EPIDEMIOLOGY, MOLECULAR CHARACTERIZATION, VIRULENCE GENE, AND ANTIMICROBIAL RESISTANCE PROFILES OF THERMOPHILIC *CAMPYLOBACTER* SPECIES IN PUPPIES IN THE NAIROBI METROPOLITAN REGION, KENYA

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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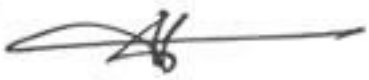

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DEDICATION

I dedicate this thesis to my late mother Naomi Kasiva may her soul rest in peace, my father Wellington Mbole, my sister June Mbindyo, my husband Nduto Mulei and my daughter; Ella Mbithe for the incredible support, love, patience and encouragement they showed me through my Ph. D journey.

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LIST OF ABBREVIATIONS & ACRONYMS

CPV	Canine Parvovirus
CCoV	Canine Coronavirus
AMR	Antimicrobial resistance
MDR	Multidrug-resistant
WHO	World Health Organization
CIBDAI	Canine Inflammatory Bowel Disease Activity Index
BPW	Buffered peptone water
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information
MAFFT	Multiple Alignment using Fast Fourier Transform

ABSTRACT

Dogs are the most popular companion animals in many parts of the world and their ownership has psychological and physical benefits. However, despite the benefits, their close contact with humans poses a major public health concern due to transmission of zoonotic agents such as *Campylobacter* species that are important gastrointestinal pathogens which cause diarrhea in puppies. *Campylobacter* species are amongst the prioritized human and animal pathogens with regards to antimicrobial resistance. Although puppies are considered an important reservoir of *Campylobacter* pathogens, the current status of *Campylobacter* species infecting puppies in Kenya remains unclear. The objectives of this study were to determine the prevalence, risk factors, virulence genes, antimicrobial resistance profiles, and molecular epidemiology of thermophilic *Campylobacter* species in puppies in the Nairobi Metropolitan Region, Kenya.

A cross-sectional study was conducted in the Nairobi Metropolitan Region, Kenya from January 2021 to August 2021 where a total of 260 puppies were randomly selected from breeding kennels, shelters and those presented to the University of Nairobi Veterinary Teaching and Referral Hospital for treatment, vaccinations, routine check-up, and boarding. Rectal swabs were collected from the puppies and a pretested questionnaire was used to collect data on potential risk factors for *Campylobacter* infection. Conventional culture and biochemical tests were done to identify *Campylobacter* species isolates. Genomic DNA was extracted and tested for presence of the *Campylobacter* DNA through amplification of the *16S rRNA* gene and species specific genes using Polymerase chain reaction (PCR). Genetic identities of the *Campylobacter* species were confirmed through BLASTn analysis and phylogenetic reconstruction. Polymerase chain reaction (PCR) was done to assess for the presence of potential virulence genes associated with

motility, adherence, invasion, and cytotoxicity. Resistant phenotypes and genotypes were determined by disc diffusion and molecular methods respectively.

In this study, 150 presumptive *Campylobacter* species isolates were identified by conventional culture and biochemical tests yielding a prevalence of 57.7% (150/260). Polymerase chain reaction (PCR) detected 64 *Campylobacter* species isolates (24.6%, 64/260) by targeting the *16S rRNA* gene specific for these microorganisms. *Campylobacter coli* was the predominant species at 13% (33/260) followed by *C. jejuni* at 4% (10/260). The frequency of the occurrence of selected virulence genes, i.e. *flaA, cadF, ciaB, iam, pldA*, as well as genes responsible for the formation of the cytolethal distending toxin (CDT), i.e. *cdtA, cdtB,* and *cdtC* was determined. *flaA, ciaB,* and c*dtB* putative genes were detected in 25, 46.9, and 43.8% of strains respectively. Resistant phenotypes were detected in *Campylobacter* species with 100% resistance to ampicillin, 96.9% to tetracyclines, 96.9% to erythromycin, and 82.8% to trimethoprim-sulphamethoxazole. The corresponding resistance genes detected in the phenotypic-resistant *Campylobacter* species isolates included *23S rRNA* gene (67.7%) for erythromycin, *gyrA* gene (31.3%) for ciprofloxacin, *tet(C)/tet(O)* genes (21%, 69.4%) for tetracycline, *blaOXA-61* gene (32.8%) for ampicillin, and *aph-3-1* gene (11.1%) for gentamicin.

This is the first study in Kenya to report on the genotyping data, virulence, and antimicrobial resistance profiles of *Campylobacter* species in puppies in Kenya; it showed that pathogenic and multidrug-resistant *Campylobacter jejuni* and *Campylobacter coli* strains are present in puppies in the Nairobi Metropolitan Region, Kenya posing potential zoonotic risk. Great attention should therefore be paid in choosing the most appropriate antimicrobial therapy in small animal practice.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Campylobacteriosis, caused by thermophilic bacteria of the genus *Campylobacter*, is a significant zoonotic gastrointestinal disease affecting humans and animals, including dogs, globally (Coker *et al*., 2002; Kaakoush *et al*., 2015; Carron *et al*., 2018; EFSA and ECDC, 2018; Elmali and Can, 2019; Igwaran and Okoh, 2019). In humans, the disease is characterized by fever, diarrhea, and pain. It is caused by *Campylobacter jejuni* and *Campylobacter coli* and rarely, by other emerging *Campylobacter* species including *Campylobacter lari*, *C. concisus*, and *C. upsaliensis* (Nachamkin and Blaser, 2000; Vandamme, 2000; Prasad *et al*., 2001; Sahin *et al*., 2002; Moore *et al*., 2005; Man, 2011; Selwet and Galbas, 2012; Kaakoush *et al*., 2015). Majority of the human infections are attributed to consumption of contaminated poultry (Wingstrand *et al*., 2006; Sahin *et al*., 2015; Tresse *et al*., 2017; Carron *et al*., 2018), raw milk (Peterson, 2003) and water (Diergaardt *et al*., 2004; Galanis, 2007; Abe *et al*., 2008).

Dogs were first associated with human campylobacteriosis in 1960 (Wheeler and Borchers, 1961) and *Campylobacter jejuni* was the first species isolated from dogs in 1977 (Skirrow, 1977). Since then, several studies have reported *C. jejuni* isolation from healthy and diarrheic dogs worldwide with pathogenic involvement occurring most frequently in puppies (less than one year) and is precipitated by factors such as crowding, stress, and concurrent diseases (Engvall *et al*., 2003; Hald *et al*., 2004; Marks *et al*., 2011).

Most studies have found that the most frequently isolated species from dog feces is *C. upsaliensis*, followed by *C. jejuni* and less frequently *C. coli, C. lari,* and *C. hyointestinalis*

(Engvall *et al*., 2003; Hald *et al*., 2004; Workman *et al*., 2005; Rossi *et al*., 2008; Acke *et al*., 2009; Koene *et al*., 2009; Parsons *et al*., 2011; Goni *et al*., 2017). Most dogs are asymptomatic and shed these pathogens in their feces ultimately infecting humans and other animals by contaminating the environment (Fox, 1990; Hald and Madsen, 1997). The prevalence of *Campylobacter* species in dogs varies widely (Acke *et al*., 2006; Parsons *et al*., 2010; Kumar *et al*., 2012; Verma *et al*., 2014; Holmberg *et al*., 2015; Lazou *et al*., 2016; Torkan *et al*., 2018; Thépault *et al*., 2020). This variation depends on the age, the method of diagnosis, study design, geographic region, housing type, the presence of infection or concomitant disease, and diarrheic versus healthy dogs (Acke *et al*., 2009; Marks *et al*., 2011; Iannino *et al*., 2017).

The isolation of *Campylobacter* species using the culture method is generally used for the diagnosis of campylobacteriosis; however, it is time-consuming and labor-intensive due to the fastidious nature of *Campylobacters* (Li *et* al., 2014). In addition, this method is biased towards the recovery of *C. jejuni* and *C. coli* (Lastovica, 2006), thereby potentially underestimating the emerging species. Due to the close phylogenetic relationship between *C. jejuni* and *C. coli*, biochemical assays cannot reliably distinguish between these two species (Miller *et al*., 2010). This is however overcome by molecular-based assays such as polymerase chain reaction (PCR) and sequencing which allow for rapid and specific detection, thus enhancing epidemiological studies (Kaakoush *et* al., 2015; Vinueza- Burgos *et al*., 2017; Ricke *et al*., 2019).

The pathogenesis of *Campylobacter* infection is complex and poorly understood. However, different studies have indicated that different virulence markers play a role in the motility and adherence of these bacteria to the intestinal mucosa, enterocyte invasion, and toxin production (Humphrey *et al*., 2007; Dasti *et al*., 2010; Wieczorek and Osek, 2013; Lapierre *et al*., 2016; Younis *et al*., 2018). This contributes to their increased occurrence and epidemiology in

comparison to other enteric bacteria (Bolton, 2015; Otigbu *et al*., 2018). The *flaA* gene is involved in motility, colonization, and biofilm formation (Park, 2002; Guerry, 2007) whereas the *cadF* gene is involved in the adherence of these bacteria to the intestinal mucosa (Carvalho *et al*., 2001; Eucker and Konkel, 2012; Pillay *et al*., 2020). The *pldA*, *ciaB*, and *iam* genes are responsible for the expression of invasion of enterocytes (Carvalho *et al*., 2001; Dasti *et al*., 2010; Hamidian *et al*., 2011; Eucker and Konkel, 2012; Shams *et al*., 2016; Pillay *et al*., 2020). *Campylobacters* also excrete several cytotoxins (encoded by *cdtA*, *cdtB*, and *cdtC* genes) that contribute to disease development (Hickey *et al*., 2000; Bolton, 2015; Tresse *et al*., 2017) .

Although majority of dogs may be subclinically infected, others especially puppies less than 6 months of age or those from stressful environments may develop mild to moderate enteritis presenting as mild to watery diarrhea or as bloody or mucoid diarrhea with tenesmus (Brown *et al*., 1999; Chaban *et al*., 2010; Weese, 2011; Acke, 2018). Additional clinical signs include anorexia, dehydration, lethargy, and rarely fever, vomiting, and abdominal pain (Brown *et al*., 1999; Sykes and Marks, 2013; Marks *et al*., 2011). Antimicrobial treatment is usually unnecessary (Kim *et al*., 2019), however, in severe cases where antimicrobial treatment is needed, macrolides (erythromycin), fluoroquinolones (ciprofloxacin) and tetracyclines are recommended (Guerrant *et al*., 2001; Iovine, 2013; Lübbert, 2016; Yang *et al*., 2019). Antimicrobials such as amoxicillin-clavulanic acid and gentamicin could be used to treat systemic *Campylobacter* infections (Dai *et al*., 2020).

A significant use of antimicrobials in humans and animals has led to an increase in antimicrobial resistant *Campylobacter* spp. (Marks, 2003; Humphrey *et al*., 2007; Luangtougkum *et al*., 2009; Agnes *et al*., 2013; Abay *et al*., 2014; Abdollahpour *et al*., 2015; Aslantaş, 2017; Issa *et al*., 2018) which has led the World Health Organization (WHO) to classify *Campylobacters* as

antibiotic-resistant 'high' priority zoonotic pathogens (WHO, 2017). There are reports of wide ranging prevalence of *Campylobacter* strains resistant to: macrolides, fluoroquinolones, tetracyclines, aminoglycosides, betalactams, cephalosporins and sulphonamides (Ishihara *et al*., 2004; Perez-Boto *et al*., 2010; Pollett *et al*., 2012; Karikari *et al*., 2017; Agunos *et al*., 2018; Ewers *et al*., 2018; Zachariah *et al*., 2021). Transmission of resistant *Campylobacter* species or their resistance genes is possible between dogs and humans via direct or indirect contact, through the environment (Iannino *et al*., 2019). Therefore, monitoring of *Campylobacter* resistance is important to public health and great attention should be paid in choosing the most appropriate antimicrobial therapy (Wieczorek *et al.*, 2018).

In Kenya, *Campylobacter* species have been reported in several studies amongst several domestic animal spp. including poultry (Conan *et al*., 2017; Mageto *et al*., 2018; Carron *et al*., 2018), cattle (Osano and Arimi, 1999), pigs, ducks, sheep, and adult dogs (Turkson *et al*., 1988). However, despite the close proximity to humans, data indicating the presence and extent of *Campylobacte*r infection in puppies is limited in Kenya.

The aim of this study was to determine the prevalence, risk factors, virulence genes, antimicrobial resistance profiles, and molecular epidemiology of thermophilic *Campylobacter* species in puppies in the Nairobi Metropolitan Region, Kenya.

1.1 Objectives of the study

1.1.1 General objective

To determine the prevalence, risk factors, molecular characterization, virulence genes, and antimicrobial resistance profiles of thermophilic *Campylobacter* species in puppies in the Nairobi Metropolitan Region, Kenya.

1.1.2 Specific objectives

a) To determine the prevalence and risk factors associated with *Campylobacter* species infection in puppies in the Nairobi Metropolitan Region, Kenya.
b) To identify and characterize *Campylobacter* species isolates derived from puppies in the Nairobi Metropolitan Region, Kenya.
c) To determine the virulence gene profiles of *Campylobacter* species isolates derived from puppies in the Nairobi Metropolitan Region, Kenya.
d) To determine the antimicrobial resistance profiles of *Campylobacter* species isolates derived from puppies in the Nairobi Metropolitan Region, Kenya.

1.2 Research problem

Dogs are the most popular companion animals worldwide and their ownership has psychological and physical benefits. However, despite the benefits, their close contact with humans poses a major public health concern due to transmission of zoonotic agents such as *Campylobacter* species. In small animal practice, few veterinarians recognise the importance of *Campylobacters* yet they cause acute diarrhea and it is difficult to differentially diagnose them from other enteric infections. Despite the clinical impact and zoonotic risk, it is difficult for veterinarians to diagnose *Campylobacter*-associated diarrhea in puppies as the clinical signs are non-specific and fecal enteric panels are expensive, require technical knowledge, and are time-consuming. Studies have also indicated puppies as an important but underestimated reservoir of genetically diverse, potentially virulent, and multidrug-resistant *Campylobacter* species (Holmberg *et al*., 2015). Acke, 2018).

1.3 Justification

Campylobacter species are important pathogens that cause gastroenteritis in humans and animals including dogs (Igwaran and Okoh, 2019). In Kenya, *Campylobacter* species have been reported in several studies amongst several domestic animal spp. including poultry (Carron *et al*., 2018), cattle (Chepkwony, 2016), pigs, ducks, sheep, and adult dogs (Turkson *et al*., 1988). However, despite their close proximity to humans and the clinical and public health impact of *Campylobacter* species, there is scanty information on the epidemiology (molecular and non-molecular) and antimicrobial resistance profiles of *Campylobacter* infections in puppies in Kenya.

This research study aimed to determine the prevalence and risk factors associated with *Campylobacter* species infection in puppies in the Nairobi Metropolitan Region, Kenya as well as the role of puppies as reservoirs of *Campylobacter* species, and potential hazard to public health based on virulence genes, antimicrobial resistance profiles, and genotyping data.

1.4 Hypothesis

1. The prevalence rate of *Campylobacter* species is high in puppies less than 5 months of age in the Nairobi Metropolitan Region, Kenya.
2. *Campylobacter upsaliensis* is the most prevalent *Campylobacter* species in puppies in the Nairobi Metropolitan Region, Kenya.
3. *Campylobacter* infection is more prevalent in kenneled puppies than in household puppies in the Nairobi Metropolitan Region, Kenya.
4. Multidrug-resistant *Campylobacter* species infections are common in puppies in the Nairobi Metropolitan Region, Kenya.

CHAPTER TWO

2.0 GENERAL LITERATURE REVIEW

2.1 Background information

Dogs are the most popular companion animals in many countries including Kenya (McNicholas *et al*., 2005) with the world dog population estimated to be 400 million (Coppinger and Coppinger, 2001). In Kenya, the dog to human population ratio is 1:8, therefore the dog population is 6 million as the human population is estimated to be 48M (NCPD, 2017) with the highest number in Nairobi County as there is an increasing need for companion, security and sniffer dogs (Weru, 2009).

2.2 Infectious causes of diarrhea in puppies

Infectious causes of diarrhea can have detrimental effects on canine health especially in puppies less than a year of age and in severe cases can cause death (Hubbard *et al*., 2007; Weese, 2011; Yagoob and Mashaei, 2011; Grellet *et al*., 2014). Several putative enteropathogens that have been associated with acute diarrhea in puppies include; viral enteropathogens like canine rotavirus, canine coronavirus (CCoV), and canine parvovirus (CPV) (Decaro and Buonavoglia, 2008; Decaro *et al*., 2011), bacterial enteropathogens especially *Campylobacter* spp., *Escherichia coli* and *Salmonella* spp. (Marks *et al*., 2011). There are also parasitic enteropathogens such as *Giardia* spp. and *Cystoisospora* spp., *Ancylostoma* spp., and *Toxocara* spp. (Claerebout *et al*., 2009, Dupont *et al*., 2013, Grellet *et al*., 2014). Age, breed, sex, breeder origin, vaccination history and/or stay in kennels are risk factors associated with acute infectious diarrhea in puppies (Stavisky *et al*., 2011; Dupont *et al*., 2013; Grellet *et al*., 2014).

2.3 *Campylobacter* spp.

2.3.1 History of *Campylobacter* spp.

Escherich first described *Campylobacter* in 1886 through a microscopic examination of vibrio-like organisms in the feces of diarrheic children. In a veterinary medical report on epizootic abortions in ewes, vibrio-like organisms were isolated from aborted fetuses (McFadyean and Stockman, 1913). Similar organisms were isolated from bovine fetuses in 1919, prompting the proposed name *Vibrio fetus* (Smith, 1919). A similar organism involved in calves' winter dysentery was described in 1931 leading to the proposed name *Vibrio jejuni* (Jones *et al*., 1931). In 1957 and 1962, King described a thermophilic *Vibrio* related to the *V. fetus* isolated in 1947 by Vincent and his colleagues. Sebald and Veron proposed the name *Campylobacter* in 1963 to distinguish vibrio-like organisms spiral organisms from Vibrio spp.

Dekeyser *et al*. (1972) used a differential filtration technique to isolate "the related vibrio" from the feces of a woman who had severe diarrhea and a fever. The fecal suspensions were filtered through 0.65 micrometer filters, and the filtrate was then inoculated onto a selective agar plate. Butzler *et al*. (1973) also isolated *C. jejuni* from 5.3% of 3800 children with diarrhea and 1.6% of 7200 healthy individuals. Butzler *et al*. (1974) reported susceptibility of isolates to erythromycin and a diagnosis of *C. jejuni* was based on the resolution of diarrhea and fecal excretion of *C. jejuni* following treatment with this antibiotic.

Butzler and his colleagues demonstrated the ability of *C. jejuni* to invade poultry as well as the close antigenic relationship between isolates from people and those from sheep, pigs, and poultry by performing agglutination and complement fixation tests (Butzler *et al*., 1974). A less difficult method for culturing *C. jejuni* and *C. coli* from stool samples was described by Skirrow (1977). The routine isolation of *Campylobacter* species was made possible by the later development of

selective media, which eliminated the need to filter fecal suspensions (Bolton *et al*., 1984; Karmali *et al*., 1986; Goossens *et al*., 1986; Endtz *et al*., 1991).

2.3.2 Taxonomy and microbiology

The genus *Campylobacter* belongs to the family *Campylobacteraceae*, order *Campylobacterales*, class *Epsilonproteobacteria*, and phylum *Proteobacteria* (Fitzgerald and Nachamkin, 2011). Numerous species are thermophilic, signifying that their optimal growth occurs at elevated incubation temperatures (Allos, 2001; Corry *et al*., 1995). The most common temperatures for in vitro growth are 37^{o}C and 42^{o}C, which correspond to the temperatures of mammalian and avian hosts, respectively (Corry *et al*., 1995).

Campylobacters are classified as 'microaerophilic' because they require less oxygen and more carbon dioxide for optimal growth. The addition of hydrogen to the gas mixture is required for *C. curvus*, *C. rectus*, and *C. concisus* (Corry and Atabay, 2001; Lastovica, 2006; Kaakoush *et al*., 2015).

They are motile due to the presence of a single or multiple flagella at one or both poles of the cell; this flagellar apparatus enables the characteristic rapid corkscrew, darting motility and colonization of the mucus lining of the gastrointestinal tract (Guerry, 2007; Lastovica *et al*., 2014). They are fastidious due to their limited nutrient utilization and slower growth rate when compared to other intestinal bacteria (Lastovica, 2006). They are 0.2-0.8μm by 0.5-5μm in size, do not form spores, and they derive their energy source from the intermediate cycle of tricarboxylic acid or amino acids (Vandamme *et al*., 2005; Hofreuter, 2014).

2.3.3 Sources of *Campylobacter* isolates

Campylobacter spp. have three main niches: the gastrointestinal tract, the urogenital tract, and the oral cavity (Man, 2011; Iraola *et al*., 2014). *Campylobacter jejuni*, *C. coli*, *C. lari*, *C. upsaliensis*, *C. hyointestinalis*, *C. hominis*, *C. ureolyticus*, and *C. helveticus* are among the *Campylobacter* spp. found in the intestine. *C. fetus* and *C. sputorum* are urogenital tract species, whereas *C. rectus*, *C. gracilis*, *C. curvus*, *C. concisus*, and *C. showae* are oral cavity species. (Man, 2011).

Campylobacter jejuni and *C. coli* are the intestinal species that have been studied the most (Man, 2011). The primary reservoirs of *C. jejuni* are poultry, wild birds, cattle, and sheep (Altekruse *et al*., 1994; Newell, 2002; Stanley and Jones, 2003; Devane *et al*., 2005). *Campylobacter upsaliensis* is most commonly found in dogs (Hald *et al*., 2004; Rossi *et al*., 2008; Koene *et al*., 2009; Marks *et al*., 2011) whereas, *C. coli* is predominantly detected in sheep and goats (Nesbakken *et al*., 2003; Brown *et al*., 2004).

Campylobacter jejuni and *C. coli* are the most studied intestinal species (Man, 2011). *C. jejuni* is mostly found in poultry, wild birds, cattle, and sheep (Altekruse *et al*., 1994; Newell, 2002; Stanley and Jones, 2003; Devane *et al*., 2005), whereas *C. upsaliensis* is mostly found in dogs (Hald *et al*., 2004; Rossi *et al*., 2008; Koene *et al*., 2009; Marks *et al*., 2011). *C. coli* is mostly associated with sheep and goats (Nesbakken *et al*., 2003; Brown *et al*., 2004).

Campylobacter spp. are pathogenic or commensal agents found in the gastrointestinal tracts of mammals and birds (Facciolà *et al*., 2017) and are shed in large numbers, contaminating the environment (Keener *et al*., 2004; Waldenström *et al*., 2010). These bacteria have been isolated from soil (Stuart *et al*., 2010), sewage (Jones, 2001; Hokajarvi *et al*., 2013), feedlots (van

Donkersgoed *et al*., 2009), and freshwater (Carter *et al*., 2009; van Dyke *et al*., 2010). The presence of *Campylobacter* spp. in these environments is thought to be due to contamination with feces carrying the species via direct and indirect routes, as the species does not grow and multiply outside of warm-blooded animals (Jones, 2001; Silva *et al*., 2011). This inability to grow and multiply below 30°C is due to a lack of cold-shock proteins, as demonstrated by *C. jejuni*, which many bacteria use to grow at lower temperatures (Hazeleger *et al*., 1998; Park, 2002). These environmental sources are thought to be vehicles for the spread of *Campylobacter* spp. between hosts, and changes in *Campylobacter* environmental loading are linked to changes in animal reservoirs (kather , 2001). Food contaminated with *Campylobacter* spp. can cross-contaminate other foods and utensils due to the handling, preparation, and storage procedures used in the food chain and kitchens (Humphrey *et al*., 2007).

2.4 Epidemiology of *Campylobacter* infections in dogs

In animals, *Campylobacter* spp. transmission is through indirect or direct fecal-oral route, with infection sources being raw or undercooked food, fresh feces from fomites, infected animals, the environment, and when in contact with animals that are infected (Acke, 2018). Numerous studies on the prevalence of *Campylobacter* spp. have been conducted in different countries and it varies depending on the age, their housing, geographic region, study design, diarrheic versus healthy dogs, the method of diagnosis, and the presence of infection or concomitant disease with enteropathogenic bacteria (Table 2.1).

Table 2. 1 Reported prevalence rates of selected *Campylobacter* species in dogs

Taxon	Prevalence (%)	Method	Country	Reference
Campylobacter jejuni	23	Rectal swab, culture	Nigeria	Salihu *et al*., 2010
	53	Rectal swab, culture	Italy	Giacomelli *et al*., 2015
	6	Fecal swab, culture	Ecuador	Toledo *et al*., 2015
	6	Rectal swab, culture	Slovakia	Badlik *et al*., 2014
	23	Rectal swab, culture	Poland	Andrzejewska *et al*., 2013
	1	Feces, culture, PCR	UK	Parsons *et al*., 2010
	11	Feces, culture	Sweden	Engvall *et al*., 2003
Campylobacter coli	7	Rectal swab, culture	Italy	Giacomelli *et al*., 2015
	4	Fecal swab, culture	Ecuador	Toledo *et al*., 2015
	9	Rectal swab, culture	Southern Chile	Fernadez and Oval, 2013
	1	Rectal swab, culture	Switzerland	Wieland *et al*., 2005
	11/40	Fecal swab, culture	Poland	Selwet *et al*., 2015
	13	Rectal swab, culture	Chile	Fernandez and Martin, 1991
Campylobacter upsaliensis	74	Rectal swab, culture	Nigeria	Salihu *et al*., 2010
	52	Feces, culture	Iran	Rahimi *et al*., 2012
	62	Feces, culture	UK	Parsons *et al*., 2011
	34	Feces, culture	Australia	Barker *et al*., 1999
	25	Feces, culture, PCR	UK	Westgarth *et al*., 2009
	59	Fecal swab, culture	Denmark	Hald *et al*., 2004
	43	Feces, culture	Sweden	Engvall *et al*., 2003

Pintar *et al*. (2015) conducted a meta-analysis research considering 34 publications that described the prevalences of *Campylobacter* spp. in pet animals and an average prevalence of 24.7% was reported in household cats and dogs. The study noted that the lack of standardization

of methods significantly complicated the interpretation of results thus confirming gaps in the knowledge which exists in the significant *Campylobacter* spp. infections and its effect on humans.

2.4.1 The Kenyan perspective

Several epidemiological studies aimed at isolating *Campylobacter* spp., establishing their prevalence and risk factors in Kenya have been conducted in humans (Kabiru, 2014; Conan *et al*., 2017; Gitahi *et al*., 2020), poultry (Conan *et al*., 2017; Mageto *et al*., 2018; Carron *et al*., 2018; Chege, 2022), cattle (Osano and Arimi, 1999), pigs, ducks, sheep, and adult dogs (Turkson *et al*., 1988). However, there is limited information on its occurrence in dogs/ companion animals.

2.5 Factors associated with *Campylobacter* species prevalence in dogs

2.5.1 Signalment

Several studies have reported high prevalence in puppies as compared to adult dogs (Parsons *et al*., 2010; Leonard *et al*., 2011; Rahimi *et al*., 2012; Kumar *et al*., 2012; Holmberg *et al*., 2015; Selwet *et al*., 2015; Thépault *et al*., 2020). This could be due to their immature immune system and underdeveloped gut microbiota, which are unable to perform competitive exclusion against pathogens (Karama *et al*., 2019). As in humans, puppies are more likely to acquire clinical campylobacteriosis than adults and more commonly shed the organism (Hald and Madsen, 1997). There are no reports of sex or animal breed predispositions in the published literature.

2.5.2 Intensive housing

Kenneled dogs that are immunocompromised are more likely to be positive to *Campylobacter* spp. on culture than pet dogs (Marks, 2003; Workman *et al*., 2005; Parsons *et al*., 2011; Acke, 2018). This is probably due to the stress, dietary variation, and close interaction with other

animals (Baker *et al*., 1999; Acke *et al*., 2009; Marks *et al*., 2011; Parsons *et al*., 2011; Badlik *et al*., 2014; Giacomelli *et al*., 2015; Leahy *et al*., 2016). Poor hygienic conditions provide an environment conducive to the propagation of *Campylobacter* spp. (Mbindyo *et al*., 2021).

2.5.3 Presence of intestinal disease

Campylobacters have been isolated from both healthy dogs (Workman *et al*., 2005; Acke *et al*., 2009; Kumar *et al*., 2012) and diarrheic dogs (Cave *et al*., 2002; Guest *et al*., 2007). Chaban *et al*. (2010) and Malayeri *et al*. (2014) reported that diarrheic dogs had a higher *Campylobacter* spp. prevalence in comparison with healthy dogs. However some studies demonstrated an insignificant difference in the prevalence of *Campylobacter* spp. in diarrheic or healthy dogs (Suchdolski *et al*., 2010; Duijvestijn *et al*., 2016), which may be an indication of the occurrence of subclinical infections.

In a study conducted by Olson and Sandstedt (1987), dogs were infected experimentally with *C. upsaliensis* and *C. jejuni*, one out of the three dogs infected with *C. upsaliensis* passed soft feces whereas one out of the three dogs infected with *C. jejuni* developed diarrhea. In another study, puppies orally inoculated with *C. jejuni* developed mild symptoms of enteritis (Macartney *et al*., 1988). Parasitic or viral enteritis may predispose dogs to *Campylobacter* spp. infection (Brown *et al*., 1999) thus clinical signs may develop in dogs with comorbidities.

2.5.4 Diet

Majority of the pet owners feed purchased or homemade raw meat diets which pose a high risk for the dogs and humans handling the meat as it is frequently contaminated with these organisms (Olkkola *et al*., 2015; Fredriksson-Ahomaa *et al*., 2017). Puppies fed on a homemade food diet are also at a risk for *Campylobacter* infections (Leonard *et al*., 2011). This increases the fecal

shedding of the organism thus increasing the risk of spread to other pets, domestic animals and humans especially those who are at a high risk due to age or immunosuppression (Mbindyo *et al*., 2021). To reduce this risk, raw meat diets should not be fed to dogs. When handling the meat prior to cooking, handwashing is encouraged together with disinfecting any surfaces that have come in contact with the raw meat (Mbindyo *et al*., 2021).

2.5.5 Infection with multiple *Campylobacter* spp.

Multiple *Campylobacter* spp. identified from fecal samples by direct polymerase chain reaction (PCR) and/or bacterial culture is relatively common (Kulkarni *et al*., 2002; Chaban *et al*., 2010; Kaakoush *et al*., 2015; Bojanić *et al*., 2017). The complex epidemiology of canine campylobacteriosis has been demonstrated by the confirmation of genetic heterogeneity within several *Campylobacter* spp. (Koene *et al*., 2009; Parsons *et al*., 2010; Amar *et al*., 2014; Bojanić *et al*., 2017; Thépault *et al*., 2020). Introgression among multiple *Campylobacter* spp. may lead to pathogen adaptation, higher pathogenicity, and antimicrobial resistance (Mbindyo *et al*., 2021).

2.5.6 Concomitant *Campylobacter* spp. infection with other organisms

Concomitant *Campylobacter* spp. infection with organisms such as parvovirus (Olson and Sandstedt, 1987; Workman *et al*., 2005) and *Helicobacter* spp. (Rossi *et al*., 2008) has been reported. Direct PCR performed on fecal samples in healthy dogs from dog parks revealed *Campylobacter* spp. co-infection with coronavirus, circovirus, *Clostridium* spp., as well as *Cryptosporidium* spp. (Hascall *et al*., 2016). Co-infection of *Campylobacter* spp. with other organisms may impact the progression of *Campylobacter* infection in dogs from mild to severe as well as increasing the incidence of multidrug-resistant organisms (Mbindyo *et al*., 2021).

2.5.7 Intermittent shedding and transient infection

The fecal shedding of *Campylobacter* spp. is either intermittent or transient. A study by Hald *et al*. (2004) reported a high shedding prevalence in puppies less than a year old. They also reported that the shedding of *C. upsaliensis* was more continuous over a long period in comparison to *C. jejuni* suggesting the commensal nature of *C. upsaliensis* while *C. jejuni* infections are transient (Hald *et al*., 2004). Parsons *et al*. (2011) noted similar observations in kenneled dogs. The intermittent shedding of homogenous *Campylobacter* spp. strains in several dogs has been determined by pulsed-field gel electrophoresis (Hald *et al*., 2004).

2.6 Clinical signs of campylobacteriosis in dogs

Campylobacter jejuni, C. coli, and C. upsaliensis are the main *Campylobacter* spp. found in dogs (Workman *et al*., 2005; Parsons *et al*., 2010). Although majority of dogs may be subclinically infected, some dogs especially puppies less than 6 months of age or those from stressful environments may develop mild to moderate enteritis presenting as mild to watery diarrhea or as bloody or mucoid diarrhea with tenesmus (Brown *et al*., 1999; Chaban *et al*., 2010; Weese, 2011; Acke, 2018). Other clinical signs include anorexia, dehydration, lethargy, and rarely fever, vomiting, and abdominal pain (Brown *et al*., 1999; Sykes and Marks, 2013; Marks *et al*., 2011). Extra-intestinal *Campylobacter* infections cause cholecystitis and cholangiohepatitis (Oswald *et al*., 1994; Center, 2009; Sykes and Marks, 2013). In addition, *Campylobacter upsaliensis* has been associated with acute polyradiculoneuritis (APN) (Martinez-Anton *et al*., 2018) while *C. jejuni* infection has been linked to abortion (Odendaal *et al*., 1994) and perinatal death (Sahin *et al*., 2014).

Barko *et al*. (2018) suggested that *Campylobacter* spp. may cause canine inflammatory bowel disease (cIBD) however, prospective studies to determine the importance of *Campylobacter* spp.

in IBD pathogenesis are warranted (Maunder *et al*., 2016). *Campylobacter* spp. have also been found in samples of saliva from dogs with oral disease, a likely indication of these organisms play a role in the pathogenesis of the condition (Yamasaki *et al*., 2012; Petersen *et al*., 2007).

2.7 Diagnosis

Campylobacter spp. are either primary or secondary pathogens therefore isolation of these organisms is not a diagnosis of canine campylobacteriosis and hence concurrent or underlying diseases should be considered (Marks and Kather, 2003; Acke, 2018). For complicated cases, blood samples should be collected for hematology and serum biochemistry (Allenspach 2013; Sykes and Marks, 2013) and diagnostic imaging done when extra-intestinal signs are present (Mapletoft *et al*., 2018).

2.7.1 Detection and identification methods

In cases where there are signs of enteritis, 2-3g of fresh feces or rectal swabs in Cary Blair or Amies transport media are submitted to the laboratory for culture (Marks *et al*., 2011). The morphological characteristics of *Campylobacter* colonies differ depending on the culture medium used. Various selective media for isolation of *Campylobacter* spp. from samples in modified atmospheric environments with a temperature range of 37^{o}C to 42^{o}C have been described, such as the modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) medium for the isolation of *C. coli* and *C. jejuni* (Gun-Munro *et al*., 1987). There is an agar with Cefoperazone, Amphotericin, and Teicoplanin (CAT) for the isolation of *C. coli, C. lari, C. helveticus, C. upsaliensis,* and *C. jejuni* (Acke *et al*., 2009; Bojanić *et al*., 2017). There is also a filtration technique with blood agar for *C. sputorum, C. curvus, C. concisus,* and *C. rectus* isolation (Lastovica, 2006; Kaakoush *et al*., 2015).

Colonies tend to spread over the agar and are typically grey in color, flat or slightly raised, with irregular margins (Nachamkin *et al*., 2000). Phenotypic tests, such as biochemical tests, growth at various temperatures, gram stain, and antibiotic sensitivity profiles, are used to characterize *Campylobacter* spp. (Bolton *et al*., 1984; Corry *et al*., 1995; On, 1996). Hippurate and indoxyl acetate hydrolysis, oxidase production, catalase activity, H2S production, and nitrate reduction are the most frequently used biochemical tests to identify *Campylobacter* spp. (Nachamkin *et al*., 2000). Basic phenotypic characteristics of selected *Campylobacter* spp. are presented in Table 2.2.

Table 2. 2 Basic phenotypic characteristics of selected *Campylobacter* species

Phenotypic test	***C. jejuni***	***C. coli***	***C. upsaliensis***
Gram stain morphology	Gram negative, curved rods	Gram negative, curved rods	Gram negative, curved rods
Test for catalase	+	+	_
Test for oxidase	+	+	+

Morphology and motility are evaluated using Gram stain or wet mount phase-contrast microscopy; if the characteristic appearance (curved rods with darting motility) is observed, *Campylobacter* spp. are presumed. If colonies are obtained from selective media (containing antimicrobial agents) and grown in a microaerobic environment, this characteristic morphological finding, combined with an oxidase-positive biochemical result, can be used to reliably identify the bacteria as *Campylobacter* spp. (Nachamkin *et al*., 2000)

In cases of *C. jejuni* enteritis, it is possible to see gram negative, slender, gull-wing rods and leukocytes on fecal gram stains (Marks *et al*., 2011) and curved bacteria with a darting motion on

phase-contrast or dark-field microscopy (Marks and Kather, 2003). However, a microscopic morphological diagnosis alone cannot be validated, and further assessment is required (Marks *et al*., 2011). Other infectious causes of diarrhea such as, helminths should also be evaluated (Allenspach, 2013).

Although phenotypic tests are easy to use, cost-effective, and widely available, they have limitations such as a lack of standardised tests, which results in variations in results of the same strains across laboratories (Fox, 2012; On, 2013). Due to the inability to differentiate closely related strains (Li *et al*., 2009), genotypic techniques such as polymerase chain reaction (PCR) have been utilized for accurate identification and characterization (On, 2013).

Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry is an inexpensive, precise, and rapid method applied in commercial diagnostic laboratories for *Campylobacter* spp. identification and typing, and the detection of antibiotic resistance (Kiehntopf *et al*., 2011; Singhal *et al*., 2015; van Belkum *et al*., 2015).

2.7.2 Molecular Diagnosis

2.7.2.1 Detection of *Campylobacter* spp. using molecular methods

These are the yardstick for the detection, speciation, and typing of organisms and they have enhanced the diagnostic capabilities of veterinary diagnostic laboratories (Adzitey *et al*., 2013; On, 2013; Cai *et al*., 2014; Kaakoush *et al*., 2015). Amplification of a DNA segment using primers and probes created for a specific genus- or species-specific sequence is the basis of the polymerase chain reaction (PCR) (Saiki *et al*., 1988; On, 1996). Polymerase Chain Reaction can be used to design a presence/absence test (conventional PCR), a test with multiple targets

(multiplex PCR), and a qualitative test (real-time PCR) (Schuurman *et al*., 2007; de Boer *et al*., 2013).

Multiplex PCR confirms *Campylobacter* spp. from fecal samples isolated on bacterial culture (Persson and Olsen, 2005; Neubauer and Hess, 2006) whereas real-time PCR detects these organisms from feces without prior bacterial culture (Chaban *et al*., 2009, Chaban *et al*., 2010). *Campylobacter* in saliva from dogs have been detected by PCR-denaturing gradient gel electrophoresis (Petersen *et al*., 2007). Primer and probe design for PCR is contingent on the available DNA sequences at the time, therefore as new species are discovered, the primers and probes should be reassessed to improve diagnostic efficacy (On, 1996; On *et al*., 2013).

2.7.2.2.1 Sub-typing of Campylobacter spp. strains

Multilocus Sequence Typing (MLST) is a "gold standard" sub-typing technique used for the identification of *C. helveticus, C. upsaliensis, C. coli,* and *C. jejuni* from clinical samples without prior bacterial culture (Dingle *et al*., 2001; Workman *et al*., 2005; Taboada *et al*., 2013; On, 2013). Amplification and sequencing of (seven) housekeeping genes are done for MLST and allelic numbers are allocated in comparison with the PubMLST databases (http://pubmlst.org/campylobacter/) (Dingle *et al*., 2001; Workman *et al*., 2005; Taboada *et al*., 2013; Holmberg *et al*., 2015). According to Cantero *et al*. (2018), whole-genome sequencing differentiates strains and also identifies the antimicrobial resistant and virulent determinants in *Campylobacter* spp.

2.8 Pathogenesis of Campylobacteriosis

The pathogenesis of *Campylobacter* infection is complex and poorly understood. There are however reports indicating that the expression of genes involved in intestinal colonization, epithelial cell adhesion and invasion, and toxin production play a crucial role in the development

of disease in animals and humans (Humphrey *et al*., 2007; Thakur *et al*., 2010; Dasti *et al*., 2010; Wieczorek and Osek, 2013; Lapierre *et al*., 2016; Younis *et al*., 2018).

The flagellin-encoding *flaA* gene is essential for small intestinal motility and colonization (Wysok and Wolfaka, 2018). According to reports, this gene may also be responsible for the expression of adhesion, colonization, and invasion of host cells in the gastrointestinal tract, therefore causing an arrest of the immune response (Farfan *et al*., 2019).

Campylobacter adhesion to fibronectin (*cadF*) gene promotes adherence to fibronectin in the gastrointestinal epithelial cells in animals and humans (Bolton, 2015). It is mediated by a 37-kDa fibronectin binding out membrane protein and is essential for *Campylobacter* adhesion and colonization of the host cell surface (Bolton, 2015; Ghunaim *et al*., 2015), and is present at a high level in *C. jejuni* isolates (Kalantar *et al*., 2017).

The Campylobacter invasive antigen B (*ciaB*), the invasion-associated marker (i*am*), and the *pldA* genes are responsible for *Campylobacter* invasion (Wieczorek *et al*., 2018). *CiaB* is involved in *Campylobacter* translocation into host cells for host invasion (O' Croinin and Backert, 2012; Wieczorek *et al*., 2018). Carvalho *et al*. (2001) detected the *iam* gene in 85% of invasive strains and 20% of non-invasive strains. The *pldA* gene which encodes for outer membrane phospholipase A, has been linked to increased bacterial invasion on host epithelial cells (Hamadian *et al*., 2011; O' Croinin and Backert, 2012; Reddy and Zishiri, 2018).

Toxins are crucial to the pathogenesis of campylobacteriosis (Méndez-Olvera *et al*., 2016). The cytolethal distending toxin (CDT) complex encodes for the cytolethal distending toxin, where cdtB is the active toxic unit and cdtA and cdtC are required for cdt binding to target cells and the

delivery of cdtB into the cell interior, where it induces cell cycle arrest and apoptosis of epithelial and immune cells in the intestines (Jain *et al*., 2008).

2.9 Antimicrobial therapy and multidrug-resistant *Campylobacter* infections

The increased use of antimicrobials in humans and animals has led to an increase in *Campylobacter* strains that are resistant to antimicrobials (Humphrey *et al*., 2007), therefore prudent use of antibiotics in animals with suspected *Campylobacter*-associated diarrhea is advised for febrile or immunocompromised patients where the infection is severe or is extra-intestinal (Marks, 2003; Marks *et al*., 2011).

Macrolides (erythromycin and azithromycin), fluoroquinolones (ciprofloxacin), and tetracyclines are recommended for effective treatment of severe *Campylobacter* infections (Marks, 2003). In dogs, erythromycin (10–15 mg/kg orally every 8 hours) (Marks *et al*., 2011; Weese, 2011), or azithromycin (5–10 mg/kg orally every 24 hours) (Marks *et al*., 2011) has been recommended in cases where susceptibility results are unavailable. Antimicrobials such as gentamicin and amoxicillin-clavulanic acid could be used as alternatives for the treatment of systemic *Campylobacter* infections (Dai *et al*., 2020).

Multi-drug resistance *Campylobacter* spp. have been isolated from dogs therefore treatment should only be administered where necessary/ supported by appropriate laboratory tests (Fitzgerald, 2008; Marks *et al*., 2011; Kumar *et al*., 2012; Cho *et al*., 2014; Rodrigues *et al*., 2015; Ahmed *et al*., 2018; Moser *et al*., 2020). In the United States, multistate outbreaks of multidrug-resistant *Campylobacter* infections have been reported in humans linked to contact with puppies from pet stores (Montgomery, 2018).

The prognosis of canine *Campylobacter* infections is good when the treatment is appropriate and in the absence of systemic complications. In cases of treatment failure, reinfection, incorrect diagnosis, the intermittent shedding of *Campylobacter* spp., strain variations, and the development of antimicrobial resistant strains should be considered (Marks *et al*., 2011; Kaakoush *et al*., 2015; Acke, 2018).

2.10 Mechanisms of drug resistance

Scientific studies have revealed that *Campylobacter* antimicrobial resistance is linked to the acquisition of resistance genes, point mutations, efflux systems, and genetic determinants of resistance which can be chromosomally encoded or can be located on plasmids (Luangtongkum *et al*., 2009).

2.10.1 Resistance to Macrolides

Macrolides act by targeting the 50S subunit of ribosomes and interrupting production of proteins (Wieczorek and Osek, 2013). Studies show the *23S rRNA* nucleotides 2058 and 2059 are of key importance in the attachment of macrolides and therefore changes in the attachment area of macrolides on the ribosome mediates their resistance (Batchelor *et al*., 2004). Replacement of nucleotides at positions 2074 and 2075 of the adenine residues in the *23S rRNA* gene in *Campylobacter* frequently occur in erythromycin resistance (Luangtongkum *et al*., 2009). The A2074C, A2074G, and A2075G mutations result in increased macrolide resistance in *C. jejuni* and *C. coli* with erythromycin resistance corresponding with resistance to all other macrolides (Avrain *et al*., 2004).

Resistance to macrolides is also facilitated by a CmeABC multidrug energy-dependent efflux pump which extrudes antimicrobials among other substances from a *Campylobacter* cell (Lin *et al*., 2002; Pubwe and Piddock, 2002; Mamelli *et al*., 2005). This pump has three components: a

periplasmic protein (encoded by *cmeA*), an inner membrane drug transporter (encoded by *cmeB*), and an outer membrane protein (encoded by *cmeC*) (Lin *et al*, 2002). Of the three genes, the *cmeB* is reported to be the best target in detecting the efflux system by polymerase chain reaction (PCR) (Olah *et al.*, 2006).

2.10.2 Resistance to Quinolones

Quinolones exert their action by targeting the enzymes topoisomerase IV (encoded by *parC* and *pare* genes) and DNA gyrase (encoded by *gyrA* and *gyrB* genes) found in bacteria which are involved in DNA duplication, transcription, repair, and recombination (Drlica and Zhao, 1997; Jacoby, 2005; Wieczorek and Osek, 2013). In *Campylobacter*, resistance to fluoroquinolones is mainly a result of *gyrA* gene mutations (Engberg *et al*., 2001). A Thr86Ile point mutation in the *gyrA* gene is reported to be responsible for high resistance to ciprofloxacin (Ge *et al*., 2005). The cmeABC efflux system responsible for multiple antimicrobial resistances also works in tandem with the gyrA mutations resulting in resistance (Lin *et al*., 2002; Pumbwe and Piddock, 2002).

2.10.3 Resistance to Tetracyclines

Tetracyclines act by attaching to ribosomes and hindering elongation of protein production (Gibreel *et al*., 2004). They use their attachment to Mg+2 cations to go through outer membrane porins (Chopra and Roberts, 2001). Ribosomal protection proteins such as the *tetO* and the tetM genes facilitate tetracycline resistance (Connell *et al*., 2003). The *tetO* is plasma mediated and is liable for tetracycline resistance in *Campylobacter* (Connell *et al*., 2003). *TetM* is the other gene that has been identified in *Campylobacter* isolates (Abdi-Hachesoo *et al*., 2014). Studies show the likelihood of C*ampylobacter tetO* originating from *Streptomyces*, *Streptococcus*, or *Enterococcus* species through horizontal genetic transmission (Batchelor *et al*., 2004).

2.10.4 Resistance to Aminoglycosides

Aminoglycosides act through the 30S ribosomal subunit, preventing precise codon-anticodon identification and disturbance of protein elongation by impeding the movement of tRNA from the A-site to the P-site (Jana and Deb, 2006). *Campylobacter* aminoglycoside resistance is mediated by a 3' aminoglycoside phosphotransferase that is encoded by *aphA-3-1* gene (Gibreel *et al*., 2004; Toth *et al*., 2010). Additionally, *Campylobacter* strains contain mosaic plasmids containing aminoglycoside resistance genes (Velazquez *et al*., 1995).

2.10.5 Resistance to Other Antimicrobial Agents

Beta-lactam resistance in *Campylobacter* is poorly defined (Stones, 2011). Most *C. jejuni* and *C. coli* isolates can produce beta-lactamases, rendering the beta-lactam particle inactive (Stones, 2011). Efflux pumps are also involved in this resistance too (Lin *et al.*, 2002).

Chloramphenicol acts by inhibition of protein elongation in bacteria (Wieczorek and Osek, 2013). Chloramphenicol resistance in *C. coli* is via an acetyltransferase encoding gene that is plasmid-mediated although this resistance is rarely seen phenotypically (Wieczorek and Osek, 2013).

Sulphonamide resistance in *C. jejuni* is a chromosome mutation with substitutions of various amino acids in the dihydropteroate synthetase (DHPS). Competition for DHPS between sulphonamides and para-aminobenzoic acid (PABA) prevents the latter from assimilation into folic acid (Engberg *et al*., 2001).

Another mechanism by which *Campylobacter* has been reported to develop resistance to multiple drugs is the CmeABC multidrug efflux pump (Pumbwe *et al*, 2004). The three fragments of the pump i.e. membrane fusion proteins inner drug transporter and outer membrane protein act to

enable the transportation of substrates from outside the cell into the cell matrix (Krishnamoorthy *et al*., 2008).

2.11 Antimicrobial resistance testing methods

Drug resistance can be tested by use of either phenotypic methods which show whether the bacterial isolate is expressing the resistance or molecular techniques which show presence of resistance genes. Phenotypic methods include:

2.11.1. Agar dilution method

Antimicrobials' minimal inhibitory concentration (MIC) is determined using the agar dilution method (Ruangpan, 2004). The minimum inhibitory concentration (MIC) of a drug is defined as the lowest concentration that will inhibit visible growth of an organism after overnight incubation (Andrews, 2001). It is carried out on Mueller-Hinton Agar (MHA), which is the preferred medium due to its reproducible results and low sulfonamide, trimethoprim, and tetracycline inhibitors, which allows most bacteria to grow satisfactorily, but other media, such as MHA supplemented with blood, may be used for bacteria with special requirements (Ruangpan, 2004).

2.11.2 Diffusion tests

2.11.2.1 Disk diffusion test

The disk diffusion susceptibility test (Jorgensen *et al*., 2007; CLSI, 2009) is an easy and relatively inexpensive test that involves inoculating isolated bacteria onto a Mueller-Hinton Agar plate and then placing antibiotic-impregnated paper disks on the agar's surface. When the plate is incubated, the antibiotics diffuse into the agar in a gradient, with the antibiotic concentration decreasing as the distance from the disk increases (Winn *et al*., 2006; Schwalbe *et al*., 2007). Antibiotic susceptibility is determined by measuring the diameter of the zones of bacterial

inhibition surrounding the antibiotic disks and comparing it to disk diffusion interpretative criteria (Schwalbe *et al*., 2007).

2.11.2.2 E-test

The E- test consists of a strip containing a known gradient of an antimicrobial agent that has been calibrated to produce results in the form of minimal inhibitory concentrations (MICs) of the antimicrobial agents (Citron *et al*., 1991; Huang *et al*., 1992).

2.12 Public health importance

Campylobacter spp. are among the leading zoonotic pathogens causing gastroenteritis worldwide (Havelaar *et al*., 2015; Kaakoush *et al*., 2015). Dog ownership has also been linked to an increased risk of human *Campylobacter jejuni*/ *Campylobacter coli/C. upsaliensis* infection (Wolfs *et al*., 2001; Labarca *et al*., 2002; Rahimi *et al*., 2012; Kittl *et al*., 2013; Mughini-Gras *et al*., 2013; Kaakoush *et al*., 2015; Bojanić *et al*., 2017; Acke, 2018).

It is estimated that approximately 9% of human *Campylobacter* infections emanate from direct contact with feces from pets (Tam *et al*., 2009; Kittl *et al*., 2013; Mughini *et al*., 2013). The presence of puppies in the household has been implicated as a risk for *Campylobacter* infection in children (Tenkate and Stafford, 2001; Mughini-Gras *et al*., 2013). A study by Wolfs *et al*. (2001) in the Netherlands showed evidence of transmission of *C. jejuni* from a household puppy to a 3-week-old infant. Transmission of the *Campylobacter* spp. can also be from infected humans to dogs. A study done in Denmark by Damborg *et al*. (2004) showed that *C. jejuni* could occur among pet dogs living with infected children. Antimicrobial resistance can be transmitted from dogs to humans and vice-versa, making dogs a potential source of antimicrobial-resistant zoonotic pathogens (Lloyd, 2007).

CHAPTER THREE

3.0 GENERAL METHODOLOGY

3.1 Study area

This study was conducted in the Nairobi Metropolitan Region which comprises Nairobi County where the capital city, Nairobi, is located; and the surrounding counties of Kajiado, Kiambu, Machakos, and Murang'a (Figure 3.1). This region is Kenya's main economic and cultural center, as well as one of Africa's largest and fastest growing cities (Mundia, 2017). Nairobi Metropolitan Region has many breeding kennels, shelters, and registered veterinary clinics.

Nairobi County lies at 1.28333 latitude and 36.81667 longitude and consists of 17 sub-counties. Kajiado County lies at -2.098075 latitude and 36.78195 longitude and its towns include Kitengela, Kiserian, Kajiado Town, Ongata Rongai, and Ngong. Kiambu County lies at -1.146188 latitude and 36.966499 longitude and its towns include Kiambu Town, Karuri, Ruiru, Limuru, Kikuyu, and Ruaka. Machakos County lies at -1.267009 latitude and 37.320177 longitude and its towns include Athi River, Machakos Town, and Kangundo-Tala. Murang'a County lies at -0.795704 latitude and 37.132202 longitude and its towns include Gatanga, Muranga Town, and Katanga.

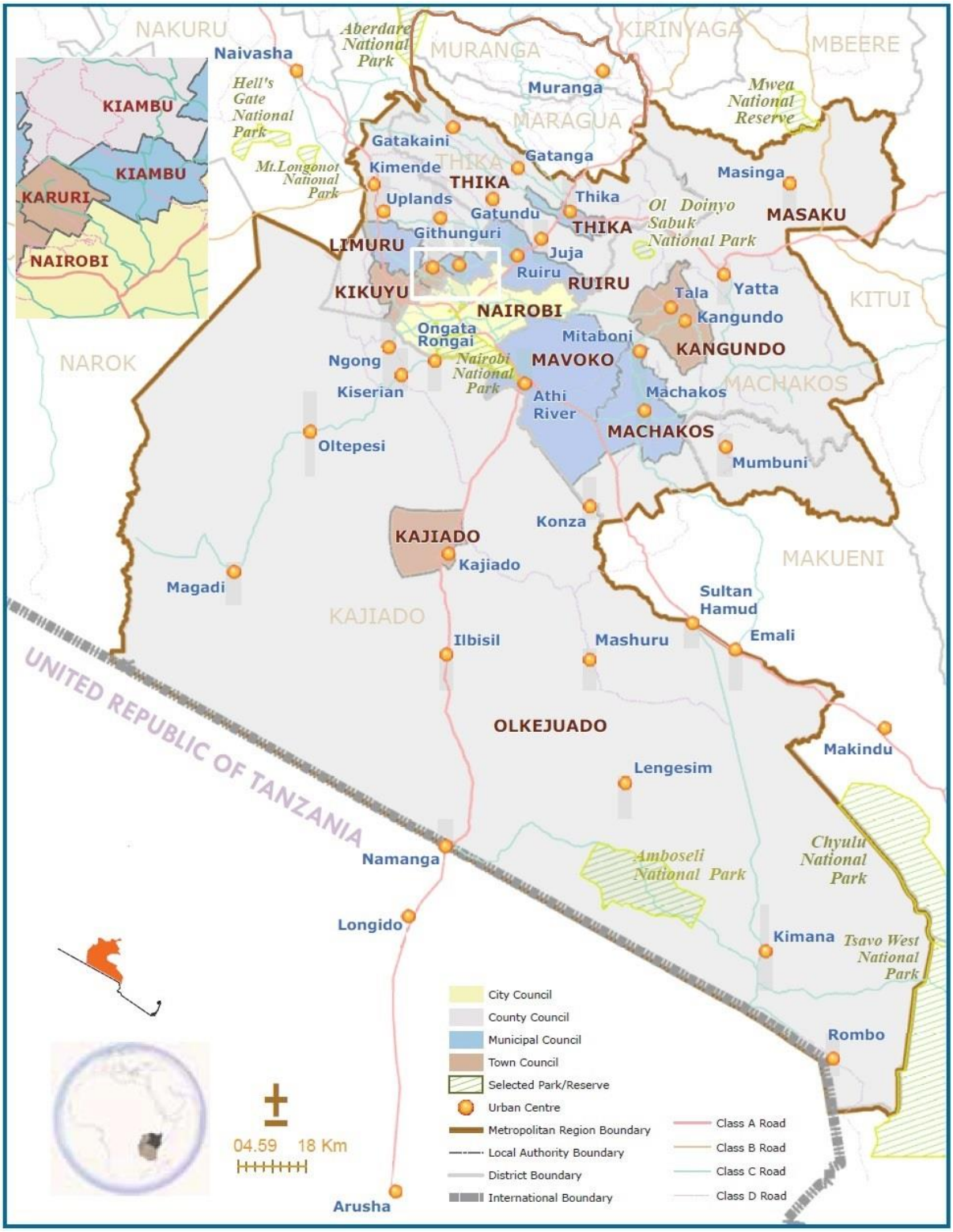

Figure 3. 1 A map of the Nairobi Metropolitan Region https://namsip.go.ke/wp-content/uploads/2018/05/The-Nairobi-Metropolitan-Region.jpg (Source: Nairobi Metropolitan Services Improvement Project (NaMSIP).

3.2 Study design

This was a cross-sectional study conducted between January–August 2021. This study was approved by the Biosecurity, Animal Use and Ethics Committee (BAUEC) of the Faculty of Veterinary Medicine, University of Nairobi, Kenya (FVM BAUEC/2019/237). Fecal collection in the puppies was done in accordance with the BAUEC guidelines, which require animal welfare and biosecurity measures to be followed when handling biological materials.

A multistage random sampling technique was used in this study. The breeding kennels, shelters and the University of Nairobi Veterinary Teaching and Referral Hospital were the primary sampling units and the randomly selected puppies were the secondary sampling units. A list of all breeding kennels in the Nairobi Metropolitan Region was obtained and used as the sampling frame. The kennels were stratified into 4 regions (North, South, East and West) based on their respective location within the Nairobi Metropolitan Region. The strata was clustered and systematic random sampling was done to include every 5th kennel from the list generated. Every selected kennel was contacted by phone and the study was described to the breeder or kennel manager. Verbal consent was given by those willing to participate. Where a breeder or kennel manager declined to participate, a replacement kennel was randomly selected from the respective category.

A detailed questionnaire (Appendix 1) was administered by the principal investigator to collect puppy-level factors (age, breed, sex, neuter status, vaccination status and deworming status), and management factors (type of food, type of housing, kennel hygiene and environmental hygiene) that were thought to be associated with occurrence of Campylobacter infections.

The puppies (in the kennels) were then randomly selected for inclusion in the study using a stratified random sampling approach. The sampling design was intended to allow for proportional sampling of puppies at the kennel level in order to ensure that each puppy age group owned by the breeder was represented (i.e. < 2 months; 2-5 months; > 5 months). Maximum of 3 puppies in each category were included in the study in each kennel. If there were more than 3 in the specified age group, then simple random sampling was used where each puppy was randomly assigned numbers written on pieces of paper and each piece of paper was folded and the breeder was asked to pick three of the papers. The puppies with the assigned numbers on the papers the breeder picked were included in the study. Study puppies were randomly selected from those presented to the University of Nairobi Veterinary Teaching and Referral Hospital for treatment, vaccinations, routine checkup, and boarding.

Each puppy was assigned a body condition score (BCS) in accordance with the Waltham Size, Health and Physical Examination (SHAPE) ScoreTM which contains seven scores from A (underweight) to G (obese) (German et al., 2006) (Appendix 2). The Canine Inflammatory Bowel Disease Activity Index (CIBDAI) clinical scoring system by Jergens et al. (2003) was used for the assessment of the puppies' general health status concerning gastrointestinal infection (Appendix 3). The numerical index assesses the severity of illness based on the presence and frequency of six cardinal signs of gastrointestinal infection. Based on the total cumulative scores, the infection was classified as follows: clinically insignificant (0 to 3), mild (4 to 5), moderate (6 to 8) or severe (9 or greater).

3.3 Sample size calculation

The prevalence of *Campylobacter* spp. was presumed to be 20% from an average of previously published studies in Africa by Salihu *et al.*, 2010 (Nigeria) and Komba, (2018) (Tanzania). Using

the sample size calculation formula by Dohoo *et al.* (2009), the sample size was calculated as follows:

$$n = \frac{Z_{0.05}^{2} * p\,q}{L^{2}}$$

(L= 0.05 margin of error, p= 0.2 is the average prevalence from published studies, q = 1-p = 0.8 and $Z_{0.05}$ is the normal deviate from the mean in Z distribution =1.96). A total of 260 puppies were included in the study.

3.4 Sampling and isolation of *Campylobacter* spp.

Sterile cotton-tipped swabs were rotated inside the rectum of the puppy for 10 seconds and then placed into screw-capped test tubes containing 10 ml of sterile buffered peptone water (BPW) (Himedia) and transported to the bacteriology laboratory in the Department of Public Health, Pharmacology and Toxicology within three hours after collecting the sample.

In the laboratory, the sample was directly streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA) (Oxoid, CM0935) plates with supplement (polymyxin B 2500IU, rifampicin 5mg, trimethoprim 5mg and cycloheximide 50mg) (Oxoid, SR0167E). The mCCDA culture media was prepared according to manufacturer's instructions.

The inoculated plates were put in an anaerobic jar with microaerophilic conditions created by a lighted candle and incubated at 42^{o}C for 48 hours. For each mCCDA plate that showed growth, four suspect colonies were subcultured onto mCCDA (Oxoid, CM0935) and incubated at 42^{o}C for 48 hours.

Characteristic colonies were examined and counted (grey/white or creamy grey in color with a moist appearance). Distinct colonies were examined for the production of the cytochrome oxidase enzyme using oxidase paper impregnated with NNN'N'tetramethyl-p-phenylene-diamine dihydrochloride (Oxoid, Basingstoke, UK). Positive response was indicated by a purple color change. By selecting a sample of the test colony with a sterile wire loop and placing it on a drop of 30% hydrogen peroxide on a clean microscope slide, the same colonies were examined for peroxidase breakdown. Effervescent air bubble production was noted as peroxidase/catalase positive. Reactive colonies were harvested and transferred into cryovials containing skim milk powder (Oxoid, LP0031). The tubes were then stored at -80^{o}C until when DNA extraction was done.

3.5 Deoyxribonucleic acid (DNA) extraction

Genomic DNA was obtained by boiling fresh *Campylobacter* cultures grown on mCCDA (Oxoid, CM0935) as described by Wang *et al*. (2002). Briefly, a loopful of 48-hour bacterial growth from plates was suspended in 1.5 ml of sterile distilled water in an Eppendorf tube. It was boiled in a 100^{o}C water bath for 30 minutes. It was allowed to cool before being centrifuged at 1500 rpm for 5 minutes at 20^{o}C. The supernatant containing DNA was aliquoted into sterile Eppendorf tubes and stored at -80^{o}C awaiting PCR analysis.

3.6 Polymerase Chain Reaction (PCR) analysis for identification of genus *Campylobacter*

Polymerase chain reaction (PCR) amplifications were performed using a thermal cycler (Bio-Rad T100^{TM} Thermal cycler)**.** To confirm members of the genus *Campylobacter*, primers (C412GF 5'-GGATGACACTTTTCGGAGC-3' and C1228R 5'- CATTGTAGCACGTGTGTC-3') (Linton *et al*., 1996) targeting the *16S rRNA* gene were used. PCR was performed in a total

volume of 12.5 µl containing mastermix of 6.25 µl and 0.25 µl each of forward and reverse primers, 5 µl of DNA template, and 0.75 µl of sterile distilled water.

The thermocycling conditions used were initial denaturation at 95^{o} C for 15 minutes, followed by 25 cycles each of denaturation of 95^{o}C for 30 seconds, annealing at 58^{o}C for 1.5 minutes, extension at 72^{o}C for 1 minute, and final heating at 72^{o}C for 7 minutes. Samples were held at 4^{o}C prior to analysis.

RNAse- free water was used in all PCR reactions as a negative control and 10 µl of amplified products were identified by electrophoresis in a 1.5% (weight/volume) agarose gel in 1X Tris-Borate-EDTA (TBE) buffer; subsequently stained with ethidium bromide and ran for 30-45 minutes at 200V and visualized by UV-illuminator (UVP GelMax 125 Imager, USA). The sizes of the amplicons were determined using 100 bp molecular ladder. Specific amplified fragments expected were of size 816 bp which corresponded to the *Campylobacter* genus.

The unit of observation corresponded to an individual sample and each sample represented an individual puppy. If *Campylobacter* was detected by PCR in a sample, the puppy was considered infected.

3.7 Primer design

The Primer Blast tool of the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye *et al.*, 2012) was used to design the primers. The *16S rRNA* gene sequences for representative pathogens were used to generate the primers. *Campylobacter jejuni* (accession no. CP030866.1), *Campylobacter upsaliensis* (accession no. AB736182.1), and *Campylobacter coli* (accession no. CP045791.1) sequences were used as reference sequences. The sequences were input into the software and the primers generated on

default parameters except for the database changed to non-redundant databases (nr) and the organism changed to *Campylobacteraceae*.

The resultant primers generated were: forward primer, Cj-cpbF1 5-AACCTTAGTCGGACAGCCTTC-3' and a reverse Cj-cpbR1 5'-CCTAGCGGAACGAGGTGTAA-3' targeting an approximately 843 bp for *C. jejuni,* forward primer, Cu-rmeF 5'- GAAAGTGGCATCCGCACAAA-3' and a reverse Cu-rmeR 5'-CCCACCAATCGCCCCTTATT-3' targeting an approximately 512 bp for *C. upsaliensis,* and forward primer, Cc-ImpF 5'- CCGCTTTATACTGCTTTCGTGG-3' and a reverse Cc-ImpR 5'-AAGCGATACTCATCCACCCC -3' targeting an approximately 386 bp for *C. coli*. The primer sequences were submitted to Macrogen Europe Laboratory, Amsterdam, The Netherlands for synthesis.

CHAPTER FOUR

4.0 PREVALENCE AND FACTORS ASSOCIATED WITH *CAMPYLOBACTER* INFECTION OF PUPPIES IN THE NAIROBI METROPOLITAN REGION, KENYA

4.1 Introduction

Campylobacter species are often found in the feces of dogs, especially in puppies (less than one year) (Parsons *et al*., 2011; Goni *et al*., 2017). Most dogs are asymptomatic and excrete *Campylobacter* in their feces which may ultimately infect humans and other animals by contaminating the environment (LeJeune and Hancock, 2001). Although a majority of dogs may be subclinically infected, some dogs especially puppies less than 6 months of age or those from stressful environments may develop mild to moderate enteritis presenting as mild to watery diarrhea or as bloody or mucoid diarrhea with tenesmus (Brown *et al*., 1999; Chaban *et al*., 2010; Weese, 2011; Acke, 2018).

Campylobacter species prevalence in dogs varies widely (Kumar *et al*., 2012; Verma *et al*., 2014; Holmberg *et al*., 2015; Lazou *et al*., 2016; Torkan *et al*., 2018; Thépault *et al*., 2020), depending on age, geographic region, housing, diagnostic method, clinical history (diarrheic versus non-diarrheic dogs), and the presence of infection or concomitant disease (Acke *et al*. 2006; Iannino *et al*., 2017; Acke, 2018). Feeding homemade and commercial diets, compost exposure, and outdoor water access have all been linked to *Campylobacter* colonization in dogs (Leonard *et al*., 2011; Procter *et al*., 2014; Karama *et al*., 2019). The infection has also been linked to purebred dogs, concurrent enteric disease, and antibiotic treatment (Carbonero *et al*., 2012; Santaniello *et al*., 2021). Furthermore, when compared to adult dogs, younger dogs are more likely to become infected with *Campylobacter* species (Holmberg *et al*., 2015).

Though, the detection of *Campylobacter* species is generally performed using conventional culture method, it is time-consuming and labor-intensive, due to the fastidious nature of the

species (Li *et al*., 2014). Hence, molecular-based assays, like polymerase chain reaction (PCR) and sequencing enable rapid and precise detection (Kaakoush *et al*., 2015; Vinueza- Burgos *et al*., 2017; Ricke *et al*., 2019).

Despite reports of puppies serving as essential reservoirs for *Campylobacter* pathogens, current data on *Campylobacter* species epidemiology in Kenyan puppies is limited. Therefore, this study aimed at determining the prevalence and associated risk factors of *Campylobacter* species in puppies in the Nairobi Metropolitan Region, Kenya.

4.2 Materials and Methods

4.2.1 Sample collection

As decribed in section 3.4.

4.2.2 Isolation and phenotypic characterization of *Campylobacter* species

As described in section 3.4.

4.2.3 Deoxyribonucleic acid (DNA) extraction

As described in section 3.5.

4.2.4 Polymerase Chain Reaction (PCR) analysis for identification of genus *Campylobacter*

As described in section 3.6.

4.2.5 Data entry and analysis

Questionnaire data, culture, and PCR results were entered into Microsoft Excel version 2016 (Redmond, WA, USA) before being exported to STATA 17.0 (StataCorp LLC, USA) for analysis. *Campylobacter* species prevalence and other demographic parameters were computed using descriptive statistics. The Chi-square test was used to compare *Campylobacter* species

carriage ratios between different categorical groups. Potential factors associated with *Campylobacter* species carriage in puppies were investigated using univariable logistic regression analysis. Covariates were retained in the model if statistically significant [$p<0.05$ (culture positive) and $p\leq0.2$ (PCR positive)] using a backward stepwise elimination procedure. All variables that showed an association with the outcome variable in the univariable logistic regression analysis ($p<0.05$) were considered in the final mixed effects logistic regression analysis. Potential clustering of puppies within kennels was controlled by including kennels as a random effect in the modeling. Model fit was assessed by checking for multi-collinearity, overall goodness of fit of the model, influential data points, and outliers. Significance of differences ($p<0.05$) between culture and PCR was analyzed using Pearson's chi-square test and correlations between culture and PCR was tested using kappa test. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated from the contingency table generated for the chi-square and kappa tests.

4.3 Results

4.3.1 Culture prevalence of *Campylobacter* species infection in puppies in the Nairobi Metropolitan Region, Kenya

Conventional culture and biochemical tests were used to identify *Campylobacter* species. The results from conventional culture (Figure 4.1) and biochemical tests revealed 150 *Campylobacter* species isolates giving a prevalence of 57.7% (150/260).

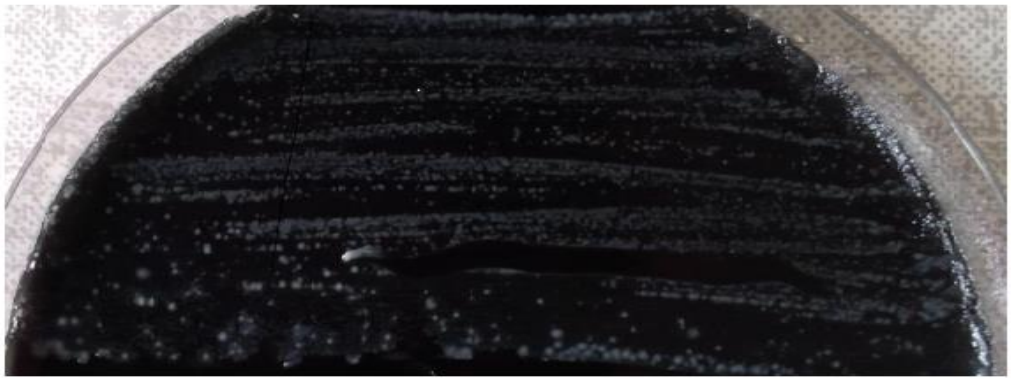

Figure 4. 1 *Campylobacter* colonies on mCCDA

4.3.2 Descriptive statistics for management variables associated with *Campylobacter* species culture positive status in puppies in the Nairobi Metropolitan Region, Kenya

A higher prevalence of *Campylobacter* species was observed in puppies from Machakos County [70% (14/20)], shelters [100% (6/6)], kept as pets [62.1% (23/37)], and those sharing a kennel [61.2% (134/219)]. These were also noted in puppies whose kennels were washed on a daily basis [61.1% (140/229)], kennels that used plain water [59.7% (77/129)], kennels with wooden floors [63.1% (53/84)], and puppies fed homemade diets [61.4% (54/88)]. The details of the management variables associated with *Campylobacter* species culture positive status are shown in Table 4.1 below.

Table 4. 1 Descriptive statistics for management variables associated with *Campylobacter* species culture positive status in puppies in the Nairobi Metropolitan Region, Kenya

Variables	Level	No. sampled	No. positive (%)
County	Nairobi	140	74(52.9)
	Kiambu	77	47(61)
	Machakos	20	14(70)
	Kajiado	23	15(65.2)
Type of facility	Veterinary Hospital	44	23(52.3)
	Breeding kennels	210	121(57.6)
	Shelters	6	6(100)
Reason for keeping puppy	Commercial	171	106(62)
	Pet	37	23(62.1)
	Breeding	17	8(47.1)
	Security	35	13(37.1)
Type of housing	Household	11	1(9.1)
	Kenneled	249	149(59.8)
Nature of housing	Individual	41	16(39)
	Grouped	219	134(61.2)
Type of floor in the kennels	Wooden	84	53(63.1)
	Concrete	176	97(55.1)
Daily washing of the kennels	Yes	229	140(61.1)
	No	31	10(32.2)
Mode of washing	Plain water	129	77(59.7)
	Soap and water	88	49(55.7)
	Disinfectant	43	24(55.9)
Type of food	Commercial	56	32(57.1)
	Homemade	88	54(61.4)
	Others	116	64(55.2)

4.3.3 Descriptive statistics of puppy level variables associated with *Campylobacter* species culture positive in puppies in the Nairobi Metropolitan Region, Kenya

The prevalence of *Campylobacter* species was high in female puppies [63.5% (87/137)], puppies less than 2 months of age [72.2% (65/90)] and local breeds [67.3% (35/52)]. Similar findings were noted in puppies whose deworming status was up to date [61.3% (95/155)], those whose vaccination status was up to date [58.4% (115/197)], those with no concurrent bacterial infections [58.3% 141/142)], and those not treated with antibiotics in the past month [57.8% 126/218].

In addition, higher prevalences of *Campylobacter* species were observed in puppies that were non-diarrheic [58.5%, (131/224)], those with a history of recent vomiting [71.4% (10/14)], those not diagnosed with parvoviral enteritis [58% (144/248)] and helminthosis [58.1% (136/234)], exposed to other pets [71% (64/90)], not exposed to poultry [61.8% (134/217)], and livestock [59% (144/244)]. The details of the puppy-level variables associated with *Campylobacter* species culture positive status are shown in Table 4.2 below.

Table 4. 2 Descriptive statistics of puppy-level variables associated with *Campylobacter* species culture positive status in puppies in the Nairobi Metropolitan Region, Kenya

Variables	Level	No. sampled	No. positive (%)
Sex	Male	123	63(51.2)
	Female	137	87(63.5)
Age	<2 months	90	65(72.2)
	2-5 months	108	52(48.1)
	>5 months	62	33(53.2)
Breed	Local	52	35(67.3)
	GSD	96	60(62.5)
	Others	112	55(49.1)
Deworming status	Not upto date	105	55(52.4)
	Upto date	155	95(61.3)
Vaccination status	Not upto date	63	35(55.6)
	Upto date	197	115(58.4)
Body condition (SHAPE)	Thin and lean	117	73(62.4)
	Ideal and moderately obese	143	77(53.8)
Recent diarrhea	Yes	36	19(52.8)
	No	224	131(58.5)
Recent vomiting	Yes	14	10(71.4)
	No	246	140(57)
Diagnosed with parvoviral enteritis	Yes	12	6(50)
	No	248	144(58)
Diagnosed with helminthosis	Yes	26	14(53.8)
	No	234	136(58.1)
Concurrent bacterial infections	Yes	18	9(50)
	No	242	141(58.3)
Recent treatment with antibiotics	Yes	42	24(57.1)
	No	218	126(57.8)
Exposure to pets	Yes	90	64(71)
	No	170	86(50.6)
Exposure to poultry	Yes	43	16(37.2)
	No	217	134(61.8)
Exposure to livestock	Yes	16	6(37.5)
	No	244	144(59)

4.3.4 Analysis of factors associated with *Campylobacter* species culture positive status in puppies in the Nairobi Metropolitan Region, Kenya

Univariable logistic regression identified 10 factors to be associated ($p<0.05$) with positive *Campylobacter* species culture status (Table 4.3). Two of the factors associated with higher *Campylobacter* species carriage were puppies that are housed together (OR: 2.4; p=0.025) and kennels that are washed daily (OR: 6.8; p=0.0001). Eight factors were associated with lower *Campylobacter* species carriage: household puppies (OR: 0.03; p=0.005), puppies 2 to 5 months of age (OR: 0.2; p=0.0001), puppies more than five months of age (OR: 0.3; p=0.044), breed of puppies (OR: 0.3; p=0.0001), puppies bred for security (OR: 0.2; p=0.045), puppies with an ideal body condition or are moderately obese (OR: 0.5; p=0.045), puppies exposed to poultry (OR: 0.3; p=0.024), and puppies exposed to livestock (OR: 0.3; p=0.043).

Multivariable logistic regression analysis revealed that the factors significantly associated with higher *Campylobacter* species positivity at $P<0.05$ were reasons for keeping puppies (OR: 14, 95% CI: 3-27.5, p=0.013), deworming status of puppies (0.R: 9.1, 95% CI: 3-27.5, p=0.0001), and puppies with a recent history of vomiting (OR: 4.7, 95% CI: 1.7-12.9, p=0.003). Protective factors identified were: household puppies (OR: 0.02, 95% CI: 0.0009-0.3, p=0.005), puppies 2 to 5 months of age (OR: 0.2, 95% CI: 0.06-0.4. p=0.0001), puppies bred for security (OR: 0.2, 95% CI: 0.04-0.7, p=0.017) and puppies with ideal body condition or are moderately obese (OR: 0.2, 95% CI: 0.1-0.5, p=0.0001). The details are shown in Table 4.4 below.

Table 4. 3 Univariable logistic regression analysis of factors associated with *Campylobacter* species positive status in puppies in the Nairobi Metropolitan Region, Kenya (p<0.05)

Variables	Description	Odds ratio	P-value
County	Nairobi	Ref	
	Kiambu	1.3	0.691
	Machakos	1.7	0.519
	Kajiado	2.3	0.509
Type of facility	Veterinary Hospital	Ref	
	Breeding kennels	1	0.736
	Shelters	1	NA
Reason for keeping puppy	Commercial	Ref	
	Pet	0.6	0.276
	Breeding	0.4	0.321
	Security	**0.2**	**0.045**
Type of housing	Kenneled	Ref	
	Household	**0.3**	**0.005**
Nature of housing	Individual	Ref	
	Grouped	**2.4**	**0.025**
Type of floor in the kennels	Wooden	Ref	
	Concrete	1.9	0.539
Daily washing of the kennels	**Yes**	**6.8**	**0.0001**
	No	Ref	
Type of food	Commercial	Ref	
	Homemade	0.9	0.91
	Others	0.7	0.651
Sex of the puppy	Male	Ref	
	Female	1.6	0.168
Age of the puppy	<2 months	Ref	
	2-5 months	0.2	**0.0001**
	>5 months	0.3	**0.044**
Breed of the puppy	Local	Ref	
	GSD	0.6	0.225
	Others	**0.3**	**0.0001**
Deworming status	Not upto date	Ref	
	Upto date	1.4	0.635
Vaccination status	Not upto date	Ref	
	Upto date	1.3	0.458
Body condition (SHAPE)	Thin and lean	Ref	
	Ideal and moderately obese	**0.5**	**0.045**
Recent diarrhea	Yes/No	1.3/Ref	0.677
Recent vomiting	Yes/No	2.1/Ref	0.077
Diagnosed with parvoviral enteritis	Yes/No	0.9/Ref	0.494
Diagnosed with helminthosis	Yes/No	0.7/Ref	0.59
Concurrent bacterial infections	Yes/No	1.2/Ref	0.818
Recent treatment with antibiotics	Yes/No	1.6/Ref	0.561
Exposure to pets	Yes/No	2.8/Ref	0.113
Exposure to poultry	**Yes**/No	**0.3**/Ref	**0.024**
Exposure to livestock	**Yes**/No	**0.3**/Ref	**0.043**

Table 4. 4 Multivariable mixed effects logistic regression analysis of significantly associated explanatory variables for *Campylobacter* species culture positive status in puppies in the Nairobi Metropolitan Region, Kenya (p<0.05)

Variables	Description	Odds ratio	95% Confidence Interval		P-value
			Lower	Upper	
Reason for puppy	Categories				0.013
	Commercial	Ref			
	Pet	3.1	0.9	11.1	0.077
	Breeding	0.6	0.1	2.2	0.406
	Security	0.2	0.04	0.7	0.017
Type of housing	Kenneled	Ref			
	Household	0.02	0.0009	0.3	0.005
Age of puppy	Categories				0.0001
	<2 months	Ref			
	2-5 months	0.2	0.06	0.4	0.0001
	>5 months	0.6	0.1	2.1	0.384
Deworming status	Not upto date	Ref			
	Upto date	9.1	3	27.6	0.0001
Body condition	Thin and lean	Ref			
	Ideal and moderately obese	0.2	0.1	0.5	0.0001
Recent vomiting	Yes	4.7			
	No	Ref	1.7	12.9	0.003

4.3.5 Molecular prevalence of *Campylobacter* species infection in puppies in the Nairobi Metropolitan Region, Kenya

Polymerase chain reaction was used to identify the presumptive *Campylobacter* species on culture. This was done by targeting the 16S rRNA gene-specific of *Campylobacter* species which produced a specific band corresponding to the expected size of 816 bp (Figure 4.2).

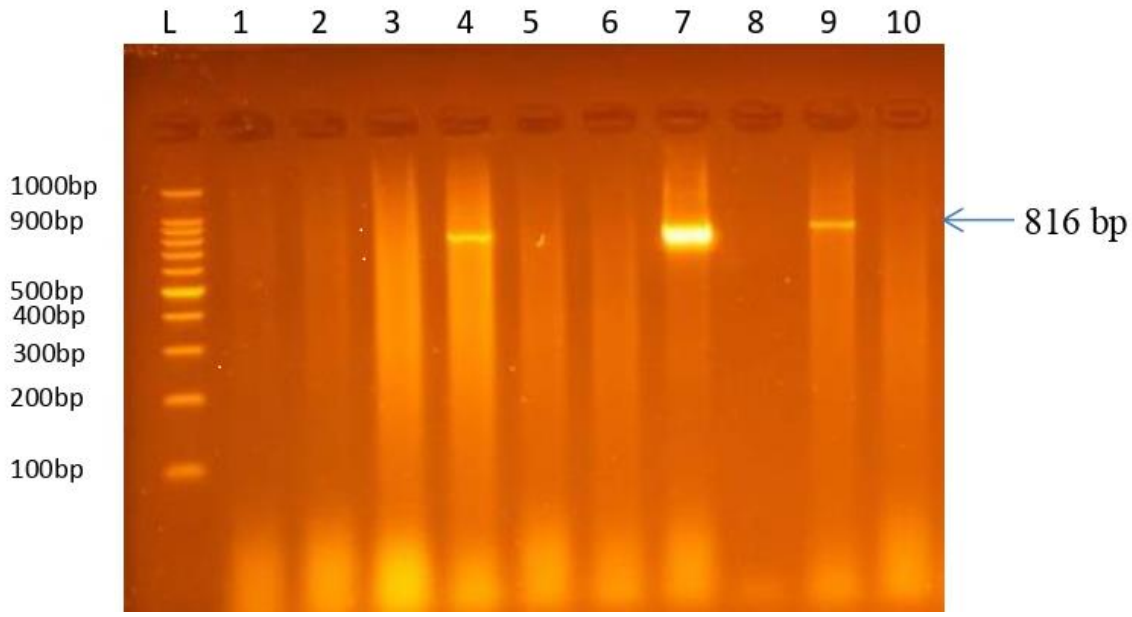

Figure 4. 2 Representative PCR amplicons of *Campylobacter 16S rRNA* gene. Lane L: Molecular ladder (100 bp); lanes 4, 7, and 9: positive samples showing amplicon at approximate 816 bp; lanes 1, 2, 3, 5, 6, and 8: no amplicons observed; lane 10: negative control.

The results from PCR analyses revealed that 64 out of the 150 presumptive *Campylobacter* species isolates were positive giving an overall molecular prevalence of 24.6% (64/260).

4.3.6 Descriptive statistics for management variables associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya

A higher prevalence of *Campylobacter* species was observed in puppies from Kajiado County [43.5% (10/23)], shelters [50% (3/6)] and those kept as pets [29.7% (11/37)]. Similar observations were noted in puppies sharing a kennel [25.1% 55/219)], whose kennels were washed on a daily basis [27.1% (62/229)], from kennels that used disinfectants [32.6% (14/43)], kennels with concrete floors [27.8% (49/176)], and puppies fed homemade diets [30.7% (27/88)]. The details of the management variables associated with *Campylobacter* species PCR positive status are shown in Table 4.5 below.

Table 4. 5 Descriptive statistics for management variables associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya

Variables	Level	No. sampled	No. positive (%)
County	Nairobi	140	30(21.4)
	Kiambu	77	19(24.7)
	Machakos	20	5(25)
	Kajiado	23	10(43.5)
Type of facility	Veterinary Hospital	44	13(29.5)
	Breeding kennels	210	48(22.9)
	Shelters	6	3(50)
Reason for keeping puppy	Commercial	171	45(26.3)
	Pet	37	11(29.7)
	Breeding	17	4(23.5)
	Security	35	4(11.4)
Type of housing	Household	11	0
	Kenneled	249	64(25.7)
Nature of housing	Individual	41	9(22)
	Grouped	219	55(25.1)
Type of floor in the kennels	Wooden	84	15(17.9)
	Concrete	176	49(27.8)
Daily washing of the kennels	Yes	229	62(27.1)
	No	31	2(6.5)
Mode of washing	Plain water	129	35(27.1)
	Soap and water	88	15(17)
	Disinfectant	43	14(32.6)
Type of food	Commercial	56	13(23.2)
	Homemade	88	27(30.7)
	Others	116	24(20.7)

4.3.7 Descriptive statistics of puppy level variables associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya

The prevalence of *Campylobacter* species was higher in female puppies [30% (41/137)], puppies less than 2 months of age [31.1% (28/90)], German Shepherd Dogs [30.2% (29/96)], puppies whose deworming status was not upto date [24.8% (26/105)], and puppies whose vaccination status was not upto date [25.4% (16/63)].

Higher prevalences of *Campylobacter* species was found in puppies that were diarrheic [25% (9/36)], had a history of recent vomiting [35.7% (5/14)], diagnosed with parvoviral enteritis [33.3% (4/12)] and helmithosis [27% (7/26)]. These were also noted in those with no concurrent

bacterial infections [25.5% (61/242)], and those treated with antibiotics in the past month [33.3% (14/42)]. Similar findings were also found in puppies not exposed to other pets [27.1% (46/170)], those not exposed to poultry [24.9% (54/217)] and livestock [25% (61/244)]. The details of the puppy-level variables associated with *Campylobacter* species PCR positive status are shown in Table 4.6 below.

Table 4. 6 Descriptive statistics of puppy-level variables associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya

Variables	Level	No. sampled	No. positive (%)
Sex	Male	123	23(18.7)
	Female	137	41(30)
Age	<2 months	90	28(31.1)
	2-5months	108	27(25)
	>5 months	62	9(14.5)
Breed	Local	52	15(28.8)
	GSD	96	29(30.2)
	Others	112	20(17.9)
Deworming status	Not upto date	105	26(24.8)
	Upto date	155	38(24.5)
Vaccination status	Not upto date	63	16(25.4)
	Upto date	197	48(24.4)
Body condition (SHAPE)	Thin and lean	117	34(29)
	Ideal and moderately obese	143	30(21)
Recent diarrhea	Yes	36	9(25)
	No	224	55(24.6)
Recent vomiting	Yes	14	5(35.7)
	No	246	59(24)
Diagnosed with parvoviral enteritis	Yes	12	4(33.3)
	No	248	60(24.2)
Diagnosed with helminthosis	Yes	26	7(27)
	No	234	57(24.4)
Concurrent bacterial infections	Yes	18	3(16.7)
	No	242	61(25.2)
Recent treatment with antibiotics	Yes	42	14(33.3)
	No	218	52(23.8)
Exposure to pets	Yes	90	18(20)
	No	170	46(27.1)
Exposure to poultry	Yes	43	10(23.3)
	No	217	54(24.9)
Exposure to livestock	Yes	16	3(18.8)
	No	244	61(25)

4.3.8 Analysis of factors associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya

Univariable logistic regression identified 11 factors to be associated ($p \leq 0.2$) with positive *Campylobacter* species PCR status (Table 4.7). Four of the factors were associated with higher *Campylobacter* species carriage. They include kennels with concrete floors (OR: 2.3; p=0.12), kennels that are washed daily (OR: 6.7; p=0.0001), puppies with a history of recent vomiting (OR: 1.5; p=0.2), and puppies treated with antibiotics in the past month (OR: 1.7; p=0.093). Seven factors were associated with lower *Campylobacter* species carriage: puppies from breeding kennels (OR: 0.6; p=0.003), puppies from shelters (OR: 3; p=0.0001), puppies bred for security (OR: 0.3, p= 0.029), kenneled puppies (OR: 0.8, p=0.005), puppies more than 5 months of age (OR: 0.3; p=0.016), puppies with an ideal body condition or are moderately obese (OR: 0.6; p=0.14), and puppies with concurrent bacterial infections (OR: 0.5, p=0.2).

Multivariable logistic regression analysis revealed that the factors significantly associated with higher *Campylobacter* species positivity at $p<0.05$ were puppies from shelters (OR: 2.6, 95% CI: 1.9-3.6, p=0.0001), kennels that are washed on a daily basis (OR: 11.4, 95% CI: 2.8-46, p=0.001), puppies with a recent history of vomiting (OR: 3.4, 95% CI: 1.01-11.4, p=0.046), and puppies treated with antibiotics in the past month (OR: 2, 95% CI: 1.11-3.6, p=0.02). Protective factors identified were: puppies from breeding kennels (OR: 0.65, 95% CI: 0.44-0.94. p=0.024) and puppies with concurrent bacterial infections (OR: 0.18, 95% CI: 0.04=0.87, p=0.033). The details are shown in Table 4.8 below.

Table 4. 7 Univariable logistic regression analysis of factors associated with *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya (p≤0.2)

Variables	Description	Odds ratio	P-value
County	Nairobi	Ref	
	Kiambu	1.6	0.420
	Machakos	1.5	0.546
	Kajiado	2.8	0.377
Type of facility	Veterinary Hospital	Ref	
	Breeding kennels	**0.6**	**0.003**
	Shelters	**3**	**0.0001**
Reason for keeping puppy	Commercial	Ref	
	Pet	0.8	0.592
	Breeding	0.8	0.704
	Security	**0.3**	**0.029**
Type of housing	Household	Ref	
	Kenneled	**0.8**	**0.005**
Nature of housing	Individual	Ref	
	Grouped	1.2	0.73
Type of floor in the kennels	Wooden	Ref	
	Concrete	**2.3**	**0.12**
Daily washing of the kennels	**Yes**	**6.7**	**0.0001**
	No	Ref	
Type of food	Commercial	Ref	
	Homemade	0.8	1.2
	Others	0.7	0.76
Sex of the puppy	Male	Ref	
	Female	1.7	0.21
Age of the puppy	<2 months	Ref	
	2-5 months	0.6	0.29
	>5 months	**0.3**	**0.016**
Breed of the puppy	Local	Ref	
	GSD	0.9	0.78
	Others	0.5	0.24
Deworming status	Not upto date	Ref	
	Upto date	0.9	0.8
Vaccination status	Not upto date	Ref	
	Upto date	0.9	0.3
Body condition (SHAPE)	Thin and lean	Ref	
	Ideal and moderately obese	**0.6**	**0.14**
Recent diarrhea	Yes/No	1.2/Ref	0.71
Recent vomiting	**Yes**/No	**1.5**/Ref	**0.2**
Diagnosed with parvoviral enteritis	Yes/No	1.4/Ref	0.51
Diagnosed with helminthosis	Yes/No	1.4/Ref	0.56
Concurrent bacterial infections	**Yes**/No	**0.5**/Ref	**0.2**
Recent treatment with antibiotics	**Yes**/No	**1.7**/Ref	**0.093**
Exposure to pets	Yes/No	0.8/Ref	0.7
Exposure to poultry	Yes/No	1.1/Ref	0.81
Exposure to livestock	Yes/No	0.7/Ref	0.63

Table 4. 8 Multivariable mixed effects logistic regression analysis of significantly associated explanatory variables for *Campylobacter* species PCR positive status in puppies in the Nairobi Metropolitan Region, Kenya (p<0.05)

Variables	Description	Odds ratio	95% Confidence Interval		P-value
			Lower	Upper	
Type of facility	Veterinary Hospital	Ref			
	Breeding kennels	0.65	0.44	0.94	0.024
	Shelters	2.6	1.9	3.6	0.0001
Daily washing of the kennels	Yes	11.4	2.8	46	0.001
	No	Ref			
Recent vomiting	Yes	3.4	1.01	11.4	0.046
	No	Ref			
Concurrent bacterial infections	Yes	0.18	0.04	0.87	0.033
	No	Ref			
Recent treatment with antibiotics	Yes	2	1.11	3.6	0.02
	No	Ref			

4.3.9 Prevalence of *Campylobacter* species infection based on the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) clinical scoring system

Fifty-four out of 260 puppies exhibited one or more of the six cardinal signs of gastrointestinal infection used to assess the degree of illness. Of the puppies whose infection status was classified as severe, 63.6% (7/11) were positive for *Campylobacter* species on culture, while 36.4% (4/11) were confirmed to be *Campylobacter* positive by PCR. Of the puppies whose infection status was classified as clinically insignificant, 36.4% (8/22) were positive for *Campylobacter* spp. on culture, while 13.7% (3/22) were confirmed to be *Campylobacter* positive by PCR (Table 4.9).

Table 4. 9 Descriptive statistics of the occurrence of *Campylobacter* species infection in puppies based on the Canine Inflammatory Bowel Disease Activity Index (CIBDAI) clinical scoring system

Infection status (CIBDAI)	No. assessed	Culture positive (%)	PCR positive (%)
Clinically insignificant	22	8(34.8)	3(17.4)
Mild	3	1(33.3)	0
Moderate	18	14(77.8)	9(50)
Severe	11	7(63.6)	4(36.4)

4.3.10 Comparison between culture and polymerase chain reaction for the *Campylobacter* species detection in puppies in the Nairobi Metropolitan, Kenya

The comparison of the culturing and PCR results is shown in Table 4.10. In comparison to culture, PCR is more sensitive (Sensitivity 100%). The two methods for detecting *Campylobacter* species are statistically different, according to the chi-squared test (p=0.0001). The coherence analysis of these two methods yielded kappa = 0.4, indicating that they are fairly consistent.

Table 4. 10 Comparison between culture and PCR in the diagnosis of *Campylobacter* species infection in puppies in the Nairobi Metropolitan Region, Kenya

		Polymerase Chain Reaction[1]		
		Positive	**Negative**	**Total**
Culture[2]	**Positive**	64[a]	86[b]	150
	Negative	0[c]	110[d]	110
	Total	64	196	260
P-value		0.0001*		
Sensitivity		100%		
Specificity		56.1%		
Positive Predictive Value (PPV)		42.7%		
Negative Predictive Value (NPV)		100%		
Level of agreement (κ)		0.4		

1-Assumed gold standard, 2-Screening test

*Statistically significant at $p<0.05$

Sensitivity: proportion of puppies with the disease who have a positive test: a/a+c; Specificity: proportion of puppies without the disease who have a negative test: d/b+d; Positive predictive value (PPV): proportion of puppies who test positive and actually have the disease: a/a+b; Negative predictive value (NPV): proportion of puppies who test negative and do not have the disease: d/c+d.

Kappa values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

4.4 Discussion

This study utilized both conventional culture and polymerase chain reaction (PCR) assays for the isolation and identification of *Campylobacter* species. For culture, modified-charcoal-cefoperazone-dexoycholate agar (mCCDA), a selective media, for the isolation and identification of *Campylobacter* species was used. Among the 150 (57.7%, 150/260) culture positive samples, PCR detected 64 *Campylobacter* species (24.6%, 64/260) by targeting the *16S rRNA* gene specific for these microorganisms. These proportions were within the range of 8.58% to 75.7% reported in studies done in the past five years (Torkan *et al*., 2018; Subejano and Penuliar, 2018; Gharibi *et al*., 2020). The reason for the 86 false positives from culture could be attributed to the overgrowth of competing fecal flora such as extended-spectrum β-lactamase (ESBL)-producing bacteria on mCCDA plates which mask the growth and confound the detection of *Campylobacter* colonies (Chon *et al*., 2016; Kim and Seo, 2020).

In the detection of *Campylobacter* species, the sensitivity and specificity of PCR were 100% and 56.1%, respectively. These findings are similar to those of previous studies (Singh *et al*., 2011; Platts-Mills *et al*., 2014). This study revealed that the agreement between the two methods for identifying *Campylobacter* species was fair (kappa = 0.4). This agreement, however, was less than the 0.95 agreement reported by Pallavi *et al*. (2015). Polymerase chain reaction assays should be used to confirm *Campylobacter* species because they offer increased sensitivity, can determine both the presence and burden of *Campylobacter* infection and avoid false positive results (On *et al*., 2013; Platts-Mills *et* al., 2014). Consequently, they should be regarded as indispensable in clinical and epidemiological research (El Baaboua *et al*., 2022). False-positive results from conventional culture can prolong potentially harmful antibiotic treatment and, more importantly, cut short the search for the infection-causing pathogen (Buss *et al*., 2019).

Nonetheless, the higher prevalence of thermophilic *Campylobacter* species observed in this study among puppies is a cause for concern, as their feces contaminate the environment and may serve as a source of infection for humans, particularly children.

Though, clinical manifestations of gastroenteritis include diarrhea and vomiting (Bhat *et al*., 2013), this study found no significant association between diarrhea occurrence and *Campylobacter* positive status, a finding that is in agreement with previous research (Parsons *et al*., 2010; Giacomelli *et al*., 2015). However, this study found a statistically significant association between vomiting in puppies and the isolation of *Campylobacter* species (both culture and PCR). This finding contradicts those of Verma *et al*. (2014), who found no correlation between *Campylobacter* species infection and the incidence of vomiting in dogs. Findings of this study, however, concur with those of Guest *et al*. (2007), who found a link between gastrointestinal signs and *Campylobacter* species infection in puppies.

Higher positivity of *Campylobacter* species carriage by culture was found in puppies deworming status was upto date (61.3%) compared with those whose deworming status was not upto date (52.4%). This could be explained by the fact that younger puppies are more likely to have their deworming status upto date due to their increased risk of suffering the negative effects from helminthosis and as mentioned above, have naïve immune systems hence increased risk of campylobacteriosis.

This study revealed that the reason for keeping puppies had a significantly higher risk for *Campylobacter* species culture positive status. Puppies kept as pets are more likely fed with homemade cooked food which may be source of *Campylobacter* species (Leonard *et al*., 2011). Poor food handling practices may also increase the chances of infection and fecal shedding of the

microorganism, spreading it to other pets, domestic animals and humans, especially those who are vulnerable due to age or immunosuppression (Mbindyo *et al*., 2021; Santaniello *et al*., 2021).

Shelter-housed puppies were at a higher risk for *Campylobacter* species PCR positive status. These findings are in agreement with findings of previous studies (Badlik *et al*., 2014; Giacomelli *et al*., 2015; Leahy *et al*., 2016). *Campylobacter* species carriage is more prevalent among puppies who share a habitat with other puppies such as in shelters (Mughini-Gras *et al*., 2013; Goni *et al*., 2017; Ahmed *et al*., 2018). This may be due to the fact that puppies are from multiple sources and the stress of comingling predisposes them to stress and vices such as coprophagia which may lead to the ingestion of these bacteria, resulting in further infection and shedding in feces, thereby contaminating the environment (Damborg *et al*., 2016). The puppies may also roll in the feces, contaminating their fur (Frenkel and Parker, 1996) and spreading the bacteria to surfaces with which they come into contact.

It is recognized that kennel hygiene is a potential risk factor for *Campylobacter* species carriage in dogs (Mbindyo *et al*., 2021). In this study, the daily washing of kennels was a highly significant risk factor for *Campylobacter* species PCR positive status in the studied puppies. *Campylobacter* species are sensitive to desiccation and do not survive in dry environments (Smith *et al*., 2016), thus daily washing increases their survivability in kennels as well as increasing the chance of contamination of water sources (Mughini-Gras *et al*., 2016), allowing water to be a vehicle for dissemination (Borck Hog *et al*., 2016). Puppies may lick the residue water, resulting in pathogen ingestion.

Treatment with antibiotics in the past month was greatly significant with the risk of *Campylobacter* species PCR positive status which was in agreement with a study by Leonard *et*

al. (2011). This could be due to the inappropriate use of antibiotics in the treatment of other systemic infections with Campylobacteriosis co-infection thus promoting the emergence of antimicrobial resistant strains of *Campylobacter* species.

4.5 Conclusions and recommendations

This study reported a high prevalence of *Campylobacter* species in puppies using conventional culture, biochemical tests and polymerase chain reaction. The deworming status of the puppies and puppies kept as pets were significantly associated with *Campylobacter* species culture positive status whereas shelter-housed puppies, the daily washing of kennels, and treatment with antibiotics in the past month were significantly associated with *Campylobacter* species PCR positive status in the studied puppies. Occurrence of vomiting was found to be significantly associated with the identification of *Campylobacter* species (both culture and PCR). Polymerase chain reaction is an ideal tool for molecular confirmation of *Campylobacter* species as it offers increased sensitivity and avoids false positive results, therefore, should be regarded as indispensable in clinical and epidemiological research. This study highlights the need to develop awareness and management strategies to potentially reduce the risk of transmitting this pathogen amongst puppies, to humans, and other animals.

CHAPTER FIVE

5.0 MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *CAMPYLOBACTER* SPECIES ISOLATED FROM PUPPIES IN THE NAIROBI METROPOLITAN REGION, KENYA

5.1 Introduction

There are 27 species and 8 subspecies in the genus *Campylobacter*, and *Campylobacter jejuni* and *Campylobacter coli* are the most prevalent causes of human campylobacteriosis. (EFSA and ECDC, 2018). Dogs are infected mainly by *C. upsaliensis*, *C. jejuni*, *C. coli*, and *C. helveticus* (Goni *et al*., 2017).

There are several different methods used for the diagnosis of *Campylobacter* infections such as direct microscopic examinations, culture, and biochemical tests (e.g. catalase test) (Marks *et al*., 2011). However, due to the fastidious nature and slow growth of *Campylobacters*, their isolation relies heavily on applied procedures (Marks *et al*., 2011).

Molecular methods such as polymerase chain reaction (PCR) are advantageous because they are simple, sensitive, and permit the speciation of isolates from culture (On, 2013; Kaakoush *et al*., 2015; Mbindyo *et al*., 2021). In addition, molecular studies, such as phylogenetic analysis, have assisted in tracing the origins of *Campylobacter* species infections by exploiting differences in the genetic properties and frequency of *Campylobacter* strains that inhabit various hosts and environments (Dearlove *et al*., 2016). Molecular techniques have also led to the discovery of *Campylobacter* genes that are conserved within a given lineage and distributed across species phylogenetically (Baig *et al*., 2015).

Data on epidemiological studies based on molecular methods for *Campylobacter* spp. in dogs in Kenya is limited. Given the clinical and public health significance of *Campylobacter* infection in

puppies, the objective of this study was the molecular characterization and phylogenetic analysis of *Campylobacter* species isolates in puppies in the Nairobi Metropolitan Region, Kenya.

5.2 Materials and Methods

5.2.1 Sampling and isolation of *Campylobacter* species

As decribed in section 3.4.

5.2.2 Deoxyribonucleic acid (DNA) extraction

As described in section 3.5.

5.2.3 Polymerase Chain Reaction (PCR) analysis for identification of *Campylobacter* species

Campylobacter species identification was conducted and isolates that were positive for the genus-specific PCR were tested by species specific PCR that targeted *Campylobacter jejuni, C. upsaliensis* and *C. coli*. Polymerase chain reaction was performed in a total volume of 12 µl containing mastermix of 6 µl and 1.5 µl each of forward and reverse primers, and 3 µl of DNA template. The thermocycling conditions for *Campylobacter jejuni*, *C. upsaliensis*, and *C. coli* were initial denaturation at 95^{o} C for 5 minutes, followed by 30 cycles each of denaturation of 94^{o}C for 30 seconds, specific Tm for each species primer for 30 seconds, extension at 72^{o}C for 1 minute, and final extension at 72^{o}C for 7 minutes. Samples were held at 4^{o}C prior to analysis.

To avoid contamination, DNA extraction, reaction set-up, PCR, and electrophoresis were done in distinct laboratory working stations. RNAse-free water was used in all PCR reactions as a negative control and 10 µl of amplified products were identified by electrophoresis in a 1.5% (weight/volume) agarose gel in 1X Tris-Borate-EDTA (TBE) buffer; subsequently stained with ethidium bromide and ran for 30-45 minutes at 200V and visualized by UV-illuminator (UVP GelMax 125 Imager, USA). The sizes of the amplicons were determined using 100 bp molecular

ladder. Specific amplified fragments expected were of size 843 bp, 512 bp, and 386 bp which corresponded to *Campylobacter jejuni, C. upsaliensis,* and *C. coli* respectively (Table 5.1).

Campylobacter isolates that were positive for the genus-specific PCR but negative for *the C. jejuni*, *C. upsaliensis*, and *C. coli* were designated as unidentified thermophilic *Campylobacter* species.

Table 5. 1 Primer sequences for the amplification of *Campylobacter* species DNA from puppy feces

PCR target	Primers	Primers (Sequence 5'-3')	Product size (bp)	Tm	Reference
Genus *Campylobacter* *16S rRNA* gene	C412F	GGATGACACTTTTCGGAGC	816	58	Linton *et al.*, 1996
	C1228R	CATTGTAGCACGTGTGTC			
Campylobacter jejuni *cpb* gene	Cj-cpbF1	AACCTTAGTCGGACAGCCTTC	843	62	This study
	Cj-cpbR1	CCTAGCGGAACGAGGTGTAA			
Campylobacter upsaliensis *rme* gene	Cu-rmeF	GAAAGTGGCATCCGCACAAA	512	52	This study
	Cu-rmeR	CCCACCAATCGCCCCTTATT			
Campylobacter coli *imp* gene	Cc-ImpF	CCGCTTTATACTGCTTTCGTGG	386	62	This study
	Cc-ImpR	AAGCGATACTCATCCACCCC			

5.3 Data analysis

5.3.1 Statistical analysis

Polymerase chain reaction results were input into Excel version 2016 (Redmond, WA, USA) before being exported to STATA 17.0 (StataCorp LLC, USA) for analysis. Descriptive statistics expressed as proportions and frequencies were computed for PCR detected and confirmed *Campylobacter* species.

5.3.2 Bioinformatics analyses of the sequences

Sanger DNA sequencing approaches were used to sequence the *16S rRNA*, *cpb*, and *imp* genes in order confirm their identities (Dey, 2018). SangeranalyseR was used to perform quality control, assembly and editing of nucleic sequence trace files (Kuan-Hao *et al*., 2021). Genetic identities of genus *Campylobacter*, *C. jejuni* and *C. coli* were confirmed by BLASTn analysis (Ye *at al*., 2012) at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Multiple Sequence alignment for *C. jejuni* and *C. coli* was done using local alignment in MAFFT version 7 (Katoh and Stadley, 2013) and visualized in Jalview version 2.11.2.4 (Kunzmann *et al*., 2020). IQtree (V2.2.0.3) (Minh *et al*., 2020) was used to generate the phylogenetic trees. IQtree's inbuilt ModelFinder was used to test 484 DNA substitution models and TPM3u+F+R2 (TPM3u Substitution model, +F base frequency and R2 rate heterogeneity) evolutionary model (Minh *et al*., 2020) was chosen as the best fit model based on Bayesian Information Criterion (BIC) (Neath and Cavanaugh, 2012). 10,000 ultrafast bootstrap replicates and 1000 replicates for SH-like approximate likelihood ratio tests were run. Visualization of the phylogenetic trees was generated in FigTree v1.4.4 (Rambaut, 2019).

5.4 Results

5.4.1 Prevalence of *Campylobacter* species infecting puppies in the Nairobi Metropolitan Region, Kenya

Polymerase chain reaction (PCR) was used to further characterize the pathogens isolated on culture. 64 isolates (24.6%, 64/260) were PCR positive for *Campylobacter* species. *Campylobacter* species detected in this study by specific-specific primers were *C. coli* (12.7%, 33/260) and *C. jejuni* (3.8%, 10/260). Mixed *C. jejuni and C. coli* were detected in four of the puppies (1.5%, 4/260). *Campylobacter upsaliensis* was not detected and twenty-three isolates were categorized as unidentified *Campylobacter* species. The primers targeting the *cpb* gene of

C. jejuni and the *imp* gene of *C. coli* produced specific bands corresponding to the expected sizes of 843 bp (Figure 5.1A) and 386 bp (Figure 5.1B) respectively

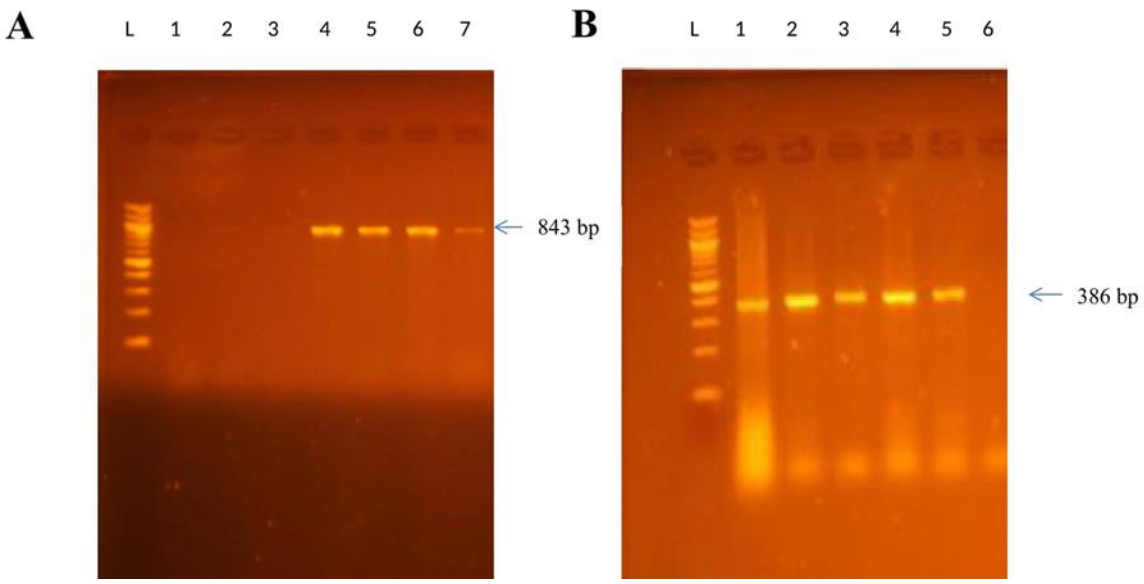

Figure 5. 1 Representative PCR amplicons of *C. jejuni* and *C. coli.* A) PCR products of *C. jejuni (cpb* gene). Lane L: molecular ladder (100bp); lane 1: negative control; lanes 2, 3: no amplicons observed; lanes 4-7: positive samples showing amplicon at approximate 843 bp. B) PCR products of *C. coli* (*imp* gene). Lane L: molecular ladder (100bp); lane 1-5: positive samples showing amplicon band at approximate 386 bp; lane 6: negative control.

5.4.2 Confirmation of genus *Campylobacter, Campylobacter jejuni* and *Campylobacter coli* detected in puppies by BLASTn analysis

Based on strong PCR bands as observed on gel electrophoresis, unpurified PCR products of representative samples of genus *Campylobacter*, *C. jejuni,* and *C. coli* were sent to Macrogen, Netherlands for purification and sequencing for confirmation of species of the detected pathogens. The stronger PCR bands imply a higher concentration of the pathogen's DNA

therefore better base-calling and consequently good chromatographs that can be analyzed. Of these, four PCR amplicons for genus *Campylobacter*, four for *C. jejuni* and twenty-five for *C. coli* had good chromatograms that were analyzed further. BLASTn analysis revealed sequence identities of between 93.66% and 99.87% for genus *Campylobacter*, of between 82.84% and 99.27% for *C. jejuni*, and of between 80.72% and 100% for *C. coli* with those of annotated sequences in the GenBank database (Table 5.2).

Table 5. 2 Confirmation of genus *Campylobacter*, *Campylobacter jejuni*, and *Campylobacter coli* detected in puppies in the Nairobi Metropolitan Region, Kenya by BLASTn analysis

***Campylobacter* species**	**Isolate no.**	**Organism**	**Accession no. of highest match**	**E-value**	**% identity**
Genus *Campylobacter (16S rRNA* gene)	48	*Campylobacter jejuni*	MZ209111.1	0.0	93.66
	143a	*Campylobacter jejuni*	MK156109.1	0.0	93.67
	167d	*Campylobacter jejuni*	CP054848.1	0.0	99.87
	216a	*Campylobacter jejuni*	MZ2091131.1	0.0	97.28
Campylobacter jejuni (*cpb* gene)	5c	*Campylobacter jejuni*	CP040607.1	0.0	98.54
	119a	*Campylobacter jejuni*	CP044162.1	0.0	99.27
	145c	*Campylobacter jejuni*	CP022080.1	0.0	99.25
	178a	*Campylobacter jejuni*	CP040607.1	7^{e-95}	82.84
Campylobacter coli (*imp* gene)	8b	*Campylobacter coli*	CP046317.1	4^{e-162}	98.79
	13a	*Campylobacter coli*	CP046317.1	2^{e-174}	99.71
	15c1	*Campylobacter coli*	CP042463.1	1^{e-146}	96.05
	16d	*Campylobacter coli*	CP044165.1	8^{e-172}	99.70
	18c	*Campylobacter coli*	CP042463.1	1^{e-171}	99.13
	19a	*Campylobacter coli*	CP006702.2	4^{e-171}	99.41
	24d	*Campylobacter coli*	CP044161.1	2^{e-168}	99.11
	25c	*Campylobacter coli*	CP046317.1	3^{e-172}	99.70
	28a	*Campylobacter coli*	CP046317.1	6^{e-169}	99.11
	49a	*Campylobacter coli*	CP046317.1	5^{e-175}	99.15
	60a	*Campylobacter coli*	CP042463.1	4^{e-171}	99.41
	75d	*Campylobacter coli*	CP038868.1	3^{e-38}	80.72
	95b	*Campylobacter coli*	CP046317.1	4^{e-165}	97.71
	121a	*Campylobacter coli*	CP046317.1	2^{e-174}	100
	126b	*Campylobacter coli*	CP046317.1	3^{e-167}	98.82
	127b	*Campylobacter coli*	CP046317.1	5^{e-175}	99.14
	131c	*Campylobacter coli*	CP046317.1	4^{e-171}	99.41
	133d	*Campylobacter coli*	CP042463.1	6^{e-170}	98.84
	136d	*Campylobacter coli*	CP046317.1	5^{e-175}	99.42
	137a	*Campylobacter coli*	CP007181.1	2^{e-73}	90.87
	138c	*Campylobacter coli*	CP046317.1	3^{e-135}	97.28
	170a	*Campylobacter coli*	CP046317.1	2^{e-172}	99.71
	189c	*Campylobacter coli*	CP046317.1	5^{e-165}	98.53
	190b	*Campylobacter coli*	CP046317.1	1^{e-171}	98.85
	254c	*Campylobacter coli*	CP046317.1	3^{e-172}	99.70

5.4.3 Sequence nucleotide polymorphisms (SNPs) detected by multiple sequence alignments of *Campylobacter jejuni* and *Campylobacter coli*

Multiple sequence alignments were done to and assess the genetic similarity of the Kenyan isolates by detecting sequence nucleotide polymorphisms (SNPs). The nucleotide sequences of one *C.jejuni* isolate (119a) were mostly conserved while two nucleotide sequences of isolates 145c and 5c had multiple single nucleotide polymorphisms indicated by black arrows (Figure 5.2). Three *C. coli* sequences indicated multiple single nucleotide polymorphisms indicated by black arrows (Figure 5.3).

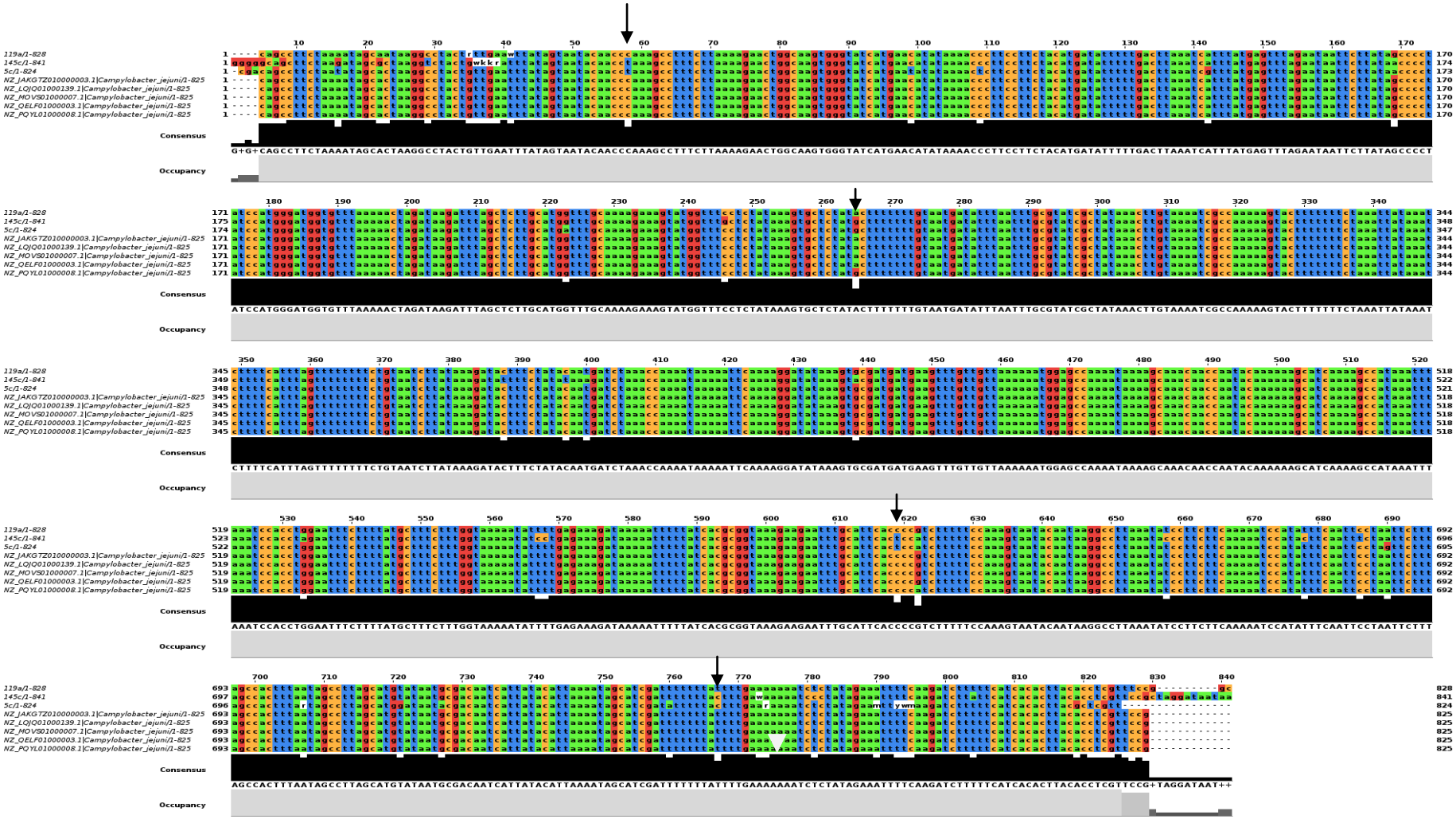

Figure 5. 2 Multiple sequence alignment for *Campylobacter jejuni* nucleotide sequences obtained from puppies in the Nairobi Metropolitan Region. The black arrows show regions of multiple nucleotide sequence polymorphism (SNPs)

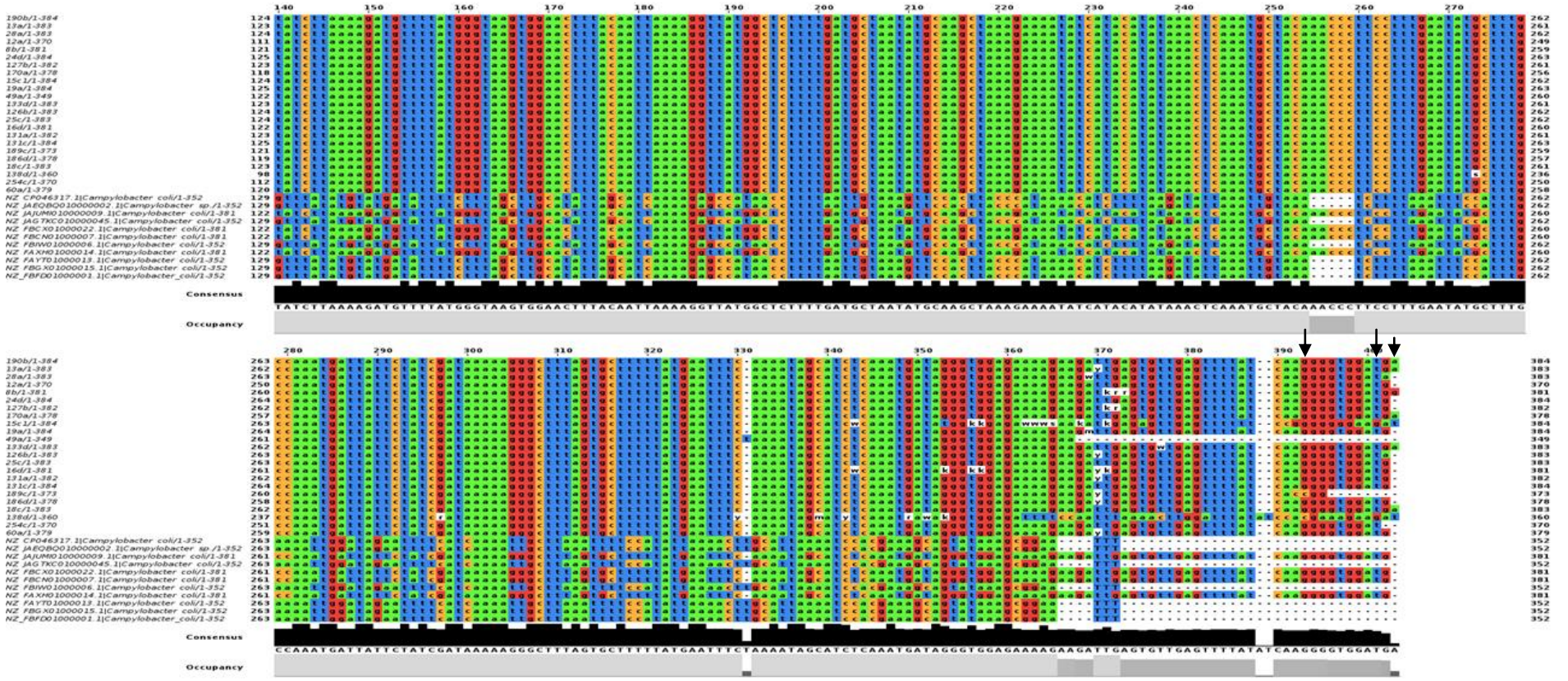

Figure 5. 3 Multiple sequence alignment for *Campylocater coli* nucleotide sequences obtained from puppies in the Nairobi Metropolitan Region. The black arrows show regions of multiple single nucleotide sequence polymorphism (SNPs) in the *Campylobacter coli* isolated from puppies in this study.

5.4.4 Phylogenetic positioning of *Campylobacter jejuni* and *Campylobacter coli* detected in puppies in the Nairobi Metropolitan Region, Kenya

Phylogenetic analysis was done to understand genetic relatedness of the Nairobi Metropolitan Region, Kenya isolates in the two species with those of annotated sequences in the GenBank database. The Nairobi, Kenya isolates of *C. jejuni* (14c and 5c) were closely related to those of *C. jejuni* isolated from wild birds (Finland) (Figure 5.4). The Nairobi, Kenya isolates of *C. coli* clustered in the same clade with those of *C. coli* isolated from cattle (UK), humans (UK), and poultry (China) (Figure 5.5-Clade 1).

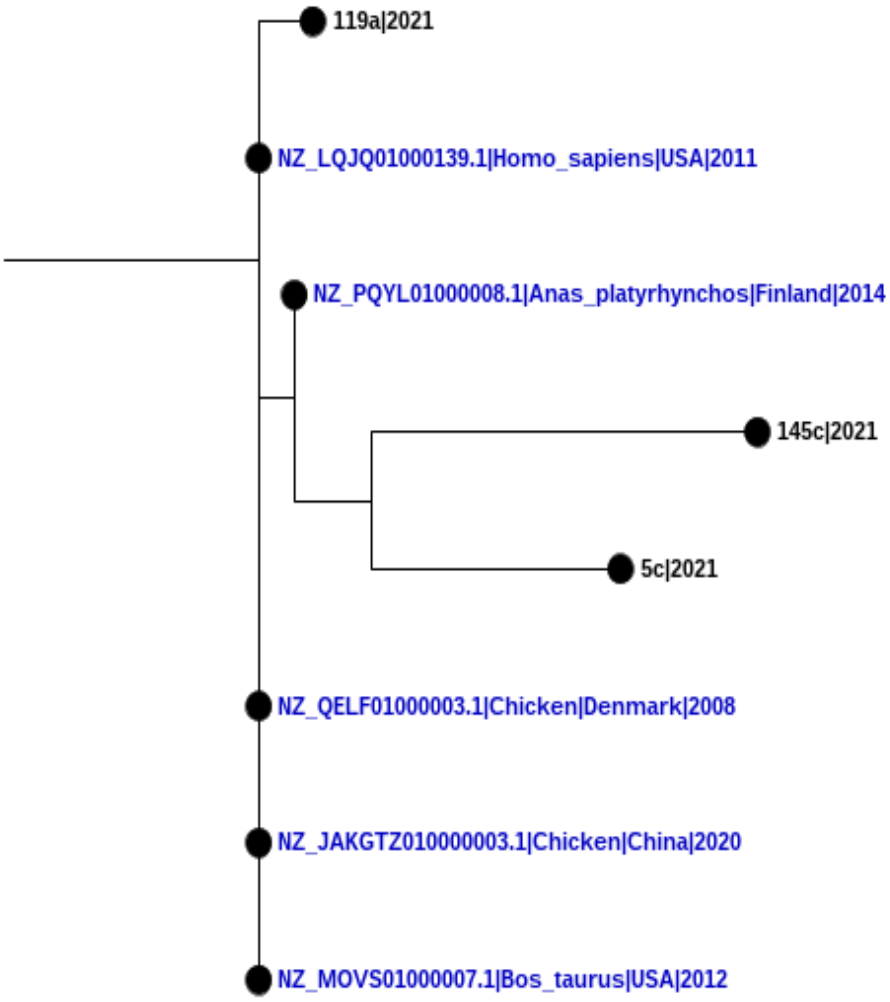

Figure 5. 4 Maximum Likelihood tree of *Campylobacter jejuni* constructed using partial sequences of the *cpb* gene. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. The analysis involved 3 nucleotide sequences from this study and 5 others obtained from Genbank (accession numbers). The tree shows the phylogenetic relatedness of *Campylobacter jejuni* isolates obtained from puppies in the Nairobi Metropolitan Region, Kenya and sequences from other countries. Bootstrap values are illustrated between most nodes and based on calculation of 1000 possible combinations.

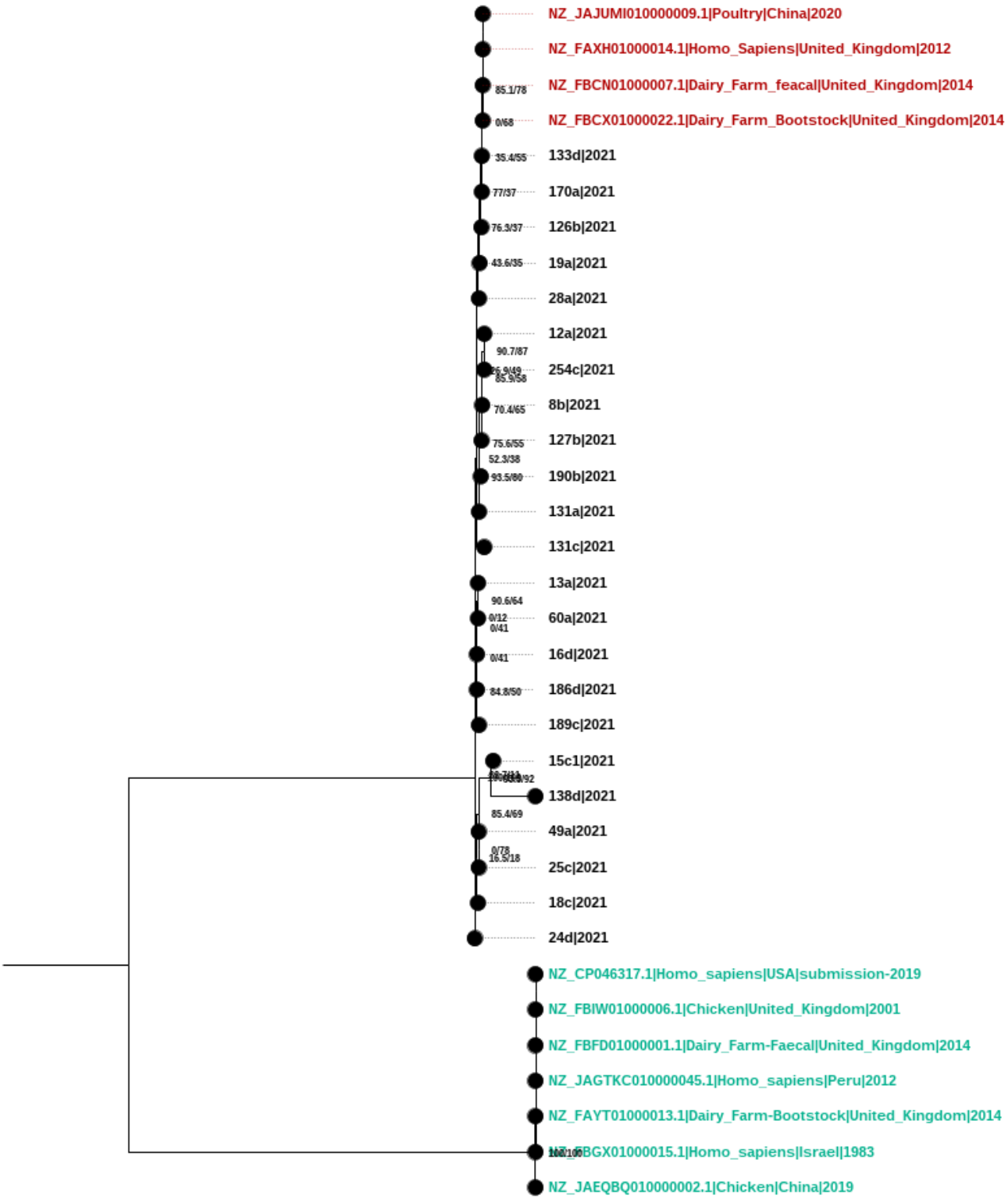

Figure 5. 5 Maximum Likelihood tree of *Campylobacter coli* constructed using partial sequences of the *imp* gene. The tree is drawn to scale with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences from this study and 11 others obtained from Genbank (accession numbers). The tree shows the phylogenetic relatedness of *Campylobacter coli* isolates obtained from puppies in the Nairobi Metropolitan Region.

5.5 Discussion

Molecular characterization of *Campylobacter* isolates to species level was necessary to confirm them in Kenyan puppies. In this study, PCR was done to identify the isolated *Campylobacter* species by targeting the *cpb* gene specific for *C. jejuni*, *rme* gene for *C. upsaliensis*, and *imp* gene for *C. coli*. Though *C. coli* is reported to be less frequent in dogs compared to *C. jejuni* and *C. upsaliensis* (Karshima and Bobbo, 2016; Karama *et al*., 2018; Thépault *et al*., 2020); this study revealed 13% (33/260) of the *Campylobacter* isolates to be *C. coli* and 4% (10/260) *C. jejuni*. These species are important causative agents of human *Campylobacter* infections and because of their close association with humans; puppies maybe an important source of human campylobacteriosis. *Campylobacter upsaliensis* was not detected in this study; although several studies have reported its predominant presence in dogs (Carbonero *et al*., 2012; Mughini-Gras *et al*., 2013; Holmberg *et al*., 2015; Bojanić *et al*., 2017).

Mixed *Campylobacter* species in the same host was detected in four of the puppies. Similar mixtures have been isolated from feces of dogs in previous studies (Hald *et al*., 2004; Wieland *et al*., 2005; Koene *et al*., 2009). Information on co-infections may be useful in epidemiological studies that involve tracing the sources of human campylobacteriosis (Koene *et al*., 2009).

Single-nucleotide polymorphisms (SNPs) are DNA sequence polymorphisms which are caused by the alteration of a single nucleotide in a specific position at the genomic level (Kim and Misra, 2007) therefore, they are powerful tools for identifying bacterial strains, their genetic diversity, and phylogenetic analysis (Rahman *et al*., 2022). In this study, single-nucleotide polymorphisms were observed in the *C. jejuni* and *C. coli* isolates. Similar findings have previously been observed by Dunn *et al*. (2018) and Jehanne *et al*. (2020).

Genetic relatedness through phylogenetic analysis between puppy *C. jejuni* isolates and those isolated from geographically distant humans and wild birds was observed in this study. Wild birds are reservoirs of potentially pathogenic *C. jejuni* strains and can be vectors of disease transmission (Ahmed and Gulhan, 2022). This study also revealed genetic relatedness through phylogenetic analysis between puppy *C. coli* isolates and those isolated from geographically distant farm environments, humans, and chicken. These study findings are similar to those of Sheppard *et al*. (2013). The genetic relatedness of *C. jejuni* and *C. coli* isolates suggests the potential role of the environment, poultry, cattle and humans in puppies' exposure to *Campylobacter* and vice-versa (Thépault *et al*., 2020).

5.6 Conclusions and recommendations

The puppies in this study were found to be carriers of *Campylobacter coli* and *Campylobacter jejuni*. The results of the phylogenetic tree analysis in this study indicated a relationship between the puppy isolates and isolates from various animal species including humans, thus highlighting the importance of puppies as a potential source of *Campylobacter* species infection to humans and other animals. Breeders and puppy owners should observe strict hand hygiene especially after handling puppies or their feces to reduce the risk of acquiring infection from their pets.

CHAPTER SIX

6.0 POLYMERASE CHAIN REACTION (PCR) DETECTION OF POTENTIAL VIRULENCE GENES IN CAMPYLOBACTER SPECIES ISOLATES FROM PUPPIES IN THE NAIROBI METROPOLITAN REGION, KENYA

6.1 Introduction

Campylobacter infection in dogs, particularly puppies, manifests as mild to watery diarrhea, or as bloody or mucoid diarrhea with tenesmus (Brown *et al*., 1999; Acke, 2018). Several virulence factors may influence these *Campylobacter* gastroenteritis clinical manifestations (Zilbauer *et al*., 2008). The *flaA* gene, which encodes the protein FlaA, is responsible for the formation of the flagellum filament and thus for the motility of *Campylobacter* species (Younis *et al*., 2018). The adherence of these bacteria to the intestinal mucosa, which is mediated by various bacterial surface adhesins like *cadF*, which encodes the outer membrane protein that interacts with fibronectin, is a crucial stage of pathogenesis (Facciola *et al*., 2017; Pillay *et al*., 2020). *pldA*, phospholipase A, *ciaB*, and *iam* are among the genes that control the expression of enterocyte invasion (Carvalho *et al*., 2001; Eucker and Konkel, 2012; Pillay *et al*., 2020). Various cytotoxins mediate subsequent cell damage, the most studied of which is cytolethal distending toxin (CDT), which is composed of three subunits (cdtA, -B, -C) (Zilbauer *et al*., 2008; Bolton, 2015; Facciola *et al*., 2017).

Since the distribution of potential virulence markers among *Campylobacter* species isolates from puppies is lacking in Kenya, this study was aimed at determining the prevalence of virulence genes associated with motility, adherence, invasion, and cytotoxicity by using Polymerase Chain Reaction (PCR).

6.2 Materials and Methods

6.2.1 Identification of virulence-associated genes using Polymerase Chain Reaction (PCR)

Deoxyribonucleic acid from cultured *Campylobacter* isolates was amplified using PCR. All confirmed isolated were screened for eight virulence-associated genes, namely *flaA*, *cadF*, *ciaB*, *iam*, *pldA*, *cdtA*, *cdtB*, and *cdtC* by polymerase chain reaction (PCR). PCR primers were sourced from Macrogen Europe. Forward and reverse primers specific for the virulence genes under investigation were designed based on the gene sequence information in the GenBank database and in previously published studies (Nachamki *et al*., 1993; Konkel *et al*., 1999; Chansiripornchai and Sasipreeyajan 2009; Rozynek *et al*., 2005; Montwedi and Ateba, 2012). Target virulence genes, primer sequences, product sizes and annealing temperatures are shown in Table 6.1.

Polymerase chain reactions were carried out using the T100™ Thermal Cycler (Bio-Rad, Singapore) for a 12.5-µL reaction. The amplification conditions for *flaA* (1728bp) and *cadF* (400bp) were as follows: an initial denaturation at 94°C for 1 minute, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. PCR conditions for *ciaB* (527bp), *iam* (518bp), *cdtA* (370bp), *cdtB* (620bp), *cdtC* (182bp) consisted of an initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, specific Tm for each primer for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. The amplification conditions for *pldA* (385bp) consisted of an initial denaturation at 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. PCR products (10ul) were electrophoresed in a 1.5% agarose gel stained with ethidium bromide in Tris-Borate-EDTA (TBE) buffer and

visualized with a UV illuminator (GelMax® Imager, Cambridge, UK). The sizes of the amplicons were determined using a molecular ladder (GelPilot 100bp Plus Ladder (100), QIAGEN, GmbH, Hilden, Germany). RNAse-free water was used in all PCR reactions as a negative control.

Table 6. 1 Target virulence-associated genes, primer sequences, amplicon sizes and annealing temperatures

Target gene	Primer sequences	Product size (bp)	Tm	Reference
flaA-F	GGATTTCGTATTAACACAAATGGTGC	1728	45	Nachamkin *et al*., 1993
flaA-R	CTGTAGTAATCTTAAAACATTTTG			
cadF-F	TGGAGGGTAATTTAGATATTG	400	45	Konkel *et al*., 1999
cadF-R	CTAATACCTAAAGTTGAAAC			
ciaB-F	TGCGAGATTTTTCGAGAATG	527	57	Chansiripornchai and Sasipreeyajan 2009
ciaB-R	TGCCCGCCTTAGAACTTACA			
iam-F	GCGCAAATATTATCACCC	518	60	Rozynek *et al*., 2005
iam-R	TTCACGACTACTACTATGCGG			
pldA-F	AAGAGTGAGGCGAAATTCCA	385	60	Chansiripornchai and Sasipreeyajan 2009
pldA-R	GCAAGATGGCAGGATTATCA			
cdtA-F	CCTTGTGATGCAAGCAATC	370	49	Rozynek *et al*., 2005
cdtA-R	ACACTCCATTTGCTTTCTG			
cdtB-F	CAGAAAGCAAATGGAGTGTT	620	51	Montwedi and Ateba, 2012
cdtB-R	AGCTAAAAGCGGTGGAGTAT			
cdtC-F	CGATGAGTTAAAACAAAAAGATA	182	51	Rozynek *et al*., 2005
cdtC-R	TTGGCATTATAGAAAATACAGTT			

6.2.2 Statistical data analysis

All statistical analyses were performed using STATA version 17.0. Descriptive statistics were used to examine the distribution of virulence genes. The Pearson's chi-square test ($\chi 2$ test) was used when applicable to compare categorical variables. Statistical significance level was set at 0.05 ($p < 0.05$).

6.2.3 Virulence gene sequencing and analysis

Unpurified PCR products of representative samples were sent to Macrogen, Netherlands for purification and sequencing. Sanger DNA sequencing approaches were used to sequence the virulence genes in order confirm their identities (Dey, 2018). SangeranalyseR was used to perform quality control, assembly and editing of nucleic sequence trace files (Kuan-Hao *et al*., 2021). Confirmation of the sequence identities was done using the Basic Local Alignment Search Tool (BLAST).

6.3 Results

6.3.1 Prevalence of virulence-associated genes in *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli* isolated from puppies

Of the *C. jejuni* isolated from puppies, 10, 30, 60, 10, 30, 90, 30 and 30% were positive for *flaA*, *cadF*, *ciaB*, *iam*, *pldA*, *cdtA*, *cdtB*, and *cdtC* respectively, and *C. coli* revealed 24.2, 36.4, 48.5, 15.2, 15.2, 78.8, 51.5, and 30.3% respectively. The details are shown in Table 6.2 below. A subset (5) of the virulence genes were sequenced and revealed a 97-100% homogeneity to those in the GenBank. Figures 6.1 and 6.2 are gel electrophoresis images of PCR- confirmed *cdtB* and *ciaB* genes.

Table 6. 2 Prevalence of virulence-associated genes in *Campylobacter* species, *Campylobacter jejuni* and *Campylobacter coli* isolated from puppies in the Nairobi Metropolitan Region, Kenya

Virulence gene	*Campylobacter* species (n=64) (%)	*C. jejuni* (n=10) (%)	*C. coli* (n=33) (%)
flaA	16(25)	1(10)	8(24.2)
cadF	20(31.3)	3(30)	12(36.4)
ciaB	30(46.9)	6(60)	16(48.5)
Iam	8(12.5)	1(10)	5(15.2)
pldA	8(12.5)	3(30)	5(15.2)
cdtA	45(70.3)	9(90)	26(78.8)
cdtB	28(43.8)	3(30)	17(51.5)
cdtC	17(26.6)	3(30)	10(30.3)

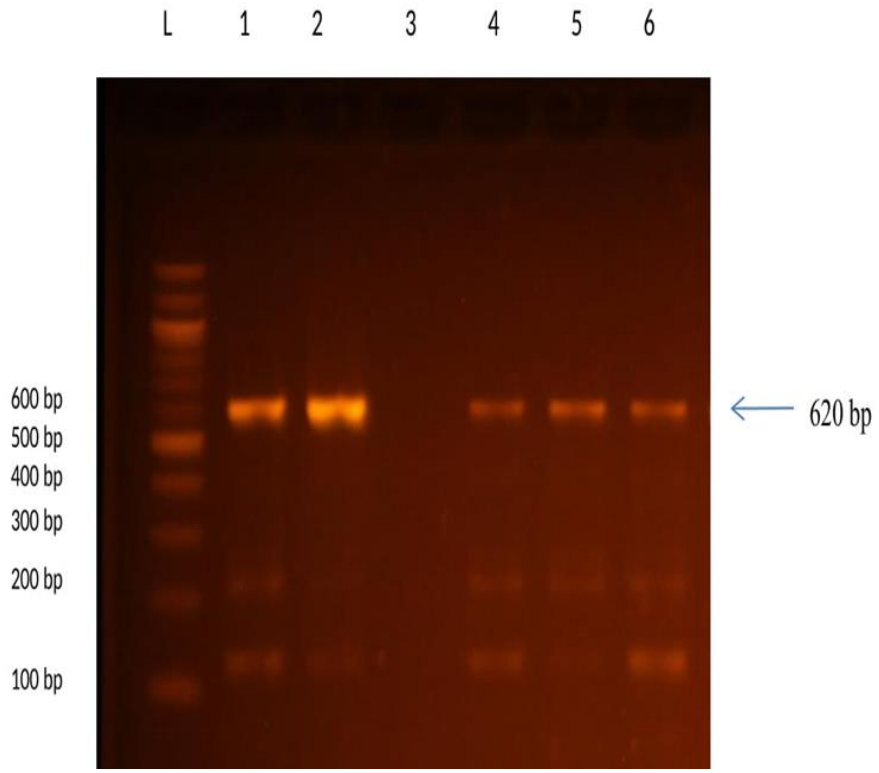

Figure 6. 1 Representative PCR amplicons of *cdtB* gene (620bp). Lane L: molecular ladder (100 bp); lanes 1, 2, 4, 5, and 6 : positive samples showing amplicons at approximate 620; lane 3: negative control.

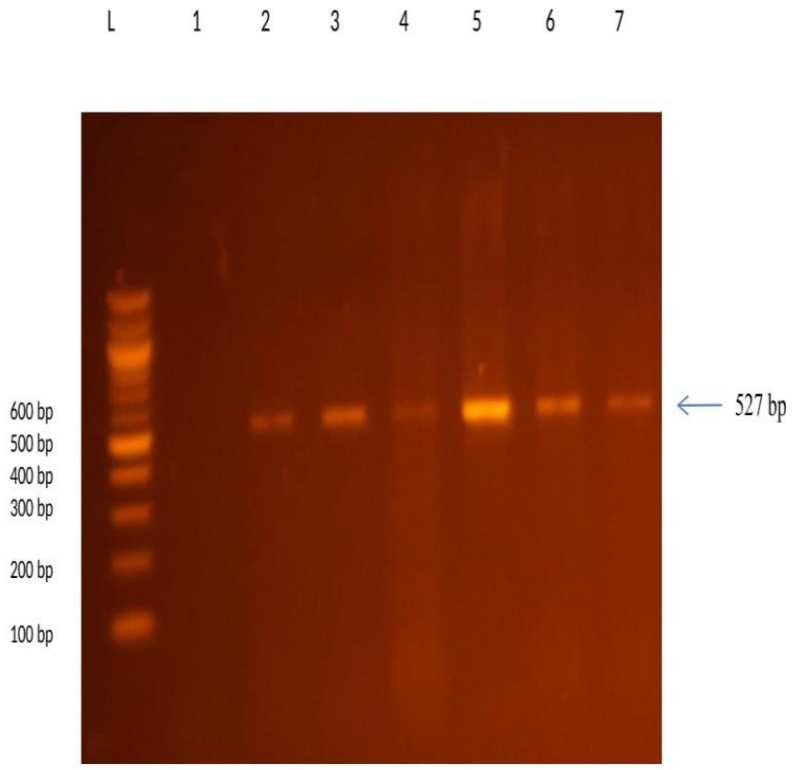

Figure 6. 2 Representative PCR amplicons of *ciaB* gene (527 bp). **Lane L: molecular ladder (100 bp); lane 1: negative control; lanes 2 to 7: positive samples showing amplicons at approximate 527 bp.**

6.3.2 Distribution of virulence-associated genes studied in puppies

A higher prevalence of *flaA*, *cadF*, *ciaB*, *iam*, *pldA*, *cdtA*, *cdtB*, and *cdtC* genes was observed in puppies less than 2 months of age at 62.5, 60, 60, 50, 62.5, 42.2, 42.9, and 53% respectively. There were statistically significant differences ($p<0.05$) observed between the presence of *flaA* and *cdtC* genes and the occurrence of diarrhea (Table 6.3).

Table 6. 3 Distribution of virulence-associated genes studied in puppies in the Nairobi Metropolitan Region, Kenya

		Virulence genes							
Factor	**Description**	***flaA***	***cadF***	***ciaB***	***iam***	***pldA***	***cdtA***	***cdtB***	***cdtC***
Age	<2 months	10(62.5)*	16(60)	16(60)	4(50)	5(62.5)	19(42.2)	12(42.9)	9(53)
	2-5 months	5(31.3)	8(26.7)	8(26.7)	4(50)	2(25)	18(40)	10(35.7)	6(35.3)
	>5 months	1(6.3)	6(20)	6(20)	0	1(12.5)	8(17.8)	6(21.4)	2(11.8)
	Total	16	30	30	8	8	45	28	17
Diarrhea	Yes	5(13.9)*	5(13.9)	3(8.3)	1(2.8)	2(5.9)	6(16.7)	5(13.9)	6(16.7)*
n=36	No	31(86.1)	31(86.1)	33(91.7)	35(97.2)	34(94.4)	30(83.3)	31(86.1)	30(83.3)

***Statistically significant at p<0.05**

6.4 Discussion

The virulome of *Campylobacter* species contributes to their pathogenicity (Han *et al*., 2019).The pathogenesis of Campylobacteriosis may be related with factors such as motility, adhesion, invasiveness, and cytotoxin production (Bolton, 2015). The present study reports on the analysis of the prevalence of virulence genes among puppy-associated *Campylobacter* isolates, where *C. jejuni* and *C. coli* were detected.

The *flaA* gene, which is essential for motility and small intestine colonization (Bolton, 2015; Wysok and Wojtacka, 2018) was detected in 24.2% of the *C. coli* and 10% of the *C. jejuni* isolates. However, higher prevalence of *flaA* gene has previously been reported in canine isolates (Andrzejewska *et al*., 2013; Selwet, 2019). The low prevalence of *flaA* gene in the current maybe attributed to the differences in primers used or genetic variation (Ngobese *et al*., 2020). There was an association between the presence of *flaA* gene and diarrhea in puppies, a finding that is consistent and noted by Rodrigues *et al*. (2015). Reports have indicated that this gene may be responsible for the expression of host cell adherence, colonization, and invasion in the gastrointestinal tract, therefore causing an arrest of the immune response (Cantero *et al*., 2018; Farfan *et al*., 2019).

The ability to adhere to and invade of host's enterocytes is also critical for the pathogenesis of campylobacteriosis (Wysok and Wojtacka, 2018). The Campylobacter adhesion protein, CadF is an outer membrane protein that initiates adhesion by binding to the fibronectin of epithelium. (Bang *et al*., 2014; Kreling *et al*., 2020). Previous studies have revealed that almost all *C. jejuni* and *C. coli* isolates possess the *cadF* gene (Konkel *et al*., 1999; Andrzejewska *et al*., 2011). This gene was observed in 30% of the *C. jejuni* strains and 36.4% of the *C. coli* strains (6.1%). However, a higher percentage has been identified among canine isolates in other studies (Selwet *et al*., 2015; Murawska *et al*., 2022). Rodrigues *et al*. (2015) noted that the *cadF* gene may act as a reporter in research on the mechanisms of *C. jejuni* pathogenicity.

Products of the CiaB, Iam, and PldA proteins are involved in the internalization of *Campylobacter* species in the host's cells (Bang *et al*., 2004; Kreling *et al*., 2020). In animals, the *ciaB* gene is important for epithelial cell invasion and colonization. The high prevalence of this gene in this study *(C. jejuni* (60%) and *C. coli* (48.5%) may indicate that these bacteria overcame stressful conditions during passage through the gastrointestinal tract and induced the disease (Guerry, 2007). The results showed that *C. coli* carried the iam gene more frequently at 15.2% than *C. jejuni* at 10%. An observation that is in agreement with Lluque *et al*. (2017) that the *iam* gene occurs more frequently in *C. coli* than in *C. jejuni*. The *pldA* gene which encodes an outer membrane phospholipase A involved in host cell invasion, was demonstrated in 30% of the *C. jejuni* strains and 15.2% of the *C. coli* strains. These were in contrast to the report of a study in Korea (Cho *et al*., 2014) that observed the pldA gene more in *C. jejuni* than in *C. coli.*

Campylobacter species virulence is also associated with the production of the cytolethal distending toxin (CDT) which consists of three subunits (CdtA, -B, -C) encoded by *cdtA*, *cdtB*, and *cdtC* genes (Bolton, 2015; Facciola *et al*., 2017). Although the CdtB subunit is responsible

for the nucleolytic effect, the role of CdtA and CdtC subunits cannot be overlooked as they are involved in the transportation of CdtB into the target cell, therefore all the three subunits are required for full toxin activity (Bolton, 2015). In this study, all the three genes were investigated and the prevalence reported was relatively high especially in the *C. coli* strains (*cdtA*-78.8%. *cdtB*-51.5%, *cdtC*-30.3%). Selwet (2019) recorded similar results in his study. In this study, *C. jejuni* and *C. coli* strains were negative for one or two of the subunits which may indicate that they were unable to express the entire CDT product or that the toxin genes were not identified with the primers used in the study, possibly due to point mutations in the coding region (Bang *et al*., 2004).

6.5. Conclusions and recommendations

High rates of virulence genes were found in *C. jejuni* and *C. coli* isolates. Given the close contact of puppies with humans, good hygiene practices should be promoted to minimize the risk of transmission especially to children and other vulnerable groups.

CHAPTER SEVEN

7.0 PHENOTYPIC AND GENOTYPIC ANTIMICROBIAL RESISTANCE PATTERNS OF THERMOPHILIC *CAMPYLOBACTER* SPECIES ISOLATED FROM PUPPIES IN THE NAIROBI METROPOLITAN REGION, KENYA

7.1 Introduction

Antimicrobial resistance of *Campylobacter* species has been identified as an increasing public health concern over the years (Whitehouse *et al*., 2018). This is emphasized by the documentation of increasing resistance to macrolides, quinolones, tetracyclines, and penicillins (Landers *et al*., 2012). Antimicrobials are increasingly being used more frequently and indiscriminately to treat a variety of bacterial infections, which encourages the development of resistance (Przdka and Osiski, 2013; Ayukekbong *et al*., 2017).

Due to the widespread use of broad-spectrum antimicrobial agents and their close contact with humans, dogs are thought to be potential reservoirs for transferring antimicrobial resistance (AMR) to humans (Lloyd, 2007). Transmission of resistant bacteria or their resistance genes between animals and humans is possible through direct or indirect contact, as well as through the environment (Larsson and Flach, 2022). The development of multidrug-resistant (MDR) *Campylobacter* isolates can occur from either the introduction of mutations or the acquisition of antibiotic resistance genes through horizontal gene transfer from other bacteria (Holmes *et al*., 2016). If AMR can be transmitted from dogs to humans, and MDR *Campylobacter* species exist among the puppies, the risk of antimicrobial treatment failure in both these animals and humans increases dramatically (Li *et al*., 2021). Understanding the prevalence of AMR among *Campylobacter* species isolates from puppies is thus required from the standpoints of both veterinary and human medicine.

This study aimed to establish the phenotypic and genotypic antibiotic resistant patterns of the *Campylobacter* isolates towards the drugs used to treat campylobacteriosis, including macrolides, quinolones and tetracyclines as well as drugs commonly used in small animal practice.

7.2 Materials and Methods

7.2.1 Antibiotic susceptibility testing (AST)

All PCR-confirmed *Campylobacter* isolates were subjected to Kirby–Bauer disc diffusion susceptibility testing per the guidelines of the Clinical and Laboratory Standard Institute (CLSI) with Mueller-Hinton Agar (Himedia) supplemented with 5% defibrinated sheep blood. Antibiotic discs from Thermo Scientific^{TM}, Oxoid (United Kingdom) were used: macrolides (erythromycin 30ug), fluoroquinolones (ciprofloxacin 5ug), tetracyclines (tetracycline 30ug), beta-lactams (ampicillin 10ug and cefuroxime 30ug), aminoglycosides (gentamicin 10ug and streptomycin 10ug), and folic acid inhibitors (trimethoprim-sulphamethoxazole 25ug). Briefly, fresh cultures of the *Campylobacter* species isolates were suspended in sterile physiological saline until turbidity equivalent to 0.5 McFarland's standard was obtained. Sterile cotton wool swabs were then used to inoculate the suspension onto the entire surface of the agar plates. The lids of the agar plates were left ajar for 3–5 minutes to allow excess surface moisture to be absorbed before the placement of drug impregnated discs using a sterile multi-disc dispenser. The agar plates were then incubated under microaerophilic conditions at 42^{O}C for 24 hours. After incubation, zone diameters around the antibiotic discs were measured and classified as susceptible, intermediate or resistant based on the CLSI breakpoints (Table 7.1). Since there were no antimicrobial susceptibility breakpoints for disc diffusion method specific with respect to *Campylobacter* for ampicillin, cefuroxime, gentamicin, streptomycin, and trimethoprim-

sulphamethoxazole provided by CLSI M45, breakpoints of *Enterobactericeae* were used (CLSI M100). In this study, all strains that read as intermediate were considered as resistant. The reference used for quality control of antibiotic discs was *Escherichia coli* ATCC 25922.

Table 7. 1 Guidelines for interpreting antimicrobial susceptibility results for *Campylobacter* spp.

			Zone diameter breakpoint (mm)			
Antibiotic class	**Antibiotic**	**Disc content (µg)**	**Susceptibility**	**Intermediate**	**Resistant**	**Reference**
Macrolides	Erythromycin	**10**	**≥ 16**	**13-15**	**≤ 12**	CLSI breakpoint for *Campylobacter*
Quinolones	Ciprofloxacin	**5**	**≥ 24**	**21-23**	**≤ 20**	CLSI breakpoint for *Campylobacter*
Tetracyclines	Tetracycline	**30**	**≥ 26**	**23-25**	**≤ 22**	CLSI breakpoint for *Campylobacter*
Beta-lactams	Ampicillin	**10**	**≥ 17**	**14-16**	**≤ 13**	CLSI breakpoint for *Enterobactericeae*
	Cefuroxime	**30**	**≥ 18**	**15-17**	**≤ 14**	CLSI breakpoint for *Enterobactericeae*
Aminoglycosides	Gentamicin	**10**	**≥ 15**	**13-14**	**≤ 12**	CLSI breakpoint for *Enterobactericeae*
	Streptomycin	**10**	**≥ 15**	**12-14**	**≤ 11**	CLSI breakpoint for *Enterobactericeae*
Folic acid inhibitors	Trimethoprim-sulphamethoxazole	**25**	**≥ 16**	**11-15**	**≤ 10**	CLSI breakpoint for *Enterobactericeae*

7.2.2 Detection of antimicrobial resistance genes

All the *Campylobacter* species isolates showing phenotypic resistance were further tested for genes encoding for antibiotic resistance using primer-specific polymerase chain reaction (Table 7.2). All *Campylobacter* isolates showing phenotypic resistance to erythromycin, ciprofloxacin, tetracyclines, beta-lactams and aminoglycosides were analyzed by PCR for genes that confer

resistance to macrolides (*23S rRNA*), fluoroquinolones (*gyrA*), tetracycline (*tet(A)*, *tet(B)*, *tet(C)*, *tet(O*), beta-lactams (*blaOXA-61*), and aminoglycosides (*aph-3-1*). Polymerase chain reaction primers were synthesized and sourced from Macrogen Europe; forward and reverse primers specific for the antibiotic resistance genes under investigation were designed based on the gene sequence information in the GenBank database and in previously published studies (Randall *et al*., 2004; Van *et al*., 2008; Obeng *et al*., 2012; Abdi-Hachesoo *et al*., 2014; Chatur *et al*., 2014; Hao *et al*., 2015). The details on the primers and annealing temperatures used in the study are shown in Table 7.2. Polymerase chain reactions were carried out using the T100™ Thermal Cycler (Bio-Rad, Singapore) for a 12.5-µL reaction. The amplification conditions for *23S rRNA* (147 bp) were as follows: an initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 7 minutes. PCR conditions for *gyrA* (382 bp) consisted of an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplification conditions for multiplex *tet (A)* (577 bp), *tet(B)* (635 bp), *tet(C)* (880 bp), and *tet(O)* (515 bp) consisted of an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. The amplification conditions for *bla-OXA-61* (372 bp) and *aph-3-1* (701 bp) were as follows: an initial denaturation at 94°C for 1 minute, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 1 minute, extension at 72°C for 2 minutes, and a final extension at 72°C for 5 minutes. PCR products (10ul) were electrophoresed in a 1.5% agarose gel stained with ethidium bromide in Tris-Borate-EDTA (TBE) buffer and visualized with a UV illuminator (GelMax® Imager, Cambridge, UK). The

sizes of the amplicons were determined using a molecular ladder (GelPilot 100bp Plus Ladder (100), QIAGEN, GmbH, Hilden, Germany). RNAse-free water was used in all PCR reactions as a negative control.

Table 7. 2 Target antimicrobial resistance (AMR) genes, primer sequences, amplicon sizes and annealing temperatures

Antimicrobial agent	Target gene	Primer sequences	Product size (bp)	Tm	Reference
MACROLIDES (Erythromycin)	*23S rRNA*-F	CGAGATGGGAGCTGTCTCAAAG	147	60	Hao *et al*., 2015
	23S-rRNA-R	CCCACCTATCCTGCACATTCTT			
QUINOLONES (Ciprofloxacin)	*gyr(A)*-F	GAAGAATTTTATATGCTATG	382	55	Chatur *et al*., 2014
	gyr(B)-R	TCAGTATAACGCATCGCAGC			
TETRACYCLINES	*Tet(A)*-F	GTGAAACCCAACATACCCC	577	56	Randall *et al*., 2004
	Tet(A)-R	GAAGGCAAGCAGGATGTAG			
	Tet(B)-F	CCTCAGCTTCTCAACGCGT	635	56	Randall *et al*., 2004
	Tet(B)-R	GCACCTTGCTGAGACTCTT			
	Tet(C)-F	ACTTGGAGCCACTATCGAC	880	56	Van *et al*., 2008
	Tet(C)-R	CTACAATCCATGCCAACCC			
	Tet(O)-F	AACTTAGGCATTCTGGCTCAC	515	56	Abdi-Hachesoo *et al*., 2014
	Tet(O)-R	TCCCACTGTTCCATATCGTCA			
BETA-LACTAMS	*blaOXA-61*-F	AGAGTATAATACAAGCG	372	45	Obeng *et al*., 2012
	bla-OXA-61-R	TAGTGAGTTGTCAAGCC			
AMINOGLYCOSIDES	*aph-3-1*-F	TGCGTAAAAGATACGGAAG	701	39	Obeng *et al*., 2012
	aph-3-1-R	CAATCAGGCTTGATCCCC			

7.2.3 Statistical data analysis

All statistical analyses were performed using STATA version 17.0. Descriptive statistics were used to examine the distribution of AMR phenotypes and genotypes. The Pearson's chi-square test ($\chi 2$ test) was used to test for statistically significant associations between genotype and

phenotype resistance of different antimicrobial drugs. Statistical significance level was set at 0.05 ($p < 0.05$). The Pearson's chi-square test ($\chi 2$ test) was also used to test for statistically significant associations between associated-virulence genes and antimicrobial resistance genes. Statistical significance level was set at 0.05 ($p < 0.05$). Kappa test was performed to evaluate the correlation between genotype and phenotype results. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were also calculated from the contingency table generated for the kappa test.

7.2.4 Antibiotic Resistance Gene Sequencing and Analysis

Unpurified PCR products of representative samples were sent to Macrogen, Netherlands for purification and sequencing. Sanger DNA sequencing approaches were used to sequence the resistance genes in order confirm their identities (Dey, 2018). SangeranalyseR was used to perform quality control, assembly and editing of nucleic sequence trace files (Kuan-Hao *et al*., 2021). Confirmation of the sequence identities was done using the Basic Local Alignment Search Tool (BLAST).

7.3 Results

7.3.1 Antimicrobial Susceptibility Testing

The results of antimicrobial susceptibility testing for *Campylobacter* species isolated from puppies against eight different antimicrobial agents are presented here. Ampicillin and cefuroxime, two of the eight antimicrobial drugs used to determine the antibiogram of *Campylobacter* species isolates, revealed no zone of inhibition suggestive of resistance in all isolates against these two drugs. They were followed by tetracyclines (96.9%), erythromycin (96.9%), streptomycin (87.5%), and trimethoprim-sulphamethoxazole (82.8%), ciprofloxacin (50%), and gentamicin (14%). All the *C. jejuni* isolates were resistant against erythromycin,

tetracycline, ampicillin, cefuroxime, streptomycin, and trimethoprim-sulphamethoxazole. A 100% of resistance of *C. coli* isolates against ampicillin and cefuroxime was observed. High level of resistance was observed against tetracycline at 96.9%, erythromycin at 93.9%, and trimethoprim-sulphamethoxazole at 78.8%. The details are shown in Table 7.3 below.

Table 7. 3 Antimicrobial resistance of *Campylobacter* spp., *Campylobacter jejuni,* and *Campylobacter coli*

Antimicrobial drug	*Campylobacter* spp. isolates	No. of isolates tested	No. resistant (%)	95% confidence interval	
				Lower	Upper
Erythromycin (10 µg)	*Campylobacter* spp.	64	62(96.9)	0.9	1
	C. jejuni	10	10(100)		
	C. coli	33	31(93.9)	0.8	1
Ciprofloxacin (5 µg)	*Campylobacter* spp.	64	32(50)	0.4	0.6
	C.jejuni	10	8(80)	0.5	1
	C.coli	33	15(45.5)	0.3	0.6
Tetracycline (30 µg)	*Campylobacter* spp.	64	62(96.9)	0.9	1
	C.jejuni	10	10(100)		
	C.coli	33	32(96.9)	0.8	1
Ampicillin (10 µg)	*Campylobacter* spp.	64	64(100)		
	C.jejuni	10	10(100)		
	C.coli	33	33(100)		
Cefuroxime (30 µg)	*Campylobacter* spp.	64	64(100)		
	C.jejuni	10	10(100)		
	C.coli	33	33(100)		
Gentamicin (10 µg)	*Campylobacter* spp.	64	9(14.1)	0.07	0.3
	C.jejuni	10	2(20)	0.05	0.5
	C.coli	33	6(18.2)	0.08	0.4
Streptomycin (10 µg)	*Campylobacter* spp.	64	56(87.5)	0.8	0.9
	C. jejuni	10	10(100)		
	C.coli	33	28(84.8)	0.7	0.9
Trimethoprim-sulphamethoxazole (25 µg)	Campylobacter spp.	64	53(82.8)	0.7	0.9
	C.jejuni	10	10(100)		
	C.coli	33	26(78.8)	0.6	0.9

7.3.2 Multidrug resistance profiles of *Campylobacter* species

The multidrug resistance (MDR) profiles for *Campylobacter* species are shown in Table 7.4. Eleven MDR phenotypes were determined with the resistance EryCipTetAmp, EryCipTetCxm,

and EryCipTet phenotypes being the most common; as they were present in 31 *Campylobacter* species isolates (48.4%). The resistance EryCipTetAmpGenSxt, EryCipTetAmpGen, EryCipTetCxmGenSxt and EryCipTetCxmGen phenotypes were the least common; as they were present in 4 *Campylobacter* spp. isolates (6.3%).

Table 7. 4 Multidrug resistance profiles of *Campylobacter* species

Drug profiles	**No. of MDR resistant *Campylobacter* spp. isolates**		
	***Campylobacter* spp. (n=64) (%)**	***C. jejuni* (n=10) (%)**	***C. coli* (n=33) (%)**
ERY-CIP-TET-AMP-GEN-SXT	4(6.3)	2(20)	3(9.1)
ERY-CIP-TET-AMP-GEN	4(6.3)	2(20)	3(9.1)
ERY-CIP-TET-AMP-STR-SXT	29(45.3)	8(80)	13(39.4)
ERY-CIP-TET-AMP-STR	29(45.3)	8(80)	13(39.4)
ERY-CIP-TET-AMP	31(48.4)	8(80)	14(42.4)
ERY-CIP-TET-CXM-STR-SXT	29(45.3)	8(80)	13(39.4)
ERY-CIP-TET-CXM-STR	29(45.3)	8(80)	13(39.4)
ERY-CIP-TET-CXM-GEN-SXT	4(6.3)	2(20)	3(9.1)
ERY-CIP-TET-CXM-GEN	4(6.3)	2(20)	3(9.1)
ERY-CIP-TET-CXM	31(48.4)	8(80)	14(42.4)
ERY-CIP-TET	31(48.4)	8(80)	14(42.4)

ERY, Erythromycin; CIP, Ciprofloxacin; TET, Tetracycline; CXM, Cefuroxime; GEN, Gentamicin; STR, Streptomycin; SXT, Trimethoprim-Sulphamethoxazole

NB: The profiles were totals for different drug combinations.

7.3.3 Antimicrobial resistance genes

The results of antimicrobial resistance genes for *Campylobacter* species isolated from puppies are presented in Table 7.5. All isolates showing resistance to erythromycin were screened for the *23S rRNA* gene. This gene was detected in 67.7% (42/62) of the *Campylobacter* spp. isolates. The *gyrA* gene was detected in 25% (2/8) of the *C. jejuni* isolates and 26.7% of the *C. coli* isolates. In the tetracycline- resistant *Campylobacter* spp. isolates, the *tet(O)* gene was detected in 80% (43/62) of the *C. jejuni* isolates and 71.9% (23/32) of *C. coli* isolates. The betalactam resistance encoding gene (*blaOXA-61*) was detected in 32.8% (21/64) of the ampicillin and

cefuroxime *Campylobacter* spp. isolates while the *aph-3-1* gene conferring resistance to aminoglycosides was detected in 11.1% (1/9) and 19.6% (11/56) of the gentamicin and streptomycin *Campylobacter* species isolates respectively. Phenotypically resistant but genotypically susceptible isolates were detected for all the drugs while phenotypically susceptible but genotypically resistant isolates were detected for erythromycin, ciprofloxacin, tetracycline, gentamicin, and streptomycin. A subset (5) of the resistance genes were sequenced and revealed a 97-100% homogeneity to those in the GenBank. Figure 7.1 is a representative gel picture of the PCR-confirmed *tet*(O), *gyrA*, *23S rRNA*, and *bla-OXA-61* genes.

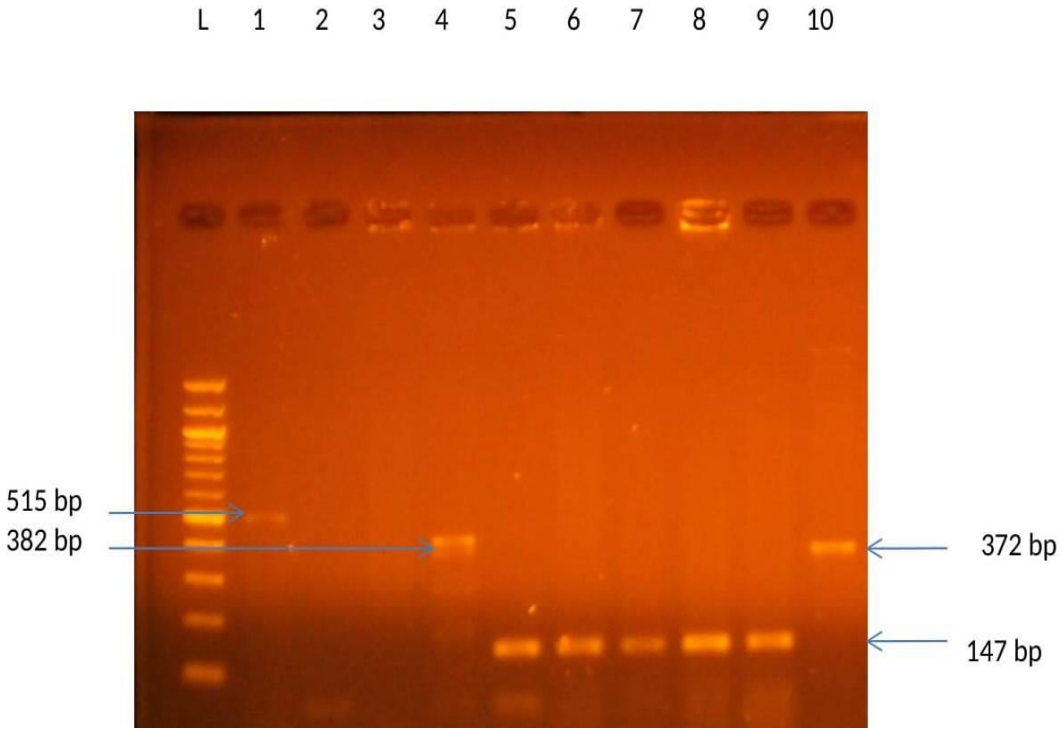

Figure 7. 1 Representative gel picture of PCR-confirmed *tet(O)*, *gyrA*, *23S rRNA*, and *blaOXA-61* genes. Lane M: molecular marker (100 bp); lanes 1: positive *Campylobacter* isolate that harboured the *tet(O)* gene (515 bp); lane 2: negative control ; lane 4: positive *Campylobacter* isolate that harboured the *gyrA* gene (382 bp); lanes 5–9: positive Campylobacter isolates that harboured the *23S rRNA* gene (147 bp); lane 10: positive *Campylobacter* isolate that harboured the *blaOXA-61* gene (372 bp).

Table 7. 5 Molecular patterns of resistance of *Campylobacter* spp., *Campylobacter jejuni, Campylobacter coli*

Antimicrobial agent	Resistance gene	*Campylobacter* spp. isolates	N^P	N^G (%)	P+/G- (%)	P-/G+
Erthromycin	*23S rRNA*	*Campylobacter* spp.	62	42(67.7)	20(32.3)	2
		C. jejuni	10	9(90)	1(10)	n.d.
		C. coli	31	23(74.2)	8(25.8)	2
Ciprofloxacin	*gyrA*	*Campylobacter* spp.	32	10(31.3)	22(68.8)	10
		C. jejuni	8	2(25)	6(75)	1
		C. coli	15	4(26.7)	11(73.3)	4
Tetracycline	*Tet(A)*	*Campylobacter* spp.	62	2(3.2)	60(96.8)	n.d.
		C. jejuni	10	n.d.	10(100)	n.d.
		C. coli	32	2(3.2)	30(93.8)	n.d.
	Tet(B)	*Campylobacter* spp.	62	11(17.7)	51(82.3)	1
		C. jejuni	10	n.d	10(100)	n.d.
		C. coli	32	5(15.6)	27(84.4)	n.d.
	Tet(C)	*Campylobacter spp.*	62	13(21)	49(79)	n.d.
		C. jejuni	10	2(20)	8(80)	n.d.
		C. coli	32	9(28.1)	23(71.9)	n.d.
	Tet(O)	*Campylobacter spp.*	62	43(69.4)	19(30.6)	n.d.
		C. jejuni	10	8(80)	2(20)	n.d.
		C. coli	32	23(71.9)	9(28.1)	n.d.
Ampicillin	*blaOXA-61*	*Campylobacter spp.*	64	21(32.8)	43(67.2)	n.d.
		C. jejuni	10	4(40)	6(60)	n.d.
		C. coli	33	11(33.3)	22(66.7)	n.d.
Cefuroxime	*blaOXA-61*	*Campylobacter spp.*	64	21(32.8)	43(67.2)	n.d.
		C. jejuni	10	4(40)	6(60)	n.d.
		C. coli	33	11(33)	22(66.7)	n.d.
Gentamicin	*aph-3-1*	*Campylobacter spp.*	9	1(14.1)	8(88.9)	11
		C. jejuni	2	n.d.	2(100)	2
		C. coli	6	n.d.	6(100)	2
Streptomycin	*aph-3-1*	*Campylobacter spp.*	56	11(20)	45(80.4)	1
		C. jejuni	10	2(20)	8(80)	n.d.
		C. coli	28	2(7.1)	26(92.9)	n.d.

N^P, No. of phenotypically resistant isolates; N^G, No. of genotypically resistant isolates; P+, phenotype resistant; P-, phenotype susceptible; G+, AMR gene detected; G-, AMR gene not detected; n.d., not detected

7.3.4 Comparison Between Genotypic and Phenotypic AMR Profiles for *Campylobacter* species

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each drug are shown in Table 7.6. Sensitivity and NPV for all the drugs were variable, while specificity and PPV for all the drugs were 50% and greater with the exception of ampicillin and cefuroxime (0% specificity) and gentamicin (8% PPV). There was statistically significant difference ($p<0.05$) observed between the presence of *tet(O)* gene and resistant tetracycline phenotype. Kappa analysis of the agreement between antimicrobial resistant genotypes and their corresponding phenotypes for the *Campylobacter* species isolates is also shown in Table 7.6. The results indicated slight agreement between resistant tetracycline phenotype and the presence of resistance genes, *tet(B)* and *tet(C)*, while no agreement was found between resistant streptomycin phenotype and the presence of the resistance gene *aph-3-1*.

Table 7. 6 Comparison between genotypes and phenotypes of antimicrobial resistance

Antimicrobial agent	Resistance gene	*Campylobacter* species isolates	Se (%)	Sp (%)	PPV(%)	NPV (%)	P-value	Kappa
Erthromycin	*23S rRNA*	*Campylobacter* spp.	68	100	95	100	0.333	0.03
Ciprofloxacin	*gyrA*	*Campylobacter* spp.	31	69	50	50	1	0
Tetracycline	*Tet(A)*	*Campylobacter* spp.	3	100	100	3	0.796	-0.03
	Tet(B)	*Campylobacter* spp.	18	50	92	2	0.25	0.1
	Tet(C)	*Campylobacter* spp.	21	100	100	4	0.468	0.1
	Tet(O)	*Campylobacter* spp.	70	100	100	10	0.04*	-0.1
Ampicillin	*blaOXA-61*	*Campylobacter* spp.	33	0	100	0	-	0
Cefuroxime	*blaOXA-61*	*Campylobacter* spp.	33	0	100	0	-	0
Gentamicin	*aph-3-1*	*Campylobacter* spp.	11	80	8	85	0.527	0.03
Streptomycin	*aph-3-1*	*Campylobacter* spp.	20	88	92	13	0.628	-0.1

*Statistically significant at $p<0.05$

Se, Sensitivity; Sp, Specificity; Positive predictive value (PPV); Negative predictive value NPV). Kappa values ≤ 0 as indicating no agreement and 0.01–0.20 as slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement.

7.3.5 Association between antimicrobial resistance genes and associated-virulence genes in *Campylobacter* species isolates

Association between antimicrobial resistance genes and virulence genes among the *Campylobacter* species isolates is described in Table 7.7. The *flaA* gene was associated with *tet(B)* resistance gene while *ciaB* and *pldA* genes were associated with *23S rRNA* and *tet(O)* resistance genes respectively.

Table 7. 7 Association between antimicrobial resistance genes and associated-virulence genes in *Campylobacter* species isolates (p<0.05)

	Resistance genes							
Virulene gene	*23S rRNA*	*gyrA*	*Tet(A)*	*Tet(B)*	*Tet(C)*	*Tet(O)*	*blaOXA-61*	*aph-3-1*
flaA	1	0.213	0.407	0.027*	0.37	0.645	0.091	0.46
cadF	0.467	0.884	0.561	0.388	0.967	0.141	0.747	0.863
ciaB	0.839	0.38	0.928	0.297	0.953	0.934	0.934	0.03*
iam	0.683	0.683	0.103	0.628	0.127	0.0001	0.651	0.628
pldA	0.041*	0.221	0.587	0.146	0.557	0.191	0.268	0.628
cdtA	0.071	0.971	0.35	0.759	0.559	0.005*	0.193	0.072
cdtB	0.683	0.221	0.103	0.259	0.667	0.524	0.331	0.872
cdtC	0.158	0.675	0.446	0.041*	0.7	0.799	0.144	0.041*

***** Indicates an association between virulence genes and antimicrobial resistance genes

7.4 Discussion

In this study, antimicrobial susceptibility testing was performed for all PCR-confirmed *Campylobacter* species isolates and the resistance genes which are associated with phenotypic drug resistant strains were investigated. A 100% and 93.9% erythromycin resistance was noted among the studied *C. jejuni* and *C. coli* strains respectively. This finding is in contrast to that reported in a previous study by Selwet *et al*. (2015) where no resistance to erythromycin in canine isolates was noted. This high resistance for erythromycin is of concern as it is one of the drugs of choice for clinical treatment of camylobacteriosis (Igwaran and Okoh, 2019).

The level of ciprofloxacin resistance of *Campylobacter* species isolates was at 50%, which may be considered moderate in comparison with varied data from a few reports on ciprofloxacin resistance in canine *Campylobacter* strains (Selwet, 2019; Moser *et al*., 2020; Lemos *et al*.,

2021). In some studies, a low ciprofloxacin level (15%) was noted (Komba, 2018); however, other studies revealed high levels of ciprofloxacin resistance of upto 90.3% (Torkan *et al*., 2018; Murawska *et al*., 2022). Resistance to fluoroquinolones, such as ciprofloxacin, is caused by mutations in the DNA gyrase *gyrA* and *gyrB* regions. (Chatur *et al*., 2014).

A very high level of tetracycline resistance (96.9%) was noted among the studied *Campylobacter* species strains. These observations are in agreement with Gitahi *et al*. (2020) and Nguyen *et al*. (2016) who reported high levels of resistance of 92.9% and 71% human and chicken isolates respectively in Kenya. Tetracycline resistance is associated with the presence of ribosomal protection proteins encoded by *tet* genes (Connell *et al*., 2003). Four tetracycline ribosomal protection proteins were detected in this study: *tet(O)* was found as the main tetracycline resistance determinant at 69.4% followed by *tet(C)* at 21%, *tet(B)* 17.7% , and *tet(A)* at 3.2%. The finding of a high prevalence of the *tet(O)* gene is similar to that in previous studies (Woz´niak-Biel *et al*., 2018; Wysok *et al*., 2020). The *tet(O)* gene occurs in *C. jejuni* and *C.coli* and can be found either on the chromosome or on plasmid (Gibreel *et al*., 2004). The presence of *tet(A), tet(B),* and *tet(C)* has been reported in some tetracycline resistant *Campylobacter* isolates (Abdi-Hachesoo *et al*., 2014; Gitahi *et al*., 2020; Béjaoui *et al*., 2022). The *tet(A)* gene encodes an efflux protein that pumps out tetracyclines out of the cell (Roberts, 2011).

All the studied *Campylobacter* species isolates were resistant to beta-lactams, ampicillin and cefuroxime. The prevalence was higher than reported in previous studies (Aslantaş, 2018, Torkan *et al*., 2018). The observed resistance in this study could be attributed to the widespread use of betalactams or beta-lactam combinations with other antimicrobials for the treatment of infections in dogs. Resistance to both ampicillin and cefuroxime were found to be associated with enzymatic activation of *blaoxa-61* in 32.8% of the isolates. *Campylobacter* species

resistance is not only associated with enzymatic activation of *blaoxa-61*, but also by other resistance mechanisms such as the production of betalactamases (Stones, 2011) or the reduced permeability of the outer wall in *C. jejuni* (Jonker and Picard, 2012), all which could explain the level of resistance noted in this study against these antimicrobials.

A total of 14.1% *Campylobacter* species isolates were resistant to aminoglycoside gentamicin. The finding of low levels of gentamicin resistance is also similar to previous studies (Rahimi *et al*., 2012; Aslantaş, 2018; Torkan *et al*., 2018). The gentamicin resistance was more prevalent in *C. jejuni* (20%) than in *C. coli* strains (18.2%). This finding was varied from those of a study by Yao *et al*. (2017) who reported that gentamicin resistance was much more prevalent in *C. coli* than in *C. jejuni* strains. However, *C. jejuni* and *C. coli* isolates displayed resistance to streptomycin at 100% and 84.8% respectively. Findings that contrast those from previous studies (Giacomelli *et al*., 2014; Marotta *et al*., 2019) that reported low resistance levels to streptomycin in poultry. Resistance to streptomycin is generally due to inactivation of the drugs by aminoglycoside phosphotransferases or adenyltransferases (Iovine, 2013).

Campylobacter species isolates had a high level of resistance to trimethoprim-sulphamethoxazole at 82.8%. This finding is similar to a study by Lengerh *et al*. (2013). The high resistance observed in this study might be due to the widespread use of trimethoprim-sulphamethoxazole for the treatment of bacterial infections in dogs (Clare *et al*., 2014). However, in this study, screening for resistance genes in the phenotypically trimethoprim-sulphamethoxazole-resistant strains was not carried out which is a limitation in this study.

Multidrug resistance (MDR), which is defined as resistance to three or more antimicrobial classes (Schwarz *et al*., 2010), was observed in all the *Campylobacter* strains. In the present

study, a higher percentage of *Campylobacter* species showed MDR phenotypes towards erythromycin, ciprofloxacin and tetracycline (80% for *C. jejuni* and 42.4% for *C. coli*). High rates of MDR phenotypes have previously been described in human and animal origin (Duarte *et al*., 2014; Santos-Ferreira *et al*., 2022).

In this study, there was a slight to no correlation between phenotypic resistance and the presence of known resistance genes to the tested antimicrobials. These findings were discordant with reports by Dahl *et al*. (2021) and Marotta *et* al. (2019). In this study, phenotypically resistant *Campylobacter* isolates were identified however no resistance genes could be detected. Interestingly, phenotypically susceptible but genotypically resistant (false positive) isolates were observed for erythromycin, ciprofloxacin, gentamicin, and streptomycin. These observations were similar with reports by Painset *et al*. (2020). These discrepancies identified in this study may be because of the presence of uncharacterized genes and/or mutations conferring resistance, the inability of detected resistance genes to be fully expressed, or the selection of the incorrect resistance genes to correlate genotype and phenotype (Enne *et al*., 2006; Painset *et al*., 2020; Maunsell *et al*., 2021). Other independent mechanisms of resistance, such as biofilm formation or point mutations, could also explain these findings (Joshua *et al*., 2006; Wang *et al*., 2014).

In this study, there was an association between resistance genes and associated-virulence genes detected in studied puppies. These virulence genes are involved in the colonization and cell invasion as well as toxin production. Similar observations were noted by Lapierre *et al*. (2016) and Gharbi *et al*. (2021). This association suggests that the resistant strains may have invasion and cytotoxicity capacities.

The high frequency of resistance for some antimicrobials observed in this study is a source of concern, given the close contact between puppies and humans (especially children), which offer favourable conditions for transmission of these bacteria by direct contact (e.g. licking, petting), or through contact with the environment (Damborg *et al*., 2016).

7.5 Conclusions and recommendations

This study showed high resistance to antimicrobials used for treatment of *Campylobacter* species infections as well as drugs commonly used in small animal practice. Due to the possible risk of transmission of MDR *C. jejuni* and *C. coli* strains to humans from puppies, there is need for routine surveillance of resistance as well as antimicrobial stewardship in small animal practice.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

The studies on the occurrence of *Campylobacter* spp. in puppies in Kenya are limited and have mainly focused on poultry (Mageto *et al*., 2018, Carron *et al*., 2018). Given the clinical and public health significance of *Campylobacter* species infection in puppies, this study aimed to: 1) determine the prevalence of *Campylobacter* species in puppies and the associated risk factors, 2) characterize the *Campylobacter* spp. isolates to assess their genetic diversity and their potential link with isolates from humans or other animals, 3) determine the prevalence of virulence genes associated with motility, adherence, invasion, and cytotoxicity by using Polymerase chain reaction (PCR), and 4) establish the phenotypic and genotypic antibiotic resistant patterns of the *Campylobacter* isolates towards the drugs used to treat campylobacteriosis, including macrolides, quinolones and tetracyclines as well as drugs commonly used in small animal practice.

In this study, 150 presumptive *Campylobacter* species isolates were identified by conventional culture and biochemical tests yielding a prevalence of 57.7% (150/260). However, polymerase chain reaction (PCR) detected 64 *Campylobacter* species isolates (24.6%, 64/260) by targeting the *16S rRNA* gene specific for these microorganisms. The PCR prevalence is in agreement with the reported range of 8.58 to 75.7% in other studies (Giacomelli *et al*., 2015; Leahy *et al*., 2016; Torkan *et al*., 2018; Ma Socorro Edden and Gil, 2018; Thépault *et al*., 2020; Gharibi *et al*., 2020). The wide range in *Campylobacter* species prevalence among puppies observed in these studies may be attributable to variations in the diagnostic methods, study methodology, management systems, and hygiene practices. The difference between conventional culture and biochemical tests and the PCR results maybe due to the fact that the molecular test is a more

sensitive test as it is uses primers that pick out the DNA of bacterial genes that encode ribosomal RNA (16S rRNA) (Janda and Abbott, 2007). It is recommended that PCR be adopted for detection of *Campylobacter* species during surveillance studies.

This study also provided data on significant factors associated associated with *Campylobacter* species culture and PCR positive status in the studied puppies with some previously reported and other findings in this study. The prevalence of *Campylobacter* species presented in this study may be a reflection of the larger puppy population of Kenya. However, a detailed study involving a larger sample size should be conducted in order to improve the understanding of the epidemiology of this disease and thus facilitate decisions about actions to reduce the risk of this infection in puppies.

Polymerase chain reaction and partial sequencing were used to detect and confirm the presence of *Campylobacter* species isolates in the puppies. The studied puppies were found to be carriers of *C. coli* at 13% (33/260) and *C. jejuni* at 4% (10/260). These results provided new information as previous studies showed *Campylobacter upsaliensis* to be the most common species in dogs (Holmberg *et al*., 2015; Giacomelli *et al*., 2015; Goni *et al*., 2017). However, in this study *C. coli* and *C. jejuni* were the most common. The *C. jejuni* and *C. coli* isolates obtained from puppies in this study had a close relationship to isolates from humans, poultry, cattle, and farm environment as shown by phylogenetic tree analysis. The study suggests the role of puppies as potential reservoirs of *C. jejuni* (cpb gene) and *C. coli* (imp gene). A One Health approach is recommended to further investigate *Campylobacter* species infections due to the close interaction between humans, animals, and the environment.

When compared to other pathogens such as *Escherichia coli*, the pathogenesis of *Campylobacter* species infection is complicated and poorly understood. However, there are reports that suggest the presence of genes involved in motility, colonization, adherence, epithelial cell invasion, and toxin development play an important role in disease development (Wieczorek and Osek, 2013; Lapierre *et al*., 2016). This study found that the isolates of *C. jejuni* and *C. coli* from puppies possessed a variety of different virulence genes associated with motility (*flaA*), adherence (*cadF*), invasion (*ciaB*, *iam*, and *pldA*), and cytotoxicity (*cdtA*, *cdtB*, and *cdtC*). These are the virulence-associated genes that could contribute to their survival and colonization in the puppy gut (Lapierre *et al*., 2016). The virulence genes reported in this study have been previously reported in *Campylobacter* strains isolated from humans (Laprade *et al*., 2016; Oh *et al*., 2017), highlighting the potential virulence of these *Campylobacter* strains in causing human infections. Future studies whether genome based or of experimental disease model disease need to take into account the various complexities of the pathobiology of *Campylobacter* species influencing the gastroenteritis clinical manifestations in both humans and puppies.

The *Campylobacter jejuni* and *Campylobacter coli* isolates from this study were highly resistant to ampicillin, cefuroxime, erythromycin, tetracycline, streptomycin, and trimethoprim-sulphamethoxazole, findings that were in agreement with those reported by previous studies (Lengerh *et al*., 2013; Aslantaş, 2018; Marotta *et al*., 2019; Gitahi *et al*., 2020; Lemos *et al*., 2021). All the *Campylobacter* isolates were found to be resistant to at least three of the antimicrobial classes tested, indicating multidrug resistance (Schwarz *et al*., 2010). Resistance genes corresponding to the tested antimicrobials were identified, however there was little to no correlation between phenotypic resistance and presence of resistance genes. These discrepancies identified in this study may be because of the presence of uncharacterized genes and/or

mutations conferring resistance, the inability of detected resistance genes to be fully expressed, or the selection of the incorrect resistance genes to correlate genotype and phenotype (Murawska *et al*., 2022). Nonetheless, the presence of most of the screened antimicrobial resistance genes suggests that *C. jejuni* and *C. coli* puppy isolates could serve as reservoirs for the horizontal gene transfer of these resistance genes to several intestinal pathogens (Béjaoui *et al*., 2022). Further screening of resistance genes and intrinsic mechanisms encoding for resistance to *Campylobacter* species should be considered in order to gain a better understanding on the genetic basis of antimicrobial resistance of these isolates.

8.1. Conclusions

From the findings of this study, it can be concluded that:

1. *Campylobacter* jejuni and *Campylobacter coli* are present in puppies in the Nairobi Metropolitan Region, Kenya.
2. Puppies kept as pets and deworming status were significantly associated with *Campylobacter* culture positive status while puppies from shelters, from kennels that are washed daily as well as those treated with antibiotics in the past month were significantly associated with *Campylobacter* PCR positive status. Occurrence of vomiting was significantly associated with both culture and PCR *Campylobacter* species positive status.
3. The occurrence of high rates of virulence genes indicate potential pathogenicity of *Campylobacter* species isolates.
4. *Campylobacter* species showed high resistance rates to critically important antimicrobials and are MDR.
5. Resistance to gentamicin was low.

8.2 Recommendations

Based on the findings of this study, the following can be recommended:

1. Breeders and puppy owners should be made aware of *Campylobacter* risk of puppies inorder to reduce the transmission from the puppies to humans especially children.
2. A detailed study involving a larger sample size should be conducted in order to improve the understanding of the epidemiology of this disease and thus facilitate decisions about actions to reduce the risk of this infection in puppies.
3. A One Health approach is recommended to further investigate *Campylobacter* species infections due to the close interaction between humans, animals, and the environment.
4. Whole genome sequencing should be done for further characterization of *Campylobacter* species isolates in order to assess the clonal diversities of the isolates.
5. Phylogenetic analysis of *Campylobacter* species isolates from diverse hosts in Kenya should be done to ascertain genetic relatedness of the isolates.
6. Further screening of resistance genes and intrinsic mechanisms encoding for resistance to *Campylobacter* species should be considered in order to gain a better understanding on the genetic basis of antimicrobial resistance of these isolates.
7. Continuous monitoring of *Campylobacter* species isolates susceptibility in combination with the judicious use of antimicrobials in small animal practice will be of great importance for public health and gentamicin should be reserved for treatment as it has low resistance.

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Appendix 1 Questionnaire

Date (day/month/year) ___/___/____

Name (Interviewee) _____

Physical address _____

PUPPY INFORMATION

Name _____

Sex _____

Age _____

Breed _____

Neuter status Entire Neutered

Please tick where appropriate and explain in the field 'other'

1. What is the reason for keeping the puppy?

a) □ Pet

b) □ Security

c) □ Breeding

d) □ Other _____

2. How was the puppy bred?

a) □ Artificial Insemination

b) □ Natural mating

3. i) What is the vaccination status of the puppy?

a) □ Upto date

b) □ Vaccinated once

c) □ Never been vaccinated

d) □ Don't know

ii) If vaccinated, which vaccine?

e) □ Parvovirus

f) □ Rabies

g) □ DHLP-R

h) □ Other _____

4. i) What is the deworming status of the puppy?

a) □ Upto date

b) □ Dewormed atleast once

c) □ Never been dewormed

d) □ Don't know

ii) If dewormed, which dewormer is used?

a) □ Pyrantel

b) □ Fenbendazole

c) □ Praziquantel

d) □ Ivermectin

e) □ Other

5. i) Any history of illness?

□ Yes □ No

ii) What signs did the puppy exhibit?

a) □ Vomiting

b) □ Diarrhea

c) □ Vomiting/Diarrhea

d) □ Other significant signs

6. What type of feed do you give your puppy?

a) □ Commercial food

b) □ Homemade food

c) □ Food for humans

d) □ Other _____

7. i) What type of housing do you have?

a) □ Individual (alone in compound/kennel)

b) □ Group (two or more in compound/kennel)

ii) What is the nature of the housing?

a) □ Kenneled throughout

b) □ Kenneled during the day and freed at night

c) □ Never kenneled

d) □ Roams outside but comes back into compound

e) □ Roams outside but does not come back into compound

8. Are there any other animals in the compound?

a) □ None

b) □ Cows

c) □ Poultry

d) □ Sheep/goats

e) □ Adult Dogs/ Cats

9. i) How often do you wash the kennels?

a) □ Daily

b) □ Other

ii) What do you use?

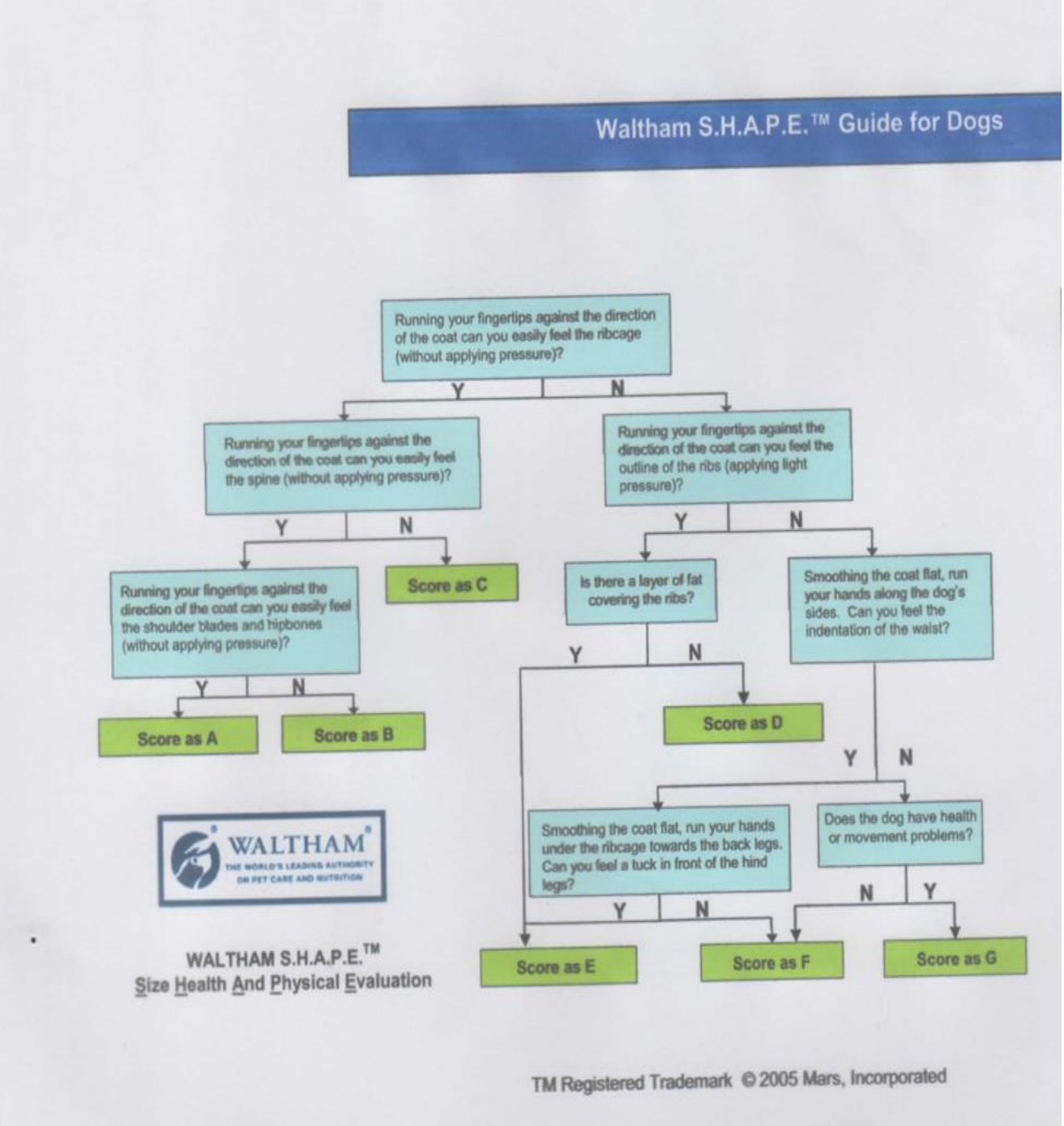
a) □ Soap and water

b) □ Disinfectant and water

c) □ Bleach

d) □ Plain water

Appendix 2 Waltham Size, Health and Physical Examination (SHAPE) Score™



Appendix 3 Canine Inflammatory Bowel Disease Activity Index (CIBDAI)

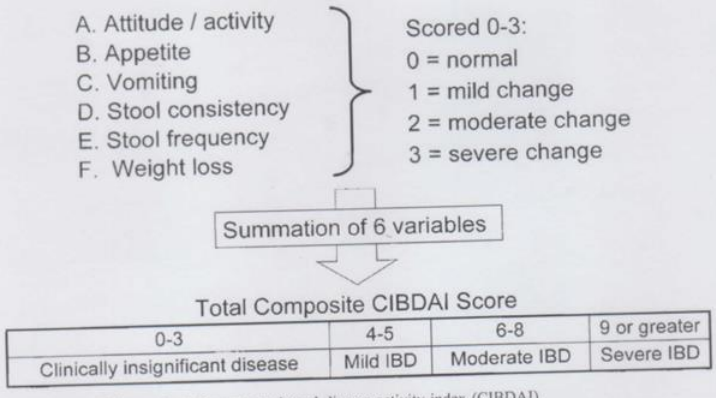


Fig 1. Criteria for assessment of the canine inflammatory bowel disease activity index (CIBDAI).

source origin that were free of gastrointestinal signs. Furthermore, control dogs were judged to be healthy on the basis of normal results on physical examination, CBC, serum biochemistry, urinalysis, multiple fecal examinations, and dirofilarial antigen assay.

Canine IBD Activity Index

Clinical disease activity was assessed by a simple numeric scoring system termed "the canine IBD activity index" (CIBDAI). Scoring criteria were modified in pilot studies until clinicians found an index that was repeatable and that correlated well among investigators. Under this system, 6 salient gastrointestinal signs were scored 0–3 by the gastroenterologist on the basis of the magnitude of their alteration from normal in a given IBD patient. Each parameter of the CIBDAI was assessed independently of the others as an average over time since the clinical signs had developed. Calculations of CIBDAI variables were as follows: (1) attitude/activity (0 = normal, 1 = slightly decreased, 2 = moderately decreased, and 3 = severely decreased); (2) appetite (0 = normal, 1 = slightly decreased, 2 = moderately decreased, and 3 = severely decreased); (3) vomiting (0 = none, 1 = mild [1 time/wk], 2 = moderate [2–3 times/wk], and 3 = severe [>3 times/wk]); (4) stool consistency (0 = normal, 1 = slightly soft feces or fecal blood, mucus, or both, 2 = very soft feces, and 3 = watery diarrhea); (5) stool frequency (0 = normal, 1 = slightly increased [2–3 times/d], 2 = moderately increased [4–5 times/d], and 3 = severely increased [>5 times/d]); and (6) weight loss (0 = none, 1 = mild [<5% loss], 2 = moderate [5–10% loss], and 3 = severe [>10% loss]). These scores then were summed, yielding a total cumulative CIBDAI score that reflected clinically insignificant disease or the presence of mild, moderate, or severe IBD (Fig 1).

Laboratory Analysis of Serum Acute-Phase Proteins

Serum acute-phase proteins (APPs) were measured by canine-specific commercial test kits or validated assays performed at research institutions.[20,a,b] The APPs evaluated included CRP, measured by enzyme-linked immunosorbent assay (ELISA) with canine CRP as a standard; haptoglobin (HAP), measured by a hemoglobin-HAP binding assay with bovine HAP as a standard; AGP, measured by radioimmunodiffusion with canine AGP as a standard; and serum amyloid A (SAA), measured by ELISA with canine SAA as a standard.

Histologic Examination of Endoscopically Obtained Biopsy Specimens

Multiple (10–15 per organ evaluated) mucosal biopsy specimens were obtained endoscopically from the stomach, small intestine, or large intestine of diseased dogs for microscopic review. Dogs having upper gastrointestinal signs (eg, vomiting, small bowel diarrhea, anorexia, and weight loss) underwent esophagogastroduodenoscopy, whereas dogs having only lower gastrointestinal signs (eg, tenesmus, hematochezia, mucoid feces, and frequent defecation) had a full colonoscopy performed to the cecum. Both upper and lower endoscopic examinations were performed in dogs having mixed signs of enterocolitis. Endoscopic lesions of increased granularity, increased friability, mucosal erosions, or some combination of these findings were observed in 52% of the IBD dogs. In these instances, multiple biopsy specimens were obtained directly from these lesions as well as from normal-appearing mucosa. Multiple biopsies from the gastric body, duodenum, or ascending, transverse, and descending colonic regions were obtained from all other dogs with IBD that had endoscopically normal mucosa. Mucosal specimens also were obtained from the stomach, small intestine, and colon of each control dog.

Histologic examination of all tissues was performed by a single pathologist (YN) who objectively graded endoscopic specimens and assigned a histologic lesion severity score for each dog. No information regarding history, clinical signs, or endoscopic observations was made available to the pathologist. A histologic grading system based on the extent of architectural disruption and mucosal epithelial changes was used.[3,b] In this scheme, mild IBD lesions were those with no mucosal disruption, glandular necrosis, immaturity (eg, delayed maturation or differentiation), or fibrosis of the lamina propria. Severe IBD was characterized by architectural distortion of the mucosa (extensive ulceration; necrosis; villus atrophy, fusion, or collapse; and glandular loss or severe glandular hyperplasia or fibrosis of the lamina propria). Moderate lesions were characterized by microscopic changes that varied in severity between these 2 extremes. No attempt was made to quantitate the number of inflammatory cells in the lamina propria of biopsy specimens. Rather, inflammatory cell types were included in the pathologist's report by means of standard classification schemes, including lymphocytic-plasmacytic, eosinophilic, suppurative, and granulomatous gastroenterocolitis based on the predominance of the cellular infiltrate.

Experimental Design and Data Collection

During an 18-month period (1999–2001), inflammatory activity (expressed as a numeric CIBDAI score) was evaluated prospectively in

Appendix 4 List of publications

1. Mbindyo SN, Kitaa JMA, Aboge GO, Abuom TO and Mulei CM, 2021. A review of Campylobacter infections in dogs. International Journal of Veterinary Science 10(4): 259-266. https://doi.org/10.47278/journal.ijvs/2021.057

2. Mbindyo SN, Kitaa JM.A, Abuom TO, Aboge GO, Muasya DW, Muchira BW, Gitahi N andMatiku CM, 2022. Prevalence and risk factors of Campylobacter species infection of puppies in the Nairobi Metropolitan Region, Kenya. International Journal of Veterinary Science 12(3): 389-394.

Appendix 5 Accession numbers

Identification	Isolate number	Accession number
Campylobacter coli	13a	SRR23609455
	131c	SRR23609458
	138d	SRR23609456
Campylobacter jejuni	119a	SRR23609462
	145c	SRR23609453
FlaA	125c	SRR23609409
Iam	127b	SRR23609406
CiaB	29d	SRR23609415
23S rRNA	12	SRR23609461
GyrA	125c	SRR23609439
TetB	201a	SRR23609427
TetO	15c	SRR23609444
BlaOXA-61	3c	SRR23609402
Aph-3-1	145c	SRR23609405

NB: These accession numbers will be released to the public in October 2023.