Molecular Epidemiological study of *Campylobacter* spp. Carriage in Mammalian Wildlife and Livestock on Southern Ontario Farms

by

Mythri Viswanathan

A Thesis presented to The University of Guelph

In partial fulfilment of requirements for the degree of Master of Science in Population Medicine

Guelph, Ontario, Canada

© Mythri Viswanathan, 2015
ABSTRACT

Molecular Epidemiological Study of Campylobacter spp. Carriage in Mammalian Wildlife and Livestock on Southern Ontario

Mythri Viswanathan
University of Guelph, 2015

Advisors:
Dr. David L. Pearl
Dr. Claire Jardine

This thesis is focused on Campylobacter carriage in mammalian wildlife and livestock in southern Ontario. Multi-level logistic regression models were constructed to investigate Campylobacter spp. and antimicrobial resistant Campylobacter spp. carriage in wildlife and livestock species on 25 farms. Samples were collected from dairy and beef cattle, swine and raccoons as well as a selection of other mammalian wildlife. Molecular subtyping data, produced by the Campylobacter–specific 40-gene comparative genomic fingerprinting assay (CGF40), were used to compare isolates from wildlife and livestock. Cluster analysis was conducted to visualize the groupings of wildlife and livestock C. jejuni isolates found. Wildlife and livestock carried Campylobacter at significantly different prevalences, had different antibiograms, and rarely shared the same CGF40 subtypes. Dendrogram and correspondence analysis indicated that the subtypes of Campylobacter circulating in livestock and wildlife populations were distinct. Combined, these results suggest that transmission between mammalian wildlife, especially raccoons, and livestock is limited.
ACKNOWLEDGEMENTS

I would like to sincerely thank the members of my advisory committee Dr. David Pearl, Dr. Claire Jardine, and Dr. Eduardo Taboada for their mentorship and guidance throughout the completion of this thesis. Dr. Pearl, thank you for your continued support and patience, I greatly appreciate the time and effort you have dedicated to teaching and mentoring me, I am extremely lucky to have had you as an advisor. Dr. Jardine, thank you for your constant understanding and your enthusiasm about this project. Dr. Taboada, thank you for all that you have contributed and your efforts in collaborating from a distance and from two hours earlier.

Thank you to the OVC for their financial support and the Public Health Agency of Canada and the Canadian Wildlife Health Cooperative for providing the data that made this project possible. I would also like to express my gratitude to the field work team who laboriously collected the samples in this study.

I would like to say a special thank you to all my friends and family who have been so gracious in dealing with my highs and lows throughout this process. You have all been there to celebrate my victories with me and also lend an ear when things didn’t appear to be going right which has made this process manageable. To my lab-mates, and friends in clinical research thank you for always making me laugh, I will miss being in this office. Last but not least, thank you to my parents who have always encouraged me to achieve, I would not have been able to do this without you.
STATEMENT OF WORK

Bacterial isolation, antimicrobial susceptibility testing and molecular subtyping were performed by Public Health Agency of Canada personnel. Specifically, sampling and isolation data were collected from the C-EnterNet (now FoodNet Canada) program, antimicrobial resistance data were obtained from the CIPARS program, and molecular subtyping data were collected from the Lab for Foodborne Zoonoses, Lethbridge.

All data cleaning, merging, assessment, and statistical analyses were performed by Mythri Viswanathan. Writing and manuscript generation were performed by Mythri Viswanathan. Editing assistance for this thesis in its entirety was received from Drs. David Pearl, Claire Jardine, and Eduardo Taboada.
# TABLE OF CONTENTS

Acknowledgements ...................................................................................................................... iii  
Statement of work .......................................................................................................................... iv  
Table of contents .......................................................................................................................... v  
List of tables ....................................................................................................................................... vii  
List of figures ....................................................................................................................................... ix  

CHAPTER 1: Introduction, literature review, study rationale and objectives ............................... 1

Introduction ......................................................................................................................................... 1  
Literature Review ............................................................................................................................... 2  
Epidemiology of *Campylobacter* species in humans ................................................................. 2  
Prevalence and epidemiology of *Campylobacter* species in agricultural and wildlife species .............................................................................................................................. 5  
Wildlife as foodborne pathogen reservoirs and raccoon pathogen transmission-associated behaviours ............................................................................................................................... 8  
Microbiology and antimicrobial resistance of *Campylobacter jejuni* & *Campylobacter coli* ........................................................................................................................................ 10  
Molecular subtyping methods for *Campylobacter jejuni* & *Campylobacter coli* ............... 11  
Cluster analysis methods for molecular epidemiology studies ............................................. 14  
Study rationale and objectives ................................................................................................. 16  
References ......................................................................................................................................... 18  

CHAPTER 2: Molecular and statistical analysis of *Campylobacter* carriage and antimicrobial resistance in wildlife and livestock from Ontario farms ........................................ 34

Abstract .......................................................................................................................................... 35  
Introduction ....................................................................................................................................... 36  
Methods .......................................................................................................................................... 37  
Results .............................................................................................................................................. 43  
Discussion ........................................................................................................................................ 46  
Conclusions ...................................................................................................................................... 50  
References ........................................................................................................................................ 51
CHAPTER 3: Cluster analysis of Campylobacter jejuni genotypes isolated from mammalian wildlife and bovine livestock from Ontario farms ....................................................67

Abstract ......................................................................................................................................68
Introduction ..................................................................................................................................69
Methods .........................................................................................................................................70
Results ............................................................................................................................................74
Discussion .......................................................................................................................................76
Conclusions .....................................................................................................................................79
References .......................................................................................................................................80

CHAPTER 4: Summary discussion and conclusions ........................................................................89

Limitations and Strengths ..............................................................................................................92
Future Studies ..................................................................................................................................93
Conclusions .....................................................................................................................................95
References .......................................................................................................................................96
LIST OF TABLES

Table 2.1: Frequency of *Campylobacter* spp. and antimicrobial resistant *Campylobacter* in wildlife and livestock samples from 8 swine, 8 beef, and 9 dairy farms in Ontario, Canada .................................................................60

Table 2.2: Univariable mixed logistic model with a random effect for location for the following outcomes; shedding of *Campylobacter* spp., shedding of *C. jejuni*, and shedding of *Campylobacter* resistant to ≥ 1 antimicrobials tested ........................................61

Table 2.3: Mixed logistic multivariable model with a random effect for location assessing the association between shedding *Campylobacter* spp. and sample type and farm type ..........63

Table 2.4: Significant contrasts obtained from univariable mixed-logistic models with a random effect for location for the following outcomes: shedding of *Campylobacter* spp. and shedding of *C. jejuni* ...........................................................................................................64

Table 2.5: CGF40 subtypes identified more than once from wildlife and/or livestock samples ........................................................................................................................................65

Table 2.6: Mixed logistic multivariable model with random effect for location assessing the association between antimicrobial resistance to ≥ 1 antimicrobials and sample type and *Campylobacter* species ..............................................................................................................66

Table 3.1: Mixed logistic models with a random effect for farm location assessing the association between sample type and being in the ‘wildlife cluster’ and association between animal species and being in the ‘wildlife cluster’ as defined by unweighted pair-group method with arithmetic mean (UPGMA) dendrogram visual identification, and multiple correspondence analysis ..........................................................................................87
Table 3.2: Exact logistic model assessing the association between each CGF40 gene and being a livestock sample versus a wildlife sample.
LIST OF FIGURES

Figure 3.1: Simple-matching unweighted pair group method with arithmetic mean (UPGMA) dendrogram of *C. jejuni* isolates with visually identified wildlife and livestock clusters .................................................................84

Figure 3.2: Two dimensional multiple correspondence analysis (MCA) scores of wildlife and livestock *C. jejuni* subtypes..................................................................................................................85

Figure 3.3: Multiple correspondence analysis (MCA) coordinate plot showing weighting of presence (1) and absence (0) of CGF40 genes by colour calculated by multiple correspondence analysis for first two dimensions .................................................................86
CHAPTER 1:

INTRODUCTION, LITERATURE REVIEW, STUDY RATIONALE
AND OBJECTIVES

INTRODUCTION

Campylobacter spp., including C. jejuni and C. coli, are the most common bacterial causes of gastrointestinal illness in humans in the developed world (Wilson et al., 2008). Infection in humans typically causes symptoms including vomiting, diarrhea and fever (Blaser et al., 1979). Although infection in humans is typically self-limiting (Blaser et al., 1983), the burden that chronic Campylobacter-associated diseases place on the healthcare system makes it a significant pathogen of concern (Public Health Agency of Canada, 2014). Campylobacter jejuni has been identified as a common antecedent to Guillain-Barré syndrome and reactive arthritis (Nachamkin et al., 2002), highlighting the importance of preventing the initial infection. Campylobacter spp. frequently asymptomatically colonize the intestinal tracts of food animals (Butzler et al., 1999), which impacts the farm-to-fork food safety continuum as contaminated meat products are the main source of human exposure (Young et al., 2007). Campylobacter spp. have also been detected in wild animals, however, the role of wildlife species in the epidemiology of Campylobacter in livestock and humans is not well understood. This thesis will explore the possible sharing of Campylobacter species between mammalian wildlife and livestock and describe the diversity of molecular subtyping of Campylobacter circulating in wildlife populations found on farms, and in beef and dairy cattle and swine populations. The ultimate aim is to understand the potential sources of Campylobacter for
human infection, the potential role of wildlife in the transmission of this pathogen to food-producing animals, and to determine if there is a role for wildlife control in farm-level strategies aimed at controlling foodborne diseases. This literature review will summarize the following topics relevant to the sharing of *Campylobacter* between wildlife populations and livestock populations:

1. Epidemiology of *Campylobacter* species in humans

2. Prevalence and epidemiology of *Campylobacter* species in agricultural and wildlife species

3. Wildlife as foodborne pathogen reservoirs and raccoon pathogen transmission-associated behaviours

4. Microbiology and antimicrobial resistance of *Campylobacter jejuni* & *Campylobacter coli*

5. Molecular subtyping methods for *Campylobacter jejuni* & *Campylobacter coli*

6. Cluster analysis methods for molecular epidemiology studies

**LITERATURE REVIEW**

**Epidemiology of *Campylobacter* species in humans**

*Campylobacter* spp. are known widely to be the foremost bacterial cause of gastroenteritis in developed nations (WHO, 2011) with an adjusted estimated incidence in Canada of 145,350 cases per year (PHAC, 2014) and 2.1-2.4 million cases per year in the United States (Alkettruse et al., 1999). This adjusted incidence accounts for underestimation due to underreporting and due to the difficulty of *Campylobacter* spp. isolation during diagnosis (Vandamme, 2000). The majority of *Campylobacter* infections in developed countries are caused by *Campylobacter jejuni* and *Campylobacter coli*
(Phillips, 1995), however, other species including *Campylobacter upsaliensis* (Labarca et al., 2002), and *Campylobacter lari* (Lastovica et al., 2000) are also implicated in human infection. When examining the age distributions and dynamics of *C. jejuni* infections in developed and developing nations, epidemiological differences are noted. In developed nations, such as Canada, campylobacteriosis is most common in children less than 1 and adults between the ages of 15 and 44 years old (Acheson et al., 2001). In contrast, in developing nations most symptomatic *C. jejuni* infections are in children less than two while adult populations are largely asymptomatic (Coker et al., 2002). The reason for these different clinical manifestations is not well known. *Campylobacter jejuni* infections usually arise as sporadic cases, but outbreaks have been identified (Wesley et al., 2000).

A variety of sources of *Campylobacter* spp. causing human infections have been identified. The most common sources of the pathogen are contaminated raw or undercooked meat products, unpasteurized dairy products as well as contaminated water and ice (Young et al., 2007). Both *C. jejuni* and *C. coli* have limited survival capabilities in environmental sources such as water, but studies by Axelsson-Olsson et al. (2005) found that *C. jejuni* strains are able to survive in water for significantly longer periods of time by associating with protozoa like the highly resilient protozoan *Acanthamoeba polyphaga*. *Campylobacter*-contaminated ice and water are a concern in developing countries as they are linked to travellers’ diarrhea (Hill et al., 2008). However, risk factors for *Campylobacter* infection of residents in the developing world have been identified as contaminants such as garbage and animals in cooking areas, and lack of proper water sanitation (Rao et al., 2001).

Symptoms of *Campylobacter jejuni* infections in humans include abdominal pain,
diarrhea and fever. These infections are usually self-limiting in healthy adults and resolve within a week (Katley et al., 2005). These campylobacteriosis cases cost approximately $1846 USD per case due to loss of productivity for both the caregiver and person with illness and medical costs (Scharff, 2011). In developing countries, most cases of adult \textit{C. jejuni} infections remain asymptomatic and are much less severe than cases in developed countries (Coker et al., 2002). In rare instances, \textit{Campylobacter jejuni} infections can give rise to severe sequelae including Guillain-Barré syndrome (GBS), reactive arthritis (ReA) and irritable bowel syndrome (IBS) (WHO, 2012).

Guillain-Barré syndrome is the most common cause of acute flaccid paralysis since control measures have been put in place for polio (Ketley et al., 2005). The syndrome generally begins with loss of motor and sensory functions in the legs and can spread to affect both the arms and the trunk of the body (National Institute for Neurological Disorders & Stroke, 2015). It is currently predicted that approximately 31\% of GBS cases are preceded by \textit{Campylobacter} infection and that these cases are more severe and have poorer outcomes, such as death and severe disability, when compared to cases not preceded by \textit{Campylobacter} infection (Rees et al., 1995). Due to the complexity and need for supportive care during recovery, the burden of disease in developed countries is considerable. Studies assessing the economic cost of \textit{Campylobacter}-associated GBS in the United States found that the mean cost per patient with GBS was approximately $320,000 USD (Frenzen, 2005) with an overall annual cost of $0.2 to $1.8 billion USD (Buzby et al., 1997).

Reactive arthritis is seen in approximately 1-5\% of campylobacteriosis cases globally (WHO, 2012) and is characterized by joint pain and swelling (Kvien et al.,
Among enteric bacterial pathogens in North America, *Campylobacter jejuni* is the most common infection preceding ReA (Townes et al., 2008). A large population-based case-control study conducted by Hannu et al. (2002) in Finland found that among 45 patients with ReA preceded by *Campylobacter* infection, 37 were shedding *C. jejuni* and the remaining 8 were shedding *C. coli*, indicating that *C. coli* infection also has importance when considering ReA (Hannu et al., 2002). Due to the unstandardized diagnostic criteria for ReA, the burden of disease has not been studied in depth. This problem persists because diagnosing campylobacteriosis cases is difficult, and in most cases cannot be established after the onset of arthritic symptoms. However, it has been established that of all ReA cases, approximately 25-50% of patients develop acute arthritis after reactive arthritis and approximately 25% develop chronic spondyloarthritis (Hannu et al., 2006). The development of chronic cases raises concern about the large health burden of *Campylobacter*-triggered ReA.

**Prevalence and epidemiology of *Campylobacter* species in agricultural and wildlife species**

Agricultural animal species have been shown to carry *Campylobacter* species with no clinical signs of infection (Gyles et al., 2008). The most common food-animal species linked to human disease cases in North America are poultry, beef cattle, dairy cattle, and swine (Young et al., 2007). Poultry has been identified as the most important source of human exposure to *C. jejuni*. The prevalence of *C. jejuni* among broiler flocks ranges from 42% to 100% in developed nations (Jacobs-Reitsma et al., 1998, Newell et al., 2003, Humphrey et al., 2009). It has been established that flocks enter processing
plants with significant amounts of *Campylobacter* which then easily spreads among chicken carcasses during processing (Keener et al., 2004), indicating that the introduction of *Campylobacter* on farm can lead to higher prevalence down the farm-to-fork food continuum. Surveillance of retail meat in Canada in 2010 found *Campylobacter* spp. in 1%, 2%, and 36% in ground beef packages, pork chops, and chicken breasts, respectively (Public Health Agency of Canada, 2014). Identical sequence types, identified by MLST, of *Campylobacter* have been isolated from wild birds and poultry sampled on farm indicating that wildlife may serve as a source or transmitter of pertinent subtypes of *C. jejuni* (Kwan et al., 2008). Few studies have focused on the sources of *Campylobacter* for mammalian agricultural species (e.g., dairy cattle, beef cattle and swine) that are also implicated in *Campylobacter* outbreaks and sporadic cases through contaminated products such as raw milk, and raw or undercooked pork and beef (Young et al., 2007).

Studies have established that both swine and cattle are highly susceptible to colonization by *Campylobacter* spp. after birth (Young et al., 2001, Wesley et al., 2000). However, these two livestock species are more likely to shed different bacterial species; swine shedding *C. coli* and cattle shedding *C. jejuni* in most cases. In dairy operations in England and the United States, the prevalence of *Campylobacter* spp. carriage among mature dairy cattle ranges between 2% and 51% (Wesley et al., 2000, Atabay et al., 1998, Meanger et al., 1989, Englen et al., 2007, Kwan et al., 2008). The prevalence of carriage can vary within this range among seasons, and dairy cattle shed more *Campylobacter* during spring and autumn (Beach et al., 2002). The prevalence of *Campylobacter* spp., measured in U.S studies, has ranged between 2 and 9.2% in raw bulk tank milk (Jayarao et al., 2001, Jayarao et al., 2006). Researchers in Canada and the U.S have found that the
prevalence of *Campylobacter* spp. in beef cattle ranges between 64% and 89.4% without seasonal variation, based on pooled pen samples and individual animal samples (Inglis et al., 2005, Guévremont et al., 2014, Beach et al., 2002, Stanley et al., 1998).

Ontario swine predominantly shed *C. coli* and studies have found the prevalence of fecal shedding ranges between 66% and 100% in grower-finishers (Varela et al., 2007). Nursery pigs did not shed measurable levels of *C. coli* in faecal samples at the time of birth, but the prevalence increased dramatically within seven days to 32.4% (Alter et al., 2005). It has also been noted that the prevalence of *C. coli* in swine has not been associated with farm management practices, but there is speculation that more intensive approaches to food-animal production favour re-infection with pathogens, leading to higher prevalences (Oporto et al., 2007).

Mammalian wildlife found on farms, and in urban areas also carry *Campylobacter* species. In a small study of eleven raccoons, 36% were shedding *C. jejuni* (Hamir et al., 2004). A study done in Sweden, examining avian and mammalian wildlife, found that the prevalence of *Campylobacter* spp. shedding in moose (*Alces alces*), deer (*Capreolus capreolus*), and hares (*Lepus* spp.) ranged between 1% and 4% (Wahlstrom et al., 2003). In the U.S., a study of small mammalian wildlife found a prevalence of 0% (Sippy et al., 2012), while similar studies have recovered *Campylobacter* spp. in small mammalian wildlife such as rabbits (Kwan et al., 2008), and house mice (*Mus musculus*) and brown rats (*Rattus norvegicus*) (Meerburg et al., 2006) at low prevalence. Many studies have focused on the role of avian wildlife in maintaining and transmitting pathogenic strains of *Campylobacter* spp. to avian livestock (Craven et al., 2000, Colles et al., 2008), but the potential of mammalian wildlife to do the same has not been studied in depth.
Wildlife as foodborne pathogen reservoirs and raccoon pathogen transmission-associated behaviours

Wildlife, including fish, mammalian, and avian species, have long been documented as reservoirs for many pathogens such as rabies, Nipah virus, Brucella spp. and Avian Influenza (Kruse et al., 2004). Urban, suburban and agricultural areas serve as habitats for both wildlife species and humans which exposes humans to wildlife and their excreta. Raccoons (Procyon lotor), specifically, are often found in close association to humans in urban areas due to their scavenging and urban-adapted behaviours. For the purposes of this review, a focus will be placed on studies involving mammalian wildlife, raccoons specifically, and their documented ability to transmit foodborne pathogens.

Studies have shown that a variety of important foodborne pathogens are maintained in many terrestrial wildlife reservoirs. For example, investigations by the CDC of an E. coli O157:H7 outbreak in 2006, linked the outbreak to wild swine maintaining the identical outbreak subtype in nature (Wendel et al., 2009). Deer also carry E. coli O157:H7 and Cryptosporidium and have been implicated in foodborne outbreaks associated with contaminated produce in the United States (Besser et al., 1993, CDC, 1997, Laidler et al., 2013). Horizontal transmission of Salmonella Typhirium between rodent and domestic cattle and swine has also been previously documented (Skor et al., 2008). It is evident that pathogens of public health significance are common in a variety of wildlife, but raccoons have the ability to inhabit a variety of habitats and their specific behaviours make them a species of particular interest in southern Ontario. The presence of Salmonella species in raccoons is well documented. Recent studies have revealed that raccoons can readily acquire different Salmonella serotypes since a variety
of subtypes can be isolated from animals sampled repeatedly (Jardine et al., 2011). These studies suggest that raccoons could play a significant role in maintaining and transmitting foodborne pathogens within a variety of settings.

Since raccoons have a broad habitat range they are often found in close proximity to humans and domestic animals in urban and agricultural settings (Rosatte et al., 2010). The flexible foraging and social behaviours of raccoons make them ideal candidates for carrying and transmitting pathogens in a variety of habitats. Their large home range sizes (3.4 km\textsuperscript{2}-3.9 km\textsuperscript{2}) enable them to potentially transmit pathogens among farms (Rosatte et al., 2010). Raccoons also share a common area for defecation, referred to as latrines, which have been recognized as potential sources for the transmission of pathogens among raccoons (Smith et al., 2008) and to other species that use latrines as sources of food (Page et al., 2001). Research by Bondo et al. (2014) has documented the presence of 
Salmonella serovars, implicated in human disease, on the paws of raccoons. This implies that mechanical transmission of foodborne pathogens by raccoons is also possible. Recent studies have also found that a small group of Campylobacter cases were associated with direct contact with raccoons at a wildlife rehabilitation centre (Saunders et al., 2014). It is highly possible that the combination of a large home-range size, latrine use, and the potential ability to transmit pathogens mechanically and biologically could mean that foodborne pathogens are frequently spread within raccoon communities, and subsequently to agricultural species and humans.
Microbiology and antimicrobial resistance of *Campylobacter jejuni* & *Campylobacter coli*

*Campylobacter jejuni* and *C. coli* have been isolated most frequently from human campylobacteriosis cases (Blaser et al., 2008). Both of these *Campylobacter* spp. are often found in similar environments due to their similar structural qualities, but research has established that there may be a difference in types of food exposures that result in human infections with *C. jejuni* and *C. coli* (Gillespie et al., 2002). Both bacterial species are microaerophilic, gram-negative bacteria that are rod-shape with a flagellum (Sanuel, 1996). The ability of *C. jejuni* and *C. coli* to thrive in microaerophilic environments makes them ideal candidates to colonize gastrointestinal tracts.

Antimicrobial resistance is a public health priority and the prevalence and patterns of resistance have been studied in a number of foodborne pathogens, including *Campylobacter* species. A study completed in Spain found the highest prevalence of antimicrobial resistant *Campylobacter* spp. occurs in agricultural species, specifically swine and broiler chickens (Sáenz et al., 1996), likely due to the use of antibiotics for disease treatment and growth promotion (Mathew et al., 2007). The prevalence of antimicrobial resistance (AMR) in isolates from swine (82%) and chickens (81%) from Belgium have been high (Sáenz et al., 2010). Both macrolide (Gibreel et al., 2006) and fluoroquinolone resistance have been documented in *Campylobacter* spp. (Aarestrup et al., 1999). The emergence of macrolide resistance in *Campylobacter* spp. has been observed mainly in swine *C. coli* isolates and poultry *C. jejuni* isolates (Van Looveren et al., 2001). While fluoroquinolone resistant *Campylobacter* has been most frequently isolated from poultry and poultry products (Nelson et al., 2007), it has also been present
in *Campylobacter* isolates from avian wildlife (Sippy et al., 2012). Tetracycline resistance is also common and has been documented in bacterial isolates from agricultural species, and is the most common type of resistance found in *Campylobacter* carried by wildlife species (Sippy et al., 2012). It has been noted that high levels of tetracycline resistance in wild species is due to common conjugative transfer of gene cassettes between *Campylobacter* strains (Velázquez et al., 1995, Ansary et al., 1992).

The emergence of resistance to macrolides and fluoroquinolones is of great public health concern as these classes of antibiotics are often used to treat human cases of campylobacteriosis. Most commonly fluoroquinolones and macrolides are used in extreme cases when the infection is not self-limiting (Aarestrup et al., 1999). Therefore, understanding if wildlife have the capacity to carry and transmit antimicrobial resistant subtypes between different farms is of great public health relevance.

**Molecular subtyping methods for *Campylobacter jejuni* & *Campylobacter coli***

A variety of methods have been developed for subtyping *Campylobacter* species. These methods are categorized as either phenotyping methods (e.g., biotyping) or genotyping methods (e.g., multi-locus sequence typing) (Fitzgerald et al., 2007). Typically in epidemiologic studies, genotyping methods are used to investigate clustered subtypes during *Campylobacter* outbreaks due to their high discriminatory power to differentiate strains, and because of their relative ease of use (Fitzgerald et al., 2001). For the purposes of this review only genotyping methods will be described further due to their relevance to this thesis.

The genotyping methods most commonly used for *Campylobacter* spp. are
multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), PCR-restriction fragment length polymorphism (RFLP) of the flagellin gene short variable region (flaA), DNA sequencing of the flagellin gene short variable region (flaA SVR sequencing), and random amplified polymorphic DNA (RAPD) (Fitzgerald et al., 2001). Previous studies have specifically identified PFGE and MLST as the most discriminatory methods for subtyping of *Campylobacter* (Ragimbeau et al., 2008) making them extremely useful in describing bacterial populations in an epidemiological context.

Before the development of MLST, PFGE with *Sma*I served as the most discriminatory form of *Campylobacter* subtyping (Nielsen et al., 1998). Briefly, this method exploits the *Sma*I macrorestriction profile of strains to identify a subtype. A standardized PFGE subtyping scheme was proposed by Ribot et al. (2001) as a robust and reproducible method that would allow for comparison of subtyping results of *Campylobacter* from multiple studies for the purpose of meta-analyses and critical appraisal. By optimizing suspension buffers and electrophoresis conditions this protocol gave high-resolution results. However, the use of this method was never universally accepted due to its vulnerability to point mutations that occur frequently in *Campylobacter* populations (Nielsen et al., 2000). Multilocus sequence typing gained prominence as the most discriminatory and reliable typing system for *Campylobacter* (Clark et al., 2012) as the vulnerabilities of PFGE became more obvious. This method explores the variance in seven housekeeping loci of *Campylobacter*, and is widely used today (Dingle et al., 2001). However, recent studies have proposed combining MLST with *flaA* short variable region sequencing to heighten discriminatory power and effectively identify sources during outbreak investigations (Sails et al., 2003). Due to the
large number of isolates obtained during surveillance initiatives, a more rapid, high-resolution, low-cost method with the potential to detect clusters of cases was in demand. The recently developed *Campylobacter*-specific 40-gene comparative genomic fingerprinting assay (CGF40) was developed to answer this demand and also designed to be highly discriminatory for use during outbreak investigations (Taboada et al., 2012).

CGF40 is a PCR-based method that assesses the absence and presence of 40 accessory genes across the whole *Campylobacter* genome (Taboada et al., 2012). This methodology was specifically created using *Campylobacter jejuni* and *Campylobacter coli* isolates of agricultural, environmental and human origin to include genes that were highly variable among samples from these sources (Taboada et al., 2012). In brief, CGF40 is a multiplex PCR that involves eight 5-plex PCRs for each individual isolate (Clark et al., 2012). Using gel electrophoresis, the presence/absence of each of the forty genes is determined and coded into binary data for further analysis. The discriminatory power and development of this method specifically for *Campylobacter* makes it ideal for epidemiological research related to source attribution. Using Simpson’s index of diversity, a calculation that indicates the probability of two unrelated strains being identified as different (Hunter et al., 1988), CGF40 has greater discriminatory power compared to any single method of *Campylobacter* subtyping including PFGE and MLST (Clark et al., 2012). It was also found that CGF40 alone was more discriminatory than any other two methods combined (Clark et al., 2012) serving as a testament to its superiority as a *Campylobacter* typing method. CGF40 also possesses a high concordance to MLST, as a number of distinct but similar CGF40 subtypes can be mapped to a single sequence type (Taboada et al., 2012). This feature will allow for future meta-analyses.
Cluster analysis methods for molecular epidemiology studies

Molecular epidemiology has gained prominence as a key tool in determining source attribution during outbreaks and providing data on strains present within a study population. By using cluster analyses to determine which strains are closely related to one another, molecular epidemiology studies add to our ability to make more informed and knowledgeable inferences. A variety of methods to create dendrograms are recommended depending on the typing method utilized during molecular subtyping. For instance, different similarity coefficients, like Jaccard and Dice, and clustering methods (e.g., neighbour-joining) are suggested for fingerprint and sequence data. However, we will focus on unweighted pair-group matching using arithmetic mean (UPGMA) methods and similarity coefficients most appropriate for the data produced from CGF40 typing subsequently used in chapters 2 and 3 of this thesis.

Dendrograms are often produced in epidemiological studies to assess and visualize the similarities between molecular subtypes (Hall et al., 2006). These methods utilize similarity matrices, developed by a specific similarity coefficient algorithm, and transform these measures into distances between strains and clusters using a clustering algorithm. In molecular epidemiology studies, the UPGMA clustering method is used most commonly (Hall et al., 2006). This method for producing dendrograms has received criticism especially when using it with sequence and fingerprint data, such as MLST and PFGE; the rooting mechanism and evolutionary distances produced can be erroneous with these types of data, but the use of UPGMA dendrograms is valid when examining groupings rather than investigating the order of descent or attempting to identify the position of a common ancestor (Hall et al., 2006). Unweighted pair group method with
arithmetic mean (UPGMA) is a hierarchical clustering method and the major assumption is that a single rate of recombination takes place among all the isolates (Gronau et al., 2007). When using the formulated tree to assess the general grouping of isolates this assumption is not a concern. When dealing with character data, it is recommended that a simple-matching similarity coefficient be used during UPGMA dendrogram creation so that presence and absence of characters are equally weighted (Mayer et al., 2004). This is why a simple-matching coefficient is recommended for the character data produced by CGF40.

Along with phylogenetic clustering methods, statistical methods to assess clustering are available. For instance, principal components analysis (PCA) is often used when continuous variables are being analyzed (Abdi et al., 2010). Typically used as a complexity reduction method, PCA assigns eigenvectors and values that capture the most variance in the data (Tipping et al., 1999). The eigenvector with the largest magnitude eigenvalue is the principal component (Tipping et al., 1999). In PCA analysis, these components represent alternative axes, to explain the data, which must be interpreted by the researcher (Tipping et al., 1999). In cases where researchers are working with categorical variables, multiple correspondence analysis (MCA) is considered the best method for dimensional analysis (Le Roux et al., 2010).

Multiple correspondence analysis is typically interpreted as a variation of PCA for categorical data (Abdi et al., 2010). Much like PCA, this method calculates eigenvectors/values based on a correspondence matrix, either the Burt matrix or the indicator matrix (Abdi et al., 2007). The indicator matrix simply contains the observations as rows and the variables to be examined as columns, the values for these
column variables can either be present (1) or absent (0) (Statacorp., 2013). The Burt matrix is often used for larger datasets, as it requires less memory, and is a cross-tabulation of the variables (Statacorp., 2013). Multiple correspondence analysis provides values called the principles of inertia which are analogous to eigenvalues produced in PCA (Greenacre, 2007). The percentage of explained variance by each dimension is also provided as the percentage of inertia, and much like PCA and factor analysis, it is most common that the first dimension captures the majority of the variance seen in the data (Johnstone, 2001). Results are often graphically displayed for ease of interpretation. The contributions of each point to each axis is calculated and is a statistic that indicates the weight of each variable to the axis (Le Roux et al., 2010). These contributions aid in the interpretation of each axis and it is of note that the distances seen are just meant to help interpret the separation of points and are not related to evolutionary relatedness (Abdi et al., 2010).

**STUDY RATIONALE AND OBJECTIVES**

*Campylobacter* is the leading bacterial cause of gastroenteritis and these infections are a significant burden of disease in Canada, with the majority of cases being caused by consumption of contaminated meat products. Food animals have a variety of pathogen sources when they are on farm, but wildlife pose an interesting threat as they can carry pathogens among farms. Therefore, understanding the role that mammalian wildlife, specifically raccoons, play in maintaining *Campylobacter* spp. and sharing subtypes with mammalian agricultural species is of great importance. In order to address this research gap this thesis has the following objectives:
1. Determine if there are significant differences in the prevalence of *Campylobacter* spp. carriage, and antimicrobial resistant *Campylobacter spp.* carriage among mammalian wildlife and livestock species (Chapter 2)

2. Assess if *Campylobacter spp.*, CGF40 subtypes, and antibiograms are similar between wildlife and livestock species that share environments (Chapter 2)

3. Assess if there are distinct groups of *Campylobacter* isolates recovered from mammalian wildlife and livestock species (Chapter 3)
REFERENCES


Clark, C. G., Taboada, E., Grant, C. C., Blakeston, C., Pollari, F., Marshall, B., rahn, K., MacKinnon, J., Daignault, D., Pillai, D. & Ng, L. K. (2012). Comparison of molecular typing methods useful for detecting clusters of *Campylobacter jejuni* and


CHAPTER 2:

MOLECULAR AND STATISTICAL ANALYSIS OF

*CAMPYLOBACTER* CARRIAGE AND ANTIMICROBIAL

RESISTANCE IN WILDLIFE AND LIVESTOCK FROM ONTARIO FARMS
ABSTRACT

The objectives of this study were to 1) compare the carriage of *Campylobacter* and antimicrobial resistant *Campylobacter* among livestock and mammalian wildlife on farms in Ontario, and 2) identify the potential sharing of *Campylobacter* subtypes between livestock and wildlife. Using data collected from a cross-sectional study of 25 farms in 2010, we assessed associations, using mixed logistic regression models, between *Campylobacter* and antimicrobial resistant *Campylobacter* carriage and the following explanatory variables: animal species (beef, dairy, swine, raccoon, other), farm type (swine, beef, dairy) and type of sample (livestock or wildlife), and *Campylobacter* species (*jejuni*, *coli*, other). Models included a random effect to account for clustering by location where samples were collected. Samples were subtyped using a *Campylobacter*-specific 40 gene comparative fingerprinting assay. A total of 92 livestock samples and 107 wildlife samples were collected, and 72% and 27% tested positive for *Campylobacter*, respectively. Pooled fecal samples from livestock were significantly more likely to test positive for *Campylobacter* than wildlife samples. Relative to dairy cattle, swine were at significantly increased odds of testing positive for *Campylobacter*. The odds of shedding *Campylobacter jejuni* was significantly greater in beef cattle compared to both dairy cattle and raccoons. Fifty unique subtypes of *Campylobacter* were identified and only one subtype was found in both wildlife species and livestock species. Livestock *Campylobacter* isolates were significantly more likely to exhibit antimicrobial resistance (AMR) compared to wildlife *Campylobacter* isolates. *Campylobacter jejuni* was more likely to exhibit AMR when compared to *C. coli*. However, *C. jejuni* isolates were only resistant to tetracycline, and *C. coli* exhibited
multi-drug resistance patterns. Based on differences in prevalence of Campylobacter spp. and resistant Campylobacter between livestock and wildlife species studies, and the lack of similarity in molecular subtypes and AMR patterns, we concluded that the sharing of Campylobacter species between livestock and mammalian wildlife was uncommon.

**Keywords**

Antimicrobial resistance, Campylobacter, livestock-wildlife interface, molecular subtyping, raccoons

**INTRODUCTION**

Campylobacteriosis is the leading bacterial foodborne illness in many developed nations (Acheson et al., 2001), including Canada (Public Health Agency of Canada, 2013). Infections in humans typically cause gastroenteritis symptoms, including vomiting, diarrhea and fever, and have been associated with severe sequelae including Guillain-Barré syndrome (Nachamkin et al., 1998). Although the majority of campylobacteriosis cases in developed nations are self-limiting, serious infections in immunocompromised individuals often require antibiotic therapy and therefore antimicrobial resistance in Campylobacter is a public health concern (Aarestrup et al., 1999). Raw and undercooked contaminated poultry or products cross-contaminated by raw poultry are the primary sources of human infection (Friedman et al., 2004). However, contaminated dairy, beef and pork products have also been shown to contribute to the incidence of campylobacteriosis (Evans et al., 1996, Jayarao et al., 2006, Lammerding et al., 1988, Kapperud et al., 2003).

Although livestock and poultry are the main reservoirs of Campylobacter, it has also been detected in a variety of wildlife species (Wilson et al., 2008). Many wild
animals use the farm environment for food and shelter and may come into contact with livestock production animals. Previous studies attempting to assess the role of wildlife in the maintenance and transmission of Campylobacter have found Campylobacter prevalence ranging from 0% in small rodents like Microtus spp. (Sippy et al., 2010); to 37.2% in wild birds (Agunos et al., 2014). Studies have also shown that genotypes of Campylobacter jejuni implicated in human disease have been isolated from wild birds (Kwan et al., 2008) further suggesting that wildlife are capable of transmitting medically important strains of Campylobacter. Due to the large focus on poultry as the major cause of campylobacteriosis, little research has been done exploring the role of non-avian wildlife and the transmission of Campylobacter to livestock species such as beef cattle, dairy cattle and swine. Understanding what role, if any, wildlife have on the on-farm epidemiology of Campylobacter is essential for the development of effective on-farm control measures (Collins et al., 2004). Our objectives were to 1) compare the prevalence of Campylobacter and antimicrobial resistant Campylobacter in livestock and mammalian wildlife trapped on farms, and 2) investigate the potential sharing of Campylobacter between domestic livestock and mammalian wildlife trapped on farms using a newly developed, highly discriminatory method for molecular subtyping of Campylobacter, Campylobacter-specific 40 gene comparative genomic fingerprinting assay (CGF40) (Taboada et al., 2012), and antimicrobial resistance profiling.

METHODS

Sample Collection

Samples were collected from livestock and wildlife on 25 farms near Guelph (43°57’N, 80°24’W), Ontario, Canada. On eight farms the predominant agricultural
species was swine, eight were primarily beef operations, and nine were primarily dairy operations. The methods used for sample collection from wildlife species were approved by the Animal Care Committee at the University of Guelph using recommendations made by the Canadian Committee on Animal Care and have been described previously by Jardine et al. (2013). Briefly, medium-sized mammals (raccoons (*Procyon lotor*), skunks (*Mephitis mephitis*), opossums (*Didelphis virginiana*), and groundhogs (*Marmota monax*)) were trapped in Tomahawk live traps (Tomahawk Live Trap Co. Tomahawk, Wisconsin, USA), and anesthetized using 0.5 mg/kg medetomidine hydrochloride and 5 mg/kg ketamine hydrochloride prior to sample collection. Animals were released at site of capture after being administered 0.25 mg/kg atipamazole an anesthetic reversal agent. Small mammals (meadow voles (*Microtus pennsylvanicus*), mice (*Mus musculus* and *Peromyscus* spp.), short-tailed shrews (*Blarina brevicauda*), and Norway rats (*Rattus norvegicus*)) were trapped in Sherman live-traps (H. B. Sherman Traps, Inc., Tallahassee, Florida, USA) and euthanized using an anesthetic overdose of halothane prior to sample collection. Fecal samples (medium sized mammals) and colon samples (small mammals) were collected onto Cary-Blair media swabs (Becton, Dickinson and Company, Mississauga, ON, Canada).

Livestock samples were collected as detailed by the National Integrated Enteric Disease Surveillance Program (Public Health Agency of Canada, 2010). In brief, three pooled samples representing all age and production groups and a pooled stored manure sample were collected during the farm visit. The pooled samples consisted of fresh fecal matter from five individual animals put into one large bag and gently mixed. A sterile scoop was used to transfer the pooled samples into specimen containers. Stored manure
samples were taken from either a liquid pit or a dry pile. Liquid pile sampling was done using a sampling pole and a bottle holder to collect 3 sub-samples from different locations around the pit at two different depths, leading to six sub-samples, if possible. Sub-samples were mixed to create a pooled sample. During dry pile sampling a sampling scoop was used to select 5 sub-samples from different locations in the pile and these were mixed to create a pooled sample.

**Campylobacter Isolation and Subtyping**

Samples were tested for *Campylobacter* by two methods employed concurrently:

1) direct plating onto modified charcoal-cefoperazone-deoxycholate agar (mCCDA) and
2) enrichment in Bolton broth followed by plating onto mCCDA. Swabs used during sample collection were used to inoculate a mCCDA plate and subsequently to inoculate a 5 mL tube of Bolton Broth. All mCCDA plates were then streaked and incubated at 42±1°C and examined at both 48 and 72 hours. Inoculated tubes were mixed and 1 mL was transferred into a new 9 mL tube of Bolton Broth, mixed and incubated at 42±1°C for 48 hours and then streaked onto a mCCDA plate. These plates were then incubated for 42±1°C and were examined at both 48 and 72 hours. All incubations were performed under microaerophilic conditions. Colonies were identified using the confirmation procedures as described in MFLP-46 (Health Canada Compendium of Analytical methods), however, catalase-negative colonies were also reported as *Campylobacter* positive. Catalase-negative isolates were also tested for susceptibility to cephalothin and nalidixic acid according to MFLP-46. Catalase-positive isolates were biotyped as *C. jejuni*, *C. coli*, and *C. lari* using methods described by Lior H. (1984) with revisions to the method by Lior et al. (1987).
Subtyping of *Campylobacter* isolates was done using the 40 gene comparative genomic fingerprinting assay (CGF40) as outlined by Taboada et al. (2012). Approximately 20-100 ng of DNA from each isolate was used in 8 multiplex polymerase chain reactions (PCRs) each using one of eight primer sets. Each 25 µl reaction contained the following: 1 µl of DNA, 1 U MP Taq polymerase (Fisher Scientific, Nepean, Canada), 1 x buffer with synthetic factor MP, 2.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 0.4 µM of each of 10 primers. PCR was conducted using the following conditions before removal from the thermocycler: initial denaturation at 94°C for 5 minutes; 35 cycles of denaturation at 94°C for 30 seconds; annealing at 55°C for 30 seconds; extension at 72°C for 30 seconds; final extension at 72°C for 5 minutes; and then held at 4°C. PCR results were analyzed using standard gel electrophoresis on 2% agarose gels using a 15- to 3,000-bp alignment marker with band sizes determined based on QX 100 to 3,000-bp DNA sized markers (Qiagen, Mississauga, Canada). Visuals of gels were produced with BioCalculator v3.0 software (Qiagen, Mississauga, Canada). All results were then recorded as binary values, indicating presence (1) or absence (0) of each marker gene.

**Antimicrobial Susceptibility Testing**

Antimicrobial susceptibility testing was conducted by the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) using procedures outlined in the CIPARS Annual Report (Public Health Agency of Canada, 2009). Each isolate’s susceptibility was tested against the following 9 antimicrobials: ciprofloxacin, telithromycin, azithromycin, clindamycin, erythromycin, gentamicin, nalidixic acid, florfenicol, and tetracycline. Isolated colonies were streaked onto Mueller Hinton agar.
plates with 5% sheep blood enrichment before incubation in microaerophilic conditions at 42 ± 1°C for 24 hours. Testing was conducted using CAMPY susceptibility plates (Sensititre™, Trek™ Diagnostic Systems) from the National Antimicrobial Resistance Monitoring System. Prior to testing, colonies were suspended in 5 mL of Mueller-Hinton broth (MHB) and mixed for 10 seconds or more on a vortex device, 10 µl of this MHB mixture was then transferred to an 11 mL MHB and laked horse blood mixture and again mixed for at least 10 seconds. This final mixture was then pipetted onto CAMPY plates at 100 µL per well. Plates were sealed and incubated in microaerophilic conditions at 42 ± 1°C for 24 hours.

**Statistical Analysis**

All statistical analyses were performed using STATA (STATA 13.0 MP for Windows, STATA Corp, College Station, TX). Descriptive statistics, including frequency tables, for all independent and dependent variables were examined to detect recording errors. In addition, to avoid issues concerning collinearity, we determined if there were any highly correlated variables using Spearman’s rank-order correlation coefficients. If Spearman’s rho ≥ |0.8| then the two variables were not included in the same model.

**Statistical Models**

Mixed logistic regression models were built using adaptive quadrature with the “melogit” command in STATA and included a random intercept for farm location. These models examined the associations between shedding *Campylobacter jejuni*, shedding *Campylobacter* species, and shedding *Campylobacter* resistant to one or more antimicrobials tested and the following independent variables: sample type (wildlife, livestock), farm type (beef, dairy, swine), animal species (beef, dairy, swine, raccoon,
other), and *Campylobacter* spp. (*Campylobacter jejuni, Campylobacter coli*, other).

Since both sample type and animal species are variables that are constructions of each other, they were never tested in the same model. In addition, the variable concerning *Campylobacter* species was only considered in the model for antimicrobial resistance. All variables were tested initially in univariable mixed logistic regression models. Multivariable models were constructed using all eligible variables and interactions were examined among all independent variables. The final models included variables that were statistically significant, part of a significant interaction term, or acted as a confounding variable; evidence of confounding was based on a 20% or greater change in a model coefficient following removal of the variable. All tests were two-tailed and an α value of 0.05 was used.

Variance components from multi-level models were used to calculate the variance partition coefficient (VPC)/intraclass correlation coefficients (ICC) using the latent variable technique (Dohoo et al., 2003).

**Residuals and Diagnostics**

To assess the fit of the multi-level models, the normality and homoscedasticity of best linear unbiased predictors were assessed graphically. Pearson residuals, for animal-level residuals, were assessed graphically to determine if there were any outliers. Outlying observations were inspected for potential recording errors. If no errors were noted, the models were refit without the outlier to assess their potential impact on the model. If the outlier changed the direction and statistical significance of a model coefficient, we noted the impact of the observation on the model.
RESULTS

_Campylobacter_ spp. shedding

_Campylobacter_ was isolated from 71.7% of livestock samples and the prevalence ranged from 87.5% for swine to 58.3% for dairy cattle (Table 2.1). _Campylobacter_ was isolated from 27.1% of wildlife samples (Table 2.1). However, the prevalence of _Campylobacter_ spp. in raccoon samples was 40.8% (Table 2.1). The small individual sample size of all wildlife captured other than raccoons led to insufficient power to analyze individual wildlife species’ risk for _Campylobacter_ spp. shedding and resistant _Campylobacter_ spp. shedding. Therefore, in subsequent statistical analysis all wildlife other than raccoons were grouped together in an “other wildlife” category.

In the model including animal species, no additional variables were included in the final model. In this model, beef and swine species had significantly higher odds of shedding _Campylobacter_ spp. compared to raccoons (Table 2.2); the random effect for location accounted for approximately 18% of the variance in the outcome (Table 2.2).

Sample type was statistically significant based on univariable analysis. In the final multivariable model, sample type and farm type were both statistically significant (Table 2.3), we found that the odds of shedding _Campylobacter_ spp. was greater on swine farms compared to dairy farms and that livestock samples had significantly greater odds of being positive for _Campylobacter_ spp. compared to wildlife samples (Table 2.3). The random effect of location accounted for approximately 9.3% of the variation seen in the outcome (Table 2.3).
Statistical analysis of *Campylobacter jejuni* shedding

Among the isolates collected 62.1% (59/95) were *C. jejuni*, 32.6% (31/95) were *C. coli* and 2.1% (2/95) were *C. lari*. The remaining three isolates found in this study were of unidentified species. All 22 *Campylobacter* isolates from beef farms were identified as *C. jejuni*. In dairy cows, 14.3% of isolates obtained were *C. coli* and 76.1% were identified as *C. jejuni*, two isolates from dairy samples were not identified to species (Table 2.1). Over 96% of isolates obtained from swine were identified as *C. coli* and the single remaining isolate could not be identified to species (Table 2.1). All 20 raccoon *Campylobacter* isolates found in this study were *C. jejuni*. Only a single wildlife isolate, from a rat, was identified as *C. coli* and was sampled from a swine farm. The prevalence of *C. jejuni* in beef, dairy, and raccoons were 70.8%, 44.4% and 40.8%, respectively (Table 2.1).

When assessing the odds of shedding *C. jejuni* (Table 2.2) swine samples were omitted from analysis since no samples tested positive for *C. jejuni* (Table 2.1). For the same reason when assessing farm type swine farms were omitted from consideration. Univariable analyses found that sample type and farm type were not significant predictors of *C. jejuni* shedding, but animal species was a statistically significant variable (Table 2.2); beef had significantly greater odds of shedding *C. jejuni* compared to dairy cattle and raccoons (Table 2.4). In this model, location accounted for 29% of the variance (Table 2.2).

**Molecular subtypes identified by CGF40**

A total of 50 CGF40 subtypes were identified among the 95 isolates collected. Of the 29 *Campylobacter* isolates cultured from wildlife samples, 17 CGF40 subtypes were
identified. Thirty-four CGF40 subtypes were identified among all livestock

Campylobacter isolates. Only 1 CGF40 subtype was seen in both wildlife and livestock
species (Table 2.5); subtype seven (C. jejuni) was found in a raccoon trapped on a dairy
farm, a skunk trapped on a swine farm, and in two beef cattle from the same farm. All
other subtypes were mutually exclusive to either wildlife or livestock (Table 2.5).

Statistical analysis of antimicrobial resistance patterns

Of all Campylobacter jejuni isolates, 50% (28/56) showed no resistance to the
antimicrobials tested, and the remaining 50% showed resistance only to tetracycline.
Resistance to tetracycline was most common, occurring in 40% of all Campylobacter
spp. isolates (38 of 95), including 25.9% (7/27) of C. coli isolates from swine and 20.6%
(6/29) of isolates from wildlife. The prevalences of antimicrobial resistant
Campylobacter carriage in this study was 41.7% in dairy and beef cattle, 37.5% in swine,
and 5.6% in wildlife (Table 2.1). The prevalences of antimicrobial resistance to ≥1
antimicrobials tested of Campylobacter isolates from beef, dairy, swine, and wildlife
were 58.8%, 71.4%, 42.9%, and 20.6% respectively. Multi-drug resistance was detected
in 5 C. coli isolates from swine. Three isolates showed resistance to telithromycin,
erythromycin, clindamycin and azithromycin. A third multi-drug resistant pattern was
exhibited by two isolates, which were resistant to telithromycin, erythromycin and
azithromycin. Two unspeciated Campylobacter isolates, obtained from dairy cattle
exhibited resistance to tetracycline, nalidixic acid and ciprofloxacin.

In univariable analysis, only sample type was a statistically significant predictor
of resistance to ≥1 antimicrobials tested. However, in the final multivariable model,
including sample type and Campylobacter species, Campylobacter jejuni isolates were
more likely to exhibit antimicrobial resistance to ≥1 antimicrobials tested when compared to *Campylobacter coli* and livestock had significantly greater odds of shedding resistant *Campylobacter* spp. compared to wildlife (Table 2.6). In this multivariable model location accounted for 42% of the variance in the outcome (Table 2.6).

**DISCUSSION**

**Prevalence of *Campylobacter* spp. shedding**

Similar to previous studies in Canada, we found that the prevalence of *Campylobacter* spp. was highest in swine followed by beef cattle and dairy cattle (Varela et al., 2007, Inglis et al., 2005, Guévremont et al., 2014). Specifically, we found that swine had higher odds of *Campylobacter* shedding than both dairy and beef cattle. *Campylobacter coli* was almost exclusively shed by swine except for one isolate from a wildlife sample. It is unclear whether this reflects differences in the microbiota of swine, or limited opportunities for transmission because of strict biosecurity measures on swine farms. Dairy, beef cattle, and raccoons tested positive for *C. jejuni*; however, beef cattle were significantly more likely to shed *C. jejuni* compared to dairy cattle and raccoons.

When considering transmission pathways, it is expected that a similar prevalence of carriage would be observed in both wildlife and livestock populations if they are readily exchanging bacteria or at least that a statistical interaction would exist between farm type and sample type or animal species. During model building, we found no significant interaction between farm type and sample type indicating there was no significant difference in the odds of shedding *Campylobacter* spp. or *C. jejuni* between livestock and wildlife among different farm types. Our finding implies that the exchange of *Campylobacter* between wildlife and livestock may not be as likely as with other
bacterial pathogens (Daniels et al., 2003, Jay et al., 2007, Jardine et al., 2011, Allen et al., 2011, Lejeune et al., 2008, Williams et al., 2011).

**Molecular subtyping of Campylobacter**

A total of 50 unique CGF40 subtypes were found in this study. Based on 100% subtype similarity, a single subtype was found in both wildlife and livestock (Table 2.5), but it should be noted that this subtype was isolated from three non-adjacent farm locations. Sippy et al. (2012) found identical subtypes of *Campylobacter* in wild birds and domestic cattle and Kwan et al. (2008) identified an identical subtype occurring in wild birds, domestic poultry, as well as human campylobacteriosis cases. However, few studies have looked at the potential for exchange of *Campylobacter* between livestock species, other than poultry, and medium sized mammalian wildlife, like raccoons. Our results, based on comparing subtype similarity in wildlife and livestock, indicate that at the time of sampling, isolates were not being readily exchanged between wildlife and livestock species. The discriminatory strength of CGF40 can be seen by the infrequent repetition of subtypes on identical farm types with similar species as well as within farms. Cluster analyses may be useful in indicating if there are well defined subgroups or groups of isolates between livestock and wildlife species (Kaufman et al., 2009). This approach may be useful in determining if different clusters of *Campylobacter* subtypes are circulating in livestock and in wildlife.

**Antimicrobial resistance profiling**

As has been reported previously, the prevalence of tetracycline resistance is higher in *C. jejuni* isolates from cattle than in *C. coli* isolates in swine (Englen et al., 2007, Sato et al., 2004, Bae et al., 2005). In this study, we found that resistance to
tetracycline and other antimicrobials tested was significantly more likely in isolates obtained from livestock than wildlife. Tetracycline-resistant *Campylobacter* was found in wildlife sampled in this study, indicating a possible cross-over event in the past, however, it is also highly likely that this resistance was gained by *Campylobacter* isolates during multi-gene plasmid uptake. Previous studies have documented the common circulation of tetracycline resistance genes in the environment, which are conferred to *C. jejuni* and *C. coli* through plasmids (Taylor et al., 1988). These plasmids may contain additional genes of selective advantage to *Campylobacter* spp. allowing for the genes conferred to persist in wildlife. The significant difference in tetracycline-resistance prevalence in wildlife and livestock indicates limited exchange which is further emphasized by the lack of multi-drug resistance crossover into wildlife *Campylobacter* isolates in this study. Swine *Campylobacter* isolates had the largest number of multi-drug resistance patterns than seen in isolates from any other livestock or wildlife species. These isolates showed resistance to macrolide-lincosamide antimicrobials and were all identified as *C. coli*, except for one unspeciated isolate. Macrolide resistance has been documented in *C. coli* isolated from swine which have shown increased resistance to macrolides compared to both *C. coli* and *C. jejuni* isolates from other food animals (Aerestrup et al., 1997). Much like macrolide resistance in swine, quinolone resistance has been documented in food animals (van Diest et al., 1999) in the past and was also found in dairy *Campylobacter* isolates in this study. The lack of quinolone or macrolide resistant *Campylobacter* in wildlife sampled in this study, and the significant difference in the prevalence of AMR *Campylobacter* suggests that exchange of *Campylobacter* subtypes between livestock and wildlife is uncommon. It should be noted that antimicrobial resistance may be exhibited
based on selective pressures and therefore a subtype in wildlife may not show resistance
due to lack of selective pressure, while showing resistance in livestock. However,
coupled with our finding of lack of subtype crossover, and lack of similarity in
antibiograms, it appears that Campylobacter strains are rarely exchanged between
mammalian wildlife and livestock.

Limitations

The relatively small sample size of this study limited our ability to examine
differences among wildlife species. Consequently, wildlife were categorized as either
raccoons or other wildlife species for statistical analyses. This study assessed fecal
samples only and therefore did not explore the possibility of mechanical transmission by
raccoons and other wildlife. As with any study attempting to isolate a pathogen, we also
must consider the possibility of culture bias (Ranjard et al., 2000). It is possible that
certain species were more likely to have Campylobacter identified while others did not
have suitable fecal samples possibly due to the smaller amount of feces produced by
some wildlife species, such as mice. It is also of note that livestock samples in this study
were pooled while wildlife samples were individual animal fecal samples. This may lead
to an exaggerated prevalence in livestock compared to wildlife.

The timeframe of sampling may have also contributed to differences in the
CGF40 subtypes collected. All sample collection took place between January 19th, 2010
and August 30th, 2010. Throughout the study, sampling of beef, dairy and wildlife took
place between the months of May 2010 and August 2010. Swine samples were taken
between the months of January-March which may indicate that the observed differences
in prevalence between swine and other species is due to season rather than species
differences. Since swine most commonly shed *C. coli*, the implication of this sampling difference is limited in the context of this research.

**CONCLUSIONS**

We found significant differences in the prevalence of *Campylobacter* and antimicrobial resistant *Campylobacter* in livestock compared to wildlife, and the CGF40 subtypes and antimicrobial resistance profiles of *Campylobacter* isolates from wildlife and livestock differed. These finding indicate that transmission of *Campylobacter* spp. between livestock and wildlife was infrequent and suggest that wildlife may harbor host specific strains of *Campylobacter* at the scale of our study. Studies examining a larger population of isolates from wildlife, livestock, and humans are still required to better estimate the risk mammalian wildlife pose to humans through the transmission of *Campylobacter* through livestock or the environment. Further studies, specifically molecular cluster analysis studies, are required to better understand the ecology of *Campylobacter* in mammalian wildlife and to determine if wildlife species carry host specific strains of *Campylobacter*. The high prevalence of *Campylobacter* spp. found in wildlife potentially pose a risk to public health, therefore studies investigating the similarity between human *Campylobacter* subtypes and wildlife *Campylobacter* subtypes is necessary.
REFERENCES


Salmonella in raccoons (Procyon lotor) in southern Ontario, Canada. Journal of 
Wildlife Diseases, 47(2), 344-351.

Jardine, C. M., Reid-Smith, R. J., Rousseau, J., & Weese, J. S. (2013). Detection of 
Clostridium difficile in small and medium-sized wild mammals in Southern Ontario, 

Jay, M. T., Cooley, M., Carychao, D., Wiscomb, G. W., Sweitzer, R. A., Crawford-
spinach fields and cattle, central California coast. Emerging Infectious 
Diseases, 13(12), 1908.

consumption among farm families in Pennsylvania. Journal of Dairy Science, 89(7), 
2451-2458.

Kapperud, G., Espeland, G., Wahl, E., Walde, A., Herikstad, H., Gustavsen, S., Tveit, 
I., Natás, O., Bevanger, L., & Digranes, A. (2003). Factors associated with increased 
and decreased risk of Campylobacter infection: a prospective case-control study in 


Table 2.1. Frequency of *Campylobacter* spp. and antimicrobial resistant *Campylobacter* in wildlife and livestock samples from 8 swine, 8 beef and 9 dairy farms in Ontario, Canada.

<table>
<thead>
<tr>
<th>Variables of Interest</th>
<th>Number of samples (n)</th>
<th># of <em>Campylobacter</em> spp. +ve (%) ; 95% CI for %</th>
<th># of <em>C. jejuni</em> isolates (%) ; 95% CI for %</th>
<th># of <em>C. coli</em> isolates (%) ; 95% CI for %</th>
<th>Frequency of antimicrobial resistance (%) ; 95% CI for %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildlife:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Raccoon (<em>Procyon lotor</em>)</td>
<td>49</td>
<td>20 (40.8); 26.9, 55.8</td>
<td>20 (40.8); 26.9, 55.8</td>
<td>0 (0)</td>
<td>5 (10.2); 3.4, 22.2</td>
</tr>
<tr>
<td>Skunk (<em>Mephitis mephitis</em>)</td>
<td>8</td>
<td>7 (87.5); 47.3, 99.7</td>
<td>5 (62.5); 4.4, 91.5</td>
<td>0 (0)</td>
<td>1 (12.5); 0.3, 52.6</td>
</tr>
<tr>
<td>Opossum (<em>Didelphis virginiana</em>)</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Shrew (<em>Blarina brevicauda</em>)</td>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Groundhog (<em>Marmota monax</em>)</td>
<td>1</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Vole (<em>Microtus pennsylvanicus</em>)</td>
<td>3</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Mouse (<em>Mus musculus</em>)</td>
<td>19</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Deer mouse (<em>Peromyscus spp.</em>)</td>
<td>20</td>
<td>1 (5); 0.1, 24.9</td>
<td>1 (5); 0.1, 24.9</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Rat (<em>Rattus norvegicus</em>)</td>
<td>4</td>
<td>1 (25); 0.6, 80.6</td>
<td>0 (0)</td>
<td>1 (25); 0.6, 80.6</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Livestock:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef cattle</td>
<td>24</td>
<td>17 (70.8); 48.9, 87.4</td>
<td>17 (70.8); 48.9, 87.4</td>
<td>0 (0)</td>
<td>10 (41.7); 22.1, 63.4</td>
</tr>
<tr>
<td>Dairy cattle</td>
<td>36</td>
<td>21 (58.3); 40.7, 74.4</td>
<td>16 (44.4); 27.9, 61.9</td>
<td>3 (8.3); 1.8, 22.5</td>
<td>15 (41.7); 25.5, 59.2</td>
</tr>
<tr>
<td>Swine</td>
<td>32</td>
<td>28 (87.5); 71.0, 96.5</td>
<td>0 (0)</td>
<td>27 (84.4); 67.2, 94.7</td>
<td>12 (37.5); 21.1, 56.3</td>
</tr>
</tbody>
</table>
Table 2.2. Univariable mixed logistic model with a random effect for location for the following outcomes; shedding of *Campylobacter* spp., shedding of *C. jejuni*, and shedding of *Campylobacter* resistant to $\geq 1$ antimicrobials tested.

<table>
<thead>
<tr>
<th>Sample Type:</th>
<th>Shedding of <em>Campylobacter</em> spp.</th>
<th></th>
<th>Shedding of <em>Campylobacter jejuni</em></th>
<th></th>
<th>Resistance to $\geq 1$ Antimicrobials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>p-value</td>
<td>Odds Ratio</td>
<td>p-value</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>Wildlife (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Livestock</td>
<td>9.56 (4.33, 21.09)</td>
<td>&lt;0.001</td>
<td>1.35 (0.65, 2.81)</td>
<td>0.419</td>
<td>6.01 (1.44, 25.12)</td>
</tr>
<tr>
<td>Location</td>
<td>ICC (95% CI)</td>
<td>Variance (95% CI)</td>
<td>ICC (95% CI)</td>
<td>Variance (95% CI)</td>
<td>ICC (95% CI)</td>
</tr>
<tr>
<td></td>
<td>0.16 (0.04, 0.44)</td>
<td>0.60 (0.14, 2.54)</td>
<td>0.22 (0.07, 0.49)</td>
<td>0.92 (0.26, 3.21)</td>
<td>0.46 (0.19, 0.77)</td>
</tr>
</tbody>
</table>

| Animal Species: | | | | | |
| Other wildlife (ref) | - | - | - | - | - | - |
| Swine         | 74.71 (15.26, 365.84) | <0.001 | - | - | 3.47 (0.18, 65.23) | 0.406 |
| Beef          | 34.42 (6.45, 183.65) | <0.001 | 94.43 (11.09, 804.18) | <0.001 | 7.32 (0.21, 252.31) | 0.270 |
| Dairy         | 15.05 (3.85, 58.85) | <0.001 | 16.69 (2.99, 92.99) | 0.001 | 33.74 (0.89, 1265.21) | 0.057 |
| Raccoon       | 8.08 (2.17, 30.05) | 0.002 | 19.81 (3.46, 113.35) | 0.001 | 1.07 (0.042, 27.08) | 0.968 |
| Location      | ICC (95% CI) | Variance (95% CI) | ICC (95% CI) | Variance (95% CI) | ICC (95% CI) | Variance (95% CI) |
|              | 0.18 (0.054, 0.44) | 0.70 (0.19, 2.64) | 0.29 (0.11, 0.59) | 1.40 (0.41, 4.78) | 0.49 (0.19, 0.79) | 3.10 (0.76, 12.63) |
Table 2.2. Continued.

<table>
<thead>
<tr>
<th>Farm Type:</th>
<th>Shedding of <em>Campylobacter</em> spp.</th>
<th>Shedding of <em>Campylobacter jejuni</em></th>
<th>Resistance to ≥ 1 Antimicrobials</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>p-value</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>Beef (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dairy</td>
<td>0.85 (0.31, 2.32)</td>
<td>0.754</td>
<td>0.57 (0.18, 1.75)</td>
</tr>
<tr>
<td>Swine</td>
<td>1.25 (0.48, 3.23)</td>
<td>0.645</td>
<td>-</td>
</tr>
<tr>
<td>Location</td>
<td>ICC (95% CI)</td>
<td>Variance (95% CI)</td>
<td>ICC (95% CI)</td>
</tr>
<tr>
<td></td>
<td>0.094 (0.02, 0.35)</td>
<td>0.034 (0.07, 1.77)</td>
<td>0.14 (0.03, 0.49)</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.:</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Other</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Campylobacter</em> spp. (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. coli</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. jejuni</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Location</td>
<td>ICC (95% CI)</td>
<td>Variance (95% CI)</td>
<td>ICC (95% CI)</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.3. Mixed logistic multivariable model with a random effect for location assessing the association between shedding *Campylobacter* spp. and sample type and farm type.

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample type:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildlife (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Livestock</td>
<td>12.07</td>
<td>&lt;0.001</td>
<td>5.34, 27.27</td>
</tr>
<tr>
<td><strong>Farm type:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Beef</td>
<td>1.92</td>
<td>0.253</td>
<td>0.63, 5.86</td>
</tr>
<tr>
<td>Swine</td>
<td>4.12</td>
<td>0.010</td>
<td>1.40, 112.14</td>
</tr>
<tr>
<td><strong>Random Effect</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>0.093 (0.015, 0.41)</td>
<td>0.34 (0.05, 2.26)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4. Significant contrasts obtained from univariable mixed-logistic models with a random effect for location for the following outcomes: shedding of *Campylobacter* spp. and shedding of *C. jejuni*.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>i. Campylobacter</em> spp.</td>
<td>Beef vs. Raccoon</td>
<td>4.26</td>
<td>0.030</td>
<td>1.15, 15.71</td>
</tr>
<tr>
<td></td>
<td>Swine vs. Raccoon</td>
<td>9.25</td>
<td>0.001</td>
<td>2.48, 34.50</td>
</tr>
<tr>
<td></td>
<td>Swine vs. Dairy</td>
<td>4.97</td>
<td>0.035</td>
<td>1.12, 21.99</td>
</tr>
<tr>
<td><em>ii. C. jejuni</em></td>
<td>Beef vs. Dairy</td>
<td>5.66</td>
<td>0.047</td>
<td>1.02, 31.21</td>
</tr>
<tr>
<td></td>
<td>Beef vs. Raccoons</td>
<td>4.77</td>
<td>0.036</td>
<td>1.10, 20.58</td>
</tr>
</tbody>
</table>
Table 2.5. CGF40 subtypes identified more than once from wildlife and/or livestock samples.

<table>
<thead>
<tr>
<th>Repeated Subtype</th>
<th>Campylobacter spp.</th>
<th>Frequency of Subtype</th>
<th>Farm Type of Isolate</th>
<th>Animal Species Sampled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Swine</td>
<td>Dairy</td>
</tr>
<tr>
<td>Subtype 4</td>
<td>jejuni</td>
<td>5</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 7**</td>
<td>jejuni</td>
<td>4</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 9</td>
<td>jejuni</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 10</td>
<td>jejuni</td>
<td>4</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 14</td>
<td>unidentified</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 15</td>
<td>jejuni</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 18</td>
<td>jejuni</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 26</td>
<td>unidentified</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 28</td>
<td>coli</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 30</td>
<td>coli</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 35</td>
<td>coli</td>
<td>7</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 36</td>
<td>coli</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 38</td>
<td>coli</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 40</td>
<td>coli</td>
<td>2</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 41</td>
<td>coli</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 42</td>
<td>jejuni</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 43</td>
<td>jejuni</td>
<td>8</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 48</td>
<td>jejuni</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Subtype 50</td>
<td>jejuni</td>
<td>3</td>
<td>●</td>
<td>●</td>
</tr>
</tbody>
</table>
Table 2.6. Mixed logistic multivariable model with random effect for location assessing the association between antimicrobial resistance to ≥ 1 antimicrobials and sample type and *Campylobacter* species.

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample type:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildlife (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Livestock</td>
<td>22.04</td>
<td>0.002</td>
<td>3.11, 156.06</td>
</tr>
<tr>
<td><strong>Campylobacter Species:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. coli (ref)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C. jejuni</td>
<td>7.33</td>
<td>0.032</td>
<td>1.19, 45.37</td>
</tr>
<tr>
<td>Unknown</td>
<td>88.16</td>
<td>0.022</td>
<td>1.91, 4058.36</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random Effect</th>
<th>ICC (95% CI)</th>
<th>Variance (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>0.42 (0.16, 0.73)</td>
<td>2.36 (0.61, 9.05)</td>
</tr>
</tbody>
</table>
CHAPTER 3:

CLUSTER ANALYSIS OF *CAMPYLOBACTER JEJUNI* GENOTYPES
ISOLATED FROM MAMMALIAN WILDLIFE AND BOVINE
LIVESTOCK FROM ONTARIO FARMS
ABSTRACT

Using data collected from a cross-sectional study of twenty-five farms (8 beef, 8 swine, and 9 dairy) in 2010, we assessed clustering of molecular subtypes of *C. jejuni* based on a *Campylobacter*-specific 40 gene comparative genomic fingerprinting assay (CGF40) subtypes, using unweighted pair group method with arithmetic mean (UPGMA) analysis, and multiple correspondence analysis. Exact logistic regression was used to determine which genes differentiate wildlife and livestock subtypes in our study population. A total of 33 bovine livestock (17 beef & 16 dairy), 26 wildlife (20 raccoon (*Procyon lotor*), 5 skunk (*Mephitis mephitis*), and 1 mouse (*Peromyscus* spp.) *C. jejuni* isolates were subtyped using CGF40. Dendrogram analysis, based on UPGMA, showed distinct branches separating bovine livestock and mammalian wildlife isolates. Furthermore, two-dimensional multiple correspondence analysis was highly concordant with dendrogram analysis showing clear differentiation between livestock and wildlife CGF40 subtypes. Exact logistic regression conducted gene-by-gene revealed 15 genes that were predictive of whether an isolate was of wildlife or bovine livestock isolate origin. Both multiple correspondence analysis and exact logistic regression revealed that in most cases the presence of a particular gene (13/15) was associated with an isolate being of livestock rather than wildlife origin. In conclusion the evidence gained from dendrogram analysis, multiple correspondence analysis, and exact logistic regression indicate that mammalian wildlife carry CGF40 subtypes of *C. jejuni* distinct from those carried by bovine livestock. Future studies focused on source attribution of *C. jejuni* in human infections will help determine if wildlife transmit *Campylobacter jejuni* directly to humans.
Keywords
Molecular epidemiology, cluster analysis, Campylobacter, livestock-wildlife interface, raccoons, bovine livestock

INTRODUCTION

_Campylobacter jejuni_ is the most common bacterial cause of human gastroenteritis in the developed world (Altekruse et al., 1999). The 2010 annual incidence of campylobacteriosis in Canada was 26.3 cases/100,000 person-years (Public Health Agency of Canada, 2010) and has since remained relatively stable. Although the majority of _Campylobacter_ infections are self-limiting, some infections can lead to a host of severe sequelae, notably Guillain-Barré syndrome and reactive arthritis (Rees et al., 1995, Acheson et al., 2001). The considerable burden of disease that _C. jejuni_ is responsible for, through its high incidence and severe sequelae, makes understanding its epidemiology and transmission dynamics a high priority. In efforts to trace common sources of _C. jejuni_ during campylobacteriosis outbreaks, many epidemiologists have employed molecular subtyping techniques (Wiedmann et al., 2002). As the benefits of molecular subtyping to epidemiological investigations became evident, a variety of methodologies were developed and used in laboratories worldwide. _Campylobacter_-specific 40 gene comparative genomic fingerprinting assay is a recent highly discriminatory method that is used for the molecular subtyping of _Campylobacter jejuni_ (Taboada et al., 2010). As the distribution of food increases both nationally and internationally, the process of identifying sources of foodborne agents in human infections has become increasingly important.
Previous studies using molecular subtyping have identified that the most common causes of campylobacteriosis are raw and undercooked meat products and unpasteurized dairy products (Corry et al., 2001, Humphrey et al., 1987). Although livestock and poultry are important reservoirs of \textit{Campylobacter jejuni}, a variety of studies have investigated the potential role of wildlife in transmitting \textit{Campylobacter} to livestock and humans (Kwan et al., 2008, Kwan et al., 2008, Petersen et al., 2001, Jensen et al., 2006). In previous studies, molecular subtyping data have been used to demonstrate transmission of \textit{C. jejuni} between wild birds and poultry (Kwan et al., 2008). However, few studies have examined the role of mammalian wildlife and the role they may play in dispersing and maintaining \textit{Campylobacter} in agricultural systems. In a previous study, we found little evidence of transmission between livestock and mammalian wildlife, based on molecular subtyping, prevalence data, and antibiograms, potentially indicating the existence of wildlife-specific \textit{C. jejuni} strains (Chapter 2). The objectives of this research are to 1) determine if unique mammalian wildlife and bovine livestock \textit{Campylobacter}-specific 40 gene comparative genomic fingerprinting assay (CGF40) groups of \textit{C. jejuni} can be identified using cluster analysis, and 2) determine which genes exist more commonly in CGF40 subtypes isolated from mammalian wildlife and bovine livestock.

\section*{METHODS}

\subsection*{Sample collection}

All farm samples were collected in 2010 from Guelph (43°57'N, 80°24'W), Ontario, Canada and surrounding areas. Of the 25 participating farms in this study, eight were predominantly swine farms, eight were predominantly beef farms and nine were predominantly dairy farms. Wildlife samples were collected in accordance to standards
set by the Animal Care Committee at the University of Guelph, based on the recommendations of the Canadian Committee on Animal Care. Wildlife sampling methods are described by Jardine et al. (2013). In brief, medium sized-mammals (i.e., raccoons (*Procyon lotor*), skunks (*Mephitis mephitis*), opossums (*Didelphis virginiana*), and groundhogs (*Marmota monax*) were trapped using Tomahawk live traps (Tomahawk Live Trap Co. Tomahawk, Wisconsin, USA) and anesthetized using 0.5 mg/kg medetomidine hydrochloride and 5 mg/kg ketamine hydrochloride to allow for sample collection. An anesthetic reversal agent, 0.25 mg/kg atipamazole, was used prior to releasing animals at the site of capture. All small wildlife (i.e., meadow voles (*Microtus pennsylvanicus*), mice (*Mus musculus* and *Peromyscus* spp.), short-tailed shrews (*Blarina brevicauda*), and Norway rats (*Rattus norvegicus*) were trapped in Sherman live-traps (H. B. Sherman Traps, Inc., Tallahassee, Florida, USA) before euthanization by anesthetic overdose with halothane, and sample collection. Fecal samples from medium sized mammals and colon samples from small mammals were collected onto Cary-Blair media swabs (Becton, Dickinson and Company, Mississauga, ON, Canada).

Livestock samples were collected and processed by the National Integrated Enteric Disease Surveillance Program (Public Health Agency of Canada, 2010). Livestock isolates of *C. jejuni* were obtained from pooled pen, liquid pit, and dry pile fecal samples. Pooled samples were collected according to the protocol detailed by the Public Health Agency of Canada (2010) and described in Chapter 2.

*Campylobacter* isolation and subtyping

All samples were tested for *Campylobacter* presence by two methods used concurrently: 1) direct plating on modified charcoal-cefoperazone-deocycholate agar
(mCCDA) plates, and 2) enrichment in Bolton broth prior to plating onto mCCDA. Swabs used during sample collection were used to inoculate both a mCCDA plate and 5mL of Bolton broth. Following inoculation mCCDA plates were streaked and incubated at 42±1ºC and examined at 48 and 72 hours. Bolton broth tubes inoculated with samples were mixed and 1 mL of solution was transferred to a new tube containing 9mL of Bolton broth, which was mixed and incubated at 42±1ºC for 48 hours prior to streaking onto a mCCDA plate. Plates were incubated and examined during the time points indicated, previously. All incubations were conducted under microaerophilic conditions. Colonies were identified using the confirmation procedures as described in MFLP-46 (Health Canada, 2014) and catalase positive isolates were speciated as *C. jejuni* using methods described by Lior H. (1984) with revisions to the method by Lior et al. (1987).

*Campylobacter jejuni* subtyping was completed using a 40-gene comparative genomic fingerprinting assay (CGF40) as developed and outlined by Taboada et al. (2012). Briefly 20-100 ng of DNA from isolates were used in 8 multiplex polymerase chain reactions (PCRs) each using one of eight primer sets. Each 25 µl reaction was composed of 1 µl of DNA and the following mastermix: 1 U MP Taq polymerase (Fisher Scientific, Nepean, Canada), 1 x buffer with synthetic factor MP, 2.5 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 0.4 µM of each of the 10 primers. PCR conditions were as follows: initial denaturation at 94º C for 5 minutes; 35 cycles of denaturarion at 94º C for 30 seconds; annealing at 55º C for 30 seconds; extension at 72º C for 30 seconds; final extension at 72º C for 5 minutes; and then held at 4º C. Results of PCRs were assessed using standard gel electrophoresis on 2% agarose gels using a 15- to 3,000-bp alignment marker with band sizes determined based on QX 100 to 3,000-bp
DNA sized markers (Qiagen, Mississauga, Canada). Visualization of gels was completed by BioCalculator v3.0 software (Qiagen, Mississauga, Canada).

**Statistical Analysis**

*Cluster analyses*

Dendrogram analysis was completed using Bionumerics (Bionumerics 6.6, Applied Maths NV, Austin, TX). Simple-matching unweighted pair group method with arithmetic mean (UPGMA) cluster analysis was done using CGF40 output for dendrogram creation. Clusters were chosen and identified visually to use for further statistical analysis.

Multiple correspondence analysis (MCA) was done using the “mca” command in STATA (STATA 13.0 MP for Windows, STATA Corp, College Station, TX) using the Burt method and principal normalization. The number of dimensions used for further analysis was determined by their ability to explain 80% or more of the variation in the data as well as their ease of interpretation. Each observation score was calculated using the “predict” command and plotted to visualize the distribution of subtypes along the dimensions.

*Multi-level logistic regression modelling*

All statistical analyses were completed using STATA. Statistical significance for all analyses was set at α=0.05. Before model building descriptive statistics were examined to ensure sufficient sample size and correct any recording errors. Multi-level logistic regression was done to assess the association between cluster identity (wildlife or livestock) based on UPGMA groupings and MCA groupings and the following independent variables: animal species (beef, dairy, raccoon, other wildlife) and sample
type (wildlife or livestock). All models included a random intercept for farm location. To assess model fit, homoscedasticity and normality of the best linear unbiased predictors were examined. Pearson residuals, at the sample level, were also examined to identify outliers. Intraclass correlation coefficients (ICC) were calculated in STATA using the latent variable technique with the “icc” command (Dohoo et al., 2003).

**Exact logistic modelling**

Exact logistic regression models were constructed to evaluate the association between genes used in MCA analysis and the following outcome variables: sample type (livestock or wildlife) and animal species (beef, dairy, raccoon, and other wildlife). Statistical significance for all models were computed using the score method.

**RESULTS**

**Descriptive statistics**

Thirty-three livestock and 26 wildlife *C. jejuni* isolates were subtyped using CGF40, of 92 and 107 samples taken from livestock and wildlife, respectively (Chapter 2). Of these isolates 17 were from beef cattle, 16 from dairy cattle, 20 were from raccoons (*Procyon lotor*), five from skunks (*Mephitis mephitis*), and one from a mouse (*Peromyscus* spp.).

**Cluster analysis**

Based on visual assessment of the dendrogram, constructed using UPGMA (Figure 3.1), we noted that the majority of wildlife subtypes were within a single group, with the exception of two isolates. In Figure 3.1, it can be seen that only 11 livestock samples, of the 33 analyzed, were found to be in what will be referred to as the wildlife
cluster. It is of note that beef and dairy cattle were equally distributed throughout the identified grouping referred to subsequently as the livestock cluster (Figure 3.1).

Of all 40 characters tested for in CGF40 testing, 35 were included in multiple correspondence analysis. Five characters were eliminated from analysis because they were completely predictive of failure or success in all observations. Two dimensional multiple correspondence analyses (MCA) revealed that 81.2% of the variation in data was explained within these first two dimensions, with the first dimension accounting for 62.6% of the variance. It can be seen that when observation scores are plotted that mammalian wildlife and bovine livestock subtypes show considerable differentiation, along dimension 1, supporting the results of the dendrogram analysis (Figure 3.2). It was also found that no specific differentiation or clustering was seen when comparing dairy cattle subtypes and beef cattle subtypes (Figure 3.2). When comparing the MCA plot (Figure 3.2) and the MCA coordinate plot (Figure 3.3) it can be seen that the presence of genes are more likely to predict bovine livestock subtypes, to the left of the x origin, along dimension one and the absence of genes are more likely to predict mammalian wildlife subtypes. The node used to define the separation of wildlife and livestock clusters using UPGMA corresponded to the origin of the dimension 1 (i.e., MCA dimension 1 score=0) using MCA.

Mixed logistic regression analysis found that isolates of *C. jejuni* collected from wildlife were at significantly greater odds of being in the wildlife cluster compared to the livestock cluster based on UPGMA and MCA (Table 3.1). The intraclass correlation coefficient indicated that the farm where samples originated from accounted for 38% of the variance (Table 3.1). We found that raccoon isolates and other wildlife isolates were
significantly more likely to be found in the wildlife cluster in comparison to beef cattle (Table 3.1) and dairy cattle (OR=54.04; 95% CI 3.25-899.80; p=0.005). In this model the farm accounted for 38% of the variance seen in the outcome (Table 3.1).

**Exact logistic regression**

Fifteen genes were identified that are significantly associated with the sample type (wildlife or livestock) of *Campylobacter* isolates (Table 3.2). It was noted that, of all of these fifteen genes only the presence of three, cj1294, cj1329, and cj1334 were positively associated with being a wildlife sample. The presence of the remaining 12 genes was positively associated with being livestock samples (Table 3.2).

**DISCUSSION**

From dendrogram analysis a clear separation of mammalian wildlife and bovine livestock subtypes was observed. Interestingly, only two wildlife isolates were not included in the single wildlife cluster. These wildlife isolates were the only two isolates with the same CGF40 subtypes as isolates found in bovine livestock (Chapter 2). A total of 11 bovine isolates, comprised of 6 subtypes, were grouped within the wildlife cluster, three of which originated from beef cattle, two from dairy cattle and one found in both. In most cases, these bovine isolates existed within sub-clusters that included wildlife isolates obtained from different farm types than the farm type of the livestock isolate. McCarthy et al. (2007), when assessing multi-locus sequence typed genotypes of *C. jejuni*, also found that the majority of transmissions occur within species. Molecular subtyping of isolates from agricultural species (poultry and bovids) provided strong evidence for host-adaptation of *C. jejuni* (McCarthy et al., 2007; Colles et al., 2003).
Ultimately, based on visual analysis of the dendrogram, strong evidence for mammalian wildlife and bovine livestock specific strains of *Campylobacter* was present.

In efforts to provide quantitative support for the distinction in wildlife *C. jejuni* isolates and bovine *C. jejuni* isolates, a multi-level logistic model was fitted. This model revealed that wildlife isolates were significantly more likely to be categorized into the wildlife cluster of the dendrogram compared to bovine livestock species when also considering the clustering that would occur with multiple isolates coming from a common farm location (i.e., autocorrelated data). It was also found that of all animal species, raccoons were most likely to be found in the wildlife cluster followed by all other wildlife. This model provided support for theories developed by visual analysis and supports the differentiation of *C. jejuni* occurring in mammalian wildlife compared to bovine livestock. It is of note that little distinction amongst species of wildlife and species of bovine livestock sampled was observed.

Multiple correspondence analyses provided the same results as those from UPGMA dendrogram analysis. We found that bovine livestock subtypes were differentiated from wildlife subtypes, specifically along the first dimension calculated. However, a distinction was not found between beef subtypes and dairy subtypes. The high degree of congruency between both forms of cluster analysis conducted in this study indicating that *C. jejuni* differentiation among these populations exists. Previous research by Lyautey et al. (2007) saw similar patterns of differentiation in *Listeria monocytogenes* between mammalian wildlife (i.e., deer, moose, raccoons and otters) and beef and dairy cattle when using *Eco*RI ribotyping, pulse-field gel electrophoresis and polymerase chain reaction-restriction fragment length polymorphism methods. Differentiation of MLST
clonal complexes of *C. jejuni* and *C. coli* obtained from wildlife (e.g., rabbits, badgers) and cattle was also found by Kwan et al. (2008).

Investigation of MCA weighting of each individual gene in cross-reference with subtype MCA scores revealed that in general the presence of genes had a greater magnitude of weight for bovine livestock isolates and the absence of genes a larger weight for wildlife isolates. To investigate this further, exact logistic regression was conducted to assess the associations that are present between individual genes and the sample type (livestock or wildlife) of the isolate. Regression analysis revealed 15 genes that had associations with sample type that were statistically significant. Of these 15 genes, 12 were positively associated with being a livestock sample and the remaining 3 were positively associated with being a wildlife sample. This was highly concordant with conclusions we made from the MCA coordinate plot that presence of genes were positively associated with a livestock sample. This study identified that only 15 genes of 40 tested were able to significantly differentiate mammalian wildlife and bovine livestock populations. It is possible that the remaining 25 genes are superfluous when comparing bovine livestock and mammalian wildlife *Campylobacter*. Since the CGF40 method was developed using agricultural, environmental and human isolates (Taboada et al., 2012), it is possible that these remaining genes are unnecessary for distinguishing mammalian wildlife *Campylobacter* isolates from bovine livestock isolates, but are important for distinguishing other sample types from one another. These models, along with MCA and UPGMA dendrogram derived results, suggest that wildlife and livestock carry largely differentiated populations of *C. jejuni*. Due to the small effective sample size we were unable to introduce a random-effect to account for farm location of isolates in these
regression models so it is possible that the p-values obtained from these analyses are too liberal (i.e., the true p-value was underestimated).

Although discrete groups of *C. jejuni* between mammalian wildlife and bovine livestock exist, future studies should examine the differences between human campylobacteriosis case isolates of *C. jejuni* and wildlife isolates. Direct contact and indirect contacts between humans and wildlife in residential, agricultural and wildlife habitats (Bradley et al, 2007, Kruse et al., 2004) makes the potential source of human infections unclear. Consequently, source attribution is a key initiative of the Canadian government (Public Health Agency of Canada, 2013). Differentiating whether human cases are the result of exposure to livestock or wildlife isolates of *C. jejuni* has important implications for efforts to control this pathogen.

**CONCLUSIONS**

We found strong evidence of differentiation between bovine livestock and mammalian wildlife isolates of *C. jejuni* obtained on southern Ontario farms based on CGF40 subtyping. The triangulation of UPGMA dendrogram analysis, multiple correspondence analysis, and regression methods strongly suggests the potential for host-adaptation of *C. jejuni* in mammalian wildlife and bovine livestock. To establish if these results reflect a similar relationship between wildlife and livestock *Campylobacter* carriage across Canada, future research should be conducted with a larger population of *Campylobacter* isolates from wildlife, livestock, and humans. Source attribution studies should also be conducted to investigate the association between contact with wildlife and human campylobacteriosis cases.
REFERENCES


Figure 3.1. Simple-matching unweighted pair group method with arithmetic mean (UPGMA) dendrogram of *C. jejuni* isolates with visually identified wildlife and livestock clusters.
Figure 3.2. Two dimensional multiple correspondence analysis (MCA) scores of wildlife and livestock *C. jejuni* subtypes.
Figure 3.3. Multiple correspondence analysis (MCA) coordinate plot showing weighting of presence (1) and absence (0) of CGF40 genes by colour calculated by multiple correspondence analysis for first two dimensions.
Table 3.1. Mixed logistic model with a random effect for farm location assessing the association between sample type and being in the ‘wildlife cluster’ and association between animal species and being in the ‘wildlife cluster’ as defined by unweighted pair-group method with arithmetic mean (UPGMA) dendrogram visual identification and multiple correspondence analysis.

<table>
<thead>
<tr>
<th>Model</th>
<th>Independent Variable</th>
<th>Odds Ratio</th>
<th>P-value</th>
<th>95% Confidence Interval*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample type:</td>
<td>Livestock (ref) - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wildlife   50.48 0.004 3.38, 754.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Animal species:</td>
<td>Beef (ref) - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dairy      2.87 0.366 0.29, 28.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Raccoons   154.86 0.004 4.95, 4847.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other wildlife 33.27 0.046 1.05, 1047.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Random Effect</td>
<td>ICC (95% CI)</td>
<td>Variance (95% CI)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Farm Location</td>
<td>0.38 (0.04, 0.89)</td>
<td>2.01 (0.15, 27.77)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Farm Location</td>
<td>0.38 (0.04, 0.89)</td>
<td>2.03 (0.15, 27.43)</td>
<td></td>
</tr>
</tbody>
</table>

*95% confidence interval of odds ratio
Table 3.2. Exact logistic model assessing the association between each CGF40 gene and being a livestock sample versus a wildlife sample.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p-value</th>
<th>Gene frequency (%) amongst livestock, n=33 (95% CI)</th>
<th>Gene frequency amongst wildlife, n=26 (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cj0057 175bp</td>
<td>11.56</td>
<td>3.08, 51.08</td>
<td>&lt;0.001</td>
<td>81.8 (64.5, 93.0)</td>
<td>26.9 (11.6, 47.8)</td>
</tr>
<tr>
<td>Cj0177 399bp</td>
<td>7.85</td>
<td>1.84, 48.75</td>
<td>0.002</td>
<td>51.5 (33.5, 69.2)</td>
<td>11.5 (2.4, 30.1)</td>
</tr>
<tr>
<td>Cj0181 486bp</td>
<td>10.85</td>
<td>2.18, 109.62</td>
<td>0.001</td>
<td>48.5 (30.8, 66.5)</td>
<td>7.7 (0.9, 25.1)</td>
</tr>
<tr>
<td>Cj0297c 300bp</td>
<td>4.00</td>
<td>1.04, 17.64</td>
<td>0.037</td>
<td>84.8 (68.1, 94.9)</td>
<td>57.7 (36.9, 76.7)</td>
</tr>
<tr>
<td>Cj0298c 198bp</td>
<td>4.00</td>
<td>1.04, 17.64</td>
<td>0.037</td>
<td>84.8 (68.1, 94.9)</td>
<td>57.7 (36.9, 76.7)</td>
</tr>
<tr>
<td>Cj0755 107bp</td>
<td>19.39</td>
<td>2.93, +Inf*</td>
<td>&lt;0.001</td>
<td>36.3 (20.4, 54.9)</td>
<td>0 (0, 13.22**)</td>
</tr>
<tr>
<td>Cj1294 160bp</td>
<td>0.08</td>
<td>0.00, 0.54*</td>
<td>0.007</td>
<td>72.7 (54.5, 86.7)</td>
<td>78.8 (61.1, 91.0)</td>
</tr>
<tr>
<td>Cj1329 307bp</td>
<td>0.26</td>
<td>0.06,0.94</td>
<td>0.029</td>
<td>51.5 (33.5, 69.2)</td>
<td>63.6 (45.1, 79.6)</td>
</tr>
<tr>
<td>Cj1334 462bp</td>
<td>0.26</td>
<td>0.06,0.94</td>
<td>0.029</td>
<td>51.5 (33.5, 69.2)</td>
<td>63.6 (45.1, 79.6)</td>
</tr>
<tr>
<td>Cj1427c 613bp</td>
<td>8.12</td>
<td>2.29, 32.69</td>
<td>&lt;0.001</td>
<td>75.8 (57.7, 88.9)</td>
<td>26.9 (11.6, 47.8)</td>
</tr>
<tr>
<td>Cj1550c 188bp</td>
<td>50.97</td>
<td>7.85,+Inf*</td>
<td>&lt;0.001</td>
<td>60.6 (42.1, 77.1)</td>
<td>0 (0, 13.22**)</td>
</tr>
<tr>
<td>Cj1551 241bp</td>
<td>50.97</td>
<td>7.85,+Inf*</td>
<td>&lt;0.001</td>
<td>60.6 (42.1, 77.1)</td>
<td>0 (0, 13.22**)</td>
</tr>
<tr>
<td>Cj1522 22bp</td>
<td>50.97</td>
<td>7.85,+Inf*</td>
<td>&lt;0.001</td>
<td>60.6 (42.1, 77.1)</td>
<td>0 (0, 13.22**)</td>
</tr>
<tr>
<td>Cj1585 630bp</td>
<td>8.54</td>
<td>1.65, 86.57</td>
<td>0.003</td>
<td>42.4 (25.5, 60.8)</td>
<td>7.7 (0.9, 25.1)</td>
</tr>
<tr>
<td>Cj1727c 369bp</td>
<td>12.21</td>
<td>2.39, 123.37</td>
<td>&lt;0.001</td>
<td>51.5 (33.5, 69.2)</td>
<td>7.7 (0.9, 25.1)</td>
</tr>
</tbody>
</table>

*one-sided, 97.5% confidence interval

**Median unbiased estimate
CHAPTER 4:
SUMMARY DISCUSSION AND CONCLUSIONS

The major objectives of this study were to determine if there are significant differences in *Campylobacter* spp. carriage in mammalian wildlife and livestock in southern Ontario. Specifically, we were interested in examining if medium and small sized mammals, such as raccoons and mice, with close contact to livestock barns and feed, have a role in maintaining and spreading *Campylobacter* spp. to livestock, or if distinct *Campylobacter* subtypes are circulating among these populations. These objectives were met using data collected as a part of a cross-sectional study of 25 farms in southern Ontario in 2010. Multi-level models were used to compare the prevalence of *Campylobacter* spp., *C. jejuni*, and antimicrobial resistant *Campylobacter* spp. in livestock and wildlife populations sampled. Cluster analysis was completed to visualize potential clustering of *Campylobacter* subtypes by farm, by individual species, and by sample type (livestock or wildlife).

This study is novel in its use of the highly-discriminatory *Campylobacter*-specific 40-gene comparative genomic fingerprinting assay (CGF40). This method is a rapid, low-cost alternative to other genotyping methods, and has high concordance to results produced by multi-locus sequence typing (Taboada et al., 2012). Based on CGF40 subtyping and cluster analysis, this study found that mammalian wildlife and livestock circulated distinct strain populations of *Campylobacter*, although crossover of one subtype was observed. This observation is echoed in previous research of smaller mammalian wildlife such as voles, mice, shrews, and moles (Sippy et al., 2011). It has also been found in previous research by McCarthy et al. (2007), using multi-locus
sequence typed genotypes of *C. jejuni*, that the majority of transmissions of *Campylobacter* occur within host species rather than between host species, and agricultural species such as sheep and chicken have been shown to shed host-adapted subtypes of *C. jejuni* (McCarthy et al., 2007; Colles et al., 2003). It is of note that mammalian wildlife species, specifically rabbits, have been shown to carry sequence types of *Campylobacter* that are also isolated from cattle as well as human campylobacteriosis cases (Kwan et al., 2008). Therefore the potential role of mammalian wildlife in the maintenance and transmission of *Campylobacter* spp. subtypes that are found in livestock should not be ruled out.

This study identified antimicrobial resistance patterns and prevalence that distinguished wildlife *Campylobacter* from livestock *Campylobacter*. Wildlife isolates in this study exhibited resistance only to tetracycline while resistance to macrolides and quinolones was found in livestock species. It was found that *C. coli* isolates from swine in this study exhibited antimicrobial resistance to the macrolides tested. These findings were supported by studies in the past that have established tetracycline resistance genes are commonly circulated in the environment and among wildlife species (Allen et al., 2010, Taylor et al., 1988), while quinolone resistance in *Campylobacter* isolates from cattle and macrolide resistance in *Campylobacter* isolates from swine are relatively common (Anderson et al., 2001, Engberg et al., 2001). In fact, *Campylobacter coli* from swine have shown a higher prevalence of resistance to macrolides compared to both *C. coli* and *C. jejuni* isolates from other food animals (Aerestrup et al., 1997). The prevalence of antimicrobial resistant *Campylobacter* spp. found in this study is consistent with literature documenting AMR *Campylobacter* in beef cattle, dairy cattle, and swine.
(Englen et al., 2007, Sato et al., 2004, Bae et al., 2005). It would be expected that if wildlife and livestock species were readily exchanging *Campylobacter* spp. the prevalence of this pathogen would be similar in wildlife and livestock. Statistical analysis found that livestock *Campylobacter* had significantly higher odds of being resistant to one or more of the antimicrobials tested, than isolates collected from wildlife. The difference in prevalence of AMR *Campylobacter* and distinct antibiograms seen in livestock and wildlife, in this study, suggests that the exchange of *Campylobacter* between these groups of animals is uncommon. This difference could reflect that resistance genes can be readily lost and acquired based on selection pressure and that the exact subtype in both populations could display different antimicrobial resistance profiles. However, coupled with evidence of lack of subtype crossover, this observation provides evidence that antimicrobial resistant subtypes of *Campylobacter* are not readily exchanged.

The limited evidence of exchange of *Campylobacter* found in this study indicate that mammalian wildlife, specifically raccoons, are of lower threat to *Campylobacter*-specific biosecurity efforts on farm than avian wildlife species who have shown more common carriage (Kwan et al., 2008). Transmission of pathogens at the wildlife-livestock interface is highly variable, and despite their apparent limited involvement in maintaining and transmitting livestock *Campylobacter* subtypes, raccoons readily share *Salmonella* serovars found in livestock species and humans (Jardine et al., 2011); whereas other species of wildlife such as wild birds are strongly associated with transmitting subtypes of *Campylobacter* to livestock that have also been isolated from human cases of campylobacteriosis (Kwan et al., 2008). This complex relationship suggests that wildlife-
livestock interactions can result in different opportunities for the exchange of foodborne pathogens depending on the bacterial and vertebrate species investigated.

**LIMITATIONS & STRENGTHS**

This study was a cross-sectional study which took place between January and August, 2010. This study design is limited because it captures only prevalence data. This limits the ability of this study to establish direction of *Campylobacter* transmission between livestock and wildlife as the study did not indicate changes in incidence of *Campylobacter* spp. shedding in these two populations. This study was also limited because data were collected over only one year which did not allow for identification of larger seasonal patterns; it is possible that behavioural changes associated with different seasons may influence *Campylobacter* spp. transmission dynamics in raccoons (Prange et al., 2004), as other pathogens carried by raccoons are influenced by season (Jardine et al., 2014). Despite these limitations, a cross-sectional study design is ideal for exploratory, hypothesis-generating studies across a large number of farms due to its relatively low time and financial commitment compared to other study designs (e.g., cohort studies).

The small number of samples collected from mammalian wildlife species, other than raccoons, in this study limited the scope considerably. Due to sample size issues and insufficient power, statistical models that included non-raccoon wildlife species could not be fitted. Consequently, research investigating a variety of mammalian wildlife species is still required. Future studies, particularly of rodents, should also account for potential culture bias since this study only found *Campylobacter* in 4.1% (2/49) of small wildlife that were sampled and previous studies have established a *Campylobacter* spp. prevalence of up to 38.6% in rodents trapped on farms (Hald et al., 2003). The relatively
small sample size of raccoons (n=49) prevented the development of raccoon specific models to examine how raccoons of different age and sex transmit and maintain *Campylobacter* species.

The use of the highly discriminatory *Campylobacter* specific 40-gene comparative genomic fingerprinting assay (CGF40) in this study has both its strengths and limitations. It is the superior genotyping method for distinguishing molecular subtypes of *Campylobacter* spp. due to its high-resolution and low-cost (Clark et al., 2012). Research has found that CGF40 alone is more discriminatory than any pairwise combination of pulse field gel electrophoresis, multilocus sequence typing, and flagellin gene short variable region sequencing for identifying *Campylobacter* subtypes (Clark et al., 2012). Comparing results obtained in studies using CGF40 cannot always be done easily with studies using other genotyping methods, making it difficult to consolidate and perform meta-analyses. However, it has been established that CGF40 and MLST are highly related, as each MLST sequence type can be broken down into a number of distinct but very similar CGF40 subtypes (Taboada et al., 2012). Future research that maps highly relevant MLST types to CGF40 subtypes will make it easier to compare molecular epidemiological studies of *Campylobacter*.

**FUTURE STUDIES**

Future research is necessary to validate and explore findings from this study. Source attribution is a growing priority of the Canadian government (Public Health Agency of Canada, 2013) and with novel highly-discriminatory methods of *Campylobacter* spp. typing there is an opportunity to understand how wildlife are involved in transmitting pathogens to humans. It is possible, as humans and wildlife
continue to share environments, wildlife may be implicated in transmitting pathogens through contaminating the environment rather than acting as a reservoir for livestock Campylobacter subtypes. Consequently, studies looking at source attribution are of key importance in determining if wildlife in general plays a role in transmitting Campylobacter spp. to humans. Future studies should use larger databases of CGF40 wildlife, livestock, and human subtypes to assist with source attribution investigations. These larger magnitude studies will also be helpful in establishing the validity of observations of distinct C. jejuni populations in wildlife and livestock in this smaller study. Other wildlife species such as skunks, rodents, cervids and wild birds are also relevant in source attribution studies. These studies are useful in distinguishing if farm-level pathogen control efforts are sufficient or if efforts should be focused on limiting wildlife contact with humans. To further understand raccoon-specific carriage of Campylobacter, studies investigating the relationship between Campylobacter spp. isolates from raccoons and human isolates of Campylobacter are necessary. With raccoons entering urban areas and people also engaging in recreational activities in conservation areas, contact between humans, raccoons, and the surfaces they share is common.

This study found that C. jejuni isolates from different species of wildlife were not differentiated from one another. However, our sample size was small and it is possible that different subtypes of Campylobacter spp. may be found in different wildlife species if more animals are examined. It is also possible that species living in urban versus rural versus suburban areas may exhibit different carriage and shedding patterns of Campylobacter spp. which may be of importance when developing intervention
strategies. Using longitudinal studies in the future would also allow investigation of seasonal patterns of *Campylobacter* carriage in wildlife and livestock. Longitudinal studies would provide incidence data which might enable a directionality of transmission to be determined between livestock and wildlife. Smaller mammalian wildlife species may also play a larger role in transmitting *Campylobacter* spp. than what was found in this study due to the small number of small animals captured. For instance, one of four rats, sampled on swine farms in this study, carried *C. coli*. *Campylobacter coli* is the predominant species of *Campylobacter* shed in swine, and in this study was not found in any other wildlife species. This suggests that rats may be worth investigating for their role in maintaining and transmitting *C. coli* in swine.

**CONCLUSIONS**

This study established that the exchange of *Campylobacter* spp. between mammalian wildlife, specifically raccoons, and livestock is infrequent, and distinctive *C. jejuni* subtypes are found in mammalian wildlife and livestock. Raccoons do not appear to play a major role in maintaining and transmitting livestock-associated *Campylobacter* spp. in southern Ontario. However, it is possible that raccoons and other wildlife that contaminate environments, including water, used by humans, could transmit *Campylobacter* to humans, making future source attribution studies extremely important. With *Campylobacter* spp. being the foremost bacterial cause of gastroenteritis (WHO, 2011) and causing severe sequelae such as Guillain-Barré syndrome, and reactive arthritis (Nachamkin et al., 2002), determining how this pathogen enters the food system and environment is of utmost importance.
REFERENCES


other animals in the immediate environment of cattle, pigs and poultry farms in Denmark. *International Journal of Medical Microbiology*, pp.140 (Suppl. no.35).


Campylobacter species. Antimicrobial Agents and Chemotherapy, 32(8), 1107.