- Prev2*((1-SeT1)*SeT2)*(1-SeT3) + (1-Prev2)*(SpT1*(1-SpT2))*SpT3
p2[2,2,1] <- Prev2*((1-SeT1)*(1-SeT2))*SeT3 + (1-Prev2)*(SpT1*SpT2)*(1-SpT3)
p2[2,2,2] <- Prev2*((1-SeT1)*(1-SeT2))*(1-SeT3) + (1-Prev2)*(SpT1*SpT2)*SpT3
SeT1approximately dbeta(1,1)
SpT1approximately dbeta(1,1)
SeT2approximately dbeta(1,1)
SpT2approximately dbeta(1,1)
SeT3approximately dbeta(92, 4.08) # mode = 0.98, 95% >0.95
SpT3approximately dbeta(152,4.08) # mode = 0.95, 95% >0.91
Prev1approximately dbeta(1,1)
Prev2approximately dbeta(1,1)
}
```

```
# data
# T1 = QC; T2 = DFA; T3 = PCR
# n1 = batch 1; n2 = batch 2
list(n1 = 96, n2 = 74, Q = 2, y1 = structure(.Data = c(0,0,0,2,0,1,0,93),.Dim = c(2,2,2)),
y2 = structure(.Data = c(1,0,1,2,1,1,3,65),.Dim = c(2,2,2)))
```

```
# initials 1
list(SeT1 = 0.86, SpT1 = 0.97, SeT2 = 0.64, SpT2 = 0.75, SeT3 = 0.96, SpT3 = 0.99, Prev1 = 0.010, Prev2 =
0.108)
```

Results

In determining the best test to use as a reference standard, neither DFA nor PCR analysis emerged as the obvious choice for either *Giardia* or *Cryptosporidium* because both tests had low detection for both organisms. However, assigning a positive finding if either PCR or DFA results were positive provided the highest proportion of correct classification of positive samples. The prevalence of *Giardia* was 38% in population 1 (institutional colony) and 49% in population 2 (vendor colony; Table II.2).

The prevalence of *Cryptosporidium* was 1% in population 1 and 9% in population 2 (Table II.2). Overall *Giardia* was significantly ($P < 0.0000$) more prevalent than *Cryptosporidium* in both populations, and *Cryptosporidium* parasites were significantly more prevalent ($P = 0.0050$) in population 2 than population 1. *Giardia* prevalence was not significantly different between the 2 populations ($P = 0.0900$).

Table II.2. Apparent prevalence of *Giardia* and *Cryptosporidium* in populations 1 and 2.

	Giardia		*Cryptosporidium*	
	Population 1	Population 2	Population 1	Population 2
Reference standard	38.5 (29.3, 48.7)	48.6 (36.9, 60.6)	1.0 (0.1, 7.2)	9.5 (3.9, 18.5)
QC	13.5 (8.0, 22.1)	27.0 (18.0. 38.4)	2.1 (0.5, 8.1)	5.4 (2.0, 13.7)
DFA	14.6 (8.8, 23.3)	31.1 (21.5, 42.7)	1.0 (0.1, 7.2)	4.1 (1.2, 12.0)
PCR	33.3 (24.5, 43.5)	25.7 (16.9, 37.0)	No data	8.1 (3.6, 17.1)

For the detection of *Giardia*, the sensitivity of the QC test was 38%, and specificity was 95% (Table II.3). For the detection of *Cryptosporidium*, the sensitivity of the QC test was 25% and specificity was 95% (Table II.4). The ROC area for QC detection was 0.67 for *Giardia* (Figure II.3), and 0.61 for *Cryptosporidium* (Figure II.4).

Table II.3. Evaluation of *Giardia* detection

	Sensitivity	Specificity	PPV	NPV	LR(+)	LR(–)
QC	38.4 [27.2, 50.5]	94.8 [88.4, 98.3]	84.8 [68.1, 94.9]	67.2 [58.6, 74.9]	7.44 [3.0, 18.3]	0.65 [0.5, 0.8
DFA	50.7 [38.7, 62.6]	100 [96.3, 100]	100 [90.5, 100]	72.9, [64.5, 80.3]	–	0.49 [0.4, 0.6]
PCR	69.9 [58.0, 80.1]	100 [96.3, 100]	100 [93.0, 100]	81.5 [73.4, 88.0]	–	0.30 [0.2, 0.4]

LR(–), negative likelihood ratio ; LR(+), positive likelihood ratio ; NPV, negative predictive value; PPV, positive predictive value. Data are given as mean percentage (95% CI).

Figure II.3. Receiver operating characteristic curve for *Giardia* analysis with QC (blue; area, 0.67), DFA (red; area, 0.75), and PCR (green; area, 0.85) assays compared with the reference standard (gray).

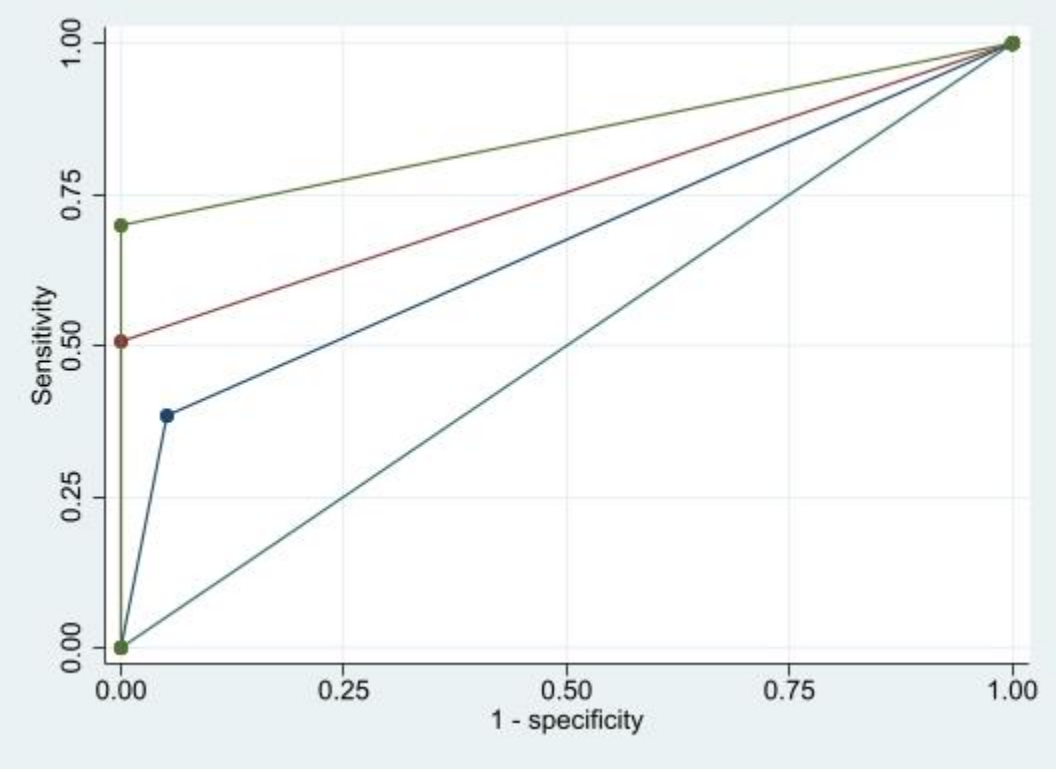
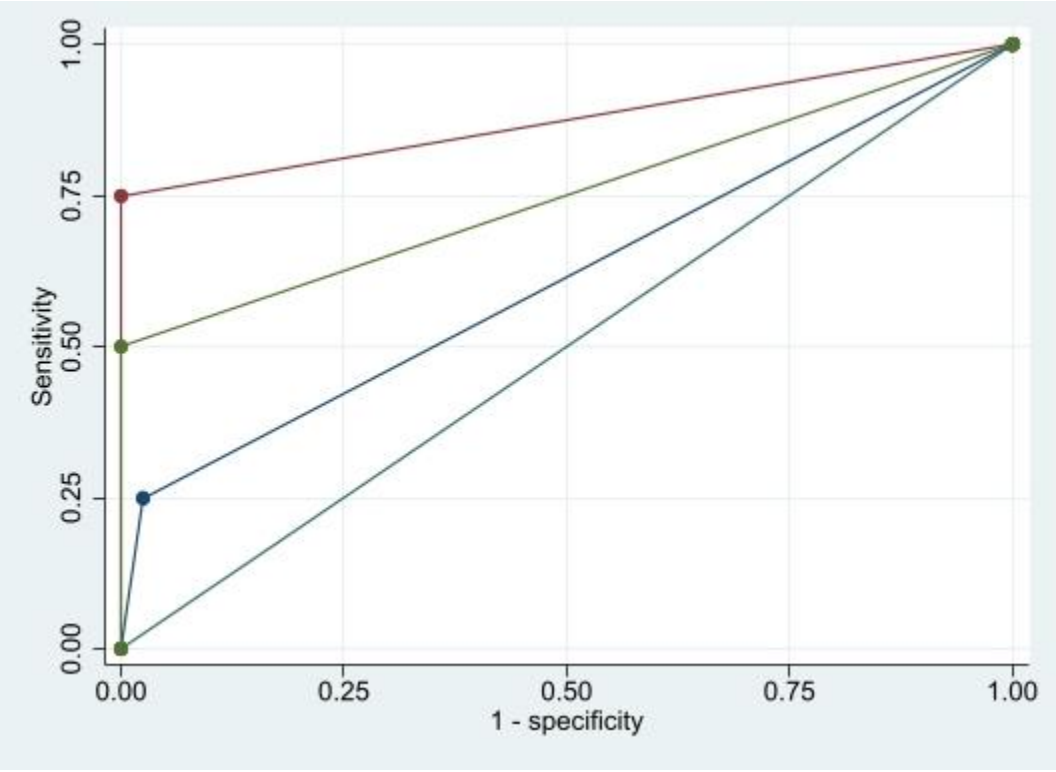


Table II.4. Evaluation of *Cryptosporidium* detection

	Sensitivity	Specificity	PPV	NPV	LR(+)	LR(–)
QC	25.0 (3.2, 65.1)	95.5 (93.8, 99.3)	33.3 (4.3, 77.7)	96.3 (92.2, 98.6)	10.13 (2.2, 47.3)	0.77 (0.5, 1.2)
DFA	50 (15.7, 84.3)	100 (97.7, 100)	100 (39.8, 100	97.6 (93.9, 99.3)	–	0.5 (0.3, 1.0)
PCR	75.0 (34.9, 96.8)	100 (97.7, 100)	100 (54.1, 100	98.8 (95.7, 99.9)	–	0.25 (0.1, 0.8)

LR(–), negative likelihood ratio ; LR(+), positive likelihood ratio ; NPV, negative predictive value; PPV, positive predictive value. Data are given as mean percentage (95% CI).

Figure II.4. Receiver operating characteristic curve for *Cryptosporidium* analysis with QC (blue; area, 0.61), DFA (red; area, 0.75), and PCR (green; area, 0.88) assays compared with the reference standard (gray).



The prevalence of *Giardia* based on Bayesian analysis was 33% in population 1 and 51% in population 2, which falls within the confidence interval (CI) of our reference standard (Table II.2). Bayesian analysis showed that for the QC test, the mean sensitivity was 48% and specificity was 98%; for the DFA test, the mean sensitivity was 51% and specificity was 99%; and for the PCR test the mean sensitivity was 63% and specificity was 92% (Table II.5).

Bayesian analysis showed that prevalence of *Cryptosporidium* was 1% for population 1 and 9% for population 2. For the QC test, the mean sensitivity was 40% and specificity was 97%; for the DFA test, mean sensitivity was 38% and specificity was 99%; for the PCR test, mean sensitivity was 93% and specificity was 99% (Table II.6).

Table II.5. Bayesian analysis for *Giardia*

	Mean %	Median % (95% CI)	1 SD	Monte Carlo error
Prevalence population 1	32.6	32.0 (16.1, 51.7)	0.0966	1.9×10^{3}
Prevalence population 2	50.6	50.7 (33.2, 68.0)	0.0896	1.4×10^{3}
Sensitivity QC	48.2	47.2 (30.4, 71.0)	0.1068	2.1×10^{3}
Specificity QC	97.9	98.4 (93.4, 99.9)	0.0363	2.0×10^{4}
Sensitivity DFA	51.4	51.4 (41.6, 61.8)	0.0515	8.7×10^{4}
Specificity DFA	99.2	99.4 (97.0, 100)	0.0081	9.4×10^{5}
Sensitivity PCR	62.8	62.9 (55.5, 69.7)	0.0363	2.9×10^{4}
Specificity PCR	92.5	92.5 (84.1, 99.7)	0.0450	9.5×10^{4}

Table II.6. Bayesian analysis for *Cryptosporidium*

	Mean %	Median % (95% CI)	1 SD	Monte Carlo error
Prevalence population 1	1.1	0.7 (0.0, 3.9)	0.0173	1.4×10^{4}
Prevalence population 2	8.9	8.54 (3.0, 16.9)	0.0356	3.0×10^{4}
Sensitivity QC	40.5	38.97 (10.4, 78.5)	0.1784	1.3×10^{3}
Specificity QC	97.1	97.26 (94.0, 99.1)	0.0132	1.1×10^{4}
Sensitivity DFA	37.7	37.38 (21.4, 56.0)	0.0895	6.1×10^{4}
Specificity DFA	98.7	97.26 (96.7, 99.8)	0.0079	6.9×10^{4}
Sensitivity PCR	93.3	93.89 (84.6, 98.6)	0.0370	2.9×10^{4}
Specificity PCR	99.2	99.44 (97.3, 100)	0.0073	8.7×10^{4}

Discussion

In this study, we evaluated the use of a human QC diagnostic test originally developed for the detection of *Giardia* and *Cryptosporidium* in dogs. We used frequentist statistics to perform standard comparison to a reference testing scheme and Bayesian statistics for comparison of tests without using a 'gold standard' as a reference. Our results indicate that the QC test provides good certainty that a positive finding for either *Giardia* or *Cryptosporidium* is a true positive. However, because the QC test has low sensitivity, confirmatory testing should be performed before concluding that a dog is negative for the presence of either *Giardia* or *Cryptosporidium*.

We collected and analyzed samples from pathogen surveillance testing of clinically normal dogs maintained in institutional and vendor colony populations. No single test stood out as a true gold standard. We therefore analyzed the data by using 2 distinct statistical methods. First, we created the reference standard test by using the results from both of our 2 near-perfect specificity tests; this approach assigns the sample as positive if either of the tests were positive. This approach increases the sensitivity of the overall testing scheme when highly specific tests, such as DFA and PCR, are used to assess low prevalence populations. Although this approach provided concise and easily interpreted results, we further analyzed the data by using Bayesian analysis. Those results closely approximated the frequentist statistical analysis, giving us confidence in interpretating these diagnostic tests for detection of *Giardia* and *Cryptosporidium* in asymptomatic canine populations.

Evaluation of a diagnostic test in asymptomatic, subclinical, or carrier subjects is the most rigorous approach to assessment of the test. Subjects with such an infection status are likely to have low concentrations of organisms in their stool relative to clinical cases [98]. This can

lead to test results that vary when different tests are applied to the same sample [99]. This variability is evident in the evaluation of our reference test in which we analyzed both PCR and DFA results in parallel. The low sensitivity demonstrated by PCR and DFA when compared with the reference standard supports the assumption that our samples had low concentrations of organisms. Furthermore, the asymptomatic status of our subjects may explain the lower sensitivity of PCR analysis and DFA in our study as compared with previous reports [75,78–83].

Compared with the QC test, the PCR and DFA tests in our current study detected more cases of *Giardia*, whereas PCR analysis detected more cases of *Cryptosporidium*. However, given the extra expense, time, and specialized equipment needed for PCR and DFA tests, the QC test performed well. The overlap in the CIs of specificity of QC, DFA, and PCR tests for both *Giardia* and *Cryptosporidium* indicate that the 3 tests perform similarly in detecting a negative dog. The overlap in the CIs of sensitivity for QC, DFA, and PCR for *Cryptosporidium*, and of QC and DFA for *Giardia* indicate that the tests perform similarly in detecting an infected dog.

Bayesian analysis agreed with our standard, frequentist analysis in the current study, with the Bayesian mean prevalences of both organisms in populations 1 and 2 falling within the CI of the frequentist analysis. Similarly, the mean sensitivities of the QC, DFA, and PCR tests fell within the CIs of frequentist analyses for both *Giardia* and *Cryptosporidium*. The mean specificities in the Bayesian analysis of the QC, DFA, and PCR tests fell within the CIs of the frequentist analysis for *Cryptosporidium*, and the specificities of the QC and DFA tests fell within the CIs of the frequentist analyses for *Giardia*. In the current study, DFA sensitivity for *Cryptosporidium* did not approach what is reported in the literature [100]. Although this difference could be due to the low organism concentrations in our samples, another possibility is

that the species of *Cryptosporidium* in our population is not *C. parvum* but rather the common dog species, *C. canis*. Further analysis will be necessary to investigate this finding.

A limitation of our study is the assumption (for the Bayesian analysis) of independence of the 3 diagnostic tests, based on their biologic characteristics. Because we did not know the true infection status of each dog in our populations, we could not reliably assess conditional dependence and therefore assumed their independence in our Bayesian analysis, as has been done previously in other studies [93,101–103]. Other limitations of the current study include the low prevalence of *Cryptosporidium* in our samples and the variability between tests that traditionally have high sensitivity and specificity. These limitations could be mitigated in future studies by performing the tests in replicate, as suggested previously [92].

In conclusion, we find the QC test is a simple, quick, and economical test that yields reliable results for both *Giardia* and *Cryptosporidium* in asymptomatic dogs. The QC test showed good specificity as compared with DFA and PCR analysis and achieved results that were close to our goals of sensitivity (50%) and specificity (90%) for both *Giardia* and *Cryptosporidium* detection.

CHAPTER III

DETERMINATION OF ASSEMBLAGES OF *GIARDIA* AND SPECIES OF *CRYPTOSPORIDIUM* IN LABORATORY DOGS POSITIVE ON FECAL SCREENING EXAMS

Introduction

Giardia duodenalis is a diplomonad protozoal pathogen found commonly in a wide array of animals. This organism was first identified in 1859 as *Cercomonas intestinalis* and subsequently renamed *Giardia lamblia* and *Giardia intestinalis* [5]. *G. duodenalis* is currently classified into 8 different genetic assemblages, designated with letters A-H, with variable clinical significance in humans [9].

Humans, domestic animals including dogs, and a large variety of wildlife have all been identified as natural hosts for assemblage A [9]. Assemblage A has the largest variety of susceptible host species and is divided into sub-assemblages (e.g., AI, AII, and AIII) [9,14]. Studies have shown assemblage AI typically is identified in domestic and wild animals including dogs and AII in humans [15]. Assemblage B is also split into sub-assemblages (e.g., BIII and BIV) and has been primarily isolated from humans [15]. Assemblage B has recently been associated with higher rates of human clinical illness than assemblage A [9]. Assemblages C and D, while normally found in dogs, have sporadically been isolated in immunocompromised humans as well [12,16]. Assemblage E is commonly found in ruminants but has also been associated with rare zoonotic infection in humans [9]. Some parasitologists recently have recommended naming each of these *G. duodenalis* assemblages as its own species [17]. Current

recommendations made by Thompson and Monis are to maintain assemblage A as *G. duodenalis* and renaming assemblage B as *G. enterica*, assemblages C and D as *G. canis*, assemblage E as *G. bovis*, assemblage F as *G. cati*, and assemblage G as *G. simondi* [17]. These would accompany other species of *Giardia* already identified, *G. muris* in rodents, *G. microti* in voles, *G. cricetidarum* in hamsters, *G. peramelis* in bandicoots, *G. agilis* in amphibians, and *G. ardeae* and *G. psittaci* in birds [17,104].

Cryptosporidium is a genus of protozoal pathogens that comprises over 25 different host associated species and numerous subspecies [28,35]. These protozoa are of the phylum Apicomplexa, which holds characteristics such as complex life cycles [35]. This complex life cycle allows for both asexual and sexual reproduction allowing for auto-reinfection [38,105]. The two species of *Cryptosporidium* that most commonly cause disease in humans are *C. hominis* and *C. parvum* [28]. *Cryptosporidium parvum* is the species most associated with zoonotic infections, and while usually identified in ruminants, it has been isolated in dogs [106]. *Cryptosporidium canis* is the dog host adapted species [12]. *Cryptosporidium canis*, while uncommon, has been associated with disease in humans, specifically those who are immunocompromised [28,107].

Currently, *Giardia* is the most common gastrointestinal parasite in humans in the United States with an incidence of 5.8 per 100,000 people [52]. *Cryptosporidium* sp. infections are on the rise in the United States with an incidence of 4.2 per 100,000 people, an increase of 241% since 2004 [54].

Testing modalities to detect *Giardia* include direct smear for patients with diarrhea, fecal centrifugation floatation for dogs with semisolid or solid feces, enzyme-linked immunoassay (ELISA), direct immunofluorescence assay (DFA), and PCR. Utilization of a combination of

these tests has been recommended in the literature [81,108]. Current recommendations for *Giardia* detection in dogs are to test for presence of the organism using a combination of microscopic testing via centrifugal floatation or direct smear and a sensitive and specific test such as fecal enzyme-linked immunosorbent assay or polymerase chain reaction if the animal is symptomatic [108]. Similar diagnostic testing modalities exist for *Cryptosporidium* detection, yet recommendations for testing schemes are less well defined in the literature.

Due to the zoonotic nature of both organisms we chose to evaluate canine stool samples that tested positive for *G. duodenalis* or *Cryptosporidium* to determine which assemblages of *Giardia* and species of *Cryptosporidium* were present in our canine population.

Materials and Methods

Animals and Sample Collection

During a previous study at our institution 170 fecal samples from male and female laboratory and kennel housed dogs, ages 3 months to 13 years, were collected and tested for the presence of *G. duodenalis* or *Cryptosporidium* via three methods [109]. Of these original samples, 45 were identified as *G. duodenalis* with the SSU rRNA locus and 5 samples were identified as *Cryptosporidium* sp. with the SSU rRNA locus. These samples were selected for the current study.

Experimental Design

Sample Preparation

Fresh samples were collected from the dogs in the original study and stored in an -80°C freezer for further processing. All samples were thawed, and cysts and oocysts were isolated via

a modification of a standard gradient centrifugation protocol [86,87]. This process began with creating an emulsion of 2 to 3 grams of feces with 0.01M PBS-EDTA and straining this mixture through a double layer of cheese cloth. The elution was then slowly pipetted into a 15 mL conical tube containing 7 mL of sucrose solution (specific gravity, 1.26). This tube was centrifuged at 800 x *g* for 10 minutes. Using a disposable pipette, the top layer of the fecal emulsion and sucrose solution interface was pipetted into a clean conical tube and centrifuged for 10 more minutes at 1,200 x *g*. The resulting pellet was isolated, resuspended, and rinsed twice with PBS-EDTA. The final pellet was resuspended in 1 mL of PBS-EDTA and stored at -80°C until DNA extraction [81,87,88]. DNA extraction was completed using a commercially available kit and stored at -20°C until further testing [110].

Giardia Testing

Samples were initially screened for the presence of *G. duodenalis* by amplifying a 292-bp fragment of the SSU rRNA gene as had been previously described [90,91,109]. To confirm the presence of *G. duodenalis* all samples showing bands on gel electrophoresis were purified with a commercially available kit and submitted for sequencing [111–113].

The resulting 45 samples were further analyzed for three genes in a multilocus approach of assemblage determination. A previously described two-step nested PCR was used to amplify a 530 bp fragment of the triosephosphate isomerase (TPI) gene using primers 5- AAA TIA TGC CTG CTC GTC G -3’ and 5’-CAA ACC TTI TCC GCA AAC C-3’ for the primary reaction and 5’-CCC TTC ATC GGI GGT AAC TT -3’ and 5’- GTG GCC ACC ACI CCC GTG CC-3’ for the secondary reaction [114]. A 432 bp fragment of the glutamate dehydrogenase (GDH) gene was amplified using primers 5’- TCA ACG TYA AYC GYG GYT TCC GT -3’ and 5’-GTT

RTC CTT GCA CAT CTC C -3’ for the primary reaction and 5’- CAG TAC AAC TCY GCT CTC GG -3’ and 5’- GTT RTC CTT GCA CAT CTC C -3’ for the secondary reaction [115]. A 384 bp fragment of the beta-giardin (BG) gene was amplified using primers 5’-AAG CCC GAC GAC CTC ACC CGC AGT GC -3’ and 5’-GAG GCC GCC CTG GAT CTT CGA GAC GAC -3’ for the primary reaction and 5’- CAT AAC GAC GCC ATC GCG GCT CTC AGG AA -3’ and 5’-GAG GCC GCC CTG GAT CTT CGA GAC GAC -3’ for the secondary reaction [116].

The primary and secondary PCR master mix included 8.75 μL of molecular grade water, 0.625 μL of 10 μM forward primer, 0.625 μL of 10 μM reverse primer, and 12.5 μL of GoTaq® Green to which 2.5 μL of sample was added for a total of 25 μL [117]. PCR of the TPI gene followed a 2 min initiation at 95 °C for the primary reaction, 40 cycles were run at 95 °C for 30 s, 53 °C for 45 s, and 72 °C for 90 s, then a final extension at 72 °C for 90 s after the cycles and before storing at 4 °C. The secondary reaction differed in that the 40 cycles were run at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 90 s. PCR of the GDH gene followed a 2 min initiation at 95 °C for the primary reaction, 40 cycles were run at 95 °C for 30 s, 51.5°C for 45 s, and 72 °C for 90 s, then a final extension at 72 °C for 90 s after the cycles before storing at 4 °C. The secondary reaction did not differ. PCR of the BG gene followed a 2 min initiation at 95 °C for the primary reaction, 40 cycles were run at 95 °C for 30 s, 64°C for 45 s, and 72 °C for 90 s, then a final extension at 72 °C for 90 s after the cycles before storing at 4 °C. The secondary reaction differed in the 40 cycles were run at 95 °C for 30 s, 65°C for 45 s and 72 °C for 90 s.

Gel electrophoresis was conducted on all resulting secondary reaction products. A 1% agarose gel, stained with GelRed® and using the Quick-Load® DNA ladder, was processed with a 100-mV procedure, for 45-75 minutes, and was visualized with the Bio-Rad GelDoc Go gel imaging system (Bio-Rad, Hercules, CA) [118–120]. Successful amplification was conditionally

assumed for TPI if a band was present at approximately 530 bp, GDH if a band was present at approximately 432 bp, and BG if a band was present at approximately 384 bp. PCR product purification was conducted as described above.

Cryptosporidium Testing

As was described in a previous study, samples were analyzed for the presence of *Cryptosporidium* DNA using a two-step nested PCR amplifying the SSU rRNA gene [92,109]. Samples were considered conditionally positive if an 800 bp fragment was visualized on gel electrophoresis. All samples that were conditionally positive were purified and sequenced as described for *Giardia*.

Analysis

All resulting genetic sequences were manually cleaned, using MEGA and SNAP GENE to read the sequences, and the forward and reverse reads aligned with ClustalW algorithm in MEGA, removing ambiguous reads [121,122]. The resulting sequence was queried with the NIH NLM NCBI nBLAST tool. This final sample sequence was aligned with ClustalW with the resulting reference sequences from NIH NLM NCBI to find genetic similarities.

Following alignment and BLAST identification, determination of the optimal nucleotide substitution model algorithm was determined using MEGA with the Maximum Likelihood test, and the model with the lowest Bayesian Information Criterion (BIC) was chosen. A phylogenetic tree was constructed using the Maximum Likelihood Tree analysis, and recommended model, in MEGA, with the Bootstrap test of phylogeny with 1000 replications.

Results

Giardia Sequencing

At the SSU rRNA locus, 45 samples yielded sequences that could be matched to *G. duodenalis*, while 2 samples at the TPI locus, 5 samples at the GDH locus, and 6 samples at the BG locus yielded sequences that could be matched to *G. duodenalis* with a total of 9 samples successfully genotyped via one or more loci (Table III.1).

On the TPI loci, 38 of the 45 samples showed bands on gel electrophoresis. Of those 38 samples, 2 generated a high-quality sequence and closely matched with a reference isolate of *G. duodenalis* (Table III.1). Sample 32 was a 534 bp sequence that matched with a 99% identification to assemblage AI (GenBank No. KR051228) and a 99% match to assemblage AII (GenBank No. KR075936). Sample 136 was a 523 bp sequence that matched with 99% identification to assemblage C. The phylogenetic relationship between samples and GenBank references is depicted in Figure III.1. The remainder of the sequences were unable to be matched.

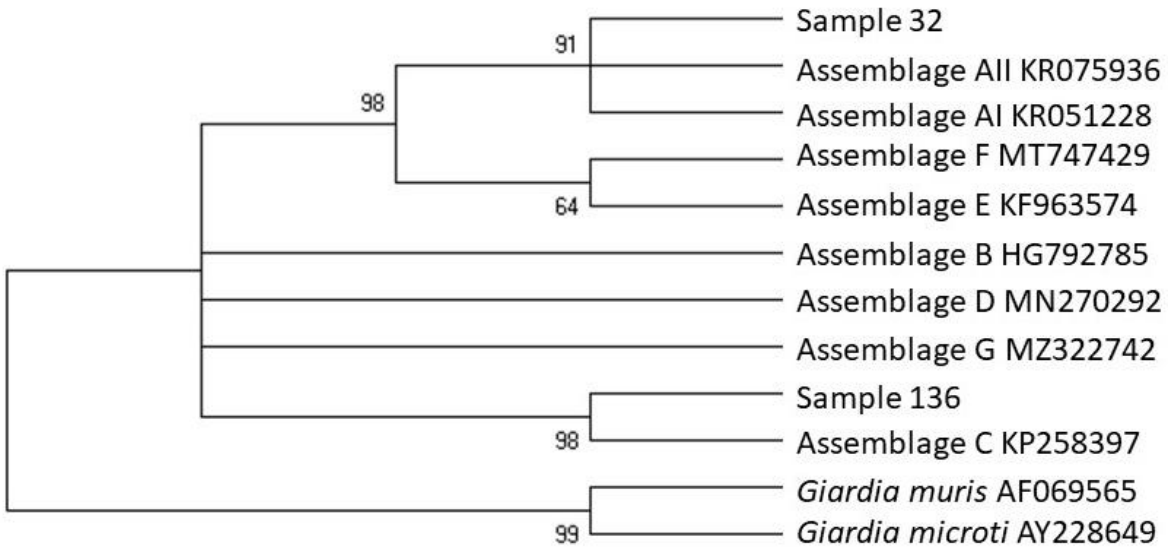
Figure III.1. Evolutionary relationships of taxa *Giardia duodenalis* TPI Locus

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [1]. The bootstrap consensus tree inferred from 1000 replicates [3] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates) are shown next to the branches [3]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6197)). This analysis involved 12 nucleotide sequences. There were a total of 563 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

1. Tamura K. and Nei M. (**1993**). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**:512-526.

2. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.

3. Felsenstein J. (**1985**). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.



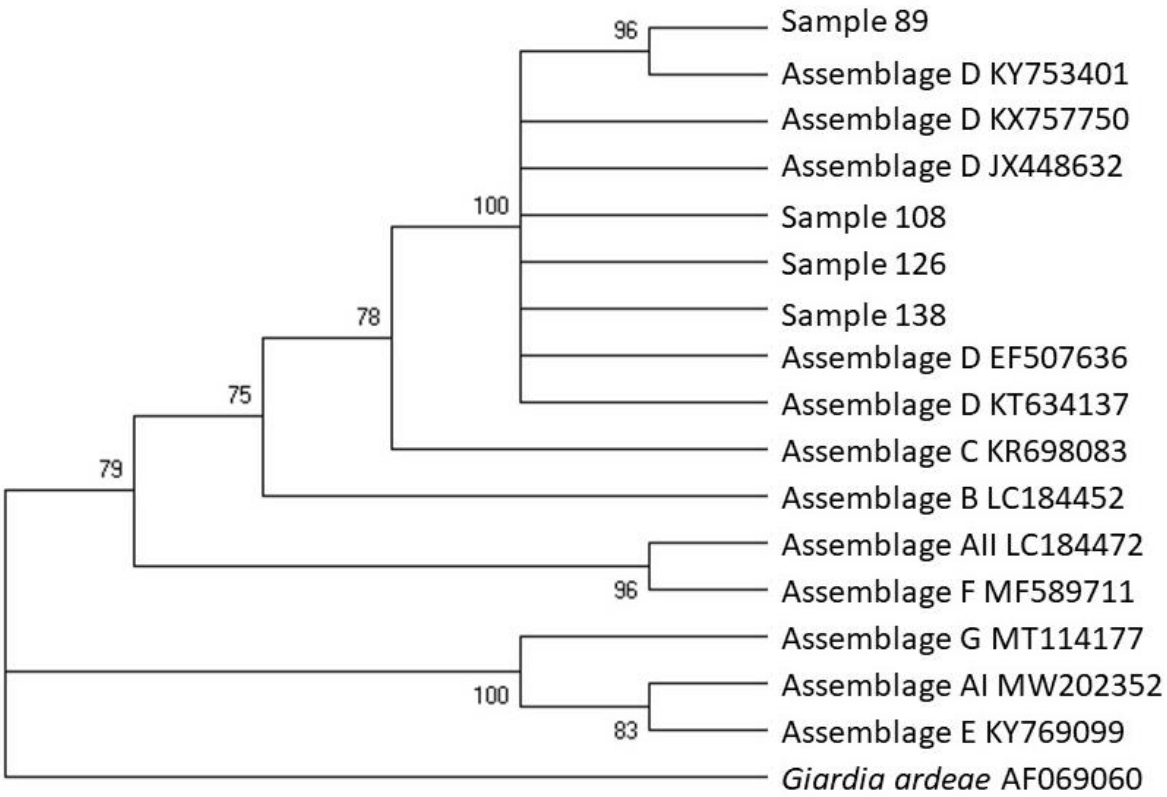
At the GDH loci, 39 of the 45 samples expressed bands on gel electrophoresis. Of those 39 samples, 5 generated a high-quality sequence and closely matched with a reference isolate of *G. duodenalis* (Table III.1). Sample 89 was a 439 bp sequence that matched with a 100% identification to assemblage D (GenBank No. KY753401). Sample 108 was a 433 bp sequence that matched with a 99% identification to assemblage D (GenBank No. KX757750). Sample 126 was a 380 bp sequence that matched with 100% identification to assemblage D (GenBank No. JX448632). Sample 136 was a 439 bp sequence that matched with 100% identification to assemblage D (GenBank No. KT634137). Sample 138 was a 428 bp sequence that matched with 100% identification to assemblage D (GenBank No. EF507636). The phylogenetic relationship between samples and GenBank references is depicted in Figure III.2. Seven of the other sequences returned as partial identifications with common gastrointestinal bacteria, and the rest were unmatched.

Figure III.2. Evolutionary relationships of taxa *Giardia duodenalis* GDH Locus

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model [1]. The tree with the highest log likelihood (-2991.14) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.1212)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 17 nucleotide sequences. There were a total of 796 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

1. Tamura K. (**1992**). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Molecular Biology and Evolution* **9**:678-687.

2. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.



At the BG loci, 32 of the 45 samples expressed bands on gel electrophoresis. Of those 32 samples 6 samples generated a high-quality sequence and closely matched with a reference isolate of *G. duodenalis* (Table III.1). Sample 7 was a 249 bp sequence that matched with 92% identification to assemblage A (GenBank No. FJ560591). Further analysis in MEGA with pairwise distance showed this was closely related to *G. microti* (GenBank No. MF185955) with a 10% difference. Sample 84 was a 359 bp sample that matched with 100% identification to assemblage A (GenBank No. FN377868). Sample 89 was a 334 bp sample that matched with 97% identification to assemblage D (GenBank No. KF736103). Further analysis in MEGA with pairwise distance showed this was closely related to *G. microti* with an 11% difference. Sample 121 was a 388 bp sample that matched with 99% identification to assemblage AI (GenBank No. KF963547). Further analysis in MEGA with pairwise distance showed this was closely related to *G. microti* with a 9% difference. Sample 136 was a 249 bp sequence that matched with 99% identification to assemblage D (GenBank No. KY979501). Further analysis in MEGA with pairwise distance showed this was closely related to *G. microti* with a 16% difference. Sample 138 was a 387 bp sequence that matched with 96% identification to assemblage D (GenBank No. AY545647). The phylogenetic relationship between samples and GenBank references is depicted in Figure III.3. Eleven of the other sequences matched partially to common gastrointestinal bacteria and the remainder were unable to be matched.

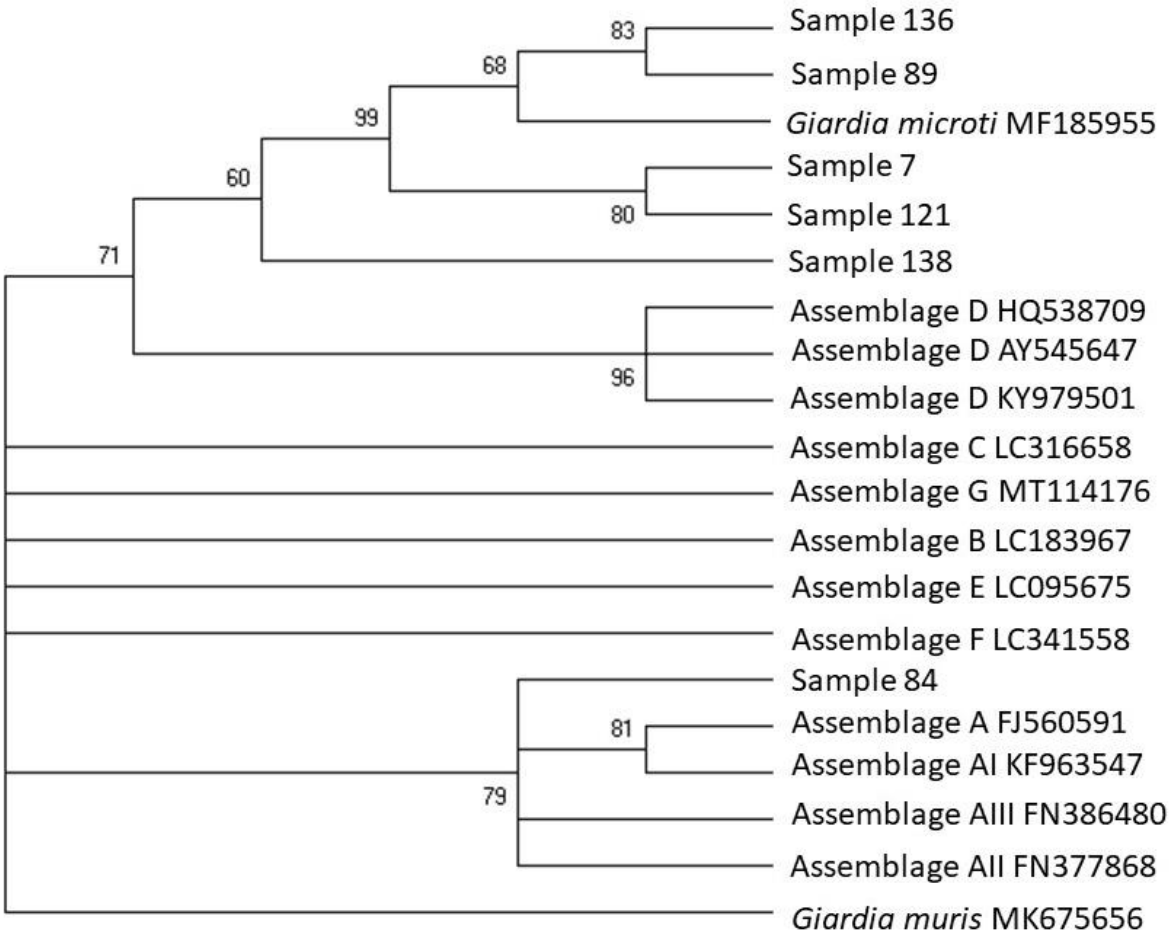
Figure III.3. Evolutionary relationships of taxa *Giardia duodenalis* BG Locus

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [1]. The bootstrap consensus tree inferred from 1000 replicates [3] is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test 1000 replicates) are shown next to the branches [3]. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.0749)). This analysis involved 20 nucleotide sequences. There were a total of 843 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

1. Tamura K. and Nei M. (**1993**). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**:512-526.

2. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.

3. Felsenstein J. (**1985**). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**:783-791.



The relationship between sequences loci and assemblage determination is depicted in the Venn Diagram in Figure III.4.

Figure III.4. Venn Diagram showing the assemblage identifications of nine *Giardia duodenalis* positive samples.

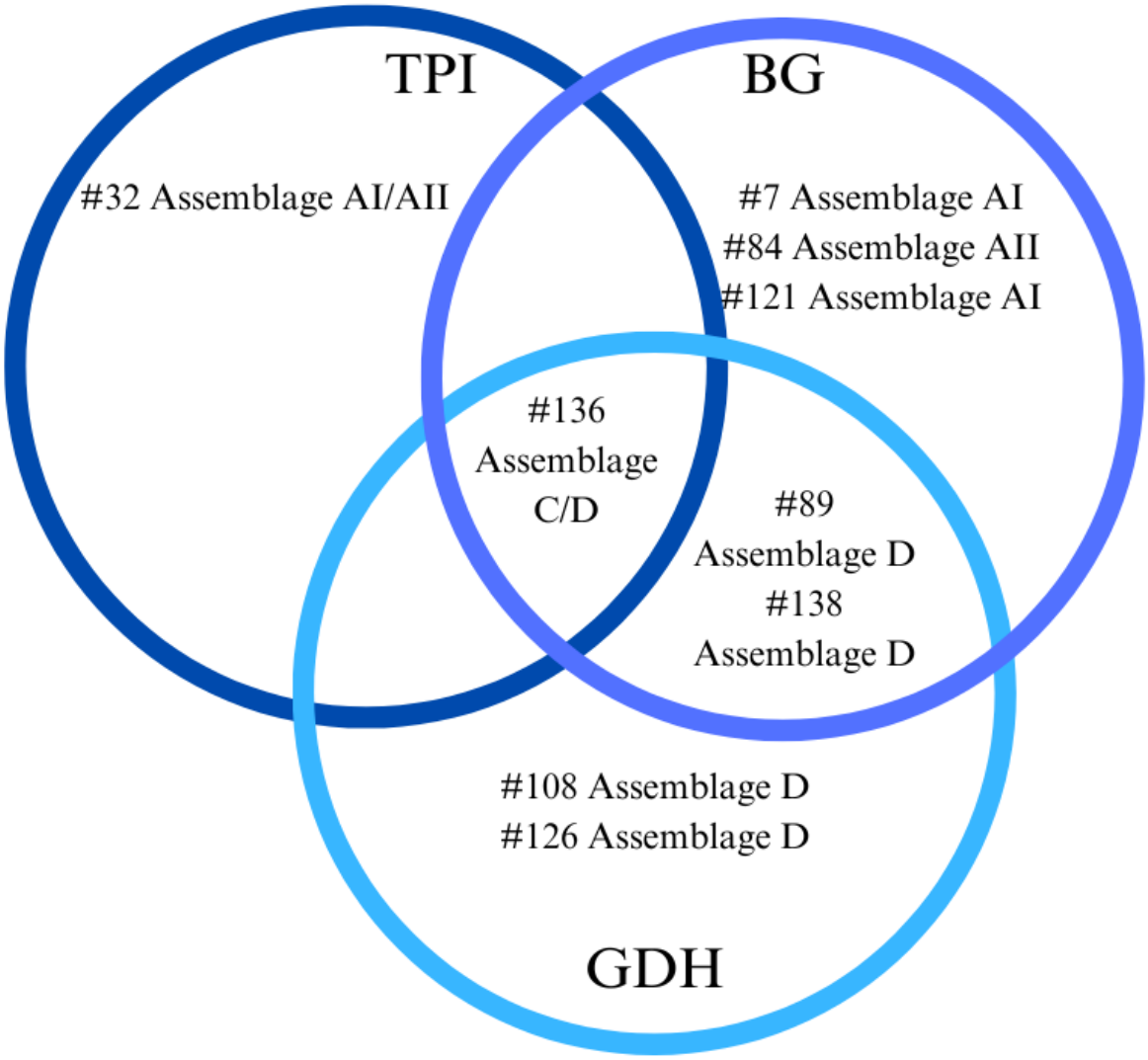


Table III1 Multiloci genotyping results for *Giardia duodenalis* at the TPI, GDH, and BG loci

Sample ID	Identification	% identity	GenBank Reference
TPI			
32	Assemblage AI	99	KR051228
32	Assemblage AII	99	KR075936
136	Assemblage C	99	KX014796
GDH			
89	Assemblage D	100	KY753401
108	Assemblage D	99	KX757750
126	Assemblage D	100	JX448632
136	Assemblage D	100	KT634137
138	Assemblage D	100	EF507636
BG			
7	Assemblage AI	92	FJ560591
84	Assemblage AII	100	FN377868.1
84	Assemblage AIII	100	FN386480.1
89	Assemblage D	97	KF736103.1
121	Assemblage AI	99	KF963547
136	Assemblage D	99	KY979501.1
138	Assemblage D	96	AY545647.1

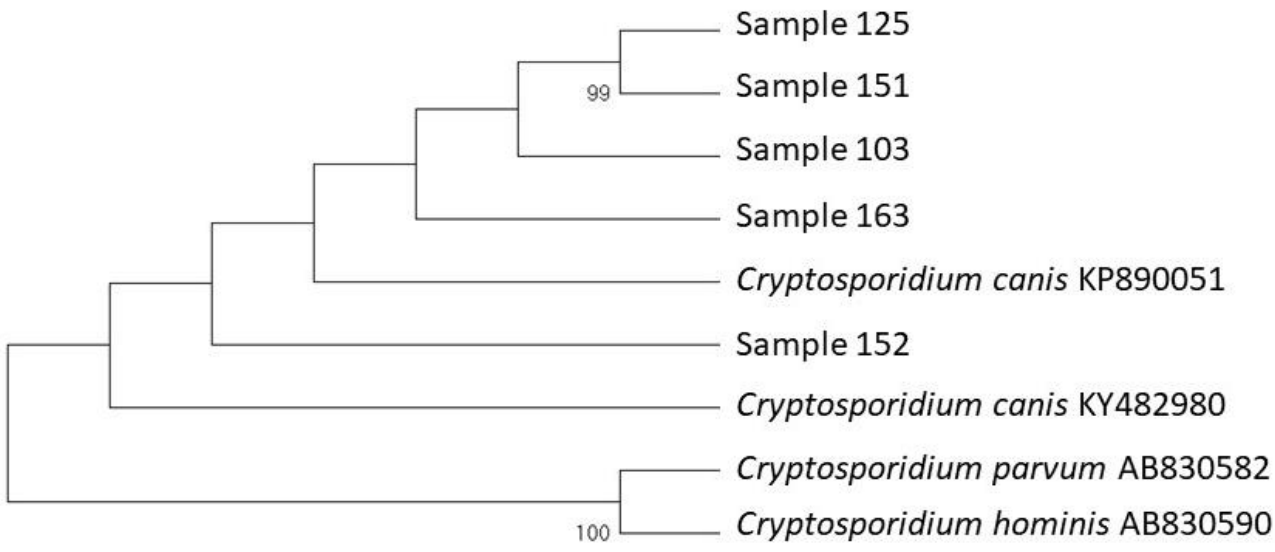
Cryptosporidium Sequencing

Fifteen samples from the *Cryptosporidium* SSU RNA PCR expressed bands on gel electrophoresis. Of these samples, five were able to be sequenced. Sample 103 was a 770 bp sequence that matched with 100% identification to *Cryptosporidium canis* (GenBank No. KP890051). Sample 125 was a 608 bp sequence that matched with 100% identification to *Cryptosporidium canis* (GenBank No. KY483980). Sample 151 was a 658 bp sequence that matched with 98% identification to *Cryptosporidium canis* (GenBank No. KY483980). Sample 152 was a 682 bp sequence that matched with 99% identification to *Cryptosporidium canis* (GenBank No. KY483980). Sample 163 was an 832 bp sequence that matched with 100%

identification to *Cryptosporidium canis* (GenBank No. KY483980). The relationship between samples and GenBank references is depicted in Figure III.5. The remainder of the samples were unable to be matched.

Figure III.5. Evolutionary relationships of taxa *Cryptosporidium canis* SSU locus
The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [1]. The tree with the highest log likelihood (-976.66) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Tamura-Nei model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 591 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

1. Tamura K. and Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512-526.
2. Tamura K., Stecher G., and Kumar S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution https://doi.org/10.1093/molbev/msab120.



Discussion

In this study we were able to isolate and identify different assemblages of *Giardia duodenalis* and *Cryptosporidium canis* from subclinical laboratory dogs who tested positive for the organisms. Of our *Giardia* identifications 44.4% (4/9) of samples were identified as assemblage A, via NCBI nBLAST, the assemblage known to affect dogs and humans alike. Of the 4 assemblage A samples identified, one was identified as assemblage AII, the human specific sub-assemblage of *Giardia*. The remaining samples, 55.6% (5/9) were identified as assemblage D, which is commonly associated with canine infections [75]. This percentage of assemblage A is higher than earlier studies [16,123]. Depicted in the Venn Diagram (Figure III.4), the BG locus amplified more assemblage A sequences and the GDH locus amplified more assemblage D sequences. This association between gene amplified and assemblage detected is not novel and has been previously noted in the literature [16]. Further research is needed into the subclassification of the canine associated assemblages to determine zoonotic potential of the subtypes. The relationship between specific subtype present and clinical disease should be investigated along with subtype zoonotic potential, since subtypes of assemblage C, based on the TPI gene, have been documented in the literature to rarely infect humans [124]. We also found 4 samples that closely matched *G. microti* on MEGA's pairwise distance calculation when the BG loci was amplified. *G. microti* has been isolated in rodents (the prairie vole, Pere Davi's vole, and the yellow-necked mouse), large cats (the leopard and cheetah), deer (the Red and Roe deer), and Bramandi fish, which could indicate a lack of host-specificity for this species [104]. To the authors' knowledge this would be the first identification of *G. microti* in a dog. For final species confirmation, further analysis should be conducted into the molecular relationship between *G.*

microti and *G. duodenalis* at the BG loci with additional refinement of the genotyping of these two species.

Of the *Cryptosporidium* identifications in this study 100% (5/5) of samples were identified as *C. canis*. This agrees with other investigations in dogs where this is the most common isolate of the protozoa [42]. The significance of this subclinical infection in our population rests in the aere infected with *C. canis* [28].

Further molecular epidemiologic investigation is needed into the subtypes of these other *Cryptosporidium* species that can infect humans to determine genetic association and distributions. Currently performing PCR of the 60kDa glycoprotein gene (gp60 otherwise known as gp40/15) is the method of determining *Cryptosporidium* species subtype. While this is a useful methodology for some species such as *C. parvum, C. hominis,* and *C. meleagridis* it has not been well established in other *Cryptosporidium* species, including *C. canis* [28]. Importantly, common primers for the gp60 gene do not amplify DNA associated with *C. canis, C. felis,* or *C. ubiquitum* [28]. This necessitates the development of primers specific to these species before further molecular epidemiologic studies can further evaluate any potential relationships.

Limitations of this study include the inability to genetically identify a high percentage of archival samples. It is well documented in the literature the difficulty that can occur when PCR is conducted on fecal samples due to the fecal microbiome that serves as PCR inhibitors [125–128]. In our study we performed cyst and oocyst isolation and washing that has been successfully used in the past. Future work will include a combination of using washed cyst and oocyst samples, techniques of adding inhibitor neutralization during extraction, and dilution based on spectrophotometry estimation of nucleic acids. While this limitation prevented us from demonstrating the genotype of some of our samples, we were still able to identify assemblages of

Giardia duodenalis associated with zoonotic infection and isolated only *Cryptosporidium canis* from our samples.

Conclusion

In conclusion, our study demonstrates the need for further molecular epidemiologic investigation into the assemblages and sub-species of *Giardia duodenalis* and *Cryptosporidium canis*. Our study found potentially zoonotic assemblages of *Giardia* during the screening of routine fecal exams of subclinical dogs, leading to the future consideration of recommendations to screen dogs who live with immunocompromised or high-risk individuals. Studies, such as this one, demonstrate how animals that we have close relationships with, such as dogs, can serve as reservoirs for disease.

CHAPTER IV EPIDEMIOLOGIC INVESTIGATION OF FACTORS ASSOCIATED WITH SUBCLINICAL INFECTIONS OF *GIARDIA DUODENALIS* AND *CRYPTOSPORIDIUM CANIS*. IN KENNEL HOUSED DOGS

Introduction

Giardia duodenalis is a protozoan parasite that can cause both clinical disease and subclinical infection in many animals including but not limited to dogs and humans. Current estimates of infection with *G. duodenalis* in dogs have a wide range. A recent meta-analysis looked at 127 papers reporting *Giardia* infection in dogs using testing methods including microscopy, ELISA, IFA, and PCR and found that there was an overall test positivity rate of 2.61% with rates as low as 0% and as high as 70% [57]. A recent investigation that sampled 3022 dogs and 288 dog parks in July and August of 2019 from major cities around the United States evaluated the zinc sulfate (specific gravity 1.24) centrifugal floatation method (processed at IDEXX, 401 Industry Rd, Louisville, KY 40208), coproantigen method (*Giardia* Test, IDEXX Laboratories, Inc., Westbrook, Maine) , or both methods together and found that 89.8% of samples positive for *Giardia* on coproantigen tested negative on fecal floatation [74]. The same study found that 13% of dogs were positive for *Giardia* using these methods. Investigations of kennel housed laboratory dogs have cited proportions of *G. duodenalis* infection in dogs ranging from 38.5%-100.0% depending on housing location [109,129–132]. Factors in kennel housed dogs that can lead to higher proportions of *Giardia* infection include frequency of cleaning the kennels, access to contaminated outdoor spaces, and group housing.

Cryptosporidium infections in dogs are reported less often than *G. duodenalis*. A recent global meta-analysis of canine *Cryptosporidium* infection revealed an 8% prevalence in studies

that conducted various microscopic modalities with and without staining (n=76), 7% in studies that conducted coproantigentic methods including immunofluorescence assays, enzyme-linked immunosorbent assays, and enzyme immunoassays (n=42), and 6% in studies that conducted molecular methods including PCR, nested PCR, real-time PCR, and RFLP-PCR (n=42) [41]. A recent study of dogs, kennel-housed in Texas, revealed proportions of infection congruent with this analysis, ranging from 1.0 to 9.5% presence of infection [109].

Infection with either *Giardia* or *Cryptosporidium* is not only a concern for our domestic animal species, as both organisms are also commonly associated with disease in humans [5,35]. *Giardia* is the most common gastrointestinal parasite in humans in the United States [52]. *Cryptosporidium*. infections are currently on the rise in humans, and the Centers for Disease Control and Prevention has recently implemented CryptoNet that uses the BioNumerics platform to enable the collection and molecular characterization of clinical isolates of *Cryptosporidium*. SSU rRNA and glycoprotein gene sequences are used to determine the specific species of *Cryptosporidium* and case investigation surveys are used to characterize disease in humans [35].

With high rates of *Giardia* infection in kenneled dogs, and low, yet consistent, rates of infection with *Cryptosporidium* in these dogs, it is important for veterinarians, researchers, facilities managers, animal rescues, and breeders to understand factors associated with the increased odds of subclinical infection with either organism.

The goal of this analysis was to provide additional knowledge for evidence-based decisions regarding housing, screening, and diagnosis of dogs in these settings. First, we hypothesized that dogs with loose feces as scored with the Purina Fecal Score chart (Nestle Purina PetCare, St. Louis, MO) would be at increased odds for subclinical *G. duodenalis*

infection and that younger dogs are at an increased odds of subclinical *G. duodenalis* or *Cryptosporidium* infection.

Materials and Methods

Animals and Sample Collection

In a previously published study, fecal samples (n = 170) were collected once from dogs housed at a Texas research facility (n = 96) and laboratory dog supplier (n = 74) between March and October of 2021 and used to evaluate the sensitivity and specificity of a human point of care fecal *Giardia* and *Cryptosporidium* test. A census of all dogs in the research facility and a convenience sample of laboratory supplier dogs were collected during daily cleaning activities and routine yearly physical exams.

Secondary analysis on this dataset was then completed, for this current study, by separating the research facility into four groups defined by either the building or group responsible for the dog and treating the laboratory dog supplier group's locations as a fifth and final group. Groups 1-4 all hold individual unique characteristics including the lab personnel working with each group and types of dry dog food fed. All dogs in groups 1-4 are housed indoors, some single housed and some group housed, with some dogs having play time allowed in grass yards outside. Kennels are cleaned daily with water, disinfected chemically with a minimal contact time of 10 minutes with activated hydrogen peroxide (Peroxigard™, Oakville, ON) disinfectant every one to two weeks depending on the building, and washed down with soap every month or as needed. Dogs included in group 5 are housed in non-climate controlled large kennels with concrete flooring and brick walls. Dogs in these facilities are handled similarly and fed similar foods. Their kennels are washed daily with water unless temperatures are too cold to

allow for this, in which case they are scraped clean that day. They are chemically disinfected weekly with an unspecified concentration of bleach and with no standardized contact time.

Samples from the original study in which sex, age, breed, laboratory, and fecal score data were available were selected for this study (n = 153). Fecal score was determined by visual assessment of the sample, with one reviewer, based on the 1 to 7 fecal score metric published by the Purina Institute where 1 is a hard and pellet like fecal mass and 7 is a watery defecation with no texture [133]. Breeds of dogs included golden retrievers, Labrador retrievers, beagles, and a variety of large hounds. Ages were determined in months based on date of birth.

Experimental Design

Samples were tested as previously described by each of the three tests: the QuickChek point of care rapid membrane enzyme immunoassay (QC) (TechLab®, Blackburg, VA), Merifluor™ *Cryptosporidium/Giardia* direct immunofluorescent assay (DFA) (Meridian Bioscience®, Cincinnati, OH), and inhouse polymerase chain reaction (PCR) with previously described primers and sequencing confirmation [90–92,109]. All samples in the data set were analyzed to determine associations between subclinical *Giardia* infection, *Cryptosporidium* infection, or coinfections with both organisms and each of the variables.

Statistical Analysis

The main outcome of this analysis was the subclinical presence of gastrointestinal protozoa, treated as 3 discrete outcomes: *Giardia*, *Cryptosporidium*, and co-infection.

Definitions and Handling of Variables

Apparent positive proportion of subclinical infection in the subpopulation was determined by interpreting the three tests, QC, DFA, and PCR in parallel: to be considered positive for either *Giardia* or *Cryptosporidium,* the sample needed to test positive on one of the three tests. A dog was considered coinfected with *Giardia* and *Cryptosporidium* if the sample was positive for both pathogens based on the criteria presented above.

Subgroup histograms of the fecal score showed that some subgroups approached a normal distribution while others did not. As a predictor of infection, the variable fecal score was categorized into hard, scores 1-2, (n=28), normal, scores 3-4, (n=108), and soft, scores 5-6, (n=17).

Age was dichotomized into two categories, selected for age-related biological characteristics involved in the maturation of the immune system: 0-18 months (n=53) and >18 months (n=100). The variable of breed was collapsed into two groups based on breed standard genetic backgrounds: hounds (n=72) and retrievers (n=81).

Variable Analysis

Descriptive statistics were reported for all variables as medians with interquartile range (IQR). We reported the positive proportion of *Giardia, Cryptosporidium,* and coinfection (presence of both detected). We reported differences in positive proportion between *Giardia* and *Cryptosporidium* overall and by group using the z-test for two proportions.

Due to the small population size in this analysis, exact logistic regression was performed, using the outcome variable *Giardia* test-positive status [134–136]. Exact logistic regression for the *Giardia* outcome was completed with a grouping variable based on location. Univariable

regression was completed with the independent variable fecal score. Results were reported as odds ratios with a 95% confidence interval.

To model demographic factors affecting *Giardia* test positivity, we evaluated potential exposure variables of interest, which include breed, sex, and age with exact logistic regression. Any independent variable analyzed with a z score p-value < 0.2 was included in the final regression equation. Results were reported as odds ratios with a 95% confidence interval and all p-values less than 0.05 are considered statistically significant.

Fisher's exact test was used to determine relationships between *Cryptosporidium* infection and coinfection and each of the measured variables due to the uncommon outcome and low positivity rate. The infection status variable was analyzed with the variables of fecal score, sex, age, and breed. Results were reported as proportion with associated p-value. All data were analyzed using STATA SE 17.0 (STATA Corp, LLC, College Station, TX).

Results

Giardia and Cryptosporidium Positive Proportion

The overall positive test proportion of *G. duodenalis* and *C. canis* as well as positive proportion by group is shown in Table IV.1. The overall positivity of *G. duodenalis* was significantly higher than *C. canis*. (p-value < 0.01). *Giardia duodenalis* was significantly higher than *Cryptosporidium* in each subgroup except for subgroup 5.

Table IV1. Positivity of *Giardia* and *Cryptosporidium*

Lab	*Giardia* % [95% CI]	*Cryptosporidium* % [95% CI]
Overall (n=153)	45.10 [37.34, 53.10]*	6.54 [3.53, 11.77]
Group 1 (n=22)	22.72 [9.26, 45.87]*	No cases detected
Group 2 (n=12)	41.67 [16.44, 72.16]*	No cases detected
Group 3 (n=41)	36.59 [23.05, 52.63]*	2.39 [0.32, 16.21]
Group 4 (n=10)	90.00 [45.33, 98.99]*	9.49 [1.01, 5.47]
Group 5 (n=68)	51.47 [39.52, 63.25]	11.8 [5.92, 22.03]
**Giardia* significantly greater than *Cryptosporidium* with p-value < 0.05.		

Giardia duodenalis

Fecal score analysis showed that there was a slight decrease in odds of dogs with hard stool, 0.34 times the odds of sub-clinical infection with *G. duodenalis* compared to normal stool. There was no difference in the odds of subclinical infection between dogs with soft stool and normal stool. Results of the *Giardia* analysis are shown in Table IV.2.

The median age of dogs infected with *Giardia* was 19.0 [IQR 7.0, 44.0] months. The median age of dogs without *Giardia* was 36.5 [IQR 19, 56] months. Being younger than 19 months was associated with 3.36 times the odds of having a subclinical *G. duodenalis* infection compared to older dogs. Due to age being the only demographic variable that was significant, a multivariable regression equation was not created.

Table IV2. Univariable Analysis of *Giardia* subclinical infection

Variable	OR	[95% CI]	p-value
Fecal Score			
Hard Stool	0.34	[0.10, 0.99]	0.049
Normal Stool	(ref)	(ref)	
Soft Stool	1.93	[0.57, 7.01]	0.358
Demographics			
Age			
>18 mo	(ref)	(ref)	
0-18 mo	3.36	[1.31, 9.17]	0.009
Breed			
Hound	(ref)	(ref)	
Retriever	1.60	[0.71, 3.67]	0.294
Sex			
Male	(ref)	(ref)	
Female	0.73	[0.34, 1.52]	0.453

Cryptosporidium canis

There was no relationship detected between infection and the categorized fecal score variable (Table IV.3). The median age of dogs infected with *Cryptosporidium* was 7.0 [IQR 5.0, 46.0] months and in those not infected was 29.0 [IQR 7.0, 50.0] months. Utilizing Fisher's exact test, the relationships between infection and the dichotomized breed variable, retriever versus hound, and sex, male versus female, showed that none were overrepresented in those infected (Table IV.3). The relationship between being infected and being younger than 19 months of age was also not significant (Table IV.3).

Co-infection

The median age of dogs with co-infection was 5.5 [IQR 3.5, 17] months, and in dogs without co-infection was 29 [IQR 7, 50] months. Only 4 of the 153 dogs sampled had co-infections. Co-infection occurred more commonly in dogs infected with *Cryptosporidium* (40%) than in dogs infected with *Giardia* (6%). Co-infection was not significantly related to breed (p-value = 1.0), fecal score (p-value = 1.0), or age (p-value = 0.12).

Table IV.3. Univariable Analysis of *Cryptosporidium* subclinical infection

Variable	Proportion infected	Percentage infected (%)	p-value
Fecal Score			0.666
Hard Stool	1/28	3.57	
Normal Stool	9/108	8.33	
Soft Stool	0/17	0	
Demographics			
Age			0.120
>18 mo	4/100	4.00	
0-18 mo	6/53	11.32	
Breed			0.301
Hound	6/72	8.33	
Retriever	4/81	4.94	
Sex			0.348
Male	6/75	8.00	
Female	4/78	5.13	

Discussion

Understanding the relationship between variables associated with subclinical infection of parasites, such as *Giardia* and *Cryptosporidium*, is important for veterinarians, breeders, researchers, and any professional who maintains large populations of dogs in a colony, breeding, performance, or other kennel setting. This study looked at a population of group, kennel housed dogs and evaluated the associations between easily obtained variables, including fecal score, age, breed, and infection with either *Giardia*, *Cryptosporidium*, or co-infection with both organisms.

There are several fecal scoring metrics available to animal care professionals. For this study we chose the widely available Purina 7-point fecal scoring system. A recent study compared the Purina 7-point and the Waltham 5-point fecal scoring scales. The Purina metric showed a kappa of 0.40 to 0.77 and the Waltham metric showed a kappa of 0.54 to 0.61 [137]. This indicates that there could be more variability of scoring with Purina than Waltham, which is reasonable due to an increased number of rating options in that metric. Despite this difference in agreement, we chose the Purina fecal scoring metric due to the wide availability and veterinarian familiarity with the scale.

It is biologically plausible that dogs harboring either *Giardia* or *Cryptosporidium* would have softer feces than those without the organism. There is disagreement in the literature regarding the effect of subclinical infection on fecal consistency. One study cited that positive test status for *Giardia*, but not *Cryptosporidium*, was significantly associated with development of loose stool in sled dogs during racing [138]. This study was limited by small sample size with only 5 of 53 dogs testing positive for *Giardia* prior to racing and 5 of 67 testing positive for it during the race. Chronic subclinical infection has been associated with dysbiosis with a recent study citing enrichment in pro-inflammatory bacterial species and opportunistic pathogens in

Giardia positive dogs [139]. While subclinical *Giardia* infection is not as likely in dogs exhibiting hard feces in this study (OR 0.3 [0.1, 1.0]), subclinical *Cryptosporidium* infection does not have a clear association with fecal score in our study. A study conducted in London, UK with a prevalence of 4.6% (31/677), also found no significant association with fecal score and *Cryptosporidium* infections [140]. Other studies have echoed these findings for *Cryptosporidium* in dogs, with no clear link to soft stools in subclinical animals [141,142].

Age was related to infection with *Giardia*. This fits with our understanding of the immune system and immune responses to *Giardia* infection. Immune system responses to *Giardia* have been demonstrated to depend on IL-17A, Th17 cells, and production of IgA [143,144]. We know that these cells, cytokines, and immunoglobulins take time to fully develop and increase during the maturation process [145]. A study in beagles revealed that IgA showed a steady age-dependent increase over 10 to 20 months [146,147]. Furthermore, future research could be done to assess involvement of long-term stimulation of the Th17 cells and the IL-17A response in parasitic infections and predisposition to autoimmune conditions such as immune mediated hemolytic anemia, irritable bowel disease, asthma, and idiopathic epilepsy [145,148–151].

There were more dogs younger than 19 months that were positive for *Cryptosporidium,* although this relationship was not significant. Age's relationship to *Cryptosporidium* infection is biologically plausible as recent investigations have led to a deeper understanding of the immune response to *Cryptosporidium* infections. There are several components essential to the immune response to this organism, including intestinal epithelial cells, innate and adaptive immunity, chemokines, cytokines, and antimicrobial peptides [48,152]. While some components of the immune system are viable early in a dog's life it is accepted that full functionality and serum

immunoglobulin levels do not reach those of adults until 12 months [153]. A recent study found that the chemokine CCL20, which is a chemokine needed for T lymphocyte recruitment, was downregulated during infection with *Cryptosporidium* in neonatal mice, likely making them susceptible to infection [154]. When researchers supplemented mice with recombinant CCL20, they found that the number of oocysts significantly decreased compared to control mice. This is an area for further research in dogs and as a potential model for human disease.

The size of our study limits the degree to which we can assess significant relationships between specific breeds, genetics, and fecal scores. Due to the limitation, many of these variables had to be dichotomized. Despite this limitation, this study provides insight that can be built upon in the future.

This study adds valuable knowledge in that we show a significant protective relationship between a hard fecal score and *Giardia* infection as well as a relationship between age and infection with *Giardia*. With this addition to available knowledge, we hope to provide managers of canine kennel facilities with the information needed to make evidence based standard operating procedures. Future studies focusing on age related biologic factors, and their association with subclinical infection, as well as studies in larger cohorts of both clinical and subclinical dogs assessing the viability of using fecal score as a predictor for infection with either of these organisms is needed.

Conclusion

In conclusion, this study shows that co-infections with *Giardia* and *Cryptosporidium* can occur in kennel housed groups of dogs. We were able to demonstrate that there is no clear link between sex or breed and infection with *Giardia* or *Cryptosporidium* in dogs. While there were

more dogs younger than 19 months with *Cryptosporidium* infection, this relationship was not significant and there was no clear link to fecal score and infection with that organism. We were able to confirm that kennel housed dogs younger than 19 months were at 3.4 times the odds of *Giardia* infection compared to older dogs, and that the presence of hard stool in associated with decreased odds (0.3 [0.1, 1.0]) of subclinical *Giardia* infection.

CHAPTER V

THE RELATIONSHIP BETWEEN SOCIAL DETERMINANTS OF HEALTH AND *GIARDIA DUODENALIS* INFECTION IN TEXAS CANINES

Introduction

The Centers for Disease Control and Prevention (CDC) estimates human giardiasis affects 5.8 per 100,000 people each year in the United States. This is a rate that has seen minimal change over the past decade [52]. The Companion Animal Parasite Council (CAPC), an independent non-profit group who is devoted to the promotion of parasite awareness in dogs and cats, currently tracks the occurrence of several common canine and feline parasites at the county, state, and national level in the US and Canada, including *Giardia*. They estimate a *Giardia* test positivity of 7.49% nationally and 5.41% in Texas [53]. Giardiasis is a nationally notifiable disease in the US [52]. Individual states may voluntarily, but are not required to report, positive human *Giardia* tests to the CDC, limiting national efforts to monitor, measure, and alert communities of *Giardia* as a potential public health threat [155]. Eight states do not report this information to the CDC, one of which is Texas [52].

Giardia duodenalis is a zoonotic protozoal pathogen that infects various species of animals, including but not limited to, dogs, cats, rodents, ruminants, horses, and wildlife, as well as humans [1]. *Giardia* infection manifests in the patient from subclinical disease to prolonged diarrhea, malnutrition, and chronic inflammation [22,25]. It is estimated that *Giardia* was responsible for 1,460 emergency room visits in the US during 2014 and 7.9% of patients were admitted to the hospital, costing an average of $21,800 per hospital stay [156]. *Giardia* infection

has been linked to long term conditions including chronic diarrhea, increased gastrointestinal co-infections, ulcerative colitis, and chronic fatigue in humans [6,20–22]

The risk that canine *Giardia* infection poses to human infection is unclear. The ability for *Giardia* to infect a specific species, human or animal alike, depends on its genetic subtype, or assemblage. The literature ranges from citing evidence for zoonotic infections to the need for continued research. Molecular epidemiologic research focusing on different geographic regions and diverse socioeconomic populations should be conducted before claims of minimal zoonotic risk from dogs are made [1,12]. *Giardia duodenalis* has not only been noted to be zoonotic, but holds the potential for anthroponosis, with a proposed method of dogs acquiring infection via exposure to human waste [157]. In humans, infection with and transmission of parasites, including *Giardia*, can be potentiated by poor socioeconomic factors, such as poor housing, air quality, water quality, or a lower economic status of an individual [66]. Social determinants of health (SDOH), such as these, are the circumstances in which we develop, mature, live, work, and die, and are usually divided into 5 categories: physical environment, social factors, economic factors, medical care, and health behaviors [68]. SDOH are often used to suggest where human health inequities and health divides may occur [67,68].

SDOH are usually discussed in the context of human health [66,69–72]. Recently, there has been an interest in determining social determinants of animal health (SDOAH) and the impact of human SDOH on domestic animal health [73,158]. Furthermore, dogs can be used as a sentinel species for risk of infectious diseases with zoonotic potential or of other importance to human and animal health [157,159,160]. Texas boasts some of the greatest diversity in the nation, making it an important setting in which to better understand the effects of societal factors on health [161]. In Texas, where data on human *Giardia* infection is not routinely reported, but

some data on canine infections is, we sought to describe SDOAH for canine *Giardia* test positivity in the context of SDOH, veterinary service indices, and human medical service accessibility at the county level.

To fill the gap in knowledge about *Giardia* epidemiology, we analyzed data on canine *Giardia* test positivity, or the proportion of dogs whose stool tested positive when submitted for laboratory testing, to identify geographical distribution, temporal trends, and the impact of socioeconomic factors on canine *Giardia* infections in a large, diverse setting.

Materials and Methods

We described canine *Giardia* infection trends and cumulative testing positivity in Texas during the years 2012 to 2022. We defined positivity as the proportion of stool samples testing positive for *Giardia* among samples submitted to veterinary diagnostic laboratories.

Data Sources

For the first objective, the total number of canine *Giardia* samples submitted for testing and the number of positive tests reported for the State of Texas and individual counties within Texas were obtained from CAPC for the years 2012-2022 [53]. CAPC obtains their data from voluntary veterinary test submissions to commercial diagnostic laboratories, including Idexx and Antech (IDEXX Laboratories, Inc. Westbrook, ME; Antech Diagnostics, Fountain Valley, CA) [53].

For the second objective, the CAPC data from 2020 to 2022 was supplemented by similar data from the Texas Veterinary Medical Diagnostic lab (TVMDL) for the years 2020 through 2022. County level data on poverty (%), population size, unemployment (%), income (categorical) and

education (%) for the State of Texas were obtained from the US Department of Agriculture (USDA), Economic Research Service (ERS), for the reported period from 2020-2021 [162]. The USDA sources this data from the Census Bureau and Bureau of Labor Statistics. County level data on veterinary service accessibility (veterinary care accessibility score [VCAS]) were obtained through The Veterinary Care Accessibility Project for the year 2021 [163]. VCAS is a complex metric consisting of the number of households, pets, and veterinary employees, along with percentage of non-English speakers (%), vehicle accessibility (%), per-capita income, and poverty (%) to provide a single numerical percentile measure of accessibility of veterinary care. Finally, data on medically underserved areas (MUA) in Texas was obtained from the Health Resources and Services Administration [164]. This classification identifies areas and populations that have a lack of human primary care services. Counties were included in the SDOAH analysis if they had a minimum of 10 tests submitted during the 2020 to 2022 time period

Data Analysis

Decade Positivity Analysis

Giardia positivity (or positive proportion [%]) was calculated as a proportion of canine stool specimens that tested positive for *Giardia* (numerator) out of the total number of *canine stool* samples that were submitted to veterinary diagnostic laboratories (denominator). Positivity was calculated for Texas as a whole and by county (n=35), per year from 2012 to 2022. The cumulative positivity (%) was calculated as the sum of positive test results (numerator) over the sum of all tests performed (denominator) over the same 11-year period. Counties were excluded from analysis if fewer than 10 tests were performed in any single year. This decision was made

to keep as many counties included in the analysis as possible while minimizing the effect of small numbers on apparent large fluctuations in proportions [53,165].

Two-way scatter plots including the linear fit line with a 95% confidence interval were created for each county and the state with *Giardia* test positivity on the y-axis and year on the x-axis. Using ordinary least squares (OLS) linear regression of yearly *Giardia* test positivity over time, we report the slope of the best linear fit line to reflect the increasing or decreasing nature of positivity for each county and for the state. A choropleth map was generated using these slopes by county. To assess if changes over time were different than the state, we calculated the difference in slopes and standard errors. Differences in the slopes were considered significant at an adjusted significance for multiple comparisons of $p \leq 0.001$ [167].

A choropleth map was generated depicting cumulative positivity by county. The cumulative positivity for each county was compared to the overall state positivity using the z test for two proportions and an adjusted significance for multiple comparisons of p-value ≤ 0.001 to determine significance [167].

Multivariable Statistical Analysis

To meet objective 2, we assessed the relationship between the 2020-2022 cumulative positivity and potential SDOAH factors. This decision was made because supplementary data on *Giardia* test positivity available from TVMDL for these years made a more robust data set for this period. Potential SDOH factors included economic indicators (poverty, household income level, county percentage of state median income), county population size, rural designation, unemployment rates, college graduation percentage, and veterinary access (measured as VCAS). Poverty is the percent of county residents classified as impoverished based on the US Census

Bureau's Small Area Income and Poverty Estimates Program and was dichotomized as poverty less than or equal to that of the state poverty level (0) or a poverty rate higher than the state (1) [167,168]. County population (continuous variable) is the number of residents in the county. Rural status is derived from the USDA's Rural Urban Continuum Codes (RUC) [169], coded by the USDA Office of Management and Budget on a scale of 1 (large metropolitan area) through 9 (completely rural). RUC was collapsed into 4 categories: large metropolitan area (1), large city (2), a non-metropolitan, populated county (3), and rural county (4). Median household income, in dollars per household, was dichotomized into a median income greater than or equal to that of the state median (0) or a median income less than the state (1). The percentage of state median income, a continuous variable calculated by dividing the county's median household income by the state median household income, was categorized: greater than or equal to (100%) (0), 80% to 99% (1), and <80% of the state median income (2). Unemployment, the proportion of job-seeking residents who are currently on the job market divided by the total labor force, was dichotomized into less than or equal to that of the state (0) and higher than the state (1). College education, the proportion of four-year college graduates living in the county, was dichotomized into greater than or equal to that of the state (0) and less than the state (1). The VCAS is a continuous variable with a scale from 0 to 100, with 0 indicating that veterinary care is near completely inaccessible, and 100 indicating that veterinary care is very accessible. The VCAS was collapsed into 4 categories: 0 to 24 (1), 25 to 50 (2), 51 to 75 (3), and >75 (4). The MUA is a categorical variable dichotomized as a county having no MUA (0) or at least 1 MUA (1).

Cumulative *Giardia* positivity was calculated for the period 2020-2022. For included counties, descriptive summary statistics (median [IQ range]) were evaluated for *Giardia* test positivity, poverty rate, county size (population), rural and urban/suburban classification,

income, unemployment classification, education status, VCAS, and MUA. Factors of interest were assessed for correlation.

To assess factors associated with *Giardia* positivity, we performed univariate regression analysis for canine *Giardia* positivity (dependent variable) and potential SDOAH indicators, as independent variables. The Poisson regression model was first tested but due to overdispersion, the negative binomial regression model using a bootstrap standard error with 1000 replicates was used. Models were evaluated using the Wald chi squared analysis, pseudo R^2, deviance dispersion, Pearson dispersion, AIC, and BIC statistics as is described in the literature [170,171]. Differences in positivity over each factor of interest were expressed as prevalence ratios (PR [95% CI]). Each factor's main effects were evaluated by regression for association with positivity [172]. Factors with a p-value <0.20 were included in a multivariable regression model building strategy. The model was developed in a bidirectional elimination stepwise method, individually dropping variables with insignificant (p-value > 0.05) association until only significant independent variables remained. Interactions between all remaining factors with a moderate (0.4-0.6) or strong (0.7-0.9) correlation coefficient were evaluated [172]. The final model that maximized the Wald chi squared and pseudo R^2 values was chosen. Margins were calculated for the final equation and margins graph generated. Associations with p-value ≤ 0.05 were considered statistically significant.

Statistical procedures were conducted in Stata SE 17.0 (StataCorp, College Station, TX) or Microsoft Excel 360, and maps were created with Arc GIS.

Results

The 11-year canine *Giardia* test positivity for Texas was 4.6% (Table V.1). Positivity was also ascertained for 35 counties for which sufficient data was available for 2012 to 2022 (Figure V.1). The highest positivity was observed in El Paso (12.1%), Parker (11.1%) and Cameron (10.2%) counties. Lower positivity was observed in Wood (0.6%), Walker (1.6%), and Harrison (1.7%) counties.

Positivity in Texas, as a whole, did not increase significantly over the 11-year period, from 4.6% in 2012 to 4.5% in 2022 (slope: 0.04, p-value: 0.07; Table V.2). Counties with significant increases in positivity, compared to the state as a whole, were: Cameron (slope: 0.91, p-value: 0.002), Hayes (slope: 0.36, p-value: <0.001), Kendall (slope: 0.42, p-value: 0.001), Lubbock (slope: 0.93, p-value: 0.002), and Williamson (slope: 0.22, p-value: <0.001) (Figure V.2 and Figure V.3). Those with a significantly decreasing positivity were: Bexar (slope: -0.30, p-value: 0.004), El Paso (slope: -0.348, p-value: 0.002), Fort Bend (slope: -0.23, p-value: <0.001), Grayson (slope: -1.54, p-value: 0.028), Guadalupe (slope: -0.80, p-value: 0.012), Montgomery (slope: -0.10, p-value: 0.010), and Parker (slope: -1.12, p-value: <0.001).

Table V.1. Cumulative canine *Giardia* test positivity, 2012-2022

	Total Tests	Positive Tests	Cumulative Positivity (%)	Difference (State - County)	p-value
State of Texas	5697082	265447	4.66		
County					
Bell	8244	421	5.11	+0.45	0.027
Bexar	468302	31494	6.73	+2.07	<0.001*
Brazoria	56483	1695	3.00	-1.66	<0.001*
Cameron	2755	281	10.20	+5.54	<0.001*
Collin	392431	18397	4.69	+0.00	0.205
Comal	56785	2599	4.58	-0.01	0.177
Dallas	863364	46276	5.36	+0.70	<0.001*
Denton	286495	15326	5.35	+0.69	<0.001*
El Paso	122368	14755	12.06	+7.40	<0.001*
Ellis	22249	1747	7.85	+3.19	<0.001*
Fort Bend	100790	2932	2.91	-1.75	<0.001*
Galveston	72694	2584	3.55	-1.10	<0.001*
Grayson	6234	249	3.99	-0.67	0.006
Greg	7305	64	3.01	-1.65	<0.001*
Guadalupe	14749	888	6.02	+1.36	<0.001*
Harrison	5682	98	1.72	-2.93	<0.001*
Hayes	78101	3095	3.96	-0.70	<0.001*
Hidalgo	19178	464	2.42	-2.24	<0.001*
Hood	34899	1775	5.09	+0.43	0.0001*
Hunt	11457	485	4.23	-0.43	0.0153
Johnson	14456	771	5.33	+0.67	<0.001*
Kaufman	21071	695	3.30	-1.36	<0.001*
Kendall	34082	1513	4.44	-0.22	0.027
Lubbock	16689	996	5.97	+1.31	<0.001*
Midland	20270	917	4.52	-0.14	0.181
Montgomery	255688	10387	4.06	-0.60	<0.001*
Navarro	4304	283	6.58	+1.92	<0.001*
Nueces	23885	1476	6.18	+1.52	<0.001*
Parker	27097	3018	11.14	+6.48	<0.001*
Tarrant	487589	23855	4.89	+0.23	<0.001*
Travis	616424	24655	3.40	-0.66	<0.001*
Victoria	11049	512	4.63	-0.03	0.450
Walker	4584	71	1.55	-3.11	<0.001*
Williamson	246970	7708	3.12	-1.54	<0.001*
Wood	15465	92	0.59	-4.06	<0.001*

Table V.2 Change in canine ***Giardia*** **test positivity, 2012-2022**

	Slope	95% CI	p-value	Difference from State	p-value
State of Texas	0.04	-0.004, 0.082	0.073		
County					
Bell	-0.14	-0.97, 0.69	0.706	-0.18	0.627
Bexar	-0.30	-0.47, -0.12	0.004	-0.34	0.001*
Brazoria	0.07	-0.03, 0.17	0.125	+0.03	0.480
Cameron	0.91	0.43, 1.38	0.002	0.87	0.001*
Collin	0.12	-0.05, 0.29	0.159	0.08	0.335
Comal	0.02	-0.16, 0.21	0.790	-0.02	0.85
Dallas	0.10	0.04, 0.17	0.007	0.07	0.081
Denton	0.42	0.12, 0.71	0.011	0.38	0.010
El Paso	-0.48	-0.74, -0.23	0.002	-0.52	<0.001*
Ellis	0.28	-0.12, 0.68	0.149	0.24	0.193
Fort Bend	-0.23	-0.28, -0.18	<0.001	-0.27	<0.001*
Galveston	0.15	0.05, 0.25	0.006	0.11	0.029
Grayson	-1.54	-2.87, -0.21	0.028	-1.58	0.015
Greg	0.08	-0.29, 0.45	0.645	0.04	0.815
Guadalupe	-0.80	-1.39, -0.22	0.012	-0.84	0.004
Harrison	0.31	-0.11, 0.73	0.135	0.27	0.171
Hays	0.36	0.28, 0.44	<0.001	0.33	<0.001*
Hidalgo	-0.13	-0.31, 0.06	0.155	-0.16	0.062
Hood	0.04	-0.16, 0.23	0.678	-0.00	0.979
Hunt	-0.08	-0.43, 0.27	0.617	-0.12	0.455
Johnson	-1.56	-2.59, -0.53	0.008	-1.60	0.003
Kaufman	0.21	0.00, 0.43	0.047	0.18	0.081
Kendall	0.42	0.23, 0.61	0.001	0.38	<0.001*
Lubbock	0.93	0.43, 1.44	0.002	0.90	<0.001*
Midland	0.10	-0.31, 0.51	0.593	0.06	0.738
Montgomery	-0.10	-0.17, -0.03	0.010	-0.14	0.001*
Navarro	-0.55	-1.41, 0.30	0.177	-0.60	0.135
Nueces	0.27	-0.05, 0.58	0.086	0.23	0.120
Parker	-1.12	-1.49, -0.75	<0.001	-1.16	<0.001*
Tarrant	0.08	-0.01, 0.18	0.087	0.04	0.370
Travis	0.06	-0.00, 0.12	0.056	0.02	0.56
Victoria	0.09	-0.20, 0.38	0.499	0.05	0.70
Walker	-0.14	-0.37, 0.08	0.174	-0.18	0.08
Williamson	0.22	0.14, 0.31	<0.001	0.18	<0.001*
Wood	-0.01	-0.07, 0.06	0.821	-0.05	0.200
*Indicates significant difference with the multiple comparison's correction of p-value ≤ 0.001					

Figure V.1. Cumulative canine *Giardia test* positivity, 2012-2022.

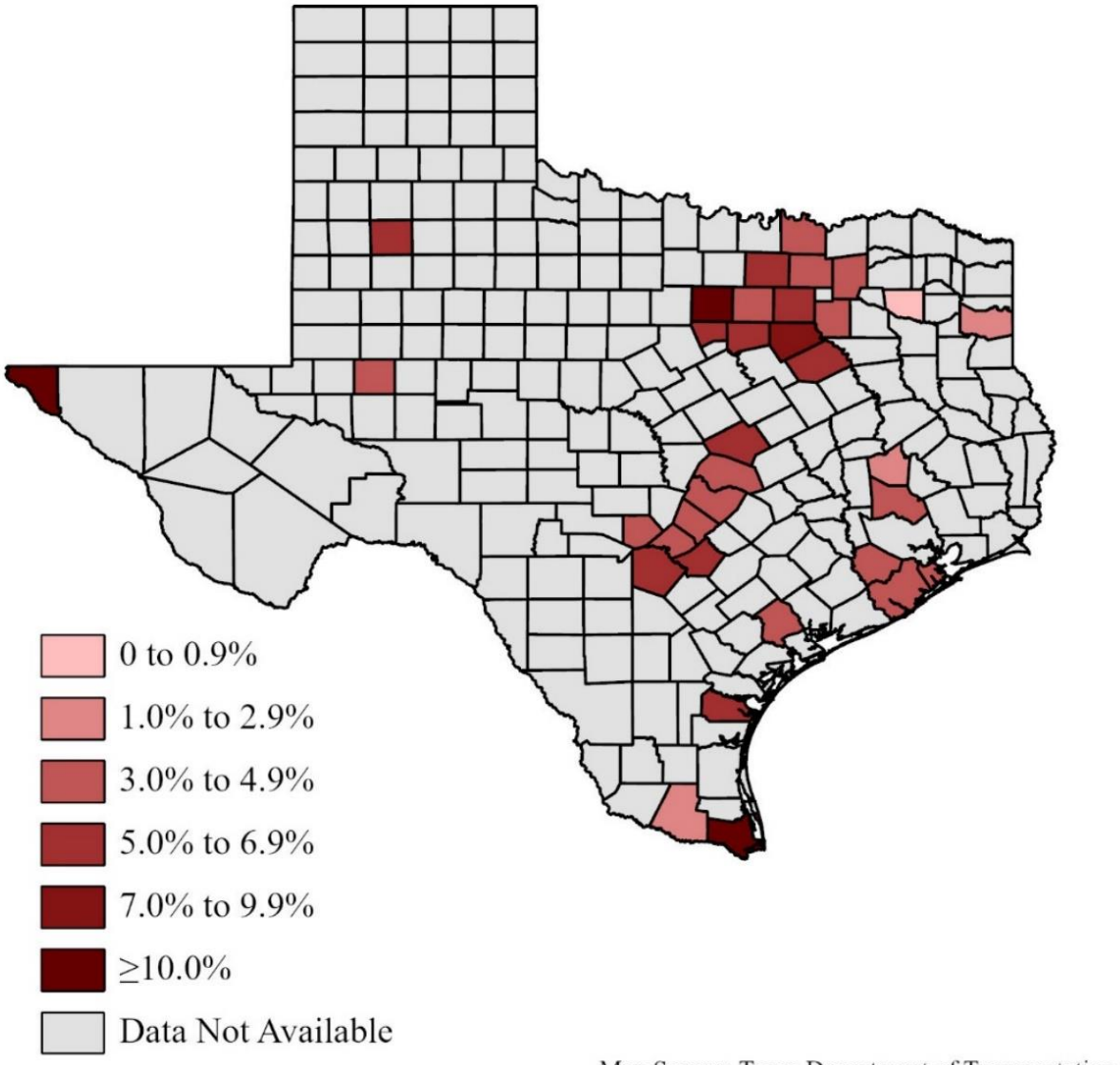
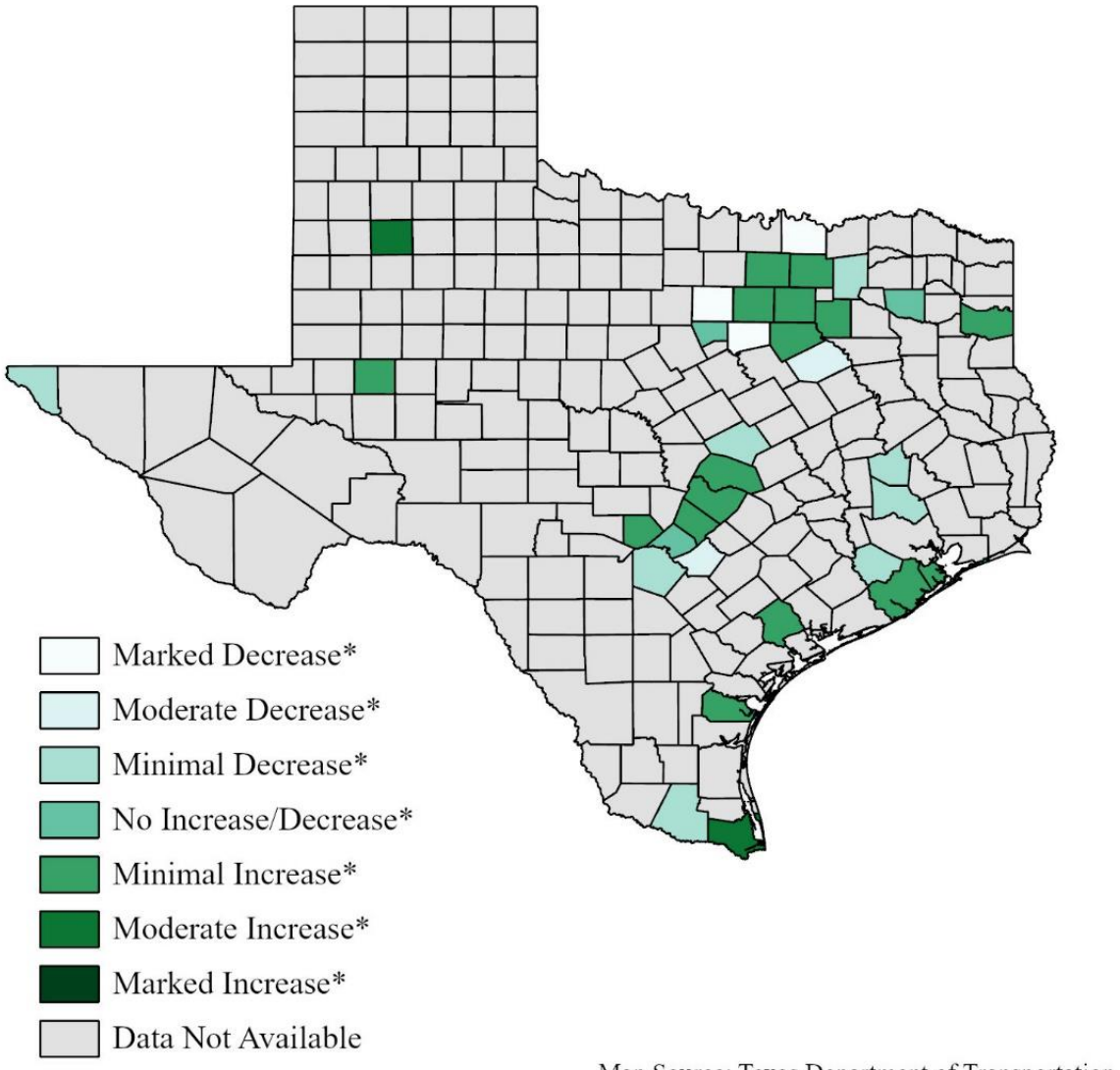
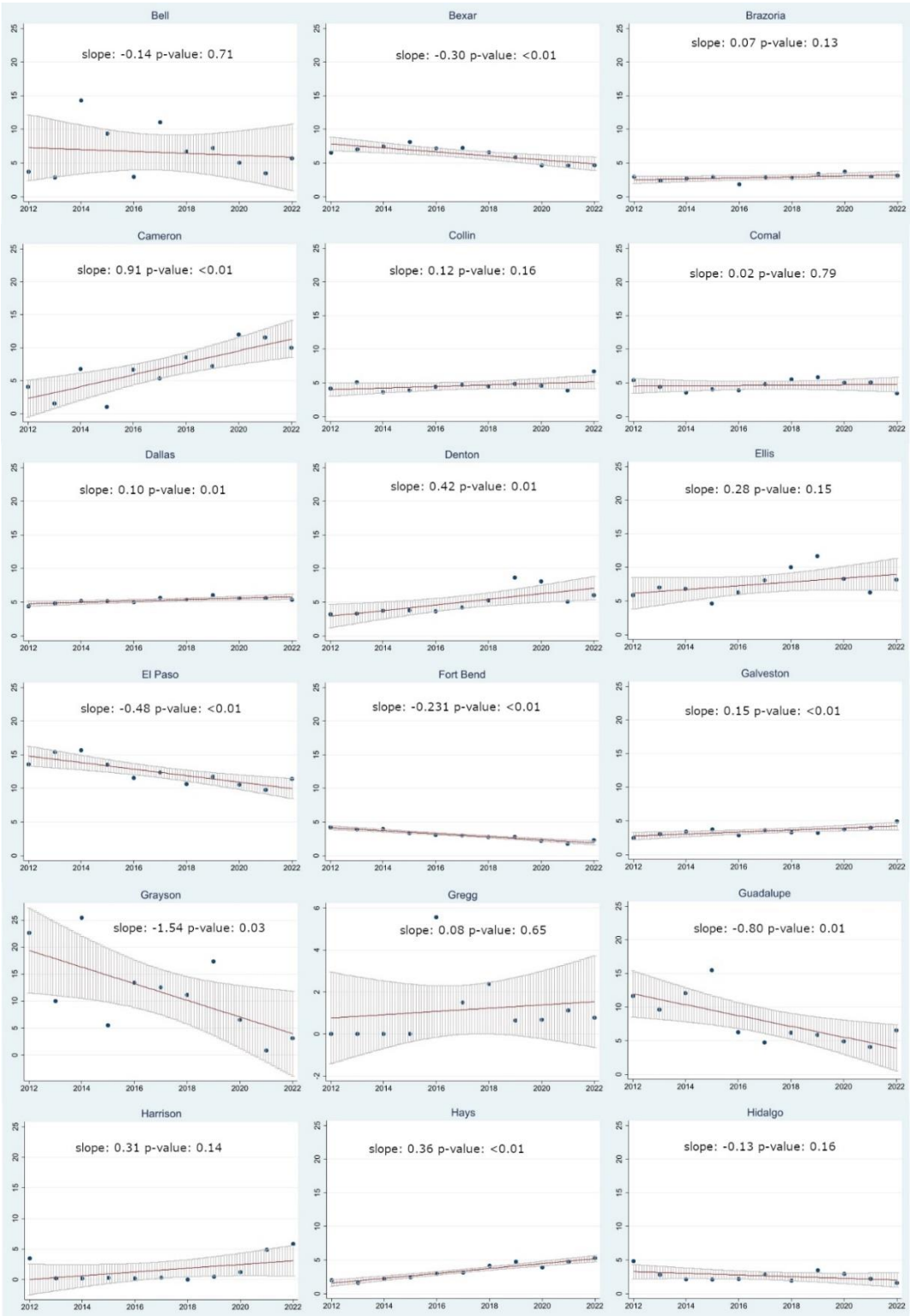


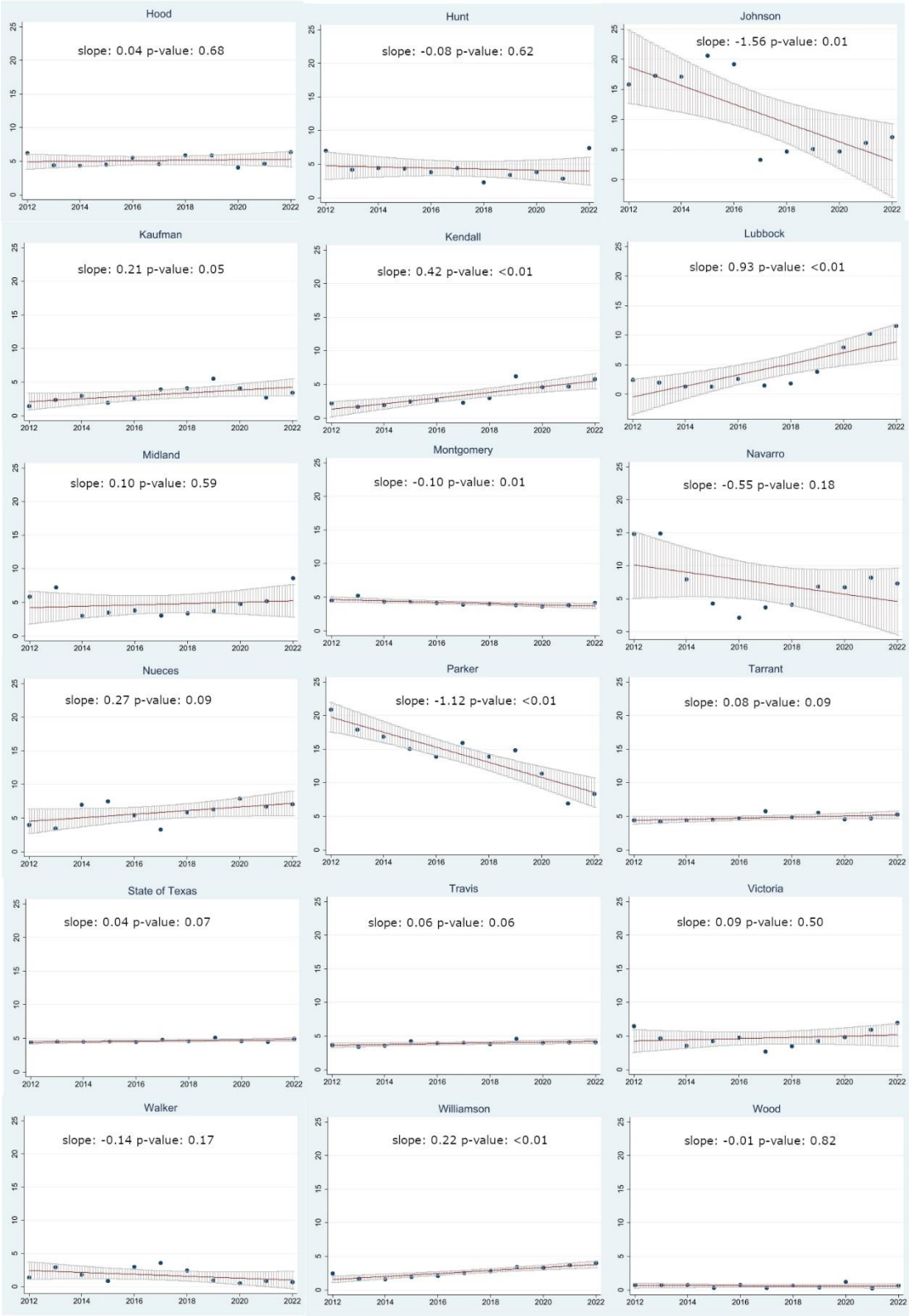
Figure V.2. Changes in canine *Giardia* test positivity, 2012-2022.



* Marked decrease indicates a slope of -2.0 to -1.0, moderate decrease indicates a slope of -0.99 to 0.5, minimal decrease indicates a slope of -0.49 to -0.05, no increase/decrease indicates a slope of -0.049 to 0.049, minimal increase indicates a slope of 0.05 to 0.49, a moderate increase represents a slope of 0.5 to 0.99, and a marked increase indicates a slope of 1.0 to 2.0.

Figure V.3. Canine *Giardia* positivity by county and state, 2012-2022.




Hood
slope: 0.04 p-value: 0.68
Hunt
slope: -0.08 p-value: 0.62
Johnson
slope: -1.56 p-value: 0.01
Kaufman
slope: 0.21 p-value: 0.05
Kendall
slope: 0.42 p-value: <0.01
Lubbock
slope: 0.93 p-value: <0.01
Midland
slope: 0.10 p-value: 0.59
Montgomery
slope: -0.10 p-value: 0.01
Navarro
slope: -0.55 p-value: 0.18
Nueces
slope: 0.27 p-value: 0.09
Parker
slope: -1.12 p-value: <0.01
Tarrant
slope: 0.08 p-value: 0.09
State of Texas
slope: 0.04 p-value: 0.07
Travis
slope: 0.06 p-value: 0.06
Victoria
slope: 0.09 p-value: 0.50
Walker
slope: -0.14 p-value: 0.17
Williamson
slope: 0.22 p-value: <0.01
Wood
slope: -0.01 p-value: 0.82

SDOH Analysis

A total of 98 counties with available data from 2020-2022 were included in an analysis comparing *Giardia* test positivity with potential SDOAH factors. An unadjusted analysis revealed VCAS and college graduation rate as potential factors associated with a higher proportion of canine *Giardia* test positivity and unemployment as a potential factor associated with lower positivity. Of all factors significant at $p<0.20$, MUA, population, unemployment, median household income, percentage of total state median household income, education status, and VCAS were included in a full model building strategy (Table V.2).

Due to confounding, MUA, population median household income, and percentage of total state median household income were dropped from the model. Because of interaction, education status and VCAS were combined into an interaction term. SDOAH that were associated with an increase in positivity include low VCAS (0-24) in the face of low college graduation (<31.5%) (PR 10.9; Table V.3). The marginal effects plot demonstrates this interaction between college graduate percentage and veterinary coverage graphically showing that at a low VCAS (0-24), counties with high college graduates have a lower positivity, near 1%, compared to those with low college graduates, near 11% (Figure V.4). The SDOAH that was associated with a decrease in positivity was unemployment (PR 0.67).

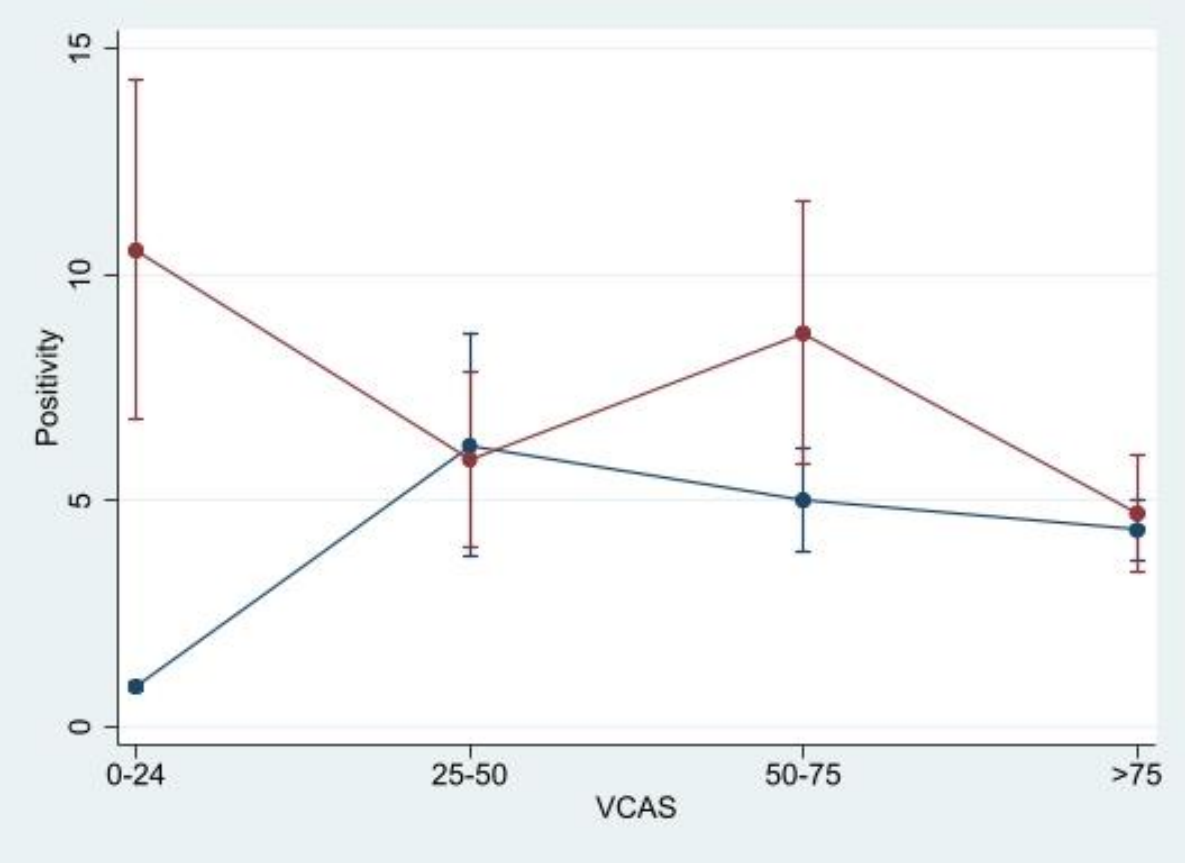
Table V.3. Univariable Association between canine *Giardia* positivity and potential SDOAH, 2020-2022.

Variable	N	PR [95% CI]	p-value
Poverty			
≥state average	51	1.05 [0.74, 1.48]	0.799
<state average	47	(Ref)	(Ref)
MUA			
present	62	0.80 [0.57, 1.13]*	0.204
absent	36	(Ref)	(Ref)
RUC			
metropolitan	44	(Ref)	(Ref)
large city	13	1.24 [0.75, 2.05]	0.392
small city	33	1.19 [0.84, 1.69]	0.324
rural	8	1.45 [0.52, 4.09]	0.474
Population	98	0.99 [0.99, 1]*	0.074
Unemployment			
≥state average	29	0.70 [0.49, 1.01]*	0.058
<state average	69	(Ref)	(Ref)
Median Household Income			
<state average	70	1.35 [0.98, 1.86]*	0.062
≥state average	28	(Ref)	(Ref)
Percentage of State Median Income			
>99%	28	(Ref)	(Ref)
80-99%	50	1.46 [1.03, 2.07]*	0.033
<80%	20	1.09 [0.71, 1.67]*	0.689
College graduates			
<state average	19	1.61 [1.24, 2.09]*	<0.001
≥state average	79	(Ref)	(Ref)
VCAS			
0-24	23	2.06 [1.38, 3.06]*	<0.001
25-50	30	1.23 [0.87, 1.73]*	0.239
51-75	27	1.76 [1.26, 2.46]*	0.001
>75	18	(Ref)	(Ref)
*included in full model-building strategy.			

Table V.4. Social determinants (SDOAH) for canine *Giardia* test positivity, 2020-2022.

Variable	N	PR [95% CI] (adjusted)	p-value
Unemployment			
≥state average	29	0.67 [0.47, 0.96]	0.027
<state average	69	(Ref)	
College graduates			
<state average	69	1.09 [0.80, 1.47]	0.593
≥state average	29	(Ref)	
VCAS			
0-24	23	0.20 [0.17, 0.24]	<0.001
25-50	30	1.43 [0.95, 2.15]	0.083
51-75	27	1.15 [0.87, 1.51]	0.309
>75	18	(Ref)	
VCAS*College graduates			
0-24 * <state average	22	10.91 [6.75, 17.65]	<0.001
25-50 * <state average	27	0.88 [0.49, 1.57]	0.654
51-75 * <state average	21	1.60 [0.96, 2.66]	0.069
>75 * <state average	9	(Ref)	

Figure V.4 Marginal effects on canine *Giardia* positivity of having a college graduation rate (%) higher than (blue line) or lower than (red line) that of Texas (31.5%) at different levels of veterinary coverage and accessibility (VCAS)



Discussion

Tracking trends in *Giardia* infection and factors that may influence giardiasis at a local level can provide valuable information to public health professionals and veterinarians about its epidemiology, highlight areas of public health concern, and help inform measures. In this secondary analysis of publicly available data, we use canine *Giardia* test positivity, as a surrogate to the incidence of canine giardiasis, to highlight areas in Texas with high rates of canine *Giardia* test positivity and identify SDOAH associated with *Giardia* infection.

In general, counties in the Dallas-Ft. Worth and San Antonio areas exhibited a high cumulative positivity compared to the state while counties close to the Austin and Houston areas did not. Furthermore, counties that demonstrated a marked increase in canine *Giardia* infection were in or near major metropolitan areas of Texas. Further prospective investigation of factors and policies that could lead certain metropolitan areas, or geographical regions, in the state to possess higher proportions of canine *Giardia* positive tests, while others possess lower, is warranted. One explanation for metropolitan risk of *Giardia* infections may be a close shared physical space if metropolitan dogs living indoors and use of outdoor communal play areas to defecate, a hypothesis supported by a [2020] study that found *Giardia* present in 74% of U.S. dog parks [74]. Another important public health consideration in these shared settings is the potential for zoonotic transmission, particularly with dog owners cleaning up pet waste, and especially if methods of hand sanitization are not readily available.

Seeing an increase in this zoonotic pathogen in dogs in areas with high-risk human populations presents another potential public health concern. In this analysis, Cameron and El Paso counties had high rates of canine *Giardia* infection. These are located at the Texas-Mexico border and are the sites of border crossing and immigration detention areas. Since immigrant

populations in the United States are already at risk for health inequities, they may be particularly vulnerable to high incidence zoonoses [173,174]. Increases in canine *Giardia* positivity were marked in Cameron and Lubbock counties, while El Paso and Parker counties have demonstrated a decrease over the past decade. It would be valuable to know about specific changes or interventions in these latter counties, which could shed light on potential ways that other counties could protect their canine populations against giardiasis.

Adopting a One Health perspective for epidemiologic research, we demonstrate that human SDOH may be useful metrics to predict risks or reveal factors underlying the epidemiology of disease, not only humans, as intended, but also in domestic animal populations [73]. We identified SDOAH associated with a high incidence of *Giardia* infection that included education level and access to veterinary healthcare. There are several hypotheses about how these factors may influence *Giardia* positivity rates. Poor access to veterinary care may decrease the likelihood that pets receive routine care and client education that could prevent *Giardia* exposures and infections. While that may be true, our source of data on test positivity is from voluntarily submitted test samples, which likely do not accurately reflect *Giardia* among populations who cannot or who are not accessing veterinary care. Despite this limitation, in this study, we established that the VCAS metric was indicative of canine *Giardia* positivity. Interestingly, the MUA metric, the human counterpart to the VCAS, was not. This may have to do with the different methods by which these designations are determined [164]. There is currently no human medical coverage factor equivalent to the VCAS that has been adopted, something that could be explored as a potential health metric in the future.

In this study we showed a link between percentage of college graduates and canine *Giardia* test positivity. There are many underlying factors related to attending and graduating

college that hold potential to be inversely related to parasitism. Counties with higher percentages of college graduates are more likely to host larger cities with desirable work conditions [175]. Recent work has termed the phenomenon of college graduates' movement from rural communities to city centers as “brain drain” [175]. The economic impact of a county having a sizable percentage of college graduates is substantial. Research into this by the Georgetown University Center on Education and the Workforce cited that individuals who obtain a bachelor’s degree earn $2.8 million over their career while those working with a high school diploma earn $1.6 million [176]. That increase in income helps the county’s economy. Given this information, the percentage of college graduates as a SDOH or SDOAH could be an indirect assessment for both the county’s rural-suburban-urban status and its economic state.

We showed that in Texas counties, college graduation rates are linked with VCAS. In counties with a low veterinary coverage, those which also had a low percentage of college graduates had 11 times the *Giardia* as similar counties high percentage of college graduates. In this setting with poor access to veterinary care, one might hypothesize that less disposable income or lack of health education could play a role in *Giardia* endemicity. A closer look into this mechanism and how to alleviate the extra burden of giardiasis is warranted.

Contrary to the above, we found high unemployment to be a protective factor against *Giardia* positivity. This is counterintuitive as one would assume populations that are living on a fixed income would not be able to submit testing for this organism. While this is a reasonable assumption, in our dataset counties with higher unemployment submitted more overall tests (median 1866 [IQR 109, 7122]) compared to those with normal to low unemployment (median 734 [IQR 92, 7770]). Further investigation is needed into the underlying factors of this

relationship to explain how counties with potentially economically suppressed populations, due to unemployment, are submitting higher numbers of tests.

The main limitation of this study is that *Giardia* is not a reportable condition for animals nor humans, meaning that data are not fully accurate. We noted that neither CAPC nor TVMDL data completely covered all counties in Texas for the entire time assessed. The voluntary nature of testing and reporting may vary across veterinarians and practices. This may have preferentially sampled areas with better access to veterinarians and with clients who can afford submission of external testing. Furthermore, specimens submitted for testing may arise largely from suspected cases of disease, reflecting symptomatic infections. The labs providing this data are not veterinarians' only option for testing *Giardia* in dogs. The clinicians may also choose to use other veterinary labs or choose in-house diagnostics using their own equipment. The CAPC estimates that their data represents approximately 30% of the samples tested each year [53]. Other limitations include the lack in genotyping of the data available. Due to the lack of known assemblage present, we cannot fully assess zoonotic risk or potential correlation to human giardiasis rates. Despite our attempt to use the most complete and timely data available, the nature of available data means that we are not able to assess *Giardia* in populations who are not accessing testing or who test at non-reporting facilities, it is unlikely that our data accurately reflect the occurrence of asymptomatic or subclinical cases, and we are unable to speak to the zoonotic potential of *Giardia* infecting canines in Texas,

Conclusion

In our analysis we isolated potential areas of concern in Texas for high canine *Giardia* positivity, central Texas around the Dallas-Fort Worth and San Antonio metropolitan areas and south and west Texas along the border. All these areas occur in medically underserved (human) areas. Furthermore, this study found three SDOAH, VCAS, percentage of college graduates, and unemployment, that may be indicators of county level canine *Giardia* infection in Texas. We found that counties with low veterinary coverage (< VCAS 25) corresponded to higher *Giardia* infection (PR 11 [7, 18]) when college graduation rates were low (< Texas state average), compared to counties with average or greater graduation rates.

We have shown the current need to conduct active surveillance research to assess overall canine and human *Giardia* prevalence. This study identified some geographic areas of concern for canine *Giardia* infection but was limited in the analysis due to lack of data for all counties in Texas. For these identified areas, more research should be conducted and outreach to veterinary medical providers to encourage client education in efforts to limit the spread of *Giardia* in dogs. While this study supplies translational information for public health researchers to use during similar human studies, *Giardia* infection is currently not monitored in humans in Texas so accurate analysis of human rates of disease is needed. Future research determining how the relationships uncovered in this canine study compare to the relationships between SDOH and *Giardia* positivity in humans at the county level will be vital in achieving a translational model of this disease and establishing the role dogs play as sentinels for this disease.

This study lays a foundation for future studies evaluating the potential sentinel role that dogs play for human giardiasis in non-reporting states, such as Texas, the use of SDOAH as an

indicator of increases in *Giardia* positivity in dogs in other geographical areas, and the use of SDOH to predict an increase in *Giardia* positivity in humans.

CHAPTER VI CONCLUSIONS

The relationship between canine *Giardia* and *Cryptosporidium* infection, clinical disease, zoonotic potential, and risk factors for infection and illness is a complex one which we still do not fully understand. In this plan of research, we were able to assess a human rapid membrane enzyme coproantigen point-of-care test on canine feces, identify *Giardia duodenalis* assemblages and *Cryptosporidium canis* present in kennel housed laboratory dogs, determine what relationship was present between fecal score and canine subclinical *Giardia* and *Cryptosporidium* infection, identify individual demographic factors associated with canine subclinical *Giardia* and *Cryptosporidium* infection, identify counties in Texas experiencing high cumulative or increasing canine *Giardia* infection, and define the relationship between social determinants of animal health and county level canine *Giardia* positivity in the state.

In the evaluation of the human point-of-care coproantigen assay outlined in Chapter 2 we assessed the sample apparent prevalence of *Giardia* and *Cryptosporidium*, and evaluated sensitivity, specificity, positive predictive value, negative predictive value, positive likelihood ratio, and negative likelihood ratio for the QuikChek coproantigen membrane enzyme assay, using the MeriFluor direct immunofluorescent assay and inhouse PCR (with SSU rRNA primers) for both *Giardia* and *Cryptosporidium* in the dog. We completed this analysis using a gold standard (GS) reference of the parallel interpretation of the DFA and PCR tests and standard formulas with the 2 x 2 table as well as a Bayesian evaluation of diagnostic tests for which no gold standard (NGS) exists using established priors for QC, DFA, and PCR. In this study, screening kenneled laboratory dogs that were apparently healthy for *Giardia*, the QC test performed with a low sensitivity (GS: 38%, NGS: 48%) and reasonable specificity(GS: 95%,

NGS: 98%) We also established that in the two populations sampled, the apparent prevalence of *Giardia* was 39% and 49%, values much higher than the currently reported 5% *Giardia* positivity in the State of Texas [53]. For *Cryptosporidium* we were able to determine the sensitivity of the QC test was low (GS: 25%, NGS: 41%) and the specificity was reasonable (GS: 96%, NGS: 97%) a. The apparent prevalence of *Cryptosporidium* infection in our two populations was 1% and 9.5%, values congruent with the reported prevalence of *Cryptosporidium* in North America of 5% [41].

We showed that *Giardia* was more prevalent than *Cryptosporidium* for both populations and that *Cryptosporidium* was more prevalent in our laboratory dog supplier population. For both *Giardia* and *Cryptosporidium,* we demonstrated that testing with a single test had low sensitivity. Based on this finding we recommend testing with at least two tests to improve sensitivity of testing for these organisms when screening subclinical dogs. In this study we showed subclinical shedding of organisms, highlighting the risk of potential subclinical spread of disease, and validated the use of the QC point-of-care *Giardia* and *Cryptosporidium* tests in dogs.

For the molecular analysis conducted in Chapter 3 we were able to use multilocus PCR genotyping to identify *Giardia duodenalis* assemblages present in dogs as well as SSU RNA PCR to identify *Cryptosporidium* species. In the samples we genotyped, we found *Giardia* assemblage A in 4 of 9 samples and assemblage D in the other 5. This analysis of Giardia genotypes showed a higher proportion of assemblage A than has been previously reported, which is the assemblage associated with zoonosis. This underscores the importance of future molecular epidemiology research into this organism in diverse geographic locations and populations of humans and animals [124]. We also showed genetic similarities between *Giardia microti* and *Giardia duodenalis* at the BG loci suggesting that further research needs to be conducted

investigating the genetic relationship between these two organisms. *Cryptosporidium canis* was the only species of *Cryptosporidium* present in our canine samples. Further work to characterize the subspecies needs to be conducted as current primers for sub-species genotyping do not amplify genes in *C. canis* [28]. This study shows the need for future prospective molecular epidemiology research, focusing on high-risk populations and in areas that have not been previously investigated.

With the epidemiologic analysis of the laboratory dog population in Chapter 4, we were able to determine the potential relationship between fecal score and test positivity as well as analyze the relationship between age, breed, and sex and test positivity for both *Giardia* and *Cryptosporidium*. In this study we found a protective relationship in dogs exhibiting hard feces, they were at 0.37 times the odds of being *Giardia* test positive compared to dogs exhibiting normal feces. For kennel housed laboratory dogs, we were not able to show a statistically significant relationship between fecal score and a positive *Cryptosporidium* test result. *Giardia* test positivity was not significantly related to sex or breed but was to age. Dogs younger than 19 months had 3.4 times the odds of being infected with *Giardia* compared to older dogs. While *Cryptosporidium* positive dogs did not show a significant relationship with age, in this study we showed more dogs younger than 19 months were infected with *Cryptosporidium* than older dogs. We also showed the median age of *Cryptosporidium* infected dogs was 7 months while non-infected was 29 months. With this study we demonstrate the relationship between fecal consistency and age with *Giardia* test positivity. Further studies into the potential relationship between gastrointestinal microbiome, immune status, and test positivity could help determine the mechanism of action driving these relationships.

The Texas county level analysis of canine *Giardia* test positivity in Chapter 5 provided us the opportunity to show which counties, of those with available data, were experiencing an increase in *Giardia* positivity over the past decade as well as which counties had a significantly higher decade cumulative *Giardia* positivity than the state. This study also established the relationship between county level *Giardia* positivity and the potential non-medical factors (syn. SDOAH) of poverty, education, median household income, unemployment, population, rural status, veterinary medical coverage, and human medically underserved areas. With this analysis we found 15 counties that differed in slope either increasing or decreasing in yearly trend from the state. We found 4 counties of concern with a statistically significant increase in cumulative positivity compared to the state. Of these four counties, two are in areas with high-risk populations, at common border crossing sites. One of these counties experienced a high cumulative positivity of disease in dogs and an increase in yearly positivity over the past decade. Further establishment of the potential sentinel role these dogs are playing in regard to human disease is needed. Of the SDOAH analyzed, unemployment, college graduation, and veterinary coverage availability were related to a county's *Giardia* test positivity. Here we found that unemployment was protective against *Giardia* positivity and the interaction between veterinary coverage and college education was associated with increased *Giardia* positivity. We found that counties with low veterinary coverage corresponded to higher *Giardia* positivity when college graduation rates were lower than the Texas average.

The limitations of these studies include sample size and use of diagnostic laboratory sourced secondary data. Despite these limitations, we were able to demonstrate significant epidemiologic relationships and show important, potentially zoonotic, genotypes of organisms present. This highlights the importance of using all available data for analysis in future studies,

recognizing that while this is a limitation, important results can be generated from meager datasets.

Additional research into alternative PCR primer targets and whole genome sequencing is needed. With this we can further investigate the relationship and potential effects of *Giardia* and *Cryptosporidium*, as well as other parasites and infections, on both the gastrointestinal microbiome and presence of antimicrobial resistant genes. This would highlight the role these factors play in long-term illness.

In this study we were able to evaluate the use of an available human *Giardia* and *Cryptosporidium* point-of-care test in dogs, identify zoonotic assemblages of *Giardia* in dogs, identify a relationship between fecal score and *Giardia* subclinical infection, describe the inverse relationship between age and subclinical infection with *Giardia*, spotlight counties with both high cumulative *Giardia* positivity and increasing yearly proportions, and determine that there is a higher canine *Giardia* positivity in counties with low veterinary coverage that also experience low college graduation rates. This plan of study adds to the growing bank of knowledge on these two organisms and lays the groundwork for future One Health, multi-species, prospective molecular epidemiology studies.

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