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DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

Isolation and characterization of *E.coli* and *Campylobacter* spp. from diarrhoeal samples collected from selected hospitals in Amathole District Municipality, Eastern Cape, South Africa

BY

OMOLAJAIYE SUNDAY ABRAHAM

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SUPERVISOR: PROF. A.I OKOH

CO-SUPERVISOR: DR. BENSON C. IWERIEBOR

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CERTIFICATION

I hereby certify that this research work was carried out by **Dr. Omolajaiye Sunday Abraham** and supervised by me in the Department of Biochemistry and Microbiology, University of Fort Hare, Alice South Africa in accordance with the requirements for the award of Masters of Science degree in Microbiology and the work herein is original and has not been submitted at any other university for the award of any degree.

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PROF. A.I OKOH (Supervisor)	BENSON C. IWERIEBOR (Co-supervisor)
.....	……
Date	Date

DEDICATION

To the Lord God Almighty, for being so faithful in all,

To all who have lost their lives due to diarrhoea and its complications

To the “microscopically” big family of **OMOLAJAIYE** worldwide.

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I whole-heartedly magnify the **Lord Almighty** for enabling me to embark on and conclude this study.

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LIST OF ABBREVIATIONS

AEMREG Applied and Environmental Microbiology Research Group

AIDS Acquired Immunodeficiency Syndrome

ATP Adenosine triphosphate

ATPase Adenosine triphosphatase

BFP Bundle- forming Pilus

cAMP cyclic Adenosine monophosphate

CDC Centre for Disease and Control

CDT Cytolethal Distending Toxin

CFA Colonization factor antigen

CO_2 Carbon dioxide

CSIR Council for Scientific and Industrial Research

DAEC Diffusely adhering *E. coli*

DALYs Disability-adjusted life years

DEC Diarrheagenic *Escherichia coli*

DNA Deoxyribonucleic acid

DNAase Deoxyribonuclease

DOH Department of health

DRC Democratic Republic of Congo

DWEA Department of Water and Environmental Affair

DHIS District Health Information System

EAggEC Enteroaggregative *E. coli*

EHEC Enterohaemorrhagic *E. coli*

EIEC Enteroinvasive *E. coli*

EPEC Enteropathogenic *E. coli*

ETEC Enterotoxigenic *E. coli*

EU European Union

EFSA European Food Safety Agency

GIT Gastrointestinal tract

GHS Genral household survey

GST1 Glucose-sodium transport 1

HIV Human Immunodeficiency virus

HSP Heat shock protein

5HT 5hydroxytryptamine

HUS Heamolytic ureamic syndrome

ICTV International Committee on Taxonomy of Viruses

IMCI Integrated Management of Chilhood Illness

IVF Intravenous fluid

LOS Lipo oligosaccharides

LPS Lipo polysaccharides

LT	Heat labile
MDG	Millenium Development Goal
MOMP	Major outer membrane protein
NDDI	National Digestive Diseases Information
NGT	Nasogastric tube
ORF	Open reading frame
ORT	Oral rehydration therapy
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
SSA	Statistics South Africa
SOD	Superoxide dismutase
ST	Heat stable
UN	United Nations
UNICEF	United Nation Children's Fund
VT	Verocytotoxin
WHO	World Health Organisation

ABSTRACT

Approximately 2-4 billion cases of infectious diarrhoea occur every year, with the highest numbers recorded in sub-Saharan Africa. It remains the most common public health issue among children in developing nations. The purpose of this research was to unfold the prevalence of diarrhoeagenic *E. coli* and *Campylobacter* pathotypes as well as elucidate their antibiogram characteristics in diarrhoeal stool samples collected in some medical facilities in Eastern Cape Province, South Africa. Two hundred stool samples were collected from both inpatients and outpatients from male and females of all age groups attending selected medical facilities in the study area. Isolation and characterization of both organisms were done using culture based and molecular methods. Antibiotic susceptibility patterns of identified isolates were determined against a panel of 12 antimicrobial agents. One hundred and twenty presumptive *E. coli* isolates and 42 presumptive isolates of *Campylobacter* spp. were isolated. Eighty-two percent (82%) of the presumptive *E. coli* isolates were confirmed as *E. coli* while 46.3% belonged to *Campylobacter* spp. Pathotyping of the diarrhoeagenic *E. coli* isolates by Polymerase chain reaction (PCR) showed the following prevalences: DAEC 43 (32%), EHEC 18 (17%), EIEC 11 (10%) and EPEC 18 (17%). EAEC and ETEC were not detected, while for *Campylobacter* spp. 37 (88%) were *C. jejuni*, and *C. coli* was not detected. A total of 12 (32.4%) of the confirmed *Campylobacter jejuni* isolates were found to possess the *fli*M gene, 9 (24.3%) possessed the *flh*A gene and only 6 (16.2%) harboured the gene *flg*E2. None were positive for the *fla*A, *fla*b and *flh*B genes.The antibiotic resistance patterns observed among the *E. coli* isolates were high against ampicillin (98.1%), chloramphenicol (94.3%) and tetracycline (90.6%). For *Campylobacter* spp., resistance observed were: chloramphenicol (91.6%), tetracycline (25.2%), erythromycin (49.6%) and gentamycin (56.4%). A lesser resistance against imipenem (35.9%) and quinolone (ciprofloxacin) (45.5%) were exhibited by the *E.coli* isolates. 10.8% and 20.3% of the

Campylobacter isolates were resistant to imipenem and ciprofloxacin respectively. The presence of chloramphenicol (*Cat*A1) and tetracycline (*tet*A) resistance genes were detected in 94% and 89% of *E. coli* isolates respectively while 98% of *Campylobacter* spp. harboured the *cat*A1 resistance gene. It could be deduced from this study that *E. coli* and *Campylobacter spp.* are predomiant enteric pathogens as the etiologic agents of diarrhoea in the study community, and that their antimicrobial resistance is high in the study location. The need to develop strategies to prevent infection and control resistant organisms is evident.

CHAPTER ONE

General Introduction

1.1 Introduction

Diarrhoea is defined as frequent (3 or more), loose and watery bowel movements commonly called stools, in a 24-hour period. It remains a notable cause of illnesses and deaths, most importantly among children in developing nations, accounting for approximately 2 million deaths yearly world-wide (NDDI, 2013). The scourge is mainly connected to unhygienic conditions such as drinking faecal contaminated water, storing cooked food at room temperature, failing to dispose of faeces hygienically, failure to wash hands after defecation and bottle rather than breast-feeding in the first four to six months. Death occurs as a result of severe dehydration complicated by the loss of large volumes of body fluid through the stools. Further, diarrhoea as a result of acute gastroenteritis can affect people of any age group and is a reason for hospital admissions and morbidity even in developed countries (Scallan *et al*., 2011).

Reports from epidemiological studies have indicated that diarrhoea begins as a water and food borne related illness and it can be transmitted via the faecal-oral route especially in susceptible individuals (Scallan *et al.,* 2011). In addition to drinking unsafe or contaminated water, food substances are also an important medium through which diarrhoeal diseases are transmitted. For example, in developing countries, where the level of poverty can be high, combined with poor sanitation, there is a greater possibility of faecal contamination of home-made and public food (Jill *et al.,* 2010), and in some epidemics, the infection has been linked to consumption of foods that are faecally contaminated. Diarrhoea epidemics in developed countries have been found to be occasionally associated with the consumption of contaminated sea foods. The identification and characterisation of the major etiological agents of this disease is important for the purpose of epidemiological surveillance, design and formulation of treatment plans (Breurec *et al.,* 2016). The different diarrhoeal syndromes could be caused by either single or multiple etiologies of viral, bacterial and parasitic

infections (Nweze, 2010). Generally, bacteria are more prevalent than other etiologic agents of diarrhoea and typically peak during summer months. This may be due to the presence of bacteria in food substances (raw and undercooked), the possibility of cross-contamination of food and water in the community and in homes due to unhygienic practices, poor environmental sanitation such as unhygienic waste and faeces disposal and lack of access to potable water for domestic purposes as a result of poor socio-economic status. These organisms are able to cause pathology in the body of their host when they acquire virulent properties. The pathogenicity of a bacterial isolate involves: (1) the availability of molecules that allow a pathogen to colonize, including adhesive molecules and enzymes needed to penetrate deeper into the host tissue or cell, (2) processes that allow the organism to obtain nutrients efficiently from the host (3) production of toxins and (4) mechanisms to evade the host's immune defence, prolonging the survival of the pathogen. Clinically, these pathogens have certain strains which are resistant to the antimicrobial agents, making the scourge worse (Nweze, 2010)

Epidemiologically, diarrhoea has continued to be one of top prevailing causes of morbidity and mortality across the globe and its prevalence is still alarming despite the introduction of various medical advances, quality treatment plans and correction of fluid loss with oral rehydration therapy (ORT). Diarrhoeal diseases still play a key role among the classical five major killers of children under five years of age.

In South Africa, diarrhoea accounts for up to 24% deaths among children aged 1-5 and this may be connected with the increase in prevalence of HIV/AIDS disease in the country (Bamford, 2013)

The prevalence of infectious diarrhoea has been reported to be unusually high in South Africa. Tanih *et al.,* (2014) recorded a 40.8% prevalence of *E. coli* in some diarrhoeal stool

specimens from children of the Dzimuali community. In addition, campylobacter-related diarrhoea has been noted to have a high prevalence in South Africa. South Africa has a high prevalence of campylobacter-related diarrhea among developing countries (Platts-Mills and Kosek, 2014). Currently there is little information on the role of *E. coli* and *Campylobacter* spp. in diarrhea cases in the Amathole District Municipality of the Eastern Cape Province.

1.2 Rationale of the study

The purpose and necessity of this research will be proven by addressing these questions;

1. How high is the prevalence of *E. coli* and *Campylobacter* spp. as infectious diarrhoea agents in the study community?
2. To what extent do these organisms cause diarrhoea and what are the favourable pathogenic determinants of these organisms?
3. How effective and sensitive are the commonly used antibiotics to these agents in the study community?
4. Does the antimicrobial resistance to commonly used antibiotics contribute to the high prevalence and virulence of these organisms?
5. What is the statistical correlation of these two organisms to previous studies locally and internationally with regards to their prevalence and virulence as infectious diarrhoeal bacterial agents?

1.3 Hypothesis

The working hypothesis set for this study was that *E. coli* and *Campylobacter* spp. are generally the etiological agents for diarrhoeal cases in the Eastern Cape, South Africa.

1.4 Research aim and objectives

The main aim of this study was to evaluate incidences of *E. coli* and *Campylobacter jejuni* as the etiologic agents of diarrhoea in patients in selected hospitals in the Eastern Cape Province, South Africa. The specific objectives were:

- To collect stool samples from diarrhoeal patients and screen them for the presence of presumptive *E.coli* and *Campylobacter* spp.
- To isolate, purify and characterize the presumptive isolates and delinate them into relevant pathotypes.
- To determine the patterns of antimicrobial susceptibility of the isolates.
- To screen for the presence of antimicrobial resistance determinants among the isolated pathogens based on the observed phenotypic resistance patterns.

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CHAPTER TWO

Literature Review

2.1 Epidemiology and etiology of diarrhoea

Epidemiological studies have shown that diarrhoea can be classified into three types based on their clinical syndromes and causative agents (WGO, 2008).

Acute watery diarrhoea

Acute watery diarrhoea reffers to diarrhoeal episodes that starts acutely, usually last for 14 days or less and involve the passage of frequent loose or watery stools without visible blood. The most common causes of acute watery diarrhoea are *Rotaviruses*, enterotoxigenic *E.coli*, *Shigella* spp., *Campylobacter* spp. and *Cryptosporidium* spp.

Dysentery

Dysentery is a typical diarrhoea in which blood is identified clinically with faeces. The clinical manifestation includes weight loss, anorexia and the destruction of the intestinal mucosa membrane by invasive bacteria. The most common pathogen for dysentery is *Shigella* spp. Others include *Campylobacter jejuni*, enteroinvasive *E. coli*, or *Salmonella* spp.

Persistent diarrhea

This also occurs acutely and continuously and is usually of long duration (at least 14 days). Multiple enteropathogens could be responsible for persistent diarrhoea. Examples include enteroadherent *E. coli*, *Shigella* spp. and *Cryptosporidium* spp.

2.1.1 Epidemiology of diarrhoea

According to the World Health Organization, diarrhoea can be defined as the passing of liquid or watery stools at least 3 consecutive times in 24 hours. This clinically distinguishes it from dysentery which includes the presence of blood and or mucus in stools. However, the firmness and solidity of the stool rather than the frequency of stools is important as frequent passing of stools does not necessary imply diarrhoea (NDDI, 2013). Therefore, the

epidemiological definition of diarrhoea is passage of loose stools with an increase in volume due to physiological imbalances of the secretory and absorptive mechanism of water and electrolytes in the intestinal tracts (NDDI, 2013).

According to UNICEF and WHO, almost 2-4 billion cases of diarrhoeal diseases occur across the globe annually, mostly in developing countries, and account for 1 in 9 child deaths worldwide. According to a Centres for Disease Control analysis, 2,195 children die daily of diarrhoea and 801,000 child deaths occur from diarrhoea every year, making diarrhoea the second leading cause of death among children under the age of 5 (UNICEF/WHO, 2010).

Diarrhoea has been notably responsible for morbidity and mortality especially among children in sub-Saharan African. Africa is known to have certain factors that encourage the high prevalence of diarrhoeal disease. These include: geographic, economic, political, socio-cultural and personal factors. These factors impede the possibility of preventing this scourge in Africa (WHO, 2014).

There is urgent need to curtail this worrisome pandemic across the globe by formulating sustainable policies and embarking on research to reduce the disease and alleviate current issues with regards to infectious diseases as well as up turning the overall effects of disease associated with geographyical transition (UNICEF/WHO, 2010).

Intermittent epidemics of dysentery and cholera are prevalent in this region of the world due to poor sanitation. Increased levels of diarrhoeal illnesses and deaths from childhood diarrhoea, probably as a result of invasions by entero-pathogens such as *Shigella* and *Rotavirus*, is exacerbated by improper treatment plans and the continous misuse of antimicrobial agents (Gaedicke and Schreier, 2004).

Another notable factor that contributes to the poor outcome in the management of diarrhoea is inadequate awareness regarding new trends of diarrhoea management among health care providers on how to effectively treat the condition. Irregularities in the use of antimicrobials has been a cogent reason for an increased rate in antimicrobial resistance throughout the continent (Nguyen *et al.,* 2005) as antimicrobial resistance will increasingly affect our clinical ability to successfully treat diarrhoeal diseases. Acknowledging the clinical significance of diarrheal disease as a contributory factor to morbidity and mortality especially in childhood in Africa, provides compelling reason to implement the recently introduced programme called Integrated Management of Childhood Illness (IMCI) in order to boost the management of the disease. Development of strategic interventions are required to reduce the extent of antimicrobial resistance among bacterial enteropathogens globally (Ochoa *et al.,* 2009; CDC, 2017).

The statistical analysis of all child deaths from diarrhoea cases shows that 78% occur in Africa and South-East Asia (WHO, 2013a). Genarally, children under five years of age are inflicted with approximately three episodes of acute diarrhoea annually.

Globally, among this age group, acute diarrhoea is the second most common cause of death (after pneumonia). Children, most especially during infancy, are more prone to this disease due to low immunity and the risk reduces with age. Significant effects of diarrhea in children include retardation in growth and development, nutritional imbalances and impaired psychological and cognitive functions, especially in countries deficient in resources (Lopez *et al.,* 2006; Walker *et al.,* 2011).

Although the incidence of diarrhea is high in children it is also a lingering cause of morbidity and mortality in all ages globally, with lower mortality rates occurring among older children and adults than in children below five years of age. However, the disease still constitutes a

significant threat as approximately 2.8 billion episodes occur annually among older children, adolescents and adults (WHO, 2014).

Having understood the scourge of diarrhoea as a major determinant in morbidity and mortality indices, it will be beneficial to assess factors such as length of time of affectation and degree of severity which are valuable in analysing and assessing the overall effect and sequelae complicated by diarrhoea (Kosek *et al.,* 2003). Detailed analysis of duration of diarrhea and degree of severity will help in estimating disability adjusted life years (DALYs) with respect to the number of years that are lost to disability caused by the disease. Although many studies have reported on the degree of severity and length of time of affectation of diarrhoeal diseases, detailed estimates of the cases of prevalence are rare in literature (Gold, 2002).

Reports from various studies in South Africa show that an increasing number of children and adults (young and old) die from diarrhoea, making it the third leading cause of mortality in South Africa. Studies conducted by the CSIR show a high incidence of diarrhoea deaths in South Africa within the age range of 45 to 64 in the last 12 years (CSIR, 2010).

Diarrhoea was ranked as the tenth leading cause of death in 1998 in South Africa; by 2004 to 2005 it had become the third most common natural cause of death (CSIR, 2009). This disease has remained an important and nagging issue in public health and a serious health challenge in developing countries (Jafari, 2009). In South Africa, high mortality rates are recorded among the susceptible age groups such as children under five and the elderly.

According to a study conducted by the Council for Scentific and Industrial Research (CSIR) in 2010, the statistical data from diarrhoea cases are definitive indices of the health status of a particular locality. Diarrhoea episodes especially in young children are noted and recorded at

various health facilities and are readily available on the national health database. However, currently there is a minimal attention given to adult diarrhoea (CSIR, 2010).

Regardless of relevant inputs and advancement in recent years, over two million South Africans still do not have access to a good water infrastructure and approximately six million do not have access to a private water supply. According to the Department of Water and Environmental Affairs more than 12 million South Africans still lack access to any form of sanitation (DWEA, 2012).

According to the data from the Department of Health's National Information System, through General Household Survey (GHS) and National Population Surveying outcomes from Statistics South Africa, children living in poor environmental conditions with lack of access to potable water and those with HIV/AIDS are approximately 10 times more likely to die from diarrhea than their more privileged counterparts (SSA, 2012; WHO, 2012).

Maronel and Genthe have indicated that the risk of water contamination is higher for people who have to collect water from outside the family unit, from a community tap for example, than those that have access to a private water supply within the family unit (even though that water could also be contaminated). Therefore, having access to private water supply within the family unit is of greater benefit and reduces the chances of diarrhoea cases (CSIR, 2009).

It has been locally reported that Limpopo, Eastern Cape and KwaZulu-Natal are faced with the lowest proportion of access to a domesticated water supply in the country which puts them at risk for biological contamination of already-treated water and increases the chances of diarrhoea episodes (CSIR, 2009).

2.1.1.1 Major risk factors for diarrhoeal disease

Globally, the identified risk factors for gastroenteritis include insufficiency or inaccessibility of clean and safe water and sanitation, unsafe and unhygienic practices and inappropriate disposal practices at home (UNICEF, 2015a). In addition to these factors, other anti-social conditions such as poor housing systems, poverty and domestic and community over-crowding also contribute to the the prevalence of the disease. Moreover, it is observed that poverty contributes to reduced access to good medical services as well as an inability to achieve good nutritional status and continuous exposure to inappropriate health provision and care, increasing chances of illness (UNICEF/WHO, 2014). Some studies have identified monogamy of the father, defined residential areas (UNICEF, 2009), decent domestic settings and privacy (UNICEF 2008) as protective factors.

A report produced by United Nations on global water supply specified that Africa is presently faced with poor water supply (UN 2014). In a report released by WHO in 2014, it was indicated that the African continent has shown a decline in the regions which had gained access to good sanitary and environmental systems between 1990 and the year 2014 (WHO, 2015). Almost 55% (over 300 million people) of the entire African region lack access to safe water, and close to 67% (400 million people) lack access to a hygienic environment. Furthermore, it has been predicted, that there will be a rise in these figures to 400 million and 500 million respectively in most developing regions by the year 2020. This is due to the fact that the poorest populations in Africa are predominantly in rural areas, but a subsequent study from 2000 to 2015 there has revealed a 35% reduction in total numbers of people without access to safe water, due to the millennium development goals (MDG) programme (WHO, 2015).

2.1.1.2 Host factors that increase susceptibility to diarrhoea

Sex

According to some studies, gender differences may affect the prevalence of disease and management, especially in children. It has been indicated that young girls in developing nations are more susceptible to high mortality and mobidity than their male peers (UNICEF, 2015b). A study over the period of a year reported that the gender of an individual (Siziya *et al.,* 2013) is a factor influencing deaths among children suffering for various diseases (diarrhoea being one of them) in the African region. Information from studies carried out in selected medical facilities on selected patients admitted or on clinical attendance (UNICEF, 2015b: Gomwalk, *et al.,*1993) showed that male children are more likely to be taken to hospital for treatment as a result of diarrhoea than female children (male-female ratios are 2 to1 and 4 to1 respectively). In contrast, national gender-based studies conducted have shown no significant difference.

Inadequate breastfeeding

The nutritional and preventive benefits of breast milk to children with diarrhoea are documented. Diarrhoeal diseases such as shigellosis and cholera can be prevented due to the presence of antibodies in breast milk.

Exclusive breast feeding decreases mortality due to diarrhoeal diseases seven fold as it is associated with a 40% reduction in the disease in infancy (Kramer *et al.,* 2001), with even greater protection against hospitalization or persistent diarrhea. There is evidence of a dose-response relationship with 6 months of exclusive breast feeding giving the best protection. The mechanisms by which breast feeding protects against the need for diarrhoeal diseases are multiple and include the contents of breast milk, the better nutritional status of the child, the low cost and the promotion of mother-child bonding (Kramer *et al.,* 2001)

Presence of other infectious diseases e.g measles

Diarrhoeal diseases occur commonly and are more deadly in children currently suffering from measles or those that have previously suffered from measles. There is always a significant impairment to immune system in children affected by measles, a condition which complicates the diarrhoeal disease. Stastistical data have indicated that there is a significant increase in the rate of episodes of diarrhoea in children currently suffering or who have suffered from measles recently. Therefore, administration of the measles vaccine to all children in order to reduce diarrhoea incidences has been suggested by the World Health Organization (WHO, 2013a).

Inadequate nutrition

Inadequate nutrition or severe malnutrition has a significant effect on the frequency of diarrhoea and contributes to fatality and possibility of death from diarrhea particularly in children. Clinically, diarrhoea and malnutrition are sometimes interrelated, the former can cause malnourishment while the later accelerates episodes of diarrhoea. Further, persistent and prolonged phases of diarrhoea result in nutritional imbalances in some patients. Children who are malnourished are also prone to developing complications with diarrhoea. Various studies on the interdependency of diarrhea and malnutrition have shown that persistent or severe diarrhoeal disease has an adverse effect on the nutritional status of an individual (CDC, 2016). It is known that certain clinical conditions such as a reduction in the level of food intake due to lack of appetite, continuous fluid loss due to vomiting, intentional withdrawal of feeding, and acute digestive malfunctioning contribute to the deleterious effect of diarrhoea on nutritional status (Gadewar and Fasano, 2005).

Immunodeficiency or immune-suppression

This may be temporarily due to infections usually caused by viruses (e.g measles) and become worst in patients with chronic viral infections such as Acquired Immunodeficiency

Syndrome (AIDS). During severe immunocompromised status, prolonged opportunistic viral infections can set in and usually caused a persistent and prolonged diarrhoea. HIV infection becomes more complicated during diarrhoea episodes due to malabsorption of nutrients, and this further increases the rate of deaths.

Chronic diarrhoea from malabsorption of carbohydrates such as lactose has been regarded as a key complication in children with HIV (Coulston, *et al.,* 2013). It has been proven clinically that about 30% to 60% of children infected with HIV will have lactose malabsorption, and it is advisable to feed such children with low lactose nutrition during chronic or persistent diarrhoea.

Another possible nutritional malabsorption condition arises with fatty food. Almost thirty percent of children infected with HIV will experience fat malabsorption which results in excessive excretion of fat with faeces, a condition called steatorrhoea. Insufficient pancreatic exocrine secretions contribute to the steatorrhoea (Coulston, *et al.,* 2013).

Age

Epidemiological studies have shown that the incidence of diarrhoea episodes is high during the early years with the highest frequency between the first six to eleven months of life. (UNICEF, 2014). This is because although children in this age range still possess immunological antibodies derived from their mother, they are at a declining level. This is also the age at which they are introduced to the family food, which may be contaminated. Children also ingest contaminated food particles while crawling and this increases the chance of having diarrhoea at this age.

However, a report from South Africa has indicated that there is also a significantly high rate of diarrhea-associated death in adults within the age range of 45 and 64 (CSIR, 2010).

Seasonal variation

Studies from various regions have indicated that incidences of diarrhoea disease may be related to the seasonality of a particular region. For instance, in regions with temperate climates, diarrhoea due to bacterial pathogens occurs more often when it is warm, whereas incidences due to viruses (*Rotavirus*) are higher when it is colder (Jagai *et al.,* 2012). Epidemiological studies have also shown that diarrhoea caused by *Rotavirus* may occur year round in the tropics and frequency may be higher when the weather is drier and cooler, whereas bacterial instances are higher in warm but rainy seasons (Masukawa *et al.,* 2016). A study conducted in Kenya found a high infection rate of bacterial infectious diarrhoea during the dry season compared to the rainy season (Shah *et al.,* 2016b).

In China, a study found that diarrhoea in children under 5 was more likely to peak in autumn, while diarrhoea in people older than 5 years of age peaked in summer (Patel *et al*., 2013).

2.1.1.3 Diarrhoea outbreaks

Globally, diarrhoea outbreaks have been associated with two major enteric bacterial agents: *Vibrio cholerae* 01 and *Shigella dynsenteriae* type 1. They have been responsible for major epidemics and resulted in notable illnesses and even deaths in all age groups. The incidence was first observed in 1961 in Asia and described as cholera, which was caused by the EIT or biotype of *V. cholerae* 01. It has spread to all regions of the world.

Diarrhoea outbreaks are frequently high in numbers. A deadly occurrence was observed in July 1994 among Rwandan refugees in Goma, DRC (formerly Zaire), which claimed over 40,000 refugees (Daniel, 2004).

The African continent appears to be more adversely affected by cholera than was initially observed in Asia. The cholera obutbreaks which commenced in Asia in the early 70s

eventually reached Africa. Over 160,000 cases of cholera were reported,in which 2,500 people died (WHO, 2013b).

In 2013, an outbreak of diarrhoea was reported in the Western Cape in South Africa which led to the death of 2 children and about 2,500 people were hospitalised (DHIS, 2013.).

In Ethiopia, an outbreak of diarrhoea was reported in which 19 children died and about 16,000 people were hospitalised (WHO, 2017)

2.1.2 Etiology of diarrhoea

Before the advent of modern diagnostic methods, causative agents could only be identified from the faeces of about 24% of people suffering from acute diarrhoea. However, advances in modern diagnostic equipment in medical laboratories have increased the identification rate of pathogens to about 75% (WHO, 2013c).

The major etiologic agents of infectious diarrhoea are classified into viral, bacterial and parasitic (protozoan) agents.

2.1.2.1 Viral agents:

The viral agents that can cause diarrhoea are; *Astovirus, Adenovirus*, *Rotavirus* and *Norovirus*.

2.1.2.1.1 Astovirus

Astovirus belongs to the family of *Astoviridae.* It was first identified in 1975 in humans by electron microscope following an epidemic in the UK (Madeley and Cosgrove, 1975).

Astoviruses are about 28-35 nm in length, and are usually icosahedral viruses, characterized by five or six pointed star-like surface structure when viewed by electron microscope. (ICTV, 2015).

Globally, various studies have indicated that *Astoviruses* are relevant agents of gastroenteritis especially in the young (Ahmed *et al.,* 2011: Diem-Lan *et al.,* 2017).

Infections by astovirus can occur in all ages but the incidence is high in children and the immune-suppressed (Bosch, 2014).

2.1.2.1.2 Adenovirus

Adenoviruses are member of the family *Adenoviridae,* they are of medium size 90-100 nm, and are non-enveloped viruses with an icosahedral nucleocapsid with a double-stranded DNA genome (Woo *et al.,* 2010). They are resistant to changes in pH status, allowing them to survive for long periods both in and outside of the body. The major route of spread is through respiratory droplets, however, they can also spread by contact with contaminated faecal matter (CDC, 2001).

It has been reported that two sub-types *Adenovirus* 40 and 41 cause gastroenteritis, with diarrhoea being one of the symptoms (Curlin *et al.,* 2010).

2.1.2.1.3 Rotavirus

This belongs to the double-stranded RNA genome and is classified as a member of the family *Reoviridae*. They metabolize mainly in the GIT and cause pathological damage to enterocytes and small intestine villi, thereby causing histological and physiological alterations to the epithelial membrane (Greenberg *et al.,* 2009).

Rotavirus is frequently responsible for severely acute diarrhoea in infants and children (Patel *et al.,* 2013: Dennehy, 2015). On infection, *rotaviruses* bind to the gut of the host and cause structural and physiological damage to the gut which leads to malabsorption, and subsequently to diarrhoea. It can be transmitted by contact with contaminated objects or by ingesting faecally contaminated food (WHO, 2008). Rotavirus epidemics are common among

infants admitted to health facilities or in day care, as well as those in geriatic care (Parasher et al., 2006: Fischer *et al.,* 2007).

2.1.2.1.4 Norovirus

This virus was named the Norwalk agent after Norwalk, in Ohio when it was identified as a cause of an outbreak of acute diarrhoea that occurred among school children in 1968 in Ohio, United States. An electron microscopic study and examination of human stool samples identified this virus. They belong to the family *Caliciviridae* (Patel *et al.,* 2008).

It can be transferred directly from one individual to the other or as a result of direct contact with contaminated water and food. It is known to be highly contagious (Morillo and Timenetsky, 2011), and is responsible for about 19% of all cases of diarrhea cases from acute gastroenteritis across the globe, with high incidences in the developed world (Ahmed *et al.,* 2014).

2.1.2.2 Parasitic Agents

Parasitic agents include some protozoa, such as: *Giardia lambia*, *Entamoeba histolytica*, *Isospora belli* and *Cryptosporidium* spp. (Shah, *et al.,* 2016a)

2.1.2.3 Bacterial agents

The bacterial agents that cause diarrhoea include *Salmonella* spp*., Shigella* spp*., C. defficile, Campylobacter* spp. and *Escherichia coli*.

2.1.2.3.1 *Escherichia coli*

E. coli are Gram-negative organisms which do not retain crystal violet dye biochemically. They have the metabolic ability to make ATP by both aerobic respiration in the presence of oxygen and by anaerobic respiration if oxygen is absent. They are also non-spore forming in nature (CDC, 2012a).They are morphologically rod-shaped, and are about 2.0 μm in length

and 0.25-1.0µm in breath, (Ihssen *et al.,* 2010) and capable of living on any form of host. They biochemically produce lactate, ethanol, succinate and acetate using anaerobic fermentation.

E. coli optimally grow at temperature of about 37ºC, but some pathotypes of *E. coli* can replicate at a temperature of about 49ºC (Madinga and Martinko, 2006). The optimal growth of *E. coli* is enhanced by the ability of the organism to respire by both aerobic and anaerobic means, using large varieties of reduction and oxidation clusters such as oxidation of formic acid, hydrogen and amino acids, as well as pyruvic acid oxidation coupled with the reduction of substances such as, oxygen, dimethyl sulfoxide, fumarate, trimethylamine N-oxide and nitrate (Ingledew and Poole, 1984).

Strains that possess flagella are motile, and this helps them in the transfer of DNA through bacterial fusion, transduction or transformation and conjugation which leads to transmission of the genetic material (Brussow *et al.,* 2004).

2.1.2.3.1.1 *E. coli* pathotypes

A number of *E. coli* strains are non-pathogenic, commonly considered part of the normal flora of the human digestive tract where they are beneficial to the host by preventing the colonization of the colon by other, pathogenic bacteria. They also produce certain vitamins (e.g vitamin K) required by the host (Vogt and Dippold, 2005; CDC, 2012a).

E. coli is the only member of the *Enterobacteriaceae* family that does not occur in the natural environment such as in water, soil or on vegetation. It is exclusively situated in the gut of humans and other warm-blooded animals; its presence in water is usually taken as an indication of faecal contamination of such water. Some highly adapted strains of the bacteria cause diarrhoeal disease and other enteric illnesses along with other more serious health problems (Vidal *et al.,* 2005; Health Canada, 2006; Prescott *et al.,* 2008). The pathogenic

strains of *E. coli* that are capable of causing gastroenteritis are known as diarrhoeagenic *E. coli*, a group that includes an emerging pathogen of public health importance worldwide (Donnenberg and Whittam, 2010). The pathogenicity of this organism depends on the characterized virulence factors which enables it to invade the host defence mechanism. These virulence factors include: haemolysin, P- fimbriae, aerobactin, type 1 fimbrae and serum resistance. The presence of antigens O and K also shield *E. coli* from the antimicrobial effects and help it to escape phagocytosis in the absence of specific antibodies (Kaper *et al.,* 2004).

Six pathotypes of diarrhoeagenic *E. coli* that possess various virulence factors have been described. The specific virulence factors produced by these strains, together with the diseases they cause have been used to separate them into different pathotypes. They are enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), and diffusely adhering *E. coli* (DAEC) (Vidal *et al.,* 2005; Prescott *et al.,* 2008). However, two other pathogenic strains of *E. coli* which are extra-intestinal have been characterized. They are uropathogenic and neonatal meningitis *E. coli* (Wiles *et al.,* 2008; Dubois *et al.,* 2009). They cause nephrological infection and neonatal septicaemia and meningitis respectively (Nataro and Kaper, 1998).

The classification of diarrhoeagenic strains of *E. coli* into different pathotypes is based on their virulence factors and the specific gene patterns they possess (Palaniappan *et al.,* 2006). The diffusely adherent *E. coli* (DAEC) and enteroaggregative *E. coli* (EAggEC) are two classes that have been identified among the entero-adherent strains of *E. coli*. A stacked-bricklike attachment of bacterial cells to the Hep-2 cells is a characteristic of such aggregative adherence formations (Nataro *et al.,* 1994; Nataro and Kaper, 1998; Rajendran *et al.,* 2010). A 60 MDa plasmid often harbours the genetic characteristics responsible for the virulence factor in EAEC (Baudry *et al*., 1990). Some of the virulence factors responsible for

the pathogenicity of these strains include the aggregative adherence factor II (*aafII*), a heat stable toxin (*ast*), the traditional activator (*aggR*) and an antiaggregation protein (dispersin) encoded by the *app* gene (Czeczulin *et al.,* 1999; Vidal *et al*., 2005; Muller *et al.,* 2007; Nataro *et al.,* 1994; Sheikh *et al*., 2002; Rajendran *et al.,* 2010). EAEC predominantly colonizes the intestinal mucosa of the large bowel, followed by intestinal production of enterotoxin and cytotoxins. EAEC is also known to induce reduction in cellular volume of the intestinal villi, which results in inflammatory responses (Weintraub, 2007).

Enterotoxigenic *E. coli* (ETEC) strains have long been linked to traveller's diarrhoea as well as porcine and bovine diarrhea worldwide (Bekal *et al.,* 2003). It causes watery diarrhoea which often lasts up to a week, but can be protracted. The presence of one or more heat stable (STI and STII) and labile (LTI and LTII) toxin genes characterizes ETEC strains (Rajendran *et al.,* 2010). At the onset of infection, it binds to the outermost layer of the small bowel with the aid of one or more colonization factor antigens (CFA), thereby expressing multiple heat stable (ST) or heat labile (LT) enterotoxins (O'Sullivan *et al.,* 2006; CDC, 2012b).

Enterotoxigenic *E. coli* (ETEC) was first identified as diarrhoeagenic pathotype *E. coli* in humans in the 1960s, but it has since emerged as a causative agent of diarrhoea in travellers and children in the developing world (CDC, 2012b). ETEC in particular uniquely causes a toxin-mediated diarrhea. The two main toxins produced by ETEC include heat stable STa (STI) and STb (STII) which are plasmid mediated, and heat labile LTI and LTII which are closely related to cholera toxin. They are encoded for on the chromosomally mediated genes produced by these strains which are used to classify them into distinct groups (O'Sullivan *et al.,* 2006).

Diffusely adherent *E. coli* (DAEC) is genetically characterized by its *daaE* gene, which is responsible for the synthesis and expression of F1845 fimbriae, a putative pathogenic factor that assists in adherence (Bilge *et al.,* 1989; Vidal *et al*., 2005).

Enteroinvasive *E. coli* (EIEC) has an invasiveness property which is mediated by the presence of a 140-MDa virulence plasmid (pINv). This encodes a number of genes for invasion, including the *virF, ipaH, ipaL* genes, (Vidal *et al.,* 2005; Johnson and Nolan, 2009). The initial steps in EIEC infection comprise colonization of epithelial cells, bacterial multiplication, and the spread to adjacent cells (Sansonetti *et al.,* 1991; Colonna *et al.,* 1995).

Enteropathogenic *E. coli* (EPEC) was the first *E. coli* strain characterized as being pathogenic and the most common diarrhoeagenic *E. coli* pathotype particularly among infants in developing regions across the globe (Nataro and Kaper, 1998). Studies in many countries have shown that EPEC pathotypes are found with higher frequency in infants with diarrhea than those without (Vilchez *et al.,* 2009; Ifeanyi *et al.,* 2015). EPEC isolation from faeces samples was done by O- serotype as the major pathogenicity mechanisms could not be determined in these strains of *E. coli* (Behiry, *et al.,* 2012). However, further studies revealed that a plasmid-encoded type IV bundle-forming pilus (BFP) and the presence of intimin (*eae*) genes are critical to its virulence. The main functional subunit of BFP is bundling; this is a protein with high polymorphism that is structurally encoded by *bfp*A gene of the EAF plasmid-borne *bfp* operon subunit (Donnenberg and Whittam, 2010; Vidal *et al.,* 2005; Rajendran *et al*., 2010). EPEC is also known to produce verotoxin by means of the VT1 and VT2 verotoxic genes (Kong *et al.,* 1999). Typical EPEC is characterized by the presence of both *eae* and *bfp* genes, while atypical EPEC possess the *eae* gene only (Rajendran *et al.,* 2010).

2.1.2.3.2 *Campylobacter* spp.

In 1886, the classical features of infections caused by *Campylobacter* spp. was first noticed by Theodor Escherich (Samie *et al.,* 2007). These infections were named cholera infantum (Samie *et al.,* 2007). The genus was first described in 1963 (Debruye *et al.,* 2008) however the isolation of the bacteria occurred in the year 1972.

Campylobacter is one of the top pathogens in diseases relating to food consumption in developed countries (Ryan and Ray, 2004). Conditions such as consumption of contaminated food or drinking unclean water as well as the indiscriminate consumption of raw meat have been identified as risk factors to campylobacteriosis (CDC, 2013).

2.1.2.3.2.1 Bacteriology of *Campylobacter* spp.

The genus *Campylobacter* belongs to the family *Campylobacteriaceae*. It has about 15 species and 6 sub species by rRNA sequencing, 12 of these are responsible for human diseases.

Campylobacter jejuni causes diarrhoeal disease in poultry and humans. *C. jejuni* has the subspecies; *C. jejuni* subspecies *Jejuni* which is known to be a significant enteropathogen found in humans worldwide, *C. jejuni* subspecies *doylei* which causes gastroenteritis, gastritis and septicaemia in humans, and *C. jejuni* subspecies *fetus* which is a major veterinary pathogen.

Campylobacter coli affects pigs and humans. Other minor species include *C. lari* in birds and humans which causes gastroenteritis, diarrhoea and septicaemia, *C. concisus* which causes oral pathology in humans, *C. hyointestinalis* which causes watery or bloody diarrhoea and vomiting in humans and *C. upsaliensis* which causes acute watery diarrhoea, septicaemia and abscesses in humans (Ryan and Ray, 2004).

They are all gram negative, microaerophilic in nature, usually curved, and possess polar flagella with corkscrew shapes that facilitate motility, penetration and colonization of the mucosal environment. Prolonged exposure to substances such as oxygen cause them to change to a coccoid shape. They are non-fermenters of carbohydrate substrates. They possess immune-dominant antigens which include; lipopolysaccharides, porin molecule [(peptidoglycan-associated major outer membrane proteins (MOMP)}, flagella apparatus and adhesion proteins which help in adhering to the host bowel. *Campylobacter* spp. are generally microaerophilic and capnophilic; they grow optimally with reduced oxygen (5-7%) and increased CO_2 (10%). Some thermophilic species (*C. jejuni* and *C. coli*) grow better between 42-43ºC and produce oxidase and catalase enzymes (Adekunle *et al.,* 2009).

2.1.2.3.2.2 *Campylobacter* virulence factors

Campylobacter spp. are predicted to express certain potential virulence factors.

Motility and chemotactic factors

After gaining access to the gastro-intestinal tracts, they colonize the intestines due to the ability of the organism to translocate to the epithelial covering of the intestinal enterocytes. The motile ability of these organisms is due to the presence of polar flagella, as well as their corkscrew morphological form which helps in easy penetration and invasion of the mucus membrane layer (Gondo *et al.,* 2006). The *C. jejuni* flagellum consists of a thin layer polymer of protein called flagellins. These flagellins are genetically encoded by the flaAand *fla*B genes (Nuijten *et al.,* 1990), and are functionally responsible for both antigenic and phase variation processes (Caldwell *et al.,* 1986; Harris *et al*., 1987). The antigenic expression of *fla*A gene is higher than that of the *fla*B gene, and *C. jejuni* flagella normally consist of *fla*A protein. Non-motile phenotypes of *C. jejuni* have, however, been identified due to mutations in which short, truncated flagella were produced (Wassenaar *et al.,* 1993). These *C. jejuni fla*A^+*fla*B^-mutants showed

slightly decreased motility, indicating the role of *flaB* in their flagellar function (Wassenaar *et al*., 2000). The motility functions of the flagella of *C. jejuni* were proven as part of a subunit vaccine in mice, which further demonstrated the pathogenic function of flagella in *C. jejuni* (Lee *et al.,* 1999). *C. jejuni* contains multiple genes encoding proteins homologous to that of *E. coli* proteins involved in the synthesis of flagella. In the aggregation of the sub-type of the *C. jejuni* NCTC 11168 genome (Parkhill *et al*., 2000), 36 open reading frames (ORFs) were responsible for the biosynthesis of flagella. Studies on mutation revealed that the inability of the organism to express *flh*A and *flh*B genes makes it incapable of synthesizing *fla*A or *fla*B (Muller *et al*., 2006; Matz *et al.,* 2002). The ability of the organism to colonize and to demonstrate the significance of flagella during its pathogenicity has been described using multiple mutants of flagella and this finally confirmed that flagellar apparatus is critical to the virulence and pathogenesis of the organism (Nachamkin *et al.,* 1993; Wassenaar *et al*., 2002). Glycosylation of *Campylobacter* spp. using flagellum had been described in some genes of flagella namely, the *pgl* locus in *C. jejuni* 81-176 (Wassenaar, *et al.,* 2002), the *ptmAB* gene in *C. coli* VC167 (Grant, *et al.,* 1993), and the *neuB2* and *neuB3* genes in *C. jejuni* NCTC11168 (Linton *et al.,* 2000). Another important gene which is not particularly involved in flagella expression and function is the product of the *Cj1024c* gene of *C. jejuni* NCTC11168, which genetically resembles the *Helicobacter pylori* f*lg*R regulating flagellin (Spohn and Scarlato, 1999).

Campylobacter spp. also exhibits the ability to detect and move up or down against chemical gradients. This pathogenic process is known as chemotaxis. Motility and chemotaxis are crucial for the colonization of *C. jejuni* in the intestine. Mutational studies carried out on animals have shown that non-chemotactic mutants cannot function in intestinal colonization (Hughdahl 1988). *Campylobacter* (*C. jejuni*) is chemotactic to substrates such as L-serine, mucin and L-fucose, but repellent to bile acids (Bruce *et al.,* 1986). In a motile and non-invasive subtype, the regulating gene *che Y* was described a gene responsible for the non-motility (Yao *et al*., 1997). Several genes that encourage chemotaxis systems of *C. jejuni* have been described, they include *che*A, *che*V and *che*W genes (Parkhill *et al.,* 2000).

Ability to bind and invade the host cells

The binding and invasion ability of *Campylobacter* spp. in intestinal cells of the host as an important pathogenic feature has been extensively reviewed (Wooldrige and Ketley, 1997). On infection, *C. jejuni* crosses the membrane layer of the gastro-intestinal cells, binds to the surfaces, and gradually invades the membrane layer, causing some inflammatory changes which lead to epithelial cell damage. The capability *C. jejuni* to invade the epithelial cells has been described in a series of experiments, however, this varies in different strains of *Campylobacter* spp. (Everest *et al.,* 1992). Flagella have also been described to be the main determinants of the *Campylobacter* spp. pathogenic ability in adherence and invasion (Wassenaar *et al.,* 2002; Grant *et al.,* 1993). Binding and invasion of *Campylobacter* spp. in host cells are dependent on its ability to synthesize flagellar apparatus and initiate motility, as mutant strains of *C. jejuni* have shown some retardation to motility due to non-functional flagella (Yao *et al*., 1994). Adherence and invasion trigger inflammatory changes due to the release of pro-inflammatory factors such as cytokine and interleukin 8, which subsequently result in cellular damage. (Grant *et al.,* 1993).

Generally, the binding of bacterial pathogen is usually enabled by the presence of fimbriae. Though most *Campylobacter* spp. do not produce fimbriae, a study has confirmed the biosynthesis of fimbriae-like appendages by *C. jejuni* and *C. coli* when grown in the presence of bile salts (Mavis and Mozina, 2013). Proteins that are responsible for adhesion are PEB1 protein, which is encoded by *peb1*A, and *Cad*F protein which was classified as a fibronectin-binding protein. Mutants of this gene were unable bind this receptor protein (Konkel *et al.,* 2004).

Production of toxins

Another important virulence factor of *Campylobacter* spp. is its ability to produce toxin in the body of the host (Wassenaar, *et al.,* 2000). Cytolethal distending toxin (CDT), which encourages pathogenicity and virulence has been found in *Campylobacter* spp. The haemolytic activity has been described a pathogenicity of *Campylobacter* with the genome sequence of *C. jejuni* NCTC11168, which contains only the *cdt* gene, haemolysin proteins and a phospholipase (pld A) (Grant *et al.,* 1993). The *C. jejuni* CDT is encoded by a three-gene operon (*cdt* ABC), and there is no *cdt* activity in isogenic form of *C. jejuni cdt* mutants (Lee *et al.,* 2003; Purdy *et al*., 2000). If *cdt* causes diarrhoea it is presumed that it prevents the crypt cells from reaching their expected structural and functional status as a result of continued loss of their roles in absorbing intestinal substrates (Bang *et al.,* 2003). In enteric colonization, a mutant of the *C. jejuni cdt* B mutant was however unaffected (Purdy *et al*., 2000).

Ability to acquire Iron

The ability of *Campylobacter* spp. to obtain nutrients such as iron from the host bowel assists in its pathogenicity. This results in a reduced level of iron concentration in the host tissue which causes a retardation in bacterial growth as supportive nutrients. Elemental iron is converted into haem compounds in the host tissue, lactoferrin in mucosal surfaces and transferrin in serum; this low concentration of iron leads to a host defence mechanism which is specifically undefined. But *Campylobacter* spp., especially *C. jejuni* can survive in an environment with a low level of iron (Pesci *et al.,* 1994) and can also acquire and utilize the haem complex produced as a result of inflammation for its pathogenicity (Vliet *et al.,* 2002; Pickett *et al.,*

1992). Several studies have demonstrated that *Campylobacter* expresses some inozed iron acquisition systems to ensure its growth in conditions where iron concentration is low (Palyada *et al.,* 2004).

Presence of surface Polysaccharide

Biochemically, the outermost membrane layer of *Campylobacter* spp. is composed of the lipo-oligosaccharide (LOS) and lipopolysaccharide (LPS) which are major contents of the gram negative outer membrane layer, and crucial to the pathogenicity of the organism. LPS consists of two genes, a lipid A molecule that binds to large carbohydrates substrates called oligosaccharide, and an O-chain which is made up of repeating oligosaccharides found in some species of *Campylobacter*, especially *C jejuni* (Luo and Zhang, 2001). The manifold of a *C. jejuni* operon is thought to be relevant to the biosynthesis of the LOS/LPS cluster (Luo and Zhang, 2001), In *E. coli,* similar biosynthesis of these genes is responsible for the production of an O-antigen immunologically identified by *C. jejuni* LPS antisera (Fry *et al*., 2000). However, mutation created in this area has shown a vivid reaction in the formation of O-chains by *C. jejuni.* It also plays a key role in the glycosylation of protein (Bacon *et al.,* 2000).

Dealing with oxidative stress reaction

Being microaerophilic in nature, *Campylobacter* spp. have the ability to survive in the presence of toxic oxygen radicals released during metabolic activities or processes. When they come in contact with host immune defence systems during pathogenesis, *Campyobacter* spp. exhibit certain oxidative stress defence mechanism classified into superoxide stress defence systems and peroxide stress defence system (Kim *et al.,* 2015). The principal constituent of the *C. jejuni* superoxide stress defence is the superoxide dismutase (SOD) protein *sod*B (Atack and Kelly, 2009), which encodes by the *sod*B gene. SOD is

biochemically involved in the systemic mopping up of toxic superoxide by changing them to hydrogen peroxide. Studies on genetic mutation have revealed that a *C. jejuni* sodB mutant demonstrated a significant decrease in intracellular survival in INT-407 cells in vivo (Pesci *et al.,* 1994), and a *C. coli sod*B mutant showed significantly reduced growth in air and model food systems (Purdy *et al.,* 1999).

Ability to survive high temperature variations

Campylobacter spp. are able to deal with changes in temperature. The survival of both *C. jejuni* and *C. coli* in the intestinal tract of the avian, in which the standard temperature is 42ºC, as well as temperature in human hosts which is 37ºC, and during transmission in some food substances such as milk, meat and water with very high temperatures usually 40ºC and above, have proven the ability of the bacteria to withstand thermal stress. This thermo-tolerance is mostly achieved due to the ability of the organism to initiate and induce the expression of heat shock proteins (HSPs). These proteins pathologically function in thermo-tolerance and other stresses by assisting in the aggregation of several proteins in the cells and promoting the break down and detoxification of potentially toxic proteins. Examples of HSPs identified in *C. jejuni*, include GroESL, DnaJ, DnaK and ClpB proteins (Baserisalehi *et al.,* 2006). However, the previous studies have shown that the presence of a mutant of any of these HSPs disrupt the role in *C. jejuni* pathogenesis. The DnaJ mutant has solely been described as a reason for *C. jejuni*'s inability to colonize chicken intestine (Konkel *et al.,* 2004).

2.2 Pathophysiology of diarrhoea

There is osmotic balance in the normal physiological absorption and secretion of water and electrolytes throughout the entire gut. Any event that alters this dynamic process by either causing a decrease in the absorptive ability of the intestinal mucosal membrane or an increase in secretion will result in diarrhoea (Kleinman *et al.,* 2008). Absorption of water is directly dependent on the osmotic movement of electrolytes, especially sodium, which is aided

primarily by the system known as glucose-sodium transporter 1 (GST1). It enhances the active absorption of sodium and glucose, as well as water moving down the electrochemical gradient, which occurs due to a break down in jejunal contents (Deepak and Ehrenpreis, 2011). This can also occur in another mechanism through an active system called sodium-hydrogen exchanger. The absorption of sodium into the cells is carried out via an active sodium/hydrogen pump and subsequently pumped into the circulatory system through the active sodium/potassium ATPase located at the base of the epithelium (Deepak and Ehrenpreis, 2011).

The active ATPase Na/K pump is situated at the basal layer of the GIT cells such as the cells of the crypt and villus. These series of epithelial cells at the tips of the villi are active in gross absorption, while that of the Lieberkuhn function in water and electrolyte secretions. In the crypts, there is a Na/Cl channel which actively opens to higher concentration of cyclic adenosine monophosphate (cAMP) and calcium ions. Ionized sodium, chloride ions and water move into the lumen when these channels are actively opened. However, any minimal changes in the flow across these channels cause the secretion to be dramatically increased. Endotoxins from viral and bacterial enteropathogens are known to cause an upsurge in the levels of cAMP, thus driving a greater chloride ion flow across the brush border into the intestinal lumen, which subsequently leads to the net movement of water with it and diarrhoea (Fasano, 2002).

Disruption in the normal physiological balance that occurs between absorptive and secretory mechanism within the GIT causes diarrhoea. Generally, diarrhea can be regarded as either osmotic or secretory. In osmotic types of diarrhoea, there are greater numbers of osmotically active particles in the intestinal lumen, which cause passive movement of more fluid into the lumen down the osmotic gradient, which may then exceed the absorptive competence of the GIT cells and result in a diarrhoeal episode. This can be seen with the ingestion of substances such as osmotic laxatives which causes the presence of excessive solutes in the luminal

aspect of the intestines, inflammation within the mucosa, and motility disorder. In the secretory phase, the bowel mucosal cells secrete excessive quantities of fluid, which can be induced either by activation of a specific pathway by certain enterotoxins or may be due to the presence of an inherent abnormality in the GIT enterocytes. Clinically either scenario is possible but occasionally both exist at the same time (Powell and Jenkins 2012).

2.3 Clinical features associated with diarrhoea

Dirarhoea diseases have certain clinical signs and symptoms which can indicate the possible type of the diarrhea, however the main pathogen responsible for a particular diarrhoeal disease may be difficult to identify using only clinical acumen. In general, the following clinical features are commonly seen in patients with diarrhea.

- Fever: This is frequently seen in diarrhoea caused by invasive enteropathogens.
- Abdominal pain: This is commonly seen in parasitic diarrhoea, for example; *Entamoeba histolytica*, as well as in diarrhoea caused by bacteria such as *C. defficile*. Vomiting and/or nausea may be seen in diarrhoea caused by *Salmonella* spp*., Campylobacter* spp., *Yersinia* spp*. E. coli,* and some protozoans.
- Faecal evidence of inflammation: This is seen in *Yersinia* spp. *Vibiro* spp. and protozoans.
- Bloody stool: This is common in diarrhoea caused by *Shigella* spp. *Salmonella* spp. *Campylobacter* spp. *Yersinia* spp. *C. difficile, Vibrio* spp. and protozoan such as *E. histolytica* (WHO, 2013c).

2.4 Management of diarrhoea

For proper management of diarrhoea, there is a need for careful and complete physical and systemic examination by a clinician. The clinical evaluation of the patient will help to assess

the degree and extent of dehydration of the patients, as dehydration remains the most important clinical complication of acute diahrrhoea (IMCI, 2000). The management of acute diarrhoea is usually palliative until the complete healing process of intestinal mucosa takes place. Possible management plans are fluid management and encouragement of feeding with the objectives of preventing imminent hypovolaemia. Use of antidiarrheal agents and drugs is also important in managing diarrhoea (CDC, 2003). The management of diarrhoea is discussed as follows.

2.4.1 Rehydration

Some diarrhoeal cases are self-limiting, with mild to moderate fluid loss, particularly cases in developed countries. These episodes of diarrhoea do not call for a vigorous management plan, and can be corrected with simple oral fluids and continuous feeding most especially in adults. When significant fluid loss occurs in children, standard oral rehydration solution formulation is given if the child can tolerate oral feeding. In some children affected with diarrhoea, there may be significant fluid loss with on going vomiting. These children may not tolerate oral feeding and will require intravenous fluid. Fluids can also be given by nasogastric tube (NGT). The route of administering fluid depends on the tolerability of the patient and the degree of dehydration. In the developing world, there may be a delay in recovering from the pathological impact of diarrhoea due to malnutrition and other co-existing diseases. This may put children suffering from chronic diarrhoea at higher risk (King *et al.,* 2003).

Oral rehydration therapy (ORT) as a means of correcting dehydration resulting from diarrhea was the greatest achievement in health care services (WHO, 2014). ORT solution contains the necessary quantity of Na, glucose, K, Cl and alkali (bicarbonate or citrate) in water. These electrolytes replace the possible loss and imbalance that might occur in acute diarrhoea (Yang *et al.,* 2007).

The solution can be given orally or by nasogastric tube. There has been a remarkable improvement in the level of dehydration using this route (Manatsathit *et al.,* 2002). The alternative route of fluid administration is intravenously for severe dehydration and hypovolemic shock to urgently replace lost fluid. Patients can also be resuscitated with other fluids such as modified Ringer's Lactate or albumin in order to correct volume loss, and IVF with dextrose IVF could be used to correct hypoglycaemia in an emergency. It is important to observe the level of serum electrolytes and serum glucose as any imbalance in the electrolyte level is usually an indication of complicated diarrhoea. ORT must be introduced as soon as possible after the correction of initial hypovolaemia or shock. The deficiency in fluid loss must be corrected within the first few hours of presentation, and the patient must maintain hydration until full recovery phase (CDC, 2003).

2.4.2 Feeding and refeeding

Malnutrition and diarrhoea, especially when persistent, are interdependent. Malnutrition worsens diarrhoea and is significant when assessing the severity or prognosis of diarrhea in up to forty percent of diarrhoea-related mortality (Bhutta *et al.,* 2013). Studies have shown that malnutrition is associated with prolonged and persistent diarrheal episodes (UNICEF, 2014). In under-developed nations where episodes of diarrhoea are recurrent, there is always a vicious cycle of diarrhea and under nutrition with dire consequences. Feeding of children with severe dehydration must start as soon as it becomes clinically evident that the patients have been stabilized with ORT. Some schools of thought argue that feeding should be withheld during diarrhoea but not universally believed. Feeding and re-feeding is proven to reduce malnutrition and lower the severity of dehydration. Soft cereal based food has been advocated as it is easier to prepare and quickly tolerated during diarrhoeal episodes.

Introduction of early refeeding in addition with continued breastfeeding is advisable because it helps in maintaining certain absorptive and digestive functions of the intestine during

diarrhoea (WGO, 2008). It has been proven that instantaneous refeeding after the initial phase of rehydration is well tolerated and safe and has some clinical benefits (Mahalanabis *et al.,* 2000).

Khin *et al.,* (1985), reported that fluid and electrolytes losses due to diarrhoea in infants are significantly reduced with lowered ORT requirements by continuous breastfeeding. Reports from various studies have proven that non-breastfed infants have a higher chance of dying from diarrhoea than those who are exclusively breastfed (Chidiebere *et al.,* 2014). Nutritionally, breastmilk contains many protective factors which act at the gastrointestinal mucosal membrane to prevent microbial infection and enhance development of the immune system and therefore reduce continuous gastroenteritis (Hanson *et al.,* 2002). Promotion of exclusive breastfeeding for the first 4-6 months of life and its continuation during diarrhoeal illness cannot be over-emphasized.

In addition to breastfeeding, certain micronutrients and vitamins such as zinc and vitamin A are also helpful in the management of diarrhoea especially in children. Zinc has been reported to play an important role in immunity and wound healing while vitamin A helps in the maintenance of epithelisation (UNICEF, 2014).

2.4.3 Pharmacological substances

Some pharmacological products are also known to be useful in curtailing diarrhoea episodes. The prescription and use of drugs is not routinely advocated for the treatment of acute infectious diarrhoea, but in some chronic cases or diarrhoea due to metabolic or hormonal disorders, use of drugs may be allowed. It has been discovered that despite global circulation of this information, some clinicians still prescribe drugs for acute diarrhoea. (Mittal, 2001). Statistical reports from different studies show that antidiarrhoeal drugs, including

antimicrobials, have been prescribed in more than 90% of children with diarrhoea (Mittal, 2001). However, some drugs have been shown to be helpful in treating diarrhoea.

2.4.3.1 Antibiotics

These agents are purposefully meant to eliminate and eradicate diarrhoeal infectious agents and systemically reduce their spread in the body of the host, however, in most diarrhoeal cases they do not change the symptoms. They may even worsen the situation in some diarrhoea episodes especially in infections of *E. coli* 0157:H7 by triggering the occurrence of haemolytic uremic syndrome due to an increase in Shiga toxin (*Stx*) production. (Farthing *et al.,* 2013). Some classes of antibiotics used in the management of diarrhoea include macrolides, quinolones, co-trimazole, tetracycline, chloramphenicol, ampicillin and metronidazole.

2.4.3.2 Antidiarrhoeal agents

Loperamide, opiates, bismuth subsalicylate, kaolin, smectite and anticholinergic medications are antidiarrhoeal agents. They are recommended mostly in persistent or chronic diarrhea. Statistical data place their efficacy in doubt, particularly in acute diarrhoea. These agents are not recommended for children with acute diarrhoea (Mittal, 2001; Chowdhury *et al.,* 2001).

2.4.3.3 Immuno-suppressants

These agents function in modulating some toxic and disordered immune systems. They are steroids and immune-suppressants such as azathioprine, ciclosporin, and methotrexate. They are mostly used in deadly gastroenteropathies likely caused by allergic reaction to food substances, autoimmunity and idiopathic inflammatory bowel disease (Lundgren, 2002).

2.4.3.4 Anti-secretory agents

The possible cellular destruction that occurs as a result of the interaction of enterotoxins (produced by some bacteria during diarrhoea) with intestinal cells and parasympathetic

nervous system is a compelling reason to use pharmacological products to prevent toxin production and to help in maintaining normal intestinal epithelial structure and architecture (Lundgren, 2002).

5HT-receptor antagonists are agents which prevent the continuous loss of water and electrolytes as a result of rapid secretion. Use of Racecadotril has been described as potent in treating acute diarrhoea in children (Salazar-Lindo *et al.,* 2000).

2.4.4 Use of probiotics

These are live microbes that are believed to provide health benefits when consumed. They are characteristically parts of normal human gut micro-flora and include strains of *Lactobacillus* (e.g*, L. rhamnosus* (lactobacillus GG), *L. acidophilus* and *L. casei*), *Bifidobacterium* (e.g *B. bifidum* and *B breve*), and *Streptococcus* (e.g *S. thermophiles*) (Allen *et al.,* 2010). These organisms boost the microbial integrity of the intestinal surface and assist in normal bacterial-epithelial balancing. They usually make an environment hostile for enteropathogens by competing with the pathogens for essential nutrients and by producing antimicrobial substances. They contribute to the cellular development and cellular survival and assist in modulating the immune mechanism of the mucosa membrane (Hooper *et al.,* 2002).

2.5 Prevention of diarrhoea

Infectious diarrhoea is a preventable disease. Instances of diarrhoea are reduced in the developed world with lower morbidity and mortality due to diarrhoea. This is due to a clean and safe environment and a general high level of improvement in hygiene and health services. However, such practices are not as common in the developing world and this contributes to a high prevalence of disease. Various conferences and interventions have been instituted with a view to reducing incidences in developing nations but have only addressed a

few issues. Poverty, poor sanitation, lack of good social amenities and many more contribute to high incidence of the disease in developing countries. Regular means of preventing this scourge must be sought as reports on prevention of diarrhoea have indicated that an adequate water supply, hygienic practices, and good sanitation can reduce diarrhoea incidence by 26% and mortality by 65% (WHO, 2013c; Bhutta *et al.,* 2013).

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CHAPTER THREE

Evaluation of the incidence of *E. coli* pathotypes in stool samples of diarrhoeal patients in selected hospitals in the Eastern Cape Province, South Africa

3.1 Abstract

The incidence of acute infectious diarrhoea caused by diarrhoeagenic strains of *E. coli*. is high. Diarrhoeagenic *E. coli* is one of the leading bacterial pathogens which cause diarrheal diseases worldwide. This research aimed to determine the prevalence of diarrheagoenic *E. coli* pathotypes isolated from diarrhea samples and determine their antibiogram characteristics. A total of 120 stool samples were collected from patients with diarrhoea. Stool culture was performed using Chromogenic selective agar for isolation. Confirmation of the isolates and screening for virulence genes were determined by polymerase chain reaction (PCR) technique. Antimicrobial susceptibility was performed using disk diffusion method. The presence of antibiotic resistance genes was also determined by PCR based on observed phenotypic resistance pattern. A total of 120 presumptive isolates of *E. coli* were obtained from diarrhoeal stool samples from male and female patients of all age groups. One hundred and six (81.5%) were confirmed *E. coli* positive by polymerase chain reaction. Analyses of the confirmed isolates pathotypes delineation showed their distribution as DAEC 43 (32%), EHEC 18 (17%), EIEC 11 (10%), EPEC 18 (17%), while EAEC and ETEC were not detected. High resistance to antimicrobials was observed among the isolates and the following resistances to commonly used antibiotics were detected; ampicillin (98.1%), chloramphenicol (94.3%), trimethoprim-sulfamethoxazole (96.2%),and tetracycline (90.6%), with lesser resistance to ciprofloxacin (45.2%), and imipenem (35.9%). In addition, 94% of the isolates exhibited phenotypic resistance against chloramphenicol and 89% showed resistance to tetracycline as *cat*A1 and *tet*A genes respectively. The findings of this study revealed that DEC is a leading bacterial agent which causes diarrhoea in the study community and the bacterial isolates from this study showed a high degree of resistance to antimicrobial agents.

3.2 Introduction

Diarrhoea has been described as one of the leading causes of childhood illness and death, predominantly in under-developed countries. In addition, various investigations have confirmed that almost all cases of deaths due to diarrhoea could be prevented (Liu *et al.,* 2012). In 2010, of the 4 million deaths worldwide due to infectious diseases in children under five, infectious diarrhoeal diseases accounted for 0.801 million deaths (Liu *et al.,* 2012). However, there has been a reduction in the childhood death rates worldwide in recent years as a result of oral rehydration therapy. The prevalence of this disease has been clearly associated with contributory factors such as untimely weaning of children from breast feeding, drinking of unsafe water, encouraging bottle-feeding, and malnutrition (UNICEF/WHO, 2012).

A diversity of pathogens such as viruses, bacteria and parasites are causative agents for infectious diarrhoea (Wilson *et al.,* 2006). Various studies have confirmed that of the bacterial pathogens, diarrhoeagenic *E. coli* (DEC) is described as a regularly constant pathogen of acute infectious diarrhoea in children in developing countries (Yang *et al.,* 2009). The Global Burden Disease Report of the WHO, described diarrhoea as the second most common cause of mortality in children under five and this is caused by DEC (Jafari *et al.,* 2008). In developing nations, a high number of children suffer over a dozen episodes of diarrhoea in their first year. Children who suffer continual and persistent diarrhoea are likely to experience some psycho-social issues and growth retardation as well as loss of cognitive functions. (Petri *et al.,* 2008).

Diarrhoeal diseases have been a contributing factor in child undernourishment, and deterioration in growth and development. Besides, other typical infections and *E. coli*

diarrhoea may be even more harmful than *Rotavirus* infections in this view (Mondal *et al.,* 2009). Children are more prone to repeated diarrhoeal episodes caused by diverse strains of diarrheagenic *E. coli* as a result of inadequate immune systems. Out of all diarrhoeal pathogens, diarrhoeagenic *E. coli* is known as the leading agent of diarrhoeal diseases. In research carried out by Brooks *et al.* in 2006, diarrheagenic *E. coli* was detected as a bacterial pathogen in 20% of diarrhoeal specimens, 34% of which were from children under 5 years old. In a study of children with diarrhoea in Mozambique, the prevalence of diarrhoeagenic *E. coli* was 42% and 18% for the control, for *Rotavirus* the prevalence was 18% with 5% as control, and the prevalence of parasites was 38% with 57% as control (Rappeli *et al.,* 2005). *E. coli* is a vital member of the conventional strains of gastrointestinal (GIT) microflora of humans and other mammals which contributes greatly to metabolic activities of some GIT vitamins. The significance of non-pathogenic strains of *E. coli* has been exploited widely in recombinant DNA technology. However, when it acquires certain pathogenic factors, *E. coli* can be a highly versatile and deadly pathogen (Kaper *et al.,* 2004). *E. coli* pathogenic tendencies lie in its ability to express genetic flexibility and the acquisition and/or transfer of resistance or virulence genes from or to other strains of *E. coli*, as well as other organisms (Halawani *et al.,* 2010). The high rate of incidences of microbial resistance to antibiotics in treating diarrhoeal diseases is a concern and has been described as a major virulence factor for DEC. In this paper, we report on the incidence and antibiogram profile of *E. coli* pathotypes in stool samples of diarrhoeal patients from selected hospitals in the Eastern Cape Province, South Africa.

3.3 Methodology

3.3.1 Ethics and informed consent

Ethical clearance was granted by the University of Fort Hare research ethics committee for the study (ref no: OKO011SOMO01) and permission to collect samples was obtained from the Eastern Cape Department of Health (ref no: EC-20146RP10-487), while informed consent was obtained from patients before collecting samples from them. Strict confidentiality was maintained.

3.3.2 Sampling

Diarrhoeal stool samples were collected from patients attending or admitted to both private and public medical facilities in the Amatole District Municipality. Diarrhoeal samples were obtained directly from the diarrhoeal stools of patients who had just passed a watery stool and from the ano-rectal cavity of some patients who were still passing watery stools but not at the time of sample collection. In particular, outpatients made use of sterile swab sticks. The samples were taken from different age groups irrespective of sex and race. After collection, the samples were transported in cooler boxes to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice for analysis as soon as possible.

3.3.3 Bacteriological analyses

3.3.3.1 Isolation of *E. coli*

The bacteriological analyses of the samples collected were performed using standard methods as follows: diarrhoeal samples collected (on swab sticks) were streaked directly on *E. coli* chromogenic agar (Manufacturer: SIGMA-ALDRICH).

The plates were incubated at 37ºC for 24 h. Presumptive isolates (2-3 distinct colonies per plate) which were blue or pink coloured were picked with sterile wire loop and inoculated into 2 ml Tryptone Soya Broth (TSB) and incubated at 37ºC for 24 h. After 24 h incubation, the inoculated samples were stored in 20% glycerol at -80ºC for further analysis.

3.3.3.2 DNA extraction for *E. coli*

This was done following the description of Guion *et al.,* (2008). The glycerol stock was resuscitated in TSB for 18 h at 37ºC. About 2 ml of TSB culture was placed into a microcentrifuge tube and centrifuged at 13000 rpm for 5 min. After centrifugation, the supernatants were discarded and the cell pellets re-suspended in 200 µl of nuclease free water by vortexing. The suspended cells were then subjected to heating in a heating block at 100ºC for 15 min, allowed to cool and then centrifuged at 10, 000 rpm for 3 min. The supernatant (DNA template) was then decanted into DNase free microcentrifuge tubes and stored in -20ºC for further analysis.

3.3.3.3 Molecular confirmation of *E. coli*

Confirmation of the isolates was carried out by polymerase chain reaction (PCR) technique using the previously isolated DNA stored at -20^{o}C with the *uid*A gene.

The *uid*A genus-specific primer was used to confirm the isolates as *E. coli*. The PCR amplification was performed using one Taq 2X Master Mix with standard buffer (which consists of 20 mM Tris-HCl, 1.8 Mm $MgCl_2$, 22 Mm KCl, 22 mM NH_4Cl, 0.2 mM dNTPs,

5% glycerol, 25 units/l one Taq DNA polymerase) (Sarkar *et al.,* 1990). This was carried out in a 25 µl reaction. The above 25 µl reactions (in sterile PCR tubes) were subjected to PCR analysis using the following thermocycling conditions: 94ºC initial denaturation for 5 min, followed by 35 cycles of 94ºC in 60 sec, 58ºC (annealing temperature) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min.

Verification of PCR amplification of products was performed in 1.5% agarose gel (stained with ethidium bromide) electrophoresed at 110 V for 40 min, then viewed in an ALLIANCE 4.7 transilluminator and photographed.

Table 3.1: Primer sequence and expected band size for molecular confirmation of *E. coli* isolates

Target isolate/gene	**Primer sequences(5'-3')**	**Amplicon size (bp)**	**Reference**
E. coli (*uidA*)	AAAACGGCAAGAAAAAGCAG ACGCGTGGTTAACAGTCTTGCG	147	Tsai *et al*., 1993

***Source*:** Vidal *et al.,* 2004; Osode and Okoh, 2010.

3.3.3.4 Delineation of *E. coli* pathotypes among study isolates

Delineation of the confirmed *E. coli* isolates into their respective pathotypes was done using PCR and primers targeting the relevant virulence genes for EHEC, EPEC, ETEC, EIEC, EAEC and DAEC as listed in Table 3.2. The reaction mixture contained 1 µl of 10 pmol specific primer pairs: 12.5 µl of M/M, 5.5 µl of nuclease free water and 5.0 µl of DNA template. The cycling conditions were as follows: 94ºC initial denaturation for 5 min, followed by 35 cycles at 94ºC in 60 sec, 55ºC, 56ºC, 54ºC, 56ºC, 56ºC (annealing temperature for *Eae, Lt, Vir, aaf*ll and *daa*E primers respectively) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min. PCR product was verified

in a 1.5% agarose electrophoresis stained with Ethidium bromide and visualized in a transilluminator and photographed.

Table 3.2: Primers used to detect the various *E.coli* pathotypes among the isolates detected in this study

Genes/DEC	Primer sequence 5′-3′	Annealing temp (^{o}C)	Band size(bp)	References
Eae (EHEC)	TCAATGCAGTTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	55	482	Stacy-Philips *et al*., 1995
Lt (ETEC)	GCACACGGAGCTCCTCAGTCTCC TTCATCCTTTCAATGGCTTT	56	218	Stacy-Philips *et al*., 1995
Vir (EIEC)	AGCTCAGGCAATGAAACTTTGAC TGGGCTTGATATTCCGATAAGTC	54	618	Vidal *et al.,* 2004
Eae (EPEC)	TCAATGCAGTTCCGTTATCAGTT GTAAAGTCCGTTACCCCAACCTG	55	482	Stacy-Philips *et al.,* 1995
aafII (EAEC)	CACAGGCAACTGAAATAAGTCTGG ATTCCCATGATGTCAAGCACTTC	56	378	Vidal *et al.,* 2004
DaaE (DAEC)	GAACGTTGGTTAATGTGGGGTAA TATTCACCGGTCGGTTATCAGT	56	542	Vidal *et al* 2004

Source: Vidal *et al.,* 2004; Osode and Okoh, 2010.

3.1.3.5 Antibiotic susceptibility profile

The antibiotic susceptibility profiles of *E. coli* isolates detected was determined according to Clinical and Laboratory Standard Institute (CLIS) 2015 guidelines on Mueller-Hinton agar. The positive samples from glycerol stock were resuscitated in TSB and incubated at 37ºC for 24 h. The TSB culture matching 0.5 MacFarland standards was evenly inoculated onto Mueller-Hinton agar with sterile swab sticks, allowed to dry for 10 min and antibiotic discs

were dispensed using an antibiotic disc dispenser. Each positive sample was tested against the following 12 antibiotics: ampicillin (AP), cefotaxime, (30 µg), chloramphenicol, (30 µg), cefuroxime, (30µg), norfloxacin, (10 µg), trimethoprim-sulfamethoxazole, (25 µg), imipenem, (10 µg), erythromycin, (15 µg), gentamicin, (10 µg), tetracycline, (30 µg), ciprofloxacin, (5 µg) and doxycycline, (30 µg). Thereafter, the plates were incubated at 37ºC for 24 h and read for sensitivity. The antibiotics chosen are typically used for the treatment of diarrhoeal diseases caused by *E. coli*.

3.1.3.6 Screening for antimicrobial resistance genes

Antimicrobial resistance genes among the isolates were assessed based on the observed phenotypic resistance patterns and using PCR with specific primers targeting relevant resistance genes as presented in Table 3.3. PCR was performed in a reaction mixture containing 1 µl of specific primer pairs, 12.5 µl of M/M, 5.5 µl of nuclease free water and 5.0 µl of DNA template. The cycling conditions were as follow: 94ºC initial denaturation for 5 min, followed by 35 cycles of 94ºC in 60 sec, 52ºC, 53ºC (annealing temperature for *tet*A and *cat*A1 respectively) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min. Amplification of PCR product was verified in a 1.5% agarose electrophoresis stained with Ethidium bromide, visualized in a transilluminator and photographed.

Table 3.3: Primers used to detect the antimicrobial resistance genes of various *E. coli* pathotypes among the isolates detected in this study

Antimicrobial agents	Resistance gene	Sequence (5'-3')	Size (bp)	Annealing Temp. (°C)	Ref.
Chloramphenicol	*cat*A1	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGC C	547	53	Van *et al.* 2008

Tetracycline	*Tet*A	GGTTCACTCGAACGACGTCA CTGTAAGACAAGTTGCATGA	577	52	Momtaz *et al.* 2012

Source: Hiedary *et al.,* 2014.

3.4 Results

3.4.1 Isolation and confirmation of *E. coli*

A total of 120 presumptive isolates of *E. coli* were obtained from the diarrhoeal stool samples collected from both outpatients and inpatients attending or admitted in selected private and public hospitals in the Amatole District Municipality in Eastern Cape.

Of these 120 presumptive isolates, 106 were confirmed as *E. coli* representing 81.5% of the total presumptive isolates.

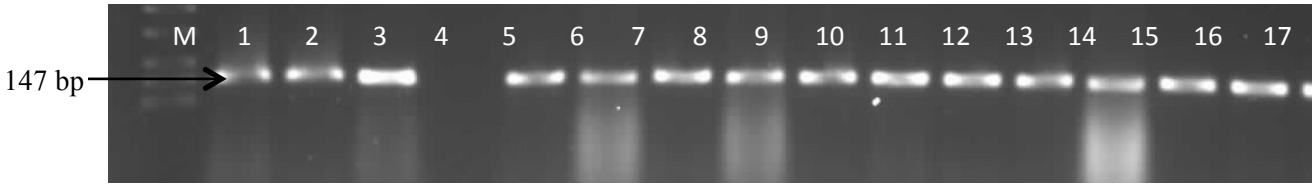

Figure 3.1: Gel picture of representative *E. coli* confirmation showing amplified *uidA* (147 bp) genes. Lane M: 50 bp Molecular weight marker; lane: 1 to 3 Positive control, (DSM 11058 strain); lane 3: negative control; lanes 4 to 17 *E. coli* isolates.

3.4.2 Pathotyping of the isolates

Table 3.4 shows the results of the detected virulence genes among the confirmed *E. coli.* The *E. coli* and pathotypes prevalence were: DAEC (32%), EHEC (17%), EIEC (10%), EPEC (17%), while EAEC and ETEC were not detected.

Table 3.4: The prevalence of detected *E. coli* pathotypes.

***E. coli* Pathotypes**	**Target genes**	**Number Positive**	**Prevalence (%)**
DAEC	*DaaE*	34	32
EHEC	*Eae*	18	17
EIEC	*Vir*	11	10
EPEC	*Eae*	18	17
EAEC	*AafII*	0	0
ETEC	*Lt*	0	0

Out of 106 confirmed *E. coli*, 32 (34% of the total confirmed *E. coli*) were identified as Diffusely Adherent *E. coli* (DAEC).

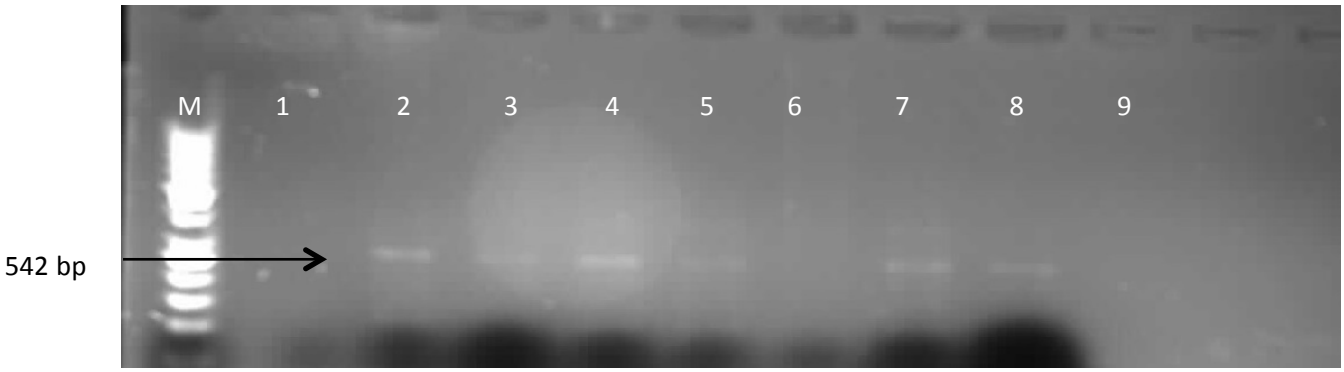

Fig. 3.2: Gel picture representative of the DAEC pathotype showing amplified *DaeE* (542 bp) gene. Lane M: 100 bp Molecular weight marker; lane 2 to 9 DAEC pathotypes.

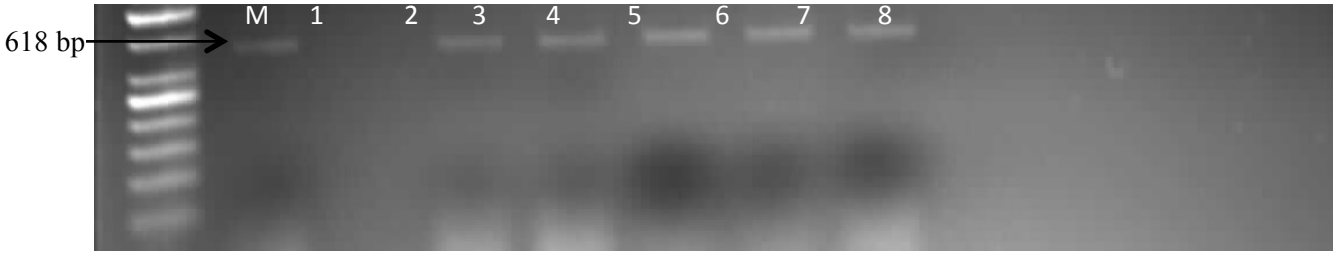

Fig. 3.3: Gel picture represensative of the EIEC pathotype showing amplified *Vir* (618 bp) gene. Lane M: 100 bp Molecular weight marker; lane 1: positive control, lane 2: negative control, lane 3 to 8 EIEC pathotypes.

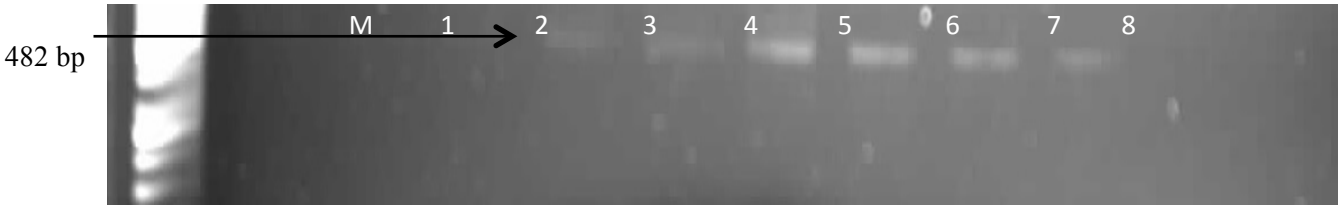

Fig. 3.4: Gel picture representative of the EHEC and EPEC pathotypes showing amplified *Eae* (482 bp) gene. Lane M: 100 bp Molecular weight marker; lane 1 to 6 *E. coli* pathotypes.

3.4.3 Antibiogram profiles of the confirmed *E. coli* isolates

The results of the antibiotic susceptibility profiles of all the confirmed *E. coli* isolates are summarized in Table 3.5. High resistance to commonly used antibiotics such as ampicillin (98.1%), chloramphenicol (94.3%), co-trimazole (96.2%), and tetracycline (90.6%), but a lesser resistance to ciprofloxacin (45.2%), and imipenem (35.9%) were observed in this study.

Table 3.5: Antibiotic susceptibility profiles of *E. coli* isolated from diarrhoeal stool samples in this study.

Antibiotics	**Disk size (µg)**	**Sensitivity (number)**	**Sensitivity (%)**	**Resistance (%)**
Ampicillin	25	2	1.9	98.1
Cefotaxime	30	4	3.8	96.2
Cefuroxime	30	12	11.3	88.7
Chloramphenicol	30	6	5.7	94.3
Trimethoprim-sulfamethoxazole	1.25/23.75	4	3.8	96.2
Imipenem	10	68	64.1	35.9
Erythromycin	15	8	7.5	92.5
Gentamycin	10	36	34.0	66.0
Tetracycline	30	10	9.4	90.6
Ciprofloxacin	5	58	54.8	45.2
Doxycycline	30	6	5.7	94.3
Norfloxacin	10	46	43.4	56.6

Results of the screening for the antibiotic resistance genes revealed that the genes encoding for resistance against chloramphenicol and tetracycline with positive for *cat*A1 and *tet*A respectively. The results showed that 94% of *E. coli* harboured *cat*A1 and 89% harboured *tet*A.

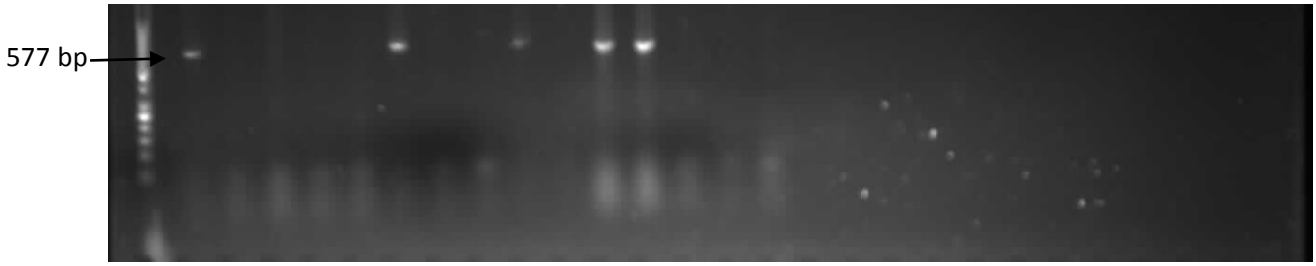

Fig. 3.5: Gel picture resistance genes for tetracycline (*tet*A). Lane M using 50 bp Molecular weight marker. Lane 1: positive control, lane 2: negative control, lane 2 to 13; *E. coli* isolates.

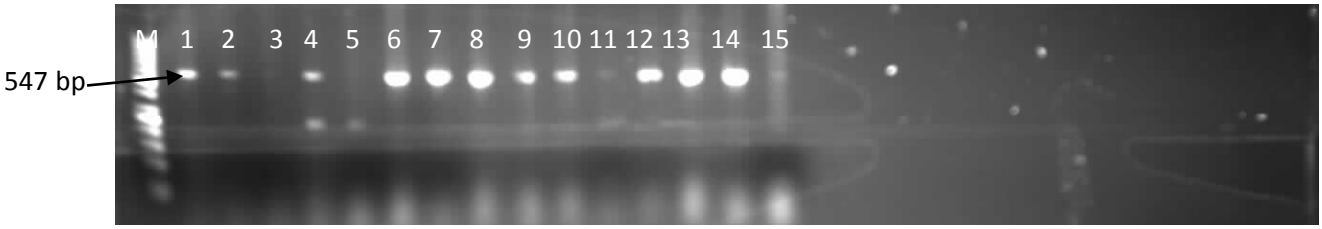

Fig. 3.6: Gel picture resistance genes chloramphenicol (*cat*A1). Lane M using 50 bp Molecular weight marker. Lane 1 to 2: Positive control, lane 3: Negative control, lane 4 to 15: *E. coli* isolates.

3.5 Discussion

In this study, the prevalence the antibiogram pattern of DEC isolated from the hospital patients were investigated. A series of studies have confirmed that bacteria are responsible for 20% to 60% of all total infectious diarrhoeal diseases (Isenbarger *et al.,* 2011: Yang *et al.,* 2009: Liu *et al.,* 2012). In industrialized nations, viral agents are more frequently identified as causative agents of acute diarrhoea, especially during the early stages of life, however, bacteria are the most common enteropathogens identified in children and adults in less developed regions (Usein, *et al.,* 2009). In this study, 106 isolates were confirmed to be DEC, which is 81.5% of the total isolates obtained from culture prior to molecular confirmation. This result is significant and shows that DEC, is a leading bacterial agent causing diarrhoea in

the study community, which corroborates previous reports by Yang, *et al.,* (2009) and the CDC, (2016). Epidemiological studies have previously confirmed DEC as the most frequently isolated enteropathogenic bacteria globally. However, a number of studies have found some differences. For instance, in Tunisia and New Caledonia, *Salmonella* spp. were reported as the most frequently identified enteropathogens (Al-Gallas 2007), while *Campylobacter jejuni* was described as the leading enteropathogens in adults in Sweden (Germani *et al*., 1999) . In South Africa, DEC remains the leading bacterial agent causing diarhoeal diseases (CDC 2016). These geographical differences in prevalence may be due to study population size and/or various microbiological techniques adopted during the process of the studies. Undeniably, in accordance with this study, DEC has been frequently isolated in developing countries such as India (Samal *et al*., 2008), Nigeria (Nweze, 2010), Thailand (Themphachana *et al.,* 2014), and Korea (Cho *et al.,* 2008). The most commonly observed pathotype of DEC in this study was DAEC (32%), followed by EHEC (17%), EPEC (17%), and EIEC (11%) while no EAEC or ETEC were detected. This conforms with reports from Maputo, Mozambique, in which DAEC frequency was higher than that of EPEC, ETEC, EIEC or EAEC in diarrhoeal stool samples (Rappelli *et al.,* 2005). Studies have shown that DAEC is widespread and may be more prevalent in HIV-positive patients (Okeke *et al.,* 2000: Mossoro *et al.,* 2002: Mwachari *et al.,* 1998). Interestingly, in a human challenge study it was discovered that some DAEC strains did not produce diarrhoea in healthy individuals; however, since the category of bacteria is heterogenous, being unable to cause diarrhoea in older people does not denote an absence of virulence in more susceptible persons (Nataro and Kaper, 2006). EPEC was also identified in this study, as corroborated in various studies in many parts of the world.

Studies in some sections of Africa suggest that EPEC is a prevailing cause of diarrhoea. In South Africa, a study conducted in Western Cape identified EPEC as the most prevailing

cause of diarrhoea during childhood (Lamprecht *et al.,* 2014). However, there has been a decline in relevance of EPEC as a pathogen in some published articles. This could be associated with the practice of certain beneficial advances such as breast feeding as it greatly prevents diarrhoea caused by EPEC (Mandomando *et al.,* 2007). The relevant decline in the prevalence of diarrhoea caused by EPEC could be linked to the UNICEF/WHO 0-6 month uninterrupted breastfeeding advocacy (UNICEF/ WHO 2012). However, it has become evident that EPEC is the prevailing cause of diarrhoea in people with HIV (Croxen *et al.,* 2013). In an inclusive study on bacterial enteropathogens carried out among hospitalized patients with diarrhoea in all age groups in India, EPEC was found to be prevailing pathotype of DEC (Lacher *et al.,* 2007). EHEC was also detected in this study as reported in previous studies.The prevalence was only 17% lower than EPEC in this study. EHEC was first documented in South Africa in 1990 in a periodic episode of haemorrhagic uremic syndrome caused by EHEC O157:H7 (Browning *et al.,* 1990). Three years later, an outbreak of EHEC which occurred in a sugar plantation in Swaziland and led to the death of approximately 2,000 people was described by a South African laboratory as one of the global EHEC epidemics (Effler *et al.,* 2001). The continuous proliferation of the organism is probably assisted by biological pollution of surface water by dead and decayed animals, or by people consuming animals killed by drought (Effler *et al.,* 2001). Germani *et al*., (1997) reported an EHEC upsurge in the Central African Republic in which 108 people presenting with bloody diarrhoea were admitted to hospital, of whom 4 died. In 2004, an outbreak of over 300 cases of diarrhoeawith bloody stool was reported in some parts of Ngoila town in Cameroon (Keusch *et al.,* 2006). Continuous isolation of EHEC has almost always been found when sought in Africa, even when there was no epidemic (Okeke, *et al.,* 2003). Even though there is a decrease in the rate of prevalence of EPEC diarrhea, it must be considered as a differential diagnosis in diarrhoea that is clinically characterized with blood-stained stool.

EHEC further causes multi-systemic pathological complications such as digestive bleeding disorder (hemorrhagic colitis), hemolytic uremic syndrome and thrombocytic thrombocytopenic puerpera, but these may be difficult to diagnose in some areas in Africa due to a common belief that such symptoms are only exhibited during endemicity of *Shigella* (Olotu *et al.,* 2008). EHEC diarrhoea epidemics have been described in North America and Western Europe due to the consumption of undercooked beef, as well as from eating vegetables fertilized with animal manure. Most importantly, EHEC is known to be transmitted zoonotically, for example in Africa, bovines are known to be a significant reservoir for EHEC. Person-to-person transmission routes have also been identified (Majalija *et al*., 2008).

In the Central African Republic, Germani (1997) linked EHEC diarrhoea to the consumption of Kanda, a snack composed of meat from the intestine of a cattle sub-species known as Zebu. The rate of prevalence of EHEC in Zebu meat was found to be almost 30% (Kaddu-Malindw *et al.,* 2001). EIEC in this study was detected in 11% of the total confirmed isolates. However, no EIEC was identified in a study conducted in Gabon, while studies from Kenya, Mozambique, Ghana and Nigeria also identified a small number of EIEC pathotypes (Opintan *et al.,* 2009). The prevalence of this pathotype of *E. coli* has been rarely reported as a diarrhoeal agent in teenagers and adults but in Ecuador, reports from one study found EIEC to be a leading cause of infectious diarrhea in all age groups. Most other studies also rarely report this pathotype in teenagers and adults (Vieira Castaneda, 2006). In this study, ETEC and EAEC, which are predominantly known to be responsible for travelers' diarrhea, (Dean and Kenny, 2009) were not detected.

The conspicuously increased rate of antimicrobial resistance of some strains of diarrhoeagenic bacterial pathogens across the globe is quite alarming more significantly in less developed regions (Hiedary, *et al.,* 2014). The indiscriminate use of antibiotics in the

treatment of diarrhoeal diseases has been noted as a cogent reason for the high rate of antimicrobial resistance (Usein *et al.,* 2009). DEC belongs to the group of GIT commensal bacteria which have been reported to be the principal determinant of resistance genes for pathogenic bacteria (Mohammad *et al.,* 2013), and *E. coli* has been often used as an important index in surveying the critical selection of antimicrobial agents and determination of genes responsible for resistance (Okeke *et al.,* 2007). Previously, some investigations have stipulated that most DEC strains have exhibited antibiotic resistance to at least ampicillin, sulfonamide or cotrimoxazole (Lamprecht *et al.,* 2014). Certain antibiotics which no longer cure diarrohea are still constantly prescribed to treat infectious diarrhea due to their accessibility and low cost (Jafari *et al.,* 2008; Bouzari *et al.,* 2007). Currently, the most frequently used antibiotics to treat diarrhoea are tetracycline, cotrimoxazole and ampicillin (Nguyen *et al.,* 2005). In this study, a high rate of DEC antimicrobial resistance to frequently used antibiotics such as ampicillin (98.1%), tetracycline (90.6%), chloramphenicol (94.3%) and trimethoprim-sulfamethoxazole (96.2%) was noticed. This finding is similar to some reports from various studies on children from Bolivia, Peru, Mozambique, Vietnam, Mexico, Argentina and Tanzania, which observed over 70% prevalence of ampicillin resistance in DEC isolates identified (Ochoa *et al.,* 2009). In another study conducted by Nguyen *et al.,* (2005) in Honoi, Vietnam, DEC pathotypes showed different stages of resistance to ampicillin (86.4%), chloramphenicol (77.2%) and trimethoprim-sulfamethoxazole (19.1%). In a study in Egypt, the prevalence of antibiotic resistance among some DEC isolates were 68.2%, 57.2% and 24.2% for ampicillin, trimethoprim-sulfamethoxazole and ampicillin-sulbactam, respectively (Putnam *et al.,* 2004). Bouzari *et al* (2007) reported a high prevalence of resistance against trimethoprim-sulfamethoxazole, tetracycline and chloramphenicol in DEC strains isolated from Tehran, Iran which is similar to the findings of this study. Several studies have found that multidrug resistant *E. coli* are common among the DEC pathotypes

and incidences of resistant DEC, which could be due possibly to environment-related conditions, transmission of pathogens from adults to children or from animals to humans (Jafari *et al.,* 2009) and occasionally as a result of unwise and indiscriminate use of different antibiotics in management and treatment of infectious diarrhoea (Usein *et al.,* 2009). In this study, resistance to imipinem, ciprofloxacin and norfloxacin were relatively lower than other studies (35.9%, 45.2% and 56.6 respectively). This ties in with certain studies which have recommended fluoroquinolones as first-line drugs to treat diarrhoea (Hiedary, *et al.,* 2014). In screening for the existence of antibiotic resistance genes in DEC strains in this study, isolates that were resistant to chloramphenicol and tetracycline were randomly selected and profiled with *cat*A1 and *tet*A for tetracycline and chloramphenicol respectively. Findings revealed that 94% and 89% harboured *cat*A1 and *tet*A genes, respectively. This concurs with a recent study conducted in North West England which also determined that the strains resistant to both chloramphenicol and tetracycline harboured *cat*A1 (91%) and *tet*A (93%) genes respectively (Ahmed *et al.,* 2010).

3.6 Conclusions

From the findings of this study, it could be deduced that *E. coli* is widely distributed and is the leading bacterial agent of diarrhoea in the study area, which corroborates previous studies that showed a high prevalence of *E. coli* in the environment which caused a variety of infections in both hospital and community settings, particularly diarrhea (Donnenberg *et al.,* 2005: Usein *et al.,* 2009). In this study, 81.5% of presumptive isolates of *E. coli* were confirmed to be positive, which supports the view that DEC remains the leading cause of infectious diarrhoea in the study area. The DEC pathotype with the highest prevalence in this study was DAEC (32%), which is similar to the results of other studies. For example in Maputo, Mozambique and Shraz in Iran, DAEC was more frequently identified than EPEC,

ETEC, EIEC or EAEC in diarrhoeal stool samples (Rappelli *et al.,* 2005; Pejman, *et al.,* 2016). This study highlighted the important role of DEAC in the epidemiology and pathogenicity of diarrhoea caused by *E. coli*. In this study, a high rate of DEC antimicrobial resistance to frequently used antibiotics such as tetracycline (90.6%), chloramphenicol (94.3%) and conventional Beta-lactams (ampicillin 98.1%) was observed, while resistance to newer generations of Beta-lactam (imipenem 35.9%) was lower. In addition, 94% and 89% of the isolates harboured *cat*A1 and *tet*A resistance genes for chloramphenicol and tetracycline, respectively. Susceptibility of the isolates was performed against 12 antimicrobial agents, but only 2 antibiotic resistance genes were screened due to financial constraints and therefore this is a significant limitation in this study. More attention should be paid to antimicrobial susceptibility of DEC first in order to guide clinicians in the selection of appropriate therapy for treating diarrhoea caused by *E. coli.*

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CHAPTER FOUR

Evaluation of the incidence of *Campylobacter specie* in stool samples of diarrhoeal patients attending selected hospitals in the Eastern Cape Province, South Africa

4.1 Abstract

Infectious diarrhoea with *Campylobacte*r spp. has been described globally as an important gastro-intestinal tract disease. The aim of this research was to determine the prevalence and antibiogram of *Campylobacter* spp. in diarrhoeal stool samples collected from hospitals in the Amathole District Municipality in the Eastern Cape Province, South Africa. A total of 80 stool samples collected from both inpatients and outpatients in the study area from different age groups, both male and female, with diarrhoea, were screened for *Campylobacter* spp. Stool samples cultures were performed using Campylobacter selective agar (charcoal based),

while confirmation of the isolates and determination of the presence of the virulence genes were performed molecularly by polymerase chain reaction (PCR). Antimicrobial susceptibility tests were done using disk diffusion method and the presence of relevant antibiotic resistance genes were determined by PCR. Out of 80 stool samples screened, only 42 (52.5%) were positive for presumptive *Campylobacter* spp. Thirty-seven (88%) of the presumptive isolates were confirmed to be *C. jejuni* while the remainder could not be delineated. A total of 12 isolates out of 37 presumptive *Campylobacter* which were confirmed *C. jejuni* (32.4%), were found to possess the *fli*M gene, 9 (24.3%) possessed the *flh*A gene and only 6 (16.2%) possessed *flg*E2. None were found to possess the *fla*A, *fla*b and *flh*B genes. Resistance to antimicrobials such as ampicillin (94.6%), chloramphenicol (91.6%), T.S (78.4%), erythromycin (49.6%) and gentamycin (56.4%) was found to be very high among the presumptive *Campylobacter* of confirmed *C. jejuni* obtained in this study. However, a lesser resistance to quinolone was also noted. The *Cat*A resistance gene was noted in 98% of presumptive *Campylobacter* of confirmed *C. jejuni* which were resistant to chloramphenicol. In conclusion, the findings of this study indicate that *Campylobacter jejuni* are prevalent among the diarrhoeal patients in the study community and a significantly high antimicrobial resistance to commonly used antibiotics for the treatment of Campylobacter disease was observed.

4.2 Introduction

Gastroenteritis related to *Campylobacter* spp. appears to be a significantly relevant digestive disease in humans. *C. jejuni* in particular has been noted to be a frequently identified bacterial pathogen of enteric infections worldwide. In 2009 over 190 000 cases of campylobacteriosis were confirmed in Europe (Wagenaar *et al.,* 2013). Results of a study conducted in Spain showed a high prevalence of *Campylobacter* spp. among children under five years (Bellido-Blasco *et al.,* 2006). In Thailand it was found that *Campylobacter* spp is one of the leading

bacterial agents to cause diarrhoea (Worada *et al.,* 2015). *Campylobacter* spp. is known to be associated with animal and human diseases (Uaboi-Egbenni, *et al.,* 2008). In Africa, diverse investigations have shown that campylobacteriosis is predominantly detected among children of a young age. For example in Nigeria, *C. jejuni* was found to be a relevant diarrhoeal agent through research conducted in Osogbo (Aboderin *et al.,* 2002). Various studies in Ethiopia have similarly noted that *Campylobacter* spp. is a significant bacterial enteropathogen responsible for diarrhoeal diseases both in both adults and children (Asrat *et al.,* 1999). In Venda, South Africa, *Campylobacter* spp. was isolated from 20% of diarrhoeal stool samples of HIV-positive patients (Obi and Bessong, 2002) and a study conducted in the Vhembe district of South Africa showed a high prevalence of *Campylobacter* spp. from the isolation of diarrhoeal specimens (Samie *et al.,* 2007).

Epidemiologically, campylobacter-related gastroenteritis is regarded as a food-borne disease commonly transmitted through the consumption of a variety of food and from drinking or the usage of contaminated water and its resources. Clinically, campylobacter infection may begin with asymptomatic presentation but later results in certain features such as watery and bloody diarrhoea. There is also a variation in the clinical presentations of the disease caused by *Campylobacter* spp. with regards to geographical location and age (Fedoroff *et al.,* 2010). Over 80% of cases are caused by *C. jejuni* while about 10% of cases are caused by *C. coli* (Mak-im *et al.,* 2012). Although the mechanism of pathogenicity of campylobacteriosis has not been fully described, reports from different investigations have found that *C. jejuni* and *C. coli* isolates from meat products expressed a series of adherence and invasive abilities to human GIT epithelial surfaces (O'Hara *et al*., 2012). Whether *Campylobacter* produces fimbriae that aids in adhering to human gut membrane layer and also in colonizing the GIT surface membrane still remains unresolved, and even with their paramount role as human pathogens, there is a minimal knowledge about their pathogenic mechanism. Various

verification studies such as hemagglutination assays, transmission electron microscopy, adherence assays and PCR have however assisted in determining the expression of fimbriae and their possible significance in the pathogenesis of campylobacteriosis (Dasti *et al.,* 2010).

The main origin of *Campylobacter* spp. infections in humans is considered to be improper handling and/or consumption of contaminated poultry meat or dairy products, as well as direct contact with some livestock and pet animals (CDCP, 2010). The principal objective of the global food hygiene and safety agencies should aim to control *Campylobacter* spp. along the food chain, as an infection contacted from food and food products is regarded globally as a public health burden (CDCP, 2010). Unfortunately, products from animals, especially raw milk which is routinely consumed by humans, are reservoirs for various pathogens such as *Escherichia coli, Mycobacterium bovis, Listeria monocytogenes, Campylobacter, Brucella* and *Salmonella* (Kishor and Gabhane, 2012; Leedom *et al.,* 2006). Of these listed pathogens, *Campylobacters* spp. remains the most common cause of zoonotic infectious diseases affecting all age groups in many regions of the world. It can also cause complication in people with underlying pathological conditions as well as in immunosuppressed individuals (Salim *et al.,* 2014). Beside the mild clinical characteristics of campylobacteriosis such as acute gastroenteritis, fever and abdominal cramps which last for a few days, there are other more deadly complications of this diseases which include septicaemia, Guillain–Barré syndrome, reactive arthritis, and even abortion (Godschalk, *et al.,* 2007: Kopyta and Wardak, 2008). Although such infections are generally self-limiting, a small proportion of cases may require medical intervention such as fluid therapy and even antibiotics such as erythromycin. Individuals with prolonged or chronic diarrhoea episodes or the immunosuppressed may require long-term antibiotic therapy (McGill *et al.,* 2006). Campylobacter-associated infections are becoming difficult to curtail due to the evolution of multidrug-resistant strains of the bacteria. In this study, we report on the incidence and antibiogram profile of

Campylobacter spp. in stool samples from diarrhoeal patients in selected hospital in the Eastern Cape Province, South Africa.

4.3 Methodology

4.3.1 Ethics and informed consent

Ethical clearance was granted by the University of Fort Hare research ethics committee for study (ref no: OKO011SOMO01) and permission to collect samples was obtained from the Eastern Cape Department of Health (ref no: EC-20146RP10-487), while informed consent was obtained from patients before collecting samples from them. Strict confidentiality was maintained.

4.3.2 Sampling

Diarrhoeal stool samples were collected from patients attending or admitted to both private and public medical facilities in the Amatole District Municipality. Samples were obtained directly from the diarrhoeal stools of patients who had just passed a watery stool and from the ano-rectal cavity of some patients who were still passing watery stools but not at the time of sample collection, especially the outpatients using sterile swab sticks. The samples were taken from different age groups irrespective of sex and race. After collection, the samples were put inside the swab stick case, tightly cork-screwed and transported in cooler boxes to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the university of Fort Hare, Alice for analysis as soon as possible.

4.3.3 Bacteriological analyses

4.3.3.1 Isolation of *Campylobacter* spp.

The bacteriological analyses of the samples were performed using standard methods: stool samples collected were streaked directly onto Campylobacter selective agar [(Charcoal-based) manufactured by Lab M Limited United Kingdom] and the inoculated plates were simultaneously incubated in a microaerophilic environment using candle jars at 37ºC for 48 h. The presumptive isolates, (2-3 per plate) which were whitish, creamy, gelatinous and mucoid colonies, were picked with sterile wire loop, into Tryptone Soya Broth (TSB) for 24 h at 37ºC, then stored in 20% glycerol at -80ºC for further analysis.

4.3.3.2 DNA extraction for *Campylobacter* spp.

This was done following the description of Leblanc-Maridor *et al.,* (2011). The glycerol stock was resuscitated in TSB for 18 h at 37ºC. About 2 mL of TSB culture was centrifuged at 13000 rpm for 5 min. After centrifugation, the supernatants were discarded and the cell pellets resuspended in 200 µL of nuclease free water by vortexing in sterile microcentrifuge tubes. The suspended cells were subjected to heating in a heating block at 100ºC for 15 min

and allowed to cool and then centrifuged at 10,000 rpm for 3 min. The supernatant containing the DNA was then decanted into DNase free microcentrifuge tubes and stored at -20ºC for further analysis.

4.3.3.3 Molecular confirmation of *Campylobacter* spp.

The isolates were confirmed as *Campylobacter* spp., using *CamJ* and *CamC* primer pairs that delineate *C. jejuni* and *C. coli* respectively, in a reaction mixture containing 1 µl of specific primer pairs (Table 4.1), 12.5 µl of M/M, 5.5 µl of nuclease free water and 5.0 µl of DNA template. The cycling conditions were as follows: 94 ºC initial denaturation for 5 min, followed by 35 cycles of 94ºC for 60 sec, 52ºC (annealing temperature for both primers) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min. PCR product was verified in a 1.5% agarose electrophoresis stained with Ethidium bromide, visualized in a transilluminator and photographed.

Table 4.1 Primer sequences for molecular confirmation of *Campylobacter* spp.

Target spp/Genes	Sequence(5'-3')	Amplicon size (bp)	Reference
C. jejuni (*CamJ*)	CATCTTCCCTAGTCAAGCCT AAGATACTCTAGCAAGATGG	735	Vaishnavi 2015
C. coli (*CamC*)	AGGCAAGGGAGCCTTTAATC TATCCCTATCTACAATTCGC	500	Vaishnavi 2015

Source: Vaishnavi 2015.

4.3.3.4 Delineation of virulence genes among *Campylobacter* isolates

Delineation of the confirmed *Campylobacter jejuni* isolates into their respective pathotypes was done using PCR and primers targeting the relevant virulence gene for the *flaA*, *flaB3, flhB-q, flgE2-q, Cjfli*M and *Cinv*A2 as listed in Table 4.2. The reaction mixture contained 1 uL of specific primer pairs, 12.5 µL of M/M, 5.5 µL of nuclease free water and 5.0 µLof DNA template. The cycling conditions were as follow: 94ºC initial denaturation for 5min, followed by 35 cycles of 94ºC in 60 sec, 50ºC, 52ºC, 58ºC, 54ºC, 59ºC and 54ºC (annealing temperature for *flaA, flaB3, flhB-q, flgE2-q, CjfliM* and *CinvA2* primers respectively) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min. PCR products were verified in a 1.5% agarose electrophoresis stained with Ethidium bromide, visualized in a transilluminator and photographed.

Table 4.2: Primer sequence for detection of virulence genes among *Campylobacter* spp in this study

Target gene	Primers	Sequence (5′-3′)	Annealing temp (□)	Amplicon size (bp)	Reference
FlaA	*FlaA1*	ATGGGATTTCGTATTAACAC	50	1713	Wassenaar and Newell 2000
	FlaA2	CTGTAGTAATCTTAAAACATTTTG			
Flab	*FlaB3F*	ATAAACACAACATCGGTGCA	52	1670	Smith *et*

	FlaB3R	GTTACGTTGACTCATAGCATA			*al.,* 1999
FlhA	*CinVA2F*	GGAAGCGGCACTTGGTTTGC	54	735	Carrillo *et*
	CinVA2R	GCTGTGAGTGAGATTATAGCAGC			*al* 2004
FlhB	*FlhB-q-F*	GCACGATTTACCAAAGCTGTTTCAA	58	101	Konkel *et*
	FlhB-q-R	CACTGGTGCTTTAGCGGGTAGA			*al* 2004
FliM	*CjfliM1*	TCATCCTCCTCTTCAGGCTC	59	1011	Konkel *et*
	CjfliM2	CACCGACACACCCATAGCCTC			*al* 2004
FlgE2	*FlqE2-q-F*	CATCTCACCAGCACCTCCTGTTC	54	132	Konkel *et*
	FlqE2-q-R	GCAAAAATCGCAATGGCTTCA			*al* 2004

Source: Konkel 2004.

4.3.3.5 Antibiotic susceptibility profile

The antibiotic susceptibility profile of diarrhoeal pathogens detected was determined according to the Clinical and Laboratory Standard Institute (CLSI, 2015) guidelines using Mueller-Hinton agar as a growth medium. The positive samples from glycerol stock were resuscitated in TSB and incubated at 37ºC for 24 h. The TSB culture was adjusted to 0.5 MacFarlan standard, and evenly inoculated onto Mueller-Hinton agar with sterile swab sticks, allowed to dry and antibiotic discs were dispensed using an antibiotic disc dispenser. The following antibiotic disks were tested against the isolates: ampicillin (25 µg), cefotaxime (30 µg), chloramphenicol (30 µg), cefuroxime (30 µg), norfloxacin (10 µg), trimethoprim-sulfamethoxazole (25 µg), imipenem (10 µg), erythromycin (15 µg), gentamicin (10 µg), tetracycline (30 µg), ciprofloxacin (5 µg) and doxycycline (30 µg). Then the plates were incubated at 37ºC for 24 h after which they were read for sensitivity according to CLSI 2015 guidelines.

4.3.3.6 Screening for antimicrobial resistance genes

Antimicrobial resistance genes among the isolates were assessed based on the observed phenotypic resistance patterns using PCR with specific primers targeting relevant resistance genes as presented in Table 4.3. PCR was performed in a reaction mixture containing 1 uL of

specific primer pairs, 12.5 µl of M/M, 5.5 µl of nuclease free water and 5.0 µl of DNA template. The cycling conditions were as follows: 94ºC initial denaturation for 5min, followed by 35 cycles of 94ºC in 60 sec, 53^{o}C (annealing temperature for *Cat*A1) for 30 sec, 72ºC (extension) for 60 sec and a final elongation step at 72ºC for 5 min. PCR product was verified in a 1.5% agarose electrophoresis stained with Ethidium bromide, visualized in a transilluminator and photographed.

Table 4.3: Primer sequence and amplicon sizes in determining the antimicrobial resistance gene in this study

Antimicrobial agent	Resistance gene	Sequence (5'-3')	Size (bp)	Annealing temp (^{o}C)	Reference
Chloramphenicol	*catA1*	AGTTGCTCAATGTACCTATAACC TTGTAATTCATTAAGCATTCTGCC	547	53	van Hoek,et al 2011

Source: van Hoek, *et al.,* 2011.

4.4 Results

4.4.1 Isolation and confirmation of *Campylobacter* spp

A total of 42 presumptive isolates of *Campylobacter* spp. were obtained from diarrhoeal stool samples collected from both outpatients and inpatients attending or admitted to selected private and public hospitals in the Amathole District Municipality in Eastern Cape.

Of these 42 presumptive isolates, 37 were confirmed as *C. jejuni*, representing 88% of the total presumptive isolates, and none were detected as *C. coli.*

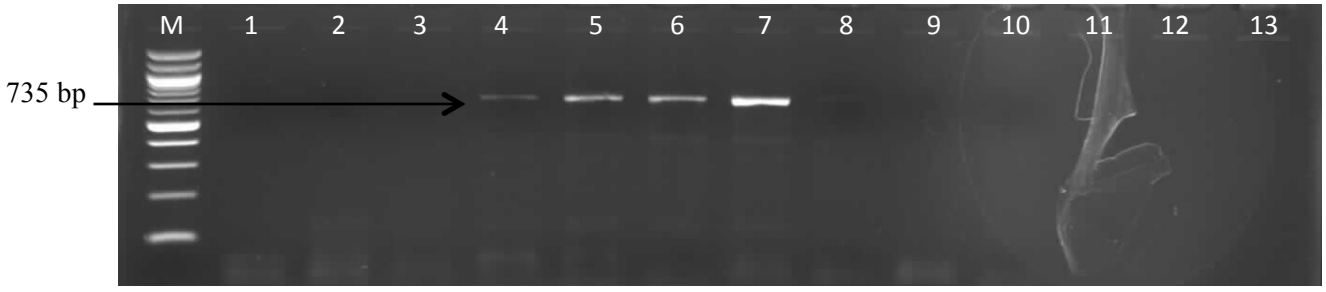

Figure 4.1: Gel picture of representative *C. jejuni*. showing amplified *Cam*J (735 bp) genes: Lane M: 100 bp Molecular weight marker; lane 1 to 13 *Campylobacter* isolates.

4.4.2 Pathotyping of the isolates

Table 4.4 shows the results of the detected virulence genes among the confirmed *Campylobacter jejuni*. The prevalence of the *Campylobacter* pathotypes were 32.4% found to possess the *fli*M gene, 24.3% possessed the *flh*A gene and only 16.2% possessed the *flg*E2. None were found to possess the *fla*A, *fla*b and *flh*B genes.

Table 4.4. The prevalence of detected *Campylobacter* spp. pathotypes

Target genes	Primers	Sequence (5'-3')	Outcome (number)	Outcome (%)
FliM	*CjfliM1*	TCATCCTCCTCTTCAGGCTC	12	32.4
	CjfliM2	CACCGACACACCCATAGCCTC		
FlhA	*CinvA2F*	GGAAGCGGCACTTGGTTTGC	9	24.3
	CinvA2R	GCTGTGAGTGAGATTATAGCAGC		
flgE2	*FlgE2-q-F*	CATCTCACCACGACCTCCTGTTC	6	16.2
	FlqE2-q-R	GCAAAAATCGCAATGGCTTCA		
FlaA	*FlaA1*	ATGGGATTTCGTATTAACAC	0	0
	FlaA2	CTGACGTTGACTCATAGCATA		
Flab	*FlaB3F*	ATAAACACAACATCG	0	0
	FlaB3R	GTGCA		
		GTTACGTTGACTCATAGCATA		
FlhB	*FlhB-q-F*	CAGGTGCGGATGTGGTGATC	0	0
	FlhB-q-R	CACTCCTTTGGCAACAACCCT		

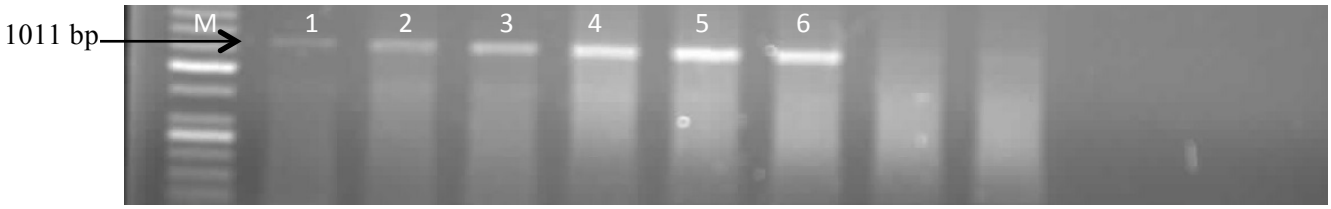

Fig. 4.2: Gel picture of the representative of *Campylobacter* pathotype showing amplified *fli*M (1011 bp) gene. Lane M: 100 bp Molecular weight marker; lane 1 to 6; *Campylobacter* isolates.

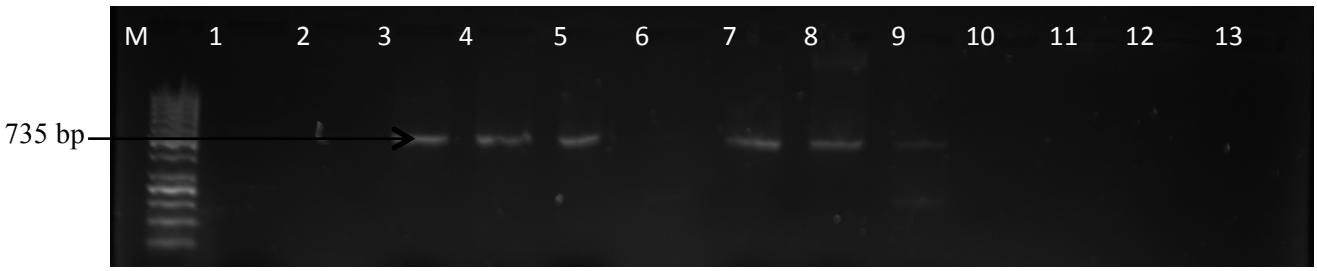

Fig. 4.3: Gel picture of the representative of *Campylobacter* pathotype showing amplified *Cinv*A2 (735 bp) gene. Lane M: 100 bp Molecular weight marker; lane 1 to 13; *Campylobacter* isolates.

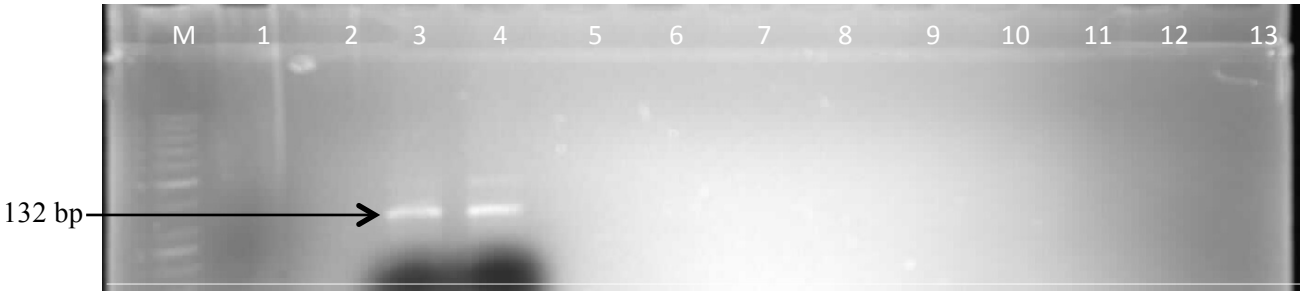

Fig. 4.4: Gel picture of the representative of Campylobacter pathotype showing amplified *flq*E2 (132 bp) gene. Lane M: 50 bp Molecular weight marker; lane 1 to 13; *Campylobacter* isolates.

4.4.3 Antibiogram profiles of the confirmed *Campylobacter* isolates

The results of the antibiotic susceptibility profiles of all the confirmed *Campylobacter jejuni* isolates are summarized in Table 4.5. There was a high resistance to commonly used antibiotics such as ampicillin (94.6%), chloramphenicol (91.9%) and co-trimazole (78.4%), but less resistance to tetracycline (25.2%), ciprofloxacin (20.3%), nofloxacin (19.0%) and imipenem (10.8%).

Table 4.5: List of antibiotics and antibiotic resistance prevalence of *Campylobacter*spp. in this study

Antibiotics	Disk size (µg)	Sensitivity (n)	Sensitivity (%)	Resistance (%)

Ampicillin	25	2	5.4	94.6
Cefotaxime	30	10	60.0	40.0
Cefuroxime	30	14	37.8	62.2
Chloramphenicol	30	3	8.1	91.9
Trimethoprim-sulfamethoxazole	1.25/23.75	8	21.6	78.4
Imipenem	10	33	89.2	10.8
Erythromycin	15	2	50.4	49.6
Gentamycin	10	16	43.2	56.8
Tetracycline	30	4	74.8	25.2
Ciprofloxacin	5	10	79.7	20.3
Doxycycline	30	4	43.8	56.2
Norfloxacin	10	27	81.0	19.0

Results of the screening for the antibiotic resistance genes revealed genes encoding resistance against chloramphenicol that were positive for the gene *cat*A1. The results showed that 98% of *Campylobacter jejuni* harboured *cat*A1.

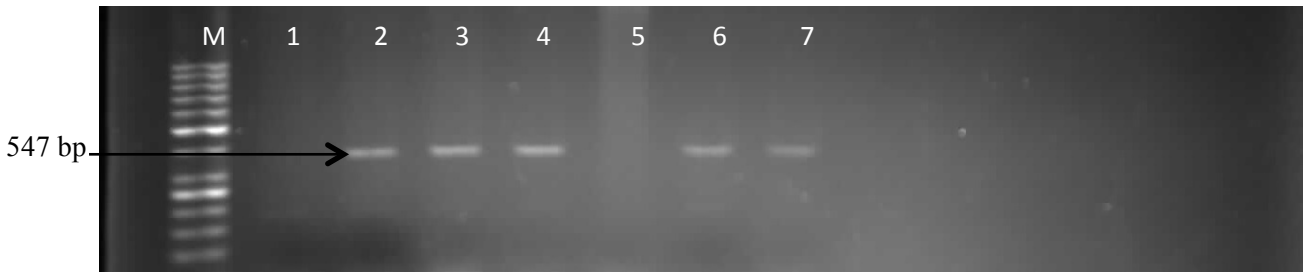

Figure 4.5: Gel picture of the representative *Campylobacter jejuni* that haboured *cat* A1 resistance gene. Lane M: 100 bp Molecular weight marker; lane 1 negative control, then 2 to 7 *Campylobacter* spp. isolates.

4.5 Discussion

In this study, the prevalence of *Campylobacter* spp. as a causative agent for diarrhoeal disease was determined. Isolation, confirmation and pathotyping was performed and the

antibiogram pattern of *Campylobacter* spp isolated from the hospital patients was investigated. Studies on the etiology of diarrhoea reveal that bacteria are the etiologic agents in about 20% to 60% of total diarrhoea incidences (Nweze, 2010). Although viral agents are found to be the most prevalent causative agents of acute diarrhoea in children in industrialized parts of the world, bacteria are the most common pathogens for diarrhoeal diseases in developing countries (Alikhani *et al.,* 2011). In this study, out of 80 samples 42 (52.5%) were isolated to be *Campylobacter* spp. based on the morphological appearance of the colonies, although only 37 were confirmed using molecular technique, (46.3% of the total sample size). This corroborates previous studies which showed that *Campylobacter* spp. is still a major cause of diarrhoeal disease. In many studies, especially in developing countries, *Campylobacter* spp. is one of the major etiologic agents of diarrhoea (Farthing *et al.,* 2013). In Africa, several studies have indicated that *Campylobacter* spp. diarrhoea is most common in young children (Adekunle *et al.,* 2009). In Durban, South Africa, *Campylobacter* spp. was found in 21% of diarrhoeal cases among children under five years and in Venda, South Africa, *Campylobacter* spp. was isolated in 20% of stool samples tested from HIV-positive individuals (Obi *et al.,* 2002). *Campylobacter* spp., especially *C. jejuni*, has been recognized as a major food borne pathogen resulting in diarrhoeal illness. In this study 88% of the total isolates were found to be *C. jejuni*. This is similar to the results obtained from a study in the Vhembe district of South Africa in which 85% of the isolates were found to be *C. jejuni* (Samie *et al.,* 2007) and also corroborates a study conducted in Italy in which no *C. coli* was isolated from diarrhoeal stools analysed (Suzuki *et al.,* 2009). Previous studies have shown high frequency in isolation of *C. jejuni* (Worada *et al.,* 2015). The prevalence of isolation of *C. jejuni* in various parts of the world varies due to the diverse standards of living conditions, water supply and eating habits. Among the studies carried out in Europe, a 9.5% isolation rate was reported from France (Bessede *et al.,* 2011) and 6.7% from UK (Linton *et al.,* 2007)

while studies from Africa showed a 9% isolation rate from Central Africa (Rizal *et al.,* 2010), 44% from South Africa (Bryner *et al.,* 2009), 18% in various reports from India (Ali *et al.,* 2003) and 43% in Thailand (Worada *et al.,* 2015), while this study reports a prevalence of 46.3% which appears to be very high compared to previous findings from anywhere in South Africa. In 2010 the European Centre for Disease Prevention and Control (EFSA) reported that campylobacteriosis is still the most common zoonotic infection in the European Union (EU) (Perko-Makela *et al.,* 2011). Previous studies in Bangladesh conducted in 2013 showed that *C. jejuni* is typically associated with predominantly watery diarrhoea and isolated in frequencies varying from 17 to 26% (Mukherjee *et al.,* 2013).

In humans the most common cause of campylobacter enteritis is *C. jejuni*, which commonly colonizes the intestinal tract of chickens (Hermans *et al.,* 2011). Alhough the pathogenic mechanism of *Campylobacter* spp is not fully understood various studies have shown that motility plays a significant role in *Campylobacter* spp. pathogenicity, as flagella aids adhesion and colonisation in the human gut. The pathogenic ability of *C. jejuni* to cause disease is a complex, multi-factorial process. Potential virulence determinants include toxin (Asakura *et al.,* 2008), adherence factors (adhesins) (Konkel *et al.,* 2013), and entry-promoting molecules (invasins) (Yamasaki *et al*., 2006). Also of interest are strain-variable genes, which encode factors involved in iron acquisition, DNA restriction/modification, sialylation, flagellar biosynthesis, LOS biosynthesis, and capsular biosynthesis (Parkhill *et al.,* 2000). Investigators have proposed that strain-variable genes encode factors that contribute to different disease presentations and enable the organism to survive in unique ecological niches. Regardless, an established set of *C. jejuni* virulence genes and a determination of their contribution to disease production have yet to emerge. Dorrell *et al.* (2001) also noted that many of the virulence genes identified to date are conserved among *C. jejuni* strains. In this study genes encoding flagellar systems were detected. The results show

that 32.4% of the isolate harboured the *fli*M gene, 24.3% harboured *flh*A and 16.2% possessed *flg*E2 genes of the flagella system. However, *fla*A, *fla*b and *flh*B were not detected in this study. Various studies conducted in different parts of the world have shown that *Campylobacter* spp possesses certain genes for flagellation which enhance adhesion and colonisation, thereby enhancing the virulence of the organism. Specific properties involved in adhesion, colonization, invasion, and toxin production appear necessary to the process of infection. In a study conducted in the USA the *fla*A gene together with others such as *cad*F, *rac*R and *dna*J were 100% present in isolates analysed (Dorell *et al.,* 2011). In a study conducted to detect the virulence pattern of *Campylobacter* spp using 26 genes (*clp*B, *cme*A, *fla*A, *fla*B, *fla*C, *flg*B, *flg*C, *flg*D, *flg*E, *flg*G, *flg*H, *flg*K, *fli*F, *fli*G, *fli*H, *fli*I, *fli*M, *fli*Y, *flh*A, *flh*B, *met*A, *rpo*N, *Cj*1514, *Cj*0667, *Cj*0668, *and Cj*0669*),* it was found that the genes of flagellar systems were expressed higher than other genes (Carillo *et al.,* 2004). This shows that mobility plays a significant role in pathogenicity of *Campylobacter* spp.

In this investigation, the antimicrobial susceptibility profile of *Campylobacter* spp. showed a high resistance to antibiotics which are commonly used to treat diarrhoea caused by *Campylobacter* spp. High resistance among the isolates to ampicillin (94.6%), chloramphenicol (91.9%), cefuroxime (62.2%) and co-trimoxazole (78.4%), were detected, while a lower resistance to ciprofloxacin (20.3%), tetracycline (25.2%), norfloxacin (27%) and imipenem (10.8%) was observed. Series of studies have shown that the drugs of choice for the treatment of human *Campylobacter* infections is erythromycin (a member of the macrolide family), followed by ciprofloxacin (fluoroquinolone family) and tetracycline (Nachamkin *et al.,* 2002), however, a dramatic increase in *C. jejuni* resistance to fluoroquinolones has been observed in the USA since the mid-1990s (Nachamkin *et al.,* 2002). Initially, during the period of 1982–1992, no isolates of *C. jejuni* showed resistance to fluoroquinolone, and only 2% were found to be resistant to erythromycin among 142 patients

(Nachamkin *et al.,* 2008). By 2001, resistance to fluoroquinolone had developed and was already widespread (40.5% among 47 *C. jejuni* isolates). This increase in resistance to fluoroquinolone in human *C. jejuni* isolates has been linked to the licensing of fluoroquinolone use in poultry production (Cardinale, *et al.,* 2006). Enrofloxacin, a related fluoroquinolone, was licensed in the early 90s in Europe and Asia, and soon after a high incidence of resistant *C. jejuni* isolates was reported from animal sources, for example from Spain (99% *C. jejuni* isolated from broiler farms), while 72% of human isolates were found to show resistance in the same period (Saenz *et al.,* 2000). A similar trend was observed in Taiwan, where 92% of *C. jejuni* isolated from chickens and 52% of human isolates were found to be resistant to fluoroquinolones by 1998–2008 (Liao *et al.,* 2012). In this study, resistance to fluoroquinolones was lower. This is similar to the results obtained from a study conducted in the Vhembe district in South Africa in which resistance to ciprofloxacin was 13% and 27% for tetracycline (Samie *et al.,* 2007). Taking into account the various groups of antibiotics, a high resistance of *Campylobacter* spp. to macrolides, fluoroquinolones, beta-lactam, trimethoprim and sulphonamides has been reported in several studies, while resistance to other antimicrobials agents including tetracycline, aminoglycoside and chloramphenicol is generally low (Aarestrup and Engberg, 2001; Yan *et al.,* 2005). In this study resistance to gentamicin (56.8%) and chloramphenicol (91.9%) were at a high level, and the isolates that were resistant to chloramphenicol were found to possess the *cat*A1 resistant gene.

4.6 Conclusion

From the results of this study, the prevalence of *Campylobacter* spp. is high and this highlights the fact that *Campylobacter* spp. is a significant agent of infectious diarrhoea and also a leading cause of gastroenteritis relating to food in the world (Ripabelli *et al.,* 2010). Isolation of *Campylobacter* spp. in various parts of the world varies due to varying standards

of living conditions, water supply and eating habits. Although other epidemiological factors that increase the risk of diarrhoea were not investigated in this study, they cannot be ignored. Among the studies carried out in Europe, a 9.5% isolation rate was reported from France (WHO, 2012) and in 44% in South Africa (WHO, 2012). In the current study, 46.3% of the diarrhoeal stool samples collected was confirmed to be *Campylobacter* spp. Results of studies in Kenya and Spain have indicated a higher prevalence of *Campylobacter* infection among children under five years (Brooks *et al.,* 2006; Bellindo-Blasco *et al.,* 2006). Recently, a study in Thailand found a high prevalence of *Campylobacter* spp. (Worada *et al.,* 2015). Although the pathogenic mechanism of *Campylobacter* spp. infection is still not clear various studies have shown that the possession of flagellar systems could assist in adhesion and invasion which in turn aid pathogenicity (Konkel *et al.,* 2001). Our results show that 32.4% of the isolates harboured the *fli*M gene, 24.3% harboured *flh*A and 16.2% possessed *flg*E2 genes of flagella system. Previous studies have proved that both *C. jejuni* and *C. coli* can harbour the genes of the flagellar secretion. The binding and colonization of human intestinal epithelial membranes by *Campylobacter* are carried out by these virulence factors (Konkel *et al.,* 2001). The major therapeutic intervention for all severe diarrhoea caused by *Campylobacter* is the use of antibiotics, but continued resistance to the drugs of choice such as macrolide and fluoroquinolones has been reported around the world. In this study, 49.6% resistance to erythromycin was noted and 20.8% to ciprofloxacin. It can be concluded that the resistance to drugs of choice is high.

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CHAPTER FIVE

General Conclusion and Recommendation

5.1 General Conclusion

The scourge of acute infectious diarrhoeal diseases is globally alarming as it has been described as one of the foremost causes of morbidity and mortality (WHO, 2014). In 2011, diarrhoea accounted for 700,000 deaths in children under five years of age worldwide, making it the second leading cause of child mortality (Bhutta *et al.,* 2013). The highest rates of death have been recorded in sub-Saharan Africa and South Asia. An estimated 1.7 billion episodes of diarrhoea, accounting for about 2.9 episodes per child per year, created health system costs of about 7 billion US dollars (Hutton *et al.,* 2004). Although this study was conducted only to isolate and characterize bacterial (*E. coli* and *Campylobacter* spp.) agents

as causes of diarrhoeal disease, epidemiological and environmental studies have shown that the majority of diarrhoeal diseases can be prevented by implementing water, sanitation and hygiene programmes, which all interrupt faecal-oral transmission pathways, commonly referred to as five "F (fluids, fields, flies, fingers and food) (WHO, 2014). This study corroborates previous studies which showed that infectious diarrhoea is one of the most important causes of morbidity and mortality worldwide (Usein *et al.,* 2009). A variety of microorganisms including bacteria, viruses and parasites can be associated with severe acute infectious diarrhoea (Daniel *et al.,* 2016) with bacterial pathogens like *E. coli* and *Campylobacter* spp. as the most commonly implicated agent (Hiedary *et al.,* 2014). DEC has been identified as one of commensally pathogenic bacteria, which cause diarrhoea (Wilson *et al.,* 2006). Many epidemiological studies have been conducted in order to document the prevalence of DEC in different countries such as in Bangladesh 40% (Albert *et al.,* 2005), Jordan 34% (Shehabi *et al.,* 2003) and in Tehran 54% (Jafari *et al.,* 2009). In this study, DEC was confirmed in 81.5% of the presumptive isolates which shows that DEC is a leading cause of infectious diarrhoea.

Campylobacter spp. is a significant bacterial agent of diarrhea both in industrialized and developing countries (Man, 2011). In this study, *Campylobacter* spp. was isolated from the diarrhoea stool samples collected in the study area and subsequently characterized. 46.3% of the total samples was confirmed to be *Campylobacter* spp. The result shows that the prevalence of *Campylobacter* spp. is high in the study area. This result is similar to that obtained from studies conducted in Barbados that indicated the highest rate of isolation of *Campylobacter* spp. (40.8%) (Workman *et al.,* 2006) and in Thailand, 43% (Worada *et al.,* 2015)

In this study, the worrisome issue is that of antimicrobial resistance which was observed in both organisms analysed in this study. Resistance to commonly used antibiotics in treating

DEC infections such as conventional Beta-lactam (ampicillin 98.1%), chloramphenicol (94.3%) and tetracycline (90.6%) was observed, as has been reported in a study in Vietnam in which DEC strains exhibited different levels of resistance to ampicillin (86.4%), chloramphenicol (77.2%) (Nguyen *et al.,* 2005). Resistance to drugs of choice (macrolide and flouroquinolone) in treating diarrhoea caused by *Campylobacter* spp. was also observed and found to be high. In this study 49.6% resistance to erythromycin and 20.8% to ciprofloxacin were observed. The results of this study show that bacterial agents are still in the forefront of pathogenic causes of diarrhoea and the prevalence is high. It is therefore important that control programmes targeting the elimination of infections due to bacterial agents should be instituted considering the burden to those most vulnerable, especially children in the study area.

In addition, increasing resistance to antibiotics constitutes a higher risk of treatment failure and underscores the importance of antibiotics monitoring, prudent usage of currently available antibiotics and the urgent need for development of more alternative strategies to treat bacterial diarrhoeal diseases. Plans must also be initiated to stop the increasing prevalence rate of multi-drug resistant pathogens by avoiding the indiscrimate use of antibiotics.

5.2 Recommendation

From this study however, lower resistance was observed in both *E. coli* and *Campylobacter* spp. to imipenem and norfloxacin which serves as a recommendation to the practitioners as best antibiotics of choice in treating diarrhea caused by these pathogens.

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