THE ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF CAMPYLOBACTER ISOLATES FROM NAIROBI, KENYA

BY

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The antimicrobial susceptibility

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DECLARATION

I, Pamela P. N. Kabiru, declare that this thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to my friend and husband Prof. Ephantus Kabiru and to our children Jimmy Wanjohi, Faith Gathoni and Jane Wangechi for their patience and understanding in course of this study.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ACRONYMS

API	Analytical Profile Index
CCDA	Charcoal, cefoperazone, deoxycholate agar
DALY	Disability Adjusted Live Year
DNA	Deoxyribonucleic acid
EPEC	Entero-pathogenic *Escherichia* coli
GBS	Guillain-Barre Syndrome
GE	Gastro-enteritis
MIC	Minimum Inhibitory Concentration
NCCLS	National Committé for Clinical Standard
PCR	Polymerase Chain Reaction
PFGE	Pulsed-field gel electrophoresis
PHD	Public Health Department
RBC	Red Blood Cells
RFLP	Restriction fragment length polymorphism
SPSS	Statistical Package for Social Scientists
USA	United State of America
WBC	White Blood Cells
WHO	World Health Organization
Stool	Human faecal material

DEFINITION OF TERMS

Diarrheic stool	Stool specimens that were soft, loose or watery.
Consistency	The texture of the stool specimen i.e softness or watery state.
Soft stool	Stool specimen that took the shape of the container and could not pour if the container was tilted.
Loose stool	Stool specimen that could pour off when the container was tilted
Watery stool	Stool specimen that contained more than 75% water and poured off like water when the container was tilted.

ABSTRACT

Campylobacteriosis is a group of infections caused by Gram negative rods of genus *Campylobacter*. It is among the most common bacterial infections of humans world-wide. Campylobacteriosis in Kenya is managed and treated presumptively according to its clinical features and manifestation. The common antimicrobial drugs used are: fluoroquinolones, macrolides and quinolones. Indiscriminate use of these drugs may lead to development of antimicrobial resistance. The main objective of this study was to investigate the antimicrobial susceptibility pattern of *Campylobacter* in Aga Khan University Hospital, Nairobi, Kenya. Four hundred and forty seven (447) diarrheic stool samples were obtained from in and out-patients seeking treatment in the Hospital. For isolation of *Campylobacter,* stool samples were cultured onto blood-free selective Campylobacter medium. The isolation rates of bacterial pathogens were 29 (6.5%) for *Campylobacter* species. Other bacteria isolated were *Shigella, Salmonella and* Enteropathogenic *Escherichia coli.* The *Campylobacter* isolates were characterized to species and sub-species by colonial morphology, staining, motility and Analytical Profile Index (API) Campy strip tests. Out of 29 *Campylobacter* isolates 28 (96.6%) were *C. jejuni* and one (3.4%) was *C. coli.* The proportional distribution of *C. jejuni* bio-type 1 accounted for six (20.7%) while bio-type 2 accounted for 23 (79.3%). There was significant difference between isolation rates of *Campylobacter* across the age groups (χ^2 = 8.825, p = 0.032. There was no significant difference in isolation of *Campylobacter* between males and females (χ^2 = 0.534, p = 0.465). Out of the 300 samples that had invasive infection 15 (5%) were due to *Campylobacter*. Antimicrobial susceptibility testing was performed by disk diffusion method and by E-test system according to Clinical and Laboratory Standards Institute (CLST). The study findings showed that there was no resistance to azithromycin, chloramphenicol, gentamicin and doxycycline. Resistance to ciprofloxacin was detected in four (13.8 %), ampicillin two (7.1 %), nalidixic acid three (10.3 %), cotrimoxazole 23 (79.3 %). Cross-resistance was detected between ciprofloxacin and nalidixic acid and between cotrimoxazole and ampicillin. Beta-lactamase production was detected in 75.5% of the isolates. The results of this study showed that the prevalence of *Campylobacter* in the patients from Aga Khan University Hospital was 6.5%. There was detection of resistance of the Campylobacter isolates to some antimicrobial drugs commonly used. Beta-lactamase production occurred in many isolates. It is therefore important for the treatment of *Campylobacter* to be instituted only after susceptibility testing has been done.

CHAPTER ONE INTRODUCTION

1.1 Introduction

Campylobacteriosis is a group of infections caused by gram negative rods of the genus *Campylobacter*. It is among the most common bacterial infections of humans worldwide that is characterized by both diarrhea and systemic illness (Martin, 1990). The major form of campylobacteriosis of public health importance is campylobacter enteritis due to *C. jejuni* and *C. coli* (Fermer and Engvall, 1999). It is estimated to cause 5 -14 % of diarrhea world wide (Karl and Yvonne, 2004). These organisms have been included amongst the most important causative agents of acute diarrhea in the industrialized world (Tauxe, 1992). There are four groups of thermophilic members of *Campylobacter* (*C. jejuni*, *C. coli*, *C. laris* and *C. upsaliensis*) that cause human enteritis, but only two are responsible for the majority of cases.

Campylobacter infections may cause acute self-limiting illness, but severe infections do occur (Allos, 2001). Fluoroquinolone, for example ciprofloxacin, is often prescribed empirically for treatment of gastroenteritis and for *Campylobacter* infections in adults (Dryden *et al.*, 1996). Macrolides such as erythromycin are also prescribed to treat *Campylobacter* infections (Allos, 2001). The rates of resistance to these drugs are increasing in both developing and developed countries (Engberg *et al.*, 2001). The objective of this study was to investigate the antimicrobial susceptibility pattern of *Campylobacter* species in Aga Khan University Hospital, Nairobi, Kenya.

1.2 Statement of the problem

Diarrhea is major problem in the world (WHO, 1999). It is particularly important in Kenya since bacterial diarrhea constitutes one of the main health problems (Shimotori *et al.*, 1986; John *et al.*, 2003). From studies (Wamola *et al.*, 1983; Mutanda *et al.*, 1985; Waiyaki *et al.*, 1986; Chunge *et al.*, 1989; John *et al.*, 2003), *Campylobacter* showed high frequency of isolation from patients with diarrhea in the country. The rate of *Campylobacter* infections worldwide has been increasing, with the number of cases often exceeding those of salmonellosis and shigellosis (Chunge *et al.*, 1989; Altekruse *et al.*, 1999; Nizar, 2001). Campylobacteriosis in Kenya is managed and treated according to its clinical features and manifestation. From these signs (fever, abdominal cramps and diarrhea with or without blood) alone empirical treatment with fluoroquinolones, macrolides and quinolones is often instituted which may lead to un-monitored development of antimicrobial resistance. Kenyan data on the *in vitro* activities of commonly used antimicrobial agents against *Campylobacter* as agent of diarrhea are scanty and sporadic and were last done in 1980s.

1.3 Justification

Campylobacter infections particularly *Campylobacter jejuni* and *Campylobacter coli*, are among the common causes of the bacterial diarrhea in humans worldwide (Martin, 1990). It is the most commonly isolated bacterial pathogen from children of less than 2 years old. No treatment is required in most cases of enteritis, as they are of short duration, clinically mild and self-limiting. However, antimicrobial treatment is necessary for systemic *campylobacter* infection, campylobacter infections in immunosuppresed

patients and severe or long-lasting campylobacter infections. Fluoroquinolones have been commonly used to treat serious campylobacter infections, they are also used as empiric therapy for travelers diarrhea (Adachi *et al.*, 2000). There have been reports of increase of antimicrobial resistance in campylobacter infections worldwide (Nachamkin, 2002; Tjaniadi *et al.*, 2003). Since diarrhea is an important problem in the world (WHO, 1999) as it is in Kenya (John *et al.*, 2003), proper control should be addressed and especially where antimicrobial therapy has to be employed. This should be guided on *in vitro* susceptibility testing because of the prevalence of antimicrobial resistance. This study was aimed at finding out the trend of antimicrobial susceptibility pattern of *Campylobacter*. The result of this study will help in management and control of Campylobacteriosis as it will provide data and information on the antimicrobial susceptibility pattern.

1.4 Null hypothesis

Campylobacter isolates from Nairobi, do not display antimicrobial resistance.

1.5 Objectives

1.5.1 Main Objective

To establish the antimicrobial susceptibility pattern of *Campylobacter* isolated from diarrheic stools received in Aga Khan University Hospital, Nairobi, Kenya.

1.5.2 Specific Objectives

i, To determine the isolation rate of *Campylobacter* from study area.

ii, To characterize the *Campylobacter* isolates from the study samples.

iii, To correlate the campylobacter infection with sex and various age groups

iv, To establish the antimicrobial response of *Campylobacter* isolates.

CHAPTER TWO LITERATURE REVIEW

2.1 Prevalence and distribution of *Campylobacter*

From many clinical and epidemiological studies, Campylobacteriosis is among the commonest bacterial infections of humans in many parts of the world (Martin, 1990; Shen and Seng, 2001; de Wit *et al.*, 2001). It is the leading cause of bacterial diarrhea illness in the developed countries, about 2.4 million cases occurring annually in United States (U.S), in United Kingdom (UK) and others (Nachamkin, 2003). *Campylobacter* causing invasive infection accounts for 5-10% of all diarrheal cases annually in the US. *Campylobacter jejuni* and C. *coli* are the two main species isolated. In developing countries, *Campylobacter* isolation rate ranges from 5-26 % (Oberhelman and Tayor, 2000). In Nigeria the prevalence rate is 5-16%, (Zaman, 1992; Coker and Adefeso, 1994), Zambia 6 % (Luo *et al.*, 1996) and Tanzania 18% (Gun-britt *et al.*, 1995). In Egypt (Rao *et al.*, 2001) and Brazil (Mangia *et al.*, 1993) prevalence rates of 9%, while in Thailand and Bangladesh prevalence rates of 13 and 17% respectively have been recorded (Echeverria *et al.*, 1989; Albert *et al.*, 1999).

In Kenya the isolation rate ranges from 12-26 % (Wamola *et al.*, 1983; Shimotori *et al.*, 1986; Waiyaki *et al.*, 1986; Chunge *et al.*, 1989; Osano and Arimi, 1999). *Campylobacter* has the highest incidence in infants and young children (DPH, 2000). In the developing countries it is the most commonly isolated bacterial pathogen from less than 2 years old children with diarrhea. Most symptomatic infections occur in infancy and early childhood and prevalence decreases with age. However, studies done in Egypt showed that infection

could be pathogenic regardless of the age as it occurs in developed countries (Rao *et al.*, 2001).

2.2 Modes of transmission

The faecal-oral route and ingestion of contaminated food and water are the principal modes of transmission of *C. jejuni* from animal reservoirs to humans. Campylobacteriosis may result from direct contact with infected animal or contact with contaminated animal carcasses (Tom, 1999).

2.2.1 Food-borne

Man becomes infected by *Campylobacter* organisms through consumption of raw or improperly handled cooked foods, primarily poultry meat and unpasteurised milk. Poultry is an important source of campylobacteriosis in developed and developing countries and they are linked not only to outbreaks but also sporadic campylobacter infection (Osano and Arimi, 1999). *Campylobacter* species were isolated from 40% and 70% retail poultry meat sold in Bangkok, Thailand and Kenya respectively (Rasrinaul *et al.*, 1988; Osano and Arimi, 1999). In Mexico, ready to eat roast chicken showed contamination with *Campylobacter* (Quinones-Ramirez *et al.*, 2000). Contamination of food may result during preparation from animals' intestinal contents or by incomplete cooking.

Cases of campylobacter infection due to consumption of raw unpasteurised milk and contaminated bottled milk where the tops have been pecked by birds have been reported

by Taylor *et al.* (1979) and Tom (1999). In some parts of the UK bird-pecked milk was thought to be an important contributory factor in the early summer peak of infection. Raw milk serves as a common vehicle of infection.

2.2.2 Animal to human

This may be through direct contact with the infected animals or through their by-products for example eggs and raw milk (Taylor, 1979; Tom, 1999; Altekruse *et al.*, 1999). Transmission can also occur from environment contaminated with *Campylobacter species* resulting from droppings of wild birds as well as domesticated and pet animals which serve as its reservoir hosts.

2.2.3 Human-to-human transmission

This may occur from infected individuals or from convalescent carriers especially in young children. High population density has also been suggested (Taylor, 1992; Rao *et al.*, 2001).

2.2.4 Water-borne

Isolates of *C. jejuni and C. coli* have been cultured from rivers, lakes and the sea from many sites in developed countries (Blaser *et al.*, 1983). Surface water gets contaminated from the excreta of wild and domestic animals. Supplies of portable water can get contaminated by sewage or untreated water. Outbreaks due to water are as a result of drinking untreated water.

2.3 Clinical features

Infections by *C. jejuni* and *C. coli* may be asymptomatic to severely ill. *Campylobacter* infection has an incubation period of between 2-10 days with a median of 4 days (Tom, 1999). Clinical features usually include fever, abdominal cramps, and diarrhea with or without blood or pus (Taylor, 1992; DPH, 2000; Oberhelman and Tayor, 2000). Initial intestinal symptoms may either be abdominal pain or nausea and vomiting. In a small number of cases, a temperature of 40°C develops, which can be associated with delirium. Diarrhea, which is of two distinct types, is an important symptom in most, but not all cases. The severity varies from the severe and prostrating to a few loose stool, but generally less than that of Salmonella infections. The two types of diarrheal symptoms observed may be due to the different mechanisms of pathogenicity predominating in different strains. The first type is secretory, with profuse watery stools which are usually bile stained and of foul odour.

The second type resembles dysentery, stools containing inflammatory cell occult blood. Colonic infection is a common feature, therefore colitis is observed. Abdominal pain is a major feature of campylobacteriosis and is usually more severity than that due to salmonella and other enteric pathogens. Vomiting is not a major symptom of campylobacter enteritis and despite its early appearance in some cases, rarely occurs more than once or twice during the course of illness. Campylobacteriosis is usually self-limiting, symptoms lasting less than a week in healthy adults. The patient has persistent weakness during recovery and abdominal pain and discomfort may persist for some time after other symptoms disappear. Recurrence of symptoms is a feature of infection in 25%

of cases and is due to campylobacter ability to undergo rapid phase variation. There are some differences in symptoms observed between adults and children, and also in developed and developing nations. In developed countries symptoms are mild; severe dehydration and fever are rare in infantile diarrhea. Bloody stools are common in industrialized nations, but elsewhere, watery stools predominate. Differences in symptoms between industrialized and developing nations are probably due to hyperendemic exposure to campylobacter and the patients are also often underweight and malnourished in the latter (Coker, 1985; Bhadra *et al.*, 1989; Rao *et al.*, 2001). *Campylobacter* infections can mimic acute appendicitis and could result in unnecessary surgery (Nachamkin, 2003). *Campylobacter* has been linked to a growing list of diseases including haemolytic uremic syndrome, hepatitis, pancreatitis, appendicitis, cholecystitis and Reiter's disease. Guillain –Barre Syndrome (GBS) is an autoimmune disorder of the peripheral nervous system which is characterized by acute flaccid paralysis and *C. jejuni* infection is the most frequently identified infection preceding GBS (Nachamkin, 1998).

2.4 Pathogenesis

Campylobacter has a low infective dose where as few as 500 organisms can cause infection (DPH, 2000). *Campylobacter* first colonizes the intestinal mucosa mediated by motility and then invades and or translocates through the epithelial surface to the underlying tissue where other putative virulence factors also occur. The presence of blood and pus in the stools of infected persons indicates that C. *jejuni* can be invasive however, a secretory form of diarrhea also occurs in children, suggesting the involvement

of an enterotoxin (George and Charlotte, 1985). *Campylobacter jejuni* expresses a cytolethal distending toxin; however the role of this toxin is not understood.

2.5 Isolation and identification of *Campylobacter*

Various different blood-based and non-blood based media containing different antibiotic supplements and growth factors are available for isolation of *Campylobacter* from stool specimens. These media include: Skirrow's, Butzler's , Blaser's, and Improved Preston blood free (Cheesbrough, 2000). These media allow growth of different species and strains of *Campylobacter* while restricting other pathogens and faecal commensals. *Campylobacter* species are microaerophilic (require decreased 0xygen) and capnophilic (require increased carbon dioxide). The primary isolation of these organisms is achieved in an atmosphere of 5% oxygen, 10% carbon dioxide and 85% nitrogen and an incubation at 36° C for 48 hours (Cheesbrough, 2000).

Campylobacter species are gram negative organisms, non-spore forming rods that may be curved, S shaped or spiral rods that are 0.2 to 0.9μm wide and 0.5 to 0.5 μm long. Some species like *C. hominis* form straight rods. *Campylobacter* species may form spherical or coccoid cells in old cultures or cultures exposed to air for a long time. The organisms are motile and monotrichus. Identification of *Campylobacter* can be done by simple colonial morphological characteristic of the organism and then microscopic appearance (Carda relli-Leite *et al.*, 1996). They are known to be catalase and oxidase positive (George and Charlotte, 1985).

For species and subspecies differentiation, further tests such as nitrate reduction, hydrogen sulphide production, tolerance to antimicrobial agents and hippurate test are done. Phenotyping and molecular methods for identifying members of this group have been done and tests such as DNA-DNA hybridization (Eyers *et al.*, 1993; Luc Dedieu et *al.*, 2 004), and 16s DNA have been used for identification (Gorkiewiecz *et al.*, 2003). Flagellin typing (Fla.typing), ribotyping and pulsed-field gel electrophoresis (PFGE) are in use for epidemiological typing (Wassenaar and Newell, 2000). A number of genetically based detection and typing methods like restriction fragment length polymorphism analysis (RFLP) of flagellin gene (fla A) in *C. jejuni* and *C. coli* (Warssenar and Newell, 2000) have also been used. A standard PCR assay based on the gene encoding for putative virulence determinants has been used for specific identification of *C. jejuni* and *C. coli* (Gonzalez *et al.*, 1997).

2.6 *Campylobacter infection* in HIV patients

Campylobacter associated diarrhea and bacteremia occur in HIV/AIDS patients worldwide. The species encountered are *C. jejuni, C. coli* and *C. upsaliensis,* often occurring together with *Arcobacter butzteri, Helicobacter fennelliae, and H. cinaelli* (Lastovica *et al.*, 2001; Germani *et al.*, 1998). The incidence of clinical manifestation is higher than in HIV negative patients, with substantial mortality and morbidity. Infants in developing countries are at risk of impaired immunity to *campylobacter* enteritis. In addition, HIV/AIDS can increase the number of cases of campylobacteriosis in the adult population in these countries.

2.7 *Campylobacter* and Travelers' diarrhea

Campylobacter jejuni is a common cause of Travelers' diarrhea. The diarrhea is more severe, and the strains are associated with antibiotic resistance (Gallardo *et al.*, 1998; Shlim *et al.*, 1999). American troops in Thailand have been afflicted with *C. jejuni* (Beecham *et al.*, 1997).

2.8 Mixed-infections involving *Campylobacter*

Co-infection of *campylobacter* with other enteric pathogens in patients with diarrhea in developing countries is very common (Wamola *et al.,* 1983; Chunge *et al.*, 1989; Mutanda *et al.*,1990; Shen and Seng, 2001) The organisms reported include *Salmonella, Shigella, Escherichia coli, Rotavirus, Giardia lamblia* and *Entamoeba histolytica.* Co-infections involving *campylobacter* are rare in developed countries.

2.9 Seasonal variation of *Campylobacter* isolation

A large number of *campylobacter* cases in developed countries are sporadic, occurring in hot (summer) months (DPH, 2000; Nachamkin, 2003). They are as a result of ingesting improperly handled or cooked food primarily poultry products. Other infections occur in cold months and are associated with contaminated food or water. There are no seasonal variations in developing countries. The isolation rates vary from one country to another and also different at times within the countries (Rao *et al.,* 2001). The absence of seasonal preference in the developing countries may be due to lack of extreme temperature variations and lack of adequate surveillance for epidemics (Taylor, 1992; Oberhelman and Taylor, 2000).

2.10 *Campylobacter* and immunity

In developing countries such as Bangladesh, Thailand, Central Africa Republic and Mexico, healthy children and adults are constantly exposed to *Campylobacter* antigen in the environment. As a result, serum antibodies to the *Campylobacter species* develop very early in life in children in developing countries than those in developed world such as United States (US) (Blaser *et al.*,1985; Blaser *et al.*, 1986; Martin *et al.*, 1989; Blaser, 1997). Breast feeding has been reported to have role in *C. jejuni* induced diarrhea. It decreases the number of episodes and duration of diarrhea (Ruiz-Palacious *et al.*,1990).

Among the Mexican children, immunity to *Campylobacter* after primary infection may prevent development of bloody diarrhea or may prevent the manifestation (Calva *et al.*, 1988). In developed countries, where most subjects are naïve, the infections are usually with more severe clinical manifestation. Immunity to campylobacter, seen among adults in developing countries, is absent in adults in developed countries (Blaser, 1997).

2.11 Socio-economic impact of campylobacteriosis

The Disability Adjusted Live Year (DALY) is the basic unit in Burden of disease (BoD) methodology to quantify the impact of the disease in a population (Murray *et al.*, 1996). It has been applied in the Dutch population to measure the mean health burden of *Campylobacter*-associated illnesses in the period of 1990-1995. The mean estimate was 1,400 DALYs per year and the main determinants were acute gastroenteritis (440 DALYs) and residual symptoms of GBS (340 DALYs). There is no DALYs data due to

Campylobacteriosis in developing countries, but diarrhea which is the manifestation of Campylobacteriosis, was one of the top three causes of death in the developing countries in 1990s (Murray *et al.*, 1996). The disease ranks as number two on WHO list of infectious diseases, each year 99 million people are stricken (WHO, 1999) and is projected to remain on top 10 by 2020. The burden of Campylobacteriosis in the developing countries may increase by the year 2020 because HIV is projected to move up to the 10^{th} from the 28^{th} position by 2020.

2.12 Control of campylobacteriosis

2.12.1 Chemotherapy

Treatment is not generally indicated because campylobacter infections are often self-limiting. In severe cases however, dehydration may occur and electrolyte replacement may be prescribed. Antimicrobial treatment is indicated in severe cases or where complications are present to kill the organisms, thus shortening the duration of illness and may be life-saving in invasive infections. Antimicrobials such as erythromycin, ciprofloxacin, tetracycline and nalidixic acid may be used to treat the infections but because of the high prevalence of resistant strains the laboratory determination of drug response should always be carried out.

2.12.2 Personal hygiene

Prevention of infection requires control measures at all stages of the food chain, from agricultural production on the farm, to processing, manufacturing and preparation of

foods in both commercial establishments and the domestic environment. Good hygienic slaughtering practices will reduce contamination of carcasses by faeces, but will not guarantee the absence of *campylobacter* from the meat and meat products. Education in hygienic handling of foods for abattoir workers and those involved in the production of raw meat is essential to keep microbiological contamination to a minimum. However, the only effective method of eliminating *campylobacter* from, contaminated food is to introduce a bactericidal treatment such as heating (cooking or pasteurization) or irradiation (WHO, 2000).

Preventive measures for campylobacter infection in the household kitchen are similar to those used against other food-borne bacterial diseases such as making sure that other foods such as fruits and vegetables do not come into contact with cutting boards and knives that have been used on raw meat and poultry. To avoid cross-contamination, cutting boards, countertops, and utensils should be all carefully cleaned with soap and hot water after preparing raw meat and poultry. Hands should be washed thoroughly using soap and water and dried completely after contact with pets especially puppies or farm animals, before and after preparing foods, especially poultry; and after changing diapers or having contact with an individual with an intestinal infection. Fruits and vegetables should be carefully washed, particularly if they are eaten raw and if possible should be peeled (WHO, 2000). Raw milk products and untreated surface water should be avoided and food kept away from insects.

2.12.3 Sanitation

Sanitation is an important control strategy for campylobacteriosis. It involves proper disposal of human solid waste. The use of latrines is usually emphasized especially in crowded settlements. In the control of campylobacteriosis there should also be proper disposal of infected slaughter material from animals and birds. This would ensure that the environment is clean and free from contamination (WHO,2000).

2.12.4 Health education

Health education is given to empower the community to control the infections at household and community level. The community should own the control programmes if there is any expected change in the attitude and in the implementation of the control strategy. The control processes should be simple, direct and easily manageable by the common person (WHO, 2000).

2.13 Antimicrobial response in *Campylobacter* isolates

Antimicrobial agents are recommended for severe infections or persons at risk such as children or immunocompromised patients (Saenz *et al.*, 2000; Engberg *et al.*, 2001), especially in Africa where HIV and AIDS has reached epidemic proportions. Erythromycin and ciprofloxacin are drugs of choice (Engberg *et al.*, 2001). The rate of resistance to these drugs is increasing in both developing and developed countries but is higher in developing countries (Steinbruckner *et al.*, 2001). Resistance to erythromycin in developed countries is often low and stable at approximately 1-2%; but this is not so in developing countries (Steinbruckner *et al.*, 2001; Feierl *et al.*, 2001), for example, in

Nigeria, in 1984, 82 % *Campylobacter* strains were sensitive but 10yrs later only 20.8% were sensitive (Coker and Adefeso, 1994). In Thailand *Campylobacter* isolates in 1994-95 were resistant to azithromycin at 7-15%.

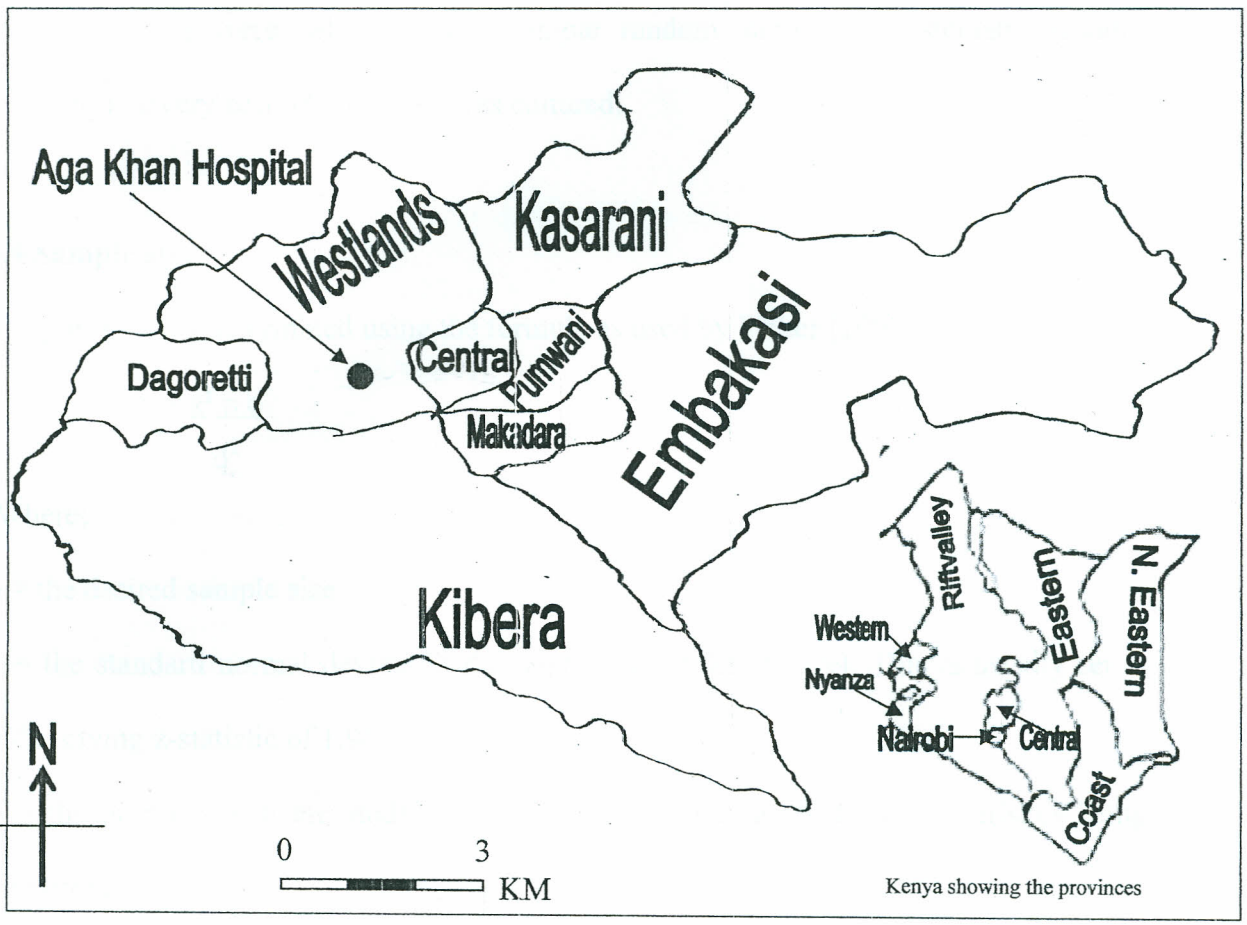
Fluoroquinolone resistant *Campylobacter* infection in human was first detected in Europe late 1980s, then around the world (Gaunt and Piddock, 1996). In Thailand, ciprofloxacin resistance increased from 0% before 1991 to 84% in 1995 (Hoge *et al.*, 1998). Recent data have shown a marked increase in resistance to quinolones in developed countries (Molina *et al.*, 1995; Kirk *et al.*, 1999). In various studies on *Campylobacter* in Kenya there appears to be no recent published data on the pathogen's antimicrobial susceptibility patterns. There is therefore need to assess the level of resistance in Kenya today to quinolones and other antimicrobial agents used for treatment of this problem in recent years.

CHAPTER THREE MATERIALS AND METHODS

3.1 Study area

The study was carried out at Aga Khan University Hospital, a tertiary care postgraduate teaching institution of 300 bed capacity. The hospital is located in Westland Division of Nairobi Province, of Kenya (Figure 1).

Figure 1: Map showing the location of Aga Khan University Hospital. In set map of Kenya showing position of Nairobi



Source: NAIROBI and ENVIRONS EDITION 3. Published by Survey of Kenya, 1978.

3.2 Study sample

The study samples were from patients both adults and children seeking treatment at the Aga Khan hospital during the months of August, September, October and November 2007. Samples were obtained from both outpatients and inpatients.

3.3 Study specimens and sampling technique

Diarrheic stools submitted to the laboratory for routine diagnosis were included in the study. Samples were selected using simple random sampling (systematic random sampling). Every second specimen was cultured.

3.4 Sample size

Sample size was determined using the formula as used by Fisher (1998)

$$n = \frac{z^2 p q}{d^2}$$

Where;

n = the desired sample size

z = the standard normal deviate at the required confidence level. This is usually set at 95%, giving z-statistic of 1.96

p = the proportion in the study population estimated to have the characteristics being measured.

q = 1-p

d = the level of statistical significance was set at 0.05.

The prevalence of *Campylobacter* infection in Kenya is not known when adults are included, hence a prevalence of 50% was used to calculate the sample size using p = 0.5, Z = 1.96, d = 0.05

$$\text{Therefore, } n = \frac{(1.96)^2 (0.5)(0.5)}{(0.05)^2}$$

$$= 384.2$$

The minimum number of study samples to be processed was 385.

3.5 Laboratory investigations

3.5.1 Macroscopic investigation

Macroscopic examination of the specimens was carried out to establish the presence of blood, mucus, adult parasites and food particles. Macroscopic examination also gave the consistency of samples as soft, loose, mucoid, watery or bloody.

3.5.2 Determination of invasive and noninvasive infections

To differentiate between invasive and noninvasive infection, the presence of white and red blood cells was determined using normal saline on un-concentrated samples by direct microscopy.

3.5.3 Isolation of *Campylobacter* spp.

In this study Blood-free medium, (charcoal cefoperazone deoxycholate agar, CCDA, Oxoid) was used according to Merino and Agulla, (1986). Immediately specimens were received in the laboratory, they were heavily inoculated using a wire loop onto the campylobacter selective culture plates (Oxoid media) which were prewarmed and surface

dried. The inoculated plates were incubated in a microaerophilic environment, in candle jar (Blaser *et al.*, 1980) at 37°C, for at least 48 hours according to Cheesbrough (2000).

Blood-free selective media was prepared according to manufacturer's instructions from campylobacter blood-free selective agar base (CM739 Oxoid) containing bacteriological charcoal, ferrous sulphates, sodium deoxycholate, sodium pyruvate, casein hydrolysate, nutrient broth and agar. CCDA selective supplement (Code SR155E Oxoid) containing cefoperazone and amphotericin B was added. One vial of supplement was used to prepare 500mls of media from which 25 plates were poured. The prepared media was stored moist at 4°C until used, maximum of two weeks.

3.5.4 Characterization of *Campylobacter*

3.5.4.1 Colonial morphology

After 48 h of incubation, plates were examined macroscopically. Where there was growth, colonies that were pinpoint in size, gray and entire round, were presumed to be *Campylobacter* according to Cheesbrough (2000).

3.5.4.2 Gram stain

From the characteristic colonies, smears were prepared, and stained by Grams stain according to George and Charlotte (1985). A characteristic morphology of slender, spirally curved rods or comma, S, or gull wing in shape was looked for.

3.5.4.3 Motility test

From pure culture on blood agar plate, few colonies were inoculated into Brain Heart Infusion broth. After 24 h of incubation at 37^0C, the organisms were checked for motility by taking a drop of broth culture, putting it on clean microscope slide, a cover slip applied and examined by use of phase-contrast microscope. *Campylobacter species* are motile with a darting motility.

3.5.5 Biochemical tests

3.5.5.1 Catalase test

Catalase test was done to establish the production of the enzyme catalase by the organism. The organisms were tested for catalase production by bringing them into contact with hydrogen peroxide according to Cheesbrough (2000). Briefly a drop of 3% hydrogen peroxide was placed on a glass slide, and with an applicator stick, culture of organism was placed on hydrogen peroxide. Release of gas bubbles (oxygen) indicated that the organism was a catalase producer referred to as "catalase positive"

3.5.5.2 Oxidase test

Oxidase testing was carried out by use of oxidase filter paper strips, impregnated with oxidase reagent (1% aqueous solution of tetramethyl-para-phenylene diamine), as described by Cheesbrough (2000). The test was performed by streaking a loopful of organism onto the oxidase filter paper strips. Development of a deep purple colour within seconds indicated a positive reaction referred to as "oxidase positive."

3.5.5.3 Analytical Profile Index (API) Campy Strip

API Campy is a two-part standardized system developed for the identification of thermophilic *Campylobacter species* (insert, 2006). The first strip consists of 10 enzymatic tests (urease, reduction of nitrates, esterase, hippurate, gamma glutamyl transferase, reduction of triphenyl tetrazolium chloride, pyrrolidonyl arylamidase, L-arginine arylamidase, L-aspartate arylamidase and alkaline phosphatase); the second strip consists of 1 enzymatic test (production of hydrogen sulphide, H_2S) 6 assimilation tests (glucose, sodium succinate, sodium acetate, propionate, malate and trisodium citrate) and 3 susceptibility tests (nalidixic acid, sodium cefazoline and erythromycin). Preparation of the two strips and the inoculum was in accordance with manufacturer's instructions. After 24 h of incubation at 35^0C under aerobic conditions, results for the first 10 enzymatic tests were obtained after the addition of appropriate reagents. Results for assimilation and inhibition tests were recorded after 24 h at 35 ^{0}C under microaerophilic conditions. Incubation was extended to 48 h if the succinate assimilation test was negative, in accordance with the manufacturer's instructions. Reading of the reaction was according to the Reading Table provided, and identification of the organisms was obtained by consulting the profile list in the package insert.

3.6 Testing for beta-lactamase producers

All isolates were tested for the production of beta-lactamase, an enzyme capable of inactivating antibiotics belonging to the β-lactam family (penicillins, cephalosporins and others). The production of such enzyme is an indication of resistance to β-lactams. The

Cefinase ™ reagent is used in the testing and is composed of paper discs impregnated with chromogenic cephalosporin, which releases a red compound on hydrolysis by a β-lactamase. The test was done according to instruction from the manufacturer. Briefly, the disc was moistened with sterile, demineralized water and then the test organism was spread over the surface of the disc. A red color was observed for the positive reaction and negative reactions were scored if no color appeared after one hour. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used as positive and negative controls respectively.

3.7 Antimicrobial susceptibility testing

3.7.1 Agar disk diffusion method

An agar disk diffusion method was performed as was originally described by Bauer *et al.* (1966). Several colonies of each isolate, obtained from a fresh culture in blood agar plate, were suspended in 5ml of Mueller-Hinton broth to achieve turbidity equal to the 0.5 McFarland standard. The suspensions were inoculated using sterile cotton swabs onto 90mm diameter Muller-Hinton 5% sheep blood agar plates and after the agar surfaces were allowed to dry, five antimicrobial disks were placed on each plate. The antimicrobials tested, together with their concentrations included azithromycin (15μg), doxycycline (10μg), nalidixic acid (30μg), gentamicin (10μg), cotrimoxazole (25μg), chloramphenicol (30μg), augmentin (30μg), nitrofuratoin (300μg), ceftazidime (30μg), ceftriaxone (30μg), cefuroxime (30μg), cefotaxime (30μg) and cefoxitin (30μg).

The plates containing the antimicrobials were incubated at 37^0C for 24 h under micro aerophilic conditions. As the bacteria on the plates grew, they were inhibited to varying degrees by the antimicrobials diffusing from the disk. Zones of inhibition were measured to the nearest millimeter using a ruler, recorded according to the guidelines of the Clinical Laboratory Standards Institute (CLSI) (2006) formerly (NCCLS). Zones of inhibition of certain diameter correlates with sensitivity or resistance to the antimicrobial tested. The raw data are in form of zone size in mm and, were interpreted on the basis of the available Clinical Laboratory Standard Institute (CLSI) data. The results obtained were reported as susceptible, intermediate, and resistant using breakpoints shown i n Table 1 (below) for each antimicrobial activity.

Table 1: Antimicrobials used and their breakpoints in susceptibility pattern testing by agar disc diffusion method

Antimicrobial Agent	Disk Content (μg)	Zone Diameter, Nearest Whole mm		
		R	I	S
Azithromycin/Erythromycin	15	≤ 13	14-17	≥ 18
Doxycycline/Tetracycline	30	≤ 12	13-15	≥ 16
Ciprofloxacin	5	≤ 15	16-20	≥ 21
Ampicillin	10	≤ 13	14-16	≥ 17
Nalidixic Acid	30	≤ 13	14-18	≥ 19
Gentamicin	10	≤ 12	13-14	≥ 15
Cotrimoxazole	25	≤ 10	11-15	≥ 16
Chloramphenicol	30	≤ 12	13-17	≥ 18
Augmentin	30	≤ 13	14-17	≥ 18
Nitrofurantoin	300	≤ 14	15-16	≥ 17
Ceftazidime	30	≤ 14	15-17	≥ 18
Ceftriaxone	30	≤ 13	14-20	≥ 21
Cefuroxime	30	≤ 14	15-17	≥ 18
Cefotaxime	30	≤ 14	15-22	≥ 23
Cefoxitin	30	≤ 14	15-17	≥18

R---Resistance, **I**---Intermediate **S**---Susceptible, **mm**----Millimeter

3.7.2 Minimum Inhibitory Concentration (MIC) method

This was done by means of E-test method. To perform the E-test, several colonies of each strain, obtained from a fresh culture in blood agar plate, were suspended in 5ml of Mueller-Hinton broth to achieve turbidity equal to the 0.5 McFarland standard. The suspensions were inoculated using sterile cotton swabs onto 90mm diameter Mueller-Hinton 5% sheep blood agar plates. Excess inoculum was removed by rotating the swab firmly against the tube above the level of liquid. The swab was streaked all over the surface of the medium three times, rotating the plates. Finally the swab was passed round the edge of the agar surface and the inoculated plates left to dry for few minutes at room temperature. The antimicrobial strips, two per plate, were placed on the inoculated plates using a pair of sterile forceps. Each strip was gently pressed down to ensure even contact with the medium.

Plates were then incubated at 37° C for 48 h under microaerophilic conditions, and the inhibitory concentrations were read at the points where elliptical zone of inhibition intersected the E-test strip. For this method, minimum inhibitory concentrations (MICs) were defined as the lowest antimicrobial concentrations yielding no growth. The antimicrobials tested in this study are as shown in Table 2.

Table 2: Antimicrobials with their ranges and breakpoints tested in the study by Minimum Inhibitory Concentration (MIC) method.

Antimicrobials	E-test MIC Ranges	Breakpoints	
		Sensitive*	Resistant**
Azithromycin/Erythromycin	0.016-256	≤ 0.5	≥ 8
Doxycycline/Tetracycline	0.016-256	≤ 4	≥ 16
Ciprofloxacin	0.002-32	≤ 1	≥ 4
Ampicillin	0.016-256	≤ 8	≥ 16
Nalidixic Acid	0.002-32	≤ 16	≥ 32
Gentamicin	0.016-256	≤ 4	≥ 16
Cotrimoxazole	0.002-32	≤ 2	≥ 4
Chloramphenicol	0.016-256	≤ 8	≥ 32

* This implied that an infection due to the isolate may be effectively treated with the usual dosage of the tested antimicrobial agent recommended for the site of infection present clinically; ** This category implied the possible failure of the tested antimicrobial agent to treat the isolate with the usually achievable systemic concentrations of the agent with normal dosage schedules and /or that demonstrate zone diameters that fell in the range where specific microbial resistance mechanisms are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.

3.8 Ethical consideration

Ethical clearance was sought from Kenyatta University, Ministry of Science and Technology and Aga-Khan University Hospital ethical committee.

3.9 Data analysis

Data was processed using SPSS. Categorical measurements were analyzed using chi-square test while the t-test was used for continuous measurements. Chi-square test was used to detect the significance of the developments of the resistance rates for all *Campylobacter species*. It was also used to test the statistical significance of the *Campylobacter species* and other aetiological agents in causing diarrhea. The t-test was used to analyze the relationship between age and infection.

CHAPTER FOUR RESULTS

4.1 Source of specimens

There were 447 specimens that were processed of which 388 (86.8%) were from outpatients and 59 (13.2%) inpatients.

4.2 Age and sex distribution of the patients

According to the laboratory request forms, the age of the patients ranged 11months to 100 years. The median age of the patients was 15 years. There were 127 (28.4%) that were below two years, 54 (12.1%) between three to five years, 44 (9.8%) between six to 16 year, and 222 (49.9%) were above 16 years. Out of 447 patients 233 (52%) were males and 214 (48%) were females with a male/ female ratio of 1.1:1 (Table 3).

Table 3: Age and Sex distribution of the patients

Age in years	Males	Females	No of patients	Percent
<2	61	66	127	28.4
3-5	26	28	54	12.1
6-16	27	17	44	9.8
>16	119	103	222	49.7
Total	233	214	447	100

4.3 Clinical presentation of the study patients

Since the study had no questionnaire the clinical conditions were recorded from the laboratory request forms submitted with the specimen to the laboratory. Most of the specimens were from patients with gastro- enteritis (GE) (77.9%). Others (5.2%) from patients with GE/Fever, (4.8%) patients with abdominal pain, (4.0%) patients with pyrexia of unknown origin (PUO), (1.2%) patients with GE/Upper resperitory tract infection (URTI), (0.8%) patients with GE/Immune suppressed syndrome(ISS) and amoebic dysentery, patients with GE/bloating, GE/malaria, GE/pregnancy, GE/post operative, salmonellosis/cryptosporidium, giardiasis, diabetes/hypertension, acute pancreatitis, TB, medical examination, organophosphate poisoning, cellulitis and sub-acute intestinal obstruction had (0.4%) each (Table 4).

Table 4: Clinical conditions of the study patients

Clinical Conditions	Frequency	Percentage
Gastroenteritis (GE)	194	78.0
GE, Fever	13	5.2
Abdominal pain	12	4.8
Pyrexia of Unknown Urigin (PUO)	10	4.0
GE, Upper Respiratory Tract Infection	3	1.2
GE, Immunosuppression syndrome	2	0.8
Amoebic Dysentry	2	0.8
GE, Bloating	1	0.4
GE, Malaria	1	0.4
GE, Pregnancy	1	0.4
GE, Post-operative	1	0.4
Salmonellosis, Cryptosporidium	1	0.4
Giardiasis	1	0.4
Diabetes, hypertension	1	0.4
Acute Pancreatitis	1	0.4
Tuberculosis	1	0.4
Medical Examination	1	0.4
Organophosphate Poisoning	1	0.4
Cellulitis	1	0.4
Sub-Acute Intestinal Obstruction	1	0.4
Total	249	**100**

Patients had clinical conditions ranging from abdominal discomfort/pain to severe upper respiratory tract infection and tuberculosis.

4.4 Consistency of the stool specimens from the study patients

Most of the specimens, 266 (59.5%) were loose while 115 (25.7%) were soft and the rest were watery (Table 5).

Table 5: Frequencies and percentages of consistency of the stool specimens from the study patients

Inclusions	Frequency	Percentage
Blood	7	1.6
Mucus	149	33.3
Undigested food particles	11	2.5
Total	167	37.4

4.5 Presence of inclusions in the stool samples

Two hundred and eighty (62.6%) specimens had no inclusions, 7 (1.6%) specimens had blood, 149 (33.3%) specimens had mucus and 11 (2.5%) specimens had undigested food particles (Table 6).

Table 6: Inclusions seen in the study stool samples

Consistency	Frequency	Percent
Soft	115	25.7
Loose	266	59.5
Watery	66	14.8
Total	447	100

4.6 Determination of invasive and non-invasive infection

Table 7 illustrates the categories of infections and their frequencies. This was based on the presence of blood cell in the study samples

Table 7: Stool microscopy for Blood Cells

Category of infection	Frequency	Percent
Non Invasive	147	32.9
Invasive	300	67.1

Invasive infection is characterized by presence of both RBCs and WBCs, while in Non-invasive infection, either or none of the RBC and WBCs are present.

4.7 Isolation of *Campylobacter*

From 447 stool specimens that were cultured 29 (6.5%) *Campylobacter* species were isolated. Out of the 29 *Campylobacter* species isolated, 28 (96.6%) were *C. jejuni* and one (3.4%) was *C. coli* (Figure 2.) The proportional distribution of *C. jejuni* biotypes was 6 (20.7%) biotype I and 23 (79.3%) biotype II (Figure 3).

Campylobacter organisms were isolated from four (13.8%) children below two years of age, six (20.7%) from children of between three to five years. No *Campylobacter* organisms were isolated from children between six and sixteen years of age, but from above sixteen years, *Campylobacter* organisms were isolated from19 (65.5%) cases (Table 8). There was significant difference in the age groups presented in the table ($\chi^2 =$ 8.825, $p = 0.032$). Isolation rate of *Campylobacter species* according to sex was 17 (58.6%) males and 12 (41.4%) females (Table 9). There was no significant difference in

the distribution of *Campylobacter* between the males and the females (χ^2=0.534, p=0.465). Other pathogenic organisms isolated from the samples are as shown in (Figure 4).

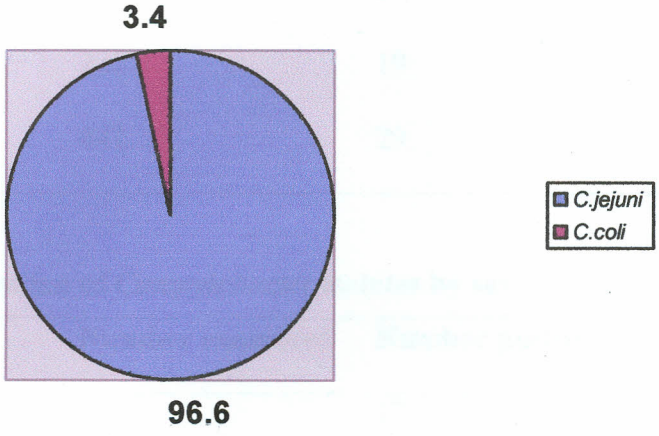


Figure 2: Proportional distribution of *Campylobacter* species (%)

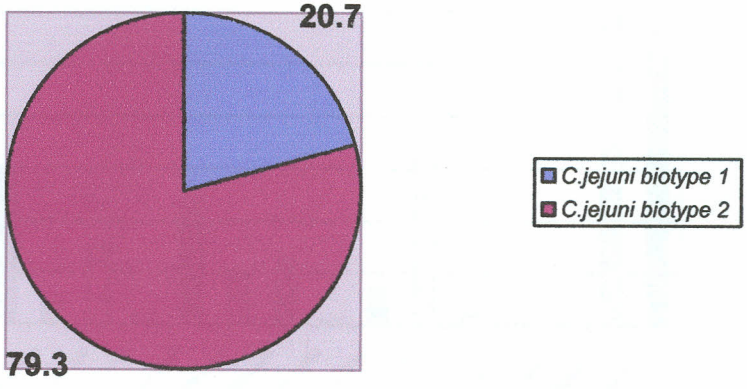


Figure 3: Proportional distribution of *Campylobacter jejuni* biotypes (%)

Table 8: Distribution of *Campylobacter* isolates by age

Age (years)	Number examined	Number positive	% Positive
0-2	127	4	3.1
3- 5	54	6	11.1
6-16	44	0	0.0
>16	222	19	8.6
Total	447	29	6.5

Table 9: Distribution of *Campylobacter* isolates by sex

Sex	Number examined	Number positive	% Positive
Males	233	17	7.3
Females	214	12	5.6
Total	447	29	6.5

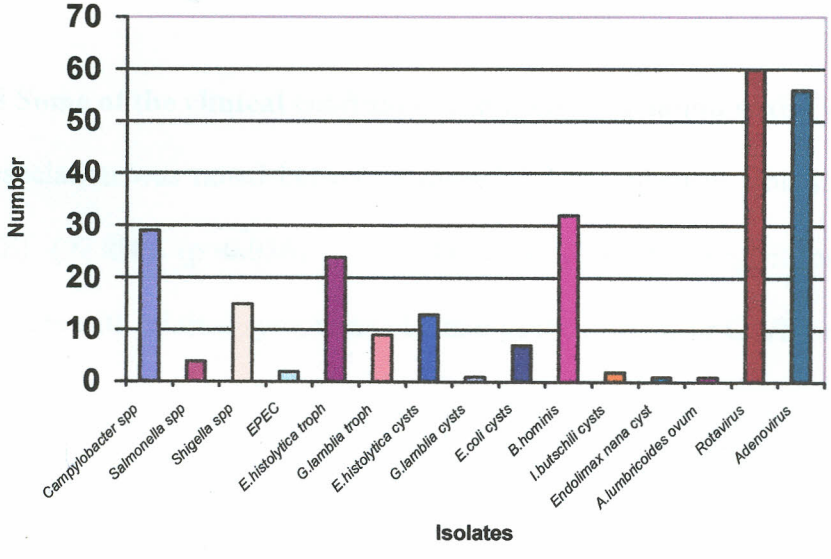


Figure 4: Pathogens isolated from study samples

EPEC----Entero-pathogenic *Escherichia coli;* *E*-Entamoeba; *G*-Giardia; *B*-Blastocystis; *A*-Ascaris; *I*-Iodomoeba

There was low mixed infections, which frequently occurred with; trophozoites of *Entamoeba histolytica*, cyst of *Blastocyst hominis* and Rota/Adeno virus (Table 10).

Table 10: Co-infection of *Campylobacter* species with other pathogens from study sample

Species*	No. of organisms
Salmonella species	0
Shigella species	0
Entero-pathogenic *Escherichia coli*	0
Trophozoite of *Entamoeba histolytica*	5
Cysts of *Entamoeba histolytica*	3
Cysts of Blastocystis *hominis*	2
Viruses (Adeno/ Rota virus)	5

Other bacterial, viral or parasitic pathogens isolated together with *Campylobacter* spp

4.8 Some of the clinical conditions and their association with *Campylobacter*

Association was noted between *Campylobacter* organism isolation with gastro-enteritis (GE) (92.8%) (p=0.036) (Table11). *Campylobacter* organisms were isolated from patients with clinical symptoms of either gastroenteritis or GE/fever.

Table 11: Clinical conditions and their association with isolation of *Campylobacter* species

Clinical conditions	*Campylobacter spp* culture results		Total
	Negative	Positive	
Gastro-enteritis (GE)	185	9 (4.6%)	194
Gastro-enteritis/Fever	10	4 (23.1%)	13
Gastro-enteritis/Bloating	0	1 (100%)	1
Pyrexia of unknown origin	9	1 (10%)	10
GE, Upper respiratory tract infection	3	0	3
Salmonella, Cryptosporidium	1	0	1
GE, Immune suppressed syndrome	2	0	2
GE, Malaria	1	0	1
GE, in Pregnancy	1	0	1
GE in Post operative	1	0	1
Abdominal pain	12	0	12
Giardiasis	1	0	1
Diabetic, Hypertension	1	0	1
Acute pancreatitis	1	0	1
Tubercullosis	1	0	1
Medical Examination	1	0	1
Organophosphate poisoning	1	0	1
Amoebic dysentery	2	0	2
Cellulitis	1	0	1
Sub-acute intestinal obstruction	1	0	1

4.9 Invasiveness and non-invasiveness of *Campylobacter*

Fifteen *Campylobacter* isolates (5%) were from stool specimens of patients with invasive infection while 14 *Campylobacter* isolates were from non invasive cases (Table 12).

Table 12: Correlation of stool microscopic findings and *Campylobacter* spp. culture results

Category	Frequency	Number of *Campylobacter* spp.	Percent
Non-invasive infection*	110	3	2.7
Non-invasive infection**	38	11	7.4
Invasive infection♦	300	15	5

♦ Invasive *Campylobacter* infection characterized by presence of both RBCs and WBCs; *Non-invasive *Campylobacter* infection characterized by absence of WBC or RBC, ** Non-invasive *Campylobacter* infection characterized by presence of either WBC or RBC in the study patients' stool samples.

4.10 Antimicrobial susceptibility patterns.

4.10.1 Antimicrobial susceptibility by disc diffusion method

Twenty nine *Campylobacter* isolates were tested against fifteen antimicrobial agents by agar disc diffusion method. There was 100% susceptibility with erythromycin, chloramphenicol, augmentin and nitrofurantoin. Good activity was observed with tetracycline and gentamicin each giving 96.6% sensitivity and so were the flouroquinolones, (ciprofloxacin and nalidixic acid) each showed 82.8% and 75.9% sensitivity respectively. Three of five cephalosporins tested showed activity of above 80% susceptibility while the other two gave activity below 50%. The antimicrobial agents tested and the result obtained is as shown in (Table 13).

Table 13: Antimicrobial susceptibility of *Campylobacter* isolates

Antimicrobials≈ (µg)	Sensitive strains N (%)	Resistant strains n (%)
Azithromycin* (15)	29 (100)	0 (0)
Doxycycline♦ (30)	28 (96.6)	1 (3.4)
Ciprofloxacin▼(5)	24 (82.8)	6 (17.2)
Ampicillin◙ (10)	26(89.7)	4 (10.3)
Nalidixic Acid▼(30)	22 (75.9)	6 (24.1)
Gentamicin• (10)	28 (96.6)	1 (3.4)
Cotrimoxazole∞ (25)	4 (13.8)	25 (86.2)
Chloramphenicol□ (30)	29 (100)	0 (0)
Augmentin◊ (30)	29 (100)	0 (0)
Nitrofurantoin▲ (300)	29 (100)	0 (0)
Ceftazidime■ (30)	24 (82.8)	6 (17.2)
Ceftriaxone■ (30)	25 (86.2)	4 (13.8)
Cefuroxime■ (30)	10 (34.5)	19 (65.5)
Cefotaxime■ (30)	27 (93.1)	2 (6.9)
Cefoxitin■ (30)	6 (20.7)	23 (79.3)

≈Categories of antimicrobial agents tested which included, *macrolides, ♦tetracyclines, ■cephalosporins, □chloramphenicol, ◙ beta lactams, •aminoglycocide, ▼quinolones, ◊beta lactamase inhibitors, ▲nitrofurans, ∞ cotrimoxazole

4.10.2 Antimicrobial susceptibility testing by minimum inhibition concentration (MIC) method

The range of MICs for each of eight antimicrobial tested with *Campylobacter* spp., MIC_{50} (concentration required to inhibit the growth of 50% of the strains) and MIC_{90} (concentration required to inhibit the growth of 90% of the strains) values, susceptibility, intermediate susceptibility, resistance levels and their accepted breakpoint values are shown in Table 14. Si milarly, the MIC distribution of each antimicrobial agent for *Campylobacter* spp. isolates are shown in Figures 5 to 12 below.

Table 14: Antimicrobial susceptibility pattern of *Campylobacter* spp. by minimum inhibitory concentration (MIC) method

Antimicrobials	Break-points	No. of Isolates	% R	% I	%S	MIC_{50}	MIC_{90}	Range
Azithromycin	S≤.5	29	0	0	100	.016	.016	.016-
	R≥8							.031
Doxycycline	S≤4	29	0.0	3.4	96.6	.016	.032	.016-6
	R≥16							
Ciprofloxacin	S≤1	29	13.8	0.0	86.2	.008	4	.002-
	R≥4							128
Ampicillin	S≤8	28	7.1	0.0	92.9	0.094	3	.016-
	R≥16							48
Nalidixic	S≤16	29	10.3	0.0	89.7	.75	32	.006-
Acid	R≥32							32
Gentamicin	S≤4	27	0	0	100	.047	.5	.016-.5
	R≥16							
Cotrimoxazole	S≤2	29	79.3	0.0	20.7	32	32	0.31-
	R≥4							32
Chloramphenicol	S≤8	28	0	0	100	.047	.5	.016-2
	R≥32							

R-resistant, S-sensitive, I-intermediate, MIC_{50}-concentration that kills 50% of the *Campylobacter* isolates, MIC_{90}-concentration that kills 90% of the *Campylobacter* isolates,

4.9.2.1 Ciprofloxacin MIC values for *C. jejuni*

The MIC values obtained for ciprofloxacin were in the range 0.002- 32µg/ml. The isolates showed 86.2% susceptibility and 13.8% resistance to ciprofloxacin. The MIC_{50} was 0.008µg/ml and MIC_{90} was 4µg/ml (Figure 5). There was no significant difference between MIC values for ciprofloxacin with regard to age and gender (p=0.583, p=0.133) respectively.

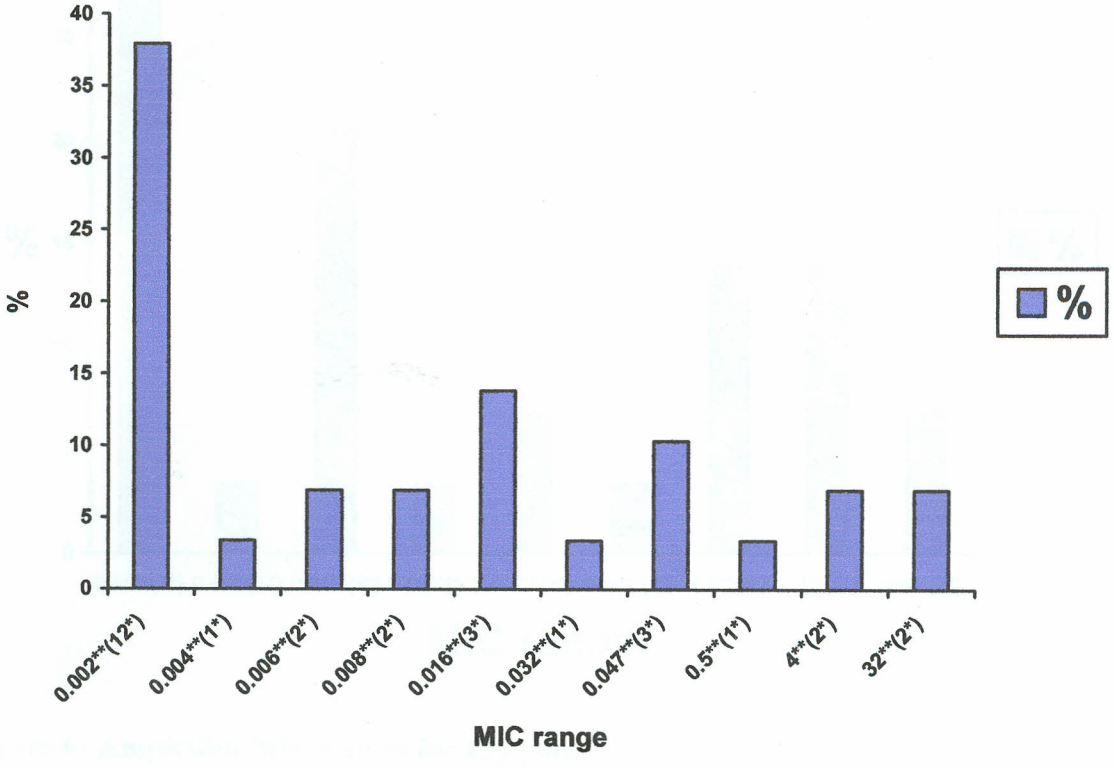


Figure 5: Ciprofloxacin MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of ciprofloxacin in µg/ml

4.9.2.2 Ampicillin MIC values for *C. jejuni*

The MIC values for ampicillin were in the range 0.016-48µg/ml. The isolates showed 92.9% susceptibility and 7.1% resistance to ampicillin. The MIC_{50} was 0.094µg/ml and MIC_{90} was 3µg/ml. Age and gender had p values of 0.655 and 0.238 respectively (Figure 6).

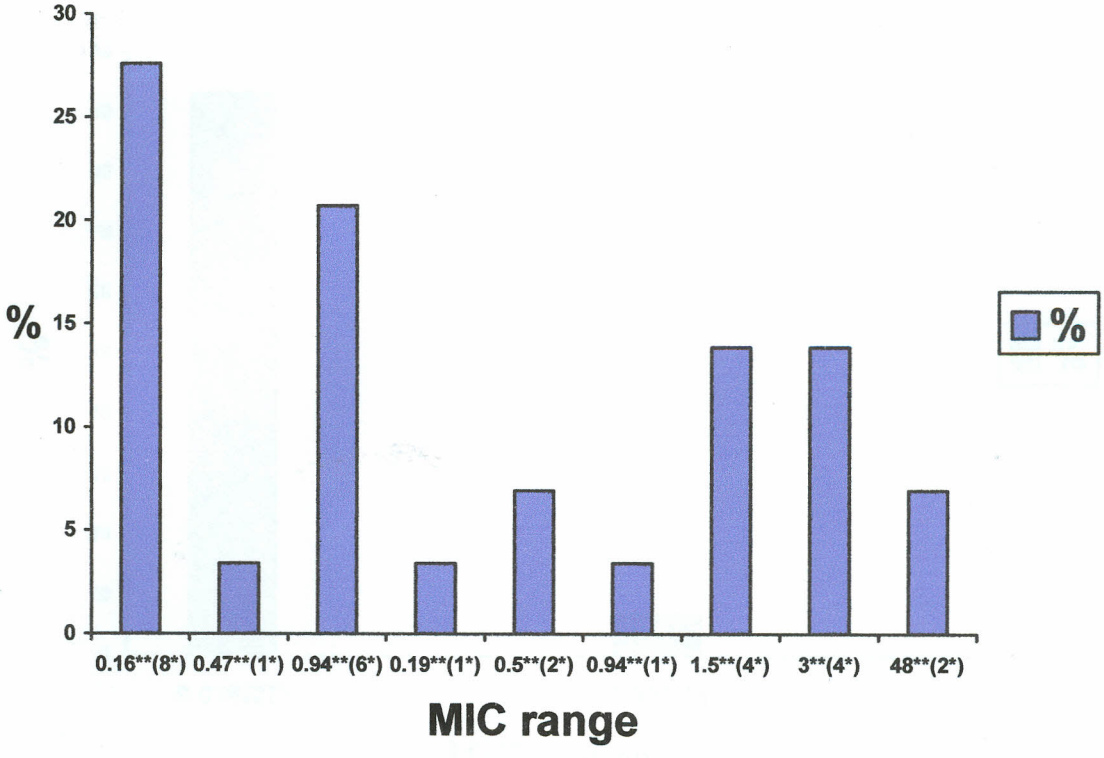


Figure 6: Ampicillin MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of ampicilin in µg/ml

4.9.2.3 Azithromycin MIC values for *C. jejuni*

The MIC values of azithromycin ranged from 0.016- 0.031µg/l. The isolates showed 100% susceptibility to azithromycin. The MIC_{50} was 0.016mg/l and MIC_{90} was 0.016mg/l. (Figure 7). There was no statistical difference between MIC values for erythromycin with regard to age and gender.

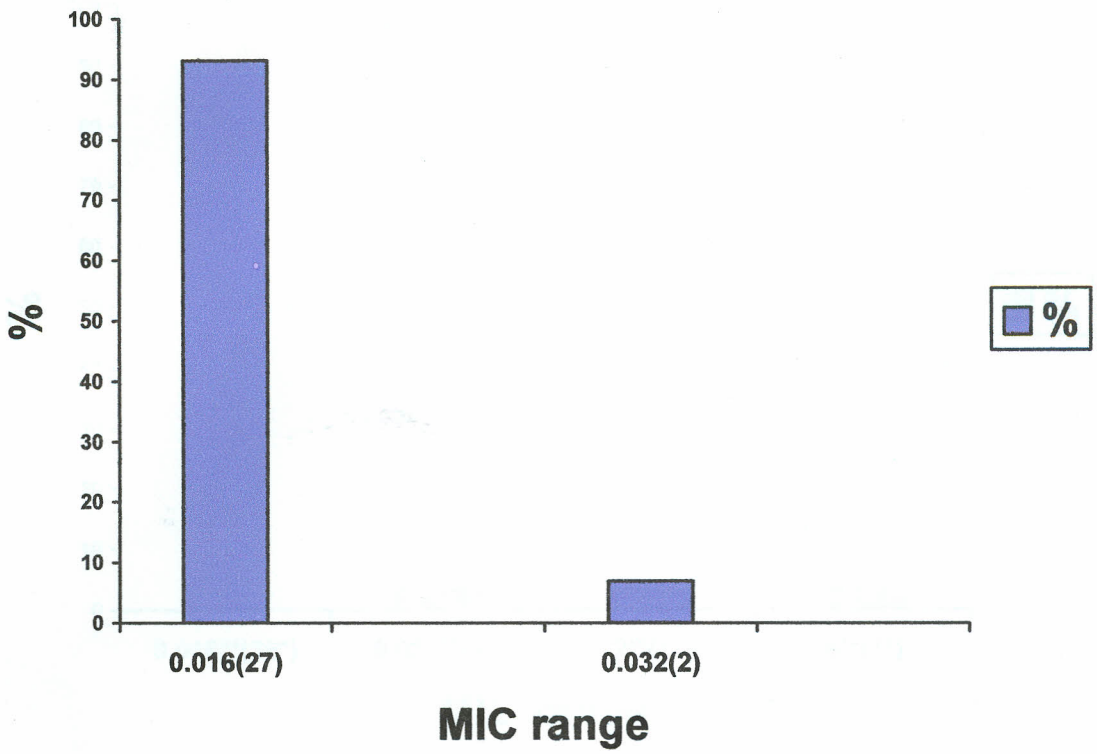


Figure 7: Azithromycin MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of azithromycin in µg/ml

4.9.2.4 Doxycycline MIC values for *C. jejuni*

The MIC values of Doxycycline ranged from 0.016- 6µg/ml.The isolates showed 96.6% susceptibility and 3.4% intermediate; no isolate demonstrated resistance to Doxycycline/Tetracycline, The MIC_{50} was 0.016µg/ml and MIC_{90} was 0.032µg/ml (figure 8). There was no statistical difference between MIC values with regard to age and gender.

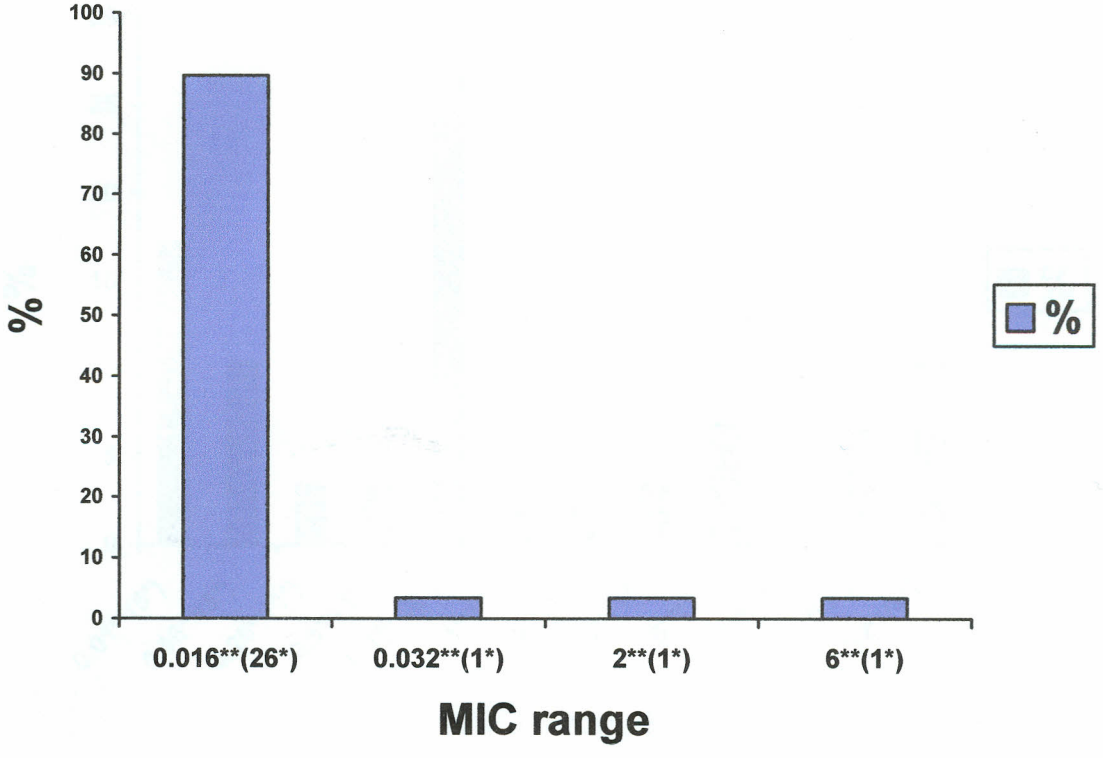


Figure 8: Doxycycline MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of doxycycline in µg/ml

4.9.2.5 Nalidixic acid MIC values for *C. jejuni*

The MIC values for Nalidixic Acid were in the range 0.006-32µg/ml. The isolates showed 89.75% susceptibility and 10.3% resistance. The MIC $_{50}$ and $_{90}$ were 0.75 and 32µg/ml respectively (Figure 9). There was no significant difference between MIC values with regard to age and gender (p=0.660, 0.418) respectively.

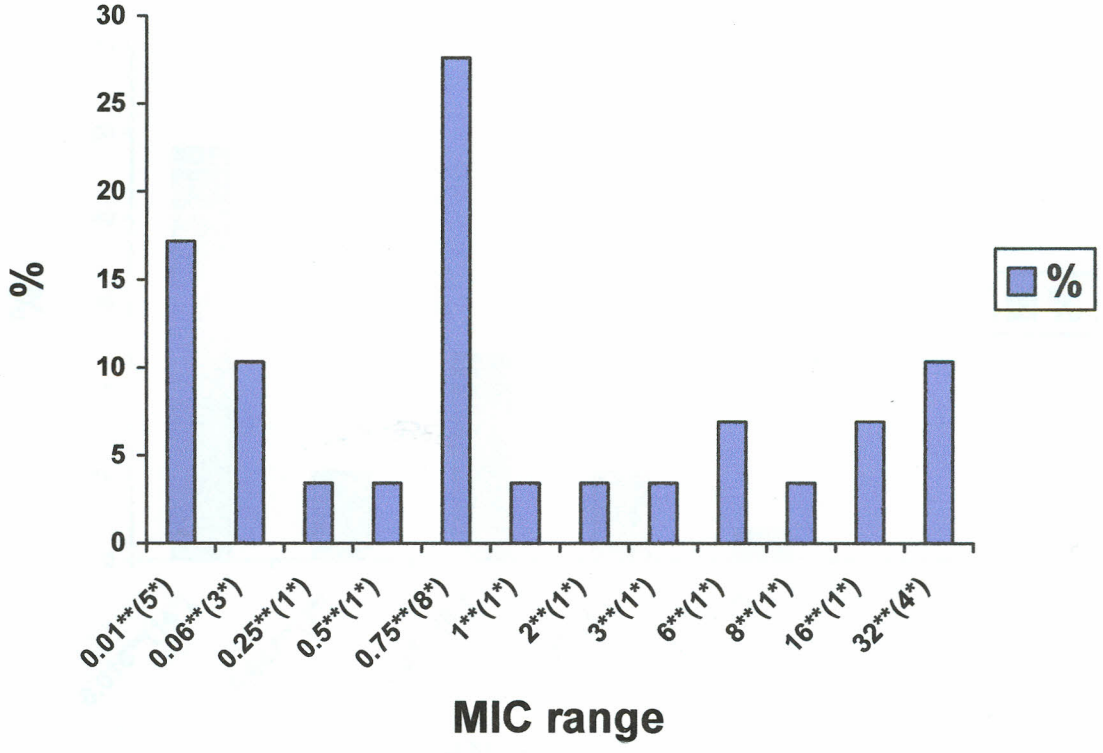


Figure 9: Nalidixic acid MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated.** Different concentrations of nalidixic acid in µg/ml

4.9.2.6 Gentamicin MIC values for *C. jejuni*

The MIC values ranged from 0.016-0.5µg/ml. The isolates showed 100% susceptibility. The MIC $_{50}$ and MIC$_{90}$ were 0.047 and 0.5µg/ml respectively (figure 10). There was significant difference between MIC values with regard to age p=0.030 but with regard to gender there was no significant difference (p=0.429).

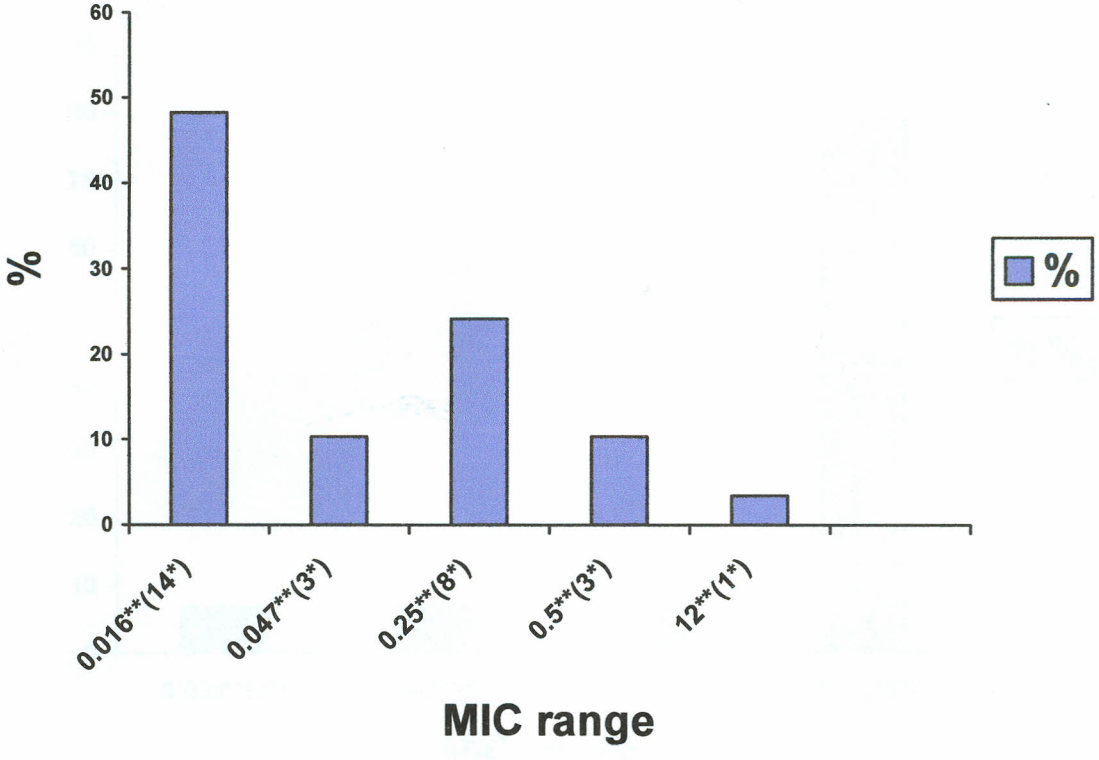


Figure 10: Gentamicin MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of Gentamycin in µg/ml

4.9.2.7 Cotrimoxazole MIC values for *C. jejuni*

The MIC values ranged from 0.31-32µg/ml. The isolates showed 20.7% susceptibility and 79.3% resistance. The MIC_{50} and MIC_{90} were both at 32µg/ml (figure11). There was no significant difference between MIC values with regard to age and gender (p= 0.434, p=0.613) respectively.

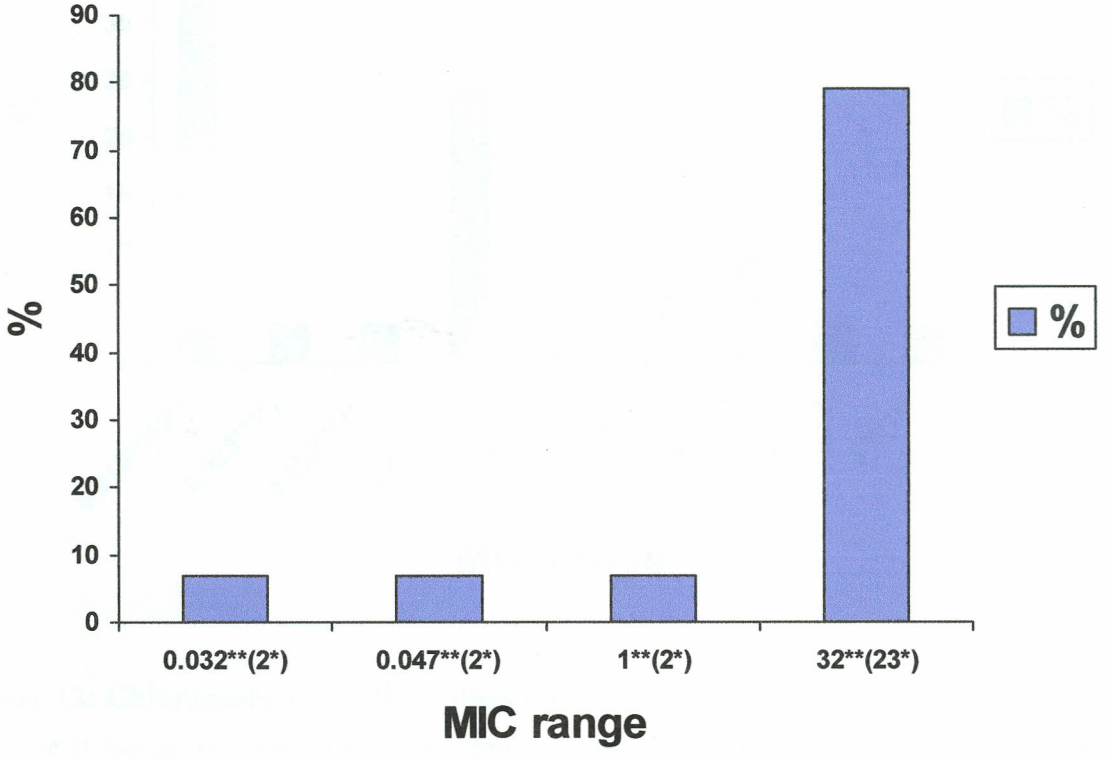


Figure 11: Cotrimoxazole MIC distribution for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of contrimoxazole in µg/ml

4.9.2.7 Chloramphenicol MIC values for *C. jejuni*

The MIC values ranged from 0.016-2µg/ml.The isolates showed 100% susceptibility. MIC_{50} and MIC_{90} were 0.047 and 0.5µg/ml respectively (figure 12). There was no statistical difference between MIC values with regard to age and gender.

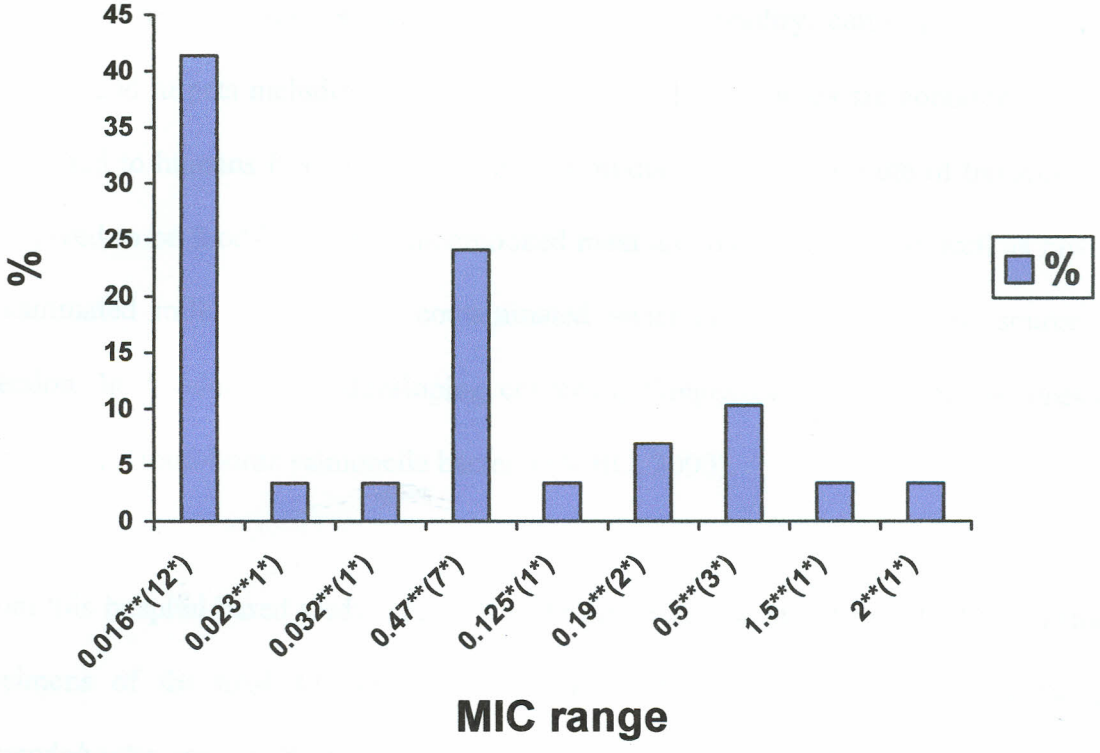


Figure 12: Chloramphenicol MIC values for *C. jejuni*

*Number of isolates that were sensitive to ciprofloxacin at the concentrations indicated. ** Different concentrations of chloramphenical in µg/ml

CHAPTER FIVE DISCUSSION, CONCLUSIONS AND RECOMMEDATIONS

5.1 Discussion

From many clinical and epidemiological studies, *Campylobacter* spp. are among the common causes of human bacterial diarrhea worldwide (Shen and Seng, 2001; de Wit *et al.*, 2001). They are prevalent in food animals such as poultry, cattle, pigs, sheep and shellfish; and in pets including cats and dogs. Campylobacterioses are zoonotic diseases transmitted to humans from animals or animal products. The main route of transmission is believed to be food-borne, via undercooked meat and meat products as well as raw or contaminated milk. Drinking of contaminated water is also a recognized source of infection. In developed and developing countries, *Campylobacter* cause more cases of diarrhea than food-borne salmonella bacteria (WHO, 2000).

From this hospital based study, bacterial pathogens were isolated from 50 (11.2%) stool specimens of the total 447 that were cultured. The isolation rates were 6.5% for *Campylobacter* spp., 4.3% for *Shigella* spp., 1.1% for *Salmonella* spp., and 0.6% for EPEC. The rest of the specimens were negative. The results showed that *Campylobacter* is a common and also leading bacterial agent of diarrhea. This pattern of isolation is similar to one obtained in Ethiopia (Beyene and Haile-Amlak, 2004). Reports from developing countries have put the prevalence rate in the range of 5-20% (Oberhelman and Taylor, 2000). This isolation rate was lower than other findings reported in the country that ranged 12-26% (Wamola *et al.*, 1983; Waiyaki *et al.*, 1986; Shimotori *et al.*, 1986 Chunge *et al.*, 1989; Osano and Arimi, 1999). It is also lower in the region

1986 Chunge *et al.*, 1989; Osano and Arimi, 1999). It is also lower in the region compared with isolation rate of 9.3% in Tanzania (Mdegela *et al.*, 2006) and 11.6% in Ethiopia (Beyene and Haile-Amlak, 2004). This could be due to difference in geographical location and study period. Although some studies have shown an increased rate of isolation of *Campylobacter* during rainy seasons (Pazzaglia *et al.*, 1993), other studies have suggested that the seasonal trends for *Campylobacter* infections may be less evident in tropical and subtropical countries (Blaser *et al.*, 1983; Moyer and Holcomb, 1988). Compared with other studies done elsewhere in the world, the isolation is higher than that reported in Italy 2.3% (Vatoli *et al.*, 1989) and Singapore 1.2% (Lim and Tay, 1992). Generally, in the developing countries *Campylobacter* infection is hyper endemic owing to poor sanitation and close contact with animals in the homes. However infections due to ingestion of organism from undercooked poultry and unpasteurized milk can not be ruled out.

Biotyping of the isolates showed more of *C. jejuni* (96.6%) than *C. coli* (3.4%). This result correlates with reports over years from Nigeria (Alabi *et al.*, 1986; Coker *et al.*, 1989; Coker and Adefeso, 1994; Samuel *et al.*, 2006), Tanzania (Mdegela *et al.*, 2006). However, report of Aboderin *et al.* (2002) showed more of *C. coli* than *C.* jejuni in Nigeria. Also *C. coli* accounts for a higher proportion of infections in Chile, Hong Kong and Central Africa Republic than elsewhere. *Campylobacter coli* are particularly associated with pigs but in some areas are also found in chicken (Mdegela *et al.*, 2006).

In this study, 75% of the isolates were β-lactamase producers. This agrees with some work done in Nigeria by Coker and Adefeso (1994) and Smith *et al.* (1997) but differ with work done in Ile-Ife Nigeria (Aboderin *et al.*, 2002) in which none of the thirty isolates was a β-lactamase producer.

In this study *Campylobacter* was not isolated with any other bacterial pathogens. A few cases, 7 out of 29 showed co-infection with parasites (*E. histolytica* and *B. hominis*) and viruses (Rota viruses and Adenoviruses). This finding lends credence to the observation that the *Camplobacter* are actually the most important causative agents of diarrhea (John *et al.*, 2003). Co -infection of *Campylobacter* with other enteric pathogens has been reported by Chunge *et al.* (1989); John et *al.* (2003) and Beyene and Haile-Amlak (2004) in developing countries which is a rare case in developed countries (Oberhelman and Tayor, 2000).

In this study most of the *Campylobacter* isolates were from specimens obtained from adults. *Campylobacter* infections in developing countries have been associated mostly with children and especially those below two years of age This finding disagrees with other studies done in Kenya and in other developing countries (Wamola *et al.*, 1983; Samuel *et al.*, 2006; Mdegela *et al.*, 2006) but agrees with a report from Egypt (Rao *et al.*, 2001) and other findings in developed countries where the infections tend to occur commonly in adults (Butzler and Skirrows, 1979). Persons at increased risk for *Campylobacter* enteritis are those with occupational exposure to cattle, sheep, and other farm animals such as pigs and poultry. Also laboratory workers and those in contact with

the excreta of infected persons as well as homosexual men are at risk (Jocelyn and Sharon, 2006). The persons involved in the above activities are usually adults and this might have been the case in this study. Closeness to animals and poor hygiene might have contributed to this high isolation of *Campylobacter* from these people.

The results of this study indicated that children that were five years and below had higher rate of *Campylobacter* isolation than children that were six years and above. This agreed with the studies done in Ethiopia (Beyene and Haile-Amlak, 2004; Mitikie *et al.*, 2000). The reason for this high isolation rate may be the fact that young individuals are frequently taken to attend health facilities and therefore higher possibility of isolating *Campylobacter*. Other factors that were likely to contribute to high infection rate in this study area are poor hygiene and sanitation, closeness to animals and low immunity because of first exposures.

The distribution of *Campylobacter* spp. between males and females was 60.5% and 39.5% respectively. It showed some male predominance among infected persons, although not different statistically, which begins during early childhood and persists until old age. These results support other studies on *Campylobacter* enteritis done in Nigeria (Coker and Adefeso, 1994; Aboderin *et al.*, 2002) and other developed countries (Friedman *et al.*, 2000). However, there are studies that have shown slight difference in Nigeria (Samuel *et al.*, 2006) and in Ethiopia (Beyene and Haile-Amlack 2004). The reason for sex distribution is not known but occupational exposure cannot be ignored.

In this study, there were 300 invasive cases 15 (5%) of which were due to *Campylobacter* spp. *Campylobacter* infections can be invasive or non-invasive. The invasive infection by *Campylobacter* occurs in every 1% of culture confirmed infections (Gerald *et al.*, 1999) and usually occurs at the extremes of life, affecting infants younger than a year and adults over 60 years (Smith *et al.*, 2002). *Campylobacter* causing invasive infection accounts for 5-10% of all diarrheal cases annually in U.SA. This agrees with the findings of this study.

In this study *Campylobacter* isolates were sensitive to azithromycin/erythromycin, doxycycline/tetracycline, chloramphenicol and gentamicin, and resistant to ciprofloxacin, ampicillin, nalidixic acid and cotrimoxazole. Similar finding with azithromycin/erythromycin and gentamicin were reported in a study by Fernandez *et al.* (2000) in Southern Chile. In the study by Sharma *et al.* (2003) gentamicin was 100% sensitive, similar with present study but disagreeing on results of azithromycin/erythromycin and doxycycline/tetracycline that gave MIC of 8µg on both antimicrobials. Harumi *et al.* (2001) on nine isolates from Kenya, reported excellent activity of erythromycin and ciprofloxacin with MIC_{90} of 0.25 and 0.0625µg/ml respectively.

A number of studies on *Campylobacter* susceptibility pattern by disc diffusion method have been reported. This pattern of susceptibility is similar to that of Aboderine *et al.* (2002) where the organisms were 100% sensitive to tetracycline, erythromycin and gentamicin. Tetracycline and erythromycin were also found 100% susceptible in a study

in Indonesia by Tjaniadi *et al.* (2003). However, it disagreed with studies done in, Nigeria (Coker *et al.,* 1994; Samuel *et al.,* 2006) and in Ethiopia, (Beyene and Haile-Amlak, 2004).

From this current study, four (13.8%) isolates were detected to be resistant to ciprofloxacin. Two (2) of the resistant strains had MIC of 4µg/ml and the others exhibited high level resistance with MICs of >32µg/ml. MIC_{50} and MIC_{90} were 0.008µg/ml and 4µg/ml respectively. These same isolates were found to be resistant to nalidixic acid and cotrimoxazole. Ciprofloxacin resistance has been widely reported worldwide. A hospital based study in Pennsylvania by Nachamkin *et al.* (2002) like the current one found a sharp increase in ciprofloxacin resistance among *C. jejuni* from 8% in1996 to 40%. Allos, (2001) reported 12% resistance in Wisconsin between 1992 and 1995, Krausse and Ullmann, (2003) reported 27.4% resistance in Germany, in Indonesia, *Campylobacter* resistance was reported to be 43% in 2000 and 35% by Tjaniadi *et al.* (2003). In Thailand where resistance to ciprofloxacin is of great concern, Hoge *et al.* (1998) reported 84% resistance and Sharma (2003) reported 2.9% resistance in the Hunter Region, New South Wales. The findings of this study were different with the findings of Harumi *et al.* (2001) which indicated excellent activity of Ciprofloxacin MIC_{90} of 0.0625µg/ml to the nine (9) isolates from Mombasa.

Resistance to fluoroquinolones in *Campylobacter* has increased in developed and developing countries. This increase came after the introduction of enroflossacin in veterinary medicine and fluoroquinolones in human medicine (Engberg *et al.*, 2001).

Other reasons for increase may be explained by differences in associations with foreign travel, method of testing and surveillance activity. Study by Smith *et al.* (1999) showed that administration of quinolone before culture resulted in a 15% increase in resistance. Other studies have demonstrated a clear relationship between antibiotic usage and the inevitable rise in resistance especially among gut-related pathogens (Levy and Marshall 2004; Wagenlehner *et al.*, 2005).

Fluoroquinolone resistance in *C. jejuni* most often appears to be due to mutations in the gene encoding subunits of DNA gyrase (topoisomerase 11) and only occasionally to topoisomerase 4 (parC) (Engberg *et al.*, 2001). Initial mutations produce high-level nalidixic acid resistance, with additional changes leading to increasing ciprofloxacin resistance. Active multi-drug efflux mechanisms for quinolone resistance in *Campylobacter* have been described (Charvalos *et al.*, 1995) and may be responsible for reduced susceptibility to quinolones, betalactams, tetracycline, chloramphenicol and other agents (Ferguson *et al.*, 1998).

Fluoroquinolones are in use for management of many forms of moderate to severe enteric infections. They are effective in both the treatment, even in single dose, and the prophylaxis of travelers' diarrhea (Salam *et al.*, 1994). Fluoroquinolones are also the agents of choice in treating invasive salmonellosis in HIV/AIDS patients (Krausse and Ullmann, 2003). The risk factors for acquiring ciprofloxacin- resistant *C. jejuni* have not been defined in our country; however, wide-spread use of fluoroquinolone in our

hospitals for treatment of many bacterial infections cannot be ignored. This may account for the emergence of this high fluoroquinolone resistance.

The MIC values of nalidixic acid ranged from 0.006- 32μg/ml. MIC $_{50}$ and MIC $_{90}$ were 0.75 and 32μg/ml respectively. The isolates showed 10.3% resistance. These results compare well with others that showed an increase in the incidence of nalidixic acid resistance among human *C. jejuni* isolates from 3.8% in1992 to 11% in 1996 and 1998 by Smith *et al.* (1999). Higher incidences of 40% and 26.3% resistance have been reported by Wasfy *et al.* (2000) and Gally *et al.* (2007) respectively. Nalidixic acid is used in treating enteropathogens and especially dysenteric illnesses in developing countries (Vinh *et al.*, 2000). It has been used as screening test to predict fluoroquinolone resistance (Smith *et al.*, 1999). The results of this study agree with that statement, for the isolates that were resistant to nalidixic acid were also resistant to ciprofloxacin.

High frequency of resistance was demonstrated to cotrimoxazole in this study, (79%). These results agree with earlier studies in Kenya by Wamola *et al.* (1983) which showed 100% resistance and by Harumi *et al.* (2001) that showed low activity with MIC $_{90}$ at 128μg/ml. Similar trend has been observed elsewhere in Africa. In Ethiopia Beyene and Haile-Amlak (2004) observed 60% resistance and in Indonesia Tjaniadi *et al.* (2003) reported 70% resistance. The resistance in Kenya may be due to the common practice of purchasing drugs in the open market and private pharmacies without doctors' prescriptions. In addition the high levels of resistance to cotrimoxazole could be due to

the wide-spread use of sulphamethoxazole-pyrimethamine (SP) for malaria treatment, (Feikin *et al.*, 2000) since these two formulations have similar modes of action.

Ampicillin showed high activity of 92.9% and resistance of 7.1%. MIC $_{50}$ and MIC $_{90}$ were at 0.094µg/ml and 3µg/ml respectively. These findings agree with the finding by Fernandez *et al.,* (2000) of 6.6% resistance with MIC $_{50}$ and MIC $_{90}$ at 1.5µg/ml and 8.0µg/ml respectively and a study by Aboderin *et al.* (2002) of 93.3% susceptibility and 3.7% resistance was reported. The study however differs from that of Sharma *et al.* (2003) 64% resistance and MIC $_{50}$ and MIC $_{90}$ at 8µg/ml and 64µg/ml respectively and that of Harumi *et al.* (2001) where MIC $_{90}$ of 64µg/ml was reported.

Although ampicillin showed such high activity *in vitro* testing, 75.6% of the isolates were β-lactamase producers. This predicted the resistance *in vivo* and therefore ampicillin would not be used for therapy. The resistance may be related to inappropriate use of Ampicillin in human therapy because it is widely prescribed and commonly available over-the-counter without prescription. Ampicillin is commonly used in treatment of acute respiratory infection.

Tetracycline/doxycycline showed no resistance in this study. Tetracycline is not used in paediatrics in the country and this probably explains why there was no resistance in children. However, it is used in poultry farming (Kariuki *et al.*, 1997) and commonly used in the treatment of skin and other systemic ailments in adults. The high resistance in *C. jejuni* to tetracycline has been reported worldwide, 56% was reported in Canada, (Gaudreau and Gilbert, 1998) and 95% in Thailand (Li *et al.*, 1998). Tetracycline

resistance in *C. jejuni* is primarily mediated by plasmid that carries the tet(O) gene. The Tet(O) protein binds to the bacterial ribosome and displaces tetracycline (Trieber *et al.*, 1998).

In this study, a high frequency of cross-resistance was detected between ciprofloxacin and nalidixic acid. All of the *Campylobacter* strains that were resistant to nalidixic acid were also resistant to ciprofloxacin a similar finding was reported by Smith *et al.* (1999) and Murphy *et al.* (1998). Another higher frequency of multi-drug resistance was observed between cotrimoxazole and the other drugs. All strains that were resistant to ampicillin, ciprofloxacin and nalidixic acid were also found resistant to cotrimoxazole. The multi-drug resistance in bacterial pathogens is common in developing countries including Kenya (Kariuki *et al.*, 2006). This circumstance is most likely related to frequent use of over-the-counter drugs with no proper medical supervision (Sack *et al.*, 1997).

The multi-drug resistance profile of most bacterial pathogens is suggestive of antibiotic resistance traits having entered the microflora of farm animals and the food produced from them, a fact demonstrated by molecular analysis of the resistance genes (Teuber, 1999). High levels of therapeutic, prophylactic and nutritional application of antimicrobials in agriculture globally have contributed to a constant influx of resistant genes into the human microflora through the food chain (Teuber, 1999). The foods that the study population is likely to eat most fall under the agricultural products that may contain antibiotics, such as poultry and poultry products, beef and milk from treated

cows. Resistance may also be brought about by not completing the antibiotic course once the patient feels better. The use of expired drugs may also contribute as pathogens on exposure to drugs of sub-lethal potency, mutate and acquire resistant traits.

Antimicrobial susceptibility testing did not reveal resistance to erythromycin, the drug of choice for treating campylobacteriosis. This report is similar to the studies earlier done in Kenya (Wamola *et al.*, 1983; Waiyaki *et al.*, 1986; Harumi *et al.*, 2001) Hungary (Varga and Fodor, 1998) and Nigeria (Aboderin *et al.*, 2002) but contrast the reports of Coker and Adefeso (1994) in Nigeria and Wasfy *et al.* (2000) in Egypt where resistance was registered.

5.2 Conclusion

In conclusion the findings of this study suggest that *Campylobacter* spp. are important aetiological agents of diarrhea in both, children and adults. The isolation rate of 6.5% compared well with those observed in other developed and developing countries. There was a significant difference in isolation of *Campylobacter* spp. between adults and children. Two isolated species, *C. jejuni and C. coli*, showed 96.6% and 3.4% prevalence, respectively. *C. jejuni biotype 2* was the predominant isolate. Co-infections with *Campylobacter* spp. and other known pathogens such as *Entamoeba histolytica*, *Blastocyst hominis* and Rota/Adeno virus were frequently observed. No mixed infections with other bacterial pathogens were reported in the present study.

Susceptibility testing revealed emergence of resistance of *Campylobacter spp.* to ciprofloxacin and multi-drug resistance to several drugs tested. In addition,

Campylobacter spp. showed very high resistance to cotrimoxazole. However, no resistance was detected to chloramphenicol, tetracycline, augmentin, nitrofurantoin and to erythromycin, the drug of choice for treating human campylobacteriosis.

5.3 Recommendations

i, This study recommends routine culture for *Campylobacter* spp. from both adults and children for better management of diarrhea cases.

ii, Routine antimicrobial resistance testing for *Campylobacter* is recommended for assessment of emerging resistance.

iii, This study recommends continued use of erythromycin as the drug of choice while gentamicin, tetracycline and chloramphenicol remain reserve drugs since no resistance was detected against them.

iv, Control strategies such as improving public hygiene and reduction of over use of antimicrobials are recommended in both veterinary and human medicine.

v, More studies are recommended to establish the actual risk factors in acquisition of infection and also the causes of drug resistance.

vi, More studies of the same in other areas of Kenya are recommended.

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