EPIDEMIOLOGY OF THERMOPHILIC CAMPYLOBACTER IN HUMANS, CHICKENS AND WATER IN MOROGORO, TANZANIA.

BY

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ABSTRACT

An epidemiological study on thermophilic *Campylobacter* spp in chickens, humans and water was carried out in urban and rural areas of Morogoro, Tanzania. The objectives of the study were to establish the prevalence of thermophilic campylobacter in humans, chickens and water in Morogoro, Tanzania and utilize polymerase chain reaction analysis as the definitive identification method of thermophilic campylobacters. *Campylobacter* spp were isolated in 70% of the 536 cloacal swabs and intestines examined in local and broiler chickens sampled from urban and rural areas. *C. jejuni* was identified in 91.2% of the 374 campylobacter positive samples while *C. coli* was identified in 33 (8.8%). *C. lari*, was not isolated in this study. Local chickens in rural areas had a significantly higher campylobacter prevalence rate (76%, n=223) than in urban areas (60%, n=112) ($p<0.05$). This was thought to be due to scavenging nature of rural local chickens. Of the 622 human with gastrointestinal problems screened for *Campylobacter* spp, 59 (9.3%) were positive. 96.6% of all the isolates were *C. jejuni* and the rest (3.4%) were *C. coli.* The frequency of isolation of *Campylobacter* spp was significantly ($p<0.001$) higher in children (16%, n=175) than in adults (7%, n=475) showing age predisposition. The study further observed that *C. coli* was only isolated from one child and one single adult patient indicating how rarely does this species infects humans in Morogoro. 312 (7.4%) males compared to 320 (7.1%) female were positive for *Campylobacter*. However, this difference was not significant ($p>0.05$), therefore sex was not a risk factor for *Campylobacter* infection. Assessment of location of residence also showed that there was no significant difference between proportion of rural human infected with campylobacter (11%, n=134) and those

in urban areas (9%, n=498) (P>0.05). Thermophilic *Campylobacter* spp were not isolated from any of the water samples from urban and rural Morogoro studied (n=146) although the risk factors for water contamination were obvious. This warrants further investigation. Definitive identification of *C. jejuni* isolates by polymerase chain reaction (PCR) revealed that 74.1% of the 243 isolates were positive and 25.9% were negative. Positive isolates to biochemical tests turning negative to PCR poses a challenge to biochemical identification methods for *C. jejuni* and calls for further studies utilizing PCR able to detect all species of thermophilic *Campylobacter* group.

DECLARATION

I, Hezron Emmanuel Nonga do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has not been submitted for a degree award at any other University.

Signature:.....

Date:13/07/2025

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DEDICATION

This work is dedicated to my wife Elizebeth Mpejiwa Daudi for her patience during the period of my study.

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ABREVIATIONS AND SYMBOLS

Abbreviation	Descriptive meaning
%	Percent
µ	Micro-10^{-6}
µm	microlitre
®	registered trade mark
<	less than
>	greater than
µg/ml	microgram per litre
AIDS	acquired immuno-deficiency syndrome
bp	base pair
CFU/ml	Colon forming unit per millitre
CI	confidence interval
cm	centimeter(s)
DNA	deoxyribonucleic acid
dNTP's	deoxyribonucleoside phosphate
e.g.	Example
et al.	and others
EDTA	ethylenediaminetetra acetic acid
ELISA	enzyme linked immuno-Sorbent assay
g	Gramme
HCL	hydrochloric acid

HIV	Human immunodeficiency virus
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
i.e.	that is
IU	international unit
Kg	kilogram(s)
KCl	Potassium chloride
Km	Kilometer
Ltd	Limited
M	Molar (concentration)
Mg	Milligram
mg/l	milligram per litre
ml	Millitre
MM	Millimole
mm	Millimeter
n	number of samples
^{o}C	degree Celsius
p	probability value (for statistical significance)
PCR	polymerase chain reaction
pH	hydrogen ion concentration
pmoles	Picomoles

RAPD	restriction amplified polymorphic DNA
REA	restriction enzyme analysis
RFLP	restriction fragment length polymorphism
rpm	revolution per minute
RR	risk ratio (for statistical significance
RNA	Ribonucleic acid
SUA	Sokoine University of Agriculture
TBE	tris-HCL, boric acid and EDTA
TEMED	N,N,N',N',-tetramethylethylenediamine
U	Unit
UV	ultra violet
V	Volts
v/v	volume by volume
V/w	volume by weight
WHO	World Health Organization
X	Times

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

Campylobacteriosis is a collective description for infectious diseases in humans and animals caused by members of the bacterial genus *Campylobacters.* In humans, it is a food- and water-borne disease characterized by acute gastroenteritis and sometimes associated with severe systemic diseases and is associated with Guillan Barre's Syndrome (GBS) (Jackson *et al.*, 1997; Dingle *et al.*, 2000; Nachamkin *et al.*, 2000; Yolanda *et al.*, 2002). Thermophilic campylobacters namely *Campylobacter jejuni, C. coli* and *C. lari* are the major causes of human campylobacter enteritis. Although, *Campylobacter upsalinensis* is not in the group of thermophilic campylobacters, it is frequently isolated from children with diarrhoea (Lastovica and Skirrow, 2000). Campylobacters (i.e. *campylos=curved* and *bacter=rod*); are a group of tiny strictly micro-aerophilic spirally curved, gram-negative pleomorphic motile bacteria formerly classified as vibrios but closely related to spirilla (Sebald and Veron, 1963; Carter and Chengappa, 1991). Campylobacters were first associated with human illness since 1970s, but had been known by veterinarians since 1913 for causing diseases in domestic animals (Skirrow and Butzler, 2000; Mareno *et al.*, 2002).

Reports have shown that children, old and immunocompromised individuals are mostly affected by the disease (Sorvillo *et al.*, 1991; Jackson *et al.*, 1997; Floreani *et al.*, 2002; Coker *et al.*, 2002). Recent studies have shown that the incidence of human campylobacteriosis is even higher than that of salmonellosis and shigellosis,

the commonly famous bacterial food borne diseases worldwide (Koenraad *et al.*, 1997; Frost, 2001; Coker *et al.*, 2002).

Campylobacteria demonstrate a considerable ecological diversity and they can be isolated from food producing animals, wild animals, birds, pet animals as the normal flora and from environments (On, 1996; Sandberg, 2002). Prevalence studies of the bacteria have shown that avian spp in particular poultry are the frequently colonized warm blooded animals (Yogasundram *et al.*, 1989), and campylobacters species have been isolated from chicken, turkeys, ducks, quail and pigeons. Free range chickens have been found to have high prevalence of up to 97.1% due to considerable exposure to carrier insects, vermin and animal excreta (Saleha *et al.*, 1998; Harvey *et al*, 2003). The prevalence of free-range domestic poultry has been incriminated for an increase of children's exposure to *C. jejuni* in the tropical countries (Marquis *et al.*, 1990). Chickens under intensive management system are at higher risk of rapid spread of the bacteria to all birds once introduced in the flock and may be facilitated by contaminated old litter, feed, water, walls and surfaces around the house (Genigeorgis *et al*, 1986; Stern, 1992).

In the transmission cycle of the thermophilic campylobacters to humans, contaminated drinking water accounts for a number of sporadic infections and large outbreaks of the human campylobacteriosis worldwide (Mentzing, 1981; Palmer *et al.*, 1983; Koenraad *et al.*, 1997; Dingle *et al.*, 2000; Jones, 2001; Diergaardt *et al.*, 2004). Outbreaks, involving up to 3500 individuals, have been related to drinking of untreated or inadequately chlorinated water (Vogt et al., 1982).

In waterborne epidemics associated with campylobacters, the drinking water source has been shown to be fecally contaminated either by runoff of surface water after rain or by leakage of a sewage pipe close to the drinking water pipeline (Marja-Liisa *et al.*, 2003). The prevalence and dynamics of the bacteria in water obtained from the lakes, rivers, dams and wells is usually associated with contamination from animals and humans faeces, water and biological wastes from slaughter plants drained to water sources by rainfall (Marja-Liisa *et al.*, 2003). The isolation frequency and contamination level may differ based on the type of water sources, water purity and season (Obiri-Danso and Jones, 1999). Although numerous methods are available for isolation of campylobacter, there are problems concerning detection of campylobacters in water. This study used the filtration and selective pre-enrichment to enhance recovery of campylobacters from environmental water samples as described by Tenover and Fennell, (1992).

The pathogenicity in humans and animals makes campylobacters important from clinical and economic perspectives as it has been reported in developed and developing countries (Coker *et al.*, 2002). Meeta and others (2001) reported an increase of human campylobacteriosis in England and Wales by 66% in one decade. High infection rates have also been reported in the USA, Netherlands, Belgium, Spain and other European countries (Nortemans *et al*, 1992; Fitzgerlad *et al.*, 2001; Schlundt, 2002). Reports of the disease in humans from the underdeveloped countries have shown the prevalence of campylobacter diarrhoea ranging from 5% to 20% (Tauxe, 1992; Oberhelman and Taylor, 2000). In the UK, treatment cost per case of campylobacteriosis in human is up to £273 (about USD 480). The loss

of productivity with respect to human resource for work and the cost of control of the bacteria have not been estimated but is relatively higher (Skirrow and Blaser, 1992).

Surveillance and control of diseases of public health importance in developing countries have focused on diseases such as malaria, tuberculosis, trypanosomosis, onchocerciasis, cholera and schistosomiasis (Lambrechts *et al.*, 1999). Though limited information is available in human campylobacteriosis from African countries, there have been reports on isolation from Ethiopia, Kenya, Algeria, Cameroon, Zimbabwe, South Africa, Nigeria and Tanzania (Asrat *et al.*, 1997; Jiwa *et al.*, 1997; Coker *et al.*, 2002). However, national surveillance programs for campylobacteriosis generally do not exist in most developing countries despite the substantial burden of disease.

Campylobacteriosis may be diagnosed by either conventional or molecular techniques. Bacterial culture is considered to be the 'gold standard' for routine isolation of campylobacters (Sandberg, 2002). Serology is another method of diagnosis, which gives the seroprevalence of the disease. It is also a useful tool of serotyping based on heat stable (HS) and heat labile (HL) antigens as well as phage typing and biotyping (resistotyping). Biochemical tests also identify campylobacters phenotypically and enhances biotyping (Bolton *et al.*, 1984). Though phenotypic typing provides large numbers of isolates, their general applicability is limited by difficulty of obtaining standard antisera and phage reagents, and lack of standardization of protocols between laboratories (On, 1996; Owen and

Leeton, 1999). The advents of molecular biology techniques in recent years are reported to be accurate and precise for diagnosis, genotypic identification and typing methods for campylobacters (Nachamkin *et al.*, 1993; Nachamkin *et al.*, 1996). In this study, the biochemical tests were used for preliminary identification of thermophilic campylobacters and PCR analysis was used as a definitive isolate identification method.

In Tanzania, several studies on *Campylobacter* spp. in animals and humans have been conducted and showed the occurence of such pathogens. Studies on thermophilic *Campylobacter* spp in commercial chickens, ducks, turkeys and goats have been reported elsewhere in Tanzania (Kazwala *et al.*, 1992a; Jiwa *et al.*, 1997). However, the prevalence of these pathogens in chicken and water in Morogoro district is still unknown. More so, despite the potentially significant health hazard posed by waterborne *Campylobacter spp*, data regarding their occurrence and prevalence in drinking and environmental water sources in warmer, developing countries like Tanzania is lacking. For example, Lindblom and other workers (1995) reported a prevalence of 18% of *Campylobacter* spp. (*C. jejuni* and *C.coli*) in humans in Dar es Salaam. These studies mentioned above gave some baseline data on *Campylobacter* spp. in humans and animals for use in further studies of the disease in Tanzania. Nonetheless, studies regarding the zoonotic implications of these pathogens and the transmission dynamics of the disease in the country have not been conducted.

The previous studies on *Campylobacter* spp. in Tanzania were conducted using conventional (phenotypic) methods of bacterial identification, which are sometimes cumbersome and inaccurate. The difficulties with phenotypic identification of campylobacters sometimes are due to their fastidiousness, asaccharolytic nature and possess few distinguishing phenotypic characteristics (Goosens and Butzler, 1992). The difficulties associated with biochemical identification methods make these organism ideal candidates for PCR as the definitive identification methods in the present epidemiological study in Tanzania. However, the definitive campylobacter identification using molecular techniques in Tanzania has not been done though they are relatively easy to use, rapid and have higher discriminatory power (Waegel and Nachamkin, 1996).

1.2 Objectives

1.2.1 Main objective

To establish the magnitude of thermophilic campylobacter in humans, chickens and water in Morogoro utilising molecular biology methods.

1.2.2 Specific objectives

(i) To determine the prevalence of thermophilic campylobacters in humans in selected areas.

(ii) To determine the prevalence of thermophilic campylobacters in local and commercial chickens.

(iii) To compare the use of cloacal swabs and intestinal contents in the isolation of thermophilic campylobacters in chickens.

(iv) To determine the occurence of thermophilic campylobacters in water.

(v) To use polymerase chain reaction analysis as the definitive identification method of thermophilic campylobacters.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition of the disease

Campylobacteriosis caused by thermophilic *Campylobacter* spp. is an infectious disease known to cause gastroenteritis in humans worldwide. neurological disorders such as Guillain Barre Syndrome (GBS) and Miller- Fisher syndrome are important post infection complications (Nachamkin, 2002). Almost all warm blooded mammals and the avian species habour the potentially pathogenic species of campylobacter as normal flora and a potential source of these pathogens and thereby are a threat to human health (Nachamkin and Blaser, 2000). Therefore, thermophilic Campylobacters may be regarded as food - and water – borne zoonotic organisms.

2.2 Actiologic agent

The groups of campylobacters, which are thermotolerant, are the major cause of the disease. Thermophilic campylobacters comprise three species namely *Campylobacter jejuni, Campylobacter coli* and *Campylobacter lari.* Of the three species, *C. jejuni* is the frequently isolated species from humans with campylobacterial diarrhoea (Blaser, 1997) and accounts up to 90% of all human cases of campylobacteriosis (Koenraad *et al.*, 1995). Moreover, *Campylobacter jejuni* is recognized as the most cause of the diseases preceeding to Guillain-Barre's Syndrome (GBS) in humans (Nachamkin, 2002). GBS currently is the leading cause of flaccid paralysis after the eradication of polio in developed countries (Nachamkin, 2002). *Campylobacter jejuni* has also been implicated in a number of conditions affecting the animal kingdom, such as diarrhoea in dogs, cats, zoo animals, calves and horses (McOrist, 1986; Sandberg, 2002; Aquino *et al.*, 2002; Misawa *et*

al., 2002) abortion in sheep and cows (Welsh, 1984; Skirrow and Butzler, 2000), mastitis in cows (Morgan *et al.*, 1985; Misawa *et al.*, 2002) and avian infectious hepatitis (Clark and Bueschkens, 1988; Carter and Chengappa, 1991).

Campylobacter jejuni and *Campylobacter coli* acquired their names from the pathological changes they cause on specific sites of the intestinal tract of cattle and pigs, respectively. *C. jejuni* causes enteritis on the jejunal mucosa of calves hence its name while *C. coli* causes colitis leading to dysentery in hogs (Jones *et al.*, 1991). The distinction between the two is achieved by hippurate hydrolysis test described by Harvey (1980) and Skirrow and Benjamin (1980) whereby *C. jejuni* hydrolyses sodium hippurate while *C. coli* does not. The *C. lari* was formerly known as nalidixic acid resistant thermophilic campylobacters (NARTC), and was first identified by Skirrow and Benjamin (1980). Leaper and Owen (1982) later renamed it as *Campylobacter lari* since the organism was frequently been isolated from seagulls of the genus *Larus*.

2.3 Classification

According to On, (1996), Nachamkin, (1997) and Blaser, (1997) campylobacters belong to the family Campylobacteraceae with the three closely related genera, Campylobacter, Arcobacter and Helicobacter.

Table 1. Current classification and pathogenicity of the genera *Campylobacter*, *Arcobacter* and *Helicobacter* in the family Campylobacteraceae

Known pathogenicity for humans	Reported but with unknown pathogenicity for humans	No known pathogenic role in humans
Campylobacter jejuni subspecies *jejuni*	*Campylobacter jejuni* subspecies *doylei*	*Campylobacter fetus* subspecies *venerialis*
Campylobacter coli	*Campylobacter hyointesinalis* subspecies *hyointesinalis*	*Campylobacter hyointesinalis* subspecies *lawsonii*
Campylobacter lari	*Campylobacter curva*	*Campylobacter hyoilei*
Campylobacter fetus sub species *fetus*	*Campylobacter rectus*	*Campylobacter helveticus*
Campylobacter upsaliensis	*Campylobacter concisus*	*Campylobacter showae*
Arcobacter butzleri	*Campylobacter mucosalis*	*Campylobacter nitrofigilis*
Arcobacter cryaerophila	*Campylobacter gracilis*	
Arcobacter skirrowi	*Campylobacter sputorum*	
Helicobacter cinaedi		
Helicobacter fenneliae		

Source: Nachamkin, 1997; Blaser, 1997

Campylobacter jejuni, C. coli and *C. lari* form a group of thermophilic campylobacters out of the 19 species and subspecies within the family (Vandamme and De Ley, 1991; Vandamme *et al.*, 1991; Nachamkin, 1995). Campylobacteraceae can further be classified into those that are known human pathogens, those that are possibly pathogenic, and those without known human pathogenicity (Table I).

2.4 Growth requirements of thermophilic Campylobacters

Thermophilic campylobacters are typically microaerophilic and requires concentration of oxygen between 3-15%, carbondioxide 3.5-10% and nitrogen 85% in microaerobic gas generator packages, tris gas incubator or anaerobic candle jars (Luechtefeld *et al.*, 1982; Wang *et al.*, 1983; Morris and Patton, 1985; Thompson *et al.*, 1990). The samples should be incubated for 48 to 72 hours at 42°C to 43°C and pH range of 6.5 – 7.5 for optimal recovery (Holt *et al.*, 1994). Bacterial culture is usually done on either solid media or enrichment broth. A number of selective media can be used for isolating the thermophilic campylobacters. They include blood-containing media (Skirrow medium) (Skirrow, 1977), Blaser campylobacter blood agar formulation (Blaser Campy BAP) (Blaser *et al.*, 1979) and campy-cefoperazone-vancomycin-amphotericin medium (Campy-CVA), Preston and Butzler's (Reller *et al.*, 1983). The blood free media includes, modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA), (Bolton *et al.*, 1984; Hutchinson and Bolton, 1984), charcoal based selective medium (CSM) (Karmali *et al.*, 1986) and the blood free semisolid motility medium (SSM) (Goossens *et al.*, 1989). Most of the recommended selective media have one or more antimicrobial agents, mainly cefoperazone, as a primary inhibitor of enteric flora. Most of these media contain growth supplements and antifungal components to inhibit contamination by fungi.

Enrichment methods are designed to isolate *Campylobacter* organisms from samples containing low numbers of organisms. A number of enrichment broths are used to recover *Campylobacter* organisms from samples. These include Preston enrichment broth (Bolton and Robertson, 1982; Ribeiro and Prince, 1984),

Campylobacter enrichment broth (Bolton *et al.*, 1984), Doyle and Roman, Camp Thio broth (Blaser *et al.*, 1979) and other formulations (Doyle and Roman, 1982; Sjogren *et al.*, 1987; Korhonen and Martikainen, 1990).

2.5 Identification

2.5.1 Micromorphology

Campylobacter spp and related bacteria are gram negative, vibrioid or bacilli cells with a single polar unsheathed flagellum at one or both ends (Figure 1). They are pleomorphic with various shapes including a curved, slender *S*-shaped or spiral rod with a size of 0.2 – 0.5μm wide and 0.5 – 5 μm long. They are non-spore formers, non-acid fast and they stain weakly and this necessitates higher concentrations of staining reagents. On wet smear and under dark field microscope, campylobacters appear as active, motile cells with a characteristic darting and cork screw-like motion, which is aided by a single polar flagellum at one end or both ends of the cells. (Smibert, 1978; Carter and Chengappa, 1991; Holt *et al.*, 1994). When the bacteria are stressed especially old culture of more than 48 hours, undernourished or exposed to normal atmospheric conditions they develop a coccoidal morphology which are regarded as degenerative forms of the bacteria (Karmali *et al.*, 1981; Moore, 2001; Park 2002). The coccoid forms are the minicells formed from the fragmentation of the flagellae and poles of the cells at a late cycle of growth (Brock and Murray, 1987). This form is known as viable but non-culturable state (VBNC), meaning that the organism is unable to grow on media that normally support growth (Rollins and Colwell, 1986). In this state, the organisms still exhibit metabolic activities and are thought to be capable of causing infection. Some trials have shown that oral administration of VBNC to mice or chicks lead to campylobacter

infection (Jones *et al.*, 1991; Stem *et al.*, 1994), although this capability is strain dependant.

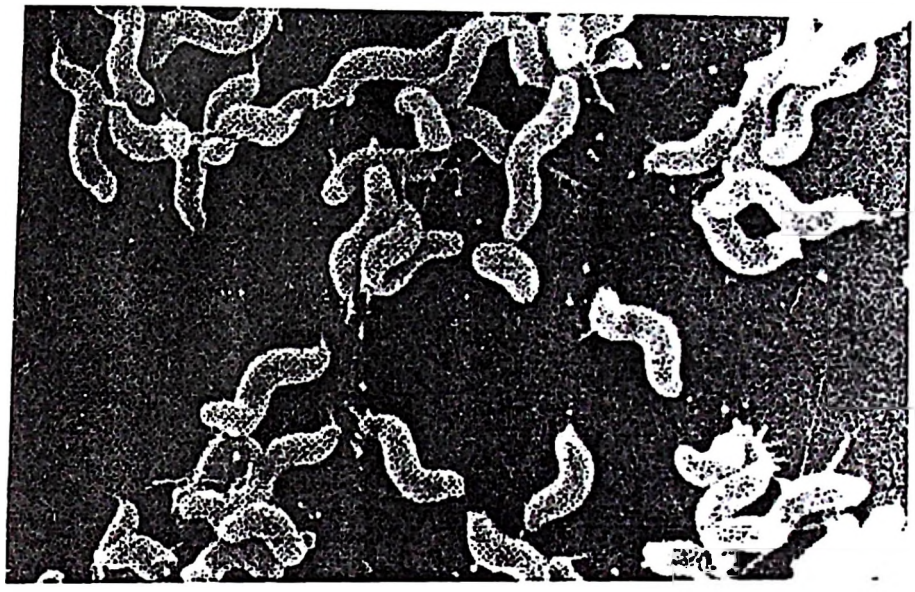

Figure 1. Micromorphology of *Campylobacter jejuni.*

2.5.2 Macromorphology

The colonial morphology of campylobacters shows some variation within each species (Smibert, 1978). Thermophilic *Campylobacter spp.* colonies on solid media are low convex or rather flat, grayish in colour and translucent in appearance measuring 2-4 mm in diameter (figure 2). The colonies are mucoid, sticky and slippery on media making it difficult to take a loopful of colonies for different purposes. The colonies have a swarming appearance and when on moist fresh agar that tend to coalesce and may extend along the line of inoculation (Skirrow and Benjamin, 1980). When the culture stays for more than 48 hours or when exposed to atmospheric conditions appear as metallic sheen (Holt *et al.*, 1994).

Figure 2. Colonies of thermophilic *Campylobacter* spp. on mCCDA.

2.5.3 Biochemical tests and biotyping

All thermophilic campylobacters are oxidase and catalase positive, negative to urease, methyl red and Voges Proskauer tests and do not ferment carbohydrates (Holt *et al.,* 1994). *C. jejuni* strains hydrolyse sodium hippurate while *C. coli* and *C. lari* do not. Recent reports have shown that about 10% of *C. jejuni* isolates do not hydrolyse sodium hippurate (Wallace, 1997). *C. coli* hydrolyses nitrate to nitrite, a test, which differentiates them from *C. jejuni,* and *C. lari*, which are negative. Again, *C. laridi* is nalidixic acid resistant unlike *C. jejuni* and *C. coli*, which are sensitive (Skirrow and Benjamin, 1980). Through biochemical tests, *C. jejuni* strains were divided into biotypes 1 and 2 depending on their ability to produce hydrogen sulphide in a medium containing ferrous sulphate, sodium metasulphite and sodium pyruvate (Skirrow and Benjamin, 1980).

2.5.4 Serology and serotyping

There are a number of serological techniques used in identification of campylobacters. The immune response to campylobacter infection is similar to that of other infectious diseases. Serum IgG and IgM levels rise in response to infection and remain elevated for 3-4 weeks before declining to baseline levels (Blaser and Duncan, 1984), whereas serum IgA levels rise during the first few weeks of infection and then fall rapidly (Kaldor *et al.*, 1983; Mascart-Leone *et al.*, 1987). Antibody mediated agglutination of *Campylobacter* cellular antigens is routinely used in diagnosis. A variety of antibody assays for detecting isotype-specific antibodies include Enzyme Linked Immunosorbent Assay (ELISA) (Blaser and Duncan, 1984), diffusion-in gel ELISA (Svedhem *et al.*, 1983), immunoblot analysis (Speed *et al.*, 1987), agglutination tests (Watson and Kerr, 1982) and complement fixation test (Jones and Robinson, 1981).

Serotyping is one of the phenotypic tests devised to study the epidemiology of *Campylobacter* infections. Two major serotyping schemes are used worldwide, and they detect heat labile "H" flagella antigens and soluble heat stable "O" (somatic) antigens (Penner and Hennessy, 1980; Lior *et al.*, 1982). The heat labile serotyping scheme originally described by Lior and others (1982), can detect >100 serotypes of *C. jejuni, C. coli* and *C. lari.* Uncharacterized bacterial surface antigens and in some serotypes, flagella, are the serodeterminants for this serotyping system (Alm *et al.*, 1991). Penner O serotyping scheme (Penner and Hennessy, 1980), detects 60 types of *C. jejuni* and *C. coli* and is based on detection of lipopolysaccharide antigens (Patton and Wachsmuth, 1992). Each system uses latex particles coated with

immunoglobulin developed against several *Campylobacter* species (On, 1996). Furthermore, Tylor and Chang (1987) produced an antibody specific for the thermophilic campylobacters outer membrane proteins (OMPs) detected by either ELISA or Immunoblot techniques.

2.5.5 Molecular techniques and genotyping

2.5.5.1 Molecular detection techniques

The use of PCR, which allows in vitro enzymatic amplification of a target gene using a specific DNA probe, offers the possibility of decreasing test time while increasing the sensitivity and specificity of detection. PCR has been used extensively to detect infectious agents directly from the specimens (Eisenstein, 1990). Phenotypic identification of campylobacters sometimes is difficult since they are fastidious, asaccharolytic bacteria and possess few distinguishing phenotypic characteristics (Goossens and Butzler, 1992). The difficulties in making routine identification of *Campylobacter* species by biochemical methods make these organism ideal candidates for PCR as the definitive identification methods (Oyof e*t al.*, 1992; Jackson *et al.*, 1996; Chuma *et al.*, 1997).

2.5.5.2 Molecular typing (Genotyping)

This offers genetic diversity of *Campylobacter* strains. By using PCR based techniques, it has been possible to discriminate *Campylobacter* strains from different sources (Burnett *et al.*, 2002). In recent years various genotyping methods for *Campylobacter jejuni* have described. These include flagellin gene typing (Nachamkin *et al.*, 1996), DNA-DNA hybridization (Van Camp *et al.*, 1993),

16S rRNA gene sequencing, nucleotide accession numbers (Burnett *et al.*, 2002), ribotyping (Fitzgerald *et al.*, 1996), pulsed field gel electrophoresis (Yan *et al.*, 1991), restricted endonuclease analysis (Penner *et al.*, 1983) and many others.

Of the methods outlined above, the simplest and cost effective method for investigation of large numbers of isolates is flagellin gene typing in particular *fla* typing (Nachamkin *et al.*, 1993). The tandem flagellin (*fla*) genes of, *fla*A, *fla*B, and *C. jejuni* demonstrate regions of conservation surrounding regions of variability. The flagellin *fla*A in *Campylobacter* spp. appears to have more significant sequence heterogeneity (Thornton *et al.*, 1990; Fischer and Nachamkin, 1991), such that molecular analysis of the flagellin gene serves as a good epidemiological marker. *Fla*-typing method involves PCR to amplify the *Fla*A gene and the restriction fragment length polymorphism (RFLP) analysis of this gene (Nachamkin *et al.*, 1993). This method has a greater stability and discriminatory power (Nachamkin *et al.*, 1993).

2.6 Pathogenicity

Thermophilic *Campylobacter* spp. are now the leading cause of enteritis in humans worldwide, and the infection is often the result of consumption of contaminated food, particularly chicken meat (Altekruse *et al.*, 1999). There is sufficient evidence available to date that healthy poultry are the major carriers of campylobacters due to presence of the organism in their intestine. The level of colonization is relatively high broiler chicken faeces ranging from 5.6×10^4 to 1.2×10^7 cfu/g (Grant *et al.*, 1980). Domestic and wild animals also carry

campylobacters as the normal intestinal flora. In chickens, *Campylobacter* spp. can also be isolated from spleen, gall bladder and blood (Beery *et al.*, 1988). Some strains of *Campylobacter* in chickens are invasive and/ or toxigenic and may cause distension of the intestine, liver abnormalities and diarrhoea (Clark and Bueschkens, 1988). Usually, the organisms are concentrated on the mucus layer in the crypts of the villi, where they are free living and able to utilize mucin and energy sources (Berry *et al.*, 1988). However, the intestinal carriage has been found to contribute significantly to the contamination of the food chain and the origin of faecal contamination of surface water, environment and humans (Blaser, 1997).

Adherence, invasion and cytotoxin production appear to be possible virulence factors of campylobacters. The *Campylobacter* spp. has colonization related factors, such as flagella, chemotoxins and adhesins (Wallis, 1994; Ziprin *et al.*, 1999). These factors play a major role in virulence, colonization and host response in *Campylobacter* spp. infections. The single polar flagellum of *Campylobacter* organisms imparts usual rapid darting motility, which is characteristic of the genus. This motility is absolutely necessary for campylobacters to colonize the gastrointestinal tract of humans and animals (Pavlovskis *et al.*, 1991). *C. jejuni* is able to move through viscous environments at speeds of up to 75 μm/s and demonstrates an increased ability to adhere and invade intestinal cells under these conditions (Carrillo *et al.*, 2004). However, non-motile mutants are unable to establish colonization and consequently cannot cause disease, hence *Campylobacter* flagella are considered as virulence factors. Non-flagellated mutants and flagellated motile mutants are also unable to invade eukaryotic cells in vitro (Yao *et*

al., 1994). Moreover, flagellin is the immunodominant antigen recognized during infection and is absolutely required for colonization in vivo (Carrillo *et al.*, 2004). Some studies have shown that development of antibodies to flagellin correlate with development of protection against disease (Martin *et al.*, 1989). The flagella filament of *Campylobacter* spp is unusual both in terms of its structure and antigenicity (Power *et al.*, 1994). The filament is composed of two flagellin subunits, *Fla*A and *Fla*B, which are almost 93% identical to one another, with most of the amino acid changes occurring at the amino and carboxyl ends of the protein (Power *et al.*, 1994).

Lipopolysaccharides (LPSs), also forming part of the endotoxins are constituents of the outer membrane of most gram-negative bacteria, including *Campylobacter* spp. (Rietschel *et al.*, 1990). They are potent immunostimulators and strongly activate B-lymphocytes, granulocytes and mononuclear cells (Rietschel *et al.*, 1990). Low doses of LPSs are considered to be beneficial to the host by causing immunostimulation and enhanced resistance to infections and malignancy. However, LPSs posses a broad spectrum of endotoxic activities (e.g. pyrogenicity and lethal toxicity), which contribute to the pathogenic potential of gram-negative bacteria (Rietschel *et al.*, 1990). Furthermore variations in the saccharide moiety of LPSs may prevent efficient complement activation and phagocytosis, thereby contributing to the virulence of bacterial infections (Liang-Takasaki *et al.*, 1982).

Recently, some phenotypic traits of infecting strains of campylobacters have been associated with the clinical presentation. In enteritis, three

pathogenic mechanisms have been proposed; production of a cholera-like enterotoxin (Ruiz-Palacios *et al.*, 1983), production of a cytotoxin (Wassenaar, 1997) and the ability to adhere to and invade epithelial cells, as demonstrated in vitro (Fauchere *et al.*, 1986; Melo and Pechere, 1990; Russel and Blake, 1994). The latter is considered essential for intestinal infection and production of disease (Ketley, 1997). There is a good correlation between the clinical presentation of diarrhoea and the isolation of *Campylobacter* strains that adhere to and invade HEp-2 cells. Variability in the clinical expression and in the phenotypic traits of isolates may be related to genetic diversity of *Campylobacter* strains.

Molecular studies have shown that a number of genes are responsible for the expression of pathogenicity in terms of adherence and colonization, which include *flaA*, *cadF*, *racR* and *dnaJ* (Datta *et al.*, 2003). While, the *virB11*, *ciaB* and *pldA* are pathogenic genes responsible for the expression of invasion and *cdtA*, *cdtB* and *cdtC* as pathogenic genes responsible for the expression of cytotoxin production (Datta *et al.*, 2003). The *wlaN* gene in *C. jejuni* is a pathogenic gene responsible for the expression of Guillain–Barré syndrome (Datta *et al.*, 2003).

2.7 Epidemiology

2.7.1 Prevalence in poultry

Poultry have been known since the 1950s to harbour campylobacters (Peckman, 1958). Although reports have shown that *C. jejuni* does not play a significant role as a causative agent of avian vibrionic hepatitis, chickens are still considered to be the major carriers of thermophilic campylobacters (Engvall *et al.*, 1986;

Petersen *et al.*, 2001). Among the avian species, poultry, including chickens, turkeys, ducks, quails and pigeons, are the most frequently colonized and usually excrete large amounts of bacteria leading to contamination of food and in particular poultry carcasses, surface water, environment and infection to humans (Saleha *et al.*, 1998; Uyttndaele *et al.*, 1999). Studies on the prevalence of *Campylobacter* spp in poultry have shown that the species most frequently isolated is *C. jejuni*, while *C. coli* is less common and *C. lari* is rarely found (Engvall *et al.*, 1986; Kazwala *et al.*, 1990). Aho and Hirn (1988) reported the prevalence of the *Campylobacter* spp. in slaughter chickens examined in sevaral countries from 1977 to 1986. Prevalence ranged from 6% in Sweden and 10% in Finland and Norway to 100% in Italy. However the prevalence appeared to be lower in Scandinavian countries when compared with other developed countries like UK and USA (Saleha *et al.*, 1998). Free-range chickens are reported to carry *Campylobacter* spp. with prevalence similar to commercial chickens (Adekeye *et al.*, 1989).

Table 2. The prevalence of thermophilic campylobacter in the poultry flocks of different countries

Country	% Positive flocks	Reference
Israel	85.0	Juven and Rogol (1986)
Netherlands	92.0	Oosterom *et al.*, (1983)
Nigeria	13.6	Adekeye *et al.*, (1989)
Malaysia	72.7	Joseph *et al.*, (1989)
South Africa	10.0-87.0	Richardson *et al.*, (1979)
Norway	10.0	Rosef *et al.*, (1982)
Northern Ireland	91.6	Neill *et al.*, (1984)
United States	14.5-86.7	Smitherman *et al.*, (1984)
West German	52.7	Altmeyer *et al.*, (1985)
Sweden	50.0	Engvall *et al.*, (1986)
Tanzania	35.3	Jiwa *et al.*, 1997
Australia	74.0	Shanker *et al.*, (1986)
Yugoslavia	71.7	Anna-Prah *et al.*, (1988)

In Malaysia, a tropical country, *C. jejuni* was found in both broilers and village (free-range local chickens) with the prevalence ranging from 81.9% to 97.1% (Saleha *et al.*, 1996), which is similar to that of temperate countries. In Tanzania, a study conducted by Jiwa *et al.*, (1997) revealed a prevalence of 35.3% in apparently healthy poultry. Table 2 shows the prevalence of *C. jejuni* in poultry and in various countries with differing climatic conditions.

A number of sources of infection to poultry under different management systems have been reported. In local scavenging chickens, exposure to carrier insects, vermin, dirty water, kitchen wastes, abattoir effluents, contaminated environments and possibly other animals increases the chances of infections (Saleha *et al.*, 1998). Although many studies have been undertaken, the sources of *C. jejuni* infection in commercial poultry during growing stage remain unclear. Engvall and others (1986) and Kazwala, (1988) studied the actual age of colonization of campylobacter in broilers and found that during first 2-3 weeks chicks were free from *C. jejuni* and some flocks remained free from the bacteria up to six weeks of age (Jacob-Reitsma *et al.*, 1992). However, once *C. jejuni* is introduced in the intensively managed chickens, it spreads rapidly to almost all birds in the flock. Vertical transmission of *C. jejuni* via the eggs is usually considered not common because of the low rate of isolation of campylobacters from naturally or experimentally infected eggs (Van de Giessen *et al.*, 1993; Jacobs-Reitsma *et al.*, 1995). However, a vertical transmission is possible in poultry since Jacobs-Reitsma *et al.*, 1994) isolated *C. jejuni* from ovaries of apparently healthy laying hens. Recently Cappelier and Collegues (1999) found that viable but non-culturable *C. jejuni* could be resuscitated by inoculation in embryonated hens' eggs. Earlier studies have shown that faecal contamination of egg contents may lead to infection of the newly hatched chickens (Shane *et al.*, 1986).

The mode of transmission and the exact sources of infection are still uncertain. Among the possible sources are contaminated feed, water, reuse of old litter, and inadequately cleaned and disinfected houses (Kazwala *et al.*, 1990; Van

de Giessen *et al.*, 1993). In addition, rodents and free flying birds (feral birds) have been shown to be infected with *C. jejuni* and may transmit the organism to poultry if they gain access to the poultry house (Genigeorgis *et al.*, 1986). Research has shown that insects like houseflies, darkling beetles, lesser mealworm beetles and cockroaches harbours the *C. jejuni* and remain the potential sources of infection to poultry (Jacobs-Reitsma *et al.*, 1995). Infected personnel at the farm may be the other source of infection to chickens. *C. jejuni* can be spread by hands, boots/footwear and clothing of the workers (Kazwala *et al.*, 1990). Seasonal variations have been also reported to influence the prevalence of *C. jejuni* in poultry. Annan-Prah *et al.*, (1988), Tauxe, (1992) and Nachamkin and Blaser, (2000), reported higher infection rates of the bacteria in poultry during summer (warmer months) as compared to the months of cold winter and spring. However, isolation peaks vary from one country to another and also within countries (Coker *et al.*, 2002). In developing countries *Campylobacter* infections usually has no seasonal preference probably due to lack of extreme temperature variation as well as lack of adequate surveillance for epidemics (Taylor, 1992).

2.7.2 Prevalence of the thermophilic campylobacters in other animals

Thermophilic *Campylobacter* spp. occurs in the intestinal contents of a wide variety of animals (Nachamkin and Blaser, 2000). Although, *C. jejuni* and *C. coli* cause acute enteritis in humans, in other mammalian species are mostly present in apparently healthy carrier state (Nachamkin *et al.*, 1993). Food producing and companion animals have frequently been found to harbour campylobacters as normal flora (On, 1996). In particular *C. jejuni* has been isolated from the intestinal

flora of healthy animals used for food production, and also from food and products (Pezzotti *et al.*, 2002).

In other domestic livestock other than poultry, different carriage rates ranging from 0% to 100% (Stern, 1981) have been reported, with isolation most frequently seen in confined herds of dairy cattle (Atabay and Corry, 1998). Food producing animals have been reported to have higher prevalence of the thermophilic campylobacters. The prevalence study conducted in Italy by Pezzotti *et al.*, (2002) revealed thermophilic campylobacter prevalence of up to 53.9% in cattle and 63.5% in pig. In Brazil, Stern (1981) encountered 24% of contamination with *C. jejuni* in unwashed sheep carcase. A study by Franco (1988) in pig carcasses showed that they were frequently contaminated with campylobacters than cattle or sheep carcasses.Aquino and others (2002) isolated *C. coli* from 9% of pigs sent for slaughter. However, the rate of contamination from pig carcasses varies widely from 2.9% in Poland (Kwiatek *et al.*, 1990) to 95% in Sweden (Svedhem and Kaijser, 1981).

The close association of man and companion animals has lead to a number of epidemiological studies on the prevalence of campylobacters in household pets. Dogs and cats have been implicated as a source of infection for human cases of campylobacteriosis (Sandberg, 2002). Sandberg, (2002) further conducted a study of *C. jejuni* in Norway and reported a prevalence of 18% and 23% in healthy cats and dogs respectively. Dogs being major sources for human infection in developed

countries probably because of their closeness to people (Sandberg, 2002).

2.7.3 Prevalence of the thermophilic campylobacters in humans

Generally, developing countries do not have national surveillance programs for campylobacteriosis therefore; incidence values in terms of number of cases for a population do not exist. Most estimates of incidence in developing countries are from laboratory based surveillance of the pathogens responsible for diarrhoea. Thermophilic *Campylobacter* spp. isolation rates in developing countries range from 5 to 20% (Oberhelman and Taylor, 2000). Table 3 shows isolation rates for some countries according to WHO regions from studies of diarrhoea in children <5 years old.

Despite the lack of incidence data from national surveys, case-control community-based studies have provided estimates of 40,000 to 60,000 per 100,000 for children <5 years of age (Oberhelman and Taylor, 2000). In developed countries, *Campylobacter* enteritis affects people of all ages and the incidence is highest in young children of <1 year old (Tauxe *et al.*, 1988). While in developing countries, the infection is virtually limited to young children and immunocompromised individuals like HIV victims (Solvillo *et al.*, 1991; Skirrow and Blaser, 1992; World Bank, 1993; Jackson *et al.*, 1997). The estimated infection rate for *C. jejuni* in USA and the UK is 960-1080 per 100 000 of the population (some 2.2-2.4 million infections per year) and 58 per 100 000, respectively (Saleha *et al.*, 1998). The rate in USA is considerably higher than the combined rates of infections from

Salmonella (21 per 100 000) and *Shigella* (7 per 100 000) (Tauxe, 1992; Blaser, 1997). Deaths attributed to *Campylobacter* infection in USA have been estimated at 680-730 per year. Worldwide, an estimated 4-5 million people die annually of diarrhoea caused by *Campylobacter jejuni* infection (Synder and Merson, 1980). *Campylobacter* infection is also common in other developed countries with the prevalence of up to 4-15% (Tauxe, 1992). Despite of great importance of the disease, many hospital laboratories do not routinely culture for this organism and therefore many infections with *C. jejuni* are substantially unreported (Tauxe, 1992; Allos and Blaser, 1995). The few published reports on enteritis in countries like Malaysia and Singapore give a low isolation rate for *Campylobacter* but according to Puthucheary and others, (1994) the true incidence may be 5-10 times greater than that of industrialized countries. The incidence of *Campylobacter* in Mexico and Thailand is 40, 000 per 100, 000 population (Taylor, 1992). This large number of enteritis due to *Campylobacter* infection is relevant to considerations of how frequently GBS follows *C. jejuni* infections.

Table 3. Isolation rates of *Campylobacter* from dirrhoea specimens from <5 year olds in selected developing countries

WHO region and country	Isolation rate (%)	Reference
Africa		
Algeria	17.7	Megraud *et al.*, (1990)
Cameroon	7.7	Koulla-Shiro *et al.*, (1995)
Ethiopia	13.8	Gedlu and Aseffa, (1996)
Nigeria	16.5	Coker and Adefeso, (1994)
Tanzania	18.0	Linblom *et al.*, (1995)
Zimbabwe	9.3	Simango and Nyahanana, (1997)
Americas		
Brazil	9.9	Mangia *et al.*, (1993)
Guatemala	12.1	Ramiro *et al.*, (1994)
Eastern Mediterranean		
Egypt	9.0	Rao *et al.*, (2001)
Jordan	5.5	Na'was and Abo-Shehada, (1991)
South-east Asia		
Bangladesh	17.4	Albert *et al.*, (1999)
Thailand	13.0	Echeverria *et al.*, (1989)
Western Pacific		
Cambodia	12.1	Yamashiro *et al.*, (1998)

2.7.4 Status of the disease in humans in Tanzania

The occurrence of human campylobacteriosis in Tanzania has been studied and showed existance of infection. Jiwa and other workers (1997) reported a campylobacter prevalence of 22% in apparently healthy children in Morogoro municipality. Lindblom and others (1995) isolated *C. jejuni/coli* from 18% of faecal samples taken from 394 diarrhoeic children. 12% of 278 asymptomatic children and a lower prevalence of 1% in diarrhoeic and asymptomatic adults in Dar es salaam. The findings may be linked with the higher prevalence of the thermophilic campylobacter reported in chickens, ducks, turkeys, goats and water (Kazwala *et al.*, 1992a; Jiwa *et al.*, 1997). This signified the zoonotic nature of the disease and may suggest the link of infection from humans to animals or *viceversa* and water may be considered as the intermediate reservoir.

2.7.5 Environmental reservoir of thermophilic campylobacter

Thermophilic campylobacters have a wide ecological diversity and isolation has been made from different environments. The pathogens have been isolated from water, roots of plants, pasture, anaerobic sludge and sewage, and abattoir effluents (On, 1996; Linton *et al.*, 1999; Cool *et al.*, 2003). Thermophilic campylobacters appear ubiquitous in untreated surface water and water is thought to be one of the main transmission routes of campylobacteriosis in humans and reported as the major source of a number of large outbreaks of human campylobacteriosis (Melby *et al.*, 1990; Jones, 2001). The study by Brennhovd *et al.*, (1992) documented a high prevalence of thermophilic campylobacters in untreated drinking water in Norway. Untreated drinking water is extensively used in third-world

countries and may be a potential transmission route for thermophilic campylobacters. The number of studies has shown the ability of campylobacters to survive in simple water systems up to four months (Rollins and Colwell, 1986; Stern *et al.*, 1994; Uyttendaele *et al.*, 2001; Cools *et al.*, 2003). Campylobacters are also common in natural water, such as streams, rivers, and lakes, due to discharges from wastewater treatment plants, runoff from pastures after rain, and direct contamination by wild birds (Jones, 2001; Diergaardt *et al.*, 2004). Campylobacters are able to survive for several days in moist, cool environments, including wells and groundwater (Jones, 2001). The study by Cool *et al.* (2003), reported the ability of *C. jejuni* to survive at 4^0C for up to 52 days and this feature could contribute to the persistence spread of *C. jejuni* in the environment. The frequency of human infection may be due to the low infectious dose of thermophilic campylobacters, which, based on human volunteer studies, ranges from 100 to 500 organisms (Black *et al.*, 1988). The prevalence and dynamics of campylobacters in drinking water obtained from the lakes, rivers and non-protected wells could probably vary from location to location.

The introduction of campylobacters into the water sources may be associated with animal manure and human excreta, water and biological wastes from abattoir and kitchens being drained to water bodies by excess water during rainfall (Sandberg, 2002). The study by Linton *et al.* (1999) on isolates of *C. coli* obtained from river water showed that the isolates were phenotypically, serologically and genotypically similar to *C. coli* isolates obtained from human providing the

contamination from human excreta. Also other studies showed that strains isolated from humans, poultry and water were phenotypically and genotypically correlated, confirming that water has a role in the transmission dynamics of the disease (Coker *et al.*, 2002).

2.7.6 Sources of infection to humans

Campylobacter infection is hyperendemic in developing countries and endemic in developed countries (Blaser, 1997; Coker *et al.*, 2002). The major sources of infection are food and environmental contamination. Human infection sources include contact with and consumption of chicken meat, consumption of raw milk, transmission from pet and other animals and contaminated water (Buswell *et al.*, 1998; Friedman *et al.*, 2004). Contaminated products particularly poultry meat and raw milk, are recognized as the primary vehicles of infection (Shane, 2000). The meat carcasses are normally contaminated during slaughter process (Koenraad *et al.*, 1995; Saleha *et al.*, 1998). As a consequence, contamination of poultry meat has been widely described, while lower contamination rates (5.1%) have been observed in red meat (Stern *et al.*, 1985). However, several studies have shown that poultry and poultry products are the leading potential sources of infection in human (Dominguez *et al.*, 2001; Coker *et al.*, 2002). For example, campylobacters were isolated from 40% and 77% of retail poultry meat sold in Bangkok, Thailand and Nairobi, Kenya respectively (Rasrinaul *et al.*, 1988; Osano and O, Arimi, 1999). Other sources include rodents, houseflies, cockroaches, feral birds, abattoir effluents and faeces of asymptomatic carrier animals (Carvalho *et al.*, 1990; Saleha *et al.*, 1998).

2.7. 7 Mode of transmission to human

Normally thermophilic campylobacters are normal flora of both wild and domestic animals including the food producing animals like poultry, cattle, sheep and swine (Blaser, 1997). Similarly, it can be carried to humans by pets like dogs, cats and birds, which are used as companion animals. Because of its widespread reservoir in animal kingdom, thermophilic campylobacters may contaminate both surface water and soils. Transmission to humans occurs by ingestion of contaminated foods of animal origin, contaminated water that has not been treated, and by direct contact with the infected animals, especially pets (Figure 4). Human to human transmission as a result of prolonged convalescent-phase excretion and high population density have also been suggested (Taylor, 1992; Rao *et al.*, 2001), although observations from developed countries show these as less likely factors (Altekruse *et al.*, 1999). The principal modes of transmissions for recognized outbreaks of *C. jejuni* infection and the endemic spread are as listed in Table 4.

Table 4. Principle modes of transmission of *C. jejuni* to humans, by epidemiological settings

Outbreaks	Endemic infection
Ingestion of unpasteurized milk	Ingestion of undercooked poultry
Ingestion of contaminated water from a municipal supply	Ingestion of contaminated surface water
Ingestion of undercooked poultry	Ingestion of unpasteurized milk and milk products
Ingestion of undercooked poultry	Contacts with pets
	Contact with infected persons
	Travel to developing countries

(Source: Blaser *et al.*, 1997)

Most outbreaks occur due to ingestion of unpasteurized milk or contaminated water and occasionally to ingestion of undercooked or raw poultry and other foods (Blaser *et al.*, 1983). Reports have shown that ingestion of poultry is responsible for 50% to 70% of all endemic cases (Blaser, 1997). Risk factors for acquiring campylobacters in the developing countries include poor hygiene in the kitchen, inadequate cooking of food, eating raw food and lack of education to people on public health principles which lead to unhygienic food preparations (Kazwala *et al.*, 1992a; Saleha *et al.*, 1998; Altekruse *et al.*, 1999). Presence of animals, cockroaches, flies and rodents in the kitchen, uncovered garbage in cooking areas and lack of piped water are other factors responsible for the disease occurrence (Saleha *et al.*, 1998; Rao *et al.*, 2001).

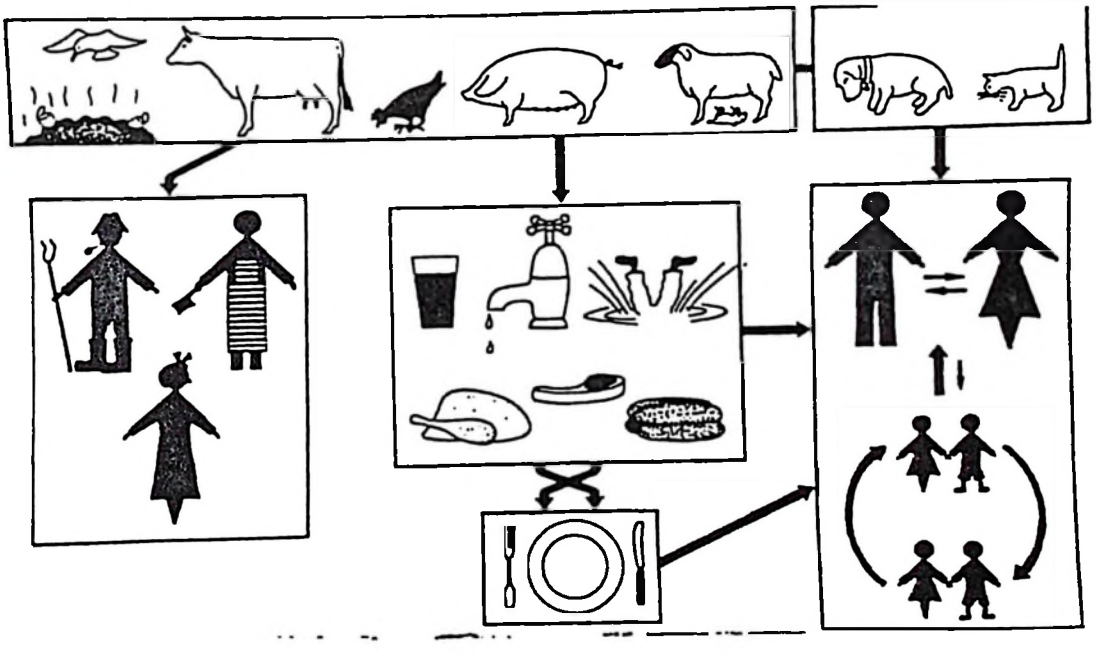

Figure 3. The transmission routes of *C. jejuni.*

2.8 Public health implications and estimates of human impact of the disease in developing countries

2.8.1 Public health implications of the disease

In developed countries, *Campylobacter* enteritis affects people of all ages and the incidence is highest in both children and young adults, while in developing countries, the infection is virtually limited to children (Skirrow and Blaser, 1992). *C. jejuni* infection is much common in children of developing than in developed countries, but many characteristics are different (Table 5). In both cases infection rate and the duration of intestinal excretion declines with age (Taylor *et al.*, 1993). Similarly, inflammatory diarrhoea is much less common among the affected persons in developing countries (except among travelers from developed countries), and symptomatic infection among adult is uncommon (Blaser, 1997). All these characteristics are markers for the development of immunity. The best explanation for the clinical and epidemiologic features of *C. jejuni* infection in developing countries is that because of high level exposure to the organism early in life, there is a gradual development of protective immunity. Nevertheless, *C. jejuni* infections contracted in early life cause substantial morbidity and mortality (Calva *et al.*, 1988).

Table 5. Comprison of the features of *C. jejuni* infections in developed with those in developing countries

Feature	Developed countries	Developing countries
Average number of infections/lifetime	0 – 1	>5
Principal age group Affected	Young adults	Children <2 years old
Principal manifestations of illness	Inflammatory diarrhoea	Non inflammatory diarrhoea
Widespread immunity among adults	Absent	Present
Principle vehicle	Poultry	Unknown

Blaser, 1997

2.8.2 Estimates of impact of human campylobacteriosis in developing countries

The Disability Adjusted Life Year (DALY) is the basic unit used in Burden of Disease (BoD) methodology to quantify the impact of disease on a population (Murray and Lopez, 1996). DALYs have been applied in some countries like Holland and it was used to measure the mean health burden of *Campylobacter* associated illness for five years. The mean estimate was 1,400 DALYs per year and the main determinants of health burden were acute gastroenteritis, gastroenteritis related mortality and residual symptoms of GBS (Havelaar *et al.*, 2000). Although there is paucity of data in developing countries to be estimated interms of DALYs, diarrhoea as a main clinical manifestation of the campylobacteriosis

remains to be one of the top three causes of mortalities (Murray and Lopez, 1996). The burden of campylobacteriosis in developing countries is estimated to increase more by the year 2020 because of the daily new infections of HIV, a disease which is normally associated with campylobacter enteritis (Coker *et al.*, 2002). Considering the higher incidence of campylobacteriosis in developing countries, DALY for the disease in these countries will likely be higher than those of the Dutch population (Coker *et al.*, 2002).

2.9.1 Clinical features of human campylobacteriosis

The usual incubation period after ingestion of campylobacters in particular *C. jejuni*, calculated from outbreak investigations, is 24 – 72 hours, but incubation periods of one week or longer occur (Skirrow and Blaser, 1995), and may be more common when low inocula (e.g. below 100 organisms) are ingested (Black *et al.*, 1988). Prodromal symptoms often are non specific (e. g. headache, myalagia, chills, vomiting and fever) and can last > 24 hours. The major clinical manifestation is an acute diarrhoeal illness, often with an acute abdominal cramping, fever and this results to dehydration, loss of weight and the patient becomes malnourished and weak (Coker *et al.*, 2002). The diarrhoea may initially be watery, with more than eight bowel movements on the worst day of illness, and it frequently becomes bloody. The abdominal pain may be so severe as to mimic appendicitis. The peak of the illness usually lasts for 24 – 48 hours before gradually resolving over a week, but it may be more prolonged. In the absence of treatment, relapse can occur in 20% of affected persons. The diarrhoea accompanying *C. jejuni* infections is considered as inflammatory because of the prominent expression of fever and constitutional

symptoms, and the stool usually contains polymorphonuclear leucocytes and gross or microscopic blood (Blaser *et al.*, 1982). Colonoscopy and biopsy reveal a diffuse inflammatory invasion resulting in colitis, and enteritis may also be present (Blaser *et al.*, 1982).

Extra intestinal infection occurs and may affect contiguous organs, causing cholecystitis, cystitis or septic abortion. Bacteraemia may occur, especially in immunocompromised individuals, and may lead to seeding of distant organs (Blaser *et al.*, 1986). Non-suppurative extraintestinal complications include reactive arthritis especially in human leukocyte antigen-B27-positive persons. Miller- Fisher syndrome and Guillan-Barre Syndrome (GBS) (Bremell *et al.*, 1991).

2.10 Diagnosis of the disease

The diagnosis of infection is usually based on the isolation of the *C. jejuni* from culture of faeces or, occasionally from another site (Blaser and Reller, 1981). A rapid presumptive diagnosis can be made by visualization of the organism in stools by Gram' stain or darkfield or phase contrast microscopy. *C. jejuni* infection leads to an elevation in serum antibodies, but a diagnostic rise usually occurs after symptoms have resolved. The median duration of convalescent fecal excretion is <3 weeks (Taylor *et al.*, 1988), so that by the time GBS develops, serology may be more sensitive than culture for identifying the presence of recent *C. jejuni* infection.

In recent years, the non-culture methods, in particular polymerase chain reaction and amplification of campylobacter DNA have been described for detection of *C.*

jejuni and other species in faecal samples of human and animal origin, and in food samples (Altekruse *et al.*, 1999). The PCR method can be combined with molecular typing methods to detect and characterize strains of thermophilic campylobacters in stool samples. The PCR has the potential to provide a rapid and specific diagnosis of Campylobacter infections (Waegel and Nachamkin, 1996).

2.11 Treatment

Although the campylobacter-associated gastroenteritis is normally self-limiting, antimicrobial treatment is normally reserved for patients with severe and advanced infections. Erythromycin is currently the treatment of choice, but fluoroquinolones and tetracycline are used as alternatives. Intravenous aminoglycoside therapy may also be considered in more serious cases of *Campylobacter* infection such as bacteraemia and other systemic infections (Aaerstrup and Engberg, 2001). Several studies have recently signaled an increasing incidence of antimicrobial resistance among *Campylobacter* spp. isolates (Aquino *et al.*, 2002). Resistance to trimethoprim is intrinsic and increasing resistance trends for other agents including sulphonamides have been reported (Schwarz and Chaslus-Dancla, 2001). Significantly, over the past decade there has been an increase in the number of quinolone resistant and to a lesser extent macrolide resistant strains reported, and identified from human infections (Piddock *et al.*, 2003). The increasing frequency of resistance to these agents is probably related to their use both in human populations and in animals raised for food production (Reina *et al.*, 1992; Segreti *et al.*, 1992).

2.12 Control

Better understanding of the risk factors for *Campylobacter* transmission may provide a means to prevent infections. New approaches in regard to food safety should include the "principle of commensurate effort" i.e. applying effort to risks in order of or their probable impact (Hall, 1997). This principle insists that the mitigation strategies should be risk targeted (quantitative) in order to control and prevent campylobacteriosis in humans. However, targeting the hazard (qualitative) in the control measures have been widely used in many countries (Sandberg, 2002). The use of Hazard Analysis of Critical Control Point (HACCP) as the control tool in production, processing and distribution of poultry products may reduce the likelihood of the problem. This aims at preventing the introduction and prevents growth of microorganism in all stages of food production, processing, distribution and consumption.

To control the disease in humans, sometimes might be difficult if the aetiologic agent is not controlled in the sources. Carrier animals, in particular poultry, food producing animals and pets should have low intestinal load or completely free from thermophilic campylobacters. The food should be properly cooked, cleanliness of the kitchen, personal hygiene, pasteurization of milk and avoid faecal contamination of the carcasses during slaughter of meat animals (Saleha *et al.*, 1998). Where possible, pets and pests (insects, rodents and feral birds) should be kept away from kitchen and food producing and processing industries. The use of potable water for drinking and all domestic activities is highly recommended.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The field study was conducted in both urban and rural Morogoro district. Morogoro municipality represented urban area while the villages of Mkuyuni, Changa and Kibwaya represented rural Morogoro. They are located about 40 km East of Morogoro town. The villages were purposively selected due to their easy accessibility and convenience of transporting samples to the University laboratory as well as availability of Mkuyuni missionary health center, which was the source of human specimen. Morogoro district is situated at the latitude 5.7 to 10°S and longitude 35.6 to 39.5°E, with an elevation of 1200 m above sea level and is about 200km west of Dar es salaam. Annual average rainfall ranges between 500 and 1800 mm and ambient temperature ranges between 18 to 28°C. The district has a bimodal rain pattern, with about 83% of the rain falling between late February and end of May, and short rains between November and January.

Human stool specimens from urban Morogoro were obtained from patients attending Morogoro region hospital, Sokoine University health center and Upendo laboratory (a private laboratory in municipality). Chicken samples were collected from different flocks with broilers and local chickens within the municipality and villages respectively. Slaughter centers within Morogoro municipality were identified and selected for sampling of chicken intestines. Chicken samples from the rural areas were also from live and slaughtered birds. Water samples were collected from different sources supplying the town and villages. All the laboratory work was

conducted in the laboratories of the Faculty of Veterinary Medicine, Sokoine University of Agriculture.

3.2 Study chickens

When selecting birds for sample collection; chicken types, age, management system and locations were considered as among the risk factors for campylobacter infections. Two types of chickens, namely, broilers and local chickens were examined in this study. The broilers were from the commercial chicken farming sector in Tanzania, which is characterized by intensification under deep litter management system whereby feeding and watering is through common feed and water troughs. Broilers are fed on commercial feeds and mostly fed on chick and broiler mash. The farmers usually use the wood shavings or rice husks as litter materials and the birds are kept up to the age of six to eight weeks before slaughter. The litters are not changed for the whole period from the first day of chicks' introduction in a house to the slaughter stage. Vaccination and other veterinary interventions are common in the broiler farms. Chickens considered for sampling were from the age of four to seven weeks.

Local chickens on the other hand are managed under traditional extensive management system. Under this system, the chickens are allowed to scavenge around homestead in the daytime. The chickens are housed only during the night hours normally in poorly constructed houses of which cleanliness is also difficult. The other form of housing is where chickens are kept in kitchens or human quarters at night. In this type of poultry management little or no health care is

provided compared to broiler birds. Chickens considered for sampling were from the age of six months and above.

3.3 Study humans

Human stool specimens were collected and labeled by medical personnel from patients with enteric complaints (diarrhoea, stomach cramps, nausea, vomiting and fever) attending health facilities in Morogoro municipality and Mkuyuni. The biodata of the sampled people such as age, gender and place of residence were recorded. The age was stratified into two categories purely for convenience and easiness of recording. Patients below 15 years old were recorded as children and 15 years and above were recorded as adult. The positive results were given to the responsible medical personnel for appropriate medical attention. Result confidentiality for the study participants was strictly adhered to and whatever findings recorded were known by the patient and his medical doctor. The Ethical Clearance permitting to collect human samples was applied for and granted by National Institute for Medical Research in Tanzania before the study began.

3.4 Study water sources

3.4.1 Morogoro municipal and rural water sources

Different sources were considered for sampling. Water samples were collected from the water distribution points, taps, ponds, shallow wells, rivers and streams (Tables 6 and 7, Figure 4).

Table 6: List of water sources sampled in Morogoro Municipality

Type of water source	Source's name
Rivers	Ngerengere, Kikundi, Morogoro, Mzinga, Lukulunge, Konga, Bigwa, Nongeni, Mgolole, Mungalugome, Kilakara
Dams	Mindu, Chamwino kwa mugulasi
Streams	Mzinga, Kilakara, Nongeni, Kichangani
Sewege treatment system	Sewage pond number 1A and B up to number 6, Sewege pond inlet.
Water collection points	Mambogo – water from Morogoro river, Maji club- water from Mindu dam, Mzinga SUA water sources- water from Mzinga river, Bigwa – water from Bigwa river and Mgolole – water from Mgolole river.
Tap water	At various points (Mazimbu, Modecco, Chamwino, Kihonda, Kilakara, Ninja, Kichangani, Kilakara, Kigurunyembe, Bigwa, Vibandani, Sultan area, Boma roaad, SUA and Forest).

Table 7. List of waster sources sampled in Mkuyuni division

Type of water source	Source's name
Rivers	Madamu, Mwaganga, Msua, Msumbara
Shallow-wells	Dispensary, msikitini, mwalimu, kibungo, mbanu
Streams	Kibungo, shuleni
Tape water	Mkuyuni dispensary, kibungo kanisani

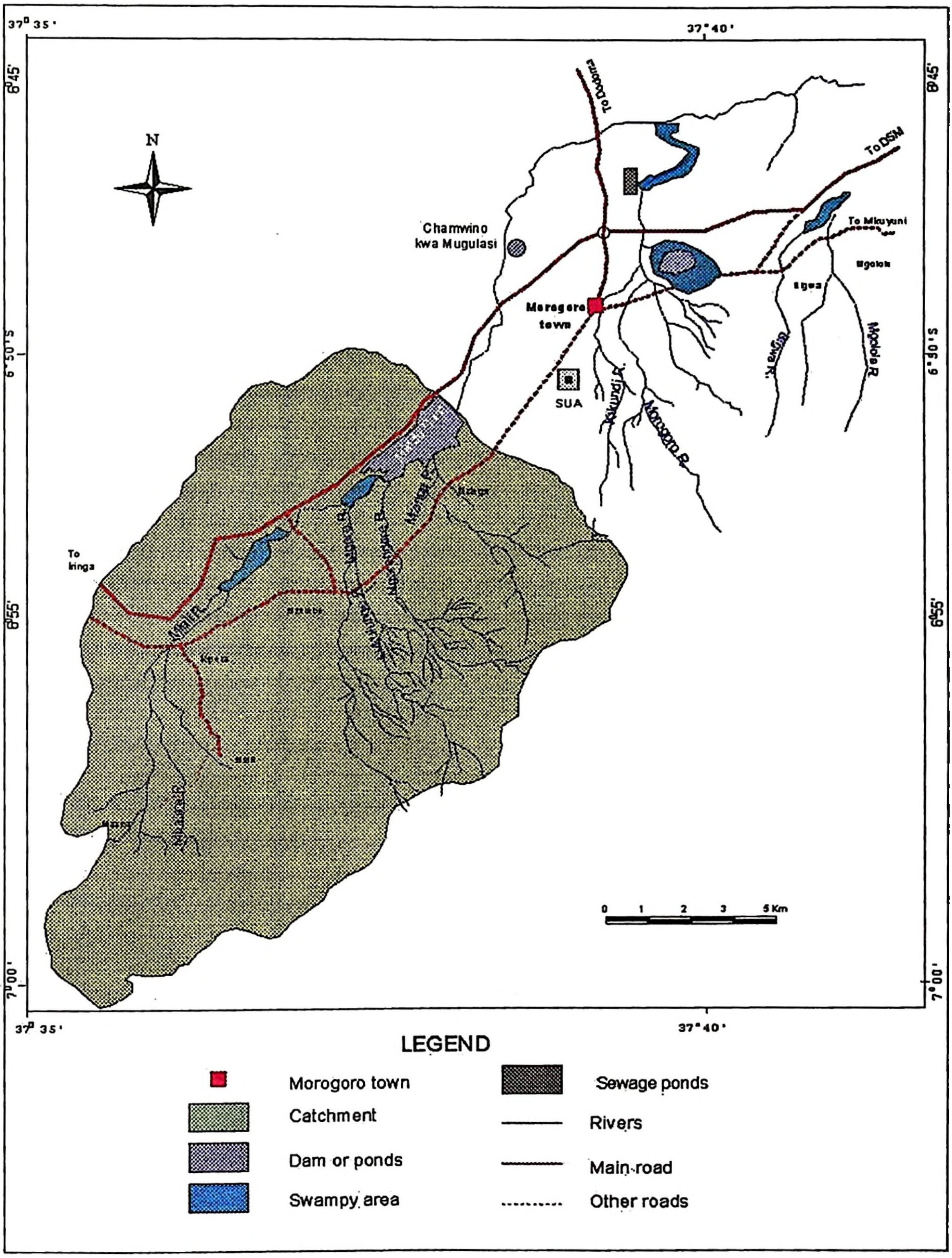

Figure 4. Morogoro water sources used for study

3.5 Sample selection

3.5.1 Chicken selection for cloacal samples

For collection of cloacal swabs, live birds were selected from chicken flocks in urban and rural Morogoro; and also from the two markets of Morogoro central and Sabasaba within Morogoro municipality. The two markets were included in the study since they are the major places where local chickens from rural places are sold. Based on size, chicken flocks were classified as small, medium or large. The classification criteria differed between the local and broiler chickens and were purely for convenience (Table 8).

Table 8. Chicken flock size classification

Type of chicken	Flock size classification		
	Small	Medium	Large
Broilers	< 200	200 - 300	> 300
Local chickens	< 40	40 – 70	> 70

Study birds were randomly selected from each flock where approximately 5-10% of the flock size was sampled as described by Noordhuizen *et al.*, (2001). The randomization of study birds was carried out by devising a transect walk within poultry house with birds picked randomly from the four corners and center of the flock.

3.5.2 Selection of chicken slaughter centers

During this study, fresh intestines were collected from slaughtering points in urban and rural Morogoro district. The slaughtering points included chicken flocks, food vending premises, bars and chicken market points where slaughtering of chicken usually takes place.

Within Morogoro municipality, the slaughter centers that participated in this study were chicken flocks (13), bars (3), food vending (3) and marketing points (2). The 13 flocks involved were chicken farms from eight wards constituting 42.1% of the 19 wards within Morogoro municipality. Specifically, these wards included Kihonda, Mazimbu, Mbuyuni, Mji Mkuu, Kingo, Sabasaba, Boma and Kilakala. The study wards selected have many flocks of chicken where slaughtering is also done. Flocks involved in this study were purposively selected based on cooperation from farmers. The bars and food vending points were also selected based on cooperation of owners. Both Morogoro central and Sabasaba markets were included in the study since they are the major places where local chicken from rural places are sold and usually slaughtered.

Intestines from rural Morogoro were collected from six slaughter centers all of which were food vending points at Kibwaya (1), Chang'a (1) and Mkuyuni (4) villages. All these centers are involved in slaughtering and selling chicken meat within study areas of rural Morogoro.

3.5.3 Selection of water sources

All major sources of water for domestic and agricultural uses in the study areas including dams, rivers, streams, sewage treatment systems, water collection points before distribution and tap water were included in the study (Tables 6 and 7, Figure 4)

Sampling points from dams were identified by devising a transect sailing in a locally made boat and at least 5-13 peripheral sampling points used to collect water samples. In addition, another sample from the center of the study dam was collected aseptically. Each water sample from every sampling point was submitted to the laboratory and cultured separately. For water samples from rivers, sampling points were identified along each river at an interval of approximately 300 to 500 metres making a range of 8 to 16 water samples collected from each river. For streams, similar approach as for the rivers was adopted during water sampling. The exit point of water at the sewage treatment system was used as a sampling point in this study and only one sample was collected for each system. All water collection points within Morogoro municipality were sampled where exit tap at each collection point was used as a sampling point. In order to have representative samples from taps, simple random selection was used to identify sampling points using streets within the study area in urban Morogoro with tap water. In total 15 streets were sampled (Table 6 and Figure 4)

In rural Morogoro, water samples were obtained from rivers, shallow wells, streams and taps (Table 7). Sampling procedure was as for the urban Morogoro

described above except for the shallow well where a sampling bottle was used to collect one sample per well with the aid of a rope used to draw water from the study well.

3.6 Sample size estimation

The sample size for live chicken was computed using a Statcal package of the Epi Info 6 for the cross-sectional study (Coulombier *et al.*, 2001). A total of 332 birds (with confidence interval of 95%, Power of 80.0%, Relative Risk=3.0, Frequency of disease in exposed group=15% with a compliance of 95%) was obtained and sampled. The same statistical package was used to estimate sample size for intestines. At the compliance of 80% and prevalence of 27% in exposed group, a total of 204 intestines were obtained and sampled (confidence interval=95%, power=80%, Relative risk=3).

The sample size for human stools was computed using Statcal package. A total of 622 was obtained at the prevalence in exposed group of 9%, compliance of 90%, confidence interval of 95%, power of 80% and relative risk of 3.0.

For water samples, the study aimed at establishing evidence of existence of contamination of water samples. The sample size was therefore computed using the Statcal package for population survey or descriptive study. A total of 146 samples was obtained (infinity population, confidence interval=95%, prevalence of 5% according to Jiwa *et al.*, (1997) and worst acceptable level of 1.5%).

3.7 Sampling and sampling methods

3.7.1 Sampling techniques

The techniques described below were used to collect samples throughout this study.

3.7.1.1 Chicken cloacal swabs and intestines samples

Moistened sterile cotton wool swabs were carefully inserted via the vents to the recta/ cloaca, and gently arolled therein and then scoop used to out the cloaca contents present. These swabs were immediately placed in the sterile universal bottles containing 10 mls of fresh Cary Blair broth, along with the cloacal contents and immediately the bottles were placed in a cool box after proper capping and transported under ice to the University laboratory. For the case of intestinal contents, the intestines were collected from the slaughter centers. The intestines were collected immediately after separation of the viscera from the carcass. The whole gastrointestinal tract was taken and each set of intestine was placed into a separate clean plastic bag and immediately stored in a cool box ready for shipment to the laboratory. A total of 13 and 7 broiler and local chicken flocks were respectively sampled from Morogoro municipality and 17 flocks of local chickens from rural areas. Two types of samples were collected from chickens, which included 332 cloacal swabs collected from the live birds and 204 intestines of slaughtered chickens and analysed.

3.7.1.2 Human stool specimens

Human stool samples from patients suffering from enteric problems were collected from medical laboratories by using sterile universal bottles on daily

bases for five months consecutively. Sample containers were being provided to patients for stool collection and waterly, bloody, mucoid and even normal stool specimens were all considered for laboratory analysis. Immediately after sampling, sterile universal bottles were securely closed and the specimens were stored under refrigeration temperature before were shipped to University laboratories for analysis. Samples were shipped in a cool box to the laboratory and analyzed within eight hours post sampling. A total of 498 and 134 stool specimens were collected and analysed from urban and rural human patients respectively.

3.7.1.3 Water samples

Water samples were collected aseptically from identified sources, used by animals and humans for different purposes. Sterile duran bottles were used to collect water sample of approximately 500ml volume. Immediately after sampling, each bottle with the sample was well labeled with sample number, date, time and location and were placed in cool box under ice and shipped within 3 hours to the University laboratory for analysis. A total of 125 and 21 water samples from Morogoro municipality and rural areas respectively were analysed.

3.8 Materials

3.8.1 Laboratory media

3.8.1.1 Cary Blair transport medium

Following cloacal swab sample collection, the samples were immediately placed in sterile Cary Blair transport medium (Lipfilchem s.r.l, Roseto, d. A. (TE)-Italy). The media contains 1.1 g – disodium hydrogen phosphate, 1.5 g –

sodium thioglycollate, 5.0 g – sodium chloride, 0.09 g – calcium chloride and 5.6 g – agar. The media was prepared according to manufacturer's instructions. Briefly the transport media was prepared by dissolving 13.3 g of the media in one litre of distilled water, boiled gently to dissolve and aliquoted into bijou bottles and sterilized in the autoclave at 121^0C for 15 minutes and cooled for use. Each time of sampling process, the freshly prepared media was used.

3.8.1.2 Nutrient broth

The nutrient broth (Oxoid® Ltd., Basingstoke, U.K.) was prepared according to manufacture's instructions and briefly was done by dissolving 6.5 g of the powder in 500 ml of distilled water, followed by gentle boiling and was sterilized in the autoclave at 121^0C for 15 minutes and cooled to 50^0C. One vial of Modified Preston antimicrobial supplements (Oxoid® Ltd., Basingstoke, U.K.) was constituted with 2 ml sterile distilled water and absolute acetone at the ratio of 1:1 and be added in the broth and mixed thoroughly. Then, the broth was dispensed at a volume of 2 ml in the universal bottles ready for use for human stool specimens and water samples enrichment. The active ingredients of the broth includes 5.0 g of peptic digest of animal tissue, 5.0 g of sodium chloride, 1.5 g of beef extract and 1.5 g of yeast extract.

3.8.1.3 mCCDA media

Primary isolation of thermophilic campylobacters was carried out on a modified charcoal, cefoperazone, deoxycholate, agar (mCCDA) (Oxoid® Ltd., Basingstoke, U.K.). The media consisted of 25 g - nutrient broth, 4 g -

bacteriological charcoal, 3 g – casein hydrolysate, 0.25 mg – sodium pyruvate and 12 g – agar. The 50g of the media powder was dissolved in 500 ml distilled water, autoclaved at 105°C for 15 minutes, and then cooled to 50°C. One vial of cefoperazone (32mg/l) supplements (Oxoid® Ltd., Basingstoke, U.K.) was constituted with 2 ml sterile distilled water and aseptically added in the molten agar and mixed thoroughly before pouring. Then, the media was poured at the volume of 20 to 30 mls in each petri dish and left at room temperature to solidify for two hours. The solidified media was incubated at 43°C for 24 hours to verify the sterility before culturing.

3.8.1.4 Blood media

Pure colonies of presumptive *Campylobacter* isolates were obtained after subculture on blood agar base (Oxoid® Ltd., Basingstoke, U.K.). This media contains 10 g – lab-lemco powder, 10 g peptone, 5 g – sodium chloride and 15 g – agar. The 40 g of the media was dissolved in 1000 ml distilled water, autoclaved at 121°C then cooled to 50°C and before pouring. Then about 100 mls of horse blood was added in the molten media, mixed thoroughly and poured in the sterile glass petri dishes at the volume of 20 to 30 mls per petri dish. The plates were left at room temperature for two hours for the media to solidify then incubated for 24 hours at 37°C to check for sterility.

3.9 Laboratory procedures

3.9.1 Sample preparation and inoculation for qualitative analysis

3.9.1.1 Chicken samples

In the laboratory, each universal bottle with a cloacal swab was opened aseptically, and the cloacal swab samples were taken aseptically using a sterile forceps and gently smeared on one side of the sterile mCCDA petridish. Streaking was done with a sterile wire loop onto well-labeled mCCDA plates as per sample number. Each sample was streaked in a separate plate and incubated as indicated in 3.9.2 below.

3.9.1.2 Human samples

The universal bottle/match boxes with samples were opened aseptically, and the samples were first put in universal bottles containing 5 ml of sterile enriched nutrient broth for the purpose of enrichment and incubated at 37^0C for 24 hours. Then the universal bottles with enriched samples were properly shaken and then by using a sterile wire loop, a loopful of enrichment culture was then subcultured onto the mCCDA media and incubated as indicated in 3.9.2 below.

3.9.1.3 Water samples

The microbiological analysis of the water samples followed the method described by Tenover and Fennell, (1992). Duran bottles with water sample were opened aseptically, and the water was filtered using a nitrocellulose filter paper (Millipore Corporation Bedford, USA) with the pore size of 0.45µm, which was fixed on a sterile filtration unit. By using sterile forceps the filters with the water filtrate

were placed in the universal bottles containing 9 ml of nutrient broth with Preston supplements for enrichment, shaken properly and incubated at 37^0C for 24 hours. After incubation, the enriched culture was shaken again; and a loopful of the culture was then subcultured onto the mCCDA media and incubated as indicated in 3.9.2 below.

3.9.2 Incubation

After inoculation, all the petri dishes were loaded in the anaerobic jars (Coldstream Engineering Ltd, 18-10, Arista Sweden) containing a lit candle and closed tightly. The jars were placed in incubators at 43°C for 48 hours as recommended by Skirrow and Benjamin (1980). *Campylobacter* suspected colonies were purified by sub culturing on blood agar base (Oxoid® Ltd., Basingstoke, U.K.) and re-incubated at 43°C under microaerophilic environment for 24 hours ready for identification to species level.

3.9.3 Bacterial identification

3.9.3.1 Morphology

The isolates were identified as *Campylobacter* spp. on the basis of their colonial morphology as described by Skirrow and Benjamin (1980). Typically, flat, low convex, mucoid grey, glossy and sticky colonies were recorded as campylobacter suspects. Wet smear was prepared on a sterile glass slide using normal saline from the suspect colonies and examined on light microscopy under times 10 mignification. Organisms, which revealed spiral shaped, highly motile with a darting or cork screw-like motion were indicative of campylobacters. The

Gram stain of the bacterial colony was done on the sterile glass slide as per Carter and Chengappa, (1991). Gram-negative short and slender spiral shaped organisms were identified *Campylobacter* spp.

3.9.3.2 Growth at 43^0C on mCCDA media

As had been documented by Skirrow and Benjamin (1980), which all thermophilic campylobacters have the ability to grow at 43^0C when incubated under microaerophilic environment. Therefore, colonies with the features described in 3.9.3.1 above following incubation at 43^0C were assumed to be of thermophilic campylobacters.

3.9.3.3 Biochemical tests

The protocal for biochemical tests used for the confirmation and identification of thermophilic campylobacters was similar to that described by Skirrow and Benjamin (1980).

(i) **Catalase tests:** A thick smear of each presumptive *Campylobacter* isolates was made on a sterile glass slide to which a drop of 5% hydrogen peroxide was made. A positive catalase reaction was based on appearance of effeversence within few seconds.

(ii) **Oxidase test:** A thick smear of a colony was put on the bloating paper wetted in 1% aqueous solution of *tetramethyl-p-phenylenediamine* and left for few seconds. A positive oxidase reaction was

based on immediate development of a deep purple colour around the smear.

(iii) **Hippurate hydrolysis test:** A 0.3 ml of sterile normal saline was added in a plastic test tube containing a powder of sodium hippurate (Lipfilchem s.r.l, Roseto, d. A. (TE)-Italy). Using a sterile wire loop, one well-isolated colony was added in the solution. The tubes were then incubated at 37^0C for 24 hours. Then four drops of 7% nihydrin solution were added and a blue purple colouration was indicative of a positive tes for *C. jejuni.*

(iv) **Nitrate test:** A 0.3 ml of sterile normal saline was added in a plastic test tube containing nitrate (Lipfilchem s.r.l, Roseto, d. A. (TE)-Italy) reagent. Using a sterile wire loop, a well-isolated colony was added in the solution. The tubes were then incubated for 24 hours at 37^0C. Then a drop of *alpha-naphtylamine* and one drop of sulfanilic acid was added and mixed gently for a few minutes. The development of red orange colouration signified a positive test for *C. coli.*

(v) **Susceptibility to cephalothin test:** Colonies, which were negative to hippurate and nitrate tests, were re-cultured on the blood agar. Using a sterile forceps a cephalothin antibiotic disc disk of 30µg/ml (Lipfilchem s.r.l, Roseto, d. A. (TE)-Italy) was placed on the agar's surface and incubated at 37^0C for 24 hours under microerophilic environment. All the isolates, which showed resistance to the cephalothin test, were recorded as *C. lari.*

3.9.4 Bacterial storage for molecular work

The identified *C. jejuni* isolates were stored in nutrient broth mixed with 50% v/v glycerol at -20^0C for molecular work.

3.9.5 Identification of thermophilic campylobacter isolates by polymerase chain reaction (PCR) techniques

Only isolates for *Campylobacter jejuni* was confirmed genetically since is the major cause of human campylobacter enteritis (Engvall *et al.*, 1986; Kazwala *et al.*, 1990, Koenraad *et al.*, 1995, Blaser, 1997, Nachamkin, 2002). The limitations of resources also made it not possible to perform molecular work to all *C. jejuni* isolates and *C. coli*. About sixty percent (243) of all 398 phenotypically confirmed *C. jejuni* strains from field studies in humans and chickens were randomly selected and used in molecular work. The stored isolates were cultured on blood agar as described above in 3.9.4. The PCR was performed as described by Nachamkin *et al.* (1993).

3.9.5.1 DNA extraction

The first step towards carrying out PCR typing was the successful extraction of DNA from campylobacter cells. Isolates of *C. jejuni* from the stored stock were grown at 43°C overnight on blood agar under microaerophilic environment. Then a loopful of bacteria colonies from a fresh culture was washed with 1 ml of sterile double distilled water and resuspended in 200 µl of distilled water in an eppendorf tube. The suspension was heated at 100°C for 20 to 30 minutes in a boiling water bath, then placed on ice for 5 minutes, and then centrifuged for 2 minutes in a microcentrifuge at 13,000 × *g* to extract DNA as was described by Nachamkin *et*

al., (1993). Five microlitre of the supernatant was then used for the PCR procedures.

3.9.5.2 Preparation of the master-mix

The PCR amplification was performed in a final volume of 50µl containing 1 x reaction buffer (Promega - Madison USA); (50mM KCl, 10mMTris-HCL; pH 9.0 at 25°C and 0.1 triton x-100), 3.0 mM magnesium chloride, 200µM of each deoxynucleoside triphosphates, 2.5U of Taq DNA polymerase (Promega - Madison USA) and 50 pmol of each of the primers (Integrated DNA Technologies, Inc.). The forward primers sequence was 5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3', corresponding to nucleotides 1 through 26 in *flaA* gene, and the reverse primer 5'-CTG TAG TAA TCT TAA AAC ATT TTG-3' corresponding to nucleotides 1705 through 1728 of *flaA* on bases of previous sequence data (Fischer and Nachamkin., 1991) was used. The reaction mixture was then overlaid with 50µl of mineral oil (Sigma chemical co. reagent, St Louis, USA) to prevent evaporation during PCR.

3.9.5.3 Optimization of PCR conditions

Precautions were taken to avoid cross contamination of template DNA, master mix reagents and PCR product by carrying out laboratory work in four different rooms. In order to get good PCR results necessary adjustments to the methodology were done with regard to the amount of reagents used as suggested by Wagers, Jr. and Fowler (1993).

3.9.5.4 Preparation of DNA templates

A 20µl of template DNA in a 0.5µl-eppendorf tube (Anachem - UK) was first denatured by incubation in boiling water for 10 minutes. Then immediately quenched on ice before adding 5µl of it in the 45µl of the master-mix in 0.5ml eppendorf tube to make a final volume of 50µl of the reaction mixture.

3.9.5.5 DNA amplification

The DNA amplification was done by using an automated thermal cycler machine (Crocodile II Appligene Inc.-Pleasanton CA, USA). The amplification process started with denaturation at 94°C for five minutes, then cycle 35 times at 92°C for 30 seconds followed by annealing at 55°C for one and half minutes and extension at 72°C for two and half minutes, then incubate at 72°C for 5 minutes and maintained at 4°C until analysed. After amplification, 10 µl of the amplicon was analysed on either 0.8% agarose gel or 7.5% polyacrylamide gel. The presence of 1720bp band indicated the positive isolate for *Campylobacter jejuni.*

3.9.5.6 Preparation of agarose gel

The agarose gel was prepared by mixing 0.8 gram of agarose powder with 0.5X TBE buffer and made up to 100 ml to obtain a 0.8% concentration of the gel. Agarose was completely dissolved by heating the solution on a hot plate. A volume of 5µl of 0.5µg/ml ethidium bromide solution was added to every 100 ml of cooled molten agarose and mixed thoroughly by a mixer. Molten agarose was then poured into the horizontal electrophoresis gel casting equipment and left for 40 minutes to set.

3.9.5.7 Loading of PCR products in agarose gel and electrophoresis

A volume of 10µl of the PCR products was mixed thoroughly with 2µl of blue/orange 6X loading dye (Promega - Madison USA) on a laboratory parafilm. The PCR products were loaded in the wells of the agarose gel and 5 µl of a 100 bp molecular weight marker (Promega - Madison USA) was also mixed with 2µl of loading dye and run in a parallel track. The horizontal gel ectrophoresis was carried out at a constant voltage of 120 for one hour in a 0.8% (w/v) agarose gel containing one micogram of ethidium bromide per millilitre in 1XTBE buffer. The gel was then examined by transillumination under UV light to determine the presence or absence of the *FlaA* amplicon of the size of 1720bp.

3.9.5.8 Preparation of polyacrylamide gel

The gel was prepared according to Sambrook *et al.*, (1989). A 7.5% gel was made by mixing 2.5ml of 30%(V/V) acrylamide (Scotlab-Scotland, UK), 2ml of 5xTBE (445mM Tris-HCL; 445mM Boric acid; 12.5mM EDTA), 100µl of 0.1%W/V Ammonium per sulphate and 10µl of 0.1(V/V) N,N,N',N'-tetramethylene (TEMED-Sigma chemical Co. St. Louis, USA) and 5.5 ml of distilled deionized water. The gel was cast using the mini Protean II™ gel kit (Biorad USA) for about 15 minutes.

3.9.5.9 Polyacrylamide gel electrophoresis of PCR products

A volume of 10µl of PCR amplicons was loaded in the wells and a 100 bp molecular weight marker was run in a parallel track. Electrophoresis was carried out at a constant voltage of 200V for one hour using a vertical mini protean II™ gel electrophoresis apparatus (Biorad USA) filled with 1XTBE buffer. When

electrophoresis of the polyacrylamide gel was complete, the gel was stained by silver nitrate as described by Herring *et al.* (1982).

3.9.5.10 Silver nitrate staining of polyarylamide gel

The gel was first fixed for 5 minutes in 100ml of 10%(V/V) ethanol and 0.5%(V/V) glacial acetic acid with gentle agitation. After five minutes, it was washed with 100ml of a 0.19% (W/V) silver nitrate ($AgNo_3$) solution for another five minutes followed by two washings with distilled water. The DNA fragments were visualized by agitating the gel in 100ml solution of 3% (W/V) NaOH and 0.75% (V/V) formaldehyde for six minutes. The colour development was stopped using 0.75 (W/V) Sodium Carbonate (Na_2CO_3) solutions as soon as the bands started appearing. The DNA bands in a gel were interpreted with reference to the DNA ladder. All the gels were well labelled and preserved at 4°C in sealed nylon bags for further references.

3.10 Statistical analysis

The bacteriology data were analysed by using Epi Info version 6 (2001) software. The Chi-squire and confidence intervals was used to compare proportions at probability $P>0.05$. The relative risk (prevalence ratio) was used to assess the strength of associations between the prevalence and risk factors. For the molecular identification data, the analysis was done using percentage proportions of isolates testing positive by PCR confirmatory test.

CHAPTER FOUR

4.0 RESULTS

4.1 Infection rate based on conventional campylobacter identification methods

4.1.1 Campylobacter infection rate in chickens

The prevalence of thermophilic campylobacters from the chickens with regard to type of poultry, management and location are presented in Table 8. Overall *Campylobacter* spp were isolated in 70% (n=374) of both cloacal and intestine samples examined. Specifically, *C. jejuni* were identified in 91.2% (n=374) campylobacter positive samples tested and the rest were *C. coli*. No *C. lari* was identified in any of the samples studied.

Considering type of poultry as a risk factor of infection, data on isolation rate in local and broiler chickens were compared. There was no significant difference in campylobacter infection between these two types of chickens ($P>0.05$) Table 8. Moreover, significantly more *C. jejuni* (86%, n=203) than *C. coli* (14%, n=33) were isolated from local chickens ($p<0.05$) while the 138 isolates in broiler chickens were all *C. jejuni*.

Comparison of management systems as another factor to campylobacter infection showed no significant difference in chicken infection under extensive management from that of intensive system ($P>0.05$). All the 141 isolates obtained from chickens under intensive system were *C. jejuni* while 200 (85%) and 33 (14.2%) of the isolates under extensive management system were *C. jejuni* and *C. coli* respectively (Table 9).

Location being a risk factor of infection in chickens was also considered only in local chickens handled from urban and rural areas. It was found that local chickens from rural areas were significantly exposed to campylobacter infection ($P<0.01$, RR=1.3, 95%CI=1.25-3.51) compared to those screened in the urban areas. The prevalence of thermophilic campylobacters from the local chickens in rural and urban areas was 76% (n=223) and 60% (n=112) respectively. Furthermore, a total of 148 (87.6%) and 21 (12.4%) *C. jejuni* and *C. coli* respectively were isolated in rural local chickens while 55 (82.1%) and 12(17.9%) were *C. jejuni* and *C. coli* respectively from local chickens in urban.

Table 9. Risk factors for *Campylobacter* infections in chickens

Risk factors	Category	N	Prevalence %	RR	95% CI	P-value	Number (%) of positive	
							C. jejuni	*C. coli*
Type of poultry	Local	335	71		0.73,		203 (86)	33 (14)
	Broilers	201	69	1.1	1.62	0.66	138 (100)	0 (0)
Management	Extensive	325	72		0.85,		200 (85)	33 (14.2)
	Intensive	211	67	1.1	1.86	0.23	141 (100)	0 (0)
Location (local chickens)	Rural	223	76		1.25,		148 (87.6)	21 (12.4)
	Urban	112	60	1.3	3.51	0.002	55 (82.1)	12 (17.9)

Key: n = sample size, RR= risk ratio, CI=Confidence interval at 95%, P = probability

Broiler chickens of four weeks and above were sampled in this study. It was found that age was the risk factor for *Campylobacter* spp in broilers. Young ages had lower thermophilic campylobacters infections compared to adults (Figure 5).

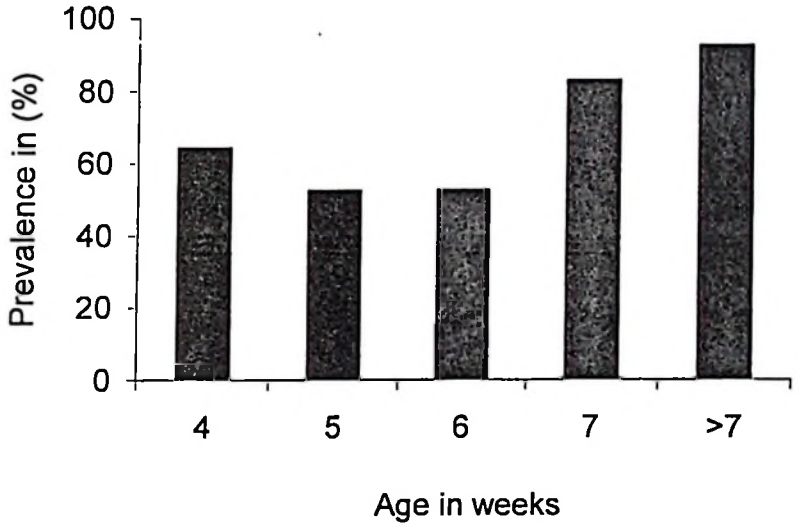

Figure 5. Infection trends of campylobacters in broilers of different weeks of age

The recovery of *Campylobacter* spp from cloacal and intestines samples is summarized in Table 10. There was a significantly ($p<0.01$) higher proportion of *Campylobacter* positive intestinal samples (82%, n=204) than that isolated from cloacal samples (62%, n=332). It was also observed that most of the chicken slaughter centers were providing the intestines to low income people and was used as meat. Clearly the risks of humans getting infection through chicken intestines were high.

Table 10. *Campylobacter* isolation rate from intestines and cloacal samples in chickens

Sample type	n	Prevalence	RR	95%CI for PR	*P*-value
Intestines	204	0.82	1.33	1.84,	0.01
Cloacal swabs	332	0.62		4.49	

Key: n = sample size, RR= risk ratio, CI=Confidence interval at 95%

Comparison of large, medium and small flock size in both local and broiler chickens did not show any significant trend interms of infection proportions ($p>0.05$) (Figure 5). In local chickens, large, medium and small flocks had the isolation rate of 69.8% (n=53), 80% (n=95) and 70.8% (n=106) respectively. In broilers 43.2% (n=44), 77.3% (n=66) and 74.7% (n=91) of chicken sampled were positive for campylobacter in large, medium and small broiler flocks respectively (Figure 6).

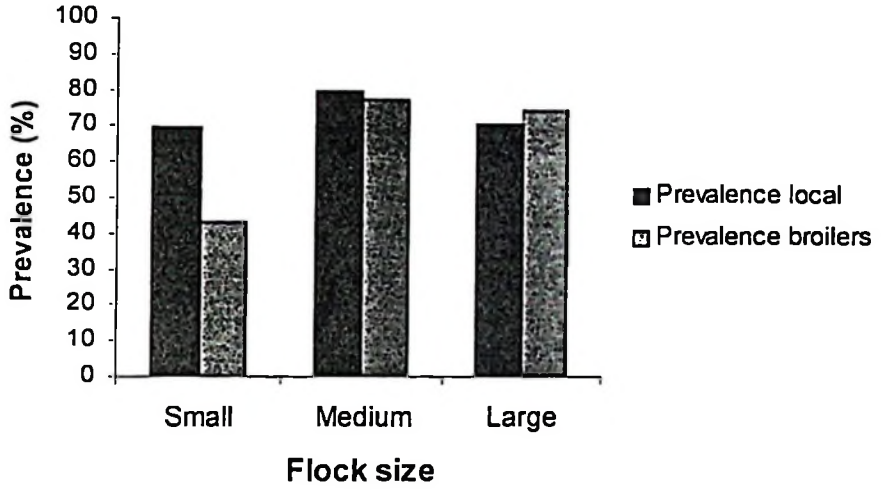

Figure 6. Distribution of *Campylobacter* infections in chickens according to flock sizes

4.1.2 Water sample results

A total of 125 and 21 water samples from Morogoro municipality and rural areas respectively were analysed for thermophilic campylobacters and were all negative.

4.1.3 Campylobacter infection rates in humans

In the present study, 622 stool specimens from humans living in urban and rural areas were analyzed for thermophilic campylobacters and gave the isolation rate of 9.3%. Of the 59 thermophilic campylobacter isolated, 96.6% were *C. jejuni* and the rest 3.4% were C. *coli.*

Data on age related proportions of infection are shown in Table.11 The risk of campylobacter infection in children was significantly (RR=2.3; $p<0.01$) higher in children (15 years and below) than in adults (above 15 years old). *C. jejuni* was

a dominant species in children (96.3%) and adults (96.9%) sampled, whereas *C. coli* was only isolated from a child and single adult patient.

Sex was found not to be a significant factor in adult patients, (P>0.05) despite males having a slightly higher proportion of infection 7.4% (n=312) compared to females 7.1% (n=320). The species of campylobacters isolated in males were *C. jejuni* and *C. coli* with the number isolated being 31 (96.9%) and 1 (3.3%) respectively. All isolates (16) in females were *C. jejuni* (Table 11).

Assessment of location showed that there was no significant difference between proportion of rural human infected with campylobacter and those in urban areas (P>0.05). All individuals who were campylobacter positive in rural areas 11% (n=134) were infected with *C. jejuni* while 43 (95.6%) and 2(4.4%) patients in urban areas were infected with *C. jejuni* and *C. coli* respectively (Table 11).

Table 11. Risk factors for *Campylobacter* infections in humans

Risk factor	Category	n	Prevalence %	RR	95% CI	P-value	Number (%) of *Campylobacter* spp	
							C. jejuni	*C. coli*
Age	Children	175	16.0	2.34	1.44, 4.63	0.01	26 (96.3)	1 (3.7)
	Adult	457	7.0				31 (96.9)	1 (3.1)
Sex (for adults)	Male	216	7.4	1.04	0.51, 2.47	0.74	16 (100)	0 (0)
	Female	241	7.1				15 (93.8)	1 (6.3)
Location	Rural	134	11.0	1.22	0.53, 2.27	0.61	14 (100)	0 (0)
	Urban	498	9.0				43 (95.6)	2 (4.4)

Key: n = sample size, RR= risk ratio, CI=Confidence interval at 95%, P = probability

4.2 Molecular identification of *Campylobacter jejuni* isolates.

A total of 61% (n=243) out of 398 randomly selected *C. jejuni* strains from field studies in humans and chickens were used in molecular work. *C. jejuni* isolates identified by hippurate hydrolysis test were subjected to *C. jejuni* specific PCR as a further confirmation method, 49 of these were from humans and 194 from chickens (Table 12). Of the 243 isolates, DNA from 74.1% was successfully amplified after a simple water extraction of the chromosomal DNA (Table 12). The results showed that 24.5% and 26.3% of the 243 human and chicken isolates, respectively, tested negative for *Campylobacter* DNA on PCR.

Table 12. Biochemically identified *C. jejuni* isolates confirmed by *C. jejuni* specific PCR

Source of isolates	n	Positive (%)	Negative (%)
Chickens	194	73.7	26.3
Human	49	75.5	24.5

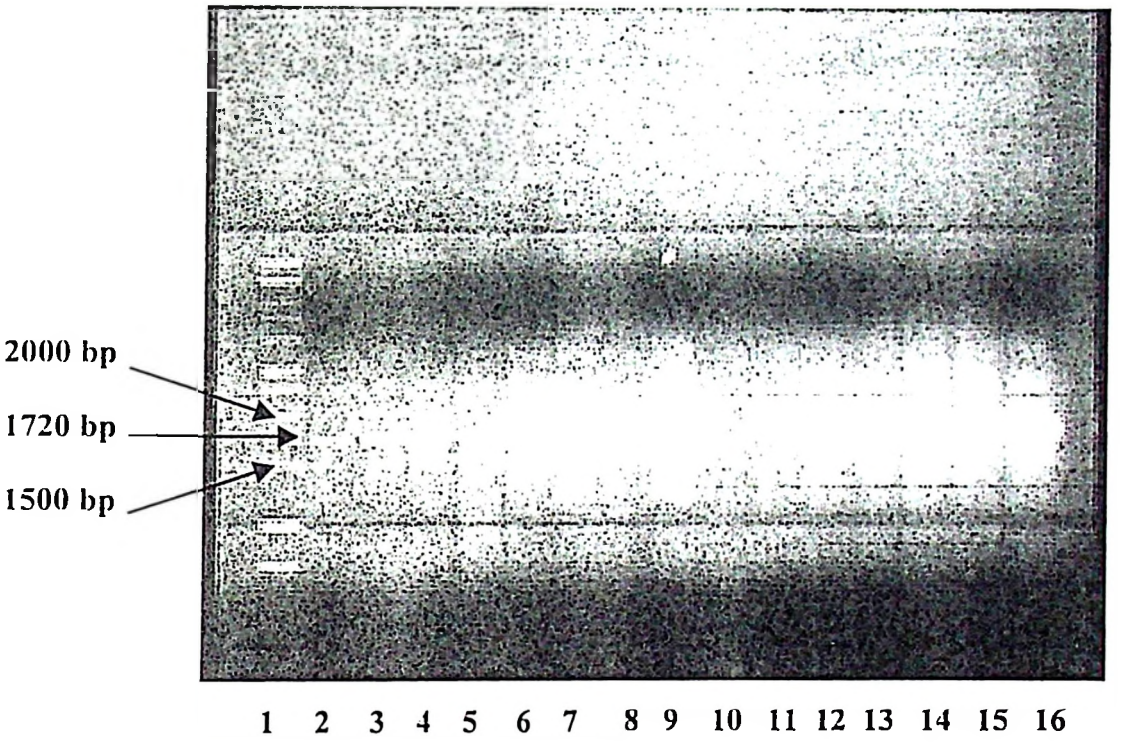

Figure 7. Agarose gel showing amplification products of *C. jejuni* as isolated from humans and chickens The desired product is at 1720. Lane 1is a molecular weight marker (1kb ladder), lanes 2, 3, 4, 5, 6, 7, 8 and 9 are chicken *C. jejuni* positive amplicons and lane 10 is a negative human *C. jejuni* samples while lane 11, 12, 13, 14, 15 and 16 are positive human *C. jejuni* amplicons.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Prevalence of thermophilic campylobacters in Morogoro

The study has demonstrated that campylobacter infections are prevalent in humans and chickens in the study areas; but none of the water samples was campylobacter positive. This suggests the zoonotic nature of the disease, which is likely to be caused by cross infection between chickens and humans. Similar results have been reported in other studies (Saleha *et al.*, 1998, Magistrado *et al.*, 2001, Harvey *et al.*, 2002, Ringoir and Karolin, 2003, Sandberg, 2002). For all water samples being negative in this study, similar findings were also reported by Diergaardt *et al.*, (2004) who didn't isolate any campylobacter from tap and ground water in South Africa. The predominant *Campylobacter* spp in human was *C. jejuni*, the aetiology for campylobacter enteritis in man. This indicates that campylobacter infection may be one of the major causes of enteritis of man in Tanzania as also reported in other studies (Lindblom *et al.*, 1995; Jiwa *et al.*, 1997). These findings are in agreement with the report by Saleha *et al.*, (1998), Aterkruse *et al.*, (1999) and Friedman *et al.*, (2004) who observed a significantly higher prevalence of *C. jejuni* than *C. coli* in human samples. It is noteworthy that *C. coli* was found at a low prevalence and *C. lari* was not isolated in all samples tested. This finding indicates that probably the two *Campylobacter spp* are not common in Morogoro as reported by Jiwa *et al.*, 1997. In another study by Lindblom *et al.*, (1995) reported very low prevalence of *C. coli* and *C. lari* in Dar es salaam, Tanzania. Likewise in other countries, isolation of *C. coli* and *C. lari* from campylobacter positive human enteritis is rare (Sandberg, 2002). Therefore *C. jejuni* is an established cause of enteritis and is frequently isolated from human infections.

5.1.1 Prevalence of thermophilic campylobacters in chickens

Findings from this study also showed that thermophilic campylobacters were prevalent in both local and broiler chickens at an overall prevalence of 70%. It has also been shown that *C. jejuni* was more prevalent than other species of thermophilic campylobacters. This species was isolated from different chicken types of different locations under different management systems. Previous similar studies in Tanzania also found a higher prevalence of *C. jejuni* in chickens than *C. coli* and *C. lari* is rarely found (Kazwala *et al.*, 1992a). The present study is in agreement with other studies elsewhere which reported *C. jejuni* to be the most prevalent thermophilic campylobacters in chickens (Saleha *et al.*, 1998; Harvey *et al.*, 2003). However, other studies reported higher prevalence of *C. coli* in broilers than *C. jejuni* (Marchados *et al.*, 1994; Pezzoti *et al.*, 2002). Further more other studies reported that there was no difference in prevalence between *C. jejuni* and *C. coli* (Madden *et al.*, 1998; Aquino *et al.*, 2002). The significant high isolation of *C. jejuni* from chickens in this study signifies the role of such birds as carriers for the infection to man. Among the avian species, poultry, in particular chickens are the most frequently colonized. They usually excrete large number of campylobacter leading to contamination of food in particular chicken carcasses which are poorly eviscerated, surface water, environment and infection to humans (Saleha *et al.*, 1998; Uyttndaele *et al.*, 1999).

The study showed that the difference in prevalence of *Campylobacter* spp in local chicken (71%) and broilers (69%) was statistically not significant ($P>0.05$). The local chicken are on risk probably due to exposure to carrier insects, vermin,

rodents, dirty water, kitchen wastes, contaminated environments and possibly other animals which increases the chances of infections (Adekeye *et al.*, 1989; Kazwala *et al.*, 1992a; Saleha *et al.*, 1998). On the other hand, broilers, which are always confined with a controlled feeding and watering thus making them more exposed to campylobacter infection especially under poor housing hygiene. This is because once the campylobacter infection is introduced in the flock chances of infecting each other are high within a short time.

According to FAO, (2002), the local chickens in Tanzania are estimated to be 26,065,000, which constitute up to 98.3% of the chicken population. Meat from local chickens is more preferred than that from broilers because of generally perceived taste (Maeda, 1994). Local chicken meat is served extensively in homes, restaurants, food vendors and in wedding ceremonies of which sometimes its poorly prepared and cooked. Risk factors for acquiring campylobacters include poor hygiene in the kitchen, inadequate cooking of food, eating raw food and lack of education to people on public health principles which lead to unhygienic food preparations (Kazwala *et al.*, 1992; Saleha *et al.*, 1998; Altekruse *et al.*, 1999). However, presence of animals, cockroaches, flies and rodents in the kitchen, uncovered garbage in cooking areas and lack of piped water are other factors responsible for the disease occurrence (Rao *et al.*, 2001).

The findings of the present study have shown that there was no significant difference between extensive management (prevalence=72%) and intensive system (prevalence = 67%). This tally with findings of other studies

carried out in Malaysia and Nigeria (Adekeye *et al.*, 1989; Saleha *et al.*, 1996). Extensive management system predisposes chicken to *Campylobacter* infection from the environment as in intensive system. In the later management system, once the infection is introduced in the flock, it spreads rapidly to almost all birds within a short period (Kazwala *et al.*, 1990. Most of the chicken houses visited in this study were dirty and the litters used were old, and therefore giving a higher chance of being sources of *Campylobacter* infections to chicken. For instance in five intensively managed flocks, the prevalence was 100% and these farms had a chicken house with a wet dirty floor with decomposing old litters mixed with chickens droppings. All these were evidences that suggest the equal exposure of intensively managed as that under extensive ones.

This study showed that local chickens from rural areas were significantly more infected with campylobacter than those from the urban areas ($P<0.01$). This again may be explained by scavenging nature of rural local chickens. Moreover, there is always no any intervention in terms of treatment or feeding in rural areas. Therefore the interaction of chickens, with other campylobacter carrier animals, contaminated environment and human is high in rural areas than in urban. Confinements during rain seasons, feed interventions and sometimes uses of antibiotics are common practices to urban chickens and might have contributed to the low isolation rate.

Although the minimum age of birds studied was 4 weeks, the isolation rate of *Campylobacter* spp in broiler chicken was found to increase with age of the birds. This tally with findings of other studies carried out in Ireland (Kazwala *et*

al., 1992b). Chicks can become colonized with campylobacter within one to seven days of hatching and the bacterial burden may reach up to 1.2 X 10^7CFU/g as the age increases (Saleha *et al.*, 1998). In the present study, it was found that the *Campylobacter* infection rate in chicken was increasing with age. For chickens, which were four weeks of age, the infection rate was 64.3% but it was observed to be significantly higher up to 93% in broilers with the age of 39 weeks. This was probably attributed by prolonged exposure to *Campylobacter* spp infectious agent. As chicken lived for a longer period in the contaminated environment, the possibilities of increased infection rate among individual chickens in the flock were high.

The other important finding in this study was observed on the type of samples used from chickens. The isolation of thermophilic campylobacters was significantly higher in intestinal samples than cloacal swabs ($P<0.001$). From these results, it is evident that intestinal positive samples give a good indication of whether the chickens are infected or not. Moreover, chances of false negative are high with cloacal swabs especially during early stages of infection and this may be a shortfall of the method. Beery *et al.*, (1988) found that the best site of intestinal colonization by campylobacter is the cecum. The mucin in the mucus layers in crypts of villi offers the best source of energy for campylobacter. Therefore during very early stages of infection in chickens, it is possible to recover campylobacters in the intestinal samples but rarely with cloacal swabs because the birds have not yet started shedding the bacteria in faeces as was also reported by Kazwala, *et al.*, (1992b). Therefore repeated cloacal sampling from live birds with duplicate

samples at specified intervals may be the best way of studying status of campylobacters in chicken flocks.

The significantly higher isolation of campylobacter in intestinal samples poses more threat to humans. Intestines give more risk of chicken carcass contamination during evisceration and consequently more likely to cause *Campylobacter* infections in humans. Moreover, when the intestines were been collected from the chicken slaughter centers, it was observed that low income people had a routine of collecting these intestines and be used as meat. This may predispose them more to campylobacter infection especially when intestines are not handled in hygienic way before cooking or when they are undercooked.

5.1.2 Prevalence of thermophilic campylobacters in water

Waterborne gastroenteritis caused by campylobacters has been reported in countries where unchlorinated surface and underground water are commonly used as drinking (Melby *et al.*, 1991; Kramer *et al.*, 1996). In Morogoro, almost all water treatment plants use surface and ground water as a raw water source (Mtaita, 2003 Personal communication). In this study, water samples from surface, underground and tap water from urban (Morogoro municipality) and rural areas were tested for *Campylobacter* spp. However, none of the water samples tested positive for these pathogens. In previous study (Jiwa *et al.*, 1997), 5% of water samples from Morogoro municipality were positive for Campylobacter spp. although the bacteria have not been isolated from chlorinated or unchlorinated underground water.

Finding from the present study suggests that water sources in Morogoro municipal and rural areas were free from campylobacters. However, interpretation of these results should be taken with caution given that during sample collection the water environments were suggestive for gross contamination. For example in some areas human and animal faecal material were seen on the ground during sampling suggesting that runoff of surface water after rain was likely to lead to faecal water contamination. *Campylobacter* spp are also reported to be common in natural water, such as streams, rivers, and lakes, due to discharges from wastewater treatment plants, pastures or agricultural runoff after rain, and through direct human, bird and animal contamination (Marja-Liisa *et al.*, 2003). The rivers, ponds and other sources of water for Morogoro have all the above mentioned risk factors for contamination. Reports elsewhere have shown that water as a vehicle contributes significantly to outbreaks of human campylobacter enteritis (Stern *et al.*, 1994; Stephen, 2000; Cools *et al.*, 2003; Diergaardt *et al.*, 2004). The fact that the study used detection methods with limitations, further studies need to be carried out before concluding the absence of campylobacters in water in Morogoro. Previous similar epidemiological studies in Finland indicated a strong association between consumption of infected drinking water and human campylobacter illness (Marja-Liisa *et al.*, 2003). Although *C. jejuni* were isolated in humans during the waterborne enteritis outbreaks, none was isolated in water samples.

The survival of campylobacters in aquatic environment is important both directly and indirectly in the causation of human campylobacteriosis. The ability of *C. jejuni* to survive in aquatic nutrient poor and well aerated environment may differ

between strains (Clive *et al.*, 1998). The non-isolation of campylobacters in this study may also have been related to low survival potential of campylobacters in aquatic environment. For example, it has been reported that dissolved oxygen tension, predation by and competition with other microorganisms reduce the survival times of *C. jejuni* and *C. coli* in water (Korhonen and Martikainen, 1991). Moreover, Survival of campylobacters in water is enhanced at low temperatures particularly around 4^0C (Cools *et al.*, 2003). The Morogoro climate with ambient temperature of up to 28°C and longer sunlight periods, may contribute to lower survival and concomitant isolation rates of *Campylobacter* spp. Ultraviolet rays and higher temperatures have been shown to destroy and even kill campylobacter cells (Sevill *et al.*, 2001, Terzieva and McFeters, 1991).

Campylobacters have been reported to adopt a viable but non-culturable (VBNC) state under stressful condition of prolonged exposure to nutrient poor aquatic environments (Rollins and Colwell, 1986), which might have been the case with samples from this study.

The choice of the media for campylobacter isolation from water either selective or non-selective is said to influence much on the results (Cools *et al.*, 2003). It has been shown that recovery of campylobacter from water on selective media containing inhibitory compounds to eliminate background bacteria is reduced when the bacteria are stressed or injured (Uyttendaele *et al.*, 2001). Therefore, stress effects to the cells seriously affect the recovery and enumeration of campylobacters from water when the isolation is done under conventional culture methods (Stephen *et*

al., 2000). In the present study, the media used was selective mCCDA. Therefore, the failure to isolate campylobacters from 146 samples collected might have been due to selectivity of the media.

The studies by Marja-Liisa and others (2003) stipulated that low or non detection of waterborne bacterial pathogens could be attributed to small sample size from each source and low sample volumes as opposed to what is recommended in most water quality control regulations. In big water bodies or in running water, usually there are dilution effects, which cause low water campylobacter concentration per unit volume up the level, which is below the detection limit for enumeration (10 CFU/ml). This occurs mostly when there is intermittent or transient water contamination and by the time the water samples are taken for analysis, chances of getting false negative results are high. This might have been another reason for not isolating any campylobacter while the risk factors of contamination were all suggestive.

Based on these findings, it is recommended that further longitudinal studies be conducted with justifiable sample sizes, large water sample volumes that should be collected repeatedly at different locations. However, because of the drawbacks of culture techniques, alternative methodologies, such as those based on molecular techniques may be considered.

5.1.3 Prevalence of thermophilic campylobacters in humans

The prevalence of *Campylobacter* spp of 9.3% in humans in Morogoro is significant especially in this era of HIV/AIDS. The campylobacter associated diarrhoea and bacteremia is reported to occur frequently in HIV/AIDS patients and the incidence of clinical manifestations is higher than in HIV negative patients, with substantial mortality and morbidity (Coker *et al.*, 2002). For example, Alterkruse and others (1999) reported incidence of campylobacteriosis in patients with HIV/AIDS was reported to be 39 times higher than the rate in the general population in United States. Moreover, HIV/AIDS cases have been predicted to double by the year 2020 and so is the number of campylobacter cases in Southern Sahara of Africa (Coker *et al.*, 2002).

This study has shown that children were significantly more affected by the disease. Blaser, (1997) found that in developed countries the highest age specific attack rates are in people less than 15 years as has been observed in this study. Normally, *Campylobacter* infection decreases as the age increases. Factors that may contribute to high infection rate in the young children may include poor hygiene and sanitation, eating habits and closeness to pet animals. During childhood in Tanzania children play excessively on the ground, consume any kind of edible materials thereon and prefer playing with dogs and cats, which are the good sources of campylobacter infections in humans. Moreover most children in the rural areas of developing countries are malnourished with poor immune status, which may increase the risk for infection (Coker *et al.*, 2002). Another possible explanation of high isolation rate in children may be attributed in part to susceptibility on first

exposure. The health and age of the host and *C. jejuni* specific humoral immunity from previous exposure influence clinical outcome after infection (Altekruse *et al.*, 1999, Friedman *et al.*, 2004). In adults, high level of exposure to the organisms early in life causes the gradual development of protective immunity. Nevertheless, *C. jejuni* infection contracted in early life cause substantial morbidity and mortality (Calva *et al.*, 1988).

The present study has shown no difference in *Campylobacter* infection rate between males and females (P>0.05). This showed that both males and females are equally predisposed to infection. Moreover, the study further showed that people screened in rural areas had no differences on campylobacter infection rate compared to those in urban areas (P>0.05). The relatively similar prevalence in urban and rural areas shows no difference in living style and therefore, both urban and rural people were equally predisposed to *Campylobacter* infection.

5.1.4 Molecular identification of *Campylobacter jejuni* isolates as a definitive method

Definitive identification of *C. jejuni* isolates from humans and chickens is needed for both epidemiological and therapeutic purposes. Routine diagnosis of campylobacteriosis in particular, which usually isolates *Campylobacter* strains more often, should be able to perform the identification in order to detect epidemiological changes. The results of the present study have shown that of all 243 *C. jejuni* isolates confirmed positive by biochemical tests, 25.9% were negative on molecular identification by PCR.

Identification of campylobacters is well known to be problematic principally because of their complex taxonomy, biochemical inertness, and fastidious growth requirements (On, 1996; On, 2001). Biochemical tests are useful for routine identification of campylobacters since they are cheap, rapid, and easy to perform than the molecular techniques and hence they have been adopted in most laboratories involved in the routine identification of *Campylobacter spp.* The main disadvantages of these tests are their relatively low discriminatory power, instability of phenotypic markers and may give false results. Steinbrueckner *et al.*, (1999) reported campylobacter strains, which were biochemically, determined as *C. coli* but proved to be hippurate negative *C. jejuni* by PCR. Likewise in this study, some isolates, which were positive on biochemical tests, were found negative on PCR, indicating the shortfalls of the tests. Positive reaction by *C. jejuni* to hippurate hydrolysis test is the only traditional biochemical test for differentiating the species from other *Campylobacter spp.* However, hippurate-negative strains of this species have been reported (Morris *et al.*, 1985; Totten *et al.*, 1987), and problems with false positive test results for non-*C. jejuni* species have also been described (Denis *et al.*, 1999; Steinbrueckner *et al.*, 1999). Therefore, biochemical differentiation is suitable for confirmation of the preliminary colonial identification of the isolate as *Campylobacter* spp. However, because of the limitations of biochemical tests (or phenotypic), there has been considerable interest in the development of molecular identification methods for *C. jejuni.* PCR tests are especially preferred because of their relative ease to use, higher discriminatory power and potential application in large-scale screening (Kricka, 1998).

The biology of the organism may also influence PCR results. *Campylobacter* is a taxonomically complex genus (On, 2001), and *C. jejuni* strains have a wide genetic diversity (Newell *et al.*, 2000; On, 2001). *C. jejuni* comprises two genetically distinct but highly related subspecies, *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doyley* and both are positive to hippurate hydrolysis test. Since the latter has no known animal reservoir and is infrequently observed in human disease, most attention is focused on *C. jejuni* subsp. *jejuni*, and most PCR tests are developed for *C. jejuni* subsp. *jejuni* strains (On and Jordan, 2003) including that of the present study by Nachamkin *et al.*, (1993). The inability of many *C. jejuni* PCR tests to recognize strains of the latter taxon suggest that the genetic difference between the two subspecies may contribute to PCR negative results to *C. jejuni* isolates (On and Harrington, 2000). The finding of 25.9% negative PCR results probably may suggest that some of the biochemically identified *C. jejuni* were of the subspecies *doyley*.

Moreover, Diergaart and others (2004) in their study in South Africa found that culture isolates on mCCDA morhologically and biochemically similar to *C. jejuni* were found to be *Arcobacter* on subsequent testing using their partial 16S rDNA gene sequence as a molecular identification method. Further more, the use of supplements in the enrichment broth during culture and the selective nature of mCCDA are supposed to be capable of inhibiting the growth of all non *Campylobacter* species (Bridson, 1995). The 25.9% *C. jejuni* negative isolates on PCR might not be real *C. jejuni* spp. Given these drawbacks of culture techniques and biochemical identification, alternative methodologies such as those based on the characteristics of these bacteria should be pursued.

5.2 Conclusion and recommendations

5.2.1 Conclusions

Based on the findings of the present study, the following conclusions are made: -

1. There was no sex predisposition to infection with campylobacters in humans. Both males and females were equally predisposed to infection.
2. Both urban and rural people were equally predisposed to *Campylobacter* infection showing that there was no difference in living style and feeding habit.
3. Human campylobacter infection occured more frequently in children than adult.
4. Extensive and intensive chicken management systems were not a contributing factor to campylobacter infections in chickens.
5. Local scavenging chickens from rural areas were significantly exposed to campylobacter infection than those in urban areas
6. Flock size has no role in spread of campylobacters in chickens
7. On screening for campylobacter infections in chickens, intestinal samples give a good indication of the flock status of campylobacter infection compared to cloacal swabs
8. There was no campylobacter isolation from water in Morogoro. This does not gurantee that Morogoro water is safe and free from campylobacters
9. A range of biochemical reactions should be used for the preliminary identification of suspect isolates of *C. jejuni*. However, the PCR identification method should be used to confirm the *C. jejuni* genotypically

5.2.2 Recommendations

From the conlusions drawn, it is therefore recommended that:-

1. The repeated cloacal sampling from live birds with duplicate samples at specified intervals may be the best way of studying status of campylobacters in live chicken flocks.
2. On studying campylobacter in water, a longitudinal study with justifiable sample sizes, large water sample volumes, collected repeatedly at different locations may conclude the status of the pathogens in Morogoro water.
3. Given the drawbacks of biochemical identification methods for campylobacters, alternative methodologies such as those based on the genotypic characteristics of these bacteria should be pursued.

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