MOLECULAR EPIDEMIOLOGY AND ANTIMICROBIAL RESISTANCE OF THERMOPHILIC *CAMPYLOBACTER* INFECTIONS IN HUMANS AND ANIMALS IN TANZANIA

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A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

2015

EXTENDED ABSTRACT

Members of the genus *Campylobacter* are known to cause more cases of human gastrointestinal illness than any other bacterium worldwide. The organisms exist as normal flora in the intestinal tracts of domestic and wild animals, more so in avian species. Humans acquire *Campylobacter* infections from contaminated animal products, particularly poultry meat, either directly or through cross-contamination of other food products. Human infections are mostly attributed to *Campylobacter jejuni* and *C. coli*, the former causing a larger proportion (85-90%) of all cases reported. In addition to infections, campylobacteriosis is also associated with the emerging threat of antimicrobial resistance as evidenced in isolates derived from different sources. An accurate picture of the epidemiology of infections caused by *Campylobacter* and other aetiological agents is lacking in developing countries due to the absence of regular surveillance programmes. Consequently the present study was conducted in Morogoro Municipality, Eastern Tanzania, to determine the molecular epidemiology and antimicrobial resistance of thermophilic *Campylobacter* isolates from humans and animals. Specific objectives were; 1) To establish the prevalence of thermophilic *Campylobacter* infections in humans and animals, 2) To determine the genetic relatedness of chicken and human derived thermophilic *Campylobacter* isolates using DNA-based typing methods, 3) To evaluate the antimicrobial resistance patterns in thermophilic *Campylobacter* isolates derived from humans and animals; and 4) To identify risk factors for thermophilic *Campylobacter* infections in humans. Stool samples were collected from 1195 human subjects; and fecal samples from 1511 farm animals, 466 laboratory animals and 112 wild birds (Indian house crows). Farm animals constituted chickens (n=1267), cattle (n=98), goats (n=81), sheep (n=57), horses (n=5) and camels (n=3); whereas laboratory animals were composed of guinea pigs (*Cavia porcelllus*, n= 30), mice (*Mus musculus*, n=160), rabbits (*Oryctulagus*

cuniculus, n=34) and rats (*Rattus rattus*, n=242). The Cape Town protocol was used for isolation of thermophilic *Campylobacter* from stool and fecal samples. *Campylobacter* isolates were identified by phenotypic and molecular techniques. The isolates were tested for resistance against several antimicrobial agents using the disc diffusion method. Risk factors for human infections with thermophilic *Campylobacter* were determined in an unmatched case control study. Selected human and chicken derived *Campylobacter jejuni* isolates were genotyped using flagellin A gene sequencing. In humans the prevalence of thermophilic *Campylobacter* was 11.4% (n=1195). Symptomatic (12.9%) and young individuals (16.7%) were more infected than asymptomatic (6.7%) and adults (10%), respectively. Most (84.6%) of the isolates were *C. jejuni* and the remaining were *C. coli*; and the difference was statistically significant at $p \leq 0.05$. Isolates had highest resistance (95.6%) for colistin sulphate and lowest for ciprofloxacin (22.1%). Proportions of resistant isolates for other antibiotics (azithromycin, erythromycin, tetracycline, cephalethin, gentamycin, nalidixic acid, ampicillin, amoxycillin, norfloxacin and chloramphenicol) ranged from 44.1% to 89%. Human infections with thermophilic *Campylobacter* were associated with young age; and consumption of chicken meat, barbecue and pre-prepared salad. In avians, thermophilic *Campylobacter* spp. were isolated from 44.0% and 20.5% of the sampled chickens and crows respectively. The majority of isolates from both chickens (87.6%) and crows (56.5%) were *C. jejuni* and the remaining were *C. coli.* The observed difference in proportions of *C. jejuni* and *C. coli* isolates was statistically significant ($p \leq 0.05$) in chickens but not in house crows. Chicken isolates had highest resistance to Colistin sulphate whereas crow isolates showed highest resistance to azithromycin and erythromycin. Lowest resistance was observed for gentamycin and ciprofloxacin for crow and chicken isolates respectively. Among chicken isolates significantly high proportions of *C. coli* were resistant to gentamycin, cephalothin, tetracycline, colistin sulphate and chloramphenical. On the other hand a high proportion *C. jejuni* isolates were resistant to

nalidixic acid. Crow derived *C. jejuni* had significantly higher resistance to nalidixic acid, cephalothin and ciprofloxacin than *C. coli* isolates from the same hosts. Among farm animals thermophilic *Campylobacter* were detected from 18 (31.6%) sheep and 3/5 (60%) of horses. Of the isolates 12 (57%) were *C. jejuni*; the remaining (43%) were *C. coli*. Of the laboratory animals 8 (26.7%) guinea pigs and 3 (1.2%) rats were colonized with *Campylobacter*. Four isolates from the guinea pigs were *C. jejuni* and the other 4 were *C. coli.* From the rats two isolates were *C. jejuni* and one was *C. coli*. The isolates showed high levels of antimicrobial resistance to erythromycin, norfloxacin colistin sulphate and nalidixic acid in ascending order; whereas low levels of resistance were observed for ciprofloxacin and gentamycin. Out of 55 sequenced isolates obtained from sporadic cases of human illness and different categories of chickens, nine different *flaA* types (7, 36, 41, 51, 61, 62, 64, 105 and 111) were detected. Both *C. jejuni* isolates from humans and chickens displayed a high degree of genetic diversity thereby suggesting weak clonality among the tested isolates. Genetic relatedness of some isolates from human and avian sources was however evident as on phylogenetic analysis some clusters contained both human and chicken *C. jejuni* isolates. The work contained in this thesis contributes significantly to the limited, available information on epidemiology and antimicrobial resistance of human and animal *Campylobacter* infections in Tanzania. For the first time the occurrence of *Campylobacter* infections in laboratory animals, antimicrobial resistance of human derived *Campylobacter* isolates, risk factors for human *Campylobacter* infections; and the population structure and relatedness of *Campylobacter jejuni* isolates from humans and chickens in the country are provided. The observed clusters containing isolates from human and avian sources confirm interspecies transmission of this zoonotic pathogen. Information on antimicrobial resistance of *Campylobacter* isolates derived from avian species in the country is also complemented. Control measures for colonization of animals and occurrence of infectons in humans with this particular bacterium species are

warranted. Similarly strategies to stem emergency and spread of antimicrobial resistant *Campylobacter* strains should be put in place.

DECLARATION

I, Erick Vitus Gabriel Komba do hereby declare to the Senate of Sokoine University of Agriculture that this thesis is my own original work done within the period of registration and that has neither been submitted nor concurrently being submitted in any other institution for a degree award.

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The above declaration is confirmed by

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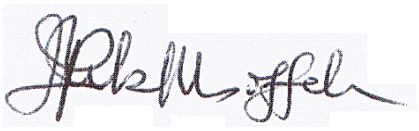
(1st Supervisor)



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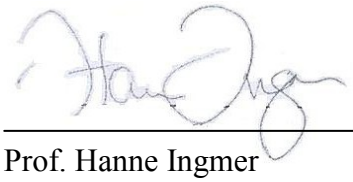

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ACKNOWLEDGEMENTS

I would first of all like to thank God, the almighty, for his blessings and giving me a chance to understand some of the basic principles of a part of his boundless creation. My sincere thanks go to my supervisors Profs. Robinson H. Mdegela, Peter L. Msoffe and Hanne Ingmer for their intellectual guidance, motivation and constructive criticisms throughout this research work. A gesture of appreciation goes to Lene Nørby Nielsen, Ewa Maria Kuninska and Sissel Skovgaard for their kind support, co-operation and hospitality during laboratory work in Denmark. Heart felt thanks are directed to the management of the Pest Management Centre of Sokoine University of Agriculture (SUA), Tanzania, for allowing me to use their laboratory facilities during my research. I am gratefull to the Staff at the centre; Dr. Georges Mgode, Mariam Kitojo and Ginethon Mhamphi to mention a few, for their friendliness and cooperation. My fellow PhD students under the same project; Abdul Katakweba, Athumani M. Lupindu, Kejeri Gillah and Maliwaza Mbwana deserve my special gratitude as they contributed in one way or another towards making this work a success. I would like to thank the chicken keepers around Morogoro Municipality for cooperation and accepting the use of their birds in this research. The managements of Morogoro Regional Hospital, Upendo Medical laboratory and SUA Health Centre are highly acknowledged for accepting serving as centres for human sampling. The Danish government is acknowledged for funding this project through the Danish International Development Fund in a project titled “Transforming Smallholder Livestock Farms into Profitable Enterprises (Livestock Enterprises)”. Lastly I would like to thank my lovely wife and children for putting up with me and making ends meet during the hectic times of my research. They were always loving, caring, supporting and patient.

DEDICATION

This PhD work is dedicated to my family; Bernadetha (my beloved wife); Mourine and Nourine (my twin daughters) and Elvis-Vitus Jr (My son). It is also dedicated to my mother Romana and my late father Vitus G. Komba.

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Status: Published in ***Tanzania Journal of Health Research***, Volume 15 (Supplementary 1) Doi: http://dx.doi.org/10.4314/thrb.v15i1.6

Paper II: Komba EVG, Mdegela RH, Msoffe PLM, Matowo DE and Maro MJ (2014). **Occurrence, species distribution and antimicrobial resistance of thermophilic *Campylobacter* isolates from farm and laboratory animals in Morogoro, Tanzania.**

Status: Published in ***Veterinary World*,** Volume 7. (Supplementary 8) pages 559-565.

Paper III: E. V. G. Komba, R. H. Mdegela, P. L. M. Msoffe, L. N. Nielsen and H. Ingmer (2015). Prevalence, Antimicrobial Resistance and Risk Factors for Thermophilic Campylobacter Infections in Symptomatic and Asymptomatic Humans in Tanzania

Status: Published in ***Zoonoses and Public Health***: doi: 10.1111/zph.12185

Paper IV: Erick V. G. Komba[a]*, Robinson H. Mdegela[a], Peter L. M. Msoffe[a] Lene N. Nielsen[b] and Hanne Ingmer. **Prevalence and antimicrobial resistance of thermophilic *Campylobacter* isolated from chickens and house crows (*Corvus splendens*) in Tanzania.**

Status: Manuscript (submitted to ***Veterinary Microbiology*** peer reviewed journal).

Paper V: E. V. G. KOMBA, R. H. MDEGELA, P. L. M. MSOFFE, G. MISINZO, H. N. TUNTUFYE, L. N. NIELSEN AND H. INGMER. **Diversities and similarities in *fla A* types of *Campylobacter jejuni* isolated from chickens and humans in Tanzania**

Status: **Manuscript (submitted to** *Epidemiology and Infection* **peer reviewed journal).**

DECLARATION

I, ERICK V. G. KOMBA, do hereby declare to the Senate of Sokoine University of Agriculture that the listed papers above that make this thesis summarize my independent work efforts, it is my own original work and will not be part of another thesis in the "Published Paper" format in any other institution.

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LIST OF ABBREVIATIONS

Ab	Absorbance
ADP	Adenosine dinucleotide
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immunodifficieny syndrome
AP	Alkaline phosphatase
BA	Blood agar
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
dNTP	Dinucleotide phosphate
EDTA	Ethylenediaminetetraacetic acid
et al	and others
flaA	flagellin gene subunit A
g	force of gravity
gDNA	Genomic DNA
Kb	Kilo base pair
kDa	Kilo Dalton
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MEGA	Molecular Evolutionary Genetics Analysis
MH	Muller Hinton
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multilocus sequence typing
MS	Mass spectrometry

MS	Microsoft
N	number
NIMR	National Institute for Medical Research
ORF	Open reading frame
PAGE	Polyacrylamide gelelectrophoresis
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RE	Restriction enzyme
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
SUA	Sokoine University of Agriculture
TAE	Tris Acetate EDTA
TEMED	N,N,N,N –Tetramethyl-Ethylenediamine
tRNA	Transport RNA
U	Unit
UK	United Kingdom
UV	Ultraviolet

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Taxonomy and History of *Campylobacter*

The history of *Campylobacter* species dates back to early 1900s when they were originally described as Vibrio-like bacteria and first recognized as a cause of infectious abortion in sheep (McFadyean and Stockman, 1913). At that time, they were classified as "*Vibrio fetus*" (*V. fetus*) because their morphology was similar to those of *Vibrio* species (Smith and Taylor, 1919). The organism was later found to be responsible for fertility problems in bovines and ovines; and also human disease i.e. unintended abortion. Several years later Jones and colleagues revealed that a "vibrio" called "*V. jejuni*" was associated with bovine dysentery (Jones *et al.,* 1931). The organism was then detected in blood culture of humans with gastroenteritis. Similarly during the mid-1940s, Doyle isolated another microaerophilic "vibrio", which he called "*V. coli*", from swine with diarrhea. He proposed that this organism was the cause of swine dysentery (Doyle, 1944). In 1886 Theodor Escherich identified a spiral shaped bacterium in stool sample from neonates with diarrhoea and this was the first description of *Campylobacter* (Skirrow and Butzler, 2000). In 1906 two British veterinary surgeons reported the presence of several novel "vibrio" species in a range of animals and in 1947 Vincent and colleagues isolated the organism (Vincent *et al.,* 1947). *Campylobacter* were initially classified as vibrio species due to spiral morphology; but due to their low DNA base composition, their non-fermentative metabolism and their microaerophilic growth requirements, Sebald and Veron postulated in 1963 a new genus, *Campylobacter* (Sebald and Veron, 1963). A later study by Butzler *et al.* (1973) raised the interest in *Campylobacter* by noting their involvement in human diarrheal disease (On, 2001). Members of the genus have experienced significant taxonomic changes such that the current genus taxonomy require further investigation for some parts (On, 2001; Debruyne *et al.,* 2005). The genus contains 15 species and six

subspecies and belongs to the epsilon class of proteobacteria which also comprises *Helicobacter*, *Arcobacter* and *Wolinella*. *C. jejuni* which is the most important member of the genus *Campylobacter* is divided into two subspecies; *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei*.

1.2 Biology and Biochemical Characteristics

Campylobacter species are Gram negative curved or spiral slender rods (Silva *et al.,* 2011) measuring 0.2 to 0.8 μm wide and 0.5 to 5 μm long. When two or more bacterial cells are grouped together, they form an "S" or a "V" shape of gull- wing. The organisms do not form spores and most are featured with a corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell (Silva *et al.,* 2011). Two species are however exceptional, one which is non motile (*Campylobacter gracilis*) and the other having multiple flagella (*Campylobacter showae*) (Debruyne *et al.,* 2005). Except for *C. gracilis* cytochrome oxidase activity is present in all other members of the genus (Vandamme, 2000; Silva *et al.,* 2011). Members of the genus *Campylobacter* have relatively small genomes (1.6–2.0 megabases) and can establish long term associations with their hosts (Young *et al.,* 2007); sometimes with pathogenic consequences (Young *et al.,* 2007). The organisms obtain energy from amino acids, or tricarboxylic acid cycle intermediates as they are not capable of fermenting or oxidizing carbohydrates (Vandamme, 2000). The organisms are catalase positive but urease negative. One member of the genus, *C. jejuni,* hydrolyzes hippurate, indoxyl acetate and reduces nitrate (Silva *et al.,* 2011). Most strains are resistant to cephalothin and susceptible to nalidixic acid (Silva *et al.,* 2011). Under unfavourable growth conditions, these microorganisms have the ability to form viable but non-culturable cells (VBNC; Portner *et al.,* 2007). Cappelier (1997) observed under laboratory conditions, that *Campylobacter* strains isolated from the soil around the broiler house may have been transformed into viable but non-cultivable forms and might have become cultivable after passing through the intestinal tract of

chickens. Many questions have been raised on whether non-culturability equates to non-viability (McKay, 1992), whether it is possible to convert the VBNC form to a culturable form (Stern *et al.,* 1994), and whether the VBNC form of *Campylobacter* exists (ACMSF, 2004).

1.3 Growth Requirements and Survival at Different Environmental Conditions

Campylobacter spp. are fastidious bacteria sensitive to environmental factors like oxygen, drying and heating (Kovačić *et al.,* 2013). The organisms are able to grow at temperatures between 30°C and 44°C (van Putten *et al.,* 2009), the optimum temperature being 42°C. Since they do not exhibit true thermophily (growth at 55˚C or above), Levin (2007) suggested that these organisms should be referred to as "thermotolerant". However, a study by De Cesare *et al.* (2003) revealed that *C. jejuni* survived for more than 4 h at 27˚C and 60-62% relative humidity on some common clean or soiled food contact surfaces. They are micro-aerophilic, growing best in an atmosphere with low oxygen tension (5% O_2, 10% CO_2, and 85% N_2) (Garénaux *et al.,* 2008). These characteristics reduce the ability of *Campylobacter* spp. to multiply outside of an animal host as well as in food during their processing and storage (Mahon *et al.,* 2007). Growth does not occur in environments with water activity (*a*w) lower than 0.987 (sensitive to concentrations of sodium chloride (NaCl) greater than 2% w/v), while optimal growth occurs at *a*w =0.997 (approximately 0.5% w/v NaCl). *Campylobacter* spp. are easily inactivated by heat treatments with their *D*-value being less than 1 min. Freezing–thawing also reduces the population of *Campylobacter* spp. (Stern and Kazmi, 1989). In pure cultures, *Campylobacter* spp. are normally inactivated by frozen storage at −15˚C in as few as 3 days (Stern and Kotula, 1982); however, freezing does not eliminate the pathogen from contaminated foods (Lee *et al.,* 1998). Hazeleger *et al.* (1995) revealed that aged *C. jejuni* cells survived the longest at 4˚C. The organisms do not survive below a pH of 4.9 and above pH 9.0 and grow optimally at pH 6.5–7.5.

1.4 Host Range

Campylobacter organisms have a broad host range inhabiting multiple animal hosts and environmental reservoirs (Waldenström *et al.,* 2010; Griekspoor *et al.,* 2013), but are thought to be particularly well adapted for survival in birds (Waldenström *et al.,* 2002; French *et al.,* 2005). They have been isolated from surface and ground waters, domestic and wild mammals, pet animals, rodents, insects and wild birds (Luechtefeld *et al.,* 1980; Kapperud and Rosef, 1983; Hald *et al.,* 2004; Miller and Mandrell, 2005; Strother *et al.,* 2005; Jacopanec *et al.,* 2008; Colles *et al.,* 2008). Animals usually carry high bacterial loads asymptomatically, suggesting commensal adaptations of the bacterium to their guts (Cody *et al.,* 2012). The most prominent source for human infections is consumption of chicken meat, either directly or through cross-contamination with other food items (Dingle *et al.,* 2002). Their growth characteristics at different temperatures (i.e. able to grow at 37–42 °C) and frequent isolation from domestic and wild birds suggest that it is primarily an avian bacterium.

1.5 Important Factors for Host Interactions

For colonization and causing infection (sometimes systemic) *Campylobacter* species express several virulence and survival mechanism factors. These include;

1.5.1 Adherence factors

Campylobacter spp. posses several proteins responsible for adhesion to the epithelial cells an important step for long term establishment and invasion. These include *Campylobacter* adhesion to fibronectin (CadF), fibronectin like protein A (FlpA), *Campylobacter* adhesion protein A (CapA), major outer membrane protein (MOMP), capsular polysaccharide (CPS), lipooligosaccharide (LOS) and protein Pei, Ellison and Blaser 1 and 4 (PEBI 1 and 4) (van Putten *et al.,* 2009; Naito *et al.,* 2010).

1.5.2 Invasion

Members of the genus *Campylobacter* have been shown in different studies to have the ability to invade gut tissues (Oelshlaeger *et al.,* 1993; Ketley, 1997). These bacteria gain entry into the tissues is through disrupted tight junctions of epithelial cells (MacCallum *et al.,* 2005; Chen *et al.,* 2006). They then get through the epithelial cell barrier via transcellular or paracellular movements. Following internalization the organisms reside within a membrane bound compartment with time changing into an unculturable state (Russel and Blake, 1994). Additional respiratory pathways are also believed to contribute to the ability of these bacteria to survive within epithelial cells. Invasion among *Campylobacter* species has also been associated with their ability to stimulate MAP kinases, leading to the production of the pro-inflammatory cytokine IL-8 (Watson and Galan, 2005). Contact with with epithelial cells causes the organisms to secrete *Campylobacter* invasive proteins, with one of them (CiaB, 73 kDa), known to be important for internalization (Konkel *et al.,* 1999). Its secretion utilizes the flagellum as type III like secretion system (Konkel *et al.,* 2004). The CiaB proteins have also been found to be secreted in the presence of chicken serum and mucus (Biswas *et al.,* 2007).

1.5.3 Flagellin

Campylobacter species are motile using polar flagella either at one or both ends allowing them to penetrate mucus barriers (Szymanski *et al.,* 1995). The flagellum consists of *flaA* and *flaB* genes all involved in motility thereby facilitating colonization (Nachamkin *et al.,* 1993). *FlaA* is additionally associated with autoagglutination (Guerry *et al.,* 2006). Both *flaA* and flaB are subject to antigenic as well as phase variation (Harris *et al.,* 1987).

1.5.4 Genetic variation and natural transformation

Several intragenomic mechanisms are known to be responsible for the extensive genetic

variation displayed by *Campylobacter* (Young *et al.,* 2007). They include gene duplication, deletion, phase variation, frameshifts and point mutations. Also involved are genetic exchange between strains and DNA uptake from the environment (de Boer *et al.,* 2002).

1.5.5 Biofilm formation and quorum sensing

Biofilm formation, a feature of many bacteria, is known to increase their survival in adverse conditions (Matz *et al.,* 2005); and enhance resistance to disinfectants and antimicrobials (Fux *et al.,* 2005). The phenomenon, enhanced by aerobicity (Reuter *et al.,* 2010) is considered to play a role in propagation of *Campylobacter* in poultry farms through water supply. There exists evidence on involvement of the flagellum in biofilm formation (Reeser *et al.,* 2007). Quorum sensing among *Campylobacter* species involves synthesis of autoinducers (AI-2) which play role in chicken colonization, motility, autoagglutination, biofilm formation, transcription of Cytolethal distending toxin and sensitivity to hydrogen peroxide (Reeser *et al.,* 2007; He *et al.,* 2008; Quiñones *et al.,* 2009). The activity of these extracellular signalling molecules has been demonstrated in food products suggesting environmental adaptation (Cloak *et al.,* 2002).

1.5.6 Toxin production

Campylobacter species are known to produce cytolethal distending toxin and haemolysin. Cytolethal distending toxin (CDT) having three components (CdtA, CdtB and CdtC) is expressed during colonization of their natural host, chicken (AbuOun *et al.,* 2005). The toxin causes cell cycle arrest thereby blocking cell division (Lara-Tajero and Galan, 2001). On the other hand hemolytic activity of *Campylobacter* species (and other bacteria) is meant to liberate heme for use as iron source (Stintzi *et al.,* 2008). Hemolytic activity of *Campylobacter* is associated with *ceuE* and *pldA* genes (Stintzi *et al.,* 2008).

1.5.7 Chemotaxis

Chemotaxisis, the movement of an organism towards or away from a chemical stimulus, has been demonstrated in several studies to be an important virulence determinant in *C. jejuni* and plays an important role in the colonization of mice. A study by Yao *et al.* (1997) found that a *cheY* null mutant of *C. jejuni* displayed a nonchemotactic but motile phenotype. The mutant displayed a three-fold increase in the adherence and invasion of INT 407 cells as compared to the wild type. The mutant was unable to colonize mice or cause symptoms in infected ferrets. The authors also found that *cheY* diploid isolates showed a chemotactic behaviour and a decrease in their *in-vitro* adherence and invasion capabilities. The isolates could colonize mice, but were unable to cause disease in infected ferrets. It was suggested that these bacteria migrated towards the mucus within the crypts, but were unable to penetrate the mucus (Konkel *et al.,* 2001).

1.5.8 Bile tolerance

Gram-negative bacteria do express lipopolysaccharide (LPS) which confers resistance to hydrophobic compounds such as bile salts which are destructive to cells. Although LPS can provide advantage to bacterial cells during their passage through the small intestine of warm-blooded animals, they can be destroyed in the presence of high concentration of bile. Several researchers have reported on bacterial tolerance to the bile salts including adaptation to lethal concentrations of bile salts leading to a significant cross–protection towards heat shock (Christopher *et al.,* 1982; Flahaut *et al.,* 1996). According to Flahaut *et al.* (1996) pretreatment with bile salts can result in the induction of a subset of heat-shock proteins which have a role in the observed cross–protection effect. Heat and bile salt shock can lead to the formation of proteins that help to protect the bacterial cell against external stress factors. It is suggested that low levels of bile might be important in regulating bacterial physiology and may facilitate the host–pathogen interaction (Pace *et al.,* 1997). It

has been shown that bile can expedite growth of nutrient–deprived *Vibrio parahaemolyticus* and enhance its virulence, size of capsule and adherence to epithelial cells. Culturing of *Campylobacter* with bile may increase its capability to adhere and invade epithelial cells (Pace *et al.,* 1997). Comparing the incidence of *C. jejuni* in the viscera, gallbladder and bile of broiler chickens, the liver was the organ of choice for *Campylobacter* infection (Carvalho *et al.,* 1997). Although, the frequency of *C. jejuni* in the bile was low (6.9%), it may indicate that *Campylobacter* can survive and may be multiply in high concentrations of bile. In line with this suggestion is the fact that 21% of the bile samples from chickens contained *Campylobacter* spp. (cited by Carvalho *et al.,* 1997).

1.6 Detection Techniques for *Campylobacter S*pecies

1.6.1 Direct methods

The techniques for direct detection of *Campylobacter* species from different samples include microscopy and polymerase chain reaction (de Boer *et al.,* 2010; de Boer *et al.,* 2013). The former is less sensitive and therefore results in underestimation of the true incidence of *Campylobacter* gastroenteritis (Bessede *et al.,* 2011; On, 1996). Therefore, molecular direct detection and identification of *Campylobacter* species is increasingly being used (Logan *et al.,* 2001); to provide a means for sensitive and rapid detection. The application of molecular techniques in detection of these organisms is however limited by inhibition caused by faecal constituents (Monteiro *et al.,* 1997); and the fact that they are labour intensive, involve high costs (Kulkarni *et al.,* 2002) and the need for sophisticated equipment. A further disadvantage of these methods is the lack of an isolate and hence the inability to perform simple antibiotic sensitivity testing and further characterization of the organisms for epidemiological purposes.

1.6.2 Indirect methods

Indirect detection of *Campylobacter* species from different samples involves the use of culture media either directly or following enrichment (de Boer *et al.,* 2010; de Boer *et al.,* 2013). The samples are cultured either on selective mediaor non selective media followed by phenotypic identification (de Boer *et al.,* 2013). The methods are useful for characterization of organisms at species and subspecies levels (Persson *et al.,* 2011). Culture methods using selective media are in favor of the detection of *C. jejuni* and *C. coli* and therefore give a biased view on the prevalence of *Campylobacteraceae* in infectious gastroenteritis (Lastovica, 2006; Bullman *et al.,* 2012). The techniques fail to isolate emerging *Campylobacter* spp. due to less than optimal conditions of temperature, atmosphere, and incubation time; and sensitivity of these other *Campylobacter* spp. to antibiotics used in selective media (Lastovica, 2006). Efficient isolation of *Campylobacter* including emergent spp. is currently achieved by the use of the Cape Town protocol. The technique involves sample filtration through a membrane filter onto an antibiotic-free blood agar plate and subsequent incubation at 37°C in an H_2-enriched microaerobic atmosphere. Lastovica *et al.* (2002) found that this protocol increases both the number of isolates and the number of *Campylobacter* spp. and species of the related genera *Arcobacter* and *Helicobacter*. With adoption of the protocol the authors were able, for the first time, to isolate *C. upsaliensis, C. concisus, C. hyointestinalis, C. rectus, Helicobacter fennelliae, Helicobacter cinaedi,* and *Arcobacter butzleri* from diarrheic stools and blood cultures of pediatric patients.

1.7 Identification and Typing of *Campylobacter*

The identification of *Campylobacter* species involves both phenotypic and genotypic techniques. The two groups of typing methods are often used to discriminate between bacteria at the species and subspecies level and are often used to identify pathogenic organisms (Eberle and Kiess, 2012). Most phenotypic and all genotypic typing methods

allow for the differentiation of *Campylobacter* isolates to the species and subspecies level. The identification of the commonly isolated species, *C. jejuni* and *C. coli*, by phenotypic methods particularly biochemical testing is hindered by the inertness of these species and by the fact that they differ only by their ability to hydrolyse hippurate (Razi *et al.,* 1981; Patton *et al.,* 1991; Debruyne *et al.,* 2008). The limitations of phenotypic methods in making routine identification of *Campylobacter* species has prompted the use of complementary molecular techniques to accurately differentiate between *Campylobacter* species.

1.7.1 Phenotypic methods

The classic techniques for differentiating isolates phenotypically are based on the presence or absence of biological or metabolic activities expressed by the organism (Arbeit, 1995). To identify *C. jejuni* and *C. coli* several phenotypical tests have been described. The most popularly used phenotypic methods to differentiate *Campylobacter* isolates include macromorphology, micromorphology, biotyping, Resistotyping, serotyping, Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and multilocus enzyme electrophoresis (Fitzgerald *et al.,* 2008; Murray, 2010). Although most of these methods lack discriminatory power, they are still used and are efficient in characterizing bacterial food-borne pathogens (Wiedmann, 2002).

1.7.1.1 Biotyping

Biotyping is the identification of bacterial isolates through the expression of metabolic activities. These metabolic activities include environmental tolerances and biochemical reactions. Species differentiation between *Campylobacter* using these methods is limited due to the relatively low activity in several conventional metabolic activities and due to special growth requirements. Biochemical tests for members of the genus include hippurate hydrolysis test, catalase test, oxidase test, indoxyl acetate hydrolysis and

production of H_2S (Fitzgerald *et al.,* 2008). The hippurate hydrolysis test has been used for differentiation between C. *jejuni* and *C. coli*. However, some hippurate negative *C. jejuni* isolates or false negative reactions make interpretation of the results of this test uncertain (Nakari *et al.,* 2008). Commercial tests for identifying *Campylobacter* species, like the bacterial identification test strip API Campy, are also available and have been a step forward in enhancing standardization, accuracy and reproducibility (Steinhauserova *et al.,* 2000). Special growth requirements for *Campylobacter* species include low oxygen tension (5% O_2, 10% CO_2, and 85% N_2) (Garénaux *et al.,* 2008) and the temperature range between 30°C and 44°C (Fitzgerald *et al.,* 2008; van Putten *et al.,* 2009), the optimum temperature being 42°C. Biotyping methods are easy to perform and are relatively inexpensive, making them an ideal method to quickly identify bacterial isolates for further testing.

1.7.1.2 Macromorphology

Campylobacter spp. are primarily be identified on the basis of their colonial morphology as described by Skirrow and Benjamin (1980). Colonies are typically small to medium (1-2 mm in diameter), flat, low convex, mucoid grey, glossy, sticky, swarming with metallic sheen appearance on fresh media. The colonies have smooth edges but irregular shape. The colonies can also be larger and spread to a continuous film ("swarming").

1.7.1.3 Micromorphology

Morphology by Gram staining and motility tests are also performed in primary identification of *Campylobacter* spp. Gram staining is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. Members of the genus *Campylobacter* are Gram-negative appearing as short, slender and curved (seagull wing shaped) or spiral shaped small rods (0.2-0.8 x 0.5-5 μm). On wet smear, under dark-field microscope at times 10 magnification, *Campylobacter* spp. display a characteristic rapid

rotating corkscrew-like or darting motility. Sometimes where daughter cells remain joined, long spiral forms are seen.

1.7.1.4 Resistotyping

This is the typing of *Campylobacter* isolates by the use of antibiotic discs. Intrinsically *Campylobacter* cells are either resistant or sensitive to cephalothin and nalidixic acid (Koneman *et al.,* 1997).

1.7.1.5 Serotyping

Serotyping has a long history of use in the typing of *Campylobacter*. Two serotyping methods are available for *Campylobacter* isolates, the Lior and Penner serotyping methods. The two methods differ on the basis of either using of heat-labile (HL) (Lior *et al.,* 1982) or of soluble heat-stable (HS) antigens (Penner and Hennessy, 1980). The Penner serotyping method was described in 1980 (Penner and Hennessy, 1980). It is well evaluated and accepted and is recognised as the "gold standard" for serotyping *Campylobacter* (McKay *et al.,* 2001). This method requires less time than other methods of serotyping (McKay *et al.,* 2001). The Lior serotyping method which was developed in 1981 based on slide agglutination (Lior *et al.,* 1981), is not much favoured as a typing method). The major disadvantages of both of these techniques are the high number of un-typeable strains and the time-consuming and technically demanding requirements. According to Mills *et al.* (1985), up to 36% *C. jejuni* isolates can be un-typeable. Other strains do cross react with multiple antigens for due to similarities in the core oligosaccharide structures (Moran and Penner, 1999). Also antiserum reagents required for serotyping are not widely available (Wassenaar and Newell, 2000). Serotyping alone does not exhibit a high discriminatory power, but could be improved in combination with a DNA-based method (Fussing *et al.,* 2007). Despite the drawbacks, Penner Serotyping has been accepted as a tool for characterising *Campylobacter* when used simultaneously with other methods such as MLST or PFGE (McTavish *et al.,* 2007).

1.7.1.6 Multi-locus enzyme electrophoresis

Multi Locus Enzyme Electrophoresis (MLEE) was first used to characterize *E. coli* isolates (Milkman, 1973). Aeschbacher and Piffaretti (1989) adopted the method to characterize *Campylobacter* spp. to determine the relationships of *C. jejuni* and *C. coli* populations between strains from nonhuman and human sources. In multilocus enzyme electrophoresis (MLEE), organisms are distinguished by variations in the electrophoretic mobility of different constitutive enzymes by electrophoresis under non-denaturing conditions (Wiedmann, 2002). Each intracellular enzyme represents a discrete, independent characteristic of an isolate; they differ in size, electrical charge, and conformation, resulting in slight alterations in migration rates across a gel. Enzyme activities are determined by locating their position on a gel after adding color-generating substrates to the enzymes (Arbeit, 1995; Downes, 2001). The variation of the electrophoretic mobility of an enzyme is dependent on mutations at the gene locus that causes amino acid substitutions, which alter the charge of the protein. Comparison studies found that MLEE is one of the methods with high discriminatory power (Bolton *et al.,* 1996). It has been shown to discriminate between isolates within the same species (Manning *et al.,* 2003) and between different species of the same genus (Meinersmann *et al.,* 2002). Drawbacks of the method include low reproducibility, difficulty of comparison of results between laboratories and its ability to generate untypeable alleles (Klena and Konkel, 2005).

1.7.1.7 Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS)

MALDI-TOF mass spectrometry (MS) is a powerful tool for the species and subspecies classification of a broad spectrum of Gram-positive and Gram-negative bacteria including *Campylobacter* and *Helicobacter*. It is a spectrophotometric method that detects a large spectrum of proteins and the technique is therefore able to discriminate between closely related species and to classify organisms at the subspecies level (Murray, 2010).

1.7.2 Genotyping methods

A number of different genotyping methods have been used for the typing of *Campylobacter* (Wassenaar and Newell, 2000). *Campylobacter* is genetically very diverse and the genome is susceptible to genomic instability. This can confound molecular epidemiological investigations over an extended time period (Hänninen *et al.,* 1998; Ridley *et al.,* 2008). Thus, combining two independent genotyping methods may have a greater discriminatory value than using only a single method (Wassenaar and Newell, 2000). Available genotyping methods for *Campylobacter* include; polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE), *flaA* PCR restriction fragment length polymorphism (RFLP), sequencing of flaA gene, amplified fragment length polymorphism (AFLP), ribotyping and multilocus sequence typing (MLST) (Ragimbeau 2008; On *et al.,* 2008; Pavlic and Griffiths, 2009; Eberle and Kiess, 2012).

1.7.2.1 Polymerase chain reaction (PCR)

Polymerase chain reaction, also known as molecular photocopying, is a typing method that amplifies target segments of DNA (Atlas and Bej, 1994). By using oglionucleotides (primers) that bind to the target segments of DNA at the 5′- and 3′-ends, DNA polymerase can be directed to the targeted site for amplification. With repeatitions the process leads to an exponential increase in the total amount of DNA. The technique provides a rapid and highly sensitive method for the detection of species specific DNA sequences. The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA (Dieffenbach and Dveksler, 2003). Several variations to the original PCR technique have been developed that are useful in identifying *Campylobacter* spp. These include reverse-transcriptase PCR, multiplex PCR and quantitative real-time (QRT)-PCR (Eberle and Kiess, 2012). Many studies involving *Campylobacter* spp. have used these variations of PCR to identify and

differentiate isolates. These techniques are easy to reproduce, highly discriminatory and available in most laboratories. Although these techniques can be expensive, they are still one of the most commonly used genotypic methods for typing *Campylobacter* spp (Eberle and Kiess, 2012). They are frequently used in screening programmes (Linton *et al.,* 1997; Lübeck *et al.,* 2003). A number of PCR assays have been developed and used to detect and identify *Campylobacter* (Linton *et al.,* 1997; Miller *et al.,* 2007). The presence of inhibitory compounds may affect the PCR reaction and give false negative results. The use of an internal standard as a control of the PCR reaction increases the reliability of the technique (Denis *et al.,* 2001). It is important to be aware that the PCR method may detect dead as well as viable bacteria (Waage *et al.,* 1999). Real-time PCR assays are becoming of increasing importance since they assess the level of contamination with a given pathogen (Lübeck *et al.,* 2003). Real-time PCR is based on the principles of conventional PCR but with continuous monitoring of product accumulation (Higuchi *et al.,* 1992).

1.7.2.2 Pulsed-field gel electrophoresis (PFGE)

This method was developed in 1983 by Schwartz *et al.* (1983) to overcome the inability of conventional agarose gel electrophoresis to separate and resolve of large DNA molecules (<30 kb) (Townsend and Dawkins, 1993). It involves the digestion of genomic DNA into pieces with restriction enzymes. A pulsing electric field applied across the gel drives the DNA pieces into the gel over a period of hours. The most common PFGE method used is contour-clamped homogeneous electric field electrophoresis (CHEF) (Chu and Gunderson, 1991). CHEF was developed by Chu *et al.* (1986) to resolve large DNA molecules. The CHEF-PFGE current field is arranged in a hexagonal shape, with electrodes delivering pulses from an angle of 120°. The current pulsed at 120° angles pushes larger DNA molecules through the gel matrix in a uniform manner, allowing better resolution than other electric fields generated using with 60-90° angles (Chu and

Gunderson, 1991). The smallest pieces slip through the pores of the agarose gel more quickly. So the pieces are separated as distinct bands in the gel, based on the size. The resulting pattern of bands is the DNA “fingerprint". PFGE has proven to be useful and discriminatory for investigation of outbreaks of *C. jejuni* (Fitzgerald *et al.,* 2001). PFGE has been shown by many studies to be an effective tool in molecular epidemiology (Kuusi *et al.,* 2005; Sails *et al.,* 2003) and is generally recognised as the gold standard for tracking outbreaks of pathogenic bacteria due to its high discriminatory power (Nielsen *et al.,* 2000). It has been used extensively for typing *Campylobacter* in studies associated with poultry (Posch *et al.,* 2006; Klein *et al.,* 2007; Lienau *et al.,* 2007). The disadvantages of PFGE include high costs, time requirement and is a labour intensive method of indirect genotyping the whole genome of bacterial isolates (Fiett *et al.,* 2004). The method requires skilled operators to get consistent, reproducible results. When different methodologies are used in different laboratories and between studies PFGE profiles are often unable to be compared in a way hampering molecular epidemiological investigations into the spread of pathogenic bacteria between countries (Swaminathan *et al.,* 2001). Distinct electrophoretic conditions may influence obtained profiles, different restriction enzymes are used to digest DNA and furthermore some *Campylobacter* isolates cannot be typed by PFGE (Wassenaar and Newell, 2000). The Pulsenet standard PFGE method was created to allow inter-laboratory molecular epidemiological investigations, particularly concerning food borne bacterial diseases (Swaminathan *et al.,* 2001). The widely-used restriction enzyme SmaI generates four to ten fragments. KpnI digest has more fragments than SmaI and is thus more discriminatory and it is often used as a secondary enzyme but has also been suggested as a primary choice for epidemiological studies (Michaud *et al.,* 2001).

1.7.2.3 *FlaA* PCR restriction fragment length polymorphism (*RFLP)*

The *Campylobacter* flagellum has been identified as a virulence factor which enables movement towards, and colonisation of the mucous layer (Young *et al.,* 2007). It is

complex and its proteins are encoded by a major flagellin gene, *flaA*, and a minor flagellin gene, *flaB* (Guerry *et al.,* 1991). The two subunits are different from one another and highly conserved making the flagellin gene locus suitable for detection by RFLP from PCR products. In flagellin gene typing, the *fla*A gene is first amplified by PCR and the amplified DNA is then digested with specific restriction endonucleases (Nachamkin *et al.,* 1993). Restriction fragment length polymorphism probes hybridize to the digested fragments, and the fragments are viewed by using agarose gel electrophoresis, giving distinguishable patterns. The discriminatory power of flagellin gene typing is determined by the type of restriction enzyme used (Fitzgerald *et al.,* 2001). Although *flaA* PCR-RFLP is quick, reproducible and most isolates are typeable, the inherent genetic instability observed at this locus (Wassenarr *et al.,* 1995; Harrington *et al.,* 1997) suggests that *flaA* PCR-RFLP should be used as a confirmatory technique as opposed to a sole technique used in epidemiological grouping of isolates (Harrington *et al.,* 1997). The use of the technique for typing has also been questioned due to the intra- and intergenomic recombination within the flagellin genes.

1.7.2.4 Sequencing of flaA gene

Analysis of the DNA sequence variation of the short variable region (SVR) of the flagellin gene A has proven to be a useful typing method for *Campylobacter* allowing relatively high sample throughput at reasonable cost (Meinersmann *et al.,* 2005). Sequence-based *flaA* typing avoids difficulties inherent in methods that rely on restriction fragment length polymorphisms of the flagellin genes (Wassenaar and Newell, 2000). Since *flaA*-SVR is limited to analysis of variations in a single and highly variant gene, long-term time–location trends cannot be examined. However, this method can be very useful for discriminating more closely related *Campylobacter* isolates (Hiett *et al.,* 2007). Several authors have found the *flaA*-SVR typing method useful in their epidemiological studies concerning *Campylobacter* from different sources (Ragimbeau *et al.,* 2008; Wassenaar *et al.,* 2009).

1.7.2.5 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP), developed by Vos *et al.* (1995), is a technique capable of genotyping genomic DNA from any origin. It is based on selective amplification of restriction fragments of chromosomal DNA. Target DNA is digested with two or more restriction enzymes. The digested DNA is then ligated with double-stranded adaptors and amplified using primers complementary to the adaptors and restriction site sequences. Subsets of the restriction fragments are amplified a second time using selective primers that incorporate a label onto the products. The products are separated using electrophoresis and denaturing polyacrylamide gel before being visualized (Vos *et al.,* 1995). The limitation of AFLP is that the system is technically demanding and requires expensive equipment to run. There are also problems associated with analysis due to complex banding patterns, which are PCR-based and are prone to variation. Another disadvantage of this method is that it requires the organism of interest to be pure enough as the DNA from other organisms can disturb the AFLP pattern. However, this technique is sensitive, reproducible and highly discriminatory and has been broadly applied for the identification and typing of *Campylobacter* in diverse animal and environmental studies including poultry (Duim *et al.,* 2001; Schouls *et al.,* 2003; Alter and Fehlhaber, 2003). The other advantage that AFLP provides is that data can be analyzed to determine the genetic relatedness among bacterial strains as well as identify or type strains. The method is also able to generate typing of any DNA regardless of origin and complexity (Vos *et al.,* 1995).

1.7.2.6 Ribotyping

Ribotyping is a ribosomal (r) RNA approach for the identification of bacterial isolates (Williams *et al.,* 1998). It involves the cleaving of genomic DNA with a frequently cutting restriction enzyme, subsequent hybridization with a labelled ribosomal gene probe, and visualization of the resulting labelled patterns (Grimont and Grimont, 1986). Although

ribotyping has a high level of typeablility for *Campylobacter* spp., its low number of ribosomal genes gives it low discriminatory power (Wassenaar and Newell, 2000; Eberle and Kiess, 2012). The other limitation of the method is that it is tedious, time-consuming, and expensive, making it unsuitable for routine genotyping (Wassenaar and Newell, 2000; On *et al.,* 2008). Automation has made ribotyping more useable as it helps decrease labor and increase sensitivity in identifying food-borne pathogens. The systems combine the molecular steps into a single efficient machine, making the testing method faster and more reliable (Pavlic and Griffiths, 2009). With *Campylobacter* spp., AR systems are used in conjunction with other genotyping methods to amplify the discriminatory power of the typing method.

1.7.2.7 Multilocus sequence typing

Multilocus sequence typing (MLST) was initially designed for *Neiserria*, but has been used for the characterisation of many bacterial populations including *Campylobacter* species (Miller *et al.,* 2005; Miragaia *et al.,* 2007; Tanabe *et al.,* 2007). It is a sequence-based typing method based on partial sequence information at seven housekeeping loci (Maiden *et al.,* 1998). It is a subtyping method that assumes that housekeeping genes are not under selective pressure (Maiden *et al.,* 1998). The multiple unlinked housekeeping genes were chosen for this subtyping scheme with the expectation that if recombination occurred at one locus then the increased number of gene loci would lessen the likelihood incorrect assumptions about bacterial relationships would be made (Maiden *et al.,* 1998). Genetic variation observed in these genes should be less than genes associated with selective pressures such as the human immune system (Maiden *et al.,* 1998). For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). MLST has been proven useful for population

characterization, lineage identification, and epidemiology of *C. jejuni* (Dingle *et al.,* 2001, Kärenlampi *et al.,* 2007). A previously proposed MLST system for *C. jejuni* (Dingle *et al.,* 2001) has since been updated to include *C. coli* with both species using the same gene fragments. The gene loci suggested for *C. jejuni* were: aspA (aspartase), glnA (glutamine synthetase), gltA (citrate synthase), glyA (serine hydroxy methyl transferase), tkt (transketolase), pgm (phospho glucomutase) and uncA (ATP synthase alpha subunit) (Dingle *et al.,* 2001, http://pubmlst.org/.). The unlinked gene loci chosen were distributed throughout the *C. jejuni* chromosome and provided a suitable level of variation within the alleles (Dingle *et al.,* 2001). Dingle *et al.* (2001) based their primer design on the previously published *C. jejuni* genome (Dingle *et al.,* 2001). The primers used amplified a 450-550 bp gene fragment dependant on the gene loci (Dingle *et al.,* 2001). The gene fragments from each of the seven loci are sequenced and the derived alleles given a number based on when they were identified (Dingle *et al.,* 2001). The seven gene loci form a seven-integer sequence (ST), which is also given a number based on when it was identified. The MLST scheme has since been extended to characterise a wider range of *Campylobacter* species including *C. lari*, *C. upsaliensis*, *C. fetus* and *C. helviticus* (Miller *et al.,* 2005).The method is highly reproducible, scalable, and data are electronically portable between laboratories, enabling comparison of isolates via the internet MLST appears best in population genetic study but it is expensive. MLST has been known to overcome the inherent difficulties associated with Multi-Locus Enzyme Electrophoresis *(*MLEE*),* providing accurate, reproducible and importantly portable evidence of bacterial relationships (Maiden *et al.,* 1998). Due to the sequence conservation in housekeeping genes, MLST sometimes however lacks the discriminatory power to differentiate bacterial strains, which limits its use as a singular subtyping tool in outbreak investigations (Clark *et al.,* 2005; Fakhr *et al.,* 2005). The discriminatory ability of MLST is less than PFGE.

1.8 Campylobacteriosis in Humans

1.8.1 Sources of infections and modes of transmission

The understanding of sources and means of transmission of *Campylobacter* is an essential factor in order to reduce the incidence of *Campylobacter* related gastroenteritis in man (Engberg *et al.,* 2001). Humans are exposed to *Campylobacter* spp. in a range of sources via both food and environmental pathways. Many reports found that, human infections occur principally via the consumption of contaminated, undercooked meat (especially poultry) and cross-contaminated foods (McCarthy *et al.,* 2007; Davies and Conner, 2007; Gormley *et al.,* 2008; van Asselt *et al.,* 2008; Janssen *et al.,* 2008; Denis *et al.,* 2008; Lyhs *et al.,* 2010). Poultry products are therefore regarded as important transmission vehicles to humans (Griekspoor *et al.,* 2013). Other modes of transmission include direct contact with contaminated animals or animal carcasses (Figueroa *et al.,* 2009), direct and indirect contact with animals, people and environment (Horrocks *et al.,* 2006; Doorduyn *et al.,* 2010; Cody *et al.,* 2012; Friesema *et al.,* 2012) and consumption of contaminated water (Sopwith *et al.,* 2008; Figueroa *et al.,* 2009). Human exposure to these sources is spatially heterogeneous and therefore the spatial pattern of infection is heterogeneous (Bessell *et al.,* 2010).

1.8.2 Risk factors for human infections

Previously identified risk factors for *Campylobacter* enteritis include international travel; ingestion of poultry, red meat, unpasteurized milk, and untreated water; use of contaminated water; contact with pets and farm animals; eating in restaurants and from fast food outlets; use of antimicrobial drugs and acid-suppressing medication; and diabetes (Eberhart-Phillips *et al.,* 1997; Neal and Slack, 1997; Effler *et al.,* 2001; Rodrigues *et al.,* 2001; Neimann *et al.,* 2003; Kapperud *et al.,* 2003; Friedman *et al.,* 2004; Stafford *et al.,* 2007; Unicomb *et al.,* 2008; Gallay *et al.,* 2008; Gormley *et al.,* 2008; Sopwith *et al.,* 2008; Danis *et al.,* 2009). Numerous studies implicate chicken consumption as an important risk factor for *Campylobacter* enteritis (Ikram *et al.,* 1994; Adak *et al.,* 1995;

Eberhart-Phillips *et al.,* 1997; Evans *et al.,* 2003; Friedman *et al.,* 2004; Michaud *et al.,* 2004; Stafford *et al.,* 2007). However, some studies report associations specifically with eating undercooked or raw chicken (Ikram *et al.,* 1992; Eberhart-Phillips *et al.,* 1997; Stafford *et al.,* 2007); others, with any type of chicken; and some authors found chicken consumption to be protective (Adak *et al.,* 1995; Eberhart-Phillips *et al.,* 1997). Other studies have found increased risks only with consumption of commercially prepared chicken (Evans *et al.,* 2003; Friedman *et al.,* 2004; Michaud *et al.,* 2004). Furthermore, the consumption of undercooked beef was identified as a risk factor in a recent French study, but the consumption of chicken was not (Gallay *et al.,* 2008). Reports also exist associating variations in *Campylobacter* incidences with the age and gender of the individual e.g. male children are 1.5 folds more at risk of infection than their female counterparts (Strachan *et al.,* 2008; Unicomb *et al.,* 2008).

1.8.3 Clinical presentation and sequelae

Contrary to mammals and birds in which gut colonization is asymptomatic (Haddad *et al.,* 2012), many *Campylobacter* infections in humans cause clinical disease. This is due to the fact that the infections elicit an inflammatory response (Hu *et al.,* 2006; Borrmann *et al.,* 2007; Zheng *et al.,* 2008). Some human infections are however asymptomatic. The clinical presentation varies profoundly ranging from mild watery to severe inflammatory diarrhoea which may be complicated by post infectious sequelae such as Guillain-Barre´ Syndrome, reactive arthritis and irritable bowel syndrome (Doorduyn *et al.,* 2008; Haagsma *et al.,* 2010; Silva *et al.,* 2011; Havelaar *et al.,* 2012) with considerable health and economic consequences (Haagsma *et al.,* 2010; Havelaar *et al.,* 2012). Specifically signs of infection in humans include watery to bloody diarrhoea, fever, headache, abdominal pain and general myalgia, nausea, presence of leukocytes and red blood cells in faeces and/or vomiting (Young *et al.,* 2007; Heymann, 2008; Haddad *et al.,* 2012; Xia *et al.,* 2013). These symptoms may persist for a week or even longer (Butzler and Oosterom, 1991). The

frequently isolated species as a cause of human diarrheal disease is *C. jejuni* contributing to more than 80% of all reported cases (Moore *et al.,* 2005; Fisher *et al.,* 2006).

1.8.4 Treatment

Most of campylobacteriosis cases involving immuno-competent human individuals are self-limiting and therefore antimicrobial therapeutic intervention is rarely indicated (Moore and Goldsmith, 2001; Elviss *et al.,* 2009). Severe/systemic and recurrent cases, cases involving vulnerable individuals (immuno-compromised patients and the elderly) and any other predisposing complication may however require treatment (Moore and Goldsmith, 2001; Elviss *et al.,* 2009). Erythromycin and ciprofloxacin are often the drugs of choice (McDermott *et al.,* 2004). Antimicrobials are known to reduce the period of fecal shedding although they have no large impact on the duration of disease symptoms (Williams *et al.,* 1989). Some clinical benefit has however been observed in early treated cases (Salazar-Lindo *et al.,* 1986). The current concern is an increase in resistance to antimicrobials in both human and animal isolates (Kist, 2002; Van Pelt *et al.,* 2004).

1.8.5 Human campylobacteriosis in developing countries

Though not routinely searched for in the laboratory investigations for etiology of diarrhea (Bonkoungou *et al.,* 2013), several studies have reported *Campylobacter* infections in humans in developing countries (Lindblom *et al.,* 1995; Gasco´n *et al.,* 2000; Brooks *et al.,* 2003; Mdegela *et al.,* 2006; Brooks *et al.,* 2006; Mshana *et al.,* 2009; Beatty *et al.,* 2009; Ewnetu and Mihret, 2010; Jacob *et al.,* 2011; Bonkoungou *et al.,* 2013). Consistently the studies have reported higher prevalence in young individuals, particularly those under the age of 5 years, as compared to adults. Using bacterial culture technique, the prevalence of *Campylobacter* infection in young children with diarrhoea in Sub Saharan Africa has been estimated to range from 1.5% to 18%. It has been mentioned previously that in these countries campylobacteriosis is a disease of young children (Khalil

et al., 1993; Molbak and Hojlyng, 1988). This observation can however partly be contributed by the fact that in developing countries children are urgently taken to health facilities even for mild symptoms as opposed to adults. In some of these countries male preponderance has been shown in human *Campylobacter* infections (Wamola *et al.,* 1983; Chuma, 2008). However a study carried out in Mombasa, Kenya (Shimotori *et al.,* 1986), found no significant differences in prevalence between males and females. Studies involving symptomatic and asymptomatic human subjects found no significant differences in prevalence of *Campylobacter* infections between the two groups (Wamola *et al.,* 1983; Mégraud *et al.,* 1990; Lindblom *et al.,* 1995; Gasco´n *et al.,* 2000; Shapiro *et al.,* 2001). However studies involving children under the age of 18 months found a high prevalence of *Campylobacter* infection in symptomatic subjects as opposed to asymptomatic counterparts (Ringertz *et al.,* 1980; Georges-courbot *et al.,* 1987; Steele *et al.,* 1988; Haq and Rahman 1991; Lindblom *et al.,* 1995). The reason for a high prevalence of *Campylobacter* infection in symptomatic subjects under 18 months as opposed to asymptomatic counterparts is induced immunity (Blaser *et al.,* 1985). A study in Burkina Faso revealed occurrence of *Campylobacter* nearly exclusively in symptomatic children (Bonkoungou *et al.,* 2013). In most of the studies carried out in developing countries, *C. jejuni* was the dominant species isolated and *C. coli* was less frequently isolated, with the ratio of *C. coli* to *C. jejuni* varying considerably among studies as well as among countries. According to Tadesse *et al.* (2011) *Campylobacter* species that are most commonly associated with human illness are *C. jejuni* and *C. coli*. The authors further point out that *C. jejuni* is responsible for up to 90% of the cases of human infections, whereas *C. coli* is responsible for the majority of the remaining human cases.

1.8.6 Human campylobacteriosis in developed countries

Campylobacteriosis is the most common food-borne bacterial cause of gastro-intestinal illness in the developed world (Friedman *et al.,* 2000). In some of these countries the disease is considered notifiable (Sears *et al.,* 2011). Unlike in developing countries where

the organism is detected mostly in young individuals (Khalil *et al.,* 1993; Molbak and Hojlyng 1988); in industrialized countries infections increase throughout childhood (Amar *et al.,* 2007); and are more common in young adults (Kaijser, 1988). A study by Bellido-Blasco *et al.* (2006), however, recorded a higher prevalence of *Campylobacter* spp. among children aged less than five years. Similar to observations in developing countries the majority of human campylobacteriosis cases in developed countries are caused by *C. jejuni* and *C. coli* (Gillespie *et al.,* 2002).

1.9 *Campylobacter* Infections in Animals

Campylobacter spp. are carried in the intestines of many wild and domestic animals, particularly avian species including poultry (Silva *et al.,* 2011). Intestinal colonization in most of these animals results in a commensal relationship thereby producing carriers (Humphrey *et al.,* 2007; Silva *et al.,* 2011; Hermans *et al.,* 2011). Some studies have however associated *Campylobacter* infections in wild animals with disease manifestations (Waldenström *et al.,* 2010; Ngotho *et al.,* 2006). While a study by Waldenström *et al.* (2010) noted a reduction in body mass in infected wild birds (European robins); Ngotho and colleagues (2006) linked *Campylobacter* infection to a fatal outbreak in the vervet monkeys. Several other previous research reports showed that *Campylobacter* spp. were associated with disease outbreaks in a variety of semi-wild and wild animals, with negative impacts on their health, productivity and welfare (Taema *et al.,* 2008). In avians, particularly chickens, the principal site of colonization is within the mucus overlying crypts of the caeca, large intestine and cloaca (Beery *et al.,* 1988). It is suggested that horizontal transmission plays a major role in the spread of C. jejuni within and between poultry flocks (Sahin *et al.,* 2002). Probable sources of infection include colonized birds, contaminated faeces, feed, litter, water, equipment and transport vehicles, or even wild birds and insects (Shanker *et al.,* 1990; Berrang *et al*., 2004).

1.10 Seasonal Trends in *Campylobacter* Infections

Understanding seasonal trends, a characteristic of many infectious diseases (Fisman, 2007; Naumova *et al.,* 2007), is important for improving disease surveillance, analysis of the seasonal differences in the risks for contracting disease as well as planning and implementing control strategies (Lal *et al.,* 2012). Evidence of seasonal peaks in human *Campylobacter* infections has been observed in several European countries (Thompson *et al.,* 1986; Hudson *et al.,* 1999; Nylen *et al.,* 2002; Patrick *et al*., 2004; Kovats *et al*., 2005; Meldrum *et al*., 2005; Sopwith *et al.,* 2006; Nachamkin *et al.,* 2008; McCarthy *et al.,* 2012). In these temperate countries seasonal peaks in human *Campylobacter* infections has been consistently observed during late spring or early summer (Nachamkin *et al.,* 2008; Thompson *et al.,* 1986; Kovats *et al.,* 2005; Nylen *et al.,* 2002) with some variation in this time point among countries (Kovats *et al.,* 2005; Louis *et al.,* 2005; Nylen *et al.,* 2002). The observed seasonality may be an attribute of increased *Campylobacter* prevalence in reservoirs for infection and seasonal changes in human behaviour affecting exposure (Nylen *et al.,* 2002). Though not universal (Humphrey *et al.,* 1993), some studies have found *Campylobacter* infections in various reservoirs (particularly poultry) to correspond to the seasonal pattern of human campylobacteriosis with peaks observed in the summer (Berndtson et al., 1996; Wedderkopp *et al*., 2000; Wilson, 2002; Evans *et al.,* 2002; Bang *et al*., 2003; Bouwknegt *et al*., 2004; Hansson *et al.,* 2004; Hofshagen and Kruse, 2005; Sopwith *et al.,* 2008). Moreover studies have shown seasonality in the isolation rate of *Campylobacter* from retail chicken (Kang *et al.,* 2006; Jore *et al.,* 2010; Ishihara *et al.,* 2012). These findings suggest that climatic factors may be important for *Campylobacter* infections in chickens and humans in these countries. In the tropics studies on *Campylobacter* have found no any seasonal variation on human infections with the organisms (Shimotori *et al.,* 1986; Lindblom *et al.,* 1995; Vargas *et al.,* 2004). Studies carried out in different seasons have yielded more or less similar isolation rates.

Previously, however, a study in Zaire (a Central African country) had reported high prevalence of *Campylobacter* infections in humans during the wet season (Blasser and Barth, 1981).

1.11 Antimicrobial Resistance Profiles

Antimicrobial resistance in bacteria originated from food of animal origin, including *Campylobacter*, is becoming a major public health concern all over the world (Isenbarger *et al.,* 2002; Nachamkin *et al.,* 2002). Increasingly *Campylobacter* isolates have developed resistance to many antimicrobials including fluoroquinolones and macrolides which are the drugs of choice for treatment of Campylobacteriosis (Fliegelman *et al.,* 1985; McNulty, 1987; Fitzgerald *et al.,* 2008). Apart from macrolides and fluoroquinolones, resistance in *Campylobacter* isolates has been described against penicillins, trimethoprim, sulfamethoxazole, rifampicin, vancomycin, aminoglycosides, betalactams and most of the cephalosporins. *Campylobacter* resistance to macrolides and fluoroquinolones is conferred largely by target modification (Gibreel and Taylor, 2006; Hao *et al.,* 2009; Iovine, 2013) and antibiotic efflux pumps (Guo *et al.,* 2008; Iovine, 2013). Resistance mechanisms for other antimicrobials include modification of the antibiotic, enzymatic inactivation of the antibiotic, target modification, efflux pumps and decreased membrane permeability (Iovine, 2013).

1.12 Study Area

This study was carried out in Morogoro Municipality, Morogoro region, Eastern part of Tanzania. As of the official 2012 population and housing census the Municipality had a population of 315,866; 14.24% of the total population of Morogoro region (n= 2,218,492). The Municipality is featured by commercial (broiler and layer) and free ranging chicken production, mainly as backyard activities involving family members. Broiler and free

ranging indigenous chickens are either sold live, frozen, cooked or sometimes as barbecue. Layer chickens produce eggs which are sold direct to consumers or to collectors who eventually practice retail or whole selling. Upon exhaustion of production layer chickens are also sold as spent layers for consumption. At Sokoine University of Agriculture, also located in Morogoro Municipality, different animal species including farm and laboratory are kept for production, teaching and research purposes. Dar es Salaam city, sharing the same zone with Morogoro (Eastern zone), was also involved in this study as a source of house crows.

1.13 Study Subjects

The present study involved collection of samples from humans, farm animals, laboratory animals, chickens and house crows. From humans stool specimens were collected from both symptomatic and asymptomatic individuals of different age groups. Morogoro regional hospital, Sokoine University of Agriculture (SUA) health centre and Upendo medical laboratory served as centres for human sample collection. Chicken samples were collected from different categories of birds to include broiler chickens, layer chickens and free range indigenous chickens. Farm (cattle, goats, sheep, horses and camels) and laboratory (guinea pigs, mice, rabbits and rats) animals kept at SUA for teaching and research purposes were also involved in this study. For the purpose of this study house crows (*Corvus splendens*) were also sampled to represent wild birds. Collected samples were analyzed in microbiology laboratories of the Pest Management Centre of SUA.

1.14 Problem Statement and Justification of the Study

As with other developing countries data on various aspects of infectious diseases in Tanzania is scant and fragmented. A review conducted on available data for human and animal campylobacteriosis revealed substantial gaps as those on epidemiology were

limited, those on risk factors for human infections were lacking and those on antimicrobial resistance were only available for *C. jejuni* isolates from ducks.

1.15 Rationale of the Study

The process of planning and developing control strategies for infectious agents, including *Campylobacter*, requires gathering information in different aspects. The aim of the present study was to provide information on molecular epidemiology and antibiotic resistance of thermophilic *Campylobacter* isolated from humans and different animal species in Eastern part of the country. Generated data would complement the available in providing a platform for public health professionals to dwell on in devising control measures for control of *Campylobacter* infections and propagation of antibiotic resistance.

1.16 Study Hypotheses

- In Tanzania humans and animals are infected with thermophilic *Campylobacter* species
- *Campylobacter* isolates from humans and animals display different levels of resistance to commonly used antibiotics
- Some *Campylobacter* isolates from humans and animals are genetically related
- Human thermophilic *Campylobacter* infections are precipitated by some factors

1.17 Objectives of the *S*tudy

1.17.1 Main objective

To determine the molecular epidemiology and antibiotic resistance of thermophilic *Campylobacter* isolates from humans and different animals in Tanzania

1.17.2 Specific objectives

1. To establish the prevalence of thermophilic *Campylobacter* infections in humans and animals

2. To evaluate antibiotic resistance patterns of thermophilic *Campylobacter* isolates derived from different hosts
3. To determine the genetic relatedness of chicken and human derived thermophilic *Campylobacter* isolates using DNA-based typing methods
4. To identify risk factors for thermophilic *Campylobacter* infections in humans

1.18 Organization of the Thesis

This thesis is organised in three chapters preceded by an extended abstract which summarizes the objectives, materials and methods, principal research findings and conclusions of this study. Chapter one covers an introduction, problem statement, justification of the study, study area, study subjects and study objectives. Chapter two presents the results obtained from each specific objective which are synthesised into either published papers (papers I, II, and III) or prepared manuscripts (papers IV and V) submitted for publication in peer reviewed scientific journals. The format and writing style of each paper is according to the targeted peer reviewed journal. Chapter three provides the conclusions and recommendations based on the findings of this study.

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CHAPTER TWO

PAPER ONE

Human and animal *Campylobacteriosis* in Tanzania: A review

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Status: Published in ***Tanzania Journal of Health Research***, Volume 15 (Supplementary 1) Doi: http://dx.doi.org/10.4314/thrb.v15i1.6

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PAPER TWO

Occurrence, species distribution and antimicrobial resistance of thermophilic *Campylobacter* isolates from farm and laboratory animals in Morogoro, Tanzania

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Status: Published in ***Veterinary World*****,** Volume 7. (Supplementary 8) pages 559-565.

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PAPER THREE

Prevalence, Antimicrobial Resistance and Risk Factors for Thermophilic Campylobacter Infections in Symptomatic and Asymptomatic Humans in Tanzania

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Status: Published in ***Zoonoses and Public Health***: doi: 10.1111/zph.12185

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PAPER FOUR

Prevalence and antimicrobial resistance of thermophilic *Campylobacter* isolated from chickens and house crows (*Corvus splendens*) in Tanzania.

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Abstract

Thermophilic *Campylobacter* are a leading cause of zoonotic gastroenteritis worldwide. A cross-sectional study was conducted to determine the prevalence and antimicrobial resistance of *Campylobacter* in chickens and house crows in Tanzania. Isolation of *Campylobacter* from fecal samples adopted the Cape Town protocol. Identification of isolates involved phenotypic and molecular techniques. Resistance testing of the isolates adopted the disc diffusion method. Thermophilic *Campylobacter* were isolated from 44% and 20.5% of the screened chickens and crows respectively. Majority of isolates from both chickens (87.6%) and crows (56.5%) were *C. jejuni* and the remaining were *C. coli.* Both chicken and crow derived isolates showed higher resistance to Colistin sulphate, Azithromycin, Erythromycin and Nalidixic acid. Crow derived isolates also had higher resistance to Amoxycillin. The least resisted antimicrobials were Gentamycin and Ciprofloxacin for crow and chicken derived isolates respectively. Among chicken isolates significantly higher proportions of *C. coli* were resistant to gentamycin, cephalothin, tetracycline, colistin sulphate and chloramphenical. *C. jejuni* on the other hand had a significantly higher proportion of antimicrobial resistant isolates for Nalidixic acid. Crow derived *C. jejuni* had significantly higher resistance rates to Nalidixic acid, cephalothin and Ciprofloxacin than *C. coli* isolates from the same hosts. Higher frequencies of crow derived isolates were resistant to Gentamycin, amoxicillin, erythromycin, azithromycin and ciprofloxacin than the chicken isolates. Our results emphasize the need for regular surveillance on the prevalence and antimicrobial resistance of *Campylobacter* in both domesticated and wild birds. Controlling indiscriminate use of antimicrobials, both in livestock and humans is highly recommended.

Key words: Cape Town protocol, wild birds, MALDI-TOF; polymerase chain reaction; Disc diffusion, Morogoro

1.0 Introduction

Campylobacter mediated diarrhoeal disease is a leading bacterial cause of gastroenteritis worldwide (Haruna et al., 2012). Of the *Campylobacter* species, *C. jejuni* causes more than 83% of human symptomatic infections; majority of the remaining proportion being caused by *C. coli*. The members of the genus *Campylobacter* have been isolated from a range of sources, including surface and ground waters, domestic and wild mammals, wild birds insects and air (Kapperud and Rosef, 1983; Waldenström et al., 2002; Miller and Mandrell, 2005; Strother et al., 2005; Colles et al., 2008). The organisms are transmitted to humans mainly by contaminated food of animal origin, with raw/undercooked poultry meat being more incriminated (Lindmark et al., 2009). Animals and birds are frequently colonized with *Campylobacter* organisms asymptomatically whereas in humans the organisms cause acute and self-limiting intestinal infection (Takamiya et al., 2011). The frequently isolated species, *C. jejuni*, has been identified as a cause of a neuromuscular disorder, Guillain-Barre´ syndrome, and reactive arthritis (Blaser, 1997).

Campylobacter enteritis is in most cases self-limiting. However, antimicrobial therapy may be required in complicated cases of enteritis and systemic infections, in which erythromycin and ciprofloxacin are the drugs of choice (McDermott et al., 2004). A recent trend shows an increase in resistance to erythromycin and ciprofloxacin; and other antimicrobials among *Campylobacter* isolates derived from both humans and animals (Ghosh et al., 2013). This creates a need for regular monitoring of resistance among members of the genus to provide useful information to guide antimicrobial therapy when required.

The development of health programs aimed at controlling diseases, including campylobacteriosis, requires sufficient information on the disease including its

epidemiology. In developing countries where surveillance of many diseases including zoonoses are not regular, such information is scant making it difficult to provide an accurate picture of infection rates in some host populations, including potential sources of human infections. As a consequence the present study assessed the epidemiology and antimicrobial resistance of chicken and crow derived *Campylobacter* isolates in Tanzania. The generated information will provide inputs for planning control strategies for chicken colonization, human infections and propagation of antimicrobial resistance.

2.0 Materials and Methods

2.1 Study Area

This study was carried out in Morogoro Municipality, Morogoro region, Eastern part of Tanzania. As of the official 2012 population and housing census the Municipality had a population of 315,866; 14.24% of the total population of Morogoro region (n= 2,218,492). The Municipality is featured by commercial (broiler and layer) and free ranging chicken production, mainly as backyard activities involving family members. Broiler and free ranging indigenous chickens are either sold live, frozen, cooked or sometimes as barbecue. Layer chickens produce eggs which are sold direct to consumers or to collectors who eventually practice retail or whole selling. Upon exhaustion of production layer chickens are also sold as spent layers for consumption. Chicken samples were collected from these different groups of birds. The house crows (*Corvus splendens*) which were also involved in this study were trapped at Mabibo compound of the National institute for Medical research, Dar es Salaam city. Chicken and crow samples were analyzed in microbiology laboratories of the Pest Management Centre of the Sokoine University of Agriculture (SUA).

2.2 Study Design and Sample Sizes

A cross-sectional study design was adopted to establish the prevalence of *Campylobacter* in chickens and crows in the study areas. Sample sizes for different categories of chickens to be involved in the study were calculated using the formula $n=Z^2p\ (1-p)/\ d^2$ (Thrusfield, 1995) where: n is sample size; Z is the multiplier from the normal distribution, p is the expected prevalence and d is the desired absolute precision. The known prevalence of *Campylobacter* infections (p) reported in previous studies in the country are 69%, 22.7%, 71% for broiler chickens, layer chickens and free ranging indigenous chickens respectively (Mdegela et al., 2006). With Z value of 1.96 at 95% confidence interval (CI) and desired precision (d) of 0.05, the calculated sample sizes (n) were 329, 270 and 317 for the respective groups of study birds. The sample size for crows was dependent on their availability during the study period.

2.3 Sample Collection

2.3.1 Chicken samples

Cloacal swabs were collected form different categories of chickens in randomly selected farms in urban and peri-urban areas of the study area. In each study farm chickens were randomly picked and sampled while making a transect walk across the chicken house. To avoid repeating sampling the same bird, sampled chickens were temporarily separated until the end of the sampling process. Free ranging indigenous chickens were also confined during sample collection. Collected samples were placed in Bolton broth and conveyed on ice to laboratories for analysis within 8 h post-sampling.

2.3.2 Crow samples

Crows were trapped in an optimized trap at the National Institute for Medical Research compound at Mabibo, Dar es Salaam. During their time in captivity the crows were

observed for clinical signs of disease. Following euthanasia intestinal samples were obtained and transported to SUA, Morogoro, for isolation of *Campylobacter*.

2.4 Isolation of Campylobacter

In the laboratory collected samples were first incubated at 42°C for 24 hours while in Bolton broth. Isolation of *Campylobacter* was then carried out using the Cape Town protocol with some modifications as indicated by Jacob et al. (2011). The technique involves culture of the organisms on an antibiotic free blood agar after a filtration step. Briefly a 0.45µm pore size nitrocellulose filter (Sartorius Stedim Biotech GmBH 37070, Goettingen, Germany) was overlaid on the surface of blood agar (Oxoid LTD., Basingstoke, Hampshire, England) onto which 200µl of enrichment broth containing the sample was dispensed and allowed to filter passively for 45 min at room temperature. The filter was then carefully removed with sterile forceps and discarded. Thereafter the filtrate was spread evenly across the medium followed by incubation of the inoculated plates in microaerophilic atmosphere at 37°C for up to 72 hours while checking for growth after every 24 hours. Suspect *Campylobacter* colonies were subsequently sub-cultured on blood agar under similar conditions to obtain pure cultures which were then subjected to identification methods.

2.5 Identification of Campylobacter isolates

2.5.1 Preliminary identification

Campylobacter isolates were preliminarily identified based on phenotypic tests namely growth atmospheric requirements, colonial characteristics, testing for Gram negativity using the KOH String Test (3% potassium hydroxide on a glass slide), motility test and the sodium hippurate hydrolysis test for differentiation of *C. jejuni* from others.

2.5.2 Confirmation of campylobacter isolates

Campylobacter isolates obtained in this study were further confirmed using a genome-based method, species specific polymerase chain reaction (PCR) as well as a spectroscopic method, Matrix assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (Bruker Daltronics with Bruker Daltonics MALDI biotyper software 2.0, Billerica, Massachusetts, USA). Genomic DNA to be used for PCR was extracted from bacterial suspensions by boiling at 100°C for 10 min. Primers F,5'CTATTTTATTTTGAGTGCTTGTG3' and R, 5'GCTTTATTTGCCATTTGT TTTATTA3' (TAG COPENHAGEN A/S, Denmark) were used to amplify the *mapA* gene of *C. jejuni*, where as primers F,5'ATTTGAAAATTGCTCCAACTATG3' and R,5'TGATTTTATTATTTGTAGCAGCG3' (TAG COPENHAGEN A/S, Denmark) were used to amplify the *ceuE* gene of *C. Coli.* Each reaction was performed in a 50µl total volume containing 10µl primer mix (12 pmol of each primer), 25µl Green master mix, 2µl DNA template and 13µl milli Q water. Amplification reactions were run in Biometra T3 thermocycler (Fisher Scientific, UK), with the following program: an initial denaturation at 95°C for 5 min followed by 34 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s and polymerization at 72°C for 1min. A final extension was performed at 72°C for 5 min. Samples were then maintained at 4°C until processed. The amplification generated 589 bp and 462 bp DNA fragments corresponding to *Campylobacter jejuni* and *Campylobacter coli* respectively. The PCR products were analyzed on a 1.5% agarose gel stained with 0.3 g/ml ethidium bromide and were visualized under UV light. A 100bp ladder was used as a molecular size standard.

2.6 Evaluation of Antimicrobial Resistance of Campylobacter Isolates

In this study antibiotic resistance testing of *Campylobacter* isolates adopted the disc diffusion method on Muller Hinton (MH) Agar (Oxoid Ltd, Basingstoke, UK) as

described by Luangtongkum *et al.* (2007), using *C. jejuni* NCTC11168 for quality control purposes. The method is known to be reliable and easy tool for monitoring the prevalence of resistance in *Campylobacter* in poultry and a suitable alternative method to agar-based MIC methods (Yang *et al.,* 2008). Briefly, *Campylobacter* suspensions were prepared in sterile normal saline and adjusted to a turbidity equivalent to a 0.5 McFarland standard. Sterile cotton-tipped swabs were inserted into the standardized inoculums, drained of and then used to transfer the inoculums onto well dried Mueller-Hinton plates. Inoculated plates were dried in incubator for five minutes and antibiotic discs were distributed over them using a BBL Sensi-disc dispenser. The plates were then incubated at 37°C for 48 hours under microaerobic conditions. After 48 hours of incubation, the diameters of zones of inhibition were measured using a ruler. In this study the most commonly used antimicrobial agents in livestock and humans in the study area and others that have been used and tested elsewhere were tested for resistance. They included twelve (12) different antimicrobials; Nalidixic acid (NA, 30 μg), Ciprofloxacin (CIP, 5 μg), Gentamycin (CN, 10 μg), Ampicillin (AMP, 10 μg), Cephalethin (CL, 30 μg), Amoxycillin (AML, 25 μg), Norfloxacin (NOR, 10 μg), Erythromycin (E, 15 μg), Tetracycline (TE, 30 μg), Colistin (CT, 10 μg), Azithromycin (AZM, 15 μg) and Chloramphenicol (C, 30 μg). A total of 225 chicken derived and 23 crow derived isolates were tested for resistance to these different antimicrobials. An isolate that resisted more than two antimicrobials was defined as multi-drug resistant.

2.7 Data Analysis

Collected data in this study were analysed using Epi Info statistical software Version 7. For prevalence and antimicrobial resistance data descriptive statistics (frequencies and cross tabulations) were computed to determine proportions for different items. The Chi-square test was used to determine the significance of differences in proportions at $p \leq 0.05$.

2.8 Ethical Considerations

Permission to conduct the study in the study areas was granted by the Regional and Municipal authorities. Farmers also provided verbal consents. Involvement of crows in this study received permission from the Wildlife division in the Ministry of natural resources and tourism. Trapped crows were humanely sacrificed using standard procedures before sample collection.

3.0 Results

3.1 Sample Collection

Between September 2011 and March 2012 cloacal swabs were collected from a total of 1267 chickens of different categories in the following order: broilers (409), layers (510) and free ranging indigenous chickens (348). The sampled birds, aged 3 weeks and above, came from 31 flocks (10 of broilers, 12 of layers and 9 of free ranging indigenous chickens). Six of the layer flocks were under the deep litter system and the other 6 used cages. Chickens less than three weeks of age were excluded from this study to avoid effects of maternal immunity. During the same period caecal samples were collected from a total of 112 house crows. Contents from the walls of these caeca were cultured for isolation of *Campylobacter*.

3.2 Prevalence of Campylobacter Organisms in Study Subjects

3.2.1 Prevalence of the organisms in chickens

Different proportions of sampled flocks for different groups of chickens were positive for *Campylobacter* as shown in Fig. 1. At bird level the overall prevalence of *Campylobacter* colonization was 44% (n=1267). *C. jejuni* accounted for 87.6% of the isolates. The colonization levels of the organism in individual groups of birds are presented in figure 2. The prevalence was significantly lower in free ranging indigenous chickens. Within

individual flocks the prevalence ranged from 0-100% for broilers, 0-94% for layers and 0-65% for free ranging indigenous chickens

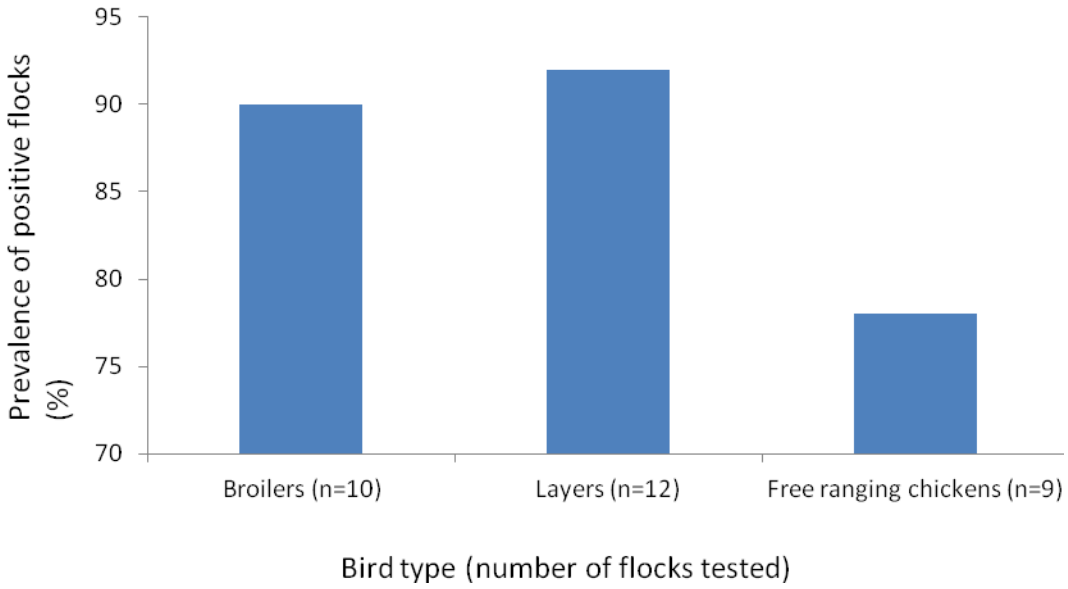

Figure 1: Prevalence of positive flocks for thermophilic *Campylobacter* in different groups of chickens in Morogoro, Tanzania

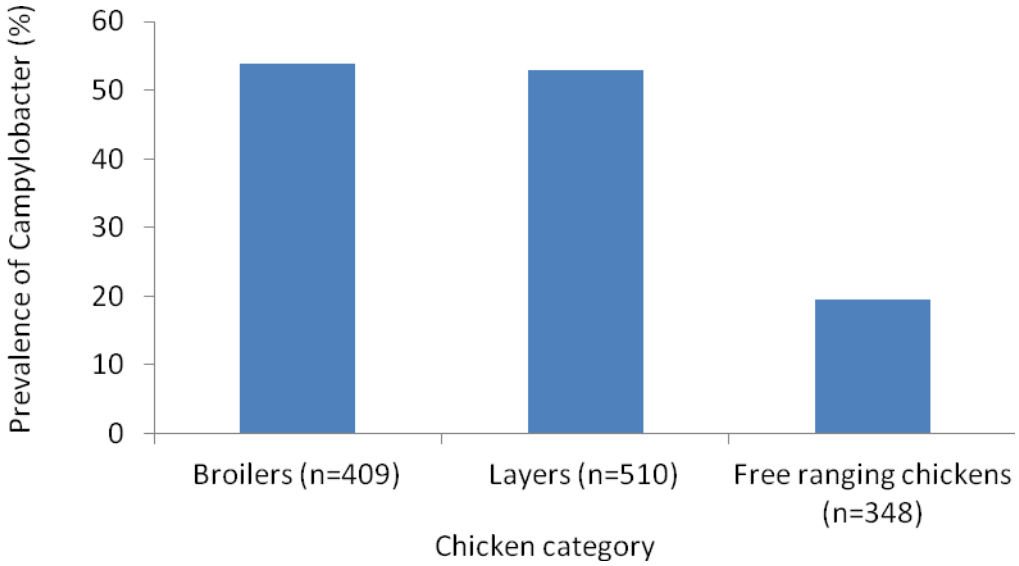

Figure 2: Isolation of thermophilic *Campylobacter* from different chicken categories in Morogoro, Tanzania

3.2.3 Prevalence of the organisms in crows

Out of 112 sampled crows, 23 carried *Campylobacter* organisms in their intestines, giving a prevalence of 20.5%. Species distribution was such that 13 isolates (56.5%) were *C. jejuni* and 10 isolates (43.5%) were *C. coli.*

3.3 Antimicrobial resistance profiles for Campylobacter isolates

3.3.1 Chicken isolates

A total of 225 chicken derived *Campylobacter* isolates were tested for susceptibility to 12 different antimicrobials. The proportion of resistant isolates were highest for Colistin sulphate (CT), Azithromycin (AZM), Erythromycin (E) and Nalidixic acid (NA); and lowest (4.9%) for Ciprofloxacin (CIP) as shown on figure 3. The proportion of resistant isolates to the remaining antimicrobials ranged from 26.2% to 36.9% (figure 3). Eighty three percent of the test isolates displayed multi-drug resistance with 23.1% resisting more than half of the test antimicrobials. Resistance to more than two antimicrobials was in the following order; resistance to three antimicrobials (17.0%), resistance to four antimicrobials (13.0%), resistance to five antimicrobials (16.0%), resistance to six antimicrobials (14.2%), resistance to seven antimicrobials (9.8%), resistance to eight antimicrobials (7.6%), resistance to nine antimicrobials (1.3%), resistance to ten antimicrobials (3.1%), resistance to eleven antimicrobials (1.3%). All *C. coli* isolates were multidrug resistant. There were significantly higher proportions of resistant *C. coli* isolates than *C. jejuni* isolates for gentamycin, cephalothin, tetracycline, colistin sulphate and chloramphenical. *C. jejuni* on the other hand had a significantly higher proportion of antimicrobial resistant isolates for Nalidixic acid (Table 1).

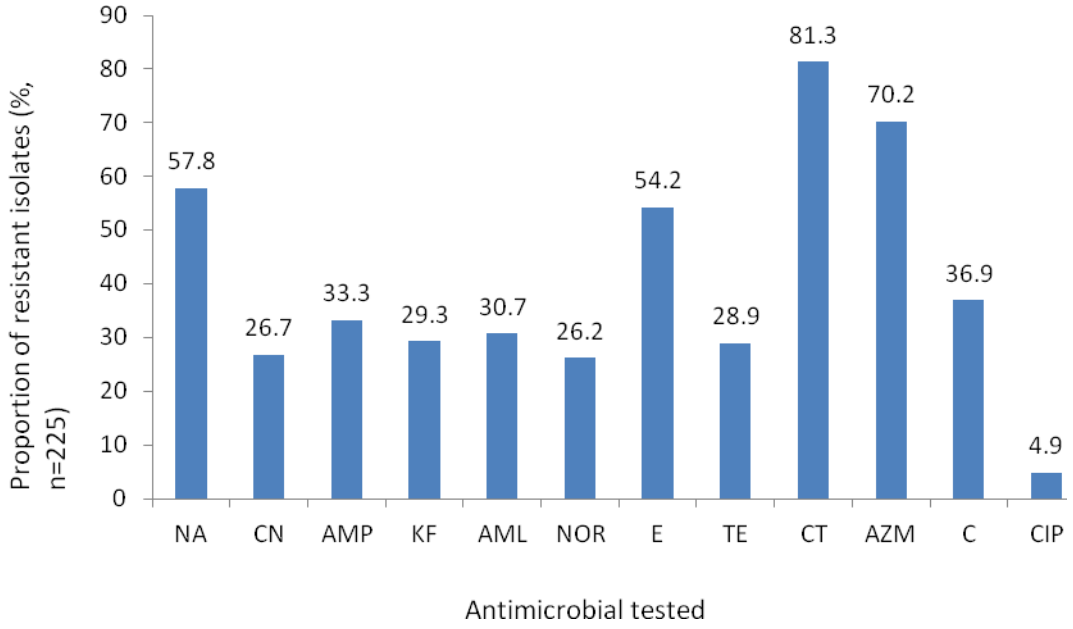

Figure 3: Antimicrobial resistance of chicken derived thermophilic *Campylobacter* isolates

Table 1: Species distribution of antimicrobial resistance among chicken derived thermophilic *Campylobacter* isolates

Antimicrobial agents	Proportion of resistant isolates (%)		P value
	***C. jejuni* (n=197)**	***C. coli* (n=28)**	
NA	60.40	39.3	0.03
CN	21.83	60.71	0.00
AMP	31.47	46.43	0.12
CL	25.90	53.57	0.00
AML	29.40	39.30	0.29
NOR	25.90	28.57	0.76
E	51.80	71.43	0.05
TE	26.40	46.43	0.03
CT	78.68	100	0.01
AZM	71.07	64.29	0.46
C	32.00	71.43	0.00
CIP	5.08	3.57	0.73

3.3.2 Antimicrobial resistance of crow derived isolates

A total of 23 crow derived *Campylobacter* isolates were tested for susceptibility to 12 different antimicrobials. The proportion of resistant isolates were higher (>50%) for Erythromycin (E), Colistin sulphate (CT), Azithromycin (AZM), Amoxycillin (AML) and Nalidixic acid (NA); and lowest (8.7%) for Gentamycin (CN) as shown on figure 4. The proportion of resistant isolates to the rest of the antimicrobials ranged from 30.4% to 47.8% (figure 4). All the test isolates displayed multi-drug resistance with 13.0% resisting more than half of the test antimicrobials. Resistance to more than one antimicrobials was in the following order; resistance to three antimicrobials (4.3%), resistance to four antimicrobials (34.8%), resistance to five antimicrobials (26.1%), resistance to six antimicrobials (21.7%), resistance to eight antimicrobials (8.7%), resistance to ten antimicrobials (4.3%). *C. jejuni* had significantly higher proportions of resistant isolates than isolates for Nalidixic acid, cephalothin and Ciprofloxacin (Table 2).

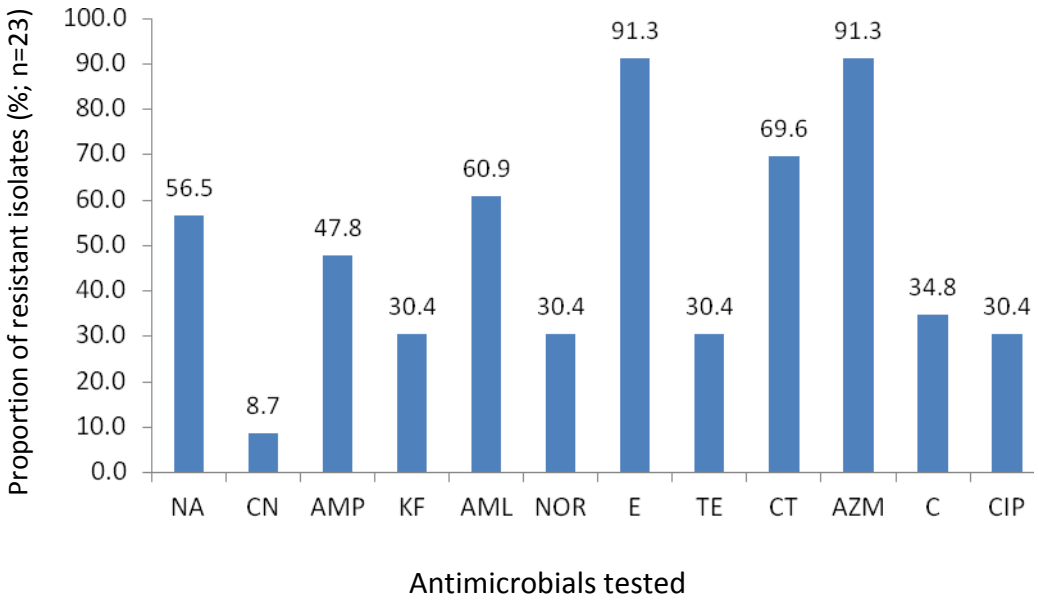

Figure 4: Antimicrobial resistance of crow derived thermophilic *Campylobacter* isolates

Table 2: Species distribution of antimicrobial resistance among crow derived thermophilic *Campylobacter* isolates

Antimicrobial agents	Proportion of resistant isolates (%)		P value
	C. jejuni (n=13)	*C. coli* (n=10)	
NA	100.0	0.0	0.00
CN	15.4	0.0	0.19
AMP	38.5	60.0	0.31
CL	53.8	0.0	0.01
AML	61.5	60.0	0.94
NOR	38.5	20.0	0.34
E	84.6	100.0	0.19
TE	23.0	40.0	0.38
CT	61.5	80.0	0.34
AZM	100.0	80.0	0.09
C	38.5	30.0	0.67
CIP	38.5	20.0	0.34

4.0 Discussion

Our study reports the prevalence and antimicrobial resistance of thermophilic *Campylobacter* isolated from chickens and crows in Tanzania. The detected extent of chicken colonization with the organism is comparable to what was reported by Kazwala et al. (1993), but lower than the prevalence observed in a study by Mdegela and others (2006); all conducted in the country. Mdegela and colleagues (2006) also reported a significantly higher prevalence of thermophilic *Campylobacter* in crows (72.8%, n=22) than what was obtained in this study (20%; n=112). Similar to these other studies, *C. jejuni* was prevailing in both chickens and crows.

An association of *Campylobacter* prevalence with chicken production systems has been reported in previous studies (Mdegela et al., 2006; Näther et al., 2009). In these studies a

higher prevalence of the organism was seen in flocks of free range farming systems. Näther et al. (2009) attributed the observation to probable exposure of the birds to potential environmental sources of *Campylobacter*. Contrary to their findings in this study free-ranging indigenous chickens were found to have a lower prevalence of *Campylobacter*. A study by Wittwer et al. (2005) could not identify a certain production type as a risk factor for *Campylobacter* colonization. The different observations in these different studies could be an attribute of differences in environmental and other sources of infections for the birds.

The present study detected *Campylobacter* in egg producing layer chickens. The layers were found to be colonized with *Campylobacter* at a similar level to broilers. These birds are rarely involved in *Campylobacter* studies as eggs are not regarded as vehicle of food-borne human *Campylobacter* infections; and also due to the fact that the risk of vertical transmission of *Campylobacter* organisms from breeder flocks to the progeny is minimal (Cox et al., 2009). In our setting where these birds are consumed at the end of laying period, their involvement in investigations on potential meat borne zoonoses such as *Campylobacter* is of importance.

Campylobacter organisms are zoonotic pathogens colonizing a range of hosts including domestic animals and wild birds (Griekspoor et al., 2013). Playing a reservoir role, wild birds have higher chances of contaminating water sources, environment and food; in a way transmitting the pathogens to humans and poultry (Altekruse et al., 1999). Several studies in developed and developing countries have reported occurrence of *Campylobacter,* particularly *C. jejuni,* in wild birds including crows (Adegbola et al., 1990; Waldenström et al., 2002; Mdegela et al., 2006). Some authors have found phenotypic and genotypic similarities among *C. jejuni* isolates from free flying birds and humans (Adegbola et al.,

(1990). In this study *Campylobacter* organisms were detected in 20% of sampled house crows (n=112). The detection of the organisms in wild birds in this and other studies is of epidemiological and public health significance, as it highlights on the possibility that crows are among sources of thermophilic *Campylobacter* to humans and chickens. It has been suggested that, free flying birds may transmit thermophilic *Campylobacter* to chickens if they get access to the rearing houses (Altekruse et al*.,* 1999).

Many studies have reported existence of resistant *Campylobacter* isolates to a number of antimicrobials including drugs of choice for treatment of human *Campylobacter* infections (Ghosh et al., 2013). In this study there was a remarkably high resistance rate of *Campylobacter* isolates to macrolides (azythromycin in chickens; and azythromycin and erythromycin in crows). Colistin sulphate was also highly resisted by *Campylobacter* isolates from both chickens and crows. Resistance to Ciprofloxacin was at low levels in both chicken and crow isolates. Our observation contradicts other authors' who reported resistance to Ciprofloxacin at frequencies as high as 100% (Zhang et al., 2010). High frequency resistance to macrolides and low level resistance to ciprofloxacin among *Campylobacter* isolates have also been reported in the country by Nonga and Muhairwa (2009). The low level resistance to ciprofloxacin suggests its potential for continual use as a drug of choice in patients with *Campylobacter* mediated gastroenteritis in our setting. Varied frequencies of resistance were obtained for other antimicrobials as reported by other authors (Taremi et al., 2006; Zhang et al., 2010).

Our results indicate a moderate resistance to tetracyclines among both chicken (28.9%) and crow (30.4%) derived *Campylobacter* isolates. This differs from what was reported in another study conducted in the country (Nonga and Muhairwa, 2009) in which resistance to tetracyclines among duck derived *Campylobacter* isolates was at a rate of 74%.

Researchers elsewhere have also reported frequencies of tetracycline resistance among *Campylobacter* isolates with a wide variation (Pratt and Korolik, 2005; Dasti et al., 2007). The observed differences in resistance rates among studies may either be attributable to differences in sources of the organisms or differences in exposure frequencies to the antibiotics among the study subjects.

Members of the genus *Campylobacter* are known to be intrinsically resistant to cephalosporins including cephalothin, with rates up to 100% (Pezzotti *et al.,* 2003). Several studies have reported higher resistance rates of *Campylobacter* isolates to cephalosporins particularly cephalothin. These include studies by Serichantalergs et al., 2010 who reported resistance at 100% and Mansouri-najand et al. (2012) who reported resistance at 93.5-97.2%. In our study cephalothin resistance was at 29.3% for chicken isolates and 30.4% for crow isolates. A relatively low resistance rate to cephalothin has also been reported by Pigrau et al. (1997).

C. coli isolates are generally known to be more resistant to antimicrobials than *C. jejuni* strains (de Jong et al., 2012). Different studies have reported higher resistance to ciprofloxacin among *C. coli* isolates compared to *C. jejuni* (Pezzotti et al., 2003; Taremi et al., 2006). In a study by de Jong et al. (2012) *C. coli* isolates were seen to display higher resistance to erythromycin compared to *C. jejuni*. In the present study this was observed with chicken isolates in which *C. coli* had significantly higher resistance rates for gentamycin, cephalothin, erythromycin, tetracycline, colistin sulphate and chloramphenical. *C. jejuni* on the other hand showed a higher resistance rate to nalidixic acid. With crow isolates resistance rates were more or less similar between the two types of isolates except for cephalothin and nalidixic acid in which the rates were higher for *C. jejuni*.

Conclusion

Findings from this study indicate a high prevalence of antimicrobial resistant thermophilic *Campylobacter* in particular *C. jejuni* in chickens and crows. The findings emphasize the need for routine surveillance and monitoring of the prevalence and antimicrobial resistance of *Campylobacter* in both domesticated and wild birds. Controlling indiscriminate use of antimicrobials, both in livestock and humans is highly recommended.

Acknowledgements

This work was conducted under the project titled "Transforming Smallholder Livestock Farms into Profitable Enterprises" (Livestock Enterprises); which received funding from Denmark through the Danish International Development Agency (grant number 66-08-LIFE). We highly value their support. Farmers involved in the study are acknowledged for their cooperation.

Conflict of interest statement

The authors declare that they have no competing interests.

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PAPER FIVE

Diversities and similarities in fla A types of Campylobacter jejuni isolated from chickens and humans in Tanzania

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Status: Manuscript (submitted to **Epidemiology and Infection** peer reviewed journal).

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Running head: *fla A* types of *C. jejuni* in Tanzania

SUMMARY

The flagellin subunit A (*flaA*) gene typing is one of the most commonly used genotyping methods for thermotolerant *Campylobacter* species. This study genotyped 55 *C. jejuni* isolates obtained from chickens and humans in Tanzania using *flaA* gene sequencing. Nine different *flaA* sequence types (7, 36, 41, 51, 61, 62, 64, 105 and 111) were detected, with types 41, 51 and 62 being most prevalent. All the *flaA* types contained isolates from both chickens and humans except for types 36 and 105, and 61 found in isolates from broiler chicken and humans only, respectively. Phylogenetic analysis revealed six different clusters, with three of them containing isolates from both chickens and humans. The observed population structure of the isolates from different sources was diverse and weakly clonal. The *flaA* types containing isolates from both chicken and human sources suggest possible interspecies transmission of *C. jejuni* in Tanzania.

Key words: *Campylobacter jejuni*, Fagellin gene, *flaA* Typing

INTRODUCTION

Campylobacter is one of the most common causes of acute diarrhoeal illness in humans [1, 2], with almost 85% of human cases caused by *Campylobacter jejuni*, and a large proportion of the remaining attributed to *C. coli* [3, 4]. Campylobacter is common in animals causing asymptomatic infections; and a major risk factor for food-borne campylobacteriosis in most sporadic cases of human illness is raw or undercooked poultry, either directly or through cross-contamination of foods that are ready to eat [5-8].

A thorough understanding of various aspects of the epidemiology of infectious agents such as *Campylobacter* species, including sources of infections, transmission routes and pathogen-host interactions, are very crucial for planning control measures for human

illness [9]. Detection of sources of infections and transmission routes requires reliable strain differentiation techniques. Various strain typing methods, both phenotypic and genotypic, have been developed and used for the epidemiological typing of campylobacter isolates from various sources [10-14]. The most commonly used genotypic methods with enhanced sensitivity and discrimination include ribotyping, pulsed–field gel electrophoresis (PFGE), flagellin gene typing (*flaA* typing), random amplification of polymorphic DNA (RAPD), amplified fragment length polymorphism fingerprinting (AFLP), restriction endonuclease analysis and multilocus sequence typing (MLST) [10, 15-17]. Large strain type diversity has been observed with application of these different genotyping methods, the degree of which is attributable to the discriminatory power of the method [10]. Similarly, genotypes have been observed in most studies to overlap for human and animal isolates, with human- and animal-specific genotypes also observed [18-23].

Flagellin gene typing is based on the restriction analysis of PCR-amplified fragments or sequencing of the flagellin-encoding gene (10, 11, 24, 25]. These genes vary between isolates, thus providing the basis of a typing scheme. This genotyping method has emerged as the most suitable target for rapid investigation of a large numbers of isolates [26] because it is quick, simple and cost-effective [27]. When concurrently used with other sequence-based typing techniques such as MLST, the additional variable target (the flagellin-encoding gene), increases the discriminatory power of sequence-based typing. The most frequently used gene for this purpose is *flaA* (23-25, 28-31], although *flaB* is also used [32]. In Tanzania no attempts have been made so far to genetically characterize *Campylobacter* species isolated from different species. This paper describes the molecular typing of a collection of *C. jejuni* isolates from chickens and humans of Morogoro in Tanzania using *flaA* gene sequencing.

METHODS

Bacterial isolates

The present study involved a total of 55 *C. jejuni* isolates (20 from humans and 35 from chickens) collected in a prevalence study during the period 2011–2012. Human isolates were obtained from sporadic cases of gastroenteritis from 3 health facilities in Morogoro Municipality. Chicken isolates were obtained from backyard production systems involving broilers, layers and free ranging indigenous birds around the Municipality. The isolates were identified with conventional bacteriological standard procedures and eventually confirmed using species-specific polymerase chain reaction (PCR) and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

Extraction of genomic DNA

Extraction of DNA from pure campylobacter isolates was carried out by boiling bacterial cells from an agar plate. Briefly, 250 µl of phosphate buffered saline (PBS) were aliquoted into an eppendorf tube and labelled appropriately. Fresh bacterial growth was scrapped from the plate with an inoculation loop and re-suspended in PBS to achieve a turbidity of 0.5 McFarland standard. The mixture was briefly vortexed and immediately placed in a heating block pre-heated to 100°C, and boiled for 10 minutes. Boiled organisms were spun at 13000 *g* for 10 min. The supernatant (containing DNA) was moved to a clean, labelled tube and stored at -20 °C until used for molecular work.

PCR amplification and purification of PCR products

PCR of the *flaA* gene was performed according to the technique of Nachamkin et al. [26]. Whole cell lysate [33] was used as the template, a reference strain NCTC *C. jejuni* 11168 was used as a positive control, and sterile water was substituted for the DNA template as a negative control. Primers Fla4F 5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3';

and FLA625RU 5'-CTG TAG TAA TCT TAA AAC ATT TG-3' (TAG COPENHAGEN A/S) were used to amplify the *flaA* gene of *Campylobacter* strains. Each reaction was performed in a 27µ total volume containing 3 µ primer mix (12 pmol of each primer), 12.5µ Green master mix (Thermo Fisher Scientific Inc., 81 Wyman Street Waltham, MA USA), 5µ DNA template and 6.5µ mill Q water. Each PCR was run in a T3 thermocycler (Biometra, Fisher Scientific, UK) under the following conditions: 5 min of initial denaturation at 95°C, followed by 34 cycles of denaturation at 95°C for 45 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 2 minutes. This was followed by final extension at 72°C for 10 min and then the products maintained at 4°C until processed. To confirm the presence of the 1.7 kb *flaA* amplicon, the PCR products were subjected to gel electrophoresis followed by ethidium bromide staining and UV trans-illumunation. To enzymatically purify the PCR products, 10µ of PCR product was transferred into new reaction tubes followed by the addition of 4µl of Fast Alkaline Phosphatase (Thermo Fisher Scientific Inc., 81 Wyman Street Waltham, MA USA) to degrade residual deoxynucleotides and 1µl of exonuclease I (Exo I) (Thermo Fisher Scientific Inc., 81 Wyman Street Waltham, MA USA) to remove excess primers. The PCR products and enzymes were incubated for 30 min at 37°C and then for 20 min at 80°C to inactivate the enzymes using the 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA).

Sequencing of PCR products

The purified PCR products were shipped to Macrogen (South Korea) and directly sequenced using Fla4F and FLA625RU primers after performing a dideonucleotide cycle sequencing PCR reaction. The obtained nucleotide sequences were deposited in the internet accessible *flaA* sequence (*flaA* (CAMP 1255)) database [34] and allele numbers were assigned by sequence comparisons against the existing *flaA* sequences.

Data analysis

FlaA gene nucleotide sequences were analysed in MEGA 5.05 [35]. Briefly, nucleotide sequences were translated and aligned using ClustalW algorithm. The aligned sequences were subjected to phylogeny test by neighbor joining method using the Kimura-2-parameter option to create a dendrogram with 1000 bootstrap replications.

Nucleotide sequence accession numbers

The sequences were submitted to the GenBank for provision of accession numbers. Accession numbers KF846011-KF846065 were provided for the 55 sequences.

Ethical considerations

Ethical clearance (NIMR/HQ/R.8a/Vol. IX/1106) was sought from the Health Research Ethics Review Sub-Committee of the National Institute for Medical Research, Ministry of Health and Social Welfare, Dar es Salaam, Tanzania. Human stool samples were collected by qualified medical personnel in the respective health facilities after explaining the purpose of the study and obtaining written consent of the subject to be screened or his/her parent/guardian. Confidentiality of the study participants is adhered to.

Results

In this study a total of 55 *Campylobacter* isolates were subjected to *flaA* gene sequencing. Out of these 20 were human derived isolates and 35 chicken derived isolates (fig 3). The chicken isolates were recovered from different bird categories in the following order; broiler chickens (n=23), layer chickens (n=11) and free range indigenous chickens (n=1). A total 9 genotypes resulted from fla A sequencing of the 55 isolates. Fla A sequence types 7, 36, 41, 51, 61, 62, 64, 105 and 111 were detected. Of these sequence types 62 (21.8%), 41 (23.6%) and 51 (23.6%) were the most prevalent (fig 1). All the fla types

constituted isolates from both chickens and humans except for types 36 and 105 (broiler chicken only) and type 61 (human only) (fig 2). Fla type 36 was detected only once. Phylogenetic analysis revealed six (6) different clusters, three of them composed of isolates from both chickens and humans (fig 3). Two clusters contained isolates from chickens alone and one from humans alone. Some isolates sharing similar clusters had different fla A sequence types.

Table 2: Overall and source specific distribution of *flaA* alleles among *C. jejuni* isolates

flaA allele type	Detection frequency				
	Overall	Humans	Broiler chickens	Layer chickens	Free ranging indigenous chickens
7	6	1	4	1	0
36	1	0	1	0	0
41	13	4	4	4	1
51	13	3	6	4	0
61	3	3	0	0	0
62	12	6	4	2	0
64	2	1	1	0	0
105	3	0	3	0	0
111	2	2	0	0	0

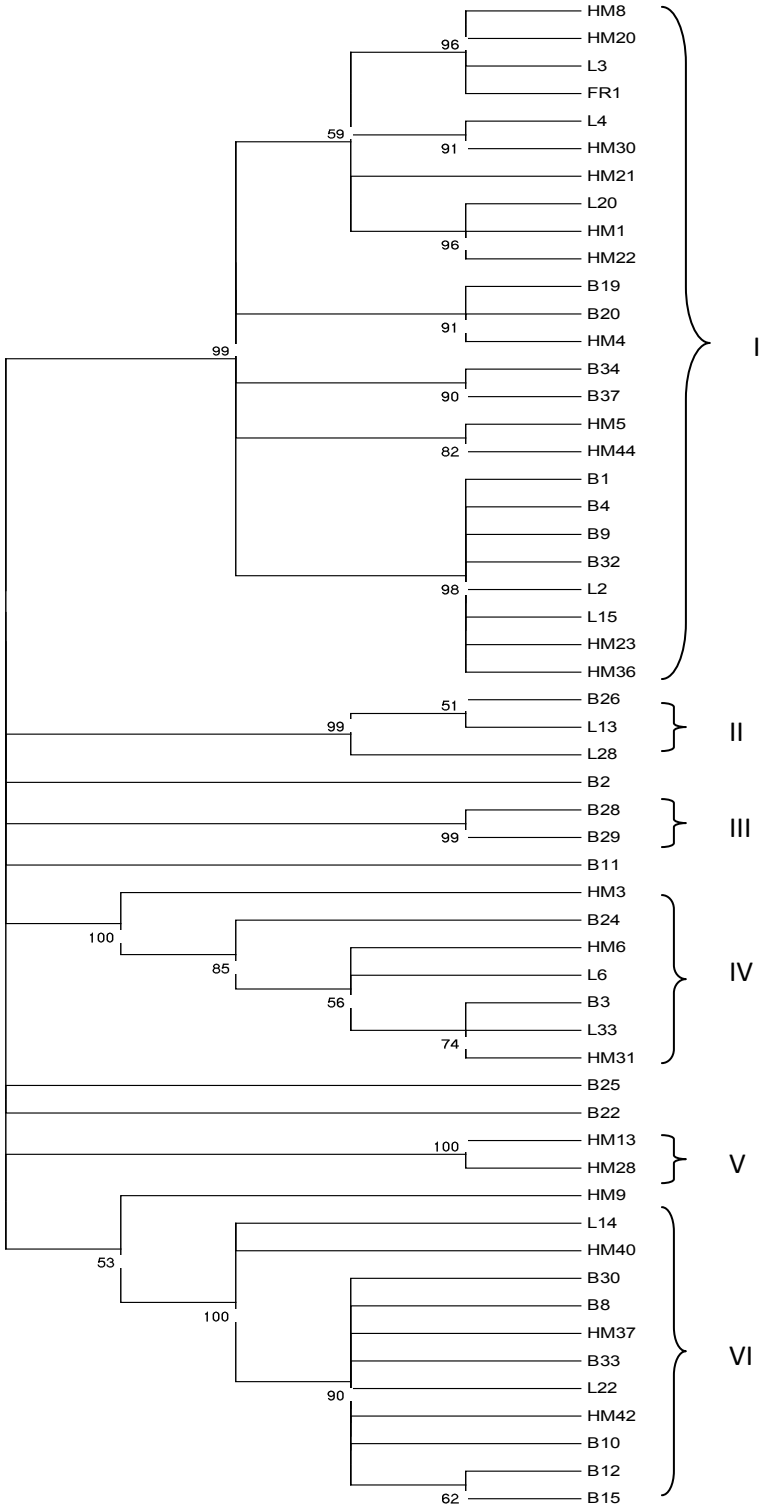

Figure 1: Relationships within 55 *C. jejuni* isolates based on *flaA* sequences (H=human, B=broiler chicken, L=layer chicken and FR=free range indigenous chicken)

DISCUSSION

The aim of the study was to subtype a selection of *C. jejuni* isolates obtained from humans and chickens to gain a better insight into the population structure of these organisms in Tanzania. It was also to compare chicken derived isolates with those from infected humans to detect genetic relatedness and eventually confirm zoonotic potential of the organisms.

Typing and comparison of *Campylobacter* isolates from different sources, including human and animal, is an important step for intervention, infection control and risk assessment of human campylobacteriosis [36]; as it makes it possible to produce estimates for the number of human cases attributable to a specific source. A wide range of phenotypic and genotypic typing methods which vary in discriminatory power, cost, rapidity, and practicality [10] have been developed and are used to that effect. Genotyping techniques are widely used for typing *Campylobacter* spp., as they are featured with higher typeability and greater discriminatory power compared with phenotyping methods [16]. The methods are available in most parts of the world. Flagellin (fla) gene typing is one of the commonly adopted genotyping methods during epidemiological investigations of the two most commonly isolated thermotolerant *Campylobacter*, *C. jejuni* and *C. coli* [10, 16]. It is considered by several authors as a relatively simple and quick typing method. In the present study the method was used for typing human and chicken derived *C. jejuni* isolates to create, for the first time in the country, a database with *flaA* sequence types, which could be used for future epidemiological and clinical studies.

Several previous studies adopting genotypic methods have revealed that *Campylobacter* spp. have a high level of genetic diversity and are weakly clonal (17, 27, 37-41]. This finding has also been observed in the present study for both human and chicken derived

isolates. This may be an attribute of rapid adaptive changes during infection or colonization cycles [42]. The suggested mechanisms to be responsible for the evolution of novel variants include natural transformation, genomic recombination and rearrangements; and chromosomal point mutations (42-48]. The displayed genetic diversity of these organisms complicates the challenge of managing their population dynamics within the different reservoirs [41]. Moreover, different lineages and the relatedness among isolates are difficult to determine, particularly when conducting long-term epidemiological studies. Such studies may need to apply more than one sub-typing methods to carefully describe the population dynamics of these organisms [41].

In this study some *C. jejuni* isolates derived from chickens shared common clusters with those derived from humans. This finding suggests that these isolates are genetically related, further highlighting that poultry are an important source of *Campylobacter* infections in humans as previously revealed in case-control studies [49-56]. Our study also indicates that while broiler chickens remains an important source, layer and free ranging indigenous chickens could be an underestimated reservoir of human *C. jejuni* cases.

Some *C. jejuni* isolates in the same cluster had different *fla* types. This finding has previously been observed by O'Mahony et al. [41]. The observed diversity in *fla A* sequence types among apparently related isolates could probably reflect genetic instability involving the flagellin locus, reported by several authors [40, 45, 57], and may have significant contribution to antigenic diversity and host evasion strategies of these bacterial species.

In conclusion, this study highlights the high level of genetic diversity that exists among *Campylobacter* strains confirming the weak clonality of this species. The results further

reveal genetic relatedness of some human and chicken derived isolates. The roles of chickens other than broilers need to be further investigated in large epidemiological case control studies with the aid of increasingly available genotyping information; in order to better determine their relative contributions to human campylobacteriosis. Though not suitable to be used alone as a typing method for long-term epidemiological studies as the method is based on a single gene prone to recombination and re-arrangement, *flaA* typing provides a useful rapid preliminary characterization of strains for short term epidemiological studies.

ACKNOWLEDGEMENTS

The government of Denmark is acknowledged for supporting this research through the Danish International Development Agency (DANIDA), (grant 66-08-LIFE).

DECLARATION OF INTEREST

None.

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CHAPTER THREE

3.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

With the findings of this study it has been shown that thermophilic *Campylobacter* are prevalent among humans and animals in the study area. Both symptomatic and asymptomatic human subjects; and farm animals, laboratory animals and wild birds are infected with *Campylobacter*. The most frequently detected species in previous studies, *C. jejuni* and *C. coli*, also featured in this study; with the former occurring at higher frequencies. The study has also been able to indicate that there exists antibiotic resistance among thermophilic *Campylobacter* isolates derived from both humans and animals; including resistance to macrolides and fluoroquinolones, the drugs of choice for the treatment of human campylobacteriosis. Furthermore the population structure, featured by weak clonaltiy; and relatedness of human and chicken derived *C. jejuni* isolates has been shown. It is recommended that there should be continuous and regular monitoring of infections with these organisms and their antimicrobial resistance trends both in humans and animals so as to provide useful information for devising control measures and guide antimicrobial therapy in cases requiring treatment. Control strategies combining intergrated efforts involving veterinary and medical professionals, a feature of one health approach, should be put in place to stem infections in animal reservoirs as well as human beings. Approaches aimed at preventing the increase and spread of antimicrobial resistance among the organisms should be adopted.

APPENDICES

Appendix 1: Informed Consent Form

Principal investigator: Dr. Erick V. G. Komba

Name of the organization: Sokoine University of Agriculture, Morogoro, Tanzania

Research Title: Molecular epidemiology and antimicrobial susceptibility of Thermophilic *Campylobacter* in the poultry production chain in Tanzania

Purpose of the study

Thermotolerant *Campylobacter* spp are most frequently isolated from diarrhoeal patients in both developed and developing countries. Poultry has been recognized as the primary reservoir of the organism and play an important role in the transmission of *Campylobacter* enteritis to humans.

In Tanzania limited work has attempted to establish relatedness between *Campylobacter* isolates from humans and chicken. The role of chicken in human infections has not been characterized. Furthermore, antimicrobial susceptibility for both human and animal isolates is an area which needs further investigation.

The present study is therefore designed to determine the prevalence of *Campylobacter* infections in humans and chicken and also establish the relatedness of isolates from humans and chicken. Antimicrobial susceptibility of the isolates will also be determined.

Procedures (Methodology)

This study will be conducted in Dar es Salaam and Arusha regions. Permission to do the research will be requested from Regional and District administrative officers. Preliminary survey of the study areas will be conducted to identify the key players in the chicken production chain i.e. producers, processors. Information on antimicrobial use, chicken population densities and farmers' knowledge on health risks associated with chicken production will be recorded during the survey. Collection of stool samples will involve drawing portions from samples presented to the health centers laboratories for diagnosis following doctor’s requests. The samples will be drawn after obtaining a written consent from the patient.

Benefits from the study

The present study, upon its completion, will provide a range of useful data including the extent of the problem, zoonotic potential of the pathogen and its susceptibility to different antimicrobials. The data will be useful in guiding the formulation of strategies to combat the problem of infections in humans and poultry; and that of antimicrobial resistance.

Confidentiality
The present research will be conducted with high degree of confidentiality. Participants' information including names and test results will be kept with secrecy.

Incentives to research participants
Free of charge consultations will be provided to the study participants at all levels of the production chain. These will include both veterinary and public health related, within the researcher's capacity.

Participation rights
Participants will be involved into the study following their own will to participate. There will be no barriers for the participants to withdraw from the research in case they want to do so.

Contact person

Principle investigator: Dr. Erick V. G. Komba
Mobile phone number: 0713 584 054
E-mail addresses: babagrid@yahoo.com
ekomba@suanet.ac.tz

Certificate of consent
I have read the above information/the information has been read to me and I have understood it. I have had the opportunity to ask question/questions about the research. The question/questions has/have been answered to my satisfaction. I consent voluntarily that I will participate in this study.

Name: Date and signature:

_____ _____

___/___/___ (*dd/mm/yy*)

If illiterate

Name of independent literate witness: Date and signature of witness:

_____ _____

___/___/___ (*dd/mm/yy*)

Name of researcher: Date and Signature of Researcher:

_____

Dr. Erick V. G. Komba ___/___/___ (*dd/mm/yy*)

Molecular epidemiology and antimicrobial susceptibility of Thermophilic *Campylobacter* in the Poultry production chain in Tanzania

Appendix 2: ***Questionnaire for patients in the case-control study of campylobacteriosis in Dar es salaam, Tanzania***

Definition: A case will be defined as a person inhabiting in Dar es salaam, ill with acute clinical signs attending to the municipal hospital. A face- to- face interview will be conducted on the day of stool sample collection.

A. Personal and demographic data:

Name of patient ……...Age……...Sex……..

Residence: Municipality……Ward……...

Address……..

Phone number……

Name of interviewer……..Date of interview……

B. Clinical information and economic impact data:

1. When did you become ill (illness onset date)? ……...
2. Which of the following symptoms did you experience?

Table 1: Clinical signs for campylobacter infection

Clinical signs	Yes	No	Unsure	When started	Duration (Days)
Nausea					
Vomiting					
Abdominal pain					
Diarrhoea					
Blood in stool					
Tenesmae					
Fever					Maximum:
Other symptoms					

3. When was the stool sample collected……..
4. Do you know any person who had similar clinical signs in two weeks before or in the two weeks after you become ill?

   Yes…….No……..Unsure……. date of illness onset……..

Is this person(s) member of your household? Yes……No……

5. Have you presently recovered from your illness? Yes…..No……..Unsure ……...
6. How many days were lost from school or work due to this illness…….
7. How many visits/consults to the doctor were made for this illness? .....
8. Did you receive drugs for this illness? Yes…….No……Unsure……Date started……Duration……Name of drug(s)……..
9. Were you hospitalized? Yes…….No…… Date started……..Duration…….
10. Do you have any chronic diseases?

Table 2: List of chronic diseases/symptoms

	Yes	No	Unsure	Comments
Anaemia				
Gastrointestinal disorder				
Recurrent diarrhoea				
Liver disorder				
Other chronic disease				

11. In the month before the onset of illness, did you take

Antibiotics: Yes …… No …….. Unsure …… Comments …….

Antacids regularly: Yes …… No …….. Unsure …… Comments …….

Ulcer medication, regularly: Yes ….. No …….. Unsure … Comments ……

Other regular medication: Yes ….. No ….. Unsure …… Comments …….

C. Data on travel and what the patient ate in the last two weeks before onset of illness

12. Did you travel during this period? Yes……..No…….

Dates (from-to) Destination

…… ……

…… ……

Did you develop diarrhoea illness during this period? Yes……No……Unsure……Date of illness onset…….. Duration…… Treatment…….....

13. Are there other persons in your household who travelled during the last month before you become ill? Yes……..No……..Unsure…….

    Did they develop diarrhoea? Yes….. No….. Date of illness…….

14. Did you eat barbecue or meals in either of the following places?

Table 3: Places where barbecue and/or other meals were consumed

	Yes	No	Unsure	Dates and type of meal	No. of meals
Bar					
Restaurant					
Cafeteria					
Barbecue					
Local brew club					
Bus stand, train					
Fast food pub					
Hotel					

15. Did you eat any poultry or poultry products at home? Date.....
    Please specify whether it was bought: (1) Live bird, (2) raw and fresh (3) raw and frozen (4) precooked.

Table 4: Consumption of poultry products

	Yes	No	Unsure	No. of meals	Specify how it was brought (1,2,3,4)
Hen					
Chicken					
Turkey					
Ducks					
Other poultry meat					

16. Did you eat any of the following meat products? Please specify whether it was bought: (1) raw and fresh, (2) raw and frozen, (3) Precooked.

Table 5: Consumption of meat products

	Yes	No	Unsure	No. of meals	Specify how it was bought
Roast beef					
Boiled beef					
Pork					
Sausages					
Game					
Other meat items (specify)					

17. Did you eat at barbecue?

Yes….No…..Unsure….. No. of meals eaten…….

What kind of food was served?

Table 6: Type of food that was served at the barbecue

	Yes	No	Unsure	No. of Meals	Comments
Poultry					
Beef					
Pork					
Fish					
Raw vegetables, Kachumbari					
Other food e.g. eggs					

18. Did you eat any meat products which was raw, rare done, or undercooked?

Table 7: Consumed meat products

	Yes	No	Unsure	No of meals	Comments
Poultry					
Pork					
Beef					
Goat					
Sausages					
Other meat (specify)					

19. How do you prefer the following meat to be cooked?

	Raw	Rare	Medium	Well
Poultry	…….	…….	……	……
Beef	…….	…….	……	……
Pork	…….	…….	……	……
Fish	…….	…….	……	……

20. Do you handle or prepare raw meat, including raw poultry in the kitchen?

Yes ….. No……. Unsure ……. No. of times …… Comments ……...

Raw poultry …….

Raw red meat ……..

21. During meat preparation, how are the following items handled after contact with raw meat before preparation of other foods?

Table 8: Handling of items used for meat handling before preparation of other food

	Hands	Chopping knives	Countertop	Board
Not used with other items				
Washed with soap and water				
Washed with hot water				
Wiped with a cloth				
Not cleaned between items				
Washed with cold water				
Do not know				

22. Did you eat any of the following items?

Table 9: Other food items that may serve as source of Campylobacter infection

	Yes	No	Unsure	No of meals	Comments
Fresh fish					
Frozen fish					
Shellfish, muscles					
Prepared salad					
Unpasteurized milk					
Pasteurized milk					
Ice cream					
Yoghurt					
Others (specify)					

23. Did you eat raw vegetables, mushrooms or unpeeled fruits

Table 10: Consumption of raw food (vegetables, mushroom and fruits)

	Yes	No	Unsure	No. of meals	Comments
Lettuce					
Raw carrots					
Green beans					
Other raw vegetables					
Raw mushrooms					
Unpeeled fruits					
Others					

24. Were you in contact with animals or birds?

Table 11: Types of animals in-contact with humans

	Yes	No	Unsure	No. of days/times	Comments
Dog					
Cat					
Other pets					
Cattle					
Pig					
Sheep or goat					
Horse					
Poultry					
Wild birds					
Wild animals or game					
Other animals or birds					

25. Did you attend or work in hospital, health facility, kindergarten or day-care center?
    Yes……..No……Unsure……..

26. What kind of drinking water supply do you have at home?

Table 12: Type of water supply and sources

Supply		Source	
Public supply		Surface source (lake, stream)	
Private water work		Ground water, bore hole	
Other private supply		Dug-out well	
Unknown		Unknown	

27. Is your drinking water treated (e.g. chlorinated) Yes……..No…….Unsure…….
    If no do you treat drinking water?.....

28. Did you drink water from any other places? Yes……No……..Unsure……
    If yes specify where and what kinds of water supply and whether it was

treated:…….

29. Did you drink water directly from a tape, lake, river, pond, well etc.?
    (for example during farming, grazing animals, meeting, hunting etc):
    Yes…….No……Unsure……

30. Do you use one of the following toilet services?

Table 13: Types of toilets/latrines

Toilet type	Yes	No
Pit latrines		
Western type		
Squat toilets		
Both types		
Others (specify)		

31. Do you use any of the following after using a toilet? (Yes or No)

Soft tissue …….. Water …….

Both ……... None ……

32. How do you wash hands after toilet use?

…….

33. Are you involved in any of farming activities listed below

Table 14: Farming (crop and livestock) activities

Activity	**No**	**Yes**	**When started**	**Responsibility (owner, attendants)**
Crop cultivation				
Gardening				
Keeping commercial layers				
Keeping broilers				
Keeping local chickens				
Keeping dairy cattle				
Keeping small ruminants				
Keeping pigs				
Keeping indigenous cattle				
Other farming related activities (specify)				

34. Do you know that there is a possibility of getting gastrointestinal infections from such activities mentioned above? Yes…… No…….
35. If Yes in (34) mention infections or diseases that humans can get from activities in 34

Table 15: Infections and diseases that can be acquired through involvement in farming

Infections/diseases	Source of infection	Control of transmission	Where did you get health education
1.			
2.			
3.			
4.			

36. Do you know any gastrointestinal diseases that humans can acquire through contact with/consumption of poultry meat? YES……NO…….……..

37 If the answer is YES in 36 mention the diseases; the pathogens; transmission; control measures and how you acquired the control skills

Table 16: Gastrointestinal diseases acquired through consumption of poultry and control measures

GIT disease	Aetiology	How is transmitted	Control measures	Source of health education

38 Do you have any comments about this campylobacter research and other zoonoses?
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.....
.....
.....

THANK YOU FOR YOUR COOPERATION