AN EPIDEMIOLOGIC INVESTIGATION OF *SALMONELLA* AND *CAMPYLOBACTER* SHEDDING AMONG SHELTER DOGS IN TEXAS

A Dissertation

by

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ABSTRACT

Salmonella and *Campylobacter* are bacterial pathogens frequently affecting both humans and animals. Even so, there is limited information concerning the epidemiology of these pathogens among canines. Dogs are capable of shedding the bacteria in their feces, often without overt signs of infection. As a result of their close contact with people, dogs could thus play a significant role in disease transmission, exposing their human companions to the bacterial pathogens. In order to fully assess this risk and implement efficacious means of disease control and prevention, however, it is essential to have a more comprehensive understanding of pathogen distribution and determinants as they relate to canine populations. To that end, the present research investigated the epidemiology of *Salmonella* and *Campylobacter* fecal shedding among shelter dogs across Texas using both culture-based and molecular methods. Culture detected *Salmonella* in 27 (5%) of the 554 samples. *Salmonella* isolates were of many different serotypes, five of which have been among top 10 serotypes isolated from human patients with laboratory-confirmed salmonellosis in the United States. Antimicrobial resistance among the isolates was minimal. Data analysis suggested that fecal consistency may be an indicator of the fecal presence of *Salmonella*, and that the effects of dog neuter status and dog origin deserve further attention.

For *Campylobacter*, qPCR suggested a bacterial prevalence of 76% (140 of the 185 samples). Nonetheless, prevalence of two main human pathogens—*Campylobacter coli* and *Campylobacter jejuni*—was undetected or low, at 0% and 5.4%, respectively.

Statistical assessment of putative shedding indicators demonstrated a possible relationship between the presence of fecal *Campylobacter*, dog sex, and dog duration of stay in shelter.

Campylobacter detection was heavily dependent on the detection method(s) employed. The qPCR method used in the present research proved to be much faster and more sensitive relative to various culture-based techniques. As such, qPCR may be most useful as an adjunct to bacterial culture, perhaps serving to guide selection of an optimal approach to culture. Integral to any approach, however, must be the recognition that canines are clear participants in *Salmonella* and *Campylobacter* epidemiology, and their contributions hold substantial import for their close companions.

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NOMENCLATURE

AFLP	Amplified Fragment-Length Polymorphism
AMP	Ampicillin
AMR	Antimicrobial Resistance
ASPCA	American Society for the Prevention of Cruelty to Animals
AT	Additional Targets
AUG	Amoxicillin/Clavulanic Acid
AVMA	American Veterinary Medical Association
AXO	Ceftriaxone
AZI	Azithromycin
CAT	Cefoperazone, Amphotericin B, Teicoplanin Selective Supplement
CDC	Centers for Disease Control and Prevention
CEF	Campy Cefex Agar
CHL	Chloramphenicol
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
FDA	Food and Drug Administration
FIS	Sulfisoxazole
FOX	Cefoxitin
GEN	Gentamicin

mCCDA	Modified Charcoal-Cefoperazone Deoxycholate Agar
MIC	Minimum Inhibitory Concentration
NAL	Nalidixic Acid
NARMS	National Antimicrobial Resistance Monitoring System
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field Gel Electrophoresis
qPCR	Quantitative Polymerase Chain Reaction
STR	Streptomycin
SXT	Trimethoprim/Sulfamethoxazole
TET	Tetracycline
WHO	World Health Organization
XNL	Ceftiofur

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The dissertation contents are solely the responsibility of the author and the dissertation committee and do not necessarily represent the official views of Texas A&M University, the Bernice Barbour Foundation, Inc., or the U.S. Food and Drug Administration's Veterinary Laboratory Investigation and Response Network.

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1. INTRODUCTION

1.1 Overview of Zoonotic Diseases

Accounting for an estimated 1–26% of all disability-adjusted life years lost due to infectious diseases (Grace et al., 2012), zoonoses are among the most pressing of public health concerns. They are also among the most difficult concerns to adequately address. There are over 200 recognized zoonoses which are, collectively, attributable to over 868 unique pathologic agents (Taylor et al., 2001) each with their own vectors, reservoirs, and ecological niches. For any given zoonosis, understanding such complexity requires focused, systematic study of its individual elements, alone and in combination, in the laboratory and in the field. On account of its role in disease prevention, one of the most fundamental elements of a zoonosis is its mode of transmission.

1.1.1 Zoonotic Disease Transmission

Zoonotic disease transmission takes a number of different forms. In aerosol transmission, the disease agent passes from its site of residence to the human respiratory tract by means of airborne particles (Fernstorm and Goldblatt, 2013). Lymphocytic choriomeningitis (LCM) provides an interesting example. Human cases of the disease have been associated with inhalation of aerosolized rodent excrement, particularly in

occupational (laboratory personnel, vendors) or domestic (house mouse-infestation) contexts (Emonet et al., 2007).

Aerosolized particles are not the only means of pathogen transmission through air. Many vector-borne zoonoses result from the bites of flying insects. Bites from infected mosquitoes, tsetse flies, and sandflies can lead to malaria, sleeping sickness, or vesicular stomatitis, respectively. Other disease vectors, like fleas and ticks, take less aerial approaches, moving opportunistically (directly or indirectly) from one organism to a (usually proximal) other.

Proximity and a lack of a third party vector define transmission via direct contact. Ebola is a particularly prominent illustration of this mode of transmission. Spread of the hemorrhagic fever often requires close contact between an infected individual (or that individual's bodily fluids) and a susceptible individual (Bausch et al 2007). Health care workers are especially vulnerable to infection when performing procedures ranging from routine physical exams to intubations and laparotomies (Muyembe-Tamfum et al., 1999; Osterholm et al., 2015).

Devices integral to a variety of medical procedures can mediate disease transmission. This manner of disease dissemination, termed fomite transmission, occurs when pathogen-contaminated, non-living objects—medical devices, doorknobs, faucets— introduce the pathogen to a new host (Boone and Gerba., 2007). A contaminated duodenoscope was the fomite implicated in a fatal outbreak of carbapenem-resistant *Enterobacteriaceae* in California between 2014 and 2015 (CDC, 2015a). Notably, fomite transmission primarily refers to pathogen exposure and

infection resulting from direct insertion of contaminated, non-living objects into an area normally protected from external elements (e.g. an artery, vein, or organ). Voluntary consumption of a contaminated inanimate product or beverage, however, falls under a different classification.

Oral or foodborne disease transmission generally encompasses diseases resulting from the consumption or imbibition of contaminated products. Colloquially known as "food poisoning," this type of transmission hospitalizes approximately 128,000 Americans each year (Scallan et al., 2011). Apart from its ubiquity and considerable social burden, however, foodborne transmission is especially remarkable for the ways in which it relates to and reflects the complexities of zoonotic diseases.

1.1.2 Foodborne Zoonoses

As with zoonotic diseases in general, foodborne zoonoses are attributable to a wide variety of pathologic agents, social, and environmental conditions. Potential pathogens include prions (PrP^c), viruses (norovirus, hepatitis viruses A and E), toxins (botulinum toxin), and bacteria (*Listeria*, *Salmonella*, *Campylobacter*). These agents can be present in food-producing animals, and can thus gain access to the food chain at all stages along the pathway from production ("farm") to consumption ("fork"). Consequently, when it comes to foodborne zoonoses, the dynamics between pathogen and zoonotic transmission are intricate and multifaceted. Furthermore, although consumption or imbibition are the ultimate mode of disease transmission, the pathway

from animal to human frequently involves food contamination through other modes of zoonotic transmission. Airborne zoonotic pathogens can contaminate beef briskets during slaughterhouse preparations, and fomite transmission can occur via knives that contact both animal hide and meat product (Burfoot et al., 2006). Likewise, houseflies ("filth adulterants" according to the U.S. Food and Drug Administration) can transfer bacteria from animal sources to foods for human consumption, as evident from an *Escherichia coli* O157:H7 case from a Japanese nursery school in 1996 (De Jesus et al., 2004; Moriya et al., 1999). In the end, however, no matter how food contamination occurs, by definition, foodborne zoonoses are attributable to oral ingestion of a pathogen of animal origin.

For foodborne zoonoses, investigations into pathogen source illustrate three principal categories of animal-related food contamination: industrial, institutional, and domestic. Industrial contamination occurs at a farm and factory with national or multi-state distribution channels, when a vast quantity of animal foodstuffs experience pathogen exposure during animal husbandry, food preparation, production, and packaging. Industrial contamination often results in widespread disease outbreaks. *Salmonella*-contaminated, Foster Farms brand chicken infected at least 634 people from 29 states and Puerto Rico from 2013-2014 (CDC, 2014). By contrast, institutional and domestic contamination usually affects a smaller number of individuals. Most instances of institutional contamination involve food contamination at an individual or regional food supplier which distributes to select institutions (restaurants, hospitals, clinics, etc.). In 2005, consumption of raw milk from a cow-sharing program in two northwestern U.S.

states was a factor in 18 cases of *Escherichia coli* O157:H7 infection (LeJeune and Rajala-Schultz, 2009). Alternatively, contamination of this category could occur at the food-serving institution itself, through faulty food preparation practices or frequent animal-human interactions. Similar situations can also arise on a more domestic level. Within any given household, lapse of adequate personal hygiene (failure to satisfactorily hand wash) subsequent to direct animal contact or contact with pet food could result in pathogen exposure and illness. Improper food storage, failure to cook foods thoroughly, and cross-contamination (raw meats coming into contact with produce eaten uncooked) are other reasons for domestic risk. Unfortunately, research into the circumstances surrounding pet-related foodborne zoonoses remains underdeveloped.

1.1.3 Companion Animals as Reservoirs for Foodborne Zoonotic Pathogens

To date, most research relating to foodborne zoonoses centers on food-producing animals such as cattle and poultry. When it comes to pets, or companion animals, however, the research is less comprehensive. Reptiles such as turtles and lizards have received considerable attention, particularly with regard to their carriage and transmission of *Salmonella*, but more common pets such as cats and dogs remain relatively overlooked. This oversight is particularly concerning since the existing literature on the subject demonstrates pathogen carriage among and zoonotic transmission from companion animals. More than 20 common zoonotic pathogens, for example, can be found among dogs or cats (Elchos et al., 2008; Ford, 2008), including

Pasteurella, *Leptospira*, *Staphylococcus*, *Salmonella*, and *Campylobacter* (Damborg et al., 2015). Recognizing the public health risks and research gaps relating to companion animal zoonoses, in 2011, the Food and Drug Administration's (FDA) Center for Veterinary Medicine (CVM) launched a funding initiative to address the pet food aspect of this issue (FDA, Center for Veterinary Medicine 2011). Focusing primarily on the population of pet cats and dogs arriving at clinics, however, the FDA data will, like previous studies, overlook a critical population: shelter dogs.

Roaming the interface between the wild and the domestic, shelter dogs are prime candidates for pathogen transmission. Broadly defined, a shelter dog is any canine that spends time in facility (e.g. a shelter) dedicated to the care, refuge, rehabilitation, and/or placement of found, surrendered, homeless or abandoned animals (ASPCA, 2017). Socially and medically neglected, shelter dogs may receive exposure to pathogens from a variety of sources (wild flora and fauna, livestock, pets, excrement, waste products, etc.), pathogens which they could then carry from one milieu to another. However, while there are reports of shelter dogs harboring pathogens such as *Salmonella* and *Campylobacter* (Chang et al., 2011, Kocabiyik et al., 2006, Tsai et al., 2007) and some even tracing cases of human infection to canine contact (Morse et al., 1976, Varga et al., 2012), very few studies actually describe pathogen epidemiology or molecular characteristics among this canine population.

1.2 Salmonella *Shedding Among Shelter Dogs*

To date, there are only a handful of published studies describing the relationship between *Salmonella* and stray or shelter dogs. Varying considerably in regard to year of publication, study duration, and sample location, the studies provide a broad overview of the subject. Even so, the considerable variations leave many questions unanswered.

The earliest study here reviewed dates back to 1976. Shimi et al (1976) took rectal swabs from 19 stray dogs in Tehran, Iran, detecting *Salmonella* in 15.8% of the samples. Many years later and many miles away, other researchers obtained strikingly similar results. Taking rectal swabs from over 1000 dogs in 5 animal shelters in Taiwan, Chang et al (2011) found *Salmonella* in 15.98% of samples. Yet, while well within the documented prevalence range of 1–36% for the general canine population (Sanchez et al., 2002), the Chang et al (2011) prevalence of ~16% is high relative to other, comparable studies at other sites.

In the years between the Shimi et al (1976) and Chang et al (2011) studies, other researchers have reported much lower *Salmonella* prevalence among stray or shelter dog populations. Examining fresh feces or rectal swabs from 82 dogs in 6 dog shelters in the Bursa province of Turkey, Kocabiyik et al (2006) obtained a *Salmonella* prevalence of 11%. In dogs from shelters in Taiwan and Florida, prevalence was around 6% (Tsai et al., 2007; Tupler et al., 2012). And, at least two other studies (in Trinidad and California) did not detect *Salmonella* in any samples from shelter dogs (Seepersadsingh et al., 2004; Sokolow et al., 2005). Thus, using only the data from such studies, prevalence of

Salmonella in stray or shelter animals appears to range from 0–16%. Influencing this prevalence range, however, are multiple factors worthy of consideration.

Nonetheless, while important for its use in tracking spatial and temporal fluctuations, prevalence has its limitations when it comes to cross-study comparisons. For one, prevalence varies according to differences in geography, environment, sample size and nature, method, and technique (Kocabiyik et al., 2006, Seepersadsingh et al., 2004). As a result, prevalence data from any given study is limited in its generalizability. For another, the overall prevalence of a given bacterial pathogen genus provides insufficient information about the pathogen's level of risk. All *Salmonella* bacteria are not equal when it comes to threatening human health or animal health (Hoelzer et al., 2011). Consequently, having detailed information, especially information about pathogen phenotypic and molecular characteristics, is an essential complement to knowledge about general pathogen prevalence. Fortunately, some information about the specific nature of stray or shelter dog *Salmonella* is available.

Much of the research on stray or shelter dog *Salmonella* at the genetic and molecular level focuses on pathogen serotype (serotype) and antimicrobial resistance (AMR). On these topics, cross-study comparison shows two, very general, consistencies. First, as with the canine population in general (Sanchez et al., 2002), stray and shelter dogs can harbor multiple varieties of *Salmonella* (including *S.* Corvallis, *S*. Dusseldorf, *S*. Enteritidis, *S*. Heidelberg, and *S*. Newport), many of which pose a risk to both human and animal health. Second, resistance to one or more clinically-used antimicrobial agents

is common among *Salmonella* isolates. Upon close analysis, however, nuances appear in the two prevailing similarities.

When it comes to specifics about serotype frequency and antimicrobial resistance profiles, there is notable, cross-study variation. Chang et al (2011) and Steneroden et al (2011) found *S*. Newport to be among the most common serotypes, while for Seepersadsingh et al (2006) and Tsai et al (2007), *S*. Heidelberg and *S*. Dusseldorf, respectively, were more frequent. Prevalence of antimicrobial resistance was equally varied. Resistance to tetracycline was most common in Chang et al's isolates (2011), while resistance to streptomycin, sulfonamide, and azithromycin was most common in the respective isolates from Kocabiyik et al (2006) and Seepersadsingh et al (2004), Steneroden et al (2011), and Tsai et al (2007). Considering the inter-study differences in sample origin, collection, and testing methodology, such variations are unsurprising. They are not, however, insignificant; they limit the ability to make evidence-based deductions about *Salmonella* in specific contexts.

1.3 Campylobacter *Shedding Among Shelter Dogs*

As is the case for *Salmonella*, there is considerable variation in the literature pertaining to *Campylobacter* shedding among shelter dogs. Although the canine capacity for fecal shedding of the pathogen is well-established, the prevalence and circumstances surrounding shedding are less certain. One study examining cases of human campylobacteriosis in Denmark detected *Campylobacter* in only 16 percent of

fecal samples from patients' pets (Damborg et al., 2004). An earlier study of healthy puppies presenting to Danish veterinary clinics found evidence of the bacteria at a slightly higher prevalence of 21 percent (Hald and Madsen, 1997). Using a similar study design with a larger sample size, researchers in Switzerland reported a canine *Campylobacter* prevalence of nearly 42 percent—two to three times greater than that of the Danish studies (Wieland et al., 2005). Several other studies, however, make even the prevalence of the Swiss study seem small. Between 76 and 77 percent of canine fecal samples were positive for *Campylobacter* in research from 2004 (Hald et al., 2004; Koene et al., 2004). Such study-to-study variation in *Campylobacter* detection is evident in the prevalence ranges put forth in relevant publications, with one report noting a range of 10–29.6 percent (Hald and Madsen, 1997), another of 1.6–34 percent (Wolfs et al., 2001), and another of 21–75 percent (Hald et al., 2004). While the overlap in ranges is of great consequence for human and animal health, the substantial width of the ranges reflects a high degree of study-to-study variation that is important to consider.

Numerous factors may account for the wide range of *Campylobacter* prevalence. Most notable among such factors are study contexts, animal characteristics, and detection methods. An obvious potential contribution to variance in *Campylobacter* detection concerns the general contexts of the detection studies; studies vary both spatially and temporally. Research reports span the globe, from Denmark and Switzerland to Canada, California, and Michigan. Publication dates span several decades, including but not limited to the 1980s, the 1990s, and the early 2000s. Although precise quantification is difficult, such vast distances in space and time could certainly

account for differences in detection. More readily quantifiable differences in prevalence estimates relate to animal-level variables. In terms of animal characteristics, male dogs seem to have higher *Campylobacter* carriage rates than female ones (Hald et al., 2004), urban-dwelling dogs higher rates than rural-dwelling ones (Hald et al., 2004), and younger dogs higher rates than older ones (Hald and Madsen, 1997; Wieland et al., 2005). Some evidence also suggests that *Campylobacter* shedding may be greater for diarrheic dogs (Hald and Madsen, 1997; Wolfs et al., 2001), though results have not always been statistically significant (Hald et al., 2004) and may vary according to a dog's age (Hald and Madsen, 1997).

In addition to animal characteristics, variation in *Campylobacter* prevalence may be attributable to variation in detection methodology. *Campylobacter* is a particularly fastidious pathogen (Chaban et al., 2009), and rates of its isolation can differ according to incubation temperature, atmospheric conditions, enrichment procedures, and specimen handling (Monfort et al., 1989). Bourke et al (1998) even goes so far as to comment that "isolation and accurate identification of *Campylobacter* species from fecal specimens by using standard phenotypic testing is problematic" (442). Fortunately, most studies try to address potential isolation and identification problems by using a combination of methods, phenotypic and molecular. Koene et al (2004), for example, used filtration, directly plated fecal samples on mCCDA, CAT, and Karmali agar plates, and also tested for 16S and 23S rRNA genes using PCR-RFLP. Unfortunately, methodological combinations vary from study to study, complicating direct study-to-study comparisons and influencing study findings. Such methodological influence may be especially

pronounced when it comes to species-level *Campylobacter* analysis. Recovery of *C. upsaliensis*, for instance, may require longer incubation times and different antimicrobial combinations or concentrations than are necessary for recovery of other *Campylobacter* species (Hald et al., 2004; Bourke et al., 1998; Labarca et al., 2002). Recovery of *Campylobacter* of any species, however, demonstrates the genuine possibility for canine-facilitated human campylobacteriosis.

Although most cases of human campylobacteriosis are attributable to poultry (Allos et al., 2001), published evidence suggests a non-negligible role for canine-to-human bacterial transmission. Pets like dogs can shed *Campylobacter* for over a year, often without clinical signs of infection or disease (Dambourg et al., 2004). Unsurprisingly, epidemiologic studies have identified pet ownership or contact as a risk factor for human disease (Hald and Madsen, 1997; Hald et al., 2004). In a study of pet-owning patients with campylobacteriosis, Dambourg et al (2004) reported probable pet-human transmission in 1 of the 45 study cases. Zoonosis may also have occurred among several dog-owning families from the province of Manitoba in Canada in the 1980s (Nayar et al., 1980). More definitive proof of transmission exists in a case report on neonatal sepsis. Clinical samples of *C. jejuni* from a three-week old girl in the Netherlands had an amplified fragment-length polymorphism (AFLP) pattern identical to that from her newly adopted (and recently ill) Labrador retriever puppy (Wolfs et al., 2001). Adoption of more specific prevention strategies would help to prevent similar cases of canine-related *Campylobacter* zoonosis. Unfortunately, the spatial, temporal, and methodological variations in literature relating to canine- carriage of *Campylobacter*

preclude all but the most general of prophylaxes. Additional epidemiologic research on the subject would do much to enhance risk assessment, guide resource allocation, and pinpoint opportunities for effective intervention.

1.4 Value of Epidemiologic Research

The value of epidemiologic research into pathogen carriage among stray and shelter dogs stems from its ability to guide prevention and control measures. The cross-study variations in epidemiologic data about prevalence, serotype, and antimicrobial profiles, however, limit data application to only the most general of recommendations. In order to help make these recommendations more precise, numerous studies have tried to identify specific risk factors for shelter dog shedding of *Salmonella* and *Campylobacter*. Unfortunately, the efforts have been more successful in potential risk determinant elimination, identifying factors that seem to have little if any conclusive effect on risk. Different studies often reach different, and, at times, contradictory, conclusions about the predictive potential of factors such as dog age, sex, and fecal consistency. Since differences in study location and/or methodology may be responsible for such discrepancies, in-depth, regional epidemiologic investigation by a specially-designated research team may yield—for the region and time-frame—more reliable results.

One of the largest and most populous American states, Texas has considerable human, bovine, and stray or shelter dog populations and a considerable number of annual salmonellosis and campylobacteriosis cases. Even so, published literature on

Salmonella and *Campylobacter* carriage among the stray or shelter dog population and the possible role of these animals as pathogen reservoirs in the state is lacking. As evident from studies of other states and countries, stray and shelter dogs can carry the bacteria, but the considerable cross-study variations mean that the studies' results are not directly applicable to shelter dogs in Texas. Moreover, these studies neither examine the influence of dog environmental histories, nor do they suggest any reliable risk factors for pathogen shedding. Through examining the epidemiologic characteristics of *Salmonella* and *Campylobacter* isolates from stray and shelter dogs around Texas, the present study promises to help fill in gaps in the literature and to expand investigation to include neglected areas of research.

1.4.1 Aims of the Present Research

The overarching hypothesis of the present research is that fecal *Salmonella* and *Campylobacter* shedding occurs among shelter canines and is related to identifiable canine-level and/or environmental variables. To test this hypothesis, the project will examine this shedding, assess putative shedding indicators, and make reference to detection methodology for *Campylobacter*. In so doing, the present research provides insight into the nature, risk factors, and prevalence of bacterial shedding, while enhancing the capacity for promoting risk assessment, resource allocation, public health, and human and animal well-being.

2. THE EPIDEMIOLOGY OF FECAL *SALMONELLA* SHEDDING AMONG SHELTER DOGS IN TEXAS*

2.1 Introduction

Companion animals can serve as reservoirs for a variety of zoonotic pathogens (Halsby et al., 2014). *Salmonella enterica* subspecies *enterica*, a zoonotic agent causing an estimated 1.2 million human illnesses per year in the United States (Scallan et al., 2011), resides in the intestinal tract of many companion animals and passes into the external environment through feces (Hoelzer et al., 2011; Salehi et al., 2013). Estimates of fecal *Salmonella* shedding can vary considerably across studies, depending on the animal population, geographic location, and time of sample collection. Among canines, the prevalence of fecal *Salmonella* has ranged from 1–5% for pet dogs to 60–80% for racing or sled dogs or dogs fed raw-food diets (Hoelzer et al., 2011). Considering that there are an estimated 69.9 million pet dogs living in 36.5% of households in the United States (AVMA, 2013), and that there is evidence of likely canine-to-human *Salmonella* transmission (Sato et al., 2000; Fukata et al., 2002; Hoelzer et al.., 2011; Polpakdee et al., 2012; Varga et al., 2012), canine *Salmonella* shedding represents a potential threat to public health. Moreover, *Salmonella* shed by dogs can demonstrate resistance to one or

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more antimicrobial agents including tetracycline, streptomycin, and chloramphenicol (Chang et al., 2011; Kocabiyik et al., 2006; Tsai et al., 2007).

Salmonella is also a cause of gastrointestinal disease in adult dogs and puppies. Clinical signs of canine salmonellosis can include vomiting, diarrhea, fever, lethargy, and abdominal pain; the diarrhea can be hemorrhagic in severe cases (Marks, 2011). However, many *Salmonella* infections among dogs remain subclinical.

Nevertheless, limited information is available concerning the epidemiology of *Salmonella* among canines in general and shelter dogs in particular. Exposures to other animals, high-stress environments, and the welfare challenges of shelters (scarcity of funding, presence of volunteers with limited disease-control training) (Turner et al., 2012) could serve to increase the susceptibility of shelter dogs to infection with zoonotic pathogens including *Salmonella*. Shelter dogs harboring such pathogens would be capable of wide pathogen dissemination due to their contact with other shelter animals, shelter personnel, and perspective adoptive families. Preventing pathogen transmission, however, requires understanding of pathogen epidemiology among reservoirs. *Salmonella* typing data can facilitate source tracking, risk assessment, and communication through its relationship to *Salmonella* host range and pathogenicity (Wain and Olsen, 2013), while trend and risk factor data allow for more effective disease control and prevention. Consequently, along with *Salmonella* prevalence estimates and isolate characterization data, elucidation of risk factors for canine *Salmonella* shedding would enhance public health. Although some studies have examined one or more of

these epidemiologic aspects for a few shelters in regions of the United States, Iran, Taiwan, Trinidad, and several other countries (Cantor et al., 1997; Morley et al., 2006; Shimi et al., 1976; Seepersadsingh et al., 2004; Tsai et al., 2007), no published study to date has looked at the canine population in shelters in Texas. Thus, the objectives of this study were to estimate the prevalence of *Salmonella* shedding among shelter dogs throughout Texas, to identify risk factors for infection, and to characterize the isolates through serotyping and antimicrobial susceptibility testing.

2.2 Materials and Methods

2.2.1 Study Design and Sample Collection

Using a repeated cross-sectional study design, research team members sampled dogs in seven animal shelters across Texas between May 2013 and December 2014. Shelters were visited for either two (two shelters) or three (five shelters) rounds of sampling during this time frame, with an interval of approximately 5 months between sampling rounds. The desired sample size of 457 was calculated for a prevalence survey with finite population correction using publicly available software (http://samplesize.sourceforge.net/iface/), assuming *Salmonella* prevalence of 5 ± 2% at an α of 0.05. Each eligible dog provided one fecal sample and, due to resource limitations, it was not feasible to perform serial sampling (collecting sample from the same dogs during subsequent shelter visits). Dogs eligible for sample collection were all

adoptable dogs healthy and well-behaved enough to provide sufficient fecal sample. Each sample consisted of fecal matter obtained directly from the rectum, during defecation, or, when fresh and directly traceable to an individual animal, from the kennel floor. When samples were collected from the kennel floor, care was taken to ensure that the sample was obtained without contacting the floor surface itself. Samples were scored to classify feces as normal (score =1), loose/semi-formed (score =2), or watery (score = 3). Samples were then placed in sterile bags or commercially-available transport media (Meridian Bioscience, Inc., Cincinnati, Ohio; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and maintained at 4°C during transport from the shelter to the lab.

2.2.2 Salmonella *Isolation and Identification*

Standard bacteriologic culture methods were used to isolate *Salmonella* from samples, as previously described (Cummings et al., 2014). Each sample was selectively enriched in 90mL of Tetrathionate Broth (BD Difco™, Sparks, MD, USA) containing 1.8 mL of iodine solution. The sample-broth mixtures were then incubated at 42°C for 18–24 h. Each mixture was then streaked onto Brilliant Green agar with novobiocin (Northeast Laboratory, Waterville, ME, USA) and Xylose Lysine Tergitol-4 (XLT-4; Northeast Laboratory, Waterville, ME, USA) plates and incubated at 37°C. After 18–24 hours, the plates were checked for growth of presumptive *Salmonella* colonies (red, lactose-nonfermenting colonies on the Brilliant Green agar with novobiocin and black, hydrogen sulfide-producing colonies on the XLT-4). From plates with such colonies, one

representative colony was selected and used to inoculate a Kligler Iron Agar slant (BBL™ Beckton Dickinson Microbiology Systems, Cockeysville, MD, USA). The Kligler slants were then incubated at 37°C for 18–24 h. Following incubation, colonies from slants displaying biochemical properties typical for *Salmonella* were streaked onto Tryptic Soy Agar with 5% Sheep blood (Northeast Laboratory, Waterville, ME, USA). After incubation at 37°C for 18–24 hours, representative colonies from each plate were placed in 3mL of Brain Heart Infusion (BHI) media (Beckton Dickinson Microbiology Systems, Cockeysville, MD, USA) and incubated at 37°C for 18–24 hours. Subsequently, 850uL of each BHI culture was placed in a sterile storage tube with 150uL pure glycerol and stored at –80°C. These frozen stocks were then later subject to additional confirmation as *Salmonella* via *invA* PCR.

For *invA* PCR, each frozen isolate stock was streaked onto Brain Heart Infusion Agar (BD Difco™, Sparks, MD, USA). The plates were then incubated overnight at 37°C. One representative colony from each plate was then placed in 100uL of nuclease-free water and microwaved for 30 seconds at full power to produce a lysate. A small volume (2uL) of each lysate was then added to a PCR tube containing 48uL of *invA* PCR master mix to give a total PCR reaction volume of 50uL. As a positive control, 2uL of *Salmonella typhimurium* ATCC 14028 was used in place of the lysate and 2uL of nuclease-water as a negative control. For each sample, the 48uL of the *invA* PCR master mix were as follows: 25uL of ProMega Master Mix (Promega Corporation, Madison, WI, USA), 2uL *invA* forward primer (5'-GAA TCC TCA GTT TTT CAA CGT TTC-3'; IDT®) at a concentration of 12.5uM, 2uL *invA* reverse primer (5'-TAG CCG

TAA CAA CCA ATA CAA ATG -3';IDT®) at a concentration of 12.5uM, and 19uL of nuclease-free water. The PCR was conducted using one 2-minute cycle of 94°C; twenty, 3-temperature cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds; and one final cycle of 72°C for 5 minutes. Upon completion of PCR, the PCR products were held at 4°C until visualization. For visualization, 2uL of each PCR product was added to 2uL of loading dye and loaded into a well of a 1.5% agarose gel. Electrophoresis proceeded at 100V for 30 minutes. Samples that yielded a 678bp band were interpreted as positive for *invA*.

2.2.3 Antimicrobial Susceptibility Testing

Antimicrobial susceptibility of *Salmonella* isolates was determined by use of the microbroth dilution method. Minimal inhibitory concentrations (MIC) were established for each isolate using the National Antimicrobial Resistance Monitoring System (NARMS) Gram-negative panel of 14 antimicrobial agents (Sensititre; TREK Diagnostic Systems, Cleveland, OH): amoxicillin/clavulanic acid (AUG), ampicillin (AMP), azithromycin (AZI), cefoxitin (FOX), ceftiofur (XNL), ceftriaxone (AXO), chloramphenicol (CHL), ciprofloxacin (CIP), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfisoxazole (FIS), tetracycline (TET), and trimethoprim/sulfamethoxazole (SXT). Clinical and Laboratory Standards Institute (CLSI) guidelines were used to interpret MIC values when available (CLSI, 2008; CLSI, 2010; CLSI, 2012). Otherwise, MIC values were interpreted using NARMS breakpoints

(FDA, 2012). Isolates were classified as being resistant or susceptible to each agent; those few isolates with intermediate susceptibility were categorized as being susceptible. Quality control testing was performed weekly using *Escherichia coli* ATCC 25922. The MIC ranges for quality control recommended by the CLSI were used, and results were accepted if the MIC values were within the expected range for this bacterial strain.

2.2.4 Salmonella *Serotyping*

Isolates were sent to the *Salmonella* Reference Center at the University of Pennsylvania for molecular serotyping. The xMAP® *Salmonella* Serotyping Assay kit (Luminex, Austin, TX) was used to identify *Salmonella* isolates. Three separate tests within the kit determine the O and H antigens simultaneously, and identify the serotype-specific markers of the AT (Additional Targets) test. *Salmonella* template DNA was extracted using the InstaGene Matrix as described by the manufacturer (Bio-Rad, Hercules, CA, USA). DNA was quantified using a NanoDrop 2000 (ThermoFisher, Waltham, MA, USA), diluted to a concentration of 100 µg/µl using nuclease free water, and stored at –20°C ± 1°C until use. The O antigen assay detected serogroups B, C1, C2, D, E, G, and serotype Paratyphi A (ParaA). The H antigen assay detected 35 antigens: a, b, c, d, j, (e,h), i, k, r, z10, z, z29, z6, y, L-complex, v, z28, EN-complex, x, z15, 1-complex, 2, 5, 6, 7, G-complex, f, (m/g,m), (m/m,t), p, s, t, z51, z4-complex, and z24. The three targets in the AT assay were *sdf*, Vi, and *flj*B. The first to targets (*sdf* and Vi) were specific for *Salmonella* Enteritidis and *Salmonella* Typhi, respectively. The *flj*B

target served as a positive control for the second motility phase of *Salmonella*. Using biotinylated primers, multiplex PCR was performed for the O antigen, H antigen, and AT assays. Following the PCR, the labeled amplicon was hybridized with the appropriate oligonucleotide probe-coupled bead mixture and then labeled with streptavidin-R-phycoerythrin (SAPE) reporter. The assay plate was analyzed on a Luminex® LX200™ platform, and the data were exported to Excel (Microsoft, Redmond, WA, USA) for analysis.

2.2.5 Epidemiologic Information

A variety of data were collected on each sampled dog. Age, breed, sex, neuter status, origin, and date of shelter admission were gathered from shelter records or, in the case of visible characteristics (age, breed, sex, neuter status), from qualified research team members at time of sample collection. Research team members also evaluated fecal sample consistency at time of collection, assigning the samples scores of 1, 2, or 3, to classify stools as normal, loose/semi-formed, or watery, respectively.

2.2.6 Statistical Analysis

Data from sample collection and laboratory analysis were entered into Microsoft Excel (Microsoft Corp, Redmond, WA, USA) and subsequently imported into a statistical software program (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) for

variable coding and analysis. Assessed variables were dog age (< 1 year old or ≥ 1 year old), sex, duration of stay in shelter (in days), origin (stray or surrendered), and fecal sample consistency. Season of sampling was also assessed; the period from March to May was defined as spring, June to August as summer, September to November as fall, and December to February as winter. Initially, bivariable analysis using the χ^2 test or Wilcoxon rank sum test (for the continuous variable, duration of stay in shelter in days) was used to assess the relationship between putative risk factors and laboratory detection of *Salmonella*. Further evaluation of all putative risk factors with $P \leq 0.25$ in this initial screen was performed according to the multivariable logistic regression model building scheme outlined by Agresti (2002, 2007), using the generalized estimating equations (GEE) method in addition to the GENMOD procedure in SAS with shelter as the random effect and with the exchangeable working correlation. Model fit was assessed using the QIC criteria. Values of $P < 0.05$ were considered significant.

2.3 Results

2.3.1 Prevalence and Risk Factors

From May, 2013 through December, 2014, we collected 554 fecal samples from dogs in seven shelters across Texas. Distribution of sampling by shelter ranged from 48 (8.7%) to 107 (19.3%). Among all sampled dogs, 263 (47.5%) were female and 290 (52.3%) were male, with sex not recorded for 1 (0.2%) dog. A total of 381 (68.8%)

were recorded as intact and 157 (28.3%) as neutered, with neuter status not available for 16 (2.9%) dogs. Among dogs with available data, 72.6% (183/252) of females and 69.2% (198/286) of males were intact. Among all sampled dogs, 474 (85.6%) were recorded as adults (≥ 1 year old) and 71 (12.8%) as puppies (< 1 year old), with age group not available for 9 (1.6%) dogs. A total of 300 (54.2%) were stray dogs and 82 (14.8%) were surrendered dogs, with origin not available for 172 (31.0%) dogs. For dogs with available data, the median duration of stay in shelter was 4 days (range: 1-370 days).

Salmonella was isolated from 27 (4.9%) of the fecal samples. Within-shelter prevalence ranged from 1.9% to 8.3% (median: 5.6%). Bivariable analysis revealed that the prevalence of fecal *Salmonella* shedding was higher ($P = 0.05$) among intact dogs (5.8%) than among neutered dogs (1.9%). The prevalence of fecal *Salmonella* shedding was also higher among stray dogs (6.7%) than among surrendered dogs (2.4%), but this finding was not statistically significant ($P = 0.1$) (Appendix Table 2.1). The median duration of stay in shelter was significantly lower ($P < 0.0001$) for *Salmonella*-positive dogs (median: 2 days) than for *Salmonella*-negative dogs (median: 5 days). *Salmonella* prevalence was significantly higher ($P = 0.03$) among watery fecal samples (14.8%) than among normal (3.9%) or semi-formed (3.7%) fecal samples. *Salmonella* prevalence did not vary significantly by age group or sex. Prevalence also did not vary significantly by season of sample collection (ranging from 3.6% in the winter to 6.9% in the summer) or by round of sample collection (ranging from 4.1% to 5.5%).

In light of the similar *Salmonella* prevalence among normal and semi-formed samples, fecal consistency was dichotomized (watery vs. other) for the multivariable analysis to facilitate interpretation. Investigation of the effects of origin (stray vs. surrendered) and duration of shelter stay were restricted to bivariable analysis because relevant data were missing for many dogs. Similarly, there was no further analysis of dog neuter status due to concerns about the potential for misclassification. There was a marginal association ($P = 0.09$) between watery feces and positive *Salmonella* status, after controlling for shelter as a random effect.

2.3.2 Antimicrobial Susceptibility

The vast majority of the *Salmonella* isolates were pan-susceptible to the 14 antimicrobial agents. Resistance to chloramphenicol was detected in only 1 of the 27 isolates (3.7%). Likewise, resistance to sulfisoxazole, was evident in 1 of the 27 isolates (3.7%). Resistance to tetracycline, however, was present in 3 of the 27 isolates (11.1%). Lone resistance to tetracycline was present in 2 of the 3 unique isolates demonstrating resistance. One of the 3 resistant isolates, however, was resistant to both chloramphenicol and sulfisoxazole in addition to tetracycline. This multi-resistant isolate was identified as belonging to the *Salmonella* Dusseldorf/Albany serotype. The two isolates with lone tetracycline resistance were identified as belonging to the *Salmonella* Anatum and *Salmonella* Derby serotypes.

2.3.3 Serotypes

Numerous *Salmonella* serotypes were represented among the 27 unique *Salmonella* isolates (Appendix Table 2.2). Serotypes Newport, Javiana, Braenderup, and Infantis were the most frequently identified, together accounting for over 50% of all isolates. An additional 14.8% were non-typeable.

2.4 Discussion

In the present study, the apparent prevalence of fecal *Salmonella* shedding among shelter dogs in Texas was approximately 5%. As fecal culture does not have perfect sensitivity for detecting the presence of *Salmonella* and serial sampling was not feasible for this study, the 5% is presumably an underestimate of the true prevalence. Nevertheless, this prevalence is near the lower end of prevalence reports for *Salmonella* among canines in general but is consistent with several studies of shelter dogs. Among canines in general, prevalence of *Salmonella* shedding can vary from a low of 1% to a high of almost 80% (Cobb and Stavisky, 2013). Differences between dog populations account for much of this variation. Domestic and pet dogs seem to have a lower prevalence of *Salmonella* shedding (around 1–5%) than do sledding dogs (up to 63%) or dogs fed raw-meat diets (up to 80%) (Hoelzer et al., 2011; Cantor et al., 1997; Joffe and Schlesinger, 2002). Fortunately, it appears that *Salmonella* prevalence ranges for shelter dogs tend to be closer to those reported for the typical pet dog than to those for racing

dogs. Studies of shelter dogs in California, Turkey, and Taiwan reported prevalences of 0%, 11%, and 16%, respectively (Sokolow et al., 2005; Kocabiyik et al., 2006; Chang et al., 2011). Differences in study geographic location; sampling and bacteriologic methodology; and social, cultural or medical practices may account for and/or influence such variations in *Salmonella* detection and distribution (Kwaga et al, 1989). Furthermore, factors like these may also contribute to differences in reported patterns of antimicrobial susceptibility and *Salmonella* serotypes.

In the literature, prevalence and patterns of antimicrobial susceptibility for canine *Salmonella* isolates are quite heterogeneous. One study detected resistance to one or more antimicrobial agent in 51% of isolates (Chang et al., 2011), another study in 67% (Kocabiyik et al., 2006), and another study in over 85% (Seepersadsingh et al., 2004). Among these same studies, differences in resistance profiles were also apparent, with tetracycline resistance being most common in the first and streptomycin resistance most common in the last two. Notably, however, for the study by Kocabiyik et al (2006), streptomycin resistance was the only resistance detected; by contrast, for the study by Seepersadsingh et al (2004), streptomycin was but one of several different antimicrobials to which resistance was detected. In the present study, antimicrobial susceptibility was quite different from the aforementioned reports, with all but three isolates being pan-susceptible to the 14 assessed antimicrobial agents and no detection of streptomycin resistance. The three resistant isolates [3/27 (11.1%)], however, were all resistant to tetracycline. Given the lack of detailed histories for the dogs yielding resistant isolates, and given the complexities of bacterial resistance itself, determining exactly how, when,

and where resistance occurred is difficult. Generally, however, for bacterial isolates from domestic animals, resistance to antimicrobial agents can be attributable to the nature of an animal's bacterial exposure and to the presence or absence of resistance-related selective pressures. Animals exposed to bacteria from environments in which antimicrobial resistance confers a survival advantage to the bacteria are more likely to acquire, carry, and shed resistant bacteria. In the context of animal shelters, detected resistance in bacterial isolates is most likely due to therapeutic uses of antimicrobial agents among shelter animals (Timoney et al., 1978) or through contaminated animal feed. It is also conceivable that bacterial exposure and resistance arose prior to shelter admittance, through direct antimicrobial administration to the animal yielding the isolate, through antimicrobial use by human or animal residents of the shelter animal's original household, or again, through contaminated animal feed. Thus, the simplest explanation for the low level of antimicrobial resistance among the isolates in the present study is the lack of exposure to resistant bacteria and/or the lack of resistance-promoting selective pressures.

As with antimicrobial susceptibility, there is considerable diversity with regard to *Salmonella* serotypes among canines (Kocabiyik et al 2006; Tsai et al., 2007; Chang et al 2011). Among the 27 *Salmonella* isolates obtained in the present study, there were 13 unique serotypes identified along with 4 (14.8%) non-typeable isolates. Interestingly, for three of the different serotypes identified herein (*Salmonella* Agbeni, *Salmonella* Idaban/Mississippi, and *Salmonella* Muenchen), there are no readily accessible, published reports describing their isolation from canine feces. Nevertheless, all have

been implicated in human salmonellosis cases or outbreaks (Stevenson, 1953; Jones et al., 2008; Taylor et al., 2012; Ashbolt and Kirk, 2006; Boase et al., 1999). Consistent with previously published reports, many of the other detected serotypes have also been responsible for cases and outbreaks of human salmonellosis. In fact, five serotypes from among the top 10 isolated from human patients with laboratory-confirmed salmonellosis in the United States (CDC, 2014) are represented in our sample of canine isolates: Newport, Javiana, Infantis, Heidelberg, and Muenchen. In 2014 alone, the Centers for Disease Control and Prevention issued reports of outbreaks attributable to *Salmonella* Newport (live poultry, contaminated cucumbers, and chia powder), *Salmonella* Braenderup (contaminated nut butter), *Salmonella* Infantis (live poultry), and *Salmonella* Heidelberg (chicken) among others (CDC, 2015).There are also reports of human cases and outbreaks in which dogs have played a role. Many describe links between human infection and pet food (Behravesh et al., 2010) such as a 2012 outbreak of *Salmonella* Infantis in the United States and Canada associated with dry dog food (Imanishi et al., 2014) and a 2013 *Salmonella* Typhimurium outbreak associated with chicken jerky pet treats (Cavallo et al., 2015). Pet food may not be the only dog-related source of human illness, however. An examination of Canadian case records from 2011 found canine contact to be a significant (OR=2.17, 95% CI 1.01 – 4.68) risk factor for *Salmonella* Enteritidis infections in Ontario (Varga et al., 2012), while an earlier report describes the contemporaneous detection of *Salmonella* Virchow with similar PFGE pattern and antimicrobial susceptibility from a diarrheic infant and two of the three household dogs (Sato et al., 2000).

Together, the detection of serotypes commonly associated with human (and, less often, animal) illness and the historical evidence for dog-related *Salmonella* zoonosis highlight the importance of having well-defined, broadly-applicable indicators of canine fecal *Salmonella* shedding. Statistical analysis of our data indicated that dog age, dog sex, and season of sampling were not statistically significant risk factors. These findings are consistent with those other studies (Sokolow et al., 2005; Tsai et al., 2007; Tupler et al., 2012). Origin (stray vs. surrendered) and duration of shelter stay appeared to be independently associated with *Salmonella* status, but missing data precluded comprehensive analysis. Nevertheless, several explanatory hypotheses are possible. Both canine origin and duration of shelter stay may affect the nature and extent of a dog's bacterial exposure(s), and, thus, the likelihood of detectable canine *Salmonella* shedding. Dogs exposed to environments containing sheep, flies, and rodents, for example, might have a higher likelihood of *Salmonella* carriage (Salehi et al., 2013; Morley et al., 2006; Snow et al., 2010). The positive association between detectable *Salmonella* and origin and between *Salmonella* and short duration of shelter stay reported in the present research, suggests that such pre-shelter *Salmonella* exposures may account for the significance of these variable. Additionally, both canine origin and duration of shelter stay may affect a dog's susceptibility to bacterial colonization, carriage, and/or shedding. Dogs from abusive households or with histories of neglect may have immune system alterations which impact their length of stay in a shelter and their physiological responses to bacteria. Moreover, physiologic responses to stress itself can greatly affect pathogenic bacterial properties like bacterial growth, motility, and virulence factor

expression (Verbrugghe et al., 2012). In order to determine the precise nature of such risk factor-bacteria-host relationships, however, more research is necessary.

Neutering (spaying of female dogs and castration of male dogs) is common for dogs in the United States, due to concerns related to aggressive behaviors, population overgrowth, and certain cancers (Hart et al., 2014). Even so, very few publications mention a role for neutering in the prevention of pathogen carriage or transmission. In most cases, such publications describe the pathogen-neutering connection in terms of canine reproductive modification. Risk of human and/or animal exposure to reproduction-associated pathogens (e.g. *Brucella canis*), for example, is much reduced when neutering renders reproduction impossible (McKenzie, 2010). However, not all pathogens are typically present in a reproductive context, and, besides the present research, at least one other study has documented a possible connection between neutering and *Salmonella*. Although not providing information about statistical significance, the study, of hospital visitation dogs in Ontario, Canada, noted that all 3 of the dogs with *Salmonella*-positive feces were sexually intact (non-neutered) (Lefebvre et al., 2006). Considering that neutering-induced changes in non-reproductive behaviors (e.g. aggression) are highly variable (McKenzie, 2010), neuter status seems unlikely to impact canine actions in a manner that would consistently alter canine pathogen exposure at a population level. Rather, two alternative hypotheses for a neuter status-*Salmonella* relationship seem more plausible. On a microscopic level, endocrine effects of neutering could have an effect. The presence or absence of gonadal hormones and other endocrine factors can influence immune responses and the host-pathogen

relationship (Klein, 2000). On a macroscopic level, neuter status could reflect inherent social or environmental differences between neutered and non-neutered canine populations. It is possible, for example, that relative to intact dogs, neutered dogs received more veterinary care prior to their entry into the shelter (hence the neutering) and/or lived in a manner that minimized *Salmonella* exposure. Additional research would help to clarify the existence of any relationship between *Salmonella* shedding and neuter status.

In contrast to the shortage of literature regarding neuter status, there is a relative abundance of published research pertaining to fecal consistency and its relationship with *Salmonella* detection. Contrary to our findings, many published reports describe the absence of any meaningful connection between diarrhea and *Salmonella* detection. In one such study, 6% of non-diarrheic dogs had feces positive for *Salmonella* while only 2% of diarrheic dogs had *Salmonella*-positive feces (Tupler et al., 2012). Notably, the latter study, like many others, had a relatively small sample size (only 100 dogs) and examined only a single animal shelter. Small sample sizes provide less reliable and less generalizable estimates of effects (dos Santos Silva, 1999). Moreover, as shelter-level variables such as dog feed can influence canine fecal consistency (Tupler et al., 2012), accounting for the variable effects of shelter in statistical assessment is quite important, and failing to account for it or studying only a single shelter may obscure variable-pathogen relationships. Featuring a large sample size and accounting for inherent shelter variability, our study may unmask a fecal consistency-*Salmonella* relationship; diarrheic feces may be more likely to be *Salmonella*-positive relative to non-diarrheic ones.

However, as with previous research, our results also show that non-diarrheic feces can contain detectable *Salmonella*. In fact, over 60% of *Salmonella*-positive dogs had grossly normal feces. Caution is thus warranted whenever fecal contact occurs or is expected, regardless of fecal consistency.

When considering the findings of the present research, it is important to note several study limitations and strengths. As with many studies, missing data limits the ability to draw conclusions. The precise origins and history of many dogs presenting to animal shelters is often unclear. Categorization of dogs as strays or surrenders, puppy (dogs <1 year) or adult (dogs ≥ 1 year) is, thus, an approximation based on shelter records or veterinary assessment, respectively. Additionally, imperfect shelter records and canine histories create the potential for neuter status misclassification. Although research team veterinarians inspected each animal, neuter status for female dogs cannot always be reliably discerned with visual and manual examination (e.g. evidence of ovariohysterectomy scars). However, the lack of significant interaction between neuter status and sex suggests that the effect of neuter status on *Salmonella* risk is the same for males (the population for which we know the neuter status data are reliable) and females. Moreover, the fact that our study even examines neuter status sets it apart from many other studies. Also, unlike much previous research on the subject, our study features a large sample size, multiple shelters throughout Texas, and an extended study time-frame. Together, these factors contribute to the strength and applicability of our findings.

In summary, detection of *Salmonella* in canine feces is positively associated with diarrheic feces. Additional research should examine the relationship between fecal

shedding and variables such as canine origin (stray vs. surrender), canine neuter status, and canine duration in animal shelters. Predictive factors aside, feces of all types from dogs of all backgrounds have the potential to harbor culturable *Salmonella* representing a variety of serotypes associated with human disease. Many of these pathogenic *Salmonella* serotypes have been previously detected in canine fecal samples. The present study, however, is perhaps the first to describe the fecal presence of *Salmonella* serotypes Agbeni, Idaban/Mississippi, and Muenchen among shelter dogs. All things considered, canines are clear participants in the epidemiology of *Salmonella*, and their contributions hold substantial import for their close companions.

3. METHODS FOR THE DETECTION OF CANINE FECAL *CAMPYLOBACTER*

3.1 Introduction

Campylobacter is one of the most predominant human pathogens, with an estimated global burden of 7.5 million disability-adjusted life years (DALYs) (WHO, 2013). Even so, many cases of human campylobacteriosis are likely to go unreported, undetected, and/or misidentified (WHO, 2013). Contributing to this lack of recognition are the difficulties surrounding *Campylobacter* cultivation methodologies and the diversity of *Campylobacter* reservoirs.

The complexities of *Campylobacter* cultivation center on the bacteria's fastidious nature and species-level heterogeneity. *Campylobacter* is particularly sensitive to atmospheric oxygen, temperature, and humidity (Koene et al., 2004; Monfort et al., 1989), and incubation typically requires the use of microaerophilic conditions at 37–42°C (Koene et al., 2004; Allos, 2001). Furthermore, the incubation itself can take several days. Adding to these complications, certain growth requirements may be species-dependent (Hald et al., 2004), so that a culture method suitable for the growth of one *Campylobacter* species may be suboptimal for that of another. Methodology, therefore, impacts not only the detection of *Campylobacter*, but also the identification of it.

On account of the barriers bacterial culture can impose, there is growing interest in molecular methods of *Campylobacter* detection and identification. The use of PCR for

direct analysis of stool samples is particularly appealing on account of the high sensitivity, relative speed and automation of the technique. Most studies on *Campylobacter* use primers for the 16S or 23S rRNA gene (On et al., 1996) for genus-level identification. Species-level detection is also possible (Kulkami et al., 2002). Moreover, because it does not depend on bacterial viability or culturability, PCR is one of the best methods for detecting bacteria in older, less fresh samples (Maher et al., 2003) or for detecting uncommon *Campylobacter* species such as *Campylobacter hyointestinalis* and *Campylobacter upsaliensis* (Kulkami et al., 2002). Even so, the detection ability of PCR may depend on the timing of DNA extraction; Kulkami et al (2002) posit that PCR detection may be inversely related to the length of time between laboratory receipt of stool samples and DNA extraction. In addition, PCR can increase the cost and labor-intensity of investigation, without the benefit of producing a bacterial isolate (Kulkami et al., 2002; On et al., 1996). Without a clinical isolate, a suspect case of campylobacteriosis cannot be confirmed to CDC standards (NNDSS Condition Case Definition 2012 Case Definition).

The clear standards for case definitions, however, do not extend to laboratory protocols for *Campylobacter* detection. Within the United States, there is considerable inter-laboratory, inter-clinic variation in detection protocols and even in the frequency with which samples are subjected to testing (Hurd et al 2012). Yet the problem persists beyond borders. A report from a 2012 expert consultation on *Campylobacter* facilitated by the World Health Organization (WHO) concluded that there is a widespread "need for standardization and validation of laboratory methods" (WHO, 2013, p.1) and, on

multiple occasions, discusses the need to do so in a variety of contexts and with consideration of *Campylobacter*'s myriad possible sources. One such possible source is the common canine companion. Thus, the aims of the present study were to assess the comparability of several different, commonly used methods (i.e. traditional culture vs molecular) of *Campylobacter* detection and to evaluate the suitability of these methods for use in an important under-researched population: shelter dogs.

3.2 Materials and Methods

3.2.1 Study Design and Sample Collection

The present study proceeded using a post-hoc analysis of results from a cross-sectional study of the fecal shedding of *Campylobacter* in dogs from animal shelters in Texas. For the cross-sectional study (unpublished data), sampling took place over a four month period (September – December 2014) at six participating shelters. Each sample consisted of fresh fecal matter directly traceable to an individual animal, with only one sample collected per animal. Less than 48 hours after sample collection, 8 mL of 1x PBS (Sigma-Aldrich, Co., St. Louis, MO, USA) was added to 2–3g of each stool sample to create fecal slurries. Fecal slurries were then subjected to three different methods of *Campylobacter* detection: PCR, direct plating, or indirect plating.

3.2.2 DNA Extraction

DNA was extracted from the fecal slurries using MO BIO PowerFecal® DNA Isolation Kits (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and following a (very slightly) modified version of the manufacturer guidelines. In brief, the extraction protocol proceeded as follows. First, 1 mL of each fecal slurry was pipetted into a labeled 1.4mm MO BIO dry bead tube. The bead tubes were then centrifuged at 12,800 rpm for 10 minutes. The resulting supernatants were removed and 750 µL of MO BIO Bead Solution added to each bead tube. The bead tubes were vortexed and 60 µL of Solution C1 added to each one. The tubes were inverted several times to allow mixture and heated in a 65°C hot water bath for 10 minutes. Using a vortex adapter tube holder, the tubes were placed horizontally on a flat-bed vortex pad and vortexed at maximal speed for 10 minutes. Next, tubes were centrifuged at 13,000 x g for 1 minute. Following centrifugation, 420 µL of each supernatant was then transferred to a new 2 mL Collection Tube and 250 µL of Solution C2 added. These tubes were vortexed then incubated at 4°C for 5 minutes. After centrifugation at 13,000 x g for 1 minute, 500 µL of each supernatant was transferred from the centrifuged tube to another 2 mL Collection Tube and 200 µL of Solution C3 was added. These tubes were vortexed then incubated at 4°C for 5 minutes. After centrifugation at 13,000 x g for 1 minute, 600 µL of each supernatant was transferred to another 2mL Collection Tube and 600 µL of Solution C4 was added. These tubes were vortexed and spun down at 13, 000 x g for 1 minute. Another 600 µL of Solution C4 was added, using pipetting to mix prior to loading 600

μL of the mixture onto a Spin Filter in a Spin Column. The Columns were then centrifuged at 13, 000 x g for 1 minute. The flow-through was discarded and another 600 μL of the Solution C4-supernatent mixture was added to the Spin Filter apparatus and centrifuged at 13, 000 x g for 1 minute. This process was repeated until all of the Solution C4-supernatent mixture had been added to and centrifuged in the Spin Filter Column. Then, 500 μL of Solution C5 was added to the Spin Filter Column and centrifuged at 13, 000 x g for 1 minute. After the flow-through was discarded, the Spin Filter Column was centrifuged at 13, 000 x g for 2 minutes. The Spin Filter was then removed from the Column and placed into a new 2 mL Collection Tube. In a biosafety cabinet, 50 μL of Solution C6 was placed onto the Spin Filter tube. After an incubation of 1 minute at room temperature, each tube was centrifuged at 13, 000 x g for 1 minute. The Spin Filter was removed from each tube and the tubes stored at –20°C to preserve the DNA.

3.2.3 Culture-Independent Campylobacter *Detection*

The DNA extracted from the fecal samples was later subjected to quantitative PCR (qPCR) using a 7900 HT Fast Real-Time PCR machine and the associated PC software (SDS 2.4, Applied Biosystems, Life Technologies, Austin, TX USA). All primers and probes used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA. The procedure followed was an adaptation of that used by Lund et al (2004). Specifically, 2 μL of each DNA sample was combined with 6.75 μL of nuclease-free

water (Ambion® Life Technologies, Austin, TX, USA), 12.5 µl of PCR Master Mix (TaqMan® Fast Universal PCR Mastermix, Applied Biosystems, Life Technologies, Austin, TX, USA), 1.25 µL (0.5mM) of forward primer for *Campylobacter* 16S rRNA gene (5'-CACGTGCTACAATGGCATAT-3'), 1.25 µL (0.5mM) of reverse primer for *Campylobacter* 16S rRNA gene (5'-GGCTTCATGCTCTCGAGTT-3'), and 1.25 µL of the TaqMan probe (5'-FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1-3') for a total reaction volume of 25 µL per sample. For a positive control, 2 µL of *C.jejuni* ATCC 33560 DNA was used in place of sample DNA, and for a negative control, 2 µL of nuclease-free water. All reaction mixtures then underwent absolute quantification according to the following amplification cycle: 1 cycle at 95°C for 20 minutes, followed by 40 cycles of 1 minute at 95°C and 20 minutes at 60°C. Based on preliminary experiments, all samples yielding C_t values between 14 and 33 were considered positive for *Campylobacter*.

Note that in nearly all cases, qPCR was performed before comparison with the culture plating results, so as to reduce the likelihood of biased interpretation.

3.2.4 Direct Plating

Using sterile, disposable, cotton-tipped applicators, swabs of each fecal slurry sample were streaked on agar plates. Two different types of agar—Modified Charcoal-Cefoperazone Deoxycholate Agar with Cefoperazone, Amphotericin B, Teicoplanin selective supplement (mCCDA-CAT) Blood Free Campylobacter Selectivity Agar

(HiMedia Laboratories, LLC, Mumbai, India) and Campy Cefex Agar (Acumedia Manufacturers, Inc., Neogen Corporation, MI, USA; abbreviated as 'CEF' throughout this manuscript), each prepared according to manufacturer instructions—were used for each sample. As soon as possible, the streaked agar plates were placed into anaerobic canisters containing AnaeroPack®-MicroAero, a microaerophilic gas generating system, (Mitsubishi Gas Chemical America, Inc., NY, USA) and incubated at 37°C. After 72 hours of incubation, the canisters were opened and the plates examined for characteristic *Campylobacter* colonies. Suspect colonies were then streaked onto Trypticase™ Soy Agar with 5% Sheep Blood (BBL™, Becton Dickinson, Franklin Lakes, NJ, USA). The blood agar plates were then incubated under microaerophilic conditions at 37°C for at least 48 hours (additional days of incubation were sometimes required to achieve enough growth for isolate characterization and preservation). Characteristic *Campylobacter* colonies were then tested using qPCR. The qPCR was performed as outlined above for the 16S *Campylobacter* ribosomal subunit (see '*Culture-Independent Campylobacter Detection*'), replacing the extracted DNA with 2 µL of a colony lysate. The colony lysate consisted of ~ 1 µL of colony placed in 50 µL of nuclease-free water (Ambion® Life Technologies, Austin, TX USA) that was microwaved at full power for 30 seconds. Again, as for the qPCR for the 16S *Campylobacter* ribosomal subunit outlined previously (see '*PCR Detection*'), all samples yielding C_t values between 14 and 33 were considered positive for *Campylobacter*.

3.2.5 Indirect Plating

The indirect plating method described in this study refers to the use of broth enrichment prior to agar plating. In this study, 1 mL of each fecal slurry sample was dispensed into a 15mL screw-cap conical tube (VWR, Sugar Land, TX, USA) containing 9 mL of Bolton Broth (HiMedia Laboratories, LLC, Mumbai, India) supplemented with Lake Horse blood (Hardy Diagnostics, Santa Maria, CA, USA) per manufacturer instructions. The slurry and broth were mixed and the caps loosened. The tubes were then placed inside anaerobic canisters containing AnaeroPack®-MicroAero, a microaerophilic gas generating system, (Mitsubishi Gas Chemical America, Inc., NY, USA) and incubated at 37°. After 48 hours, the canisters were removed from the incubators and slurry-broth mixtures plated onto mCCDA-CAT Blood Free Campylobacter Selectivity Agar (HiMedia Laboratories, LLC, Mumbai, India) and Campy Cefex Agar (Acumedia Manufacturers, Inc., Neogen Corporation, MI, USA) plates using sterile, disposable, cotton-tipped applicators and loops. Further processing then proceeded in the same manner as that described for the direct plating method.

3.2.6 Data Analysis

Data from sample collection and laboratory analysis were entered into Microsoft Excel (Microsoft Corp, Redmond, WA) and subsequently imported into a statistical software program (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) for analysis.

The kappa statistic was used to assess the level of agreement between methods and the level of test result concordance according to the formula (a+d)/n where ‘a’ represents the number of samples positive by both tests,‘d’ the number negative by both tests, and ‘n’ the total number of samples assessed (Lund et al., 2004; Martin et al., 1997; Smith, 1995). McNemar’s test was used to assess test performance and detect meaningful levels of test result discordance. Test specificity was determined as the number of samples negative by both tests divided by the sum of the number of samples negative by both tests and the number of samples positive by one test and negative by the counterpart test. For culture vs culture comparisons, sensitivity was determined as the number of samples positive by both tests divided by the sum of the number of samples positive by both tests and the number of samples negative by one test and positive by the counterpart test (Mark and Wong, 2012).

3.3 Results

3.3.1 Campylobacter *Detection*

During the study period, researchers collected and processed a total of 185 individual fecal samples, approximately 30 fecal samples from each shelter. Using qPCR for the DNA extracted from the fecal slurries (representative amplification curve appears in Appendix Figure 3.1), a total of 140 (75.7%) samples tested positive for the 16S *Campylobacter* ribosomal subunit, with prevalence varying from a low of 56.7% at

one shelter to a high of 93.3% at another. For bacterial culture overall, a total of 84 (45.4%) were identified as *Campylobacter*, with positive results ranging from a low of 25.8% at one shelter to a high of 63.3% at another. All culture-positive samples were qPCR-positive. Looking at culture results according to agar type (mCCDA-CAT or CEF), plating (direct or indirect) on mCCDA-CAT agar plates yielded slightly more *Campylobacter* positives than plating (direct or indirect) on CEF agar plates (69 [37.3%] vs 65 [35.1%]). Likewise, direct plating (on mCCDA-CAT or CEF agar plates) yielded slightly more *Campylobacter* positives than indirect plating (on mCCDA-CAT or CEF agar plates) (65 [35.1%] vs 56 [30.3%]. Direct plating on mCCDA-CAT agar plates yielded 52 (28.1%) *Campylobacter*-positives, while direct plating on CEF agar plates yielded 45 (24.3%) *Campylobacter*-positives. Indirect plating on mCCDA-CAT agar plates yielded 43 (23.2%) *Campylobacter*-positives, while indirect plating on CEF agar plates yielded 45 (24.3%) *Campylobacter*-positives.

3.3.2 Data Analysis

For all comparisons, there was significant agreement and high levels of test result concordance (Appendix Table 3.1 and Appendix Table 3.2). When comparing qPCR results to those of culture (Appendix Table 3.1), the highest levels of agreement and concordance was between the qPCR and the pooled culture (combined results of all culture methods) results (concordance= 69.7%; kappa=0.4170; $p < 0.0001$), with 84/185 (45.4%) samples positive by both methods and 45/185 (24.3%) samples negative by both

methods. Also looking at the comparison between the qPCR results and those of culture, the lowest levels of agreement and concordance was between the qPCR and mCCDA-CAT with indirect plating results (concordance= 47.8%; kappa= 0.1794; p <0.0001), with 42/182 (23.1%) samples positive by both methods and 45/182 (24.7%) samples negative by both methods. Importantly, however, for all the qPCR vs culture comparison, the level of observed agreement exceeded that expected due to chance alone (p<0.0001). Despite high agreement, however, the overall performance of qPCR was significantly different from that of culture (p values <0.0001). The probability of getting a positive result by qPCR was much higher than that of getting a positive result on culture. Specificity of qPCR relative to culture was low, ranging from a high of 44.8% (qPCR vs pooled culture results) to a low of 32.1% (qPCR vs mCCDA-CAT with indirect plating).

For culture vs culture comparisons, there was considerable similarity across all values of assessment (Appendix Table 3.2). The highest levels of agreement and concordance was observed for the comparison of indirect plating on mCCDA-CAT plates to indirect plating on CEF plates (concordance= 88.0%; kappa=0.6766; p <0.0001), with 34/184 (18.5%) samples positive by both methods and 128/184 (69.6%) samples negative by both methods. By contrast, the lowest levels of agreement and concordance was between direct plating on mCCDA-CAT plates and indirect plating on CEF plates (concordance= 71.4%; kappa= 0.2749; p <0.0001), with 23/182 (12.6%) samples positive by both methods and 107/182 (58.8%) samples negative by both methods. Nevertheless, performance was not statistically different for any comparison.

Sensitivities and specificities did vary, however, with sensitivities ranging from a high of 80.0% to a low of 44.2%, and specificities ranging from a high of 93.4% to a low of 78.9%.

3.4 Discussion

In the present study, the apparent prevalence of fecal *Campylobacter* shedding among shelter dogs in Texas was approximately 76 % according to results from qPCR, with only 45.4% of samples positive according to bacterial culture. As neither qPCR nor culture have perfect sensitivity for *Campylobacter* detection, these percentages are presumably underestimates. They are, however, in line with previous reports of canine *Campylobacter* prevalence, which document prevalence estimates ranging from 21–77% (Hald et al., 2004; Koene et al., 2004). Nevertheless, inter-study comparisons of *Campylobacter* epidemiology and prevalence are complicated by numerous factors, including differences in study geographic location, study population, and study methodology. The impact of the latter is particularly evident from the results here presented. Assessing the same samples, there was a significant difference between the performance of culture-independent qPCR and culture. This finding is at least partially attributable to the conservative criteria used for determination of a positive result according to culture; in the present study a culture-positive sample was one yielding colonies suggestive of *Campylobacter* on culture agar and testing positive for *Campylobacter* on qPCR of colony DNA. However, the performance difference between

culture-independent qPCR and culture could also be explained by the high sensitivity of qPCR, the difficulty of culturing *Campylobacter*, and the possibility of viable, non-culturable bacteria.

In contrast to the comparisons involving both culture-independent qPCR and culture, the differences evident from our culture-culture comparisons were more subtle. Overall, there were no statistically significant differences in performance for any of the comparisons, and, generally, levels of agreement and concordance were high. Even so, there were notable variations in sensitivity and specificity. Relative to CEF plates, mCCDA-CAT plates tended to have higher sensitivities and lower specificities. This observation is consistent with other reports. It is posited that blood-using media (such as CEF) can make it more difficult to visually identify *Campylobacter* colonies (Nachamkin, 1997). As to plating method, the relationship between direct plating of fecal slurry and indirect plating (plating of enriched slurry) was similar to that between the two agar types; Relative to indirect plating, direct plating tended to have higher sensitivities and lower specificities. The higher sensitivity of direct plating may result from shorter sample processing time and/or lower levels of requisite sample manipulation. Specificity may be higher for indirect plating due to the enrichment. Trends in sensitivity and specificity, however, showed evidence of interaction between plating method and plate type; Indirect plating on CEF plates resulted in higher sensitivity than direct plating on CEF plates. While slight, this elevation of sensitivity could be attributable to an increased ease of *Campylobacter* visualization on the blood-containing agar as a result of enrichment-enhanced selectivity.

Selectivity is also an important issue in a more general way. Potentially affecting all *Campylobacter* culture observations is variation among different *Campylobacter* species; certain species may be more selective in their growth requirements. Relative to *Campylobacter jejuni*, for example, *Campylobacter upsaliensis* may require longer incubation periods and lower concentrations of the antimicrobial cefoperazone (Hald et al., 2004). Thus, using multiple methodological techniques (i.e. a mix of different culture methods in combination with one or more molecularly-based approaches) may produce more robust results.

Regarding the results of the present study and their future applications, it is important to note several study limitations and strengths. As with many studies, there were some missing data. For three of the 185 total samples, plate type and/or plating method was not available and in these cases, the isolate was excluded from detailed plate type/plating method analyses. Another limitation concerns the importance of experience to *Campylobacter* culture. Although our data show no overt trend of the phenomenon (there is no linear increase in culture-positive rate over time), it is conceivable that the ability to cultivate and detect *Campylobacter* from culture improved over the duration of the study, thereby influencing results. This limitation could easily apply to other studies, past and future.

The strengths of the present research could also inform future studies. Our study features a large sample size, a unique population from multiple shelters throughout Texas, an extended study time-frame, and the head-to-head examination of multiple

bacterial detection methods. Together, these factors contribute to the strength and applicability of our findings.

In summary, use of the culture-independent and colony qPCR procedures here described is suitable for the detection of *Campylobacter* from canine feces. As with other PCR techniques, however, its cost and the fact that it does not yield a bacterial isolate must be weighed against the benefits of high sensitivity, a same-day result, and the nondiscriminatory treatment of different *Campylobacter* species. Performing culture in tandem with or under the guidance of the species information possible with qPCR could greatly enhance results. As for culture itself, all methods employed here were largely comparable, yet with some variations as to sensitivity and specificity. Future research could examine these variations and their potential relationship to different species of *Campylobacter*. In light of the relationship between canines and humans, and the impact *Campylobacter* has on both, investigations into the methodology behind bacterial detection promise to augment not only clinical diagnosis and source tracking, but also the implementation and identification of preventative strategies. By assessing multiple detection methods in an oft-overlooked population, the present study is a step in that direction.

4. THE EPIDEMIOLOGY OF FECAL *CAMPYLOBACTER* SHEDDING AMONG SHELTER DOGS IN TEXAS*

4.1 Introduction

Companion animals can serve as reservoirs for a variety of zoonotic pathogens (Halsby et al., 2014). *Campylobacter*, a zoonotic agent causing an estimated 35% of foodborne infections in the United States (CDC, 2014; Scallan et al., 2011), resides in the intestinal tract of many companion animals and passes into the external environment through feces (Workman et al., 2005). Estimates of fecal *Campylobacter* shedding can vary considerably across studies, depending on the animal population, geographic location, time of sample collection, and bacterial processing methods. Among canines, the prevalence of fecal *Campylobacter* has ranged from 2–36% to over 75% (Wolfs et al., 2001; Hald et al., 2004; Koene et al., 2004). Considering that there are an estimated 83.3 million household dogs in the United States (American Pet Products Association 2012 estimates; Humane Society), and that there is evidence of canine-human *Campylobacter* transmission (Damborg et al., 2004; Nayar, 1980; Wolfs et al., 2001), canine *Campylobacter* shedding represents a potential threat to public health. Moreover, human infection with *Campylobacter* is associated with serious, debilitating, extraintestinal complications including Guillain-Barré paralysis and Miller-Fisher

*Material in this chapter is reused with permission from Leahy, A.M., Cummings, K.J., Rodriguez-Rivera, L.D., Rankin, S.C., and S.A. Hamer. (2017). Faecal *Campylobacter* shedding among dogs in animal shelters across Texas. *Zoonoses and Public Health* 00: 1 – 5, Copyright [2017] by Blackwell Verlag GmbH.

polyneuropathy (WHO, 2013; Bourke et al., 1998). Even so, there remains a lack of information about the epidemiology of *Campylobacter* among canines in general and shelter dogs in particular.

Exposures to other animals, high-stress environments, and the welfare challenges of shelters (scarcity of funding, presence of volunteers with limited disease-control training) (Turner et al., 2012) could serve to increase the susceptibility of shelter dogs to infection with zoonotic pathogens including *Campylobacter*. Shelter dogs harboring such pathogens would be capable of wide pathogen dissemination due to their contact with other shelter animals, shelter personnel, and prospective adoptive families. Preventing pathogen transmission, however, is complicated by the fact that *Campylobacter*-shedding dogs rarely display any overt clinical signs of bacterial carriage (Damborg et al., 2004). Furthermore, once colonized by *Campylobacter*, dogs can continue to shed the bacteria intermittently for over 1 year (Damborg et al., 2004). Greater understanding of pathogen epidemiology among shelter dog reservoirs could allow for more effective disease control and prevention by facilitating risk assessment and the identification of salient indicators of bacterial shedding (Wain and Olsen, 2013). Even so, although some studies have reported on *Campylobacter* epidemiology involving dogs in regions of Denmark, Switzerland, California, and several other areas (Damborg et al., 2004; Wieland et al., 2005; Labarca et al., 2007), no published study to date has looked at the canine population in shelters in Texas. Thus, the objectives of the present study were to estimate the prevalence of fecal *Campylobacter* shedding among dogs at 6 animal shelters across Texas, to estimate the specific prevalence of *C. jejuni*

and *C. coli* shedding amongst these dogs, and to identify risk factors for such *Campylobacter* shedding.

4.2 Materials and Methods

4.2.1 Study Design and Sample Collection

The present study proceeded using a cross-sectional design to assess fecal shedding of *Campylobacter* in dogs from animal shelters in Texas. Sampling took place over a four month period (September – December 2014) in which each of the six participating shelters was visited once. In accordance with official and professional guidelines, all necessary consents and approvals were obtained. The desired sample size of 139 was calculated for a prevalence survey with finite population correction using publically available software (http://samplesize.sourceforge.net/iface/), assuming *Campylobacter* prevalence of 10 ± 5% at an α of 0.05. Each sample consisted of fecal matter obtained directly from the rectum, during defecation, or, when fresh and directly traceable to an individual animal, from the kennel floor. When samples were collected from the kennel floor, care was taken to ensure that the sample was obtained without contacting the floor surface itself. All collected samples were thus directly traceable to an individual animal. Immediately after collection, approximately 2–3g of each stool sample was placed in a labeled Whirl-Pak bag (Nasco, Fort Atkinson, WI, USA) or 50 mL screw-capped conical Falcon tube and kept on ice during transport from the shelter

to the lab. Upon arrival in the lab (less than 48 hours after sample collection), 8 mL of 1x PBS (Sigma-Aldrich, Co., St. Louis, MO, USA) was added to each stool sample to create pipette-able fecal slurries.

4.2.2 DNA Extraction

DNA was extracted from the fecal slurries using MO BIO PowerFecal® DNA Isolation Kits (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and following a modified version of the manufacturer guidelines. The modified DNA extraction protocol proceeded as follows. First, 1 mL of each fecal slurry was pipetted into a labeled 1.4mm MO BIO dry bead tube. The bead tubes were then centrifuged at 12,800 rpm for 10 minutes. The resulting supernatants were removed and 750 µL of MO BIO Bead Solution added to each bead tube. The bead tubes were vortexed and 60 µL of Solution C1 added to each one. The tubes were inverted several times to allow mixture and heated in a 65°C hot water bath for 10 minutes. Using a vortex adapter tube holder, the tubes were placed horizontally on a flat-bed vortex pad and vortexed at maximal speed for 10 minutes. Next, tubes were centrifuged at 13,000 x g for 1 minute. Following centrifugation, 420 µL of each supernatant was then transferred to a new 2 mL Collection Tube and 250 µL of Solution C2 added. These tubes were vortexed then incubated at 4°C for 5 minutes. After centrifugation at 13,000 x g for 1 minute, 500 µL of each supernatant was transferred from the centrifuged tube to another 2 mL Collection Tube and 200 µL of Solution C3 was added. These tubes were vortexed then incubated

at 4°C for 5 minutes. After centrifugation at 13,000 x g for 1 minute, 600 µL of each supernatant was transferred to another 2mL Collection Tube and 600 µL of Solution C4 was added. These tubes were vortexed and spun down quickly. Another 600 µL of Solution C4 was added, using pipetting to mix prior to loading 600 µL of the mixture onto a Spin Filter in a Spin Column. The Columns were then centrifuged at 13, 000 x g for 1 minute. The flow-through was discarded and another 600 µL of the Solution C4-supernatent mixture was added to the Spin Filter apparatus and centrifuged at 13, 000 x g for 1 minute. This process was repeated until all of the Solution C4-supernatent mixture had been added to and centrifuged in the Spin Filter Column. Then, 500 µL of Solution C5 was added to the Spin Filter Column and centrifuged at 13, 000 x g for 1 minute. After the flow-through was discarded, the Spin Filter Column was centrifuged at 13, 000 x g for 2 minutes. The Spin Filter was then removed from the Column and placed into a new 2 mL Collection Tube. In a biosafety cabinet, 50 µL of Solution C6 was placed onto the Spin Filter tube. After an incubation of 1 minute at room temperature, each tube was centrifuged at 13, 000 x g for 1 minute. The Spin Filter was removed from each tube and the tubes stored at –20°C to preserve the DNA.

4.2.3 Campylobacter *Detection*

To detect *Campylobacter*, the DNA extracted from the fecal samples was subjected to quantitative PCR (qPCR) using a 7900 HT Fast Real-Time PCR machine and the associated PC software (SDS 2.4, Applied Biosystems, Life Technologies,

Austin, TX, USA). All primers and probes used in this study were obtained from Sigma-Aldrich, St. Louis, MO, USA. The procedure followed was similar to that used by Lund et al (2004) for the detection of *Campylobacter* from chicken cloacal samples. Specifically, 2 µL of each DNA sample was combined with 6.75 µL of nuclease-free water (Ambion®, Life Technologies, Austin, TX, USA), 12.5 µL of PCR Master Mix (TaqMan® Fast Universal PCR Mastermix, Applied Biosystems, Life Technologies, Austin, TX, USA), 1.25 µL (0.5mM) of forward primer for *Campylobacter* 16S rRNA gene (5'-CACGTGCTACAATGGCATAT-3'), 1.25 µL (0.5mM) of reverse primer for *Campylobacter* 16S rRNA gene (5'-GGCTTCATGCTCTCGAGTT-3'), and 1.25 µL of the TaqMan probe (5'-FAM-CAGAGAACAATCCGAACTGGGACA-BHQ1-3') for a total reaction volume of 25 µL per sample. For a positive control, 2 µL of *C.jejuni* ATCC 33560 DNA was used in place of sample DNA, and for a negative control, 2 µL of nuclease-free water. All reaction mixtures then underwent absolute quantification according to the following amplification cycle: 1 cycle at 95°C for 20 minutes, followed by 40 cycles of 1 minute at 95°C and 20 minutes at 60°C. Based on preliminary experiments, all samples yielding C_t values between 14 and 33 were considered positive for *Campylobacter*.

4.2.4 Campylobacter *Species Identification*

In all *Campylobacter* 16S-positive DNA samples, identification of two *Campylobacter* species (*C. coli* and *C. jejuni*) proceeded using a qPCR procedure

similar to that used for genus identification (see *Campylobacter* Detection, above) with species-specific primers, probes, and controls. The reaction mix for *C. coli* consisted of 2 µL sample DNA combined with 6.75 µL of nuclease-free water (Ambion®, Life Technologies, Austin, TX, USA), 12.5 µL of TaqMan® Fast Universal PCR Master Mix (2X) (Applied Biosystems, Life Technologies, Austin, TX, USA), 1.25 µL (0.5mM) of forward primer for *Campylobacter coli* (5'-TTGAAAATATGGGTGCTTCACTTG-3'), 1.25 µL (0.5mM) of reverse primer for *C.coli* (5'-TGTGCCATCACCTGCTTGA-3'), and 1.25 µL of the probe (5'-[6FAM]AGAAGTGGCAAGCAA[BHQ1]-3') for a total reaction volume of 25 µL per sample. DNA of *C. coli* ATCC 33559 and *C.jejuni* ATCC 33560 were used as positive and negative controls, respectively. All reaction mixtures then underwent absolute quantification according to the following amplification cycle: 1 cycle at 95°C for 20 minutes, followed by 40 cycles of 1 minute at 95°C and 20 minutes at 52.5°C.

The reaction mixture for *C. jejuni* was identical to that of *C.coli*, except for the use of *C.jejuni* specific primers and probe. For *C.jejuni*, the primers and probe were as follows: 1.25 µL of forward primer (5'-TTAATGACGCGGTAAAAGTAACTATGG-3'), 1.25 µL of reverse primer (5'-TGCTTGGAGCACCAAAGCT-3'), and 1.25 µL of the probe (5'-[6FAM]CCAAGAGGACGCAATGT[BHQ1]-3'). DNA of *C.jejuni* ATCC 33560 and *C.coli* ATCC 33559 were used as positive and negative controls, respectively. All reaction mixtures then underwent absolute quantification according to the following amplification cycle: 1 cycle at 95°C for 20 minutes, followed by 40 cycles of 1 minute at 95°C and 20 minutes at 52.5°C.

Based on preliminary experiments, for both *C.coli* and *C. jejuni*, all samples yielding C_t values between 14 and 33 were considered positive for *Campylobacter coli* and those yielding C_t values between 14 and 33 positive for *Campylobacter jejuni*.

4.2.5 Data Collection and Data Analysis

In addition to actual fecal specimens, team members collected information about dog providing a fecal sample. Collected information included approximate dog age, breed, sex, neuter status, date of admission to shelter, and reason for admission. This information came from shelter records, or, with regard to visible characteristics (age, breed, sex, fixing status), from qualified research team members at time of sample collection. Fecal sample consistency was evaluated at time of collection, assigning the samples scores of 1, 2, or 3, to classify stools as normal, loose, or watery (diarrheic), respectively.

Data from sample collection and laboratory analysis were entered into Microsoft Excel (Microsoft Corp, Redmond, WA) and subsequently imported into a statistical software program (SAS version 9.4; SAS Institute Inc., Cary, NC, USA) for variable coding and analysis. Assessed variables were dog age (< 1 year old or ≥ 1 year old), sex, neuter status (neutered or intact), duration of stay in shelter (in days), origin (stray or surrendered), and fecal sample consistency (diarrheic= fecal consistency score of 3; non-diarrheic= fecal consistency scores of 1 or 2). These choices for variable categorization were made so as to minimize the chances of data separation and sparsity, as well as to

maximize the biologic plausibility, interpretability and practical applicability of the regression results.

Initially, bivariable analysis using the χ^2 test or Wilcoxon rank sum test (for the continuous variable, duration of stay in shelter in days) was used to assess the relationship between putative risk factors and laboratory detection of *Campylobacter*. Further evaluation of all putative risk factors with $P \leq 0.25$ in this initial screen was performed according to the multivariable logistic regression model building scheme outlined by Agresti (2002, 2007), using the generalized estimating equations (GEE) method in addition to the GENMOD procedure in SAS with shelter as the random effect and with the exchangeable working correlation. Model fit was assessed using the QIC criteria. Values of $P < 0.05$ were considered significant.

4.3 Results

4.3.1 Overall Prevalence and Risk Factor Analysis

A total of 185 fecal samples were collected, 133% of the planned sample size. The number of samples per shelter ranged from 30 to 33, and among all sampled dogs, 93 (50.3%) were female and 92 (49.7%) were male. A total of 162 (87.6%) dogs were adults (≥ 1 year old) and 23 (12.4%) were recorded as puppies (< 1 year old). Ninety-seven (52.4%) were stray animals, and 34 (18.4%) were surrendered; for 54 (29.2%) dogs, origin was not available.

A total of 140 (75.7%) of all samples tested positive for the 16S *Campylobacter* ribosomal subunit, with prevalence varying from a low of 56.7% at one shelter to a high of 93.3% at another (*P* value = 0.0267). For other assessed variables, the differences in *Campylobacter* detection were neither as large nor as statistically significant (Appendix Table 4.1). Detection was slightly less among adult dogs (dogs ≥1 year of age) than for young dogs (dogs <1 year of age) (75.3% vs 78.3%; χ^2 *P* value= 0.7575), and for spayed/neutered dogs than for intact dogs (71.7% vs 77.6%; χ^2 *P* value= 0.3786). Bivariable analysis indicated that none of these differences were statistically significant enough for inclusion in multivariable modeling.

Investigation of the effects of origin (stray vs. susrrendered) was restricted to bivariable analysis as the relevant data were missing for nearly 30% of dogs. On analysis, dog origin was not statistically significant (*P* = 0.6).

For more accurate analysis, fecal consistency was dichotomized (normal vs. other; fecal score=1 vs. fecal score =2 or 3) due to the small number (n=8) of diarrheic (fecal score =3) fecal samples as well as the similar *Campylobacter* prevalence among the diarrheic and semi-formed samples (prevalence of 88% and 80%, respectively). Controlling for shelter as a random effect, analysis showed a marginal association (*P*= 0.06) between abnormal fecal consistency (diarrheic or semi-formed) and positive *Campylobacter* status.

For sex and duration of stay in shelter, bivariable analysis did indicate sufficient enough association between variable and outcome to justify inclusion in multivariable modeling. Among male dogs detection of *Campylobacter* was slightly greater than it was

among female dogs (80.4% vs. 70.1%; χ^2 *P* value= 0.1335). Likewise, Wilcoxon rank sum analysis demonstrated a significant association between duration of stay in shelter and *Campylobacter* detection (*P* value <0.0004). Median duration of stay in shelter was significantly lower (*P*= 0.03) for *Campylobacter*-positive dogs (median: 6 days) than for *Campylobacter*-negative dogs (median: 9 days). Thus, using the threshold of $P \leq 0.25$ on bivariable analysis, both dog sex and dog duration of stay in shelter were eligible for inclusion in multivariable regression modeling. Assuming no interactions, the model containing the intercept as well as both of these eligible variables did not yield statistically significant *P* values for the coefficients of either variable (Appendix Table 4.1). Though not significant at the $P < 0.05$ level, the coefficient for sex suggests that *Campylobacter* detection may be less among female dogs than among male dogs (coefficient= -0.5119, odds ratio [OR] = 0.6, 95% CI 0.28 – 1.30, $P = 0.1956$). Similarly, the coefficient for duration in shelter suggests that *Campylobacter* detection decreases the longer a dog stays within a shelter (coefficient= -0.008, OR 0.99, 95% CI 0.98 – 1.00, $P = 0.2762$).

4.3.2 Campylobacter *Species*

Of the 140 samples testing positive for the 16S *Campylobacter* ribosomal subunit, 0 (0%) were positive for *Campylobacter coli* and 10 (7.1%) were positive for *Campylobacter jejuni* (Appendix Table 4.2). The *C.jejuni*-positive samples were from dogs in four of the six sampled shelters; there were three *C.jejuni*-positive samples from

each of three shelters and one *C.jejuni*-positive sample from another shelter. Overall, the prevalence of *C.jejuni*-positive samples was 5.4% (10/185; 95% CI, 2.6%–9.7%). Fecal samples from these dogs were of normal consistency (seven fecal samples) or semi-formed (three samples).

4.4 Discussion

In the present study, the apparent prevalence of fecal *Campylobacter* shedding among shelter dogs in Texas was approximately 76 percent. Though high, this prevalence is consistent with other studies. Examining fecal samples from 26 healthy pet dogs on a monthly basis, Hald et al (2004) detected *Campylobacte*r in over 76% of their 366 total fecal specimens. A similar study of household dogs in the Netherlands detected *Campylobacter* in 77% (23/30) of fecal specimens (Koene et al., 2004). Studies with larger sample sizes than those of Hald et al (2004) and Koene et al (2004), however, often report lower prevalence. A study of 72 puppies detected *Campylobacter* in 21% of the animals (Hald and Madsen, 1997), another in 41% of 261 dogs (Wieland et al., 2005), and another in almost 47% of 130 dogs (Workman et al., 2005). As the sample sizes of the latter, larger studies are closer to that of the present study (one fecal sample from each of the 185 dogs), it is important to remark on factors that can contribute to variations in reports of *Campylobacter* prevalence. Most salient among these factors are differences in study geographic location, study population, and study methodology. Reports on canine *Campylobacter* shedding hail from a variety of locations across the

globe, from Denmark to Barbados. Given that national and/or regionally-based social, cultural or medical practices may influence bacterial detection and distribution (Kwaga et al., 1989), study location may play a role in study-to-study *Campylobacter* prevalence variation. Likewise, the study populations themselves complicate direct inter-study comparisons. Some studies, for example, include only animals from specific age groups (puppies), while others include only canines from specific origins (household pets, laboratory animals, veterinary clinic clients). For bacteria like *Campylobacter*, choice of study population may be particularly salient to detection prevalence as research suggests that stray dogs have especially high levels of *Campylobacter* carriage (Workman et al., 2005) and that animal age and physiologic stress can impact animal susceptibility to bacterial pathogens (Verbrugghe et al., 2012).

Beyond the potential impacts of study location and study population, there is the not inconsiderable influence of study methodology. Due to the fastidious nature of *Campylobacter*, choice of detection method is especially significant (Monfort et al., 1989; Bourke et al., 1998; Labarca et al., 2002; Koene et al., 2004). Many studies use culture-based bacterial detection methods, rather than the more sensitive genetic methods used in the present study, and therefore may report lower bacterial prevalence. Thus, study methodology alone or in conjunction with study geographic location and study population may contribute to the variation in reports of canine *Campylobacter* prevalence. When it comes to more specific aspects of canine *Campylobacter* epidemiology, however, additional influences are worth consideration.

Beyond overall *Campylobacter* prevalence, specific information about bacterial epidemiology—from species data to shedding risk factors—greatly assist with the preservation and augmentation of public health. To this end, the present study examined the prevalence of two of the most commonly identified agents of human campylobacteriosis: *Campylobacter coli* and *Campylobacter jejuni*. *Campylobacter coli* was undetectable in our samples and the prevalence of *Campylobacter jejuni* was low (7.1%). These findings are consistent with at least two previously published studies, one of which detected *Campylobacter coli* in 0.7% of samples and *Campylobacter jejuni* in 19.4% (Hald et al., 2004), and the other which found prevalences of 1.1% and 5.7% for *Campylobacter coli* and *Campylobacter jejuni*, respectively (Wieland et al., 2005). Notably, however, not a few studies have detected higher levels (anywhere from 52-93%; Workman et al., 2005; Labarca et al., 2002) of *Campylobacter jejuni*, possibly due to study location, study population, and/or study methodology as detailed above for overall genus prevalence. Species prevalence for *Campylobacter*, however, may also reflect the influence of animal reservoirs; *Campylobacter jejuni*, for example, may be less prevalent among canines than among poultry. Additionally, some research suggests that the predominant *Campylobacter* species among canines—*Campylobacter upsaliensis*—may be the difficult to culture (Labarca et al., 2002; Hald, et al., 2004; Wieland et al., 2005). Regrettably, at the time of our study, we were unable to test for this species using a primer set. Even so, we hypothesize that *Campylobacter upsaliensis* or some of the other species found in dogs (*Campylobacter lari*, *Campylobacter helveticus*) make up a portion of the overall *Campylobacter* prevalence reported in the

present study. Significantly, as there may be species-to-species variation in risk factors for *Campylobacter* carriage (Wieland et al., 2005), *Campylobacter* species itself may underlie some of the risk factor findings in the present study.

A priori it is, at present, impossible to accurately predict whether or not *Campylobacter* or a given *Campylobacter* species will be present in canine fecal material. In order to investigate putative indicators of bacterial presence, the present study examined the relationship between *Campylobacter* detection and several different variables: dog age (< 1 year old or ≥ 1 year old), sex, neuter status (neutered or intact), duration of stay in shelter (in days), and fecal sample consistency (normal = fecal consistency score of 1; other= fecal consistency scores of 2 or 3). With the exception of dog sex and duration of stay in shelter, none of these variables showed meaningful association with detection of *Campylobacter* on preliminary analysis. Such results are both consistent with and different from previous reports. At least one study has documented a relationship between *Campylobacter* shedding, diarrhea, and young dogs (Hald and Madsen, 1997; Wolfs et al., 2001), perhaps because of age-related immune system immaturity. No such relationship was detected in the present research, possibly because of the small number of dogs less than 12 months old, the small number of diarrheic feces, and/or the lack of any true age-diarrhea-*Campylobacter* association. The observation that all diarrheic puppies (dogs less than 12 months old) in our study yielded feces with detectable *Campylobacter* casts doubt on the latter explanation, although the numbers are too small (there were only 3 diarrheic puppies out of the total sample size of 185) to allow for confidence in generalization. Looking at the relationship between age

alone and *Campylobacter* presence, however, published reports appear to allow for a stronger generalization, suggesting that younger dogs are more likely to shed the bacteria (Wieland et al., 2005; Workman et al., 2005), although the nature of the relationship may depend on the species of *Campylobacter* (Wieland et al., 2005). For diarrhea alone, the conclusions are more convoluted. A few studies remark that diarrheic feces may be more likely to harbor *Campylobacter* (Hald et al., 2004; Wolfs et al., 2001), an idea in concurrence with the bacteria's sensitivity to oxygen and desiccation (Koene et al., 2004). As in the present study, however, many published reports document no meaningful connection (Workman et al., 2005), a lack that agrees with the observation that pets rarely show clinical signs of *Campylobacter* infection (Damborg et al., 2004). Relative to diarrhea, the situation is the reverse for dog sex, with the present study and at least one other (Hald et al., 2004) detecting more *Campylobacter* among male dogs than among female dogs, while other studies report a lack of sex-*Campylobacter* relationship (Workman et al., 2005). In defense of our findings, there are two plausible explanations for why sex could matter. For one, behavioral differences between male and female dogs could result in different levels of bacterial exposure. For another, hormonal differences could influence immune responses and the host-pathogen relationship (Klein, 2000). The strong influence of other variables (study location, study population, study design, etc.) could obscure the effects of sex, thereby accounting for study-study discrepancies over its significance.

Different from many of the variables discussed above, there are relatively few discrepancies regarding neuter status and duration of stay in shelter for the simple reason

that almost no studies report findings about these factors. The lack of reporting may be attributable to study population characteristics (i.e. all dogs having the same neuter status or duration of shelter stay), failure to consider neuter status or duration of stay as variables, or publication bias (failure to report an absence of significance). For neuter status, the lack of significance found in the present study lends credence to the latter hypothesis. With regard to duration of stay in shelter, however, the present study did find an association between the variable and *Campylobacter* detection; longer stays were associated with lower detection. If replicated in future studies, this finding could serve as a positive reflection on care in Texas veterinary shelters.

Future studies may also help to address the limitations and to build on the strengths of the present research. As with many studies, missing data limits the ability to draw conclusions. The precise origins and history of many dogs presenting to animal shelters is often unclear. Categorization of dogs as puppy (dogs <1 year) or adult (dogs ≥ 1 year) is, thus, an approximation based on shelter records or veterinary assessment, respectively. Fortunately, the many strengths of our study allow for greater confidence in the analysis of other study components. Unlike much previous research on the subject, our study features a large sample size, multiple shelters throughout Texas, an extended study time-frame, and the inclusion of oft-unreported variables. Together, these factors contribute to the strength and applicability of our findings.

In summary, detection of *Campylobacter* in canine feces appears to have a marginal association with abnormal fecal consistency, and demonstrated no statistically significant relationship to dog age group or origin. Likewise, association with dog sex

and duration of stay in shelter was not statistically significant on multivariable analysis. Additional research should examine the relationship between fecal shedding, dog sex, and dog duration of stay in shelter. Predictive factors aside, feces of all types from dogs of all backgrounds have the potential to harbor culturable *Campylobacter* representing a variety of pathogenic species associated with human and animal disease. *C. jejuni* and *C. coli* are two of these pathogenic species, and while the present study did not detect any *C. coli*, it did yield several samples from different shelters which were positive for *C. jejuni*. Interestingly, the duration of canine *C.jejuni* shedding may be shorter than that of other *Campylobacter* species (Haled et al., 2004; Parsons et al., 2011), which has implications for the extent of environmental contamination, the likelihood of detection, and the window of exposure for direct transmission. Notably, the present study is perhaps the first to report on the potential influence of duration of shelter stay on shelter dog bacterial shedding. Since this variable did not show statistical significance on multivariable analysis, however, additional studies are necessary to further elucidate its role in canine bacterial shedding. All things considered, canines are clear participants in the epidemiology of *Campylobacter*, and their role has implications for humans and animals alike.

5. SUMMARY AND CONCLUSIONS

5.1 Summary

As expected, there was detectable fecal *Salmonella* shedding among the 554 Texas shelter dogs sampled in this research. Although the prevalence of *Salmonella* was relatively low (5%), over 11% of isolates demonstrated some antimicrobial resistance. Moreover, there were numerous serotypes represented among the detected *Salmonella*, many of which have been linked to human cases of disease, and a few of which may not have been previously detected in dogs. Also not widely reported elsewhere was the potential association of canine neuter status with the fecal detection of *Salmonella*. Fecal consistency likewise showed an association with fecal presence of the bacteria.

Indicators for *Campylobacter* shedding were less evident than those for *Salmonella*. Only dog sex and duration of stay in shelter showed mild association with detection of the pathogen in feces. Nevertheless, *Campylobacter* was detected at a high prevalence of over 75% in this study. While none of the detected *Campylobacter* appeared to be *Campylobacter coli*, over 7% was identifiable as *Campylobacter jejuni*, a major human pathogen.

Along with fast delivery of results and sensitivity, detection to the species level is one of the strengths of the culture-independent *Campylobacter* qPCR detection method used in the present research. The culture-independent qPCR method was far more

sensitive than the traditional culture methods used. The culture methods, however, did provide bacterial isolates able to be preserved for additional, future assessments.

5.2 Conclusions

Zoonotic pathogens can be transmitted in a variety of different ways and can reside in places near and far. For *Salmonella* and *Campylobacter*, transmission can be related to animal contact and is often fecal-oral. Potential sources of bacterial exposure are also near at hand; puppies and adult dogs are capable of carrying and shedding viable *Salmonella* and *Campylobacter*. Improved awareness, detection, and epidemiologic understanding of this shedding will enable better source tracking, allow for more targeted disease control measures, and provide for more efficacious disease prevention, for humans and canines alike.

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APPENDIX

Table 2.1: Results of bivariable analysis of potential risk factors for positive *Salmonella* shedding status among dogs at seven animal shelters across Texas, USA, May 2013 – December 2014. Reprinted with permission from (Leahy et al., 2016). Copyright 2016 by Blackwell Verlag GmbH.

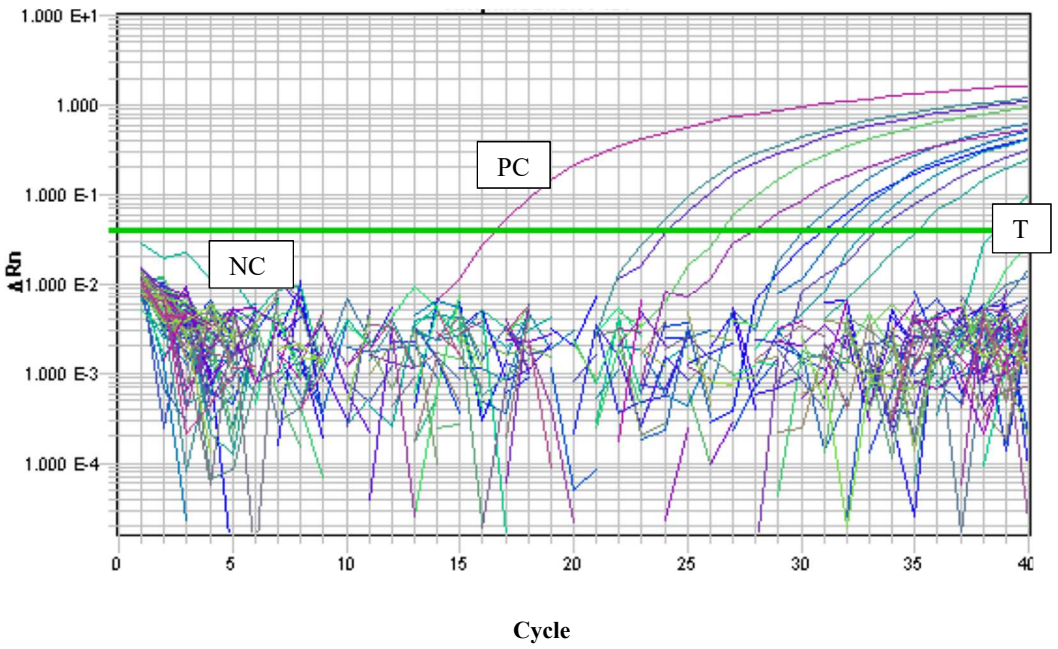
Variable	Positive results, No. (%)	Negative results, No. (%)	*P*
Fecal consistency			0.03
Normal	14 (3.9)	347 (96.1)	
Semi-formed	4 (3.7)	104 (96.3)	
Watery	4 (14.8)	23 (85.2)	
Age			0.7
Adult (≥ 1 year old)	22 (4.6)	452 (95.4)	
Puppy (< 1 year old)	4 (5.6)	67 (94.4)	
Sex			0.9
Female	13 (4.9)	250 (95.1)	
Male	14 (4.8)	276 (95.2)	
Origin			0.1
Stray	20 (6.7)	280 (93.3)	
Surrendered	2 (2.4)	80 (97.6)	

Table 2.2: Distribution of *Salmonella* serotypes identified among 27 canine isolates from Texas, USA, May 2013-December 2014. Reprinted with permission from (Leahy et al., 2016). Copyright 2016 by Blackwell Verlag GmbH.

Serotype	No. of isolates	% of isolates
Newport[a]	6	22
Javiana[b]	4	15
Braenderup[c]	2	7
Infantis[d]	2	7
Agbeni	1	4
Anatum	1	4
Derby	1	4
Dusseldorf/Albany	1	4
Heidelberg	1	4
Ibadan/Mississippi	1	4
No ID Possible	7	26

[a] Isolated from dogs in four shelters; [b] Isolated from dogs in three shelters; [c] Isolated from dogs in two shelters; [d] Isolated from dogs in two shelters

Figure 3.1 Representative amplification curve from *Campylobacter* 16S qPCR analysis of canine fecal samples from Texas, USA, September 2015 – December 2015



PC = positive control; NC = negative control; T= threshold

Table 3.1 Assessment measures comparing qPCR results to results from culture of *Campylobacter* from canine fecal samples from Texas, USA, September 2015 – December 2015

Assessment	Comparison	Specificity (%)	Exact McNemar P value	Kappa	Concordance
qPCR vs Culture	qPCR vs Culture	44.6%	<0.0001	0.4170	0.692
Plate Type (Media)*	qPCR vs CAT Plates	39.5%	<0.0001	0.3277	0.621
	qPCR vs CEF Plates	38.1%	<0.0001	0.3024	0.599
Plating Method	qPCR vs Direct	38.1%	<0.0001	0.3024	0.599
	qPCR vs Indirect	35.4%	<0.0001	0.2491	0.549
Plate-Plating Method Combinations	qPCR vs CAT + Direct Plating	34.6%	<0.0001	0.2323	0.533
	qPCR vs CAT + Indirect Plating	32.1%	<0.0001	0.1794	0.478
	qPCR vs CEF + Direct Plating	32.8%	<0.0001	0.1948	0.495
	qPCR vs CEF + Indirect Plating	33.1%	<0.0001	0.2000	0.500

* CAT= mCCDA-CAT; CEF= Campy Cefex Agar

Table 3.2 Assessment measures comparing different methods of culturing *Campylobacter* from canine fecal samples from Texas, USA, September 2015 – December 2015

Assessment	Comparison	Sensitivity (%)	Specificity (%)	Exact McNemar *P* value	Kappa	Concordance
Plate Type (Media)	CAT vs CEF	80.0	85.6	0.5847	0.6470	0.836
	CEF vs CAT	75.4	88.6			
Plating Method	Direct vs Indirect	67.9	78.9	0.2327	0.4474	0.755
	Indirect vs Direct	58.5	84.9			
Reference= CAT Direct	CEF Direct vs CAT Direct	63.5	90.8	0.2810	0.5660	0.831
	CEF Indirect vs CAT Direct	44.2	82.3	0.4885	0.2749	0.714
	CAT Indirect vs CAT Direct	50.0	87.7	0.1641	0.4000	0.778
Reference= CAT Indirect	CEF Direct vs CAT Indirect	55.8	85.0	0.8746	0.4017	0.781
	CEF Indirect vs CAT Indirect	79.1	90.8	0.5235	0.6766	0.880
	CAT Direct vs CAT Indirect	61.9	81.4	0.1641	0.4000	0.778
Reference= CEF Direct	CAT Direct vs CEF Direct	73.3	86.2	0.2810	0.5660	0.831
	CAT Indirect vs CEF Direct	53.3	86.2	0.8746	0.4017	0.781
	CEF Indirect vs CEF Direct	60.0	85.5	0.8714	0.4484	0.792
Reference= CEF Indirect	CAT Indirect vs CEF Indirect	72.3	93.4	0.5235	0.6766	0.880
	CAT Direct vs CEF Indirect	50.0	78.9	0.4885	0.2749	0.714
	CEF Direct vs CEF Indirect	57.5	86.8	0.8714	0.4484	0.792

Table 4.1: Results of bivariable analysis of potential risk factors for positive *Campylobacter* shedding status among dogs at seven animal shelters across, Texas, USA, May 2013 – December 2014. Reprinted with permission from (Leahy et al., 2017). Copyright 2017 by Blackwell Verlag GmbH.

Variable	Positive results, No. (%)	Negative results, No. (%)	*P*
Fecal consistency			0.5
Normal	100 (73.5)	36 (26.5)	
Semi-formed	33 (80.5)	8 (19.5)	
Watery	7 (87.5)	1 (12.5)	
Age			0.8
Adult (≥ 1 year old)	122 (75.3)	40 (24.7)	
Puppy (< 1 year old)	18 (78.3)	5 (21.7)	
Sex			0.1
Female	66 (71.0)	27 (29.0)	
Male	74 (80.4)	18 (19.6)	
Origin[a]			0.6
Stray	76 (78.4)	21 (21.6)	
Surrendered	25 (73.5)	9 (26.5)	

[a] Origin not available for 54 (29.2%) dogs

Table 4.2: Prevalence and species distribution of *Campylobacter* in canine fecal samples, Texas, USA, September – December 2014	
Campylobacter	**No. (%) of qPCR-positive fecal samples**
Campylobacter (all species)	140 (75.7)
Campylobacter jejuni	10 (7.1)
Campylobacter coli	0 (0.0)