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Incidence and antibiogram fingerprints of members of the Enterobacteriaceae family recovered from river water, hospital effluents and vegetables in Chris Hani and Amathole District Municipalities in the Eastern Cape Province

A thesis submitted in fulfilment of the requirements for the award of the degree of

Master of Science (MSc) in Microbiology

By

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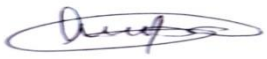
DECLARATION

I, **Lindelwa Mpaka**, declare that this thesis titled "Incidence and antibiogram fingerprints of four members of the Enterobacteriaceae family recovered from river water, hospital effluents and vegetables in Chris Hani and Amathole District Municipalities in the Eastern Cape Province" submitted to the University of Fort Hare for the degree of Master of Science in Microbiology, in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, work presented on this thesis is my original work. Where there are contributions of other researchers involved, I made every effort to specify that very clear with exemption to the citations and this thesis has none of the material submitted in the past to any other Institutein whole or in part, for the award of any other academic degree or diploma.

Signature:... 

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DECLARATION ON PLAGIARISM

I, **Lindelwa Mpaka**, student number: 201306728 hereby declare that I am completely conscious of the University of Fort Hare's policy on plagiarism and henceforth I declare that this thesis does not have any plagiarised research outputs, if there are any detected I shall be held responsible.

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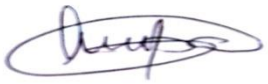

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DEDICATION

This work is dedicated to my dearest mother Babalwa Mpaka who has been the best mother I could ever ask for and she has always been the shoulder for me to cry on every time when there is something going wrong with my research. I would also like to dedicate this work to myself.

Contents

LIST OF ACRONYMS AND ABBREVIATIONS

ABR- Antibiotic resistance

ACMSF- Advisory Committee on the Microbiological Safety of Food

AIT- Austrian Institute of Technology

AMR- Antimicrobial resistance

BRICS- Acronym for the association of the main emerging five national economies which are Brazil, Russia, India, China and South Africa

CDC- Centres for Disease Control and Prevention

DDD- Daily dose per day

DM- District Municipality

DWAF- Department of Water Affairs and Forestry

EFSA- European Food Safety Authority

ESBL- Extended spectrum beta-lactamase

EUCAST- European Committee on Antimicrobial Susceptibility Testing

FAO- Food and Agriculture Organization

FDA- Food and Drug Administration

GPs- General practitioners

HAI- Hospital acquired infection

IDSA- Infectious Diseases Society of America

MRSA- Methicillin-resistant *Staphylococcus aureus*

OECD- Organisation for Economic Co-operation and Development

OIE- World Organisation for Animal Health

PBP- Penicillin-binding protein

U.S. EPA- United States Environmental Protection Agency

WHO- World Health Organisation

WRC- Water Research Commission

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The worldwide problem of antimicrobial resistance has limited the spectrum of the current affordable and effective antimicrobials. Infections associated with resistant microorganisms impose a major threat to public health and economic stability. Globally, about 700 000 deaths every year can be accredited to antimicrobial resistance. The leading mechanism of resistance amid bacterial pathogens is the extended spectrum beta-lactamases production, which inhibits spectrum activity of several antimicrobial agents. The rise in antimicrobial resistance has compelled an urgent need of developing means of combatting resistance issue amid disease-causing microbes. The main aim of this study is to evaluate the incidence and antibiogram fingerprints of Enterobacteriaceae recovered from hospital effluents, river water and vegetables in the Eastern Cape Province. A total of eighteen antibiotics from ten different antimicrobial classes were used to determine antibiogram profiles of the MALDI-TOF confirmed isolates. From the MALDI-TOF confirmed isolates, 60% of *Enterobacter* spp. and *E. coli* isolates displayed resistance against colistin, while *Citrobacter* spp. and *Klebsiella* spp. displayed 90% and 60% resistance against this antimicrobial respectively. These findings outline the need for the development of new antimicrobials. About 75.5% (25/33) of the presumptive *Enterobacter* spp. were confirmed by MALDI-TOF with 79.2% (19/24), 66.7% (2/3), 66.7% (4/6) been confirmed vegetables, hospital effluents and river water samples respectively. Likewise, about 77.8% (21/27) were confirmed as *Citrobacter* spp. of which 92.3% (12/13), 66.7% (2/3) and 63.6% (7/11) were from vegetables, hospital effluents and river water samples respectively. These results show that the selected vegetables were highly contaminated with resistant bacteria and thus unsafe to consume uncooked vegetable. Also river water was higly contaminated with resistant microbes, which also shows that these rivers are not fit to be used

as drinking water sources and recreational activities. Colistin is an antimicrobial used as a last resort of antibiotics because it exhibits broad-spectrum activity. However from the findings of the work at present, this is no longer the case. The spectrum of this antimicrobial is now reduced by Enterobacteriaceae members. To the best of my knowledge; relatively few resources have been provided to understanding, preventing, and controlling increasing antimicrobial resistance on global, national and local levels.

CHAPTER ONE

1.0 INTRODUCTION

Microorganisms develop ways to reduce or eliminate the effectiveness of medication used to treat the infections they cause and this mechanism is referred to as antimicrobial resistance occurs naturally and spread within organisms of the same species (WHO, 2016). The term antimicrobial resistance is the broad term that is used to cover the resistance of different microorganisms against antimicrobial agents. The resistance of viruses is called antiviral resistance; the resistance of parasites to antimicrobial agents is called antiparasitic resistance while resistance in fungi is called antifungal drug resistance. The production of the enzymes known as Extended Spectrum Beta-lactamases (ESBLs) by certain bacteria like *Escherichia coli* (*E. coli*) is an example of antibacterial resistance, which makes them to be resistant against the 2nd and 3rd generations cephalosporins, penicillins, monobactams and some flouroquinolones (Rupp and Fey, 2003). The principal leading cause of antimicrobial resistance and its transmission is reported to be the inappropriate and extensive usage of antimicrobial agents in human medicine, agricultural fields and veterinary medicine as well as therapeutic and non-therapeutic usage of antimicrobial agents in the production of livestock, lack or reduced hygiene and sanitation precautions, and lack of prevention and control of infection in hospital environments, and these end up affecting human health care and environment (water, soil and plants) (Aminov and Mackie, 2007; Aarestrup et al., 2008; APUA, 2008; Acar and Moulin, 2012). For example microorganisms can become resistant against an antibiotic during treatment of infection caused by the organisms if the patient does not complete the full dose of antimicrobials prescribed for treating the infection.

Increasing antimicrobial resistance has been reported in the faecal Enterobacteriaceae and because of this the family Enterobacteriaceae has become an important challenge in disease control (Pitout and Laupland, 2008; Suankratay et al., 2008; Wellington et al., 2013; Kassakian and Mermel, 2014). Common sources of faecal Enterobacteriaceae are animal as well as human faeces, agricultural labourers together with water (Slama et al., 2010). During food microbial quality analysis, the faecal Enterobacteriaceae family is used to identify the occurrence of faecal contamination in food. Faecal Enterobacteriaceae causes severe infections, and most significant members of faecal Enterobacteriaceae family are gradually becoming resistant against presently available antimicrobial agents (Paterson, 2006). Enterobacteriaceae in animals used for food production, meats, water, fresh produce, and environment could colonise human gut and cause severe infections in human beings (Walsh et al., 2011; Zheng et al., 2012; George et al., 2014). There are reports stating that faecal Enterobacteriaceae have an ability to contaminate food and donate to illness and food decay (Gundogan and Yakar, 2007; Haryani et al., 2007).

The Enterobacteriaceae members like *Salmonella* species, *Shigella* species, and pathogenic strains of *E. coli* accompanies fruits and vegetables (Brackett, 1999). These microorganisms may be found in fresh produce in agricultural environment, whereas others are associated with infected workers or contaminated water, applications of antimicrobials during cultivation, application of contaminated manures or contaminated water for irrigation, or some selection pressure occurring naturally (Levy, 1992). Because of high moisture in fresh produce, storage, temperature used during processing, absence of sterilization during processing, transport and retail display; these bacteria are favoured to grow in the fresh produce. Fresh produce have been reported to be among the groups of food that are extensively implicated as mediators that drives to enteric diseases (Beuchat, 2006), and raw meat and vegetables are the main potential carriers of large number of bacteria including faecal Enterobacteriaceae (Cooke et al., 1980).

Pollution of freshwater sources negatively impact crops as the water run through agricultural fields and freshwater is frequently used for irrigation and other processes in agriculture. The consumptions of contaminated crops and water cause exposure to pathogenic microbes such as enteric bacteria, virus and protozoa and these microorganisms have capability of causing severe human infections. According to Dekker et al. (2015), South Africa has high incidences of resistant *E. coli* in irrigation water, ground water, fresh produce and soil. Microbial qualities of some South African freshwater sources have been reported to be unsafe and poor for human consumption, especially in rural areas (Haley et al., 2009; Mugalura 2010; Onah 2010; Lobina and Akoth, 2015). According to Oluwatosin et al. (2011); irrigation water may be the primary source of fresh produce contamination throughout the world. Because of sewage irrigation system; children from communities around farms and children from families owning farms are reported to be the ones that are frequently susceptible to food and waterborne diseases caused by Enterobacteriaceae (Ait and Hassani, 1999; FDA/CFSAN, 2001). Amoah et al. (2006) conducted a research in Ghana evaluating spring onions, lettuce as well as cabbage that were grown using deprived quality irrigation water and observed that these vegetables were greatly contaminated with Enterobacteriaceae.

In food animals, antimicrobial agents play an important role as they are used for treatment and for non-therapeutic purposes. Because livestock production have been growing rapidly over the previous decade; the usage of antimicrobial agents has developed to be an essential part of production of livestock as they are used to promote growth so to produce high yields, to deter diseases amid animals and metaphylaxis (Van Boeckel et al., 2015). The application of antimicrobial agents in farms causes livestock to grow bigger, faster, and less expensive; this has been known since the late 1940s (Coates et al., 1951; Elliott, 2015). However; this phenomenon of using antimicrobial drugs for promoting growth in animal food-farming leads

to the discharge of wastes carrying antibiotic remains as well as antimicrobial resistant microorganisms into both terrestrial and marine environments (Silbergeld et al., 2008).

The dramatic increase of antimicrobial resistance has been reported throughout the world and has developed to be a global public health challenge (Levy and Marshall, 2004). However, great levels of antibiotic resistance are reported more in underdeveloped countries than in developed countries (Park et al., 2003). This might be due to several challenges that developing countries are contending which include the inappropriate use or abuse of antibiotics (Coburn et al., 2007), production of reduced quality drugs by unlicensed manufacturers (Popoff et al., 2004), and inadequate prescriptive information. Recent studies have shown that *Staphylococcus aureus* as well as *Streptococcus pneumonia* together with *Streptococcus pyogenes* also *Salmonella* species, *Mycobacterium tuberculosis* and *Vibrio cholerae* are resistant against standard antibiotic therapies and hence contribute greatly to transmittable diseases (Gandhi et al., 2006).

Paterson (2006); Ghafourian et al. (2014) reported that the main global health concern is the dramatic increase of ESBL resistance among Gram negative enteric bacteria. According to Paterson et al. (2004), microorganisms having proficiency of producing ESBL initiate infections which result in undesirable consequences such as deprived rates of clinical as well as microbiological responses to treatments, extended hospitalization, as well as sizable hospital expenditures. Gradually, food animals are recognised as potential reservoirs for ESBL-producing strains and through the food chain, these strains might be transmitted to other foods such as fresh produce and to freshwater sources. Faecal contamination in food animals might come about during the time of slaughtering of animal as well as sucking milk from the cow, or during handling and processing, and the contaminating microorganism maybe favoured to growth during shipping and storage of the products (Gundogan and Avci, 2013).

1.1 Significance of the study

Occurrence and transmission of antimicrobial resistant Enterobacteriaceae have been observed worldwide, and it is considered to be the 3rd largest threat to worldwide public health and food safety in the 21st century (WHO, 2014). In the past decade, antimicrobial resistant Enterobacteriaceae has developed to a significant challenge in control of diseases (Levy and Marshall 2004; Wellington et al., 2013). Fresh produce such as lettuce are at risk of contamination from contaminated soil, as lettuce grow very close to the soil surface and rarely undergoes any preservation process; hence, consumers of such products stand the risk of contracting infections should they come in contact with resistant pathogens on these products. Antimicrobial resistant micobes that contaminate food can cause a massive threat to public health because the resistant traits may be transmitted to other pathogenic microbes and therefore eliminating the effectiveness of several antimicrobial drugs.

Levantesi et al. (2012) and Scallan et al. (2011) reported that about 94% of food and waterborne infections are from irrigation water. Irrigation water and animal manure have been reported to be the leading transportes of pathogenic enteric bacteria to vegetables (Erickson and Doyle, 2012) and causes infections to humans with the potential to disseminate and antimicrobial resistance. The menace of antimicrobial resistant microorganisms significantly limits effectiveness of the modern antimicrobial agents, and hence can lead to the use of expensive and last resort antimicrobial agents such as colistin which developing countries can rarely afford, and consequently leading to high mortality. Even though colistin is the antibiotic of last resort, reports of resistance against this antibiotic are beginning to emerge (Bialvaei and Kafil, 2015; Hembach et al., 2017; Manohar et al., 2017;Yang et al., 2017). To the greatest of my knowledge; relatively few resources have been granted to understanding, preventing, and controlling the spread of antimicrobial resistance on global, national and local levels. Also,

while many studies have been reported on phenotypic antimicrobial susceptibility profiles of the Enterobacteriaceae family (Al-Zarouni et al., 2008; Tope et al., 2016), reports on the genotypic characteristics of antibiotic resistance determinants in the family is rarely reported.

1.2 Hypothesis

The study is based on a null hypothesis that members of faecal Enterobacteriaceae group isolated from hospital effluents, river water and vegetables do not exhibit antimicrobial resistance genes.

1.3 Aim

This study aims at evaluating the incidence and antibiogram fingerprints of four pathogens belonging to the Enterobacteriaceae family, which are *Citrobacter* spp., *E. coli*, *Enterobacter* spp.and *Klebsiella* spp. in river water, hospital effluents and vegetables in Chris Hani and Amathole District Municipalities in the Eastern Cape Province.

1.4 Specific objectives

1. To determine the occurrence of *E. coli*, *Citrobacter* spp., *Klebsiella* spp. and *Enterobacter* spp. in hospital effluents, river water and vegetables samples recovered from study sites.
2. To isolate, purify and confirm the identities of the target species using MALDI-TOF.
3. To evaluate resistance patterns of the confirmed isolates using Kirby-Bauer disk diffusion test.

4. To detect resistance genes of the MALDI-TOF confirmed isolates using polymerase chain reaction.

CHAPTER TWO

2.0 LITERATURE REIEW

2.1Background to antimicrobial resistance

The motive for the search of effective therapeutic treatments was driven by the discovery of contagious microbes in the late 19th century. Half a century later after the discovery of these contagious microbes, antibiotics were also discovered. In human antiquity, discovery of antibiotics was a massive revolving point as antibiotics transfigured the field of medicine in several aspects and saved lives countless times (Davies and Davies, 2010). However; this discovery of antibiotics was accompanied by swift appearance of resistance and this dramatically undermined the discovery of antibiotics. Antimicrobial resistance (AMR) has become a universal health problematic issue accountable for the increasing incidences of both severe and fatal ilnesses (Adefisoye and Okoh, 2016).

The emergence, spread and accumulation of resistance against the conventional antibiotics have increased and because of this increase, AMR has taken a major place in therapeutic medicine nationwide, more especially in African countries with low income (Adefisoye and Okoh, 2016). Increasing rise of AMR leads to a decreased ability of doctors to perform therapeutic techniques, which include hip arthroplasty, chemotherapy, organ transplants, hemodialysis and care for preterm babies. Illnesses linked with resistant infections donate significantly to escalating expenditures on health care as they occasion elongated hospital admissions and a necessity of additional costly drugs.

AMR has been known since 1940's when penicillin was discovered by Alexender Fleming and right after penicillin discovery, resistance against it developed (Ashley and Brindle, 1960). This was observed through several treatment failures and incidences of certain bacteria, for instance,

staphylococci which showed resistance against penicillin. When Alexander Fleming cultured *Staphylococcus* spp. he observed clear zones on the agar plate and the causative microbe for these zones was *Penicillium notatum* which was capable of excreting particular chemical in the agar. The observed growing of *Staphylococci* spp. in the presence of penicillin brought the commencement of AMR era. The exacting pressure put forth by the use of antimicrobials enlightened that antimicrobials would not last for protracted time. However this was not engaged seriously as the microbiologists between 1940s and 1970s believed that antimicrobials would always supress antimicrobial growth (Anı´bal et al., 2010).

Over the duration of the previous sixty years there have been quite a lot of unprecedented reviews unravelling the biochemistry, genetics, evolution and mechanisms of AMR. As the antibiotic era evolved around 1940's, consequently our understanding of bacteria likewise evolved (Anı´bal et al., 2010). Quinolone resistance set a prominent illustration of random sudden evolution in the field of medicine. As quinolones were introduced around 1960's, alarming growth of resistance against them was observed. Even after the establishment of fluorinated derivatives in the 1980's, resistance against quinolones and fluoroquinolones persisted and hence the resistance against this antimicrobial family was on the go since then (Fuchs et al., 1996; Carlos and Amábile-Cuevas, 2009). This emergence of resistance against fluoroquinolones was supposed to be controlled immediately after being observed, however, it wasn't and hence, the resistance continued spreading. Soon after discovery of quinolone resistance, *mar* regulon, which exists in several gram-negative bacteria as an astonishing defence mechanism was detected among isolates resistant against fluoroquinolone (Hooper et al., 1989). Fluoroquinolone resistance induced the finding of series of plasmid-borne fluoroquinolone genes which conceivably might have evolved due to microcin resistant mechanism (Tran and Jacoby 2002; Robicsek et al., 2006; Carlos and Amábile-Cuevas, 2009).

Bacteria now withstand antibiotic threat. The antimicrobial resistance mechanism is still a matter of argument in the field of medicine. Among the three communal human health threats, in 2011, the World Health Organization has adjudged AMR as a huge threat to human health (WHO, 2012). However, great attention to AMR was given way before 2011 especially around 2004 after a published report of Priority Medicines for Europe and the World. Surveillances held internationally, nationally and locally have substantiated AMR to be rising nationwide and hence also proclaimed AMR to be of major concern (Geffers and Gastmeier, 2011; Jean and Hsueh, 2011; Mattys and Opila, 2011). Gram-negative bacteria exhibit more resistance as compared to Gram-positive bacteria. Generally, the cause for more resistance in Gram-negative bacteria might be prompted by the outer membrane in these bacteria which prevents antimicrobials from entering bacterial cell.

According to WHO (2013), in Europe increasing gram-negative bacterial resistance was observed more in *E. coli* and *K. pnuemoniae* recovered from human samples such as blood or cerebrospinal fluid. Surveillance of Antimicrobial Resistance study conducted by WHO detected a very high resistance in samples collected from communal sick bays (Essack et al., 2016). In this study about 50% of *E. coli* isolates were resistant against 3rdgeneration cephalosporins and fluoroquinolones, while 50% of *K. pneumoniae* isolates were also resistant against 3rdgeneration cephalosporins. The same proportion of *S. aureus* exhibited resistance against methicillin while 25% of *Streptococcus pneumonia* was resistant against penicillin. Another 25% proportion was observed in non-typhoidal *Salmonella* and *Neisseria gonorrhoea* which were resistant against fluoroquinolones and 3rd generation cephalosporins while 25% of *Shigella* spp. exhibited resistance against fluoroquinolones (WHO, 2014). According to WHO (2014), proportions of *E. coli* that exhibited resistance against 3rdgeneration cephalosporins and fluoroquinolones varied between 0 to 87% and 0 to 98% respectively; while in *K. pneumoniae* the percentage of the isolates that were resistant against 3rd generation cephalosporins and

carbapenems were 8 to 77% and 0 to 4% respectively. Also this data revealed a range of 0 to 100% resistance against methicillin in *S. aureus*; while 1 to 100% resistance against penicillin was reported in *S. pneumoniae*. Again resistance against fluoroquinolones was detected in non-typhoidal *Salmonella* in a range of 0 to 35%, and in *Shigella* spp. in a range of 0 to 9% and while resistance of *N. gonorrhea* against third-generation cephalosporins ranged between 0 and 12%.

About ten countries have reported increasing resistance of Gonorrhoea against ceftriaxone which is the last resort of treating this organism, and currently there are no inventions of alternative treatment (WHO, 2014). This highlights that Gonorrhoea may soon become incurable. Globally, about 700 000 deaths every year can be attributed to AMR. It is estimated that about 9.5 million people can die each year if there is no immediate solution against AMR (OECD 2016). Between 2005 and 2014 the resistance of *E. coli* against 3rd generation cephalosporin increased by 10%, while resistance of *K. pneumoniae* against carbapenem increased by 5% OECD (2016). According to Essack et al. (2016), infectious diseases still cause many deaths in Africa, including deaths of kids below five years of age. In 2005, there were about 450 000 incidences of multiple drug resistant tuberculosis (XDR-TB) reported (WHO, 2012). European labs have reported growing resistance amid bacteria that are responsible for causing pneumonia, which are responsible for about 1.8 million children's deaths every year (Qazi, 2008). In Europe, mortality rates attributable to resistant bacterial hospital infections are above 25 000 a year (ECDC, 2012). In Iceland and Norway in 2003, *E. coli* and *K. pneumoniae* isolates that were resistant against cephalosporins displayed a prevalence of 27%. Resistant isolates of *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *S. pneumoniae*, *K. pneumoniae* and *Enterococcus faecium* account for more than 400 000 infections together with deaths in 2007 at Europe (ECDC/EMEA, 2009).

The Enterobacteriaceae belongs to commensal microbial flora, and they are capable of causing infections such as urinary tract, respiratory tract, bloodstream and wound infections (Kocsis and Szabó, 2013), and antibiotics are used to treat such infections. Among the families of antimicrobials used in treating Enterobacteriaceae associated infections; the most potent are the flouroquinolones, beta-lactams and aminoglycosides (Kocsis and Szabó, 2013). Bacteria are becoming more and more resistant against the currently efficient antibiotics especially members of Enterobacteriaceae because they advance quite a lot of mechanisms to resist the activity of antibiotics, and *E. coli* and *K. pnuemoniae* are the mostly implicated in infections in the Enterobacteriaceae family (Slama et al., 2010; Gundogan and Avci, 2013). These two species are also known to exhibit greater resistance against beta-lactam antibiotics amongst the Enterobacteriaceae family (Slama et al., 2010; Gundogan and Avci, 2013).

In the past decade, antibiotic resistance in Enterobacteriaceae has dramatically increased around the globe and this increase is mainly driven by an upsurge in the prevalence of Enterobacteriaceae producing ESBLs (Gundogan and Avci, 2013). This dramatically increase of antibiotic resistance in Enterobacteriaceae has steered an upsurge in the usage of the last resort of antibiotics which are the carbapenems (Kuzucu et al., 2011), which poses a potential threat to public health. Increasing resistance of *E. coli* and *K. pneumoniae* against ampicillin has been reported (Nijssen et al., 2004; Li-Kou et al., 2011; Gundogan and Avci, 2013). Most pathogenic bacteria exhibit resistance against two or more antimicrobial families. Gram-negative bacteria exhibit resistance to almost all currently existing antimicrobials (CDC, 2013), and thus pose a public health threat. Gram-negative bacteria that are mostly responsible for causing severe resistant infections include Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter*.

Between 1999 and 2013 Enterobacteriaceae exhibited 31 to 94.2% resistance against chloramphenicol and 0 to 46.5% against 3rd generation cephalosporins (Leopold et al., 2014).

Leopold et al. (2014) also reported 15.4 to 43.2% resistance of *Salmonella entericatyphi* against naladixic acid. Bacteria associated with meningitis, respiratory tract infection, and urinary tract infections display great resistance against chloramphenicol, trimethoprim as well as tetracyclines. Treating infections caused by resistant pathogens is gradually becoming common in countless hospitals (CDC, 2013). National public health institutes such as CDC have conducted assessments on antibiotic resistance threats to determine highly resistant pathogens that may pose significant threats (CDC, 2013). There is a possibility that such pathogens are not yet widely distributed, however, they display a great potential to be widespread. Pathogens that are identified as highly resistant pathogens by CDC include multidrug-resistant *Campylobacter*, Carbapenem-resistant Enterobacteriaceae (CRE), ESBL-producing Enterobacteriaceae, cephalosporin-resistant *Neisseria gonorrhoeae*, multiple drug-resistant *Acinetobacter, Clostridium difficile* (*C. difficile*) as well as Fluconazole-resistant *Candida*. Even though the identified highly resistant pathogens are not yet widespread, there is still a need to establish precaution actions on preventing the transmission of these pathogens.

Enterobacteriaceae has developed resistance against ESBL antibiotics. Resistance of Enterobacteriaceae against ESBLs has developed to be the principal threat globally. ESBL-producing Enterobacteriaceae produces numerous enzymes that reduce the spectrum activity of certain extended spectrum beta-lactam antibiotics i.e. these enzymes deactivates penicillins and cephalosporins and other beta-lactamases anatomize extended-Spectrum Cephalosporins such as Ceptazidime; monobactam and Cefotaxime, and these enzymes have been identified in numerous countries (Thenmozhi et al., 2014). According to Pitout and Laupland (2008), *E. coli* and *K. pneumoniae* are the leading microbes that display the proficiency of producing extended spectrum beta-lactamase enzymes. Developing countries such as South Africa are highly affected by antimicrobial resistance; especially in Enterobacteriaceae. South African surveillance data has perceived increasing resistance in most of bacterial strains that causes

infections. Increasing rates of resistance in Gram-negatives poses a major dilemma in South Africa as advanced antibiotics accessible are only restricted for handling Gram-positive infections, whereas antibiotics for Gram-negative are a slightly extortionate and some not yet authorised for use in South Africa. Even though Gram-negative bacteria are chief drivers of typical infections, there are no new antibiotics are expected for the next 15 to 20 years (Shisana et al., 2014).

2.2 Mechanism of resistance

Microorganisms display the outmost genetic and metabolic diversity and they have existed for more than 3.8 billion years. Microorganisms have developed several resistance mechanisms in order to respond to exacting stress that is applied by countless environmental factors. These resistance mechanisms allow microorganisms to battle against threat imposed by man seeking to strip their habitation by applying antimicrobial agents (i.e. sanitizers, disinfectants, etc.). Over the years increasing antimicrobial resistance occurrence has been observed in several countries especially in developing countries like South Africa, Gabon, and Angola and so on (Byarugaba, 2005). Developed countries have reported resistance to be frequently present on pathogens that are able to be transmitted without causing any sickness. Such pathogens can be carried for longer duration of time, and can only induce infection in certain individuals due to certain stimuli i.e. initiated by medical involvements, or in kids or individuals having compromised immune systems. AMR is a natural phenomenon, regularly caused by adaptation of infectious agents to antimicrobials. Extensive use of antimicrobials is regarded as the most important factor that is accountable for increasing AMR (Aarestrup et al., 2001; Byarugaba, 2004; Anı´bal de J et al., 2010).

Mechanism of activity of an antimicrobial is based on microbial biochemistry and mechanism of infection and must not have any negative outcome on the host. Antimicrobial agents are classified as either static or cidal. Static are those that hinder microbial growth and cidal are

those that completely eliminate bacterial growth. There are numerous manners which microbes have applied to procure resistance, however; antimicrobial chemical structure and mode of activity is the predominant factor directing development of resistance mechanism. Generally type of resistance mechanism is determined by the exact antimicrobial pathways.

There are two forms of resistance i.e. intrinsic and acquired. When a microbe naturally deprives target sites of an antimicrobial agent or naturally has restricted penetrability against an antimicrobial agent is classified to harbour intrinsic resistance. Commonly intrinsic resistance is exhibited against those antimicrobials targeting microbial cell for mode of activity and therefore pass route into the microbial cell is a prerequisite for mode of action. While acquired resistance is a type of resistance that the microbe develop due to certain adaptations against a particular microbial agent. There are five broad categories of acquired resistance mechanism of bacteria which are (i) diminished accumulation of the antimicrobial agent inside cell, this occur by weakening cell penetrability and by lessening antimicrobial agent's active efflux; (ii) alteration of enzymes to degrade antimicrobial agent; (iii) attainment of metabolic pathways to substitute to those repressed by antimicrobial; (IV) modification of antimicrobial target site; and (V) excessive target enzyme production (Fluit et al., 2001; Van Hoek et al., 2011).

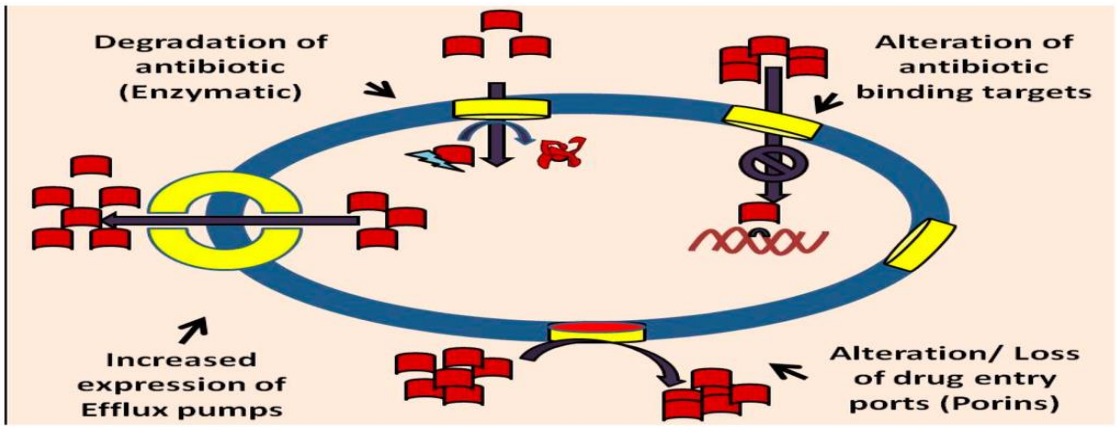


Figure 2.1: Examples of resistance mechanisms in bacteria. Source: CDC, (2013).

Resistance in Enterobacteriaceae is driven by adaptation of countless mechanisms which dodge antimicrobial static effect. High levels of resistance have been observed in members of Enterobacteriaceae family, and production of ESBLs is the prominent factor driving resistance in these organisms. This family is frequently present in communities and hospital environments. Due to the acquired ESBL resistance, this family is aligned with incompetent treatment (Pitout et al., 2005). The Enterobacteriaceae have been observed to show resistance against more than two families of antimicrobials. Fluoroquinolones, cephalosporins, beta-lactams and aminoglycosides are the most effective antimicrobials for treating infections associated with Enterobacteriaceae, however, increasing resistance of Enterobacteriaceae against these agents have been reported (Pitout et al., 2005).

2.2.1 Resistance against beta-lactam antibiotics

Beta-lactam structure is the principal factor that characterise these antibiotics as beta-lactams. Antibiotics comprised in this class are cephalosporins, carbapenems, cephamycins, oxapenams as well as penicillins. Principal task of the beta-lactam ring in these antibiotics is to disable transpeptidases that speed up the final linking interactions of peptidoglycan production in bacteria. Activity of these antibiotics is archived by getting to penicillin binding protein (PBP) and bind to it (Robicsek et al., 2006). There are three major causes of resistance to this antimicrobial family; (i) beta-lactamases deactivate beta-lactam ring, (ii) alteration of PBPs and (iii) alteration of cell penetrability. Classification of beta-lactamases is established based on proneness to inhibitors, genetic localization, hydrolytic range as well as amino acid protein order as described by Bush, Jacoby and Medeiros (1995); Anı´bal et al. (2010). Bush, Jacoby and Medeiros (1995) describe beta-lactamases into four groups: (i) cephalosporinases, those conferring resistance against clavulanic acid; (ii) penicillinases; (iii) metallo-b-lactamases and (IV) penicillinases, those that confer resistance against clavulanic acid.

These enzymes are characterized based on substrates and inhibitors (Ambler, 1980; Bush and Fisher, 2011). Ambler groupings are based on amino acid identities. They are grouped as A; B; C and D. Group A; C and D comprise beta-lactamases having serine on active site, while group B comprise metallo-beta-lactamases (MBLs). MBLs constitute zinc element at active site. There are three main classes and sixteen sub-classes identified by Bush-Jacoby-Medeiros classification (Ambler, 1980; Bush and Fisher, 2011).

2.2.2. Resistance to Aminoglycosides

Aminocyclitol ring is the prominent trait characterizing aminoglycosides. The ring is connected with amino sugars. This antimicrobial family exhibit comprehensive range of task against bacteria. Kanamycin; reptomycin; gentamycin; amikacin as well as tobramycin are examples of antibiotics representing this antimicrobial family. Both gram-negative and gram-positive infections can be cured using these antibiotics. Mode of action is achieved by binding with ribosomes on the microbial cell. Resistance against these antibiotics is an acquired resistance achieved though adaptations against these antimicrobials. Resistance against these antimicrobials depends on their modification. Aminoglycoside nucleotidyl-transferases; acetyl-transferases phosphor-transferases abbreviated as (ANT); (AAC) and (APH) respectively are the three groups of aminoglycosides categorised based on nature of their modifications (Shaw et al., 1993; Klare et al., 2001). Those that are classified as AACs, ANTs and APHs comprise modifying enzymes that speed up adjustment at −OH or −NH2 sets of the 2-deoxystreptamine nucleus. AACs speed up the acetylation of –NH2 sets in aminoglycoside molecule by means of acetyl coenzyme A acting as contributor substrate. ANTs deactivate aminoglycosides by speeding up transferral of an AMP set from the contributor substrate ATP towards hydroxil set in aminoglycoside particle (Ramirez and Tolmasky, 2010). Protein synthesis cannot be inhibited by a modified aminoglycosides either modified on amino

molecules with AAC or at hydroxyl molecules with ANT or APH enzymes. These modifications deprive aminoglycoside ribosome binding ability (Doi and Arakawa, 2007).

E. coli can resist spectrum activity of aminoglycosides because of the modification of molecule's target site. Modification of this molecule is due to methylation of 16S rRNA by Arm and Rmt methyltransferases efflux pump AcrD (Rosenberg et al., 2000). Genes coding for resistance against aminoglycosides comprise *aac*, *arm* and *rmt* which are customarily integrin members. Not only have these genes conferred resistance to aminoglycosides but also to beta-lactam as well as quinolone (Caratolli, 2009).

2.2.3 Resistance to Fluoroquinolones and Quinolones

Fluoroquinolones are modifications of Quinolones. Quinolones are synthetically manufactured, and are industrialised by upgrading 1-alkyl-1, 8-naphthyridin-4-1-3-carboxylic acid. Fluoroquinolones and quinolones are antibiotics which have a broad-spectrum activity against series of drastic diseases ever since the late 1980's. However, in the late 1980's resistance to these antimicrobials was discovered (WHO, 2007). Following the events of transmittable resistance against fluoroquinolones, in 1994 *qnrA* gene was identified in USA (WHO, 2007). This gene was isolated from *K. pneumoniae* isolate from a patient. Identification of other two quinolone resistant genes (i.e. *qnrB* and *qnrS*), then followed. These genes encrypt for a protein that hinders fluoroquinolone activity. At first this resistance was very rare but now it has spread from *K. pnumoniae* and to other species, including *Salmonella* spp. (WHO, 2007).

The *cr* modified *aac(6')Ib* gene codes for acetyltransferase resistance against ciprofloxacin. This is achieved by the addition of acetyl to the N-terminal of its piperazinyl amine. Firstly this gene was identified in *E. coli* from Shanghai, however; that is no longer the case as the gene occurred in other Enterobacteriaceae strains in several countries worldwide (Raherison et al., 2017). Moreover the gene does not only code for fluoroquinolone resistance but it has been

observed in isolates resistant against cephalosporin antimicrobials. Distribution of this gene on multiple resistance plasmids gave this gene an advantage to code for resistance against multiple antimicrobials. According to Redgrave et al. (2014), several clinical isolates exhibiting resistance against fluoroquinolones have been repeatedly reported. There are several effects associated with resistance against quinolones.

Resistance of Enterobacteriaceae against quinolones is mainly due to chromosomal mutations, and these mutations are in diverse genes that are involved in transcription and replication of DNA. Due to the upsurge usage of these antimicrobial agents, countries such as UK have developed guidelines acclaiming these agents to be only used as a second-line defence (Redgrave et al., 2014). Even though there are recommendations attempting to retain effectiveness of these antibiotics, resistance against fluoroquinolones has not yet decreased instead it is moving at an astonishing speed in countless bacterial species. Reason for this, is the deprived of dynamic investigations of fluoroquinolone resistance and information on how these agents are consumed. However; this is not the reason Europe, because ECDC have conducted satisfactory investigations that grant comparison of resistance in European countries (Redgrave et al., 2014).

Greece is the leading country which uses fluoroquinolones and hence has the maximum incidence of *E. coli* resistance against fluoroquinolone (Miriagou et al., 2010). Contrariwise, Sweden has lowest utilization rate of fluoroquinolones and henceforth has the least occurrence of resistance (Miriagou et al., 2010). Fluoroquinolones comprise of four generations, and the frequently prescribed include levofloxacin, ciprofloxacin and moxifloxacin. Resistance of *E. coli* against Fluoroquinolones in UK increased from 6% to 20% between 2001 and 2006 (Livermore et al., 2013). Gagliotti et al. (2011) reported an increased *K. pneumoniae* resistance against fluoroquinolones from 11% to 50% in Italy between 2005 and 2011, which means resistance of *K. pneumoniae* against fluoroquinolones increased yearly. Fluoroquinolones were

primarily made to target mainly gram-negative bacteria and hence it is surprising that a massive amount of data in relation to the resistance of most clinically relevant bacterial species to fluoroquinolones has become available. According to Metz-Gercek et al. (2009), there was an increasing fluoroquinolone resistance among pathogenic *E. coli* isolates in Austria from 7% to 25.5% between the years 2001 and 2007.

2.2.4 Resistance to carbapenems

Carbapenems are used in treating infections that are induced by multidrug resistant microbes. They are recommended as last defence, and are only used when a patient is suspected to be infected by a multidrug resistant organism (Bradley et al., 1999; Paterson, 2000; Paterson and Bonomo, 2005; Torres et al., 2007). When compared with other beta-lactams, carbapenems consist of a wide-ranging spectrum of effectiveness on both gram-positives as well as gram-negatives. The effectiveness spectrum of carbapenems is also reduced by the production of beta-lactamases by microorganisms and hence these enzymes are accredited to be the most main bacterial resistance mechanism. Production of carbapenemases enables a microorganism to exhibit resistance against carbapenems. Carbapenemase production has emerged and spread amongst the Enterobacteriaceae worldwide. The production of these enzymes extremely reduces the effectiveness of this lifesaving antimicrobial agent (Queenan and Bush, 2007).

There are several reports that have been documented, reporting increasing resistance against carbapenems worldwide, especially in Gram-negative bacteria (Gaibani et al., 2010; Gopalakrishnan and Sureshkumar, 2010; Chouchani et al., 2011; Livermore et al., 2011). This has grown into an exceedingly overbearing medical and public health issue. According to Paterson and Bonomo (2005), escalating resistance against carbapenems results in fewer therapeutic options. *K. pneumoniae* and *P. aeruginosa* are examples of microbes exhibiting resistance against carbapenems. The occurrence and development of carbapenemases has driven the need for investigations of resistance against carbapenems. In Europe, resistance of

K. pneumoniae against carbapenems is a leading public health concern (Redgrave et al., 2014). The ability to produce carbapenemases amongst pathogenic strains pose a strong threat to public health security as they exhibit resistance to numerous antimicrobials and thus emphasizes the need for new innovations of alternative therapeutic options (Paterson and Bonomo, 2005).

The increasing reported incidences of ESBL-Enterobacteriaceae have imposed an upsurge usage of carbapenems worldwide. Greece and U.S. were the first countries to detect *K. pnumomiae* carbapenemase-producing (KPCs), however, these strains seem to have spread as similar strains have been observed in numerous European states. In Greece it was detected that plasmids encoding *VIM* gene are mainly distributed in *K. pneumoniae* (Miriagou et al., 2010). bla_{KPC} is the responsible gene for resistance in KPCs and this gene is positioned at transposable component Tn4401. *K. pneumoniae* was the first bacteria to demonstrate KPCs, however; they have expanded to other Enterobacteriaceae (Conlan et al., 2014). KPCs have been perceived to be the principal ruthless drivers of carbapenem resistance (Gupta et al., 2011). West Europe and North Africa have reported occurrence of bla_{OXA-48} gene while bla_{VIM} was reported to be common in Mediterranean republics, and bla_{NDM} was mainly detected in Asian countries (Nordmann et al., 2011; Gustavo et al., 2017). The outmost threat in antibiotic era is CRE. *K. pneumoniae* clone, sequence type 258 which is responsible for the increase of CRE occurrence (Munoz-Price et al., 2013).

Enterobacteriaceae exhibiting resistance against carbapenems was first known in U.S. in 1966 and by now these strains have been widely distributed. These bacteria escalate swiftly and hence there is a need to develop methods aiming to prevent this increasing spread of these bacteria. Around the year 2000, increasing alarming resistance occurrence of carbapenem-resistant Enterobacteriaceae, KPCs and more of other types of carbapenemases in addition to KPC were identified (CDC, 2015). Enterobacteriaceae members like *E. coli* and *K. pneumoniae*

typically found in hospital settings turn out to be the most significant pathogens as they are the most pathogens producing ESBLs. It was revealed that ¾ of *K. Pneumoniae* from blood samples tested positive for ESBL between 2010 and 2012 (Trecarichi and Tumbarello, 2017). At present, a proportion of 9.7% to 51.3% against colistin; 5.6% to 85.4% against gentamycin and 0% to 33% against tigecycline were observed in CREs (Trecarichi and Tumbarello, 2017).

2.3 Extended spectrum beta-lactamases (ESBLs)

Based on published report by WHO (2007), incidence of ESBL-producing Enterobacteriaceae in food animals is increasing continuously and emerging nationwide. ESBLs confer resistance to beta-lactams such as extended-spectrum cephalosporins; aztreonam as well as penicillins (Bush and Fisher, 2011). ESBLs comprise of CTX-M; OXA; SHV; TEM and PER gene type. Every ESBL enzyme originates from its own antecedent. Of these enzymes, the most predominant enzyme in Europe is the SHV; whereas TEM-type is widespread in USA while CTX-M- type is prevalent all over the globe (Paterson and Bonomo, 2005).

From the TEM-types, *E. coli* exhibit two TEM-types (TEM-1 and TEM-2) of ESBLs. Both of these TEM-types confer resistance against ampicillin. Another TEM-type is TEM-3-type which exhibit ESBL activity. The slightly difference between TEM-2-type and TEM-3-type is the amino acid structure which varies by two substitutions (Sougakoff et al., 1988). Arginine amino acid situated on locus 164 and glycin amino acid located in locus 238 are the two amino acid endowing ESBL action in microorganisms. Both of glycin and arginine mutate to serine, and serine then extends the ESBL hydrolytic activity. Over 200 TEM-type have been identified at the moment and most of these identified TEM-type enzymes are ESBLs, and they have been reported to be the derivatives of TEM-1 and TEM-2 type and, these other types occur because of modifications in more than five sites from TEM1 and TEM-2. In TEM-1-type, amino acid alterations occur in loci 39, 69, 165, 182, 244, 261, 275, and 276. These modifications enable microbes to be inhibitor resistant TEM (IRT) (Chaibi et al., 1999).

The ESBL SHV (sulphydryl variable)-type is mostly harboured by *E. coli* and *K. pneumoniae.* There is a probability that 20% of resistance in these two organisms against ampicillin is due to SHV (Tzouvelekis and Bonomo, 1999). Most *E. coli* strains encompass bla_{SHV-1} in their chromosomes (Livermore, 1995). There are few derivatives of SHV enzyme. Majority of SHV-types exhibit ESBL traits. However; SHV-10 has been detected to display inhibitor resistant traits. SHV ESBL-types have originated from the chromosome of *Klebsiella* spp. and consist of a narrow beta-lactam hydrolysing activity conferring resistance against penicillin and ampicillin (Bush and Fisher, 2011). SHV-1 type encompasses two sites at its amino acid sequence where if there is just one amino acid substitution in these sites that outspreads its hydrolysis range against aztreonam and cephalosporins. The two sites are aspartate on locus 179 and glycin on locus 238 (Rasheed, 1998). SHV-1 type differs from and SHV-171 by that SHV-171 has an optimum of five amino acid loci. One example of chromosomal encrypted SHV-type comprising ESBL is SHV-38; however, it has shown a reduced activity against amoxicillin and cefalothin (Poirel et al., 2003).

The beta-lactamases such as CTX-M (cefotaximase-Munich) are one of the ESBLs that are chromosomally encoded, and originate from *Kluyvera* spp. These enzymes are well-known for their proficiency in hydrolysing cefotaxime, aztreonam as well as extended spectrum cephalosporins. Because of their capability of hydrolysing cefotaxime, these enzymes were named CTX-M. About 140 enzymes belonging to CTX-M-type were identified and all were classified as ESBLs (Peterson and Bonom, 2005). There are five sub-classes encompassed by these enzymes, which are CTX-M-1; CTX-M-2; CTX-M-8; CTX-M-9 as well as CTX-M-25. CTX-M-1 further derives and originates CTX-M- 15 which is very abundant in many microbial species worldwide (Bonnet, 2004). Two bacterial species belonging to Enterobacteriaceae family which are *Klebsiella* spp. and *E. coli* convey CTX-M on plasmids. These enzymes were

firstly well-known to be conveyed by *Salmonella enterica serovar typhimurium* and *E. coli* but now they have spread amid other Enterobacteriaceae members.

Another enzyme belonging to ESBLs is the OXA-type. OXA beta-lactamases were named after their oxacillin hydrolyzing abilities and were classified as Amblerclass D and Bush-Jacoby-Medeiros group 2d (Bush and Fisher, 2011). They are capable of inactivating benzylpenicillin, cloxacillin and oxacillin. These enzymes are frequently detected in *P. aeruginosa* (Weldhagen et al., 2003), however, more Gram-negatives have now displayed occurrence of these enzymes, especially Enterobacteriaceae family (Livermore, 1995). These enzymes are another growing family of ESBLs. There are two subclasses of OXA enzymes, which are OXA-1 and OXA-10, and have narrowed hydrolytic activity. The rest of other OXA enzymes are confirmed as ESBLs which include OXA-11; OXA-14; OXA-15; OXA-16; OXA-28; OXA-31; OXA-35 and OXA-45, and all confer resistance against aztreonam; cefotaxime as well as ceftazidime (Livermore, 1995). So far there are 311 in total of OXA-type enzymes that have been recognised for both narrow spectrum as well as ESBLs (Toleman et al., 2003). *E. coli* and *K. pneumoniae* amid Enterobacteriaceae family are the principal carriers of most ESBLs except OXA beta-lactamases. A quite number of ESBLs are originating from OXA-10 type.

Another detected ESBL enzyme is *Pseudomonas* extended resistance abbreviated as PER. This name was granted to this type of enzyme because it was initially detected in *Pseudomonas aeruginosa* (Neuhauser et al., 2003). It then subsequently expanded to *Salmonella* spp. and in *Acinetobacter* (Vahaboglu et al., 1995; Vahaboglu et al., 2001; Szabó et al., 2008). The PER enzymes have an ability of hydrolysing penicillins and cephalosporins, however; they are inhibited by clavulanic acid. Another enzyme detected displaying ESBL activity is Vietnam extended-spectrum beta-lactamase (VEB). This enzyme displays 38 percentage homology as PER. Unlike PER, VEB display resistance activity against aztreonam; ceftazidime and

cefotaxime but it is also inhibited by clavulanic acid. Surprisingly *bla*$_{VEB-1}$ is said to be located on plasmids but plasmids lack beta-lactam resistance factors (Poirel et al., 1999).

AmpC beta-lactamases type is another type that is regarded as an ESBL enzyme. This group of beta-lactamases most of them are chromosomally encoded in Enterobacteriaceae and some are plasmid encoded. AmpC that is coded by both chromosomal and plasmid genes hydrolyzes successfully broad-spectrum cephalosporins. AmpC can be induced by mutation at ampD and be expressed at great levels. These mutations drive hyper inducibility of AmpC, which then drives resistance against extended-spectrum cephalosporins and endows resistance in Enterobacteriaceae against cephalosporins and a lot of penicillins as well as clavulanic acid (Schmidtke and Hanson, 2006). AmpC is absent or not properly expressed in *P. mirabilis*, *K. pneumoniae* as well *E. coli*. Other ESBLs that are not of particular concern at the moment are SFO; BES; TLA; BEL and GES, reason is that they are merely detected (Naas et al., 2008).

2.4 Major drivers of antimicrobial resistance

The increasing health care challenge of subsequent absence of effective antimicrobials needs to be observed with an eye of an eagle and formulate means to combat this increasing public health issue. This can be achieved by understanding the nominal causes of antimicrobial resistance, for example, investigating drivers of antimicrobial resistance in community and in the environment. According to Holmes et al. (2005), the significant drivers of antimicrobial resistance can be evaluated from community settings (including the environment and agriculture) and in health-care systems. There are several factors that influence the rise and spread of AMR, however; incorrect and extensive use of antimicrobial agents on human medicine and over use of antibiotics in agricultural fields are the main drivers of AMR. This is because improper and extensive use of antimicrobial drugs provides pleasing growth conditions for resistant microorganisms to emerge and spread. Other principal factors involved in driving AMR include lack of infection prevention and control practices in healthcare

facilities; deprived hygiene and sanitation practises; lack of new antibiotics and vaccines developed; derisory national commitment to an inclusive and corresponding response, ill-defined accountability and deficient engagement of communities; derisory systems ensuring excellent and uninterrupted supply of medicines and weak or absent surveillance and monitoring systems.

2.4.1 Poor hygiene and sanitation practises

Besides the misuse of antimicrobial agents, another factor contributing to the spread of AMR is the poor hygiene practices. Resistant bacteria are transferred amid individuals through direct interaction, contaminated water and food (Smith et al., 2004). Hygienic and satisfactory treatment of potable water and disposal of human excreta and sewage are community well-beings that are related to sanitation. The principle aim of all sanitation systems is to look after human well-being by providing unpolluted and uncontaminated settings that will discontinue spread of infection, particularly through the faecal-oral route. According to Sustainable Sanitation Alliance (2008), communities with low level of sanitation can transmit diseases such as ascariasis, cholera, hepatitis, polio, schistosomiasis, trachoma and so on. According to WHO (2008), practically about a proportion of 90% of the entire deaths from diarrhoea, predominantly in children are triggered by non-availability of good potable water and basic hygiene. According to WHO/UNICEF (2010), the world has managed to provide a population of 87% with enhanced water sources. However, about 39% of the world inhabitants are still in need of improved water sources and advanced sanitation. A number of 1.1 billion residents from developing countries still eliminate wastes in the open and only 17% of them wash their hands with soap or disinfectant afterwards (Curtis et al., 2009; WHO/UNICEF, 2010). WHO/UNICEF also declared that a population of 62% lacks advanced sanitation facility which separates human excretion from direct or indirect contact with individuals.

Developing countries still lack adequate water, together with sanitation and henceforth there is a great occurrence of illnesses and death due to AMR. Each year, approximately 4 billion diarrhoeal cases are responsible for 2.2 million deaths, normally these deaths occur in kids below 5 years of age, and approximately 1.7 million children in developed countries are affected by diarrhoea. Globally, diarrhoeal cases are responsible for a proportion of 4.3% of diseases and there is a high possibility that 88% of these deaths are suspected to be caused by unsafe potable water supplies, inadequate sanitation together with poor hygiene. About 10% of the populace of developing countries is disease-ridden by duodenal worms which stimulates underdeveloped growth; underfeeding together with anaemia. About 6 million populaces are blind because of trachoma, while 500 million are at risk of being infected by trachoma, and another 300 million inhabitants are diagnosed with malaria whereas 200 million individuals are infested by schistosomiasis and 20 million of all these people suffer drastic consequences (Water Aid, 2001).

2.4.2 Inappropriate use of antimicrobials in health-care facilities

Health-care facilities play vital role in the rise of AMR, as many of their procedures are not proof-based; however they still have a very significant role in therapeutic as well as hindrance of spread of diseases. Factors that contribute to AMR in health-care facilities involve poor drug quality, inappropriate and over prescription of antibiotics by health facilitators (i.e., incorrect prescription, incorrect measures, or prescription of an antimicrobial when not needed) (Usluer et al., 2005), poor prevention of diseases mechanism in hospitals and clinics, deprived educational awareness on users, absence of appropriate dispense systems and lack of regulations.

2.4.2.1 Inappropriate and over prescription of antibiotics by health facilitators

In developing countries, there is high occurrence of infectious diseases and hence doctors work under pressure, and end up not having time for proper training and communiqué with their patients on the usage of antimicrobials and the penalties that they will face if not adhering to guidance's accordingly. According to WHO, 2007; half of all antibiotic consumption prescribed by doctors might be unnecessary. Study of Saleh et al. (2015), conducted in Lebanese reported that 52% of reported cases of AMR were attributed to incorrect measures of prescription; while 63.7% were attributed to incorrect period of consumption of treatments prescribed by physicians. Health authorities and professionals at times lack advanced information on AMR patterns, especially in developing countries. This is driven by lack of operative and reliable surveillance systems and meagre distribution of research data on AMR. Lack of distribution of updated research data about AMR challenges on health professionals on choosing appropriate antimicrobial to be applied and henceforth, they end up using broad spectrum antimicrobials (Ayukekbong et al., 2017).

Daily dose per day abbreviated as DDD pronounced that average antimicrobial intake in Organisation for Economic Co-operation and Development (OECD) goes around 20.5 per 1 000 inhabitants in the year 2014. However, this was not the initial intake, antimicrobial ingestion increased by 4% from 2005 to 2014 in OECD. Inappropriate intake of antimicrobials may be up to 90% in certain healthcare establishments (OECD, 2016). In developed countries, almost 90% of entirely all antimicrobials taken by humans are approved in general practice and usage of antimicrobials is aligned with national treatment guidelines (van Bijnen.et al., 2011).

2.4.2.2 Lack of educational awareness on users

This is one of the major causes of AMR and it is likely less noted by health-care professions. Patients do not comply with the regulations and guidelines on how to use antimicrobial drugs and this hugely contribute to the development of AMR (Malfertheiner, 1993). When patients feel better or when symptoms of infection decreases they discontinue with treatment thinking

that the pathogen is completely eliminated whereas it is still alive. Patients either fail to adhere to dosage instructions and or skip or miss doses to their own suitable times due to several reasons. These practices increase the chances of development of resistance in the remaining microorganisms to concentrations of the antimicrobial after contact (Calva and Bojalil, 1996). In African countries most patients strive for their first-line defence from out-dated witchdoctors who then give them herbal combinations of unknown usefulness and then some patients combine these medicines with antimicrobials simultaneously, while others supplement the antimicrobials with these medicines to 'improve the effectiveness' of the antimicrobials and the chemical compounds in these unknown medicines may enhance pathogen survival (Lansang et al., 1990). This rational biases and poor information in patients lead to a huge increase in AMR. All providers, such as General practitioners (GPs), pharmacologists, nurses, paramedical workers, and antimicrobial sellers, must be provided with campaigns training and teaching them on how and when to prescribe or sell the drugs and about the issues surrounding AMR. The topics that should hold in those campaigns should be about precise identification and management of communal infections, antimicrobial usage as and infection control as well as disease deterrence as these are the focal factors donating in AMR. Awareness can also assist the consumers to a clear understanding of the drugs they purchase and consume and all side effects associated with them.

2.4.3 Indiscriminate use of antibiotics in agricultural fields or non-purpose human activities

In agricultural fields antimicrobials are widely used for several factors which include preventing and treating diseases (prophylaxis in high risk animals), used as growing inducers in animal breeding (Petersen et al., 2002; Collignon et al., 2005), used as additives in plant agriculture, to shower fresh produce trees for inhibition of diseases transmission, and the usage of organic manure that contains antimicrobials on farmland and industrial processes (Vidaver,

2002). However, the same classes of antimicrobials are also applied in human medicine; raising a precise concern as this wouldn't it be a problem on human clinical activities. The use of antimicrobials in agricultural fields has significant consequences for both human together with animal health as this exercise might prime the development of resistant bacteria and also strengthens the threat for the development and spread of resistant microorganisms. These resistant microbes are then spread through ingestion of food; direct interaction with food animals or through environmental spread such as runoffs and sewage. The principal known route of spreading of resistant microorganisms between humans and animals is food, especially ESBL strains (WHO, 2007). As a result of processes such as exports, imports, migrations and immigrations, AMR among food animals from some country can be the driver of human health catastrophes to other countries. *Salmonella* spp. such as *S. Schwartzengrund*, *S. Typhimurium* DT104, and *S. Virchow* are examples of resistant microbes spreading from one country to another (WHO, 2007).

The extensive use of antimicrobials in agricultural husbandry employs an exacting pressure which support persistence of resistant strains comprising resistant genes over those that lack resistant genes, and thus prime an upsurge in resistant bacteria within microbial groups (Witte, 1998). According to Food and Agriculture Organization (FAO) (2016) the healing and non-healing purposes in animal production influence the increase in AMR. Between 2002 and 2006 tetracycline was the most antibiotic used as the growth inducer or disease-precautionary drug for livestock farming in Korea and the use of this antimicrobial agent ranged from 43% to 51% (KFDA, 2007). Korean Food and Drug Administration (KFDA) announced a rise of AMR in *E. coli* recovered from food animals and meats in 2008 even though tetracycline usage was reduced in agricultural fields after 2006. Aquaculture also plays an important role in driving AMR as prophylactic antibiotics are extremely used in this filed, especially in African countries. The extensive usage of antimicrobials in aquaculture crafts problems for human and

animal health as well as the environment. Antibiotic dose used in this field can be higher than the doses in livestock production (Cabello, 2006). Antibiotics used in fish feed have a proficiency of surviving in the water environment for a very long duration of time, through excretion, and exert selective pressure to the bacteria in the water, which spread rapidly through water systems (Meek et al., 2015). About 70% to 80% of antibiotics given to fish in aquaculture are excreted to water (Serrano, 2005; Burridge et al., 2010).

In the year 2015, Medical, food and public health organizations like WHO, (FAO) together with World Organisation for Animal Health (OIE) reported extensive misuse of antimicrobial agents in agricultural fields. Estimations by Van Boeckel et al. (2015) estimates that 67% increase in consumption of antibiotics in agriculture nationwide and 99% increase amongst BRICS between the years 2010 and 2030 is expected. Antibiotics are more used in livestock as compared to humans; approximately nearly 70% of antibiotics reasoned for significant use in humans by Food and Drug Administration (FDA) are applied in livestock. FDA report which was reported in the year 2011 states that 93% of antimicrobials which are of medical importance are added to feedstuff or water in agriculture in US, and nearly 75% to 90% of these antibiotics might be not metabolised and then excreted from animals as not metabolised like that and go in the sewage structures and water sources such as rivers, lakes and so on. Observed resistance in some major human bacteria-causing infections such as *Campylobacter* spp., *E. coli, Salmonella* spp. and enterococci; is due to farm animals (WHO, 2014). Physicians for Social Responsibility (2013) stated that £29.9 million of antibiotics were retailed for cattle and poultry production as equalled to £7.7 million of antibiotics sold for human use.

2.4.4 Lack of appropriate dispense

During production of antibiotics the active pharmaceutical ingredients might be discarded inappropriately and might cause contamination to the environment. When the antibiotics are manufactured; the contamination should be kept to a minimum. Other companies which are

manufacturing these ingredients dump their wastes any how or discharge their untreated effluent to nearby water sources. For example Swedish researchers in 2007 examined one of the sewage treatment plants of India receiving about 90 bulk of discharge from API industrialists and found that outrageous quantity of pharmaceutical active constituents were released into this plant and then discharged to a nearby river. The researchers also found that a commonly used antibiotic (ciprofloxacin) was present and exceeded toxic levels to some bacteria by 1000-fold and thus this proves industrial waste discharged from certain regions exhibit extremely high concentration of antimicrobials (Larsson, 2014). Environments contaminated by unwanted materials from antimicrobial producing companies might stand a chance to be chief reservoirs of antimicrobial resistance (Bengtsson-Palme et al., 2014; Flach et al., 2015).

Another study in China discovered that there were high levels of oxytetracycline residue from a manufacturing facility that claims to treat its waste before discharging (Li, 2008). Other antibiotics are administered to patients using needles; those needles might be dispensed directly to the environment in some healthcare facilities. Sahoo et al. (2010) states that improper dumping of pharmaceuticals or antibiotics contaminate both aquatic and terrestrial environments allowing bacteria to grow resistance and lead to the economic burden of AMR. According to Sahoo et al. (2010), wastes from hospitals, out-dated treatments from shops and household medicines wastes are incorrectly discarded and thus contaminate the environment. Aquatic contamination by pharmaceutical wastes might cause waterborne bacteria to develop resistance as they are going to be exposed to low doses of antibiotics.

2.4.5 Lack of new antibiotics invented and lack of appropriate regulations

As the current antimicrobials are becoming ineffectual, development of new antimicrobials is scarce for replacing the recently ineffective antimicrobials because of AMR. Because of economic and regulatory obstacles the pharmaceutical industries do not afford to develop new

antibiotics as a result fifteen of the eighteen biggest pharmacological corporations have deserted the industry of antibiotics (Bartlett et al., 2013). Even some researches cannot be conducted due to unavailability of funds for example another antibiotic research which was conducted in Academia was called off due to funding challenges triggered by economic misfortune (Piddock, 2012). Most of antimicrobial producing industries have switched to production of drugs that treat chronic diseases because they claim that antimicrobial production has less or no profit as compared to chronic conditions medications. The reason for this might be that antimicrobials are only used for relatively diminutive durations and are regularly therapeutic (Piddock, 2012; Gould and Bal, 2013; Golkar et al., 2014). One of the factor that contributes to the lack of new development of antibiotics is the comparatively cost of antibiotics. The overall cost of novel antimicrobials is maximally $1,000 to $3,000 per course when comparing with expenses of cancer chemotherapy that are about tens of thousands of dollars (Piddock, 2012; Bartlett, 2013; Gould and Bal, 2013; Wright, 2014).

There are still few available agents that still hold the efficiency of treating emerging, extremely resistant gram-negative bacteria like *P. aeruginosa*, Enterobacteriaceae as well as *Acinetobacter baumannii* while there are limited available antibiotics that can be used in treating methicillin-resistant *S. aureus* (MRSA) (Lushniak, 2014). Novel antimicrobials developed at present are simply improvements of the existing families of antimicrobials and these modifications bring simply a temporal solution to the AMR problem whereas this problem needs a permanent solution. WHO, 2017 also underlined the need for invention of new effective antimicrobials confirming that there is extremely limited diversity of therapeutic selections for illnesses caused by resistant bacteria such gram-negatives, together with *Acinetobacter* spp.; *E. coli* and *Klebsiella* spp., and these organisms exhibit an ability to cause drastic and normally lethal infections that pose a significant threat in hospitals and nursing care facilities. However; even if new antibiotics have been developed, the factors that

caused the dilemma of AMR will continue being practised and hence even the new antimicrobials will be ineffective at some point. Therefore, there should be strict regulations for both the production and distribution of antimicrobials. Currently there are no firm regulations protecting the use of antimicrobials for example some villages lack an effective animal healthcare systems and therefore for treating their animals they seek treatments from informal suppliers (Meek et al., 2015). This primes to insignificant usage of antimicrobials. In order for regulations to work, enterprises such as fast food stores; general producers as well as food traders need to volunteer on keeping and comply with guidelines to reduce the this crises of AMR.

2.5 Occurrence and spread of antimicrobial resistant pathogens in vegetables

Resistant microorganisms do not only occur in the environment, humans and livestock but also occur in food products such as vegetables. The part that vegetables and food plays in the dilemma of AMR is huge and is of concern (Salvador et al., 2016). Bacteria in food and fresh produce comprising resistant genes may spread resistance genes to additional pathogens in the human abdomen and then cause severe infections to humans which cannot be treated because of resistance. Food chain is another factor that contributes on AMR. As the microorganisms are found everywhere in the environment; crops are at high risk of being contaminated by irrigation water and soil and can also be in contact with the bacteria during handling, production, transportation and processing, creating health risks for the consumers. The use of polluted water discharges from human and veterinary medicine for irrigation is the most leading factor of contamination of vegetables by resistant pathogenic bacteria (Heaton and Jones, 2008). ACMSF (1999) reported that *Salmonella* (*S. typhimurium* DT104) exhibited resistant characteristics against multiple antibacterials in food at UK. Major outbreaks reported linked to fresh produce have raised a concern with regard to the fresh produce's safety (Sivapalasingam et al., 2004; Hanning et al., 2008; Hanning et al., 2009; Strawn et al., 2011).

In United States and in Europe, the latest disease outbursts reported have been aligned with leafy vegetables such as spinach, lettuce, cabbage and lettuce (Nygård et al., 2004; Soderstrom et al., 2005; Nygård et al., 2008).

Consumers are at risk of contracting diseases caused by Enterobacteriaceae members due to consumption of contaminated fresh produce with pathogenic Enterobacteriaceae, and these diseases might be difficult to treat as they might be associated with AMR (Heaton and Jones, 2008). Fresh produce is one of the major sources of ESBLs in communities (Ben-Ami et al., 2016). High occurrence of resistance genes has been observed in animals producing food (Mesa et al., 2006; Overdevest et al., 2011), and this attracts attention to food chain systems about AMR. A study of Ruimy et al. (2010) conducted in France stated that vegetables in France were often contaminated with resistance genes. The epidemic of *E. coli* capable of producing Shiga toxins around the year 2011 was triggered by *E. coli* producing beta-lactamases (Buchholz et al., 2011). Animal faeces have huge amounts of multiple antimicrobial resistant pathogens like *Listeria monocytogenes*, ESBL-producing *E. coli*, vancomycin-resistant *Enterococcus*, MRSA, *Salmonella* spp. and other Gram-negative Enterobacteriaceae (Guber et al., 2007), and these wastes are used as fertilizers in crop production. Venglovsky et al. (2009) and Marti et al. (2013) stated that fertilisers prepared using faecal droppings comprise antimicrobial resistant pathogens, and consequently they intensify the abundance of AMR genes in soils and crops. Countless incidences of vegetable contamination have been described (Ruimy et al., 2010; Reuland et al., 2014).

Vegetables are principal factors carrying resistant microbes to the human gut because they are likely used as ready-to-eat salads, smoothies and others are consumed raw or preheated and unwashed (Uzeh et al., 2009). Even if vegetables are washed, pathogens cannot be reduced or eliminated to an acceptable level by only washing, even with addition of sanitizing agents (Lynch et al., 2009). This is because pathogenic microorganisms can either adhere to plant stem

and leaves, or become internalised through the stomata, cut edges of leaves or through the roots via contaminated irrigation water (Lynch et al., 2009). Vegetables are regarded healthy as they are high in fibre and less calorie yielding food, consist of a reduced amount of protein and fat and are substantial in vitamins; and minerals but they contain a large amount of AMR microbes.

The increasing occurrence of food borne diseases is linked with the ingestion of contaminated vegetables (Andrenne et al., 2001). These food borne infections contracted through vegetables can be lethal due to AMR (Aarestrup et al., 2008). Foodborne epidemics due to consumption of raw vegetables have been repeatedly reported (Beuchat et al., 2001; Mukherjee et al., 2006; Kim and Woo, 2014). Increased occurrence and spread of AMR in microorganisms in lined with raw vegetables have been reported global (Threlfall et al., 2000; Van den Bogaard and Stobberingh, 2000; Aarestrup et al., 2002; Hayes et al., 2003; Schroeder et al., 2004). From the year 2008 to 2011 European Food Safety Authority abbreviated as EFSA, (2013) reported a rise of foodborne occurrences with 35% of cases that lead to hospitalisations and 46% of deaths from products of non-animal origin such as fresh produce.

Gbonjubola et al. (2012) isolated certain bacteria from ready to eat salads and observed that concentration of bacteria from salad samples was within a range of 6.0 x 10^4 and 2.0 x 10^6 cfu/ml, and mainly pathogens that were detected were *P. auriginosa*; together with *S. aureus*; also *E. coli* was also detected along with *Salmonella* spp. A great resistance against amoxicillin was observed in these pathogens and some were multiple resistant to the antibiotics used in the study. In the year 2008, May, in Oman at Royal Hospital an outbreak of a nosocomial outbreak with gastroenteritis caused by *Bacillus cereus* (*B. cereus*) from vegetables was reported by Al-Abri et al. (2008) and Zahra et al. (2016) which affected fifty eight people. About 2987 outbreaks of gastroenteritis were reported in May 2011 from May to July in Germany; while at the same period 855 outbreaks of hemolytic-uremic syndrome were also reported and fifty

three deaths were also reported in the same duration. All these outbreaks were driven by Shiga toxin producing *E. coli* O104:H4 recovered from fresh produce (Zahra et al., 2016).

Frequently used vegetables for the preparation of salads were studied by Nipa et al. (2011). The selected vegetables for the study encompassed beetroot, carrot, coriander leaf, cucumber, green chilli, lemon, pepper mint and tomato. In this study, the selected fresh produce were found to be greatly contaminated by faecal coliform, yeast and mold. Pathogen with the highest occurrence observed was *Enterobacter* spp. with a proportion of 21.80%; a proportion of 19.17% representing occurrence of *Pseudomonas* spp.; while *Vibrio* spp. followed by 16.92%. On the other hand *Lactobacillus* spp. showed a moderate percentage of 15.04%; yet *Staphylococcus* spp. was 10.15% present. Proportions of *Klebsiella* spp.; *E. coli* and *Citrobacter* spp. were 9.04%; 4.89% and 2.26% respectively; while *Salmonella* spp. showed the least prevalence of 0.37%. For susceptibility test, 98% of the isolates exhibited multiple drug resistance to used antibiotics like ampicillin, cephalexin, chloromphenicol, ciprofloxacin, erythromycin, gentamycin, and streptomycin. Severe worldwide epidemics have been connected with vegetables, for instance, an outbreak around 2011 in Europe was caused by fenugreek seed contaminated with *E. coli* O104:H4; while another one in America was triggered by tomato as well as spinach contaminated with *Salmonella* spp. and *E. coli* O157 (EUCAST, 2012; Olaimat and Holley, 2012; EFSA and AIT GmbH, 2013). Fresh produce have recently became prospective drivers of food borne diseases caused by antimicrobial resistant microorganisms.

2.6 Major sources of antimicrobial resistant pathogens in vegetables

The direct application of antimicrobials for crop culturing, the usage of contaminated fertilizers as well as direct usage of irrigation system can be primary drivers of fresh produce contamination by antimicrobial resistant microorganisms (Witte, 1998). Processes such as

horizontal transfer, transmit resistant genes from contaminated fresh produce to microorganisms habituated in soil and thus drives resistance again towards animals and humans thru crop ingestion (Witte, 1998; Nwosu, 2001; Sengeløv et al., 2002). Several outbreaks of *E. coli* O157:H7 that are related to fresh produce consumption have been reported from farms which use manure as the fertiliser (Chapman et al., 1997; Jiang et al., 2002). This might be because pathogens from the manure can enter into the plant root tissues and propagate throughout plant (Solomon et al., 2002). Crop exposure to antimicrobials can occur when antimicrobials are discharged in agricultural lands. Certain antimicrobial agents such as tetracycline, trimethoprim, ciprofloxacin, to mention few can be easily taken in by plant roots of some plants such as lettuce, cabbage, spinach and carrot (Franklin et al., 2015; Hussain et al., 2016). Antibiotic resistance in crops find its way to the food supply systems, and then spread to humans, environment and animals. Recently, foods that are being sold are Genetic Modified Organisms (GMO's), processes like in vitro are part of processes used to design GMO's in vitro is one of the factors causing antimicrobial resistance in crops (Rashmi et al., 2017). During this process, antimicrobial agents from different antimicrobial families such as aminoglycosides; tetracyclines, cephalosporins and beta-lactams are used. The usage of these antibiotics in the process can enable the chance of AMR to build up in the genetically modified crop.

Application of pesticides, also contributes on the occurrence of antimicrobial resistance in crops. Other pesticides constitute antibiotics on their chemical composition. When pesticides are applied to manage microorganism's occurrence, the pesticides only suppress microbial growth of receptive strains, and hence resistance to those unsuppressed strains increase and spread amid other microbes (Rashmi et al., 2017). Processes such as transduction, conjugation, transformation and horizontal gene transfer favours the spread of resistant genes amid the environment (Shistar, 2011). Study of Kabir et al. (2014) tested the effectiveness of some

antimicrobials such as imipenem, ampicillin, gentamicin, etc. against certain bacterial species such as *Shigella* spp., *Salmonella* spp., *E. coli*, etc. from vegetable samples collected from local marketplace, and *E. coli* resistance against these antimicrobials was 57.14% high resistance of 62.5% was observed from isolates recovered from vegetable samples obtained from supermarket (Kabir et al., 2014).

2.7 Occurrence and spread of antimicrobial resistant pathogens in hospitals

Hospitals have a very important role in our lives and in communities. Hospitals serve as a place of rescue from illnesses and accidents. Not only physical health that is taken care in hospitals, also emotional and mental issues are taken care of as there are social workers and centres for those needs mental healing. The very important role of all hospitals is to deliver acute in-patient and emergency care to those requiring such services. However, as helpful as hospitals are, fact remains that they are the main grounds for the establishment and transmission of antimicrobial resistant pathogens. According to Kunz and Brook, (2010); great occurrence of resistant coliform bacteria is mainly found in hospitals. AMR is an increasing troublesome issue in countless pathogens and is more dominant in hospital acquired (nosocomial) infections. Infections attained in hospital environments are transferred from one sick individual to another; such transmitted infections are recognised as hospital acquired infections. The patient might either exhibit or not exhibit any signs of the presence or occurrence of any resistant pathogen.

Countless methods have been designed to restrict transmission of microorganisms in hospital environments. Hand washing cleansing with sanitizers and disinfectants is one methods of restricting transmission of microbes around hospital settings. In hospitals it is where antimicrobials are extensively used as a result hospitals are the main sources of AMR, and antibiotic resistant bacteria mainly are transmitted in hospitals. There is great risk of transmission between direct contact of a healthcare staff together with the infected patient or

cross transmission could be from infected healthcare worker towards patient. Hospital acquired infections (HAI) and AMR are amid the greatest public health issue of the 21st century, globally (Eurosurverillance, 2010; ECDC, 2012). The Council of the European Union (2009) estimates that more than 8-12% of the people in Europe are hospitalised mainly as a result of HAI. About 37 000 deaths annually are due to HAI in Europe (WHO, 2010). These HAIs also contribute in economic burden because about 7 million Euros are spent on extra nursing care, treatment costs and for secondary operations every year (WHO, 2010). Recently HAI is no longer an issue that goes individually but it is now accompanied by the snowballing emergence of antimicrobial resistant microbe in numerous medical institutes and this dramatically restricts the spectrum range of operative antimicrobials.

The healthcare costs and productivity losses caused by HAI in line with AMR are about 1.5 billion Euros per year, and are also responsible for around 25,000 deaths annually (ECDC, 2012). In the United States expenditures each and every year experienced because of AMR are reported to be nearly $4 billion and they are not stationery but increasing with ease (U.S. Congress, Office of Technology Assessment, 1995). Communal incidence of AMR is relative a minor proportion when compared to AMR occurrence in hospitals especially intensive care units (ICUs) (McGowan et al., 1983). This great incidence of AMR extremely induces physicians to investigate means of ending or reducing AMR. NNIS obtained data indicates an alarming increase of resistance of hospital-acquired *Enterococcus* spp. against regularly applied antimicrobial i.e. vancomycin. Resistance against vancomycin was harboured by 14.2% of all enterococci linked with infection in ICU patients in December 1993. These enterococci pathogens were not only resistant to vancomycin but also displayed resistance to entirely presently effective antimicrobials (Frieden et al., 1993). An escalation of *K. pneumoniae* harbouring resistance against extended-spectrum antibiotics in hospitals was reported by Monnet et al. (1994). It was observed to increase from 1.5% in 1986 to 12.8% in

1993. The transmission of these strains crossed between one hospital to another as they were initially observed in one hospital but later were identified in other surrounding hospitals. At first Methicillin-resistant *Staphylococcus aureus* was traditionally from hospitals but now it has widely abundant outside hospitals (Layton et al., 1995).

Unreasonable antimicrobial resistance proportions from patients suffering from cancer were reported by the University of Texas in 2014 (Nesher and Rolston, 2014). Another difficult AMR was reported by the University of Warsaw from patients who underwent liver transplants (Kawecki et al., 2014). Bacterial strains such as KPC have emerged and they are highly prosperous in being transmitted in hospital settings (Mathers et al., 2015). Point prevalence survey (PPS) investigating HAIs and antimicrobial use in critical care institutes across Europe was organised by ECDC between the year 2011 and 2012. This survey detected that there were 6% of incidences of patients harbouring at least one HAI in those critical care facilities, the country range being 2.3%–10.8%. Out of 15 000 HAIs identified from this survey, the greatest HAI encountered was respiratory tract infections, 19.4% was pneumonia whilst 4.1% was lower respiratory tract, and for urinary tract infections proportion was 19.0%, whilst 10.7% was for bloodstream infections and 7.7% was accredited to gastro-intestinal infections (ECDC, 2012).

2.8 Occurrence of Antimicrobial resistant pathogens in River water

Rivers are an example of fresh surface water and are very important as they have many significant responsibilities. Rivers play an important role as they are earth's nutrients and water carriers, and henceforth, comprise a significant role on cycling of water; they are habitats for so many life forms and source of food for many organisms, act as drainage channels for surface water, used for irrigation and so on. Rivers can provide potable water (especially in undeveloped and developing countries) and many other domestic uses. However, rivers can be

contaminated by human activities such as release of sewage water to rivers, faecal contamination, runoffs of water containing pesticides and heavy metals, etc. Once the river is contaminated, the use of that river water can lead to outbreak of diseases. A free suspended bacterium in the water is one of the leading pollutants of river water (Noble et al., 1997).

Even though pathogenic microorganisms occur in every life form, however, high occurrence of faecal microbes in rivers is declared as a critical matter (Bayoumi and Patko, 2012). This is the principal reason why faecal coliforms together with intestinal enterococci are declared as excellent indicating microbes for the presence of faecal contamination together with pathogenic microbe in river water or surface water. U.S. Environmental Protection Agency (2010) states that in United States 480 000 km of rivers together with coastlines are suspected to be highly contaminated with pathogenic microorganisms while two million ha of lakes is also implicated with these microbes. Diseases that are associated with bacterial contamination are referred as water-borne diseases. These water-borne diseases are caused by different bacteria, and are connected with countless epidemics (Craun et al., 2006). Examples of water-borne illnesses include Cholera, Typhoid, Anaemia, Trachoma, Hepatitis, Diarrhea etc.

According to Fenwick, (2006) water-borne illnesses affect millions in developing countries as they still depend on river or surface waters for drinking water and other domestic uses. 3.4 million Individuals pass away each and every year because of diseases linked with contaminated water, and mostly affected are kids (WHO, 2014). On the other hand UNICEF, (2014), also announced 4000 children's death each and every day prompted by consumption of contaminated river water. Individuals needing access to treated domestic water are greater than 2.6 billion, and this causes roughly 2.2 million deaths per year; and of this 2.2 million; 1.4 million are death of children (WHO, 2010).

About 10% of Nationwide's disease epidemics are to be blamed for poor treated water, and this is the major communal health threat and this issue is highly occurring in developing countries (Pruess et al., 2008). Even though water-borne diseases are more prevalent in developing countries, they are also observed in developed countries, and they are a critical subject. From the data compiled by Arnone together with Walling in investigating AMR occurrences aligned with water, the data demonstrated 5,905 cases and 95 epidemics linked with recreational water in the U.S between 1986 and 2000 (Arnone and Walling, 2007). From this data, about 29.53% of the reported cases were gastrointestinal infection expressed by certain symptoms such as abdominal aching, diarrheal, informal throw up and nausea. From these cases, *Shigella* spp. was responsible for 27% cases; while 16.84% was due to *E. coli* 0157:H7; whilst 12.63% was to be blamed to *Naegleriafowleri* and 7.37% was caused by *Schistosoma* spp. Other than critical gastroenteritis, there are other species that responsible for several AMR occurrences of river water such *E. coli* 0157:H7; *Cryptosporadium*; *Salmonella* spp.; *V. cholerae* together with *Giarida* (Craun et al., 2006).

Satisfactory and successful running of industry (for instance, generation of food, generation of power, etc.), agriculture and domestic uses in South Africa are all made possible by stored vital water origins in dams as well as abstraction schemes (Thukela Water Project Report, 2004). About 1 298 deaths at Angola in 2006 were caused by cholera, whilst more than ten thousand individuals were infected with cholera (Thukela Water Project Report, 2004). There were investigations conducted following up these outbreaks, and it was detected that the principal driver for these outbreaks was contaminated potable water. It was suspected that the contamination of this water was due to deprived sanitation, and this cholera epidemic started in Luanda (Thukela Water Project Report, 2004). The main reason responsible for poor sanitation in Angola might be that populations from this developing capital are living under poor conditions such as situations where the communities are filled with garbage slums and

lack consistent sources of hygienic water (Timberg, 2006). In November 2008, another occurrence of cholera was reported by Zimbabwe, which comprised 6 072 cases and 294 subsequent deaths. Along with Zimbabwe, Limpopo Province of South Africa, observed cholera cases also which comprised 187 cases along with 3 deaths (one person was a South African and the other two were Zimbabweans) (Department of Health, 2008). Other cholera cases were reported in Mozambique as well as in Zambia (Department of Health, 2008). All these outbreaks were driven by inadequate supply of treated potable water and poor hygiene along with sanitation. Direct exposure of an individual to a pathogen can be through use of raw surface water as well as contaminated soil, in the course of recreation, using water contaminated by faeces, drinking of unsuccessfully treated potable water or through ingestion of fresh produce contaminated during irrigation or contaminated by soil used for cultivation (Blaak et al., 2011).

The organisms (*Vibrio* spp., *E. coli, Enterococcus* spp., Protozoa, *Salmonella*, etc.,) that normally found contaminating rivers, contain antimicrobial resistance genes. One of the factors contributing to the higher prevalence of AMR is contaminated water with antimicrobial resistant microorganisms, especially in developing countries (Okeke et al., 1999). For example, WoseKinge et al. (2010); Dekker et al. (2015), described high frequency of *E. coli* harbouring resistance isolated from treated as well as untreated water sources in South Africa. Blaak et al. (2011) studied prevalence of antibiotic resistant *Escherichia coli*, intestinal enterococci, *Staphylococcus aureus, Campylobacter spp.* and *Salmonella* spp. on New Meuse from Brienenoord accessible point; Meuse from Eijsden which is an accessible point and Rhine from Lobith which is an accessible point, these rivers are well known to be large rivers. In Meuse *E. coli* showed 48% prevalence of antimicrobial resistance against at least one antimicrobial, while *E. faecium* showed 54% prevalence and *E. faecalis* showed a prevalence of 56%. On the other hand in Rhine *E. coli* showed 32% prevalence, while a prevalence of 59% was observed

in *E. faecium*. A frequency of 50% was observed in of *E. faecalis*. New Meuse had a low prevalence of *E. faecalis* which was 18%, while *E. faecium* observed was 58% and 34% was observed from *E. coli*. For multiple resistance *E. coli* exhibited 65% resistance to more than one antimicrobial, while *E. faecium* exhibited 60% and 43% was displayed by *E. faecalis*. Resistance to five and more antimicrobials was observed in 10% of *E. coli* isolates while 8% was observed in *E. faecium*. Even though there are no detailed proportions of occurrence of *Salmonella enterica*, *Campylobacter coli* and *S. aureus*but they were detected. *E. coli* exhibiting ESBL traits was observed, enterococci extremely resistant against ampicillin as well as aminoglycosides was observed as well, while *S. aureus* showing resistance against methicillin was detected and finally *Campylobacter* spp. harbouring resistance against quinolones was also detected

South Africa is one of the developing countries that still use river water for domestic uses such as drinking water. Successful supply of hygienically treated potable water is poor to nearly 30% of people and henceforth still depend on fresh surface water sources (rivers, streams, ground water and ponds) for drinking (Venter 2010). Since the year 1997, 36% of OR Tambo District under Eastern Cape Province, RSA is been living under no accessible treated potable water, hence 28% of these people depend on rivers or streams for obtaining their domestic water, 4% of them depend on spring water while 2% put their hope of getting water in borehole and the other 2% hinge on dams (Nontongana et al., 2014). However, all of the above mentioned water sources are normally wide-opened to microbial contamination carried from human and animal faeces and environment (Nevondo and Cloete 1999; Lehloesa and Muyima 2000). This means that South African rivers may also comprise a huge number of antimicrobial resistant strains. Research conducted by Ademola et al., (2009), to detect antibiotic resistance outlines of *E. coli* from two of Durban rivers (Palmiet and Umgeni River), RSA. The research revealed 97% resistance against cephalothin in Palmiet River, while 97.1% resistance was

observed in more than one antibiotic. Umgeni River, there was 28.85% resistance against one antibiotic while 71.15% was resistant against more than one antibiotic.

2.9 Drivers of antibiotic resistant bacteria into river water

Freshwater is one of the best habitats on Earth that provide favourable conditions for a number of microbial populations (Carvalho et al., 2012; Rizzo et al., 2013). Because fresh water resources are favourable microbial habitat they might be the source of microbial resistant genes or can be receivers of such genes from human pathogens (Poirel et al., 2005; Baquero et al., 2008; Rizzo et al., 2013). Resistant genes are already abundant in many microbes found in natural water sources and these genes are then transmitted to wildlife as wildlife either reside and/ or find source of food in aquatic environments (Pruden et al., 2012). According to Messi et al. (2005) resistant bacteria exhibit resistance mainly localised in plasmid, intergron or transposon.

Because resistance is plasmid mediated it can be transmitted among water and soil microbial community with ease through horizontal gene transfer, and this resistance might be due to selective pressure (previously exposed to an antibiotic). Selective pressure could be caused by either the exposure to naturally-occurring antibiotics produced by organisms in soil or it may be due to human activities such as inappropriate dispensing of antimicrobial resistance. The study of Chee-Sanford et al. (2001), found that contamination of groundwater with antibiotic resistant bacteria was the seepage from waste lagoons and the source of resistant bacteria was waste disposal from animal agriculture. This might be the same even for river as it is also a fresh water source and therefore animal wastes, final effluents and agricultural wastes are disposed to fresh water sources such as rivers or washed away by rains to rivers.

According to Blaak et al. (2011) the chief basis of resistant microbes and AMR within rivers is the excretion of human and animal faeces that are treated with antimicrobials. Resistant

microorganisms can be driven into rivers by numerous activities which include improper treatment of sewage, leaching of soil or runoffs from surfaces. The excreted faeces regularly contaminate water used for crop irrigation as well as recreation. Humans can be infected by these resistant microbes in several ways depending on the use of the contaminated water for example ingesting raw water from rivers; ingestion of contaminated crops during irrigation; during recreational activities; during bathing and during shellfish harvesting and also the risk depends on the concentration of the pathogen in the water or on ingestion of the pathogen (Blaak et al. 2011; Ouattaraet al. 2011).

Animal faeces consist of several pathogenic microbes that can cause several diseases in humans. These pathogens can be either waterborne or food-borne. The reason why faeces have a proficiency of contaminating water sources is because there are other pathogenic microbes having the ability of living for a long time in faeces, and these faeces are then discharged onto land and carried to rivers through runoffs (FAO, 2006; WHO, 2012). Animal faeces comprise of pathogens like *E. coli* O157:H7; *Clostridium botulinum*; *Campylobacter* spp. as well as *Salmonella* spp. which have proficiency of causing several infections (Christou, 2011). Numerous documentations have highlighted outbreaks allied with *E. coli* O157 connected with waterborne transmission from potable water and water used for recreational (Effler et al., 2001). Forty thousand cases were reported in Swaziland which were prompted by *E. coli* O157 from cattle manure (Effler et al., 2001). Farm livestock which comprised *Campylobacter jejuni* was responsible for 96.6% of infections in Lancashire, UK, (Wilson et al., 2008). Other incidence in line with waterborne diseases occurred in Canada, Walkerton, where there were more than 2300 incidences of gastrointestinal infections induced by *Campylobacter jejuni* as well as *E. coli* O157:H7 which were suspected to be carried away from cattle manure to the town's water source (Auld et al., 2001).

Another driver of AMR in rivers is the release of wastewater final effluents from wastewater treatments plants. Normally before discharge of sewage water to rivers, the sewage treatments plants remove all antibiotic molecules and pathogenic microorganisms from sewage treated (Rizzo et al. 2013). However, this is not always the case due to several reasons which include poor equipment in treatment plants and pressure, or the effluent might be imperfectly treated. The untreated effluent is then released to rivers permitting the circulation of antimicrobials, and antimicrobial resistant microorganisms downstream. South African sewage treatment plants are also supposed to treat their final effluent before discharging it to the fresh water sources. This is highlighted in Act 54 of 1956 established by South African Water Act in 1956. The Act reinforces that, it is a must that effluent should be treated to reach established standards and recycled to the initial water source (Morrison et al., 2001). However this act is no longer really maintained (Turton, 2008). The reason to this is that South African population is rapidly growing and hence causing economic instability and as the population increases, so do the need for water. This leads to operation of sewage treatment plants to be extremely under stress. There should be ways developed by water and sanitation authorities to withstand water resource quality. More pressure is exerted to South Africa's capability to establish methods of enduring its water and sanitation infrastructure so as to meet Millennium Development Goals (MDGs) (Turton, 2008). Sewage effluent is the chief source of contamination to water sources, based on the conducted investigations so far (DWAF and WRC, 1995; WRC, 1997). Leaking municipal wastewater plants convey a major role in contaminating fresh water resources as the leachate is discharged to the water sources because municipal wastewater plants are designated to discharge treated effluent to lakes, ponds, rivers as well as ground water. Sewage releases from municipal wastewater treatment plants are in lined with contamination of water sources (Ngwenya, 2006). Release of untreated wastewater to fresh water sources make an excellent

way of infectious organisms to get into fresh water and the water might be used by several communities without being treated and thus initiate waterborne diseases (Craun, 1991).

South African wastewater reserve is gradually diminishing and thus strongly contributes to the experienced water contamination glitches and cause health issues. This is proved by latest cholera outbreaks (DWA, 2008). At Mpumalanga in Delmas town, there was a reported outbreak comprised of 380 incidences of diarrhoea, and also nine confirmed cases of typhoid fever, while thirty cases were suspected to be typhoid fever (Mail and Guardian, 2005). Occurrence of typhoid fever did not only end in Mpumalanga but spread throughout the country and hence Transkei in the Eastern Cape, Limpopo as well as KwaZulu-Natal were affected (Coovadia et al., 2005). The outbreak in Delmas was suspected to be linked to the town's water supply, which was thought to be contaminated by human faeces. In 2008, 94 patients were diagnosed with diarrhoea and 18 children died in the Eastern Cape Province (Ukhahlamba District Municipality Addendum, 2008). Incidence was reported to be caused by sewage spills from catchment based land activities. Municipal waste (fundamentally sewage encompassing human excretion, organic trashes and detergents) is one of the key foundations of water contaminants in the developing countries (Abbaspour 2011; Fatoki et al., 2012). Not only developing countries that are facing the challenges related to management of wastewater but the whole world does (Fatta et al., 2005). According to Naidoo and Olairan (2014), the massive amount of organic matter and nutrients in improperly treated wastewater has precarious effect on the water receiving sources. In 2012; DWA reported that some of the South African wastewater treatment plants are not treating their effluents sufficiently as a result, hundred and fifty three of wastewater treatment plants were found to be high and critical threats of Cumulative Risk Rating (CRR) (DWA, 2012). These poorly treated effluents are then discharged into the receiving water sources, which affects the downstream ecosystem and rural communities and also degrading the environment. In South Africa fresh water is already in

short supply and therefore if this problem of not treating final effluents adequately persists, the country possibly will soon damage its water resources because of loads of contamination released to water frames upon these wastewater treatment plants.

According to Chin (2010) and U.S. EPA (2012), the chief root of river contamination is the inflows of agricultural activities from agricultural lands to rivers. Also Lupo et al., (2012) has reported that rivers have developed to be the major reservoirs of antimicrobial resistant pathogenic strains driven by agricultural waste contamination. According to Paulse et al., (2012), agricultural fields are along leading sectors that utilise South Africa's water and contribute to contamination of fresh water sources such as rivers. Prophylactic antibiotics are applied in Agricultural sectors to speed up animal production to give massive yields forgetting that this lead to high levels of concentrations of antimicrobials present in rivers (Baquero et al., 2008). According to Von Baum and Marre, (2005), there are studies that have highlighted the antimicrobial resistant *E. coli* from river after being exposed to antimicrobials. The study of Sayah et al., (2005), displayed that *E. coli* that was isolated from piggery effluent of a farm was resistant to multiple ten antibiotics of different families, and the farm releases its effluent into a river. Another high antimicrobial resistance occurrence of *E. coli* was detected from food animals in a farm; resistance was mainly against streptomycin, tetracycline and sulphonamide, and also this farm discharge its effluent directly to a nearby river (Tadesse et al., 2012). In agricultural facilities antibiotics are not only used for treating infections but also widely used for prevention of the spread of infectious diseases among healthy animals such as pigs, veal calves and broiler chickens. This causes these animals to carry extremely high level of concentrations of antibiotic resistant bacteria (MARAN, 2009). An example, 88% of *E. coli* isolated from chicken faeces on a certain farm displayed multiple antimicrobial resistances in the year 2009. The faeces of these animals are washed and the effluent from this agricultural facility is discharged to rivers or flows to nearby rivers and as a result contaminates the rivers

with antibiotic resistant bacteria. About 70% of water contamination is attributed to agriculture. Agricultural facilities such as farms release agrochemicals in extremely huge amounts and massive amounts of organic matter, sediments together with drug residues and saline drainage to fresh water sources (UNEP, 2016). In the developed countries agriculture has been the leading water contamination source more than settlements and industries. About 38% of water bodies of the European Union are crucially beneath pressure from agricultural contamination (WWAP, 2015). In the rivers of the United States of America, the chief source of contamination is agriculture (US EPA, 2016). Also in China agriculture has been reported to be accountable for a large share of surface-water contamination (FAO, 2013).

2.10 Implications of antimicrobial resistance

Before introductions of antimicrobials, there were about 40% death rates attributed to pneumonia prompted by *Streptococcus pneumoniae* (Bartlettand Mundy, 1995), while for *Staphylococcus aureus* infection were at 80% (Karchmer, 1991), whereas 97% patients with endocarditis died (Newman et al., 1954). Transmission of resistance in hospitals and community surroundings introduces clinically, economically and socially implications. AMR threatens the existence of antimicrobial therapy. The use of antimicrobials to cure and inhibit infectious diseases has triggered microbes to adapt resistance mechanisms against antimicrobials applied to inhibit their survival (i.e. antiseptics, antibiotics, antifungals, anti-helminthics, etc.). AMR does not seem to diminish rather it is highly increasing, and hence poses a serious challenge globally and there is not a single strategy that has been implemented to engage the occurrence and transmission of infectious organisms exhibiting resistance against the existing antimicrobial drugs. This dramatic increase of AMR shows that the initially operative microbial agents today will be insignificant in the next coming decades.

2.10.1 Economic burden/implications by AMR

The issue of AMR is a worlwide issue which brings economic burden. Not only antibiotic resistant Enterobacteriaceae that has emerging resistance but also diseases such as tuberculosis and gonorrhoea display greater proportions of AMR. In 2013 there were approximately 480,000 reported incidences of tuberculosis displaying resistance against multiple antimicrobials in 100 countries. This type of TB is normally referred as MDR-TB representing multidrug-resistant or XDR-TB, abbreviation for extensively drug-resistant TB (WHO, 2012). Resistant diseases such as Tuberculosis (TB) double the cost of standard treatment of a normal TB which cost around $13,000- $30,000), but MDRTB can increase the costs up to $180, 000 (Wilton et al., 2001; Rajbhandary et al., 2004). More money is spent on finding alternative resources for treating infections, and hence AMR has become a leading economic and public health issue. AMR leads to more patients being admitted to hospitals due to resistant infections, and resistant infections lead to the usage of costly antimicrobials and extended hospital admissions as more attention is needed for treating these kinds of infections, and this could cost up to two times more as compared to infections caused by a susceptible pathogens. Failure on finding alternatives of antimicrobials, AMR will lead to extremely high death rates. ESBL-producing pathogens cause extremely treatment delays and hence lead to patients being admitted for lengthy durations. Infections that are due to ESBL producers are expensive to cure, consist of adverse clinical and microbiological effects and involve high mortality (Endimiani et al., 2005). There are more than 25,000 mortalities a year in Europe accredited to resistant pathogens, and this can cost up to €1.5 billion a year, while on the other hand US experiences more than 63,000 mortalities a year linked to AMR (WHO, 2012).

To prove that AMR has major economic costs, estimates indicate that about $55 billion annually in the US alone are spent on therapeutic purposes (Smith and Coast, 2013). According to the Institute of Medicine (1998) budget of infections triggered by antimicrobial resistant

organisms in the US are estimated to be about $4 to $5 million. In Europe in the year 2007 the economic costs of AMR was approximately 1.5 billion and in the USA were approximately $55 billion in 2002 and these figures included both patient and hospital costs (ECDC, 2009). Production losses in USA were roughly 64%, which is equivalent to $55 billion, while in Europe they were roughly 40%, which is equivalent to €1.5 billion (ECDC, 2009). Infections due to antimicrobial resistant organisms approximately resulted in 2.5 million extra hospital days and this cost about € 928 million, outpatient care costs were about € 10 million and productivity losses were estimated at more than € 150 million according to 2007 data of Europe (ECDC and EMEA, 2009). However, the impact of the underlying disease creates complications in evaluating exact costs acquired by antimicrobial resistance. Because of MRA, a cost to a general hospital can cost up to £500,000 in five weeks when there are outbreaks such as Methicilin-resistant *Stapylococcus aureus* (MRSA) (Cox et al., 1995).

There are additional and lengthy ICU admissions and extended hospital stays caused by AMR. Also patients might probably be transferred to long-term facilities and thus thereby accumulate costs further than hospitalization (Carmeli et al., 2002). In the study of Evans et al. (2007), expenditures for infections triggered by resistant gram-negatives were compared with infections triggered by susceptible microorganisms. For hospital admission expenditure; the difference was $51,000 while the difference for antimicrobial cost was $1,800 extra per case. Roberts et al. (2009) conducted a research to determine economic impact caused by antimicrobial resistance in the year 2008 and the findings of the study evaluated that medical costs of about 13.5% patients with antimicrobial resistant infections would range from $18,588 to $29,069 per patient in a single hospital patient cohort. About 2 million individuals in the United States get sick with antimicrobial resistant infections annually and this leads to economic cost that is up to $ 20 billion in direct healthcare costs (CDC, 2013). Due to this economic burden of AMR, in Italy in the year 2013, the pharmaceutical expenditure was

around € 26,034 million and this account for or 1.7% of GDP. The studies of Taylor at al. (2014) and KPMG LLP (2014) showed that more than 7% of GDP world's economy would probably be lost by 2050 or lose $ 210 trillion over the next 35 years because of this alarming increase of AMR.

2.10.2 Clinical implications

AMR causes adverse effects and along those adverse effects there are clinical implications which are death or treatment failure of antimicrobial agents to cure infections according to Eliopoulos et al. (2003) infectious diseases caused by resistant bacteria have more adverse clinical impact than the susceptible bacteria. The failure of treatments and delays to patients and healthcare settings causes adverse effects of antimicrobial resistance. Comparing illnesses triggered by MRSA and methicillin-susceptible *S. aureus* (MSSA), the illnesses caused by MRSA have radically high fatality rate (Rello et al., 1997; Garnacho-Monlevo et al., 2008). Also when comparing ESBL-Enterobacteriaceae associated infections and non-ESBL-Enterobacteriaceae infections, the ESBL-Enterobacteriaceae associated infections are allied with greater rates of treatment failure and death in patients (Masdell et al., 1991; Clec'h et al., 2004; Teixeira et al., 2008). Patients infected with *K. pneumoniae* producingESBLs have more treatments disappointments as compared to *K. pneumoniae* not capable of producing ESBL. The most emerging existing treat recently is the Carbapenem-resistant Enterobacteriaceae (CRE) as result infections that are due to carbapenem-resistant *K. pneumoniae* are between two and five times greater death perils compared to infections produced by strains susceptible to carbapenems as a result infections due to CRE arelinked with 48%–71% hospital mortality incidences (Krobot et al., 2004). According to Lautenbach et al. (2001) there are adverse clinical effects when treating infections produced by some associates of Enterobacteriaceae like *E. coli* and *Klebsiella* spp. with drugs such as cephalosporins. According McCarthy et al.

(1999) greater rates of clinical failure were associated with resistant microbes against co-trimoxazole as compared to susceptible ones when comparing two fluoroquinolones and co-trimoxazole.

The study of Raz et al. (2002) which was evaluating the clinical effect of co-trimoxazole resistance in complex UTI in women showed that resistant microbes have worse clinical and microbiological cures. Another study of McNulty et al. (2006) which also focussed on evaluating the problematical UTI in women detected that trimethoprim resistance stemmed lengthier time to symptom resolution, as well as massive re-consultation when compared with susceptible strains. Cephalosporins resistance due to the presence of the AmpC enzyme in some members of Enterobacteriaceae organisms such as *Enterobacter* species during antimicrobial treatment has been related with lengthier hospital days, greater expenses and augmented death (Cosgrove et al., 2002). On WHO published report in 2014 which investigated clinical effects of AMR amid six WHO areas, and it was found that, *E. coli* together with *S. aureus* had more than 50% resistance against cephalosporins (3rd generation), methicillin together with fluoroquinolones. From this report there were 45% deaths ascribed in Africa and South-East Asia to AMR. From the study *K. pneumonia*e showed 50% resistance against cephalosporins (3rd generation) as well as carbapenems and were allied 77% in Africa, 50% death rates in Eastern Mediterranean area, 81% in South East Asia while Western Pacific region had 72%.

2.11 Management of the growing AMR threat

Looking at the current state, it seems like AMR is heading the world forward post-antibiotic state, without immediate action taken, infections would kill once again. The crisis of AMR needs to be tackled with the outmost urgency. Each and every country needs to play part in combatting the alarming increase prevalence of AMR as antimicrobial resistance affects the globe and henceforth intimidates human and animal health. Penalties of infection convey

significant outcomes such as extended hospital days, failure of surgeries and many more. Not only health sectors are affected but several sectors and the whole of society is greatly affected by AMR as well. Strategies on combatting AMR dilemma need investments to support all countries financially as well as technically to develop comprehended antimicrobials, tools to diagnose threatening organisms and new effective vaccines. Other interventions are also required for a successful management of AMR. These interventions include designing guidelines for appropriate use and availability of antimicrobials so as to strengthen health systems. If the alarming increase of AMR is controlled urgently, that will enable the public health to maintain prosperous treatment and hindrance of infections with effectual and nontoxic antimicrobials with assured quality. Several steps can be taken in order to achieve the management of AMR, which include creating and advancing awareness of understanding AMR; to provide understanding on AMR through monitoring; to diminish the occurrence of infection; providing better understanding about appropriate usage of antimicrobials; as well as to establish national guidelines and regulations for the use of antimicrobials.

2.11.1 Public education about AMR

Public teaching can include activities such as enhancing campaigns such as awareness. Awareness can be advanced through operative public communication, education and training targeting different audience especially in human as well animal wellbeing and agricultural practice together with consumers. In the awareness things like washing hands especially before touching food and after using toilets, teaching about AMR (how it occurs and spread), how each and every individual contributes in the increase of AMR should be covered and also there should be researches conducted tackling on assessing how much knowledge people have about antimicrobials. The campaigns should deliver the message conveying that antimicrobials should not be used carelessly, should only be used provided there is a need (used to treat

specific infection). The issue of AMR should also be included in school curricula for instance literacy and antenatal programmes.

2.11.2 Providing the understanding on AMR through monitoring

According to WHO (2015); currently there is little knowledge about the general public's knowledge of antibiotic resistance at a global level. It is obvious that majority of people lack adequate information on important things such as incidence of AMR, choice of antimicrobial across pathogen and environmental patterns interconnected to AMR and such information is important and it should be available in an appropriate way in order to direct cure of patients, to update local, national and regional activities. Consumers should be aware of how resistance occurs and spread. There should be policies and regulations developed against AMR and for such to be achieved, there should be understanding on how resistance circulates between humans; water; animals as well as food in the environment. There should be researches and clinical researches arranged that corresponds with both national and international regulations of curing and hindering infections (WHO, 2015). According to Chief Medical Officer (CMO) (2011), health labours may lack up-to-date information and be unable to identify the type of infection, hence there should be surveillances conducted to find how much knowledge health works have.

2.11.3 Practises to reduce the occurrence of infection

Several practices such as appropriate hygiene, sanitation, vaccination, immunization and food and water safety can prevent occurrence of many microorganisms which some are resistant to current treatments. Practise of safe sex also can prevent the occurrence of infections that are transmitted through sex. Immunization is very important in the field of microbiology as it can stop diseases requiring antimicrobial treatments and can diminish the occurrence of first viral infection which are sometimes incorrectly cured using antibiotics, and which can also induce secondary infection requiring antibiotic usage (Dekker et al., 2015). Hygiene along with

sanitation measures can prevent infections such as diarrhoea and these measures can be achieved by hand washing, using safe water sources (boil the water if untreated) and using latrines in the absence of appropriate toilets. Infections such as malaria can be prohibited through the usage of netted beds infused with insecticide.

2.11.4 Providing better understanding about appropriate usage of antimicrobials

Incorrect usage of antimicrobials in medicine sector which include easy to access antimicrobials over the counter and over prescription is one of the chief factors driving an increase in AMR. This might be because antimicrobial suppliers (paramedical workers, pharmacologists, clinicians and GPs) have insufficient knowledge about AMR and hence underlining the need for them to be taught about how and when to prescribe an antimicrobial. Information on precise diagnosis and controlling and preventing of infections as well as usage of antimicrobials should be provided in all antimicrobial suppliers be either in a form of awareness or included in their both undergraduate and postgraduate curriculum. Accurate diagnosis can prevent inappropriate prescription of antimicrobials, for example the use of malaria blood smears in hospitals assist in diagnosing malaria and hence appropriate antimalarials will be used and use of sputum microscopy helps in diagnosing TB and hence patients will be treated with anti-TB rather than unnecessarily antibiotics (WHO, 2005). This can be successful provided that governments financially support all higher education institutions and national qualified associates to endow independent continuing professional development (CPD) involving AMR issues; endorse delivery of unbiased information to prescribers. Only about 50% of antibiotics are used for human activities, the rest are used in agriculture. This brings a need to liaise with agricultural fields on decreasing the use of antimicrobials and include researches for finding alternative antimicrobials that can be used in this field such as vaccines instead of antibiotics. Farmers, food producers and veterinarians

should be taught good practices such as good husbandry, safe quality of feed, hygiene, proper waste and manure management to prevent infections on farms. There should be campaigns on educating farmers to only use antimicrobials reliably by only using them to treat diseases on the advice of a veterinarian or crop specialist.

2.11.5 Establishing national guidelines and regulations for the use of antimicrobials.

It is no doubt that it is the massive usage of antimicrobials that has led to the rise of AMR and hence there should be restrictions and regulations on the usage of antimicrobials especially in non-human activities. In hospital settings a Committee for Control of Infections should be accountable for overseeing infection control programmes since AMR development and spread is greatly in healthcare facilities due to the adjacent closeness of patients who have infections and are antimicrobials course. The committee should develop and implement rules and techniques to preventing the transmission of infection. According to (WHO, 2005) governments may inspire sanatoria as well as local health sectors to have Drug and Therapeutics Committees (DTCs) by making it an accreditation requirement as DTCs have been fruitful in developed republics in endorsing more balanced, cost-effective usage of medicines as well as antimicrobials in hospitals. Rational use of antimicrobials can be achieved by updating essential antimicrobials and clinical guidelines of antimicrobials. Prescription of antimicrobials should be based on national antimicrobial list guidelines. Regulations which limit availability of antimicrobials can reduce the misuse of antimicrobials. The inappropriate prescription can be lessened provided that antimicrobial given to patients is only approved by a microbiologist. Unlicensed markets of antimicrobials should be fined as they can sell untested and reduced quality antimicrobials driving to deprived patient outcome and increase AMR. According to WHO (2005), guideline, decent obtaining practice and post-marketing monitoring is vital in encompassing AMR.

The dramatic increase of AMR is greatly threatening the spectrum of the available standard antimicrobials and also threatens worldwide economy and thus calls for a serious attention from microbiologist and physicians. AMR also reduces the microbial quality of freshwater resources and food production. The speed in which AMR is moving with shows that the soon there will be no treatment options for treating infectious diseases. All the reported cases about AMR might be just the tip of an iceberg. Common infections which were once easy to treat such as tuberculosis are now becoming impossible to treat. The world of medicine is highly under attack by AMR. Currently there are no new antimicrobials that are in development, and this seriously threatens the world of medicine. Antimicrobials save millions of lives especially those undergoing surgery, and hence there is an urgent need for the development of new policies and strategies to fight AMR. Campaigns about AMR could at least prevent further transmission and emergence of AMR. Another strategy that can reduce the emergence of AMR is to encourage policy makers to establish regulations which limit the use of antimicrobials especially in non-human medicine. Medicinal practitioners need to stop the habit of prescribing antimicrobials incorrectly. Agriculture is one of the principal contributors of MAR increase because of the extensive application of antimicrobials. This field should reduce the level of application of antimicrobials.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Permissions

Permission to collect samples from selected farms, hospitals and rivers at Chris Hani and Amathole District Municipalities (DMs) in this study was sort and obtained, during the reconnaissance visits.

3.2 Study site

The study sites are located within the Amathole and Chris Hani DMs from the Eastern Cape Province, South Africa (Figure 3.1). The study sites were chosen based on the published works that suggest them to be hotspots of antimicrobial resistant faecal Enterobacteriaceae (Blaak et al., 2011) as well as some of the unpublished findings in Applied and Environmental Microbiology Research Group (AEMREG) at the University of Fort Hare, Alice, South Africa.

Figure 3.1: Map of the Eastern Cape Province, S.A showing District Municipalities where the study was conducted (https://localgovernment.co.za).

3.2.1 Demographic Information

Table 3.1: Sampling sites on the Buffalo River

Sampling site	Description
Maden Dam (32°44′22″S; 27°17′54″E)	Situated 7 km from Buffalo River source, close to Amathola Mountains. It serves as a source of water for treatment works. Amathola Hiking Trail begins in this river. The river also serves as a

	recreational area. At the moment there are no forms of any communities found upstream the river.
Rooikrantz Dam (32°45′19″S; 27°19′35″E)	Situated about 4 km down the Maden Dam. This river has a volume of about 5 million m^3. Treatment plants supplying water to the King William's Town and its nearby communities use this river as a raw water source. Rural communities around King William's Town use water from this river for domestic purposes, recreation, herd watering and irrigation.
King William's Town (32°53′23″S; 27°23′17″E)	This sampling site of the Buffalo River is found between King William's Town and one of the rural communities known as Ginsberg. This town consist of a population of over 250 000. This site is located downstream the points in which most of the urban wastes and drains are emptied to. During sampling, the river water was found odorous. Settlements around this point do not depend directly to the river for source of water.
Eluxolzweni (32°56′16″S; 27°27′56″E)	This point is found situated just downstream of water treatments plants and these treatment plants were reported to be dysfunctional (RHP, 2004). Two communities found around this Dam which are Zwelitsha and Phakamisa. There is a great volume of water hyacinth flower and river weeds extending more than a 300 m of distance was covering the water.

Table 3.2: Sampling sites on the Ngcongcolora River.

Sampling site	Description

Ndexe (32°38′22″ S; 26°56′10″ E)	It is a rural location situated at the back of Tsomo town, the River Ngcongcolora passes through this community to Matolweni community. The river is used by nearby rural communities for recreation activities, stock watering. Members of the nearby communities irrigates their garden with the water from this river, use the river as source of drinking water for grazing animals and domestic purposes. There is a small co-orp of vegetable faming close to this point.
Matolweni (32° 4' 48.972" S; 27° 47' 9.06" E)	Matolweni is a rural community situated in Tsomo and next to Ngcongcolora River. The river is situated further downstream of Tsomo River, and consists of a number of heavily populated rural communities. The river is vital as it serves as source of drinking water to the inhabitants and their livestock, used for irrigation purposes, recreation activities and other domestic purposes. A junior secondary school situated in this community known as Wilson Mayekiso Junior Secondary School, contributes to the usage of the Ngcongcolora River. At this point this river converges with Tsomo River.

Table 3.3: Sampling sites related to hospitals, supermarkets and commercial farm samples collection spots.

Sampling site	Description
Mdantsane (32.9276° S, 27.7452° E)	This is the second largest township in South Africa in the city of East London and consists of a human population of more than 250000. The selected hospital is one of the largest hospitals in East London and is funded by the government.

Tsomo (32.0348° S, 27.8165° E)	One of the supermarkets selected for this study is situated in Tsomo. A supermarket situated in Tsomo was selected because it is the biggest franchise supermarket and villagers depend on it for their groceries. Tsomo town is situated 45km east of Qamata and 48 km west of Ndabakazi.
Alice (32°47′17″ S; 26°50′31″ E)	Two supermarkets were selected from Alice town. The town is a semi-urban town consisting of several communities both semi-urban and rural communities. On the north-west of this town there is Golf Course, while Happy Rest and Gaga is situated on the west side of the town, and Gqumashe and Ntselamantsi are found on the north side of the Alice town. Alice population consists of more than 48 000 population and about 6 000 of this population consists of University of Fort Hare's students according to UFH Internal study (2012). University of Fort Hare is located on east of Alice town and the population of the university greatly adds to the Alice population. The selected shops are the main two shops used in Alice.
Queenstown (32°48′37″ S; 26°52′20″ E)	Another hospital and farm selected are in Queenstown. This town is situated at the Middle of the Eastern Cape Province in Chris Hani District Municipality. For the surrounding town this town act as an education, commercial and administrative town and hence this hospital acts as a large hospital in Chris Hani District Municipality. This hospital is government funded. Queenstown is a centre of many agricultural activities especially vegetable and livestock farming. The selected farm distributes its vegetables to certain supermarkets across the Eastern Cape Province especially Chis Hani DM. supermarkets.

Fort Cox (32°43′48″S 27°1′32″E)	Fort Cox is a farm in Middledrift that offers Agricultural and forestry trainings and was selected for sampling.

3.3 Sample collection

3.3.1 Vegetables

Vegetables used in this study comprised lettuce, cabbage, cucumber, carrot, beetroot and spinach. The vegetable samples were collected from three selected supermarkets and two trading vegetable farms in both DMs. Samples were aseptically picked (without touching the sample to avoid possible contamination) with plastic bags and transported on ice to the AEMREG laboratory at the University of Fort Hare for microbiological analyses. The samples were analysed within six hours of collection.

3.3.2 Water and hospital effluents

Water samples were aseptically collected from two selected rivers (Buffalo River in Amathole DM and Ngcongolora River in Chris Hani DM) and two selected Hospitals (one hospital in each District). For confidentiality sake, the names of the hospitals and farms will not be mentioned. Tables 3.1 to 3.3 contain the list of the samples collection sites. Water or hospital effluent samples were aseptically collected using sterile water sampling bottles with tight lids. The samples were then carried on ice to the AEMREG laboratory for microbiological analyses. The samples were analysed within six hours of collection.

3.4 Quantification of indicator microorganisms

For the enumeration of indicator organisms in vegetables, 10 g of each selected vegetable was weighed then added in 90 ml Tryptone Soy Broth (TSB) (Laboratories CONDA, S.A.) and

placed in a sterile stomacher bag and macerated for one minute at 230 rev/min in a Seward Stomacher 400 Circulator, followed by a tenfold dilution series. About 0.1 ml of the homogenised vegetables was plated on Violet Red Bile Glucose Agar using spread plate technique and incubated between 18-24 hours at 30 °C (Laboratories CONDA, S.A.) for the total count of faecal Enterobacteriaceae. This was done in triplicates. For water samples, tenfold serial dilution was performed and for each dilution a 100 ml was filtered through sterile cellulose nitrate membrane having 0.45 µm pore size (MERK, S.A.) with the aid of a vacuum pump. This was done in triplicates. Filter membranes containing bacteria were then aseptically placed on Violet Red Bile Glucose Agar then incubated for 18-24 hours at 30 °C (Laboratories CONDA, S.A.) for the total count of faecal Enterobacteriaceae

3.5 Isolation and identification of presumptive target organisms

3.5.1 Isolation

About 100 ml of properly diluted effluent and river water samples were filtered under vacuum through sterile cellulose nitrate filter membrane. The filter membrane was then placed in a 90 ml TSB (Laboratories CONDA, S.A.) and along with the homogenized vegetables samples incubated at 37 °C for a period of 24 hours. After the incubation period, a loop-full of the enriched samples was streaked to Eosin Methylene blue agar (EMB) and incubated at a temperature of 37 °C for 24 hours for detection of the presumptive *Klebsiella* spp., *E. coli*, *Citrobacter* spp., and *Enterobacter* spp. (Laboratories CONDA, S.A.). Growth of *E. coli* colonies was observed by green metallic sheen on the surface of the EMB agar, while growth of *Klebsiella* spp. was observed by large mucoid pink to purple colonies. Growth of *Citrobacter* spp. and *Enterobacter* spp. were recognized by large mucoid red colonies. The presumptive isolates were further purified by streaking on EMB agar. The selected purified presumptive isolates were then streaked on Nutrient Agar using triple streaking technique and incubated for

a period of 24 hours at 37 °C for further purification and preservation. The purified presumptive isolates were stored in 25% glycerol stock.

3.5.2 Confirmation of the presumptive isolates

Matrix Assisted Laser Desorption Ionization-Time Of Flight (MALDI-TOF) was used for the confirmation of species for the presumptive isolates. Briefly, isolates from glycerol stocks were inoculated with inoculating loop into sterile test tubes with sterile nutrient broth then incubated at a temperature of 37 °C on a shaker overnight after which the cultures were single streaked on plates containing sterile Nutrient agar followed by incubation for a period of 24 hours at 37 °C. Using inoculating loop, colonies were pelleted and suspended in 300 µL of sterilized distilled water, vortexed and added to 100% HPLC grade alcohol. Isolates were then kept on -20 °C until ready for analysis. During MALDI-TOF analysis, using micropipette, 1 µL of each presumptive isolate was suspended in 100 mL solution containing 50% proportion of acetonitrile (ACN) and 1% of the aqueous trifluoracetic acid (TFA) then vortexed and centrifuged at a speed of 8000 rpm for 10 minutes. Supernatants of each sample solution were then transferred to new eppendorf tubes. Using micropipette, 10 µL of a solution known as the matrix, which contains 10 mg of a-cyano-4-hydroxy cinnamic acid (a-CHCA) in 1 mL of 50% ACN and 2.5% of the aqueous TFA was added into 5 µL of the solution containing the sample. The final solution was then platted on MALDI-TOF plate then after the solution was allowed to dry at room temperature. With the use of Bruker Daltonics Ultraflex MALDI TOF/TOF Mass Spectrometer, the mass spectra were obtained. Two extractions from each and every strain were carried out. Each of the two extracts was measured in quintuplicate and therefore gave ten spectra for each bacterial strain. Lists of the data which contains m/z values were extricated from the mass spectra. Identification of each bacterial species was obtained with the use of mass spectrum processing and statistical analysis with R and MassUp software, principal component analysis (PCA) and clustering.

3.6 Antibiotic susceptibility profiles

Identified isolates from water and fresh produce were screened for antibiotic susceptibility patterns using the Kirby Bauer disk diffusion technique as described by CLSI (2018). The disk diffusion method was based on CLSI guidelines. Isolates from glycerol stocks were inoculated into sterilized test tubes containing nutrient broth then after incubated overnight at 37 °C after which the cultures were streaked on plates of Nutrient agar and incubated for duration of 24 hours with 37 °C temperature. The culture was then centrifuged to pellet the cells and rinsed two times with sterilized normal saline and thereafter standardized to 0.5 McFarland standards. The solution which is standardized was then streaked on the whole surface of the Mueller Hington Agar plate (OXOID, Ltd, S.A.) to form a lawn. The antibiotic disk (Davies Diagnostics (Pty) Limited, S.A.) were then dispensed on the lawn using antibiotic Dispenser and incubated for a period of 18 hours at temperature of 37 °C. The antibiotics used were selected across ten families of antimicrobials and comprises: Amino-glycosides: amikacin (30 µg), gentamycin (10µg); cephems: cefuroxime (30 µg); carbapenems: meropenem (10µg), imipenem (10µg); fluoroquinolones: ciprofloxacin (5µg), norflaxocin (30µg); quinolones: nalidixic acid (30µg), sulfonamides: trimetroprim (25µg); nitrofuratoins: nitrofuratoin (300µg); phenicols: chloramphenicol (30µg); tetracyclines: tetracycline (30µg) doxycycline(30µg); cephalosporins: cefotaxime (30 µg); beta-lactamases: combination discs of amoxilin/clavulanate (30/10 µg) and ampicillin (25µg); polymyxins polymyxin B (300µg) and colistin (25µg). After incubation, inhibition zones diameters were measured and interpreted as described by CLSI (2018) guidelines and were interpreted as susceptible (S), intermediate (I) or resistant (R).

3.6.1 Multiple antibiotic resistance indexing (MARI) of the selected Enterobacteriaceae members

Multiple antibiotic resistance indices (MARI's) was determined for each sampling location as described by Krumperman, (1983) with the use of the formulae:-

MARI = a/b

In which "a" denotes the total number of resistance obtained

"b" denotes total number the number of the used antibiotics

3.7 Detection of antimicrobial resistance genes

3.7.1 DNA extraction

Detection of antimicrobial resistance genes was done using Polymerase Chain Reaction (PCR) (Bio –RAD). DNA was isolated with the use of boiling method as descried by Gueimonde et al. (2004). A pure colony of the isolate was suspended in a volume of 200 µL of sterilized distilled water and cell was lysed for 15 minutes at 100 °C using an AccuBlock (Digital dry bath, Labnet). Cell debris was removed by centrifugation at 13 500 ×g for 10 minutes using a MiniSpinmicrocentrifuge (Lasec, RSA). The resulting lysate DNA was used as DNA template for the PCR reaction.

3.7.2 PCR detection of resistance genes

The relevant antimicrobial resistance genes were detected using primers and PCR conditions listed in Table 3.3. After PCR amplification, the amplicons were electrophoresed where every run comprised of a negative control. From the amplicons, 5 µl of each amplicon was resolved on 1.5% agarose gel (Merck, SA) comprising of 5 µl of ethidium bromide (Sigma-Aldrich,

USA). For approximation of the expected band size, 100 bp DNA Gene Ruler (Thermo Fisher Scientific, (EU) Lithuania) was used. A 0.5 X TBE buffer was used to run gels at 100 V for duration of 45 minutes and UV trans-illumination (Alliance 4.7, France) was used to visualize electrophoresed amplicons.

Table 3.4: List of primers and PCR conditions for the detection of target antimicrobial resistance genes

Antimicrobial family	Primer	Primer sequence (5' – 3')	Expected band size (bp)	PCR conditions	Reference
SULFONAMIDES	*sul1*	F: TTCGGCATTCTGAATCTCAC R: ATGATCTAACCCTCGGTCTC	822	Initial denaturation for a period of 5 minutes at 94 °C, followed by denaturation for 1 minute at a temperature of 94 °C, 1 minute of annealing at 55 °C, 5 minutes of elongation at the temperature of 72 °C for 35 cycles; lastly 5 minutes of final elongation at a temperature of 72 °C.	Maynard et al. (2004)
	sul2	F: CGGCATCGTCAACATAACC R: GTGTGCGGATGAAGTCAG	625	5 minutes of the first denaturation at 94 °C, followed by the second denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, then elongation 72 °C for 1 minute, for a total of 30 cycles and final elongation at 72 °C for 5 minutes.	Falbo et al. (1999)
TETRACYCLINES	*tetA*	F: GCTACATCCTGCTTGCCTTC R: CATAGATCGCCGTGAAGAGG	201	Initial denaturation for a period of 5 minutes at 94 °C; then after 35 cycles of denaturation at 94 °C for 60 s, annealing at a temperature of 55 °C for 60 s and elongation at 72 °C for 60 s, and final incubation at 72°C for 5 minutes.	Ng et al. (2001)

Antimicrobial family	Primer	Primer sequence (5' – 3')	Expected band size	PCR conditions	Reference
	tetM	F: AGT GGA GCG ATT ACA GAA R: CAT ATG TCC TGG CGT GTC TA	158	Initial denaturation for a period of 5 minutes at 94 °C; then after 35 cycles of denaturation at 94 °C for 60 s, annealing at a temperature of 55 °C for 60 s and elongation at 72 °C for 60 s, and final incubation at 72°C for 5 minutes.	Strommenger et al. (2003)
AMINOGLYCOSIDES	*strA*	F: CTTGGTGATAACGGCAATTC R: CCAATCGCAGATAGAAGGC	348	First denaturation step at 94 °C for 4 minutes, then after followed by a total of 30 cycles of the second denaturation at temperature of 94 °C for 45 s, annealing for 45 s at 50 °C, elongation at 72 °C for 45 s and final elongation for 7 min.	Velusamy et al. (2007)
	strB	F: GGCACCCATAAGCGTACGCC R: TGCCGAGCACGGCGACTACC	470	First denaturation step at 94 °C for 4 minutes, then after followed by a total of 30 cycles of the second denaturation at temperature of 94 °C for 45 s, annealing for 45 s at a temperature of 50 °C, elongation at 72 °C for 45 s and final elongation for 7 min.	Velusamy et al. (2007)

Antimicrobial family	Primer	Primer sequence	Expected band size	PCR conditions	Reference
	aadA	F: GTGGATGGCGGCCTGAAGCC R: AATGCCCAGTCGGCAGCG	525	First denaturation step at 94 °C for 4 minutes, then after followed by a total of 30 cycles of the second denaturation at temperature of 94 °C for 45 s, annealing for 45 s at 50 °C, elongation at 72 °C for 45 s and final elongation for 7 min.	Velusamy et al. (2007)
	aac(3)-IIa(aacC2)a	F: CGGAAGGCAATAACGGAG R: TCGAACAGGTAGCACTGAG	428	5 min of initial denaturation at 94 °C, followed by 30 cycles of 94 °C for 30 s, 50 ° C for 30 s and 72 °C for 1 min 30 s and final incubation at 72 °C for 5 min.	Maynard et al. (2004)
	aph(3)-Ia (aphA1) a	F: ATGGGCTCGCGATAATGTC R: CTCACCGAGGCAGTTCCAT	600	5 minutes at 94°C, then after 30 cycles of denaturation, annealing and elongation at 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 minutes respectively and final incubation period at 72°C for 5 minutes.	Maynard et al. (2004)
	aph(3)- IIa (aphA2) a	F: GAACAAGATGGATTGCACGC R: GCTCTTCAGCAATATCACGG	510	5 minutes at 94°C, then after 30 cycles of denaturation, annealing and elongation at 94°C for 30 s, 50°C for 30 s and 72°C for 1.5 minute 30 s respectively and final incubation period at 72°C for 5 minutes.	Maynard et al. (2004)

Table 3. 5: List of additional beta-lactam primers and PCR conditions for the detection of target antimicrobial resistance genes

multiplex name	Primer	Expected band size	Primer sequence (5' -3')	PCR conditions	Reference
Multiplex I TEM, SHV and OXA-1-like	*bla*$_{TEM}$,	800,	F:CATTTCCGTGTCGCCCTTATTC R:CGTTCATCCATAGTTGCCTGAC	Initial denaturation for 10 minutes at 94 °C followed by 94 °C for 40 s, 60 °C for 40 s, 72 °C for 60 s for 30 cycles of denaturation, annealing and extension respectively, followed by the last step known as final extension at 72 °C for 7 minutes.	Dallenne et al. (2010)
	bla$_{SHV}$,	713,	F:AGCCGCTTGAGCAAATTAAAC R:ATCCCGCAGATAAATCACCAC		
	bla$_{OXA-1}$	564	F:GGCACCAGATTCAACTTTCAAG R:GACCCCAAGTTTCCTGTAAGTG		
Multiplex II CTX-M group group 2 and group 9	*bla*$_{CTX-M-2}$,	404	F:CGTTAACGGCACGATGAC R: CGATATGGTTGGTGGTRCCAT[b]	Initial denaturation for 10 minutes at 94 °C followed by 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 minute for 30 cycles of denaturation, annealing and extension respectively, followed by final extension at 72 °C for 7 minutes.	Dallenne et al. (2010)
	bla $_{CTX-M-9}$,	561	F:TCAAGCCTGCCGATCTGGT R:TGATTCTCGCCGCTGAAG		
Multiplex III DHA group GES	*bla*$_{DHA}$	997	F:TGATGGCACAGCAGGATATTC R:GCTTTGACTCTTTCGGTATTCG	Initial denaturation for 10 minutes at 94 °C followed by 94 °C for 40 s, 60 °C for 40 s, 72 °C for 60 s for 30 cycles of denaturation, annealing and extension respectively, followed by final elongation at 72 °C for 7 minutes.	Dallenne et al. (2010)
	bla$_{GES}$,	399	F:AGTCGGCTAGACCGGAAAG R:TTTGTCCGTGCTCAGGAT		

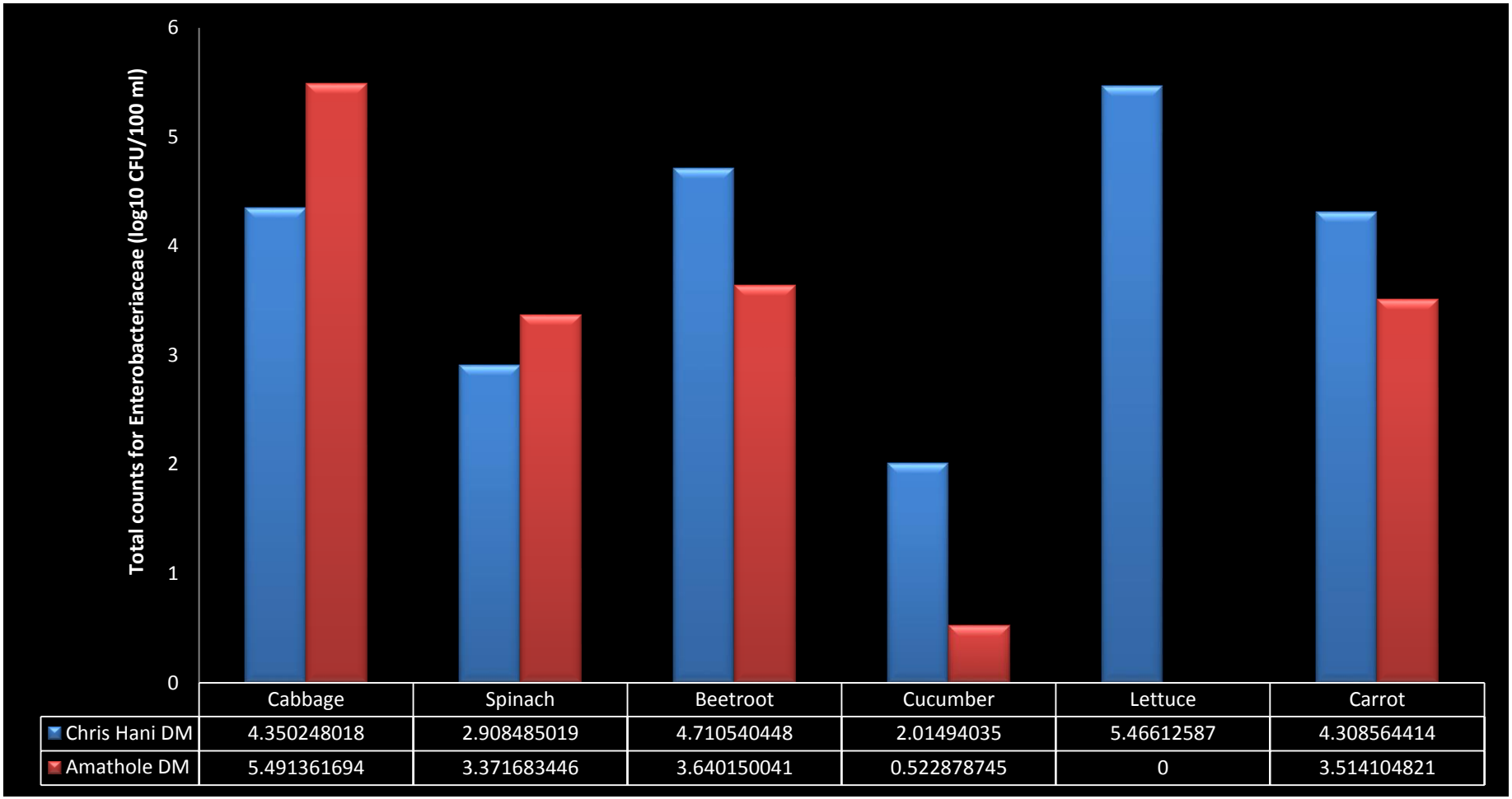
Multiplex name	*primer*	Expected band size	Primer sequence (5' – 3')	PCR conditions	Reference
Multiplex V GES and OXA-48-like	*bla*$_{GES}$,	399	F:AGTCGGCTAGACCGGAAAG R:TTTGTCCGTGCTCAGGAT	Initial denaturation for 10 minutes at 94 °C followed by denaturation, annealing and extension at 94 °C for 40 s, 60 °C for 40 s, 72 °C for 60 s for 30 cycles of respectively, followed by final extension at 72 °C for 7 minutes..	Dallenne et al. (2010)
	bla$_{OXA-48}$	281	F:GCTTGATCGCCCTCGATT R:GATTTGCTCCGTGGCCGAAA		

CHAPTER FOUR

4.0 Results

4.1 Prevalence of presumptive Enterobacteriaceae

The total Enterobacteriaceae counts obtained from the analysed vegetable samples ranged between 0 and 3×10^5 CFU/g and the highest count was obtained from cabbage sample from Amathole D.M. (Figure 4.1). Whereas total Enterobacteriaceae counts obtained from the analysed river samples ranged between 10 CFU/ 100 ml and 8×10^2 CFU/100 ml (Figure 4.2). While from the analysed hospital effluents samples, the total Enterobacteriaceae counts obtained ranged from 10 CFU/ 100 ml to 1×10^2 CFU/100 ml as shown in Figure 4.3.



	Cabbage	Spinach	Beetroot	Cucumber	Lettuce	Carrot
Chris Hani DM	4.350248018	2.908485019	4.710540448	2.01494035	5.46612587	4.308564414
Amathole DM	5.491361694	3.371683446	3.640150041	0.522878745	0	3.514104821

Figure 4.1: Relative mean counts of Enterobacteriaceae in vegetables from the selected study sites.

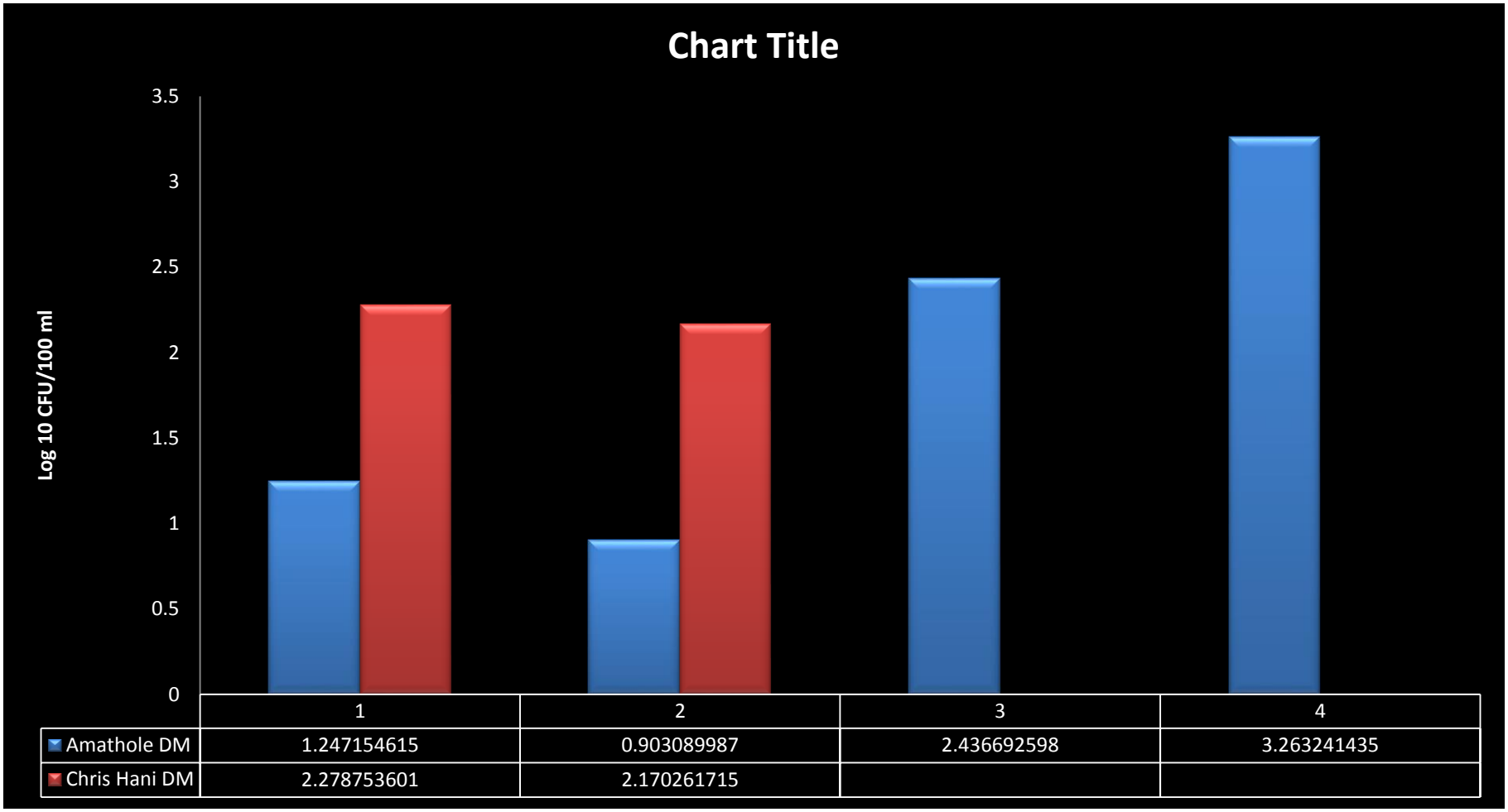


Figure 4.2: Relative mean counts of Enterobacteriaceae in river water from selected study sites.

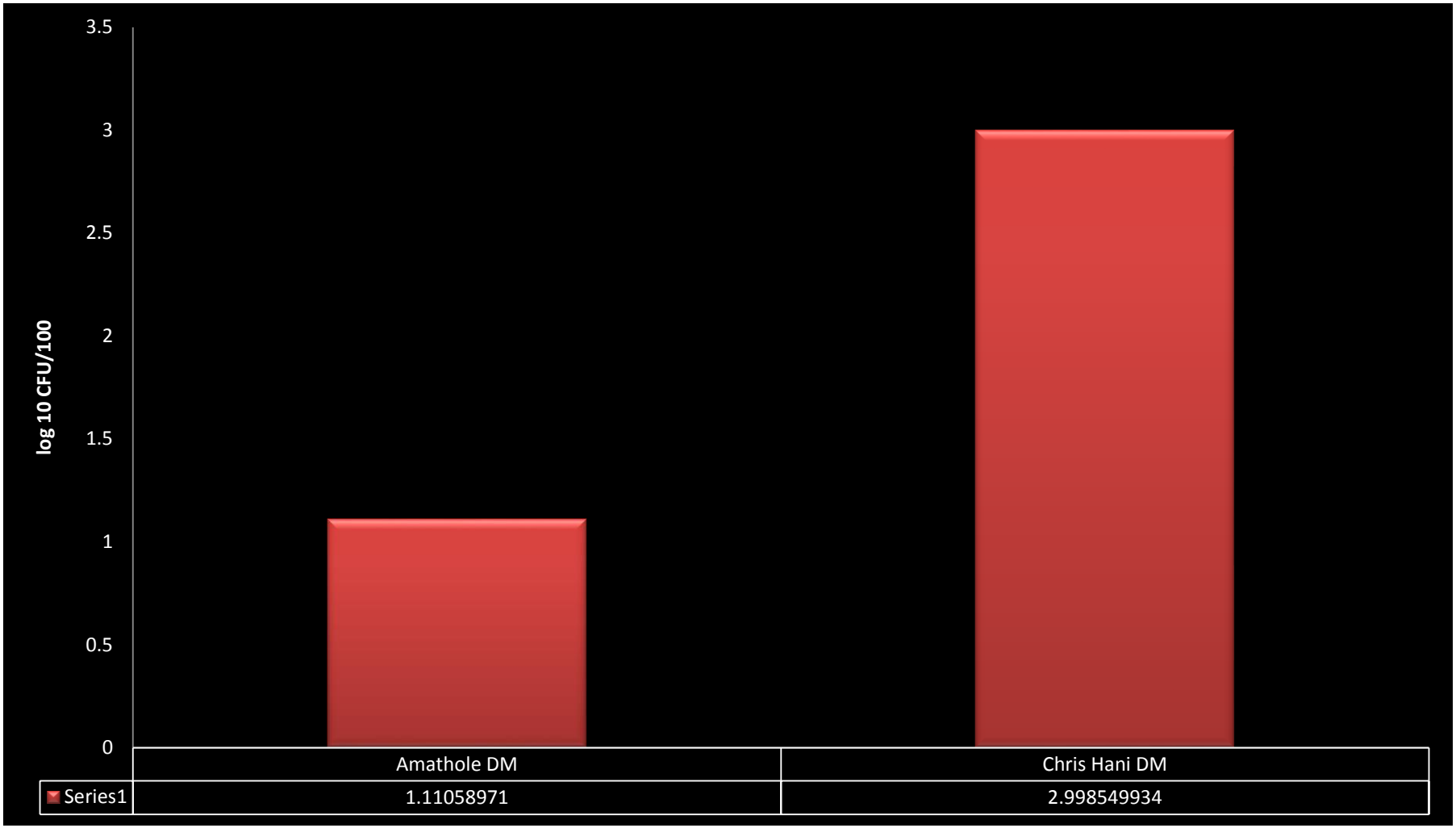


Figure 4.3: Relative mean counts of Enterobacteriaceae in hospital effluents in the selected study sites.

4.2 MALDI-TOF identification of isolates

Figure 4.4 below shows the proportions of confirmed isolates by MALDI-TOF analysis. About 81% (47/58) of the total presumptive isolates were identified as *E. coli* by MALDI-TOF. The proportions of the identified isolates from the different sample types are 78.1% (25/32) for vegetable, 84.6% (11/13) for hospital effluents and 84.6% (11/13) for river water samples. Whereas 75.5% (25/33) of the presumptive *Enterobacter* spp. were confirmed by MALDI-TOF with 79.2% (19/24), 66.7% (2/3), 66.7% (4/6) been confirmed from vegetables, hospital effluents and river water samples respectively. Likewise, about 77.8% (21/27) were confirmed as *Citrobacter* spp. of which 92.3% (12/13), 66.7% (2/3) and 63.6% (7/11) were from vegetables, hospital effluents and river water samples respectively, and as for *Klebsiella* spp. 54% (12/24) was confirmed at the genus level out of which 38.5% (5/13), 50% (2/4) and 71.4% (5/7) were from vegetables, hospital effluents and river water samples respectively.

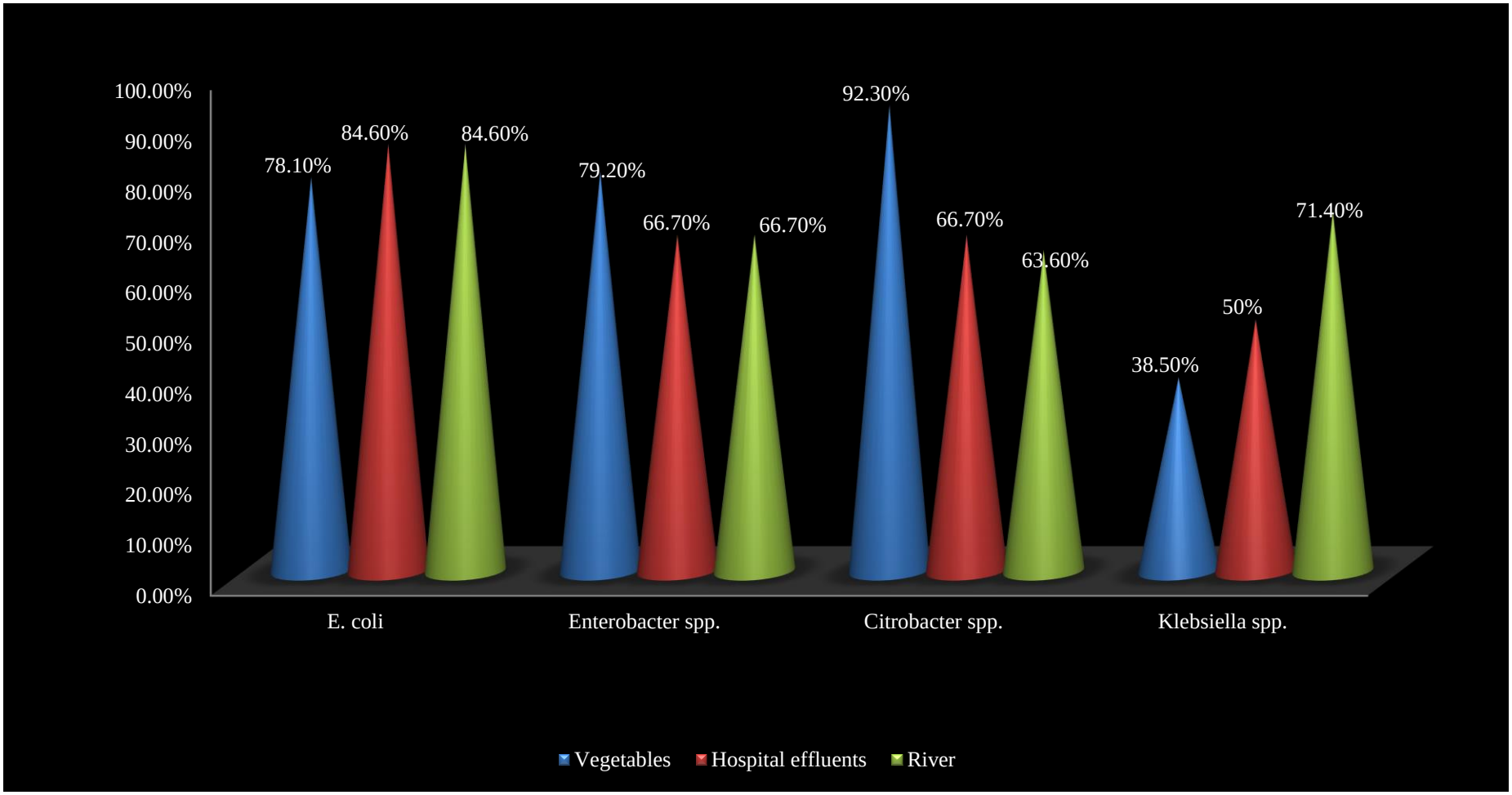


Figure 4.4: MALDI-TOF confirmed isolates for the targeted organisms from the selected samples.

4.3 Antimicrobial resistance and phenotypic characteristics

The one hundred and five (105) MALDI-TOF confirmed isolates were assessed for their phenotypic antimicobial resistance patterns against eighteen antibiotics selected across ten antimicrobial classes. For *E. coli*, high resistance was observed against Tetracycline family where a resistance of 89% (42/47) was observed against tetracycline and 87% (41/47) against doxycycline. This was closely followed by 87% (41/47) resistance against nalixidic from the quinolone family with 85% (40/47) against ampicillin, 83% (38/47) against amoxicillin cluvanate acid, 60% against polymyxin B and colistin. Lowest resistance was observed against carbapenems with a resistance proportion of 11% (5/47) and 32% (15/47) from imipenem and meropenem respectively (Figure 4.5).

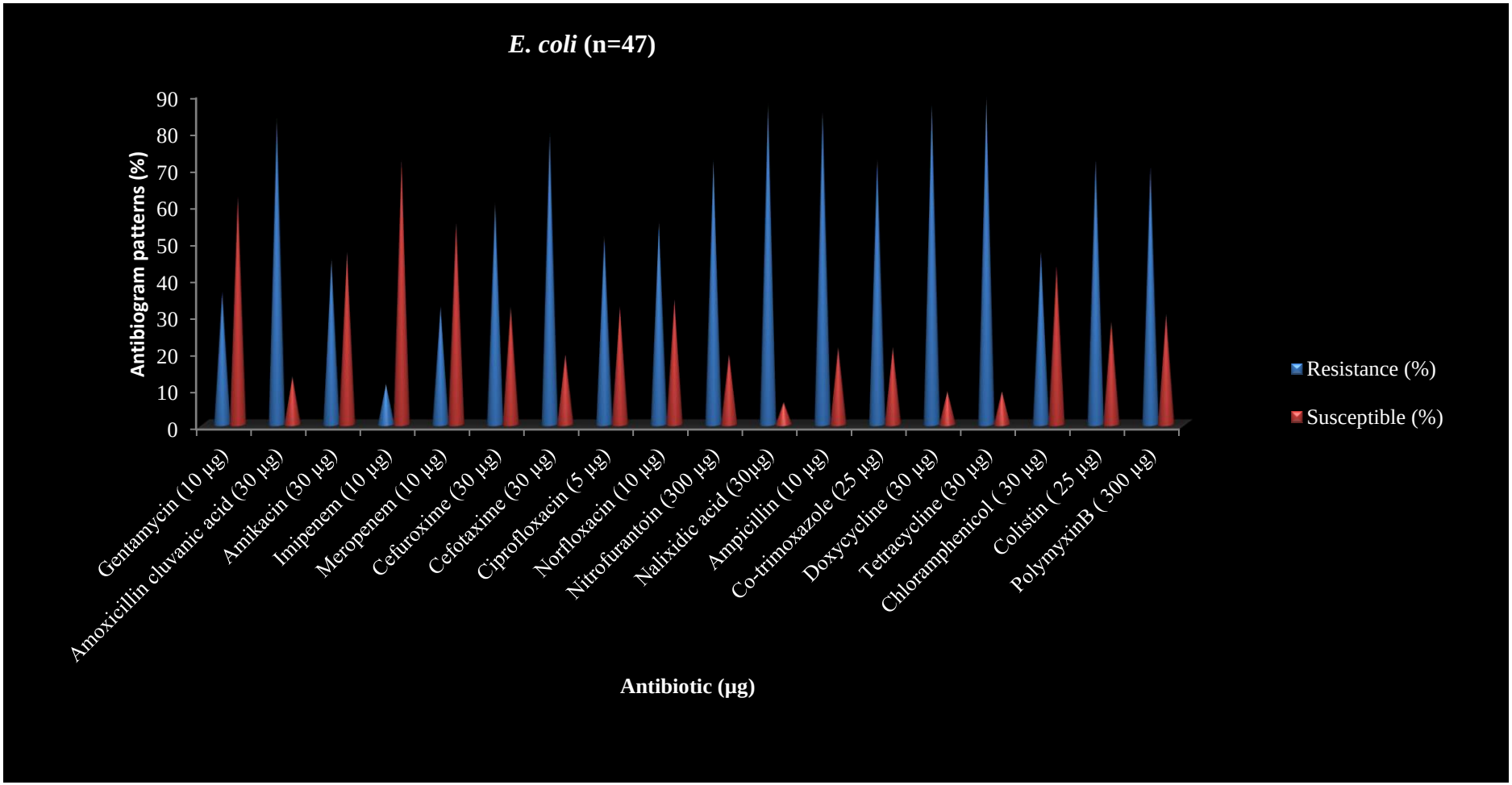


Figure 4.5: Antibiogram profiles of MALDI-TOF confirmed *E. coli* isolates from vegetables, hospital effluents and river water samples sourced in Amathole DM and Chris Hani DM.

All *Enterobacter* spp. showed 100% (25/25) resistance against amoxicillin cluvanate acid followed by 96% (24/25) against nitrofurantoin, 92% (23/25) against ampicillin, 84% (21/25) against nalixidic acid, 76% (19/25) against cefuroxime, 76% (19/25) against tetracycline, and a very low resistance was observed against amikacin with a percentage of 4% (1/25). Figure 4.6 shows the antibiogram profiles of *Enterobacter* spp.

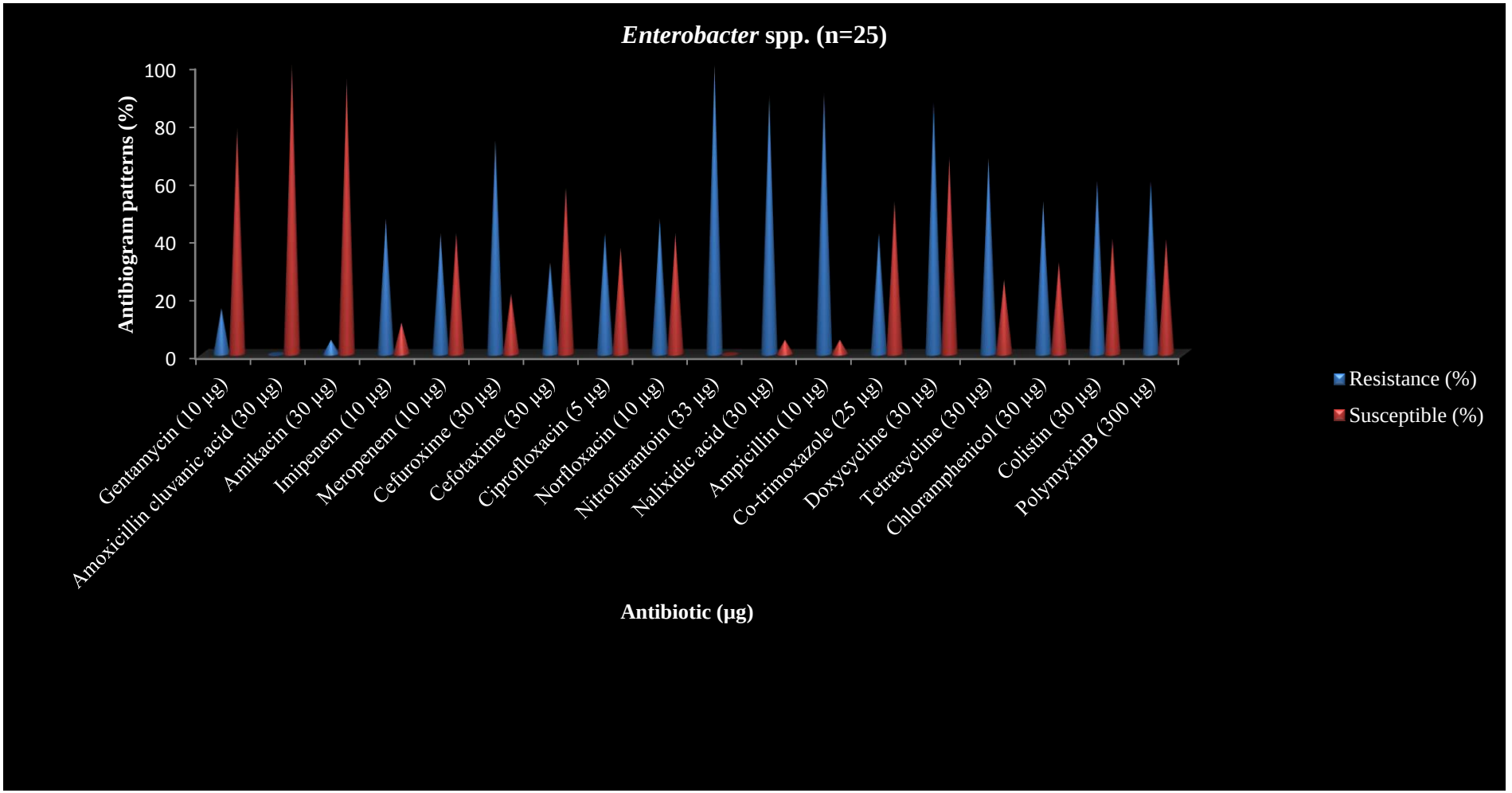


Figure 4.6: Resistance profile of MALDI-TOF confirmed *Enterobacter* spp. isolates from vegetables, hospital effluents and river water sourced in Amathole DM and Chris Hani DM.

All *Citrobacter* spp. showed 100% (21/21) resistance against ampicillin, cefotaxime, nalixidic acid and doxycycline. Resistance against other antibiotics varied between 95% (20/21) and 23% (5/21). Figure 4.7 show susceptibility profiles for *Citrobacter* spp.

The greatest resistance was observed against ampicillin, where all (12/12) *Klebsiella* spp. isolates displayed resistance against this antimicrobial agent, closely followed by 92% (11/12) against cefotaxime, 83% (10/12) against gentamycin, cefuroxime and trimethoprim. Lowest of 17% (2/12) resistance was perceived against norfloxacin and imipenem. Surprisingly 58% (7/12) and 50% (6/12) *Klebsiella* spp. isolates displayed resistance to the last resort of antibiotics as shown in Figure 4.8.

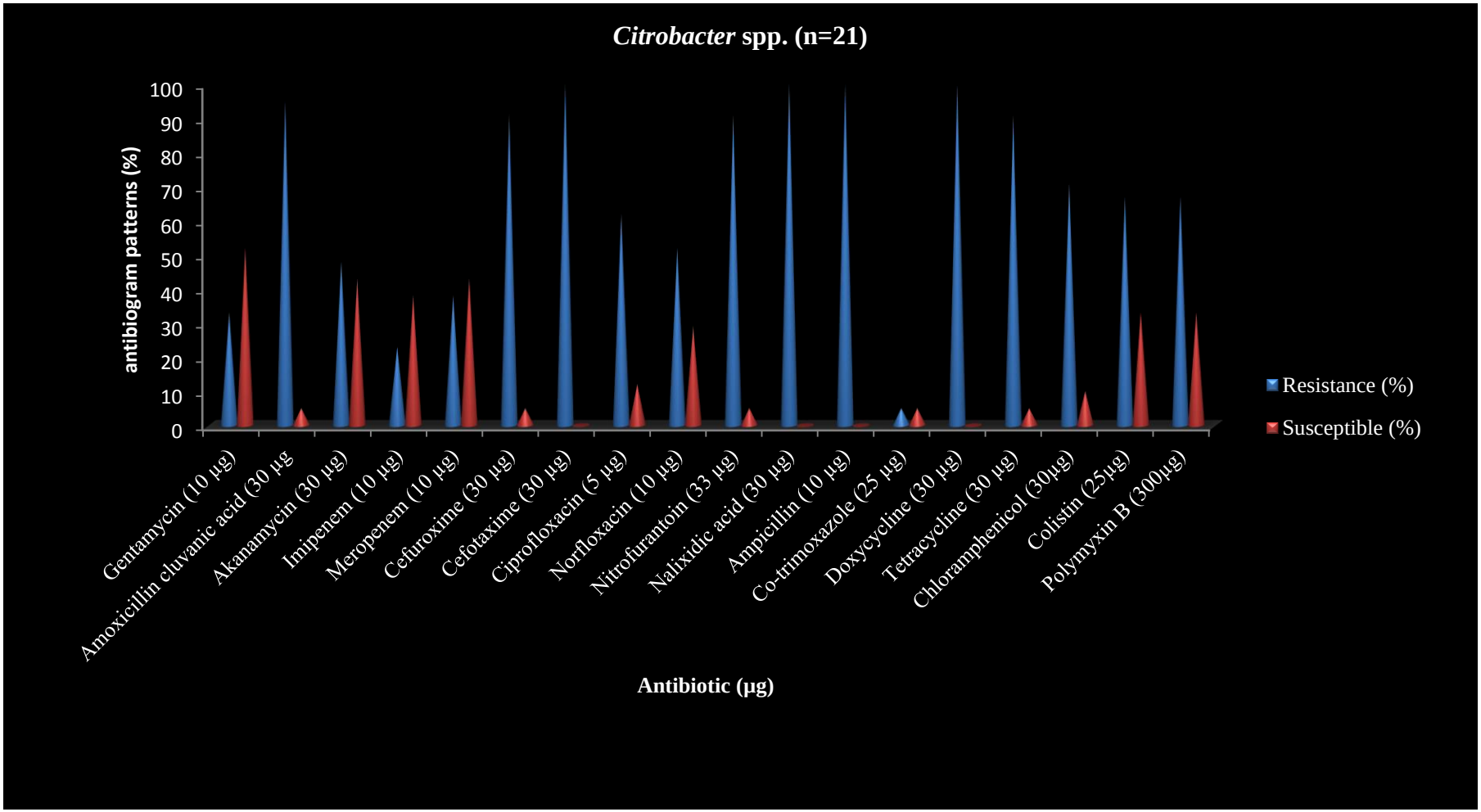


Figure 4.7: Resistance profile of MALDI-TOF confirmed *Citrobacter* spp. isolates from vegetables, hospital effluents and river water sourced in Amathole DM and Chris Hani DM.

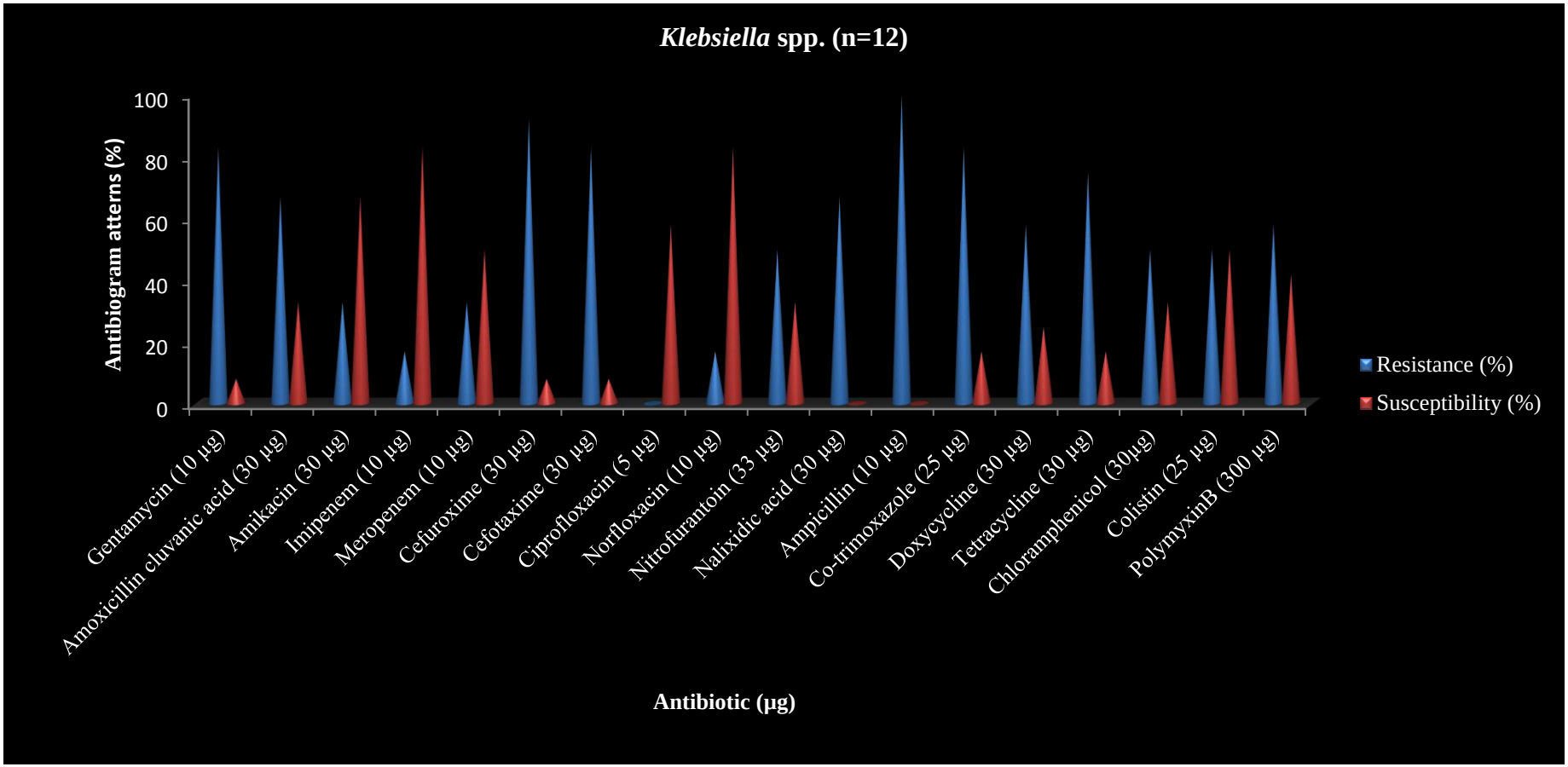


Figure 4.8: Resistance profile of *Klebsiella* spp. isolates from vegetables, hospital effluents and river water sourced in Amathole DM and Chris Hani DM.

4.4 Multiple Antibiotic Resistance Phenotypes (MARP) and Indices (MARI)

A total of 44 multiple antibiotic resistance phenotypes (MARP)-drug resistance patterns were observed for all *E. coli* isolates as shown in Table 4.1. Approximately 97.8% (44/47) of the *E. coli* isolates displayed resistance against more than two antimicrobials. The ranges of MARP included on the Table are from 3 to 17. The highest MARI values observed being 0.9 and the lowest being 0.2. Only one (2.1%) isolate which had the MARI value of 0.2, all other isolates were greater than 0.2.

Table 4.1: MAR phenotype and indices for confirmed *E. coli* isolates

***E. coli* spp. (n=47)**		
MAR phenotypes	Frequency	MARI
GM; AK; AUG; IMI; MEM; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
GM; AK; AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
GM; AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
GM; AK; AUG; AP; MEM; CTX; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
AK; AUG; AP; IMI; MEM; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; T; DXT	1	0.9
GM; AK; AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
GM; AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; IMI; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; CTX; CXM; CIP; NOR; N; C; PB; CO; NA; TS; T; DXT	1	0.8
AK; AUG; AP; IMI; CTX; CXM; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
GM; AK; AP; MEM; CTX; CXM; CIP; NOR; C; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; CTX; CXM; CIP; NOR; NI; C; NA; TS; T; DXT	1	0.7
AK; AUG; AP; CTX; CXM; NI; C; PB; CO; NA; TS; T; DXT	2	0.7
GM; AK; AUG; AP; MEM; CIP; NOR; NI; PB; CO; NA; T; DXT	1	0.7
AUG; AP; CTX; CXM; CIP; NOR; PB; CO; NA; TS; T; DXT	1	0.7
GM; AK; AUG; CTX; CXM; CIP; NOR; PB; CO; NA; TS; T; DXT	1	0.7

AUG; AP; CTX; CXM; CIP; NOR; NI; PB; CO; NA; TS; T; DXT	1	0.7
GM; AK; AUG; AP; MEM; CTX; NOR; PB; CO; NA; TS; T; DXT	1	0.7
GM; AK; AUG; AP; MEM; CTX; NI; PB; CO; NA; TS; T; DXT	1	0.7
AK; AUG; AP; CTX; CXM; NI; PB; CO; NA; TS; T; DXT	1	0.7
AUG; AP; IMI; CTX; CXM; NI; PB; CO; NA; TS; T; DXT	1	0.7
AUG; AP; CTX; CXM; CIP; NOR; NI; C; NA; TS; T; DXT	1	0.7
AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; NA; TS; T; DXT	1	0.7
GM; AK; MEM; CTX; CXM; NI; PB; CO; NA; TS; T; DXT	1	0.7
GM; AUG; AP; CTX; NI; C; PB; CO; NA; TS; T; DXT	1	0.7
GM; AUG; AP; MEM; CTX; CXM; NI; PB; CO; NA; T; DXT	1	0.7
AK; AUG; AP; MEM; CTX; CXM; NI; NA; TS; T; DXT	1	0.6
AK; AUG; AP; CIP; NOR; NI; PB; CO; NA; T; DXT	1	0.6
AUG; AP; CTX; CIP; NOR; NI; C; PB; CO; T; DXT	1	0.6
AUG; AP; MEM; CTX; CXM; CIP; NOR; NA; TS; T; DXT	1	0.6
GM; AUG; AP; CIP; NOR; NI; C; NA; TS; T; DXT	1	0.6
AUG; AP; CTX; CXM; NI; PB; NA; TS; T; DXT	1	0.6
GM; AUG; AP; CTX; NI; C; PB; CO; T; DXT	1	0.6
AK; AUG; AP; CTX; C; PB; NA; TS; T	1	0.5
AP; CTX; CXM; CIP; NOR; NA; TS; T; DXT	2	0.5
AUG; CTX; CIP; NI; C; TS; T; DXT	1	0.5
AUG; AP; NI; PB; CO; NA; T; DXT	1	0.4
AK; AP; NI; C; PB; CO; T; DXT	1	0.4
AK; AUG; AP; CTX; CO; NA; TS	1	0.4
AUG; NI; PB; CO; T; DXT	1	0.3
PB; CO; NA; TS; T; DXT	1	0.3
AUG; AP; NI; CO; NA	1	0.3
AP; CTX; CXM; CO; NA	1	0.3
AK; AP; NOR	1	0.2

The MARI values of all *Enterobacter* spp. ranged between the 0.2 and 0.9 (Table 4.2). Only 8% (2/25) of the *Enterobacter* spp. isolates displayed MARI value of 0.2. None of these isolates displayed MARI value less than 0.2. A total of 25 multidrug resistance patterns were observed for these isolates. 100% of the isolates displayed resistance to more than three antibiotics. Multiple resistances were observed up to 16 antibiotics.

Table 4.2: MAR phenotype and indices for confirmed *Enterobacter* spp.

***Enterobacter* spp. (n=25)**		
MAR phenotypes	Frequency	MARI
GM; AUG; AP; IMI; MEM; CTX; CXM; NOR; NI; C;PB; CO; NA; TS; T; DXT	1	0.9
GM; AUG; AP; IMI; MEM; CTX; CIP; NOR; NI; C; NA; TS; T; DXT	1	0.8
GM; AUG; AP; IMI; MEM; CTX; CXM; CIP; NOR; NI; C; TS; T	1	0.8
GM; AK; AUG; AP; IMI; MEM; CTX; CXM; NI; PB; CO; NA; T; DXT	1	0.8
AUG; AP; IMI; MEM; CTX; CXM; CIP; NOR; NI; C; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; IMI; MEM; CTX; CXM; CIP; NOR; NI; C; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; IMI; MEM; CTX; CXM; CIP; NI; PB; CO; NA; TS; T	1	0.8
AUG; AP; IMI; MEM; CTX; CXM; NI; CO; NA; TS; T; DXT	1	0.7
AUG; AP; IMI; MEM; CXM; NI; C; PB; CO; NA; T; DXT	1	0.7
AUG; CTX; CXM; CIP; NOR; NI; C; CO; NA; TS; T; DXT	1	0.7
AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; PB; NA; TS; T;DXT	1	0.7
AUG; AP; CTX; CXM; CIP; NI; C; CO; NA; TS; T; DXT	1	0.7
AUG; AP; CXM; CIP; NOR; NI; PB; CO; NA; DXT	1	0.6
AUG; AP; CTX; CXM; NOR; NI; C; PB; CO; NA	1	0.6
AUG; AP; MEM; CTX; NI; C; PB; CO; NA; T; DXT	1	0.6
AUG; AP; CTX; NOR; NI; C; PB; NA; TS; T; DXT	1	0.6
AUG; AP; CTX; CXM; NI; PB; CO; NA; T; DXT	1	0.6
GM; AP; CXM; NI; NA; T; DXT	1	0.4
AUG; AP; CIP; NOR; NI; CO; NA; DXT	1	0.4

AUG; AP; IMI; CXM; NI; PB	1	0.3
AUG; CTX; CXM; NI; NA	1	0.3
AUG; AP; MEM; NI; T; DXT	1	0.3
AUG; AP; NI; NA	1	0.2
GM; AUG; AP; C	1	0.2

For all *Citrobacter* spp. isolates, the highest MARI value obtained was 0.9 and the lowest was 0.5 (Table 4.3). None of the isolates exhibited less or equal to 0.2 MARI value, all the isolates were greater than 0.2. A total of 21 pattern of multidrug was observed for *Citrobacter* spp. isolates. 100% of the isolates exhibited resistance to more than nine antibiotics. Multiple resistances observed were up to 17 antibiotics.

Table 4.3: MAR phenotype and indices for *Citrobacter* spp.

***Citrobacter* spp. (n=25)**		
MAR phenotypes	Frequency	MARI
GM; AK; AUG; AP; IMI; MEM; CTX; CXM; CIP; NOR; NI; PB; CO; NA; TS; T; DXT	1	0.9
AK; AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
GM; AK; AUG; AP; IMI; MEM; CTX; CXM; NI; C; PB; CO; NA; TS; T; DXT	1	0.9
AK; AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AK; AUG; AP; MEM; CTX; CXM; CIP; NOR; NI; PB; CO; NA; TS; T; DXT	1	0.8
GM; AK; AUG; AP; CTX; CXM; CIP; NOR; NI; C; CO; NA; TS; T; DXT	1	0.8
GM; AK; AUG; AP; IMI; CTX; CXM; CIP; NOR; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; CTX; CXM; CIP; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; IMI; CTX; CXM; CIP; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
GM; AK; AUG; AP; MEM; CTX; CXM; NI; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8

AUG; AP; IMI; CTX; CXM; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
GM; AUG; AP; CTX; CXM; CIP; NOR; NI; C; PB; NA; TS; T; DXT	1	0.8
AUG; AP; CTX; CIP; NOR; NI; C; PB; CO; NA; TS; T; DXT	1	0.7
GM; AK; AUG; AP; MEM; CTX; CXM; PB; CO; NA; TS; T; DXT	1	0.7
AK; AUG; AP; CTX; CXM; NI; C; CO; NA; TS; T; DXT	1	0.7
AUG; AP; CTX; CXM; CIP; NI; C; CO; NA; TS; T; DXT	1	0.7
AUG; AP; MEM; CTX; CXM; NOR; NI; CO; NA; TS; T; DXT	1	0.7
AUG; AP; MEM; CTX; CXM; NI; C; CO; NA; TS; DXT	1	0.6
AP; CTX; CIP; NI; C; NA; TS; T; DXT	1	0.5

The lowest MARI value obtained for all *Klebsiella* spp. isolates was 0.4 and the highest was 0.8. Of all the isolates, 91.7% displayed multiple resistances against eight and more antibiotics. A total of 11 multiple antibiotic patterns were observed. Multiple resistances observed were up to 14 antibiotics. Table 4 shows the trend for MARI for *Klebsiella* spp. isolates.

Table 4.4: MAR phenotypes and indices for *Klebsiella* spp.

***Klebsiella* spp. (n=12)**		
MAR phenotypes	Frequency	MARI
AK; AUG; AP; MEM; CTX; CXM; NI; C; PB; CO; NA; TS; T; DXT	1	0.8
AUG; AP; IMI; MEM; CTX; CXM; NI; NA; TS; T; DXT	1	0.6
AUG; AP; CTX; C; PB; CO; NA; TS; T; DXT	1	0.6
GM; AK; AP; MEM; CTX; CXM; PB; CO; NA; TS	1	0.6
AK; AP; CTX; CXM; PB; CO; NA; TS; T; DXT	1	0.6
AUG; AP; IMI; MEM; CTX; CXM; NI; C NA; TS	1	0.6
AUG; AP; CTX; CXM; NI; PB; TS; T; DXT	1	0.5

AK; AUG; AP; CTX; CXM; NOR; NI; C; TS	1	0.5
AUG; AP; CTX; CXM; C; PB; NA; T	1	0.4
AUG; AP; CTX; CXM; NOR; TS; T; DXT	1	0.4
AP; MEM; CTX; CXM; PB; CO; NA; TS	1	0.4

4.5 Distribution of antibiotic resistance genes

Table 4.5 shows the distribution of resistance genes detected in the study. Nine out of the 10 beta-lactam resistant genes assayed were detected; sulphonamides, aminoglycosides and tetracyclines resistant genes were also detected.

Beta-lactams: *E. coli* isolates displayed great bla_{TEM} presence of 77.8% (35/45) proportion as compared to other investigated bacteria, while *Klebsiella* spp. isolates showed the least presence of 33.3% (4/12) proportion of this gene. Only *Klebsiella* spp. isolates exhibited bla_{SHV} in a proportion of 16.7% (2/12). Again *E. coli* isolates displayed the great proportion of bla_{OXA-1}, whereas none of the *Klebsiella* spp. isolates exhibited this gene. All isolates exhibited $bla_{CTX-M-2}$ and *Enterobacter* spp. displayed great presence of this gene with a proportion of 36% (9/25). None of the *Citrobacter* spp. isolates exhibited $bla_{CTX-M-9}$. Only *E. coli* isolates displayed the presence of bla_{DHA} with a proportion of 13.3% (6/47). *Citrobacter* exhibited the great presence of bla_{GES} and Bla_{OXA-48} with proportions 23.8% (5/21) and 12% (3/21) for bla_{GES} and Bla_{OXA-48} respectively. bla_{KPC} was not present in any of the isolates.

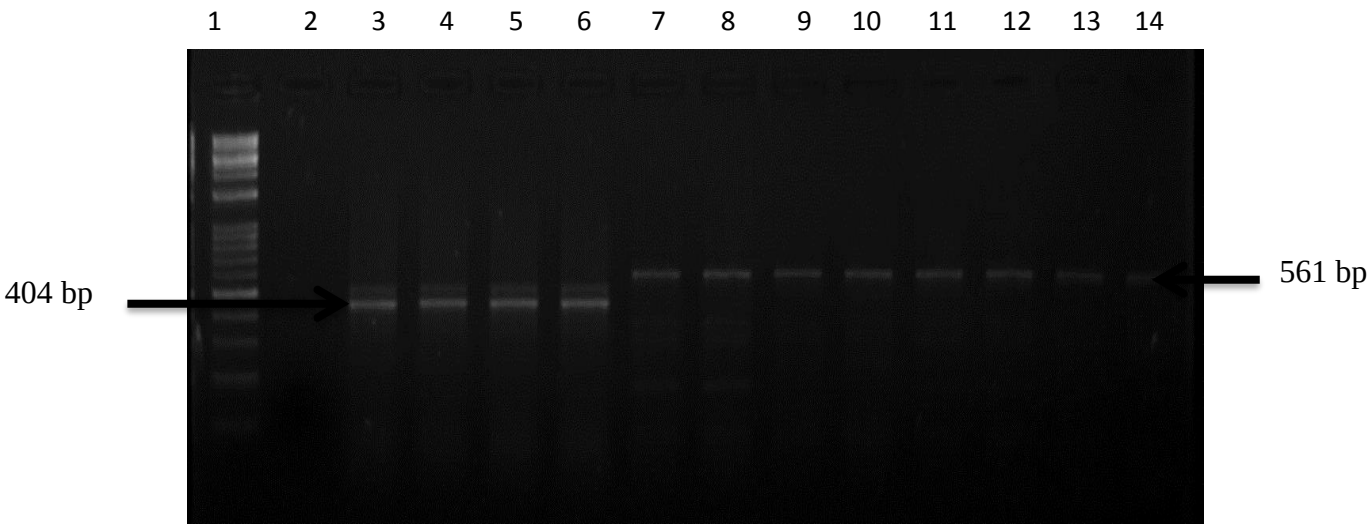


Figure 4.9: Gel picture representing molecular detection of *bla*$_{CTX\text{-}M\text{-}9}$ (404 bp) and *bla*$_{CTX\text{-}M\text{-}2}$ (561 bp) = genes. Lane 1: DNA Ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), lane 3 – 14: Isolates exhibiting resistance genes.

Sulfonamides: *sul1* resistant gene was not harboured by any of *Klebsiella* spp. isolates. *Enterobacter* spp. with a percentage of 23.1% (3/25) harboured great *sul1* presence whereas *sul2* was greatly harboured by *Klebsiella* spp. with a percentage of 70% (7/12).

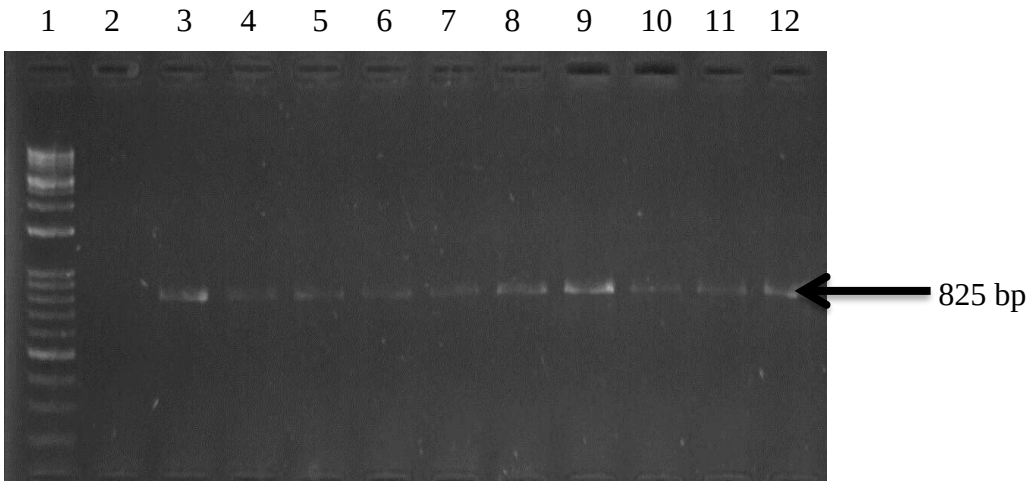


Figure 4.10**:** Gel picture representing molecular detection of *sul1* (825 bp) resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 12: isolates exhibiting resistance genes.

Tetracyclines: *tetA* was greatly abundant in *Klebsiella* spp. by 33.3% (3) while *tetM* was greatly abundant in *E. coli*.

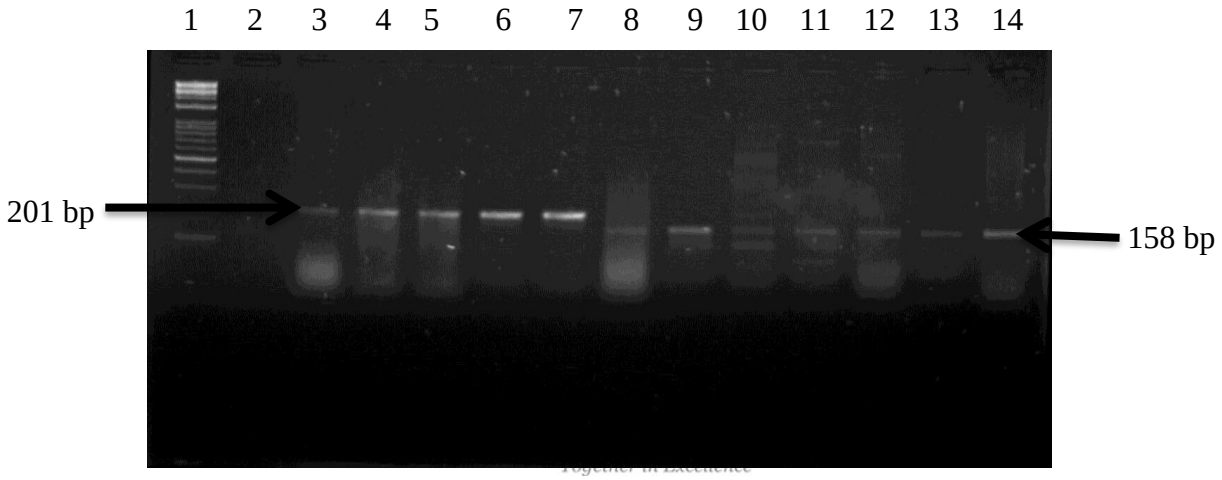


Figure 4.11**:** Gel picture representing molecular detection of *tetA* (201 bp) and *tetM* resistance genes. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 14: isolates exhibiting resistance genes.

Aminoglycosides: all of the 30 *E. coli* isolates and 4 *Klebsiella* spp. isolates showed resistance to aminoglycosides displayed 100% for *aada* gene whereas another 100% was exhibited by *Klebsiella* spp. for *strB* and *aacA2.*

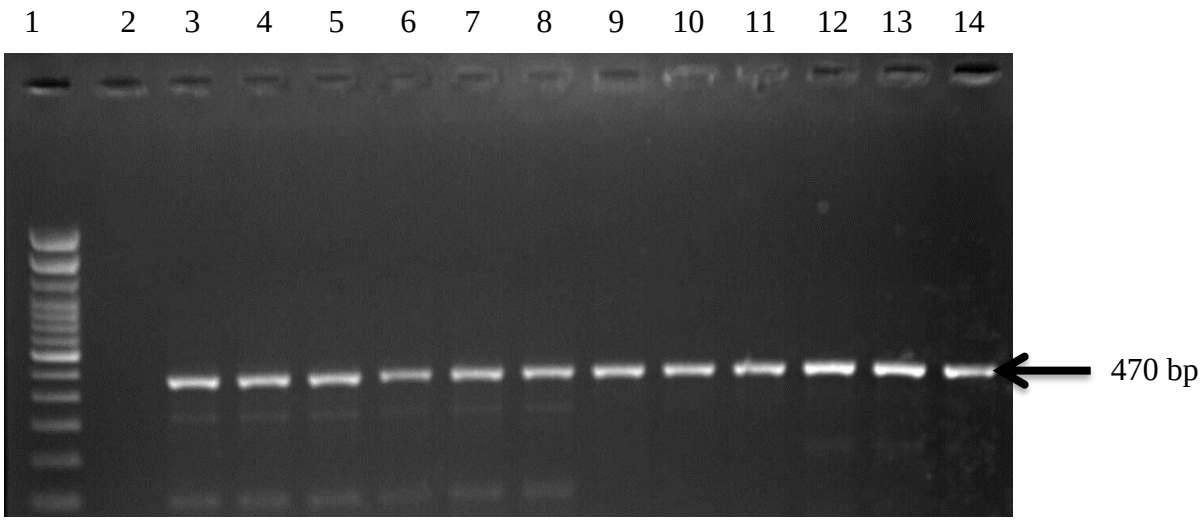


Figure 4.12: Gel picture representing molecular detection of *strB* (470 bp). Lane 1: DNA Ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), lane 3 – 14: Isolates exhibiting resistance genes.

Table 4.5: Distribution of resistance genes among analysed samples

Resistance Gene profile	*E. coli* (n=47)	*Enterobacter* spp. (n=25)	*Citrobacter* spp. (n=21)	*Klebsiella* spp. (n=12)
blaTEM	77.8% (35)	44% (11)	76.2% (16)	33.3% (4)
blaSHV	0	0	0	16.7(2)
blaOXA-1	13.3% (6)	8% (2)	4.8% (1)	0
blaCTX-M-2	11.1% (5)	36% (9)	23.8% (5)	16.7% (2)
blaCTX-M-9	8.8% (4)	8% (2)	0	8.3% (1)
blaDHA	13.3% (6)	0	0	0
blaGES	11.1% (5)	8% (2)	23.85 (5)	16.7% (2)
blaOXA-48	2.2% (1)	0	12% (3)	8.3% (1)
sul1	12.15 (4)	23.1% (3)	15% (3)	0
sul2	39.4% (13)	30.8% (4)	10% (2)	70% (7)
tetA	19.1% (8)	19% (4)	10% (2)	33.3% (3)

tetM	28.6% (12)	19% (4)	19% (2)		0
aadA	100% (30)	86% (6)	81% (17)	100% (4)	
strA	70% (21)	100% (7)	28.6% (6)	25% (1)	
strB	27% (8)	57.1% (4)	33.3 (7)	100% (4)	
aacA2	30% (9)	71.4% (5)	9.5% (2)	100%(4)	
aphA1	13.3% (4)	28.6% (2)	4.8% (1)	25% (1)	
aphA2	13.3% (4)	71.4% (5)	14.3% (3)		0

Table 4.6 to 4.8 shows the distribution of resistance genes in each of the analysed samples. In vegetables the most abundant resistant gene was *aadA* exhibited by *Klebsiella* spp. as shown in Table 4.6

Table 4.6: Distribution of resistant genes in vegetable isolates

Resistance Gene profile	*E. coli*	*Enterobacter* spp.	*Citrobacter* spp.	*Klebsiella* spp.
blaTEM	44.4% (20)	36% (9)	47.6% (9)	8.3% (1)
blaSHV	0	0	0	16.7(2)
blaOXA-1	2.2% (1)	8% (2)	4.8% %1)	0
blaCTX-M-2	6.7% (3)	24% (6)	19.1% (4)	8.3% (1)
blaCTX-M-9	4.4% (2)	8% (2)	0	8.3% (1)
blaDHA	4.4% (2)	0	0	0
blaGES	4.4% (2)	2.2% (1)	19.1% (4)	8.3% (1)
blaOXA-48	0	0	14.3% (3)	8.3% (1)
sul1	6.1% (2)	15.4% (2)	15% (3)	0
sul2	21.1% (7)	23.1% (3)	5% (1)	20% (2)

	11.9%				
tetA	(5)	14.3% (3)	5% (1)		0
	16.7%				
tetM	(7)	19.1% (4)	5% (1)		0
aadA	60% (18)	57.1% (4)	47.6% (10)	75% (3)	
strA	40% (12)	85.7% (6)	9.5% (2)	25% (1)	
	16.7%				
strB	(5)	14.3% (1)	28.6% (6)	50% (2)	
aac(3)A	20% (6)	57.1% (4)	4.8% (1)		0
(aac2)A	6.7% (2)	28.6% (2)	0	25% (1)	
aph(3)-1A	6.7% (2)	57.1% (4)	9.5% (2)		0

In the river water samples analysed, the most abundant resistant gene observed was *aac2A* and *strB*; both genes were greatly abundant in *Klebsiella* spp. (Table 4.7).

Table 4.7: Distribution of resistant genes in river water

Resistance Gene profile	***E. coli***	***Enterobacter* spp.**	***Citrobacter* spp.**	***Klebsiella* spp.**
blaTEM	20% (9)	8% (2)	23.8% (5)	8.3% (1)
blaSHV	0	0	0	0
blaOXA-1	6.7% (3)	0	0	0
blaCTX-M-2	2.2% (1)	12% (3)	4.8% (1)	8.3% (1)
blaCTX-M-9	2.2% (1)	0	0	0
blaDHA	6.7% (3)	0	0	0
blaGES	4.4% (2)	2.2% (1)	4.8% (1)	8.3% (1)
blaOXA-48	2.2% (1)	0	0	0
sul1	0	0	0	0
	12.1%			
sul2	(4)	0	0	50%

tetA	7.1% (3)	0	0	33.3% (3)	
tetM	7.1% (3)	0	0	0	
aadA	23.3% (7)	14.3% (1)	23.8% (5)	0	
strA	16.7% (5)	0	19.1% (4)	0	
strB	6.7% (2)	28.6% (2)	4.8% (1)	50% (2)	
aac2A	6.7% (2)	14.3% (1)	4.8% (1)	50% (2)	
aphA1	6.7% (2)	0	0	0	
aphA2	3.3% (1)	14.3% (1)	4.8% (1)	0	

Even in hospital effluents processed samples, *Klebsiella* spp. displayed the highest prevalence of one of the aminoglycosides resistant gene (*aac2A*) with a frequency of 50% (Table 4.8).

Table 4.8: Distribution of resistant genes in Hospital effluents

Resistance Gene profile	***E. coli***	***Enterobacter* spp.**	***Citrobacter* spp.**	***Klebsiella* spp.**
blaTEM	13.3% (6)	0	4.8% (1)	16.7% (2)
blaSHV	0	0	0	0
blaOXA-1	4.4% (2)	0	0	0
blaCTX-M-2	2.2% (1)	0	0	0
blaCTX-M-9	2.2% (1)	0	0	0
blaDHA	2.20%	0	0	0
blaGES	2.20%	0	0	8.3% (1)

	2.2%				
blaOXA-48	(1)	0	0	0	
	6.1%				
sul1	(2)	8% (1)		0	0
	6.1%				
sul2	(2)	8% (1)	5% (1)		0
tetA	0	4.8% (1)	5% (1)		0
	4.8%				
tetM	(2)	0	5% (1)		0
	16.7%				
aadA	(5)	14.3% (1)	9.5% (2)	25% (1)	
	13.3%				
strA	(4)	14.3% (1)		0	0
	3.3%				
strB	(1)	14.3% (1)		0	0
	3.3%				
aac2A	(1)	0		0	50% (2)
aphA1	0	0	4.8% (1)		0
	3.3%				
aphA2	(1)	0		0	0

CHAPTER FIVE

5.0 DISCUSSION

Klebsiella spp. and *E. coli* are members of Enterobacteriaceae and are of importance as they are excellent indicators of microbial water and food quality. Quality of potable water requires a routine monitoring for inspecting maximum microbial concentrations, *E. coli* and faecal coliforms assist in achieving this goal. The more distinct member of faecal coliform of indicating faecal contamination is *E. coli* as compared to other faecal coliform members (Odonkor and Ampofo, 2013). *E. coli* is of faecal origin. Being of faecal origin is not the only characteristic for *E. coli* to be the best organisms to be used in water and food quality. It is a very easy organism to work with, adapted to many habitats and hence allowing microbiologist to be able to manipulate its growth easily. However, as it is displaying excellent laboratory features, direct contact with it can lead to severe illnesses such as diarrhea, kidney failure, dehydration among others (Leclerc et al., 2010).

Klebsiella spp. is one of the organisms used to detect faecal contamination as well. Low numbers of *Klebsiella* exist in several individuals. They are more commonly abundant on the human intestine and widely distributed in environmental waters (João, 2010). *Klebsiella* is used as a faecal indicator in water because it is an inhabitant of several water environments and can be distributed in potable water distribution settings. They have a proficiency of inhabiting in linings of taps. They are faecal detectors of faecal origin as they are normally found in excretions of several healthy humans and animals. *Klebsiella* is of clinical importance as it is one of the organisms having a capability of causing hospital acquired infections. Infection by

Klebsiella spp. leads to severe illnesses such as soft tissue infections, urinary tract infections, and pneumonia among others. The most common *Klebsiella* species of clinical importance is *K. pnuemoniae*.

Citrobacter spp. and *Enterobacter* spp. are members of the large family of Gran negatives known as Enterobacteriaceae. Naturally *Enterobacter* spp. is a soil and water inhabitant but it may be also found in faeces, food and respiratory tract. This bacterium is responsible for several infections such as urinary tract infection and bacteraemia. *Enterobacter* spp. is one of the organisms that cause community acquired infections. *Citrobacter* spp. is normally distributed in human and animal faeces and is responsible for causing infections such as bacteraemia, peritonitis and brain abscess (Park et al., 2013). There are three pathogenic species of importance in the genus *Citrobacter* which are *C. farmer*, *C. koseri* and *C. freundii* (Clermont et al., 2015). *Enterobacter* spp. and *Citrobacter* spp. are members of coliform bacilli. Because these two pathogens are members of the coliform bacilli, they are of importance in food and water quality. Significant prevalence of these pathogens in food and water indicate faecal contamination.

5.1 Occurrence of Enterobacteriaceae in vegetables

Because these organisms are widely distributed in nature, it is therefore probable that they may be present along the food chain. This family is important in food analysis as they can cause severe illnesses such as foodborne diseases, and some are responsible for the spoilage of food (Rawat et al., 2015). Absence and presence of Enterobacteriaceae in food determine the hygiene and manufacturing concepts followed. Bacterial quality of six vegetables was investigated in the study (cabbage, spinach, carrot, beetroot, lettuce and cucumber). In the current study the total counts of Enterobacteriaceae ranged between 0 and 3×10^5 CFU/g (0 and 5.49 log10 CFU/g) and the highest count was obtained from cabbage whereas the lowest count was obtained from lettuce. The 0 log10 CFU/g count obtained from lettuce indicates that

hygiene and food manufacturing protocols were highly followed appropriately, while the high count observed from cabbage indicates lack of hygiene.

Contaminated soil might be the main factor contributing to high loads of bacterial counts in vegetables such as cabbage and spinach since they grow closer to the soil and thus stand a great chance of being contaminated by soil (Ruimy et al., 2010). Other factor might be the usage of contaminated irrigation water (Oluwatosin et al., 2011). Sujeet and Vipin (2017) observed a density of 5.8 log CFU/g from cabbage, and these findings are similar to the findings of the current work. Presence of high loads of Enterobacteriaceae observed in selected vegetables indicates that these vegetables are contaminated and may not be fit for direct consumption without further processing. The mean value from the local vegetables observed in the work of Zahra et al. (2016) was 5.2 log CFU/g, and therefore these findings are similar to the findings of the current work.

In the present study a significant occurrence of *E. coli* in a proportion of 78% was observed in vegetables samples. This may imply that, the collected vegetables from selected sites were largely contaminated by *E. coli*. Presence of *E. coli* among vegetables raises a concern, especially to consumers. There are numerous factors that can drive to contamination of fresh produce by *E. coli*. Source of contamination among these fresh produce might be due to fertilisers used that might be prepared using animal manure, during harvesting by direct contact with infected worker, during handling and processing (the organisms might be transmitted from an infected worker to the fresh produce), during irrigation (the water used for irrigation might be implicated with loads of *E. coli* which are then transferred to vegetables), during transportation (vehicle used for transportation might be contaminated by *E. coli* and then the organism will be transmitted to the fresh produce) and cultivation of fresh produce on contaminated soil (Singh et al., 2006; Pagadala et al., 2015).

The obtained findings are in line with the findings from the work ofEnabulele and Uraih (2009), who recorded a prevalence of *E. coli* with a proportion of 83.3% in vegetables, sourced form Benin in Nigeria. According to Enabulele and Uraih (2009), the use of the same vehicles used for transportation of livestock might be the source of the high occurrence of *E. coli* observed from their work. According to Solomon et al. (2002) another factor that might contribute to fresh produce contamination is the quality of irrigation water. At times, irrigation water used for irrigating fresh produce is the same water that is also used by grazing cattle. A prevalence of 53.5% of *E. coli* on vegetables was reported in the study of Dutta et al. (2014), and these findings are in line with the recorded results of the current study. The findings from this study correlates with the findings of the work of Herman et al. (2015), who detected 76% *E. coli* from vegetables in the United States.

In the work of Jensen et al. (2013), *E. coli* recovered from Lettuce was high as 54%. Lettuce is one of the vegetables that are ready-to-eat and many consumers do not consider washing them before use. Lettuce was one of the selected vegetables in the current study. High occurrence of *E. coli* from vegetables indicates a high proportion of microbial faecal contamination. High abundance of this pathogen can also represent a high risk of occurrence of zoonotic microbes such as *Salmonella* spp., *Campylobacter* spp. and other faecal excreted organisms (João, 2010). To prove that high presence of *E. coli* can represent the presence of zoonotic bacteria, an occurrence of *Shigella sonnei* in Denmark was connected with high recorded results of *E. coli* (Lewis et al., 2009). Findings from the current study are not in line with the findings of *E. coli* recorded in Iran by Hojjat et al. (2016). Hojjat et al. (2016) detected a low *E. coli* prevalence of 16.6%. Study of Campos et al. (2013) also detected a low prevalence of 4% *E. coli* in vegetables from Portugal (Porto region), which are not in line with the findings of the current study. Many factors that can cause differences between the findings of the present work and other findings of other works include applied agricultural techniques for cultivating and

harvesting of fresh produce; size of samples; identification techniques used, season, variations of geographical areas as well as and hygienic precautions.

In the present study 38.5% of *Klebsiella* spp. was detected which is lower than the recorded *Klebsiella* occurrence in a research conducted in Selangor, Malaysia by Puspanadan et al. (2012) which detected 65% *Klebsiella* in raw fresh produce. The contamination of fresh produce by *Klebsiella* may rise because of exposure of fresh produce to contaminated shelves in supermarkets, deprived hygiene in settings where fresh produce are displayed, deprived proper handling and processing techniques, use of equipment which are contaminated during cultivation and harvesting, contaminated soil, use of contaminated water during irrigation and use of contaminated vehicles during shipping. Even though there are several factors leading to contamination of fresh produce by *Klebsiella*; poor hygiene and handling are key in the contamination of vegetables by *Klebsiella* (Ponniah et al., 2010; Usha et al., 2010). Occurrence of *Klebsiella* on fresh produce raises a concern especially in vegetables such as lettuce, cucumber, carrot, cabbage and spinach as these vegetables are frequently used in preparation of salads and they are ready to eat and salads are always in high demand. Consumers stand at a great risk of contracting resistant infections through ingestion of contaminated fresh produce.

The findings of the current study are in line with the work of Falomir et al. (2013) which observed 29.2% prevalence of *Klebsiella* spp. from lettuce, carrot and tomatoes in Valencia, Spain, and again Falomir et al. (2015) in Burjassot, Spain, observed *Klebsiella* spp. occurrence of 33.3% from the same vegetables. Another work that relates to the findings of the present study is the work of Akind et al. (2016) which recorded 27.9% occurrence of *Klebsiella* spp. form vegetables collected in Ibadan, Nigeria. Low frequency of *Klebsiella* spp. represents less contamination. Therefore, during cultivation and harvesting farmers as well as workers practice excellent hygiene methods, the vegetables are not exposed to contaminated places, vegetables are not grown on contaminated soil, irrigation water used is less contaminated, shipping

vehicles are less or not contaminated at all and workers and farmers are following hygienically handling and processing methods.

High bacterial loads have been reported throughout the world (Gilbert and McBain, 2003; Obeng et al., 2007; Donkor et al., 2009). A significant occurrence of *Citrobacter* spp. in a frequency of 92.3% was observed in vegetables samples in the current study. Contamination of these vegetable samples by *Citrobacter* spp. might occur from the contact with the soil, use of contaminated irrigation water or use of contaminated manure (Golly et al., 2016). Other factors that may contribute to this significant occurrence of *Citrobacter* spp. on the processed vegetables samples might be improper handling and poor hygiene (Nipa et al., 2011). However the findings of this study are not the same as the findings of Nipa et al. (2011), who detected 12.5% *Citrobacter* spp. in vegetables from Bangladesh.

In this study high occurrence of *Enterobacter* spp. was observed from the analysed vegetable samples. The observed occurrence was 79.2% and these findings are in line with the study of Nipa et al. (2011), who detected 84.37% of *Enterobacter* spp. in vegetables. It is no doubt that any possible contamination in vegetables is mainly caused by use of irrigated water, poor hygiene practices, improper handling, use of contaminated fertilisers, use of contaminated vehicles and packaging (Beuchat, 2002; Nipa et al., 2011; Golly et al., 2016). The findings of this study are also in line with the findings of Viswanathan and Kaur (2001), who detected 55.5% of *Enterobacter* spp. from vegetable samples. However findings of Al-Kharousi et al. (2016), who detected 16.2% of *Enterobacter* spp. from vegetables, are in contrary with the findings of the current study. The findings of Chellapand et al. (2015), who detected 39.02% of *Enterobacter* spp. from vegetables samples, are in line with the findings of the present work. Another study which is in line with the findings of the present study is the study of Osamwonyi et al. (2013), who detected a high occurrence of 56% of *Enterobacter aerogenes* from vegetables used in preparing salads in Nigerian restaurants.

5.2 Occurrence of Enterobacteriaceae in river water

Enterobacteriaceae is not only used for indicating microbial quality in food but it is also an important indicating tool of indicating safety of water. Microbial quality of both surface water and drinking water is very important as consumption of microbial contaminated water is as public health concern (OECD, 2013). Members of Enterobacteriaceae family are naturally distributed in all environments and therefore that is why they are suitable indicators of microbial quality of water. The findings of the total mean counts of Enterobacteriaceae form the selected rivers on this work ranged between 10 CFU/ 100 mland 8×10^2 CFU/100 ml. surface waters are receiving basins of microorganisms from several sources like human settlements, industries, hospital effluents and agricultural farming, and this can cause high occurrence of Enterobacteriaceae in surface waters (Bouki et al., 2013). A mean count of 8.0 x 10^1 CFU/ml was observed by Abo-State et al. (2014). This value is similar to 8×10^2 CFU/100 ml observed in the current work.

Buffalo River had highest counts as compared to Ngcongcolora River. This might be due to the fact that there are some informal settlements along the banks of Buffalo River. These communities lack adequate waste removals facilities. Presence of high loads of Enterobacteriaceae in river might show the presence of other pathogenic strains such as *Vibrio* spp. *Salmonella* spp. (Figueras and Borrego, 2010). Also high loads of Enterobacteriaceae in river confirm the faecal contamination of the river. Buffalo River is used as source of portable water, and therefore faecal contamination on this river might have adverse effects.

A significant proportion of 84.6% *E. coli* occurrence was detected from the selected rivers used on the current study. The chief leading factor driving to the distribution of *E. coli* in surface water is runoffs, which carry all terrestrial wastes including human and animal faeces (Isobe et al. 2004). The alarming occurrence detected on this study raises a concern as both of the selected rivers are of community use. Individuals around these rivers still depend on these

rivers for potable water and recreational activities. Others use water from these rivers to irrigate their small gardens. This simply means that community members around these rivers stand a great risk of being infected by *E. coli*. From the recorded high incidence of *E. coli* in these rivers, there is a high possibility that there might be pathogenic strains of *E. coli* present. The occurrence of these strains can cause severe illnesses such as diarrhoea, urinary tract infection and so on. Detection of high incidence of *E. coli* in river cannot be ignored. The pathogen can be transmitted to other sources such as fresh produce and animals then transmitted to humans.

The findings of this study show that Bafallo River and Ngcongcolora River are not fit to be used for any domestic activities including recreational activities and irrigation. According to Bruce et al. (2003), in United States, the main factor contributing to transmission of *E. coli* diseases is surface water through recreational uses and drinking. Another study which had findings similar to the present work is the study of Müller et al. (2001), which detected 75% of *E. coli* from Vaal River Barrage in South Africa (between Gauteng Province and Free State Province border). High prevalence of 76% *E. coli* was recorded by Mdzivhandile (2007); the isolates were recovered from Nwanedi and Luphephe River. On the other hand, study of Chigor et al. (2010) detected a very low occurrence of *E. coli* in Nigeria from Khubanni River (2.1%). Even though *E. coli* infections are not severe as compared to *E coli* 0157:H7 infections; but that doesn't mean occurrence of *E. coli* should be ignored.

Klebsiella spp. was the least abundant pathogen as compared to *E. coli*, however; its prevalence was also high. The detected occurrence of *Klebsiella* spp. on the present work was 71.4% which is in line with the work of Abbas (2015) which detected 65.67% of *Klebsiella* spp. from Hilla River in Iraq. Also the present study recorded results that are in line with the results from the work of Barati et al. (2016), where 87% of *Klebsiella* was detected from Selinsing, Sangga Besar and Sepetang Rivers, Malaysia. A prevalence of 55% *K. pnumoniae* and 25% *K. oxytoca* were identified from surface waters in the work of Podschun etal.(2001). High

presence of *Klebsiella* spp. detected in the present work indicates the faecal contamination of the selected rivers. Occurrence of *Klebsiella* spp. lead to illnesses such as meningitis, wound infection and many more. *Klebsiella* spp. is held responsible for many infections amid South African new-borns (Ballot et al. 2012).

A significant prevalence of 66.7% was observed for *Enterobacter* spp. from river samples analysed in this study. This may imply that the two selected rivers are highly contaminated with *Enterobacter* spp. generally, the leading factors that lead to contamination of rivers by microorganisms is the discharge of agricultural, animal, domestic, human and industrial wastes into rivers (Tuckfield and McArthur, 2008; Martinez, 2009). High occurrence of *Enterobacter* spp. (32%) was observed by Karabi et al. (2015) from Ganga River. Other findings which are in line with the findings of the current study are the finding of Maravic et al (2015), who detected 33.9% of *Enterobacter* spp. in river water.

High prevalence (63.6%) of *Citrobacter* spp. from the analysed river water samples was observed in this study. High prevalence observed from the two selected rivers in the study poses a public health issue as these rivers are used for recreational activities and a source of drinking water for both humans and livestock. This high prevalence is also worrisome as *Citrobacter* spp. is an opportunistic pathogen responsible for causing several infection such as brain abscesses and neonatal (Clermont et al., 2015). These findings are in line with the findings of the study of Sabrina et al. (2016), who detected 61.7% of *Citrobacter* spp. from water and soil samples from the turtle cages. Also, the findings of this study are in line with the findings of the work of Kuczynski, (2016), who reported a prevalence of 76.4% of *Citrobacter* spp. which included *Citrobacter freundii* and *Citrobacter sakazakii* from Reconquista River in Argentina. High frequency of 47.36% of *Citrobacter* spp. occurrence from Rivers State, Nigeria was recorded by Akani et al. (2018) and these findings are in line with the findings of the current work. Several studies have recorded the occurrence of *Citrobacter* spp. and

Enterobacter spp. from river water (Mukhopadhyay et al., 2012; Guyomard-Rabenirina et al., 2017; Ribeiro et al., 2017).

5.3 Distribution of Enterobacteriaceae in hospital effluent

Hospital effluents are reservoirs of many pathogenic strains from patients and infected hospital workers. Hospital environments restricts the spread of bacteria however, admitted patients might shed bacteria with their excreta and these excretions flow to the hospital sewage drainage systems, multiply and spread to the communities (Walsh et al., 2011). In this work the obtained mean counts were 10 CFU/ 100 ml to 1×10^2 CFU/100 ml. Presence of Enterobacteriaceae in these hospitals effluents may be very hazardous to humans as these hospitals do not treat their effluents and they directly discharge their effluents to nearby rivers or municipal sewage system. These bacteria present in these effluents might be carrying resistant genes because of hospital effluents are reservoirs of high levels of antimicrobials (Brown et al., 2006, Kim and Aga, 2007, Verlicchi, 2012). Hospital effluents provide a suitable environment for pathogens carrying antimicrobial resistance determinants (Chagas, 2011). The discharge of contaminated hospital effluents to rivers if of particular concern as these organisms could persevere in the environment (Baquero et al., 2008).

Hospital wastewater act as receivers of great number of antimicrobials and human pathogens some with resistant genes and hence it is reflected as basin for antimicrobial resistant organisms and therefore contributes in the spread of AMR. *E. coli* was the dominant organism identified as compared to *Klebsiella* spp. in the present work. An extremely high prevalence of 84.6% of *E. coli* was observed from Queenstown and Mdantsane hospital effluents in this work. The findings of the current work are in contray with the work of Sood et al. (2015) which reported 9.1% of *E. coli* in Ahmedabad's tertiary-care hospital. However, findings from Mohamad et al. (2016) are in line with findings of the current study as a frequency of 70.5% *E. coli* in three hospital effluents from Iran was detected from the work of Mohamad et al. (2016). A frequency

of 46.6% *E. coli* was reported byLien et al. (2017) from Hospital Wastewater in Vietnam which corresponds with the findings from the current study. A 100% occurrence of *E. coli* was observed in six selected medical hospitals of Chittagong, Bangladesh (Hassan et al., 2015) and these findings correspond with the findings of the work at present. Presence of *E. coli* in hospital effluents can cause health problems as hospital wastes have a potential of transmitting diseases, especially if the wastes are not properly managed.

The current work detected 50% prevalence of *Klebsiella* spp. from the effluents of the selected hospitals. When comparing occurrence of *Klebsiella* to that of *E. coli* in the current work, *Klebsiella* spp. was the less abundant organism in these hospital's effluents. The recorded findings of the present study are similar to the findings of Picão et al. (2013) study which recorded 41.9% of *Klebsiella* spp. from tertiary teaching hospital in Brazil at São Paulo. Another study which has similar findings with the current study is the work of da Saúde and Paulo (2009) which detected 45.5% of *Klebsiella pneumoniae* from ten selected hospitals of Brazil at Goiânia. Findings from Ponniah et al. (2010) reported 34.9% occurrence of *Klebsiella pneumoniae* from a hospital effluent of a hospital in Rio de Janeiro city, Brazil, and these findings similar to the findings of the present work.

High occurrence of *Citrobacter* spp. with a proportion of 66.7% from the analysed hospital effluents water was recorded in this study. These findings are not surprising because hospitals are hot sport of several microorganisms (Blaak et al., 2011). This high incidence of *Citrobacter* from the analysed hospital effluents is worrisome because these bacteria will be distributed to river and communities via the discharge of hospital sewage systems. Normally these bacteria are transferred from infected patients and hospital works to sewage drainage through excretion of faeces. The hospital effluents water samples exhibited 66.7% occurrence of *Enterobacter* spp. in this study. High occurrence of this organism might imply that these hospitals consist of patients who are colonised by this organism and it passed to the hospital wastewater. This poses

a health issue as these organisms might be carrying resistant genes and antimicrobial resistant pathogens can cause severe infections that are resistant to the recently used therapeutic methods (Prasad et al., 2018). There are other studies who have previously recorded the occurrence of these organisms in hospital effluents (Moges et al., 2014; Haller et al., 2018).

5.4 Antimicrobial resistance profiles of the confirmed organisms

A significant occurrence of antimicrobial resistance among *E. coli* as well as *Klebsiella* spp. sourced from river water, vegetables and hospital effluent has been revealed in the present study. In *Klebsiella* spp. highest resistance of 100% was observed against ampicillin; followed by cefuroxime, gentamycin, cefotaxime, tetracycline and trimetroprim, while tetracycline resistance was high even in *E. coli* followed by doxycycline, nalixidic acid, ampicillin, amoxicillin cluvanate and cefotaxime and thus these antimicrobials cannot be used to treat infections caused by *E. coli* and *Klebsiella* spp. Occurrence of antimicrobial resistance in microorganisms from surface water, hospital effluents and vegetables raises a concern in public health as they can be transmitted to humans via consumption of vegetables or water and hence causes life-threatening diseases (Titilawo et al., 2015).

Camposa et al. (2013) also recorded high *E. coli* resistance against tetracycline and trimethoprim. Findings of Akther et al. (2018) revealed high *E. coli* resistance against ampicillin, tetracycline as well as cefotaxime which is corresponds with the findings of the present study. The high observed resistance against ampicillin, amoxillin, trimetroprim as well as tetracycline might be driven by the fact that some of these agents are used in human therapy (e.g. trimethoprim) and in agriculture (e.g. tetracycline). The drivers that can be attributable to implication of vegetables by multidrug resistant bacteria include irrigation water which is untreated and hence contain high loads of resistant microbes, incorrect manure fertilisers and unhygienic handling and processing. The current work recorded low resistance of *E. coli*

against gentamycin (36%) and these findings are in line with the findings of da Saúde and Paulo (2009) which detected low resistance of *E. coli* against gentamycin (40%).

Carbapenems are considered to have broad spectrum of activity and are of use as a secondary line of defence. According to the findings of the present study these antimicrobials can be still used in therapy for infections caused by *E. coli* as low resistance observed against imipenem and meropenem were low. Also the findings of da Saúde and Paulo (2009) displayed 100% potency of imipenem against *E. coli* which proves that these antimicrobials still hold their efficiency of broad spectrum activity.

Colistin and polymyxinB are said to be antimicrobials of last resort, however it is amazing how *E. coli* displayed high resistance against these antimicrobials. Also the work of Hassan et al. (2015) recorded 100% resistance of *E. coli* against colistin. There are several studies that have demonstrated resistance of *E. coli* against ampicillin, gentamycin, ciprofloxacin, trimetroprim, amoxicillin cluvanate, doxyclycline, nalixidic (Umolu et al., 2006; Enabulele et al., 2015; Hassan et al., 2015). High resistance of *Klebsiella* against ampicillin is also observed in the study of da Saúde and Paulo (2009). da Saúde and Paulo (2009), detected 100% resistance of *Klebsiella* against gentamycin and cefuroxime which is in line with findings of this study where 83% of resistance against these two antimicrobials was recorded.

All *Klebsiella* isolates were sensitive to imipenem in the study of da Saúde and Paulo (2009) which relates to the findings of the present study because high sensitivity of 83% against imipenem was observed on the work at present. Even though meropenem did not show great sensitivity (50% sensitivity displayed) against *Klebsiella* spp. on the work at present, however there was a high sensitivity of *Klebsiella* spp. against imipenem. This proves that carbapenem antimicrobial class can be reliable on treating infections induced by *Klebsiella* spp. During 1996 to 2000 *Klebsiella* spp. in China displayed 100% sensitivity to imipenem (Soo-Young et

al., 2007). From the work of Jeong et al. (2004) *Klebsiella* spp. displayed 100% sensitivity to imipenem. Even the study of Kundu and Islam (2015) displayed 100% sensitivity of imipenem against *Klebsiella* spp. High frequency of antimicrobial resistance in river water could be creditable to industrial, domestic as well as hospital wastes; runoffs or soil leaching as well as anthropogenic deeds.

High resistance of *Klebsiella* spp. against colistin and polymyxinB were observed on the present work. According to Bogdanovich et al. (2015) high resistance of *Klebsiella* against colistin was reported in Greece and South Korea hospitals. Wang et al (2018) reported 69% sensitivity of *Klebsiella* against colistin which these findings are in line to the present work findings where 50% sensitivity of *Klebsiella* against colistin was observed. Of the 92% of all Enterobacteriaceae that are resistant against carbapenem which is mediated by bla_{KPC} which also codes for colistin resistance constitute *Klebsiella pneumoniae* (CDC, 2009). In Italy, 43% of *K. pneumoniae* isolates producing carbapenemases were observed to be also resistant against colistin (Monaco et al., 2014). Findings from the work of Dallal et al. (2018) revealed high sensitivity of amikacin and imipenem to *Klebsiella* spp. and these findings are in line with the findings of the present study. In the study of Soltan Dallal et al. (2014) *Klebsiella* isolates displayed 92.5% sensitivity against imipenem respectively, and these findings are in line with the findings of the work at present.

All (100%) the *Citrobacter* spp. isolates displayed resistance against ampicillin, which is one of the penicillin's antibiotics. These findings are in line with the findings from the work of Moges et al. (2014), who also detected 100% resistance against ampicillin. This trend shows that *Citrobacter* spp. has developed resistance against ampicillin and therefore, from these findings ampicillin cannot be used to treat *Citrobacter* spp. infections such as meningitis. The resistance on *Citrobacter* spp. against ampicillin might be the result of extensive usage of

ampicillin and thus these pathogens were exposed to ampicillin and grew resistance due to selective pressure.

Also *Enterobacter* spp. isolates displayed high resistance of 92% against ampicillin. Viswanathan and Kaur (2001), recorded high frequency of 59.6% of *Enterobacter* spp. that are against ampicillin and these findings are in line with the findings of the current study. These findings suggest that ampicillin is not a considerable antimicrobial agent to be used for treating infections caused by *Enterobacter* spp. in the study area. A high frequency of 76% resistance against tetracycline and cefuroxime was displayed by the *Enterobacter* spp. on the present work. These findings are in line with the findings of Golly et al. (2016), who observed 100% resistance of *Enterobacter* spp. against tetracycline and cefuroxime.

5.5 Multiple Antibiotic Resistance Phenotypes (MARP) and Indices (MARI) of the confirmed organisms

Approximately 97.8% (44/47) of the *E. coli* isolates displayed resistance against more than two antimicrobials. Titilawo et al. (2015) also detected *E. coli* isolates that were displaying resistance to more than one antibiotic. Also *Klebsiella* isolates displayed high prevalence of multiple antimicrobial resistance which was 91.7%. According to Ramesh et al. (2010), high occurrence of multiple antimicrobial resistance might be caused by the excessive usage of antimicrobials in treating infections and hence the pathogens attained resistance against such antimicrobials. High levels of multi-drug resistance are probable in hospital effluents and to areas closer to hospitals (Sternbuerg, 1999), which is true based on the findings of the current study. Several studies have recorded high levels of multidrug resistance in *E. coli* isolates (Boerlin et al., 2005; Sayah et al., 2005; Sevanan et al., 2011). However, the study of Adefisoye and Okoh (2016) detected low levels of multiple antibiotic resistance in *E. coli* which was

32.7% which is in contradiction with the findings of the current work. The differences could be due to sampling size, geographical area and sampling methods.

Health risk that is associated with transmission of antimicrobial resistance in the environment was evaluated using multiple antibiotic resistance index (MARI). *E. coli* displayed MARI values ranging between 0.2 and 0.9, while *Klebsiella* spp. displayed 0.4 to 0.8 MARI values. Chika et al. (2017) also detected *E. coli* isolates exhibiting MARI values which were greater than 0.2. According to Krumperman (1983); Christopher et al. (2013), this data show that these bacterial isolates were from environments exposed to probable high contamination of antibiotics. The findings of this study on MARI values of *E. coli* are related with the findings of Tawab et al. (2014) and Talebiyan et al. (2015).

None of the *Klebsiella* isolates exhibited MARI value equal or less than 0.2. This suggests that the *Klebsiella* isolates were multiply resistant to several antimicrobials. These findings are similar the findings of Stanley (2017), who obtained MARI values greater than 0.4 for *Klebsiella* spp. recovered from surface waters in Nigeria.

All the *Citrobacter* spp. isolates displayed resistance to more than two antibiotics. The isolates displayed multiple antimicrobial resistance up to 17 antimicrobials. These findings are in line with the findings of Thapa et al. (2009), where *Citrobacter* spp. isolates displayed multiple antimicrobial resistance to more than two antimicrobials. Moges et al. (2014) recorded similar results, where 69.9% of the *Citrobacter* spp. on their work displayed multiple resistance from 2 to 12 antimicrobials. The MARI values obtained for the *Citrobacter* spp. in the current work were up to 0.9 and none of the isolates had MARI value less than 0.2. These findings are in line with the findings of Golly et al. (2016), who reported MARI value that was 1 from the *Citrobacter* spp. and all the *Citrobacter* spp. displayed 100% multiple antimicrobial resistance.

Viswanathan and Kaur (2001) also recorded multiple antimicrobial resistance *Enterobacter* spp. Also, the current study observed 100% of multiple resistance of the *Enterobacter* spp. to more than three antibiotics. This indicates that these samples were recovered from environments that are highly contaminated with antibiotics. 92% of the isolates displayed MARI value greater than 0.2 and these findings are in line with the findings of Golly et al. (2016), who reported a MARI value of 1 from *Enterobacter* spp. isolates. This implies that these isolates were highly exposed to antimicrobials. Both *Enterobacter* spp. and *Citrobacter* spp. displayed low resistance against carbapenems. From the findings of this work carbapenems still hold their proficiency of treating infections caused by these two organisms. According to Zhang et al. (2008) there are rare reports on resistance of *Citrobacter* spp. against Carbapenems.

5.6 Distribution of antimicrobial resistance genes in the confirmed organisms

Molecular detection of resistance genes was conducted in this study and there were eighteen antibiotic resistant genes detected. Both *E. coli* isolates and *Klebsiella* spp. isolates displayed resistance in one of the resistance genes encoding for aminoglycosides resistance which is *aadA*. Overuse of aminoglycosides might be the reason why all these isolates exhibit resistance against *aadA* gene. Long term usage of antimicrobials might genetically mediate resistance (Adefisoye and Okoh, 2016). The high existence of resistant genes in *E. coli* and *Klebsiella* spp. isolates might indicate the presence of other numerous resistant genes which were not of target on the work at present. High detection of the gene bla_{TEM} which confers resistance to beta-lactams was observed at a proportion of 77.8%. This finding corresponds with the findings of Adefisoye and Okoh, (2016), who detected 56.4% of the bla_{TEM} gene from *E. coli* isolates.

Low frequency of *tetA* and *tetM* was detected from *E. coli* isolates, which are the resistance genes coding for resistance against tetracyclines. These findings are shocking because there was a high frequency of resistance observed against tetracycline class. However these findings are not similar with the findings of Adefisoye and Okoh, (2016), who detected high frequency of *tetM* from *E. coli* isolates. The differences might be due to sampling sites, sampling seasons and sampling size. The findings on detection of antimicrobial resistance genes from *E. coli* on this study are in line with several studies (Bailey et al., 2010; Karczmarczyk et al., 2011; Titilawo et al., 2015; Adefisoye and Okoh, 2016).

The gene bla_{SHV} was only present in *Klebsiella* isolates with a frequency of 16.7% and it was only exhibited by two isolates. These findings are not in line with the findings of Mohsen et al. (2016), who detected 50% frequency of bla_{SHV}. The frequency of bla_{TEM} detected for *Klebsiella* was 33.3% and these findings are in line with the findings of Mohsen et al. (2016), who detected 53.1%. Findings of this study also relates to findings of Lim (2009), who detected 59.59% of bla_{TEM} on *Klebsiella* isolates. All *Klebsiella* spp. isolates exhibited 100% frequency on *strB*, *aadA* and *aac2A*. Occurrence of these resistant genes enables *Klebsiella* spp. to be resistant to several antimicrobials and hence poses a public health issue as it will be difficult to treat infections caused by *Klebsiella* spp. with the available antimicrobials.

Both *Citrobacter* and *Enterobacter* spp. displayed low occurrence of *sul1* and *sul2* resistant genes. These findings were surprising as there was a high resistance against trimethoprim by both organisms. High frequency of aminoglycoside coding genes such as *strA*, *aadA*, *strB* was detected in both the organisms; especially in *Enterobacter* spp. these findings were not surprising as there was a high observed frequency of resistance against gentamycin and amikacin observed in both the organisms. bla_{TEM} was the most dominant beta-lactam coding resistant gene among the other genes coding for beta-lactam resistance in both *Enterobacter* spp. and *Citrobacter* spp. This implies that bla_{TEM} was the most gene conferring resistance

against ampicillin and augmentin antibiotics. These findings are in line with the findings of Shahid, (2010), who detected 40% presence of *bla*$_{TEM}$ from *Citrobacter* spp. *bla*$_{SHV}$ was not detected in both *Citrobacter* spp. and *Enterobacter* spp. these findings are in line with the findings of Liu et al. (2017), who detected a very low frequency of 2.8% of *bla*$_{SHV}$ from *Citrobacter* spp.

CONCLUSION

Obtained results from this study evidently showed that vegetables, river water and hospital effluents are major potential reservoirs of antimicrobial resistant Enterobacteriaceae and antimicrobial resistant genes. Detected AMR genes might spread to other pathogenic strains. Infection caused by resistant pathogens cause individuals to be admitted in hospitals for longer durations than normal (Roberts et al., 2009). High occurrence of Enterobacteriaceae in these

selected samples might indicate the presence of other pathogens which are not targeted and also indicate faecal contamination.

High frequency of Enterobacteriaceae in vegetables might be attributable to soil contamination as the selected vegetables grows closer to the soil and therefore stand a high chance of being contaminated by soil. Many studies have demonstrated the occurrence of Enterobacteriaceae in vegetables (Sahilah et al., 2010; Tunung et al., 2011; Puspanadan et al., 2012). The factor that contributes to high occurrence of Enterobacteriaceae to leafy fresh produce is that the structure of these vegetables promotes the growth of microbes. Leafy greens consist of huge surface areas which enables fast utilization of nutrients plant tissues by microbes (Soriano et al., 2000). Microbes are then favoured to grow because of the moisture on fresh produce surfaces (Puspanadan et al., 2012).

A leading factor that contributes to the occurrence of antimicrobial resistant organisms in rivers is human settlements closely built next to rivers and also agricultural farming; especially small-scale farming plays a vital role in increasing the occurrence of resistant pathogens in rivers (Titilawo et al., 2015). High distribution of Enterobacteriaceae poses severe health risks especially to immune-compromised people. High levels of Enterobacteriaceae occurrence in rivers observed in this study might be the result of poor sewage disposal systems. Monitoring of water suppliers is important as it regulates the absence of faecal coliform and Enterobacteriaceae before discharging waters.

Isolates displayed high resistance against ampicillin, tetracycline, doxycycline and trimethoprim. Increasing antimicrobial resistance poses a major challenge in human medicine as several therapeutic practises involve the use of antimicrobials. However, findings of this study indicate that the carbapenems can be still antibiotics of choice for treating a number of infections caused by Enterobacteriaceae in the study area. According to Bogdanovich et al.

(2015) for many decades colistin was not recommended to be used because of its toxicity and there were other safe antimicrobials present such penicillins, now the use of colistin has been implemented because of the rise in AMR.

Our findings have showed that the selected rivers can pose a significant health problem as they are highly contaminated with bacteria. These bacteria might have gain entrance to the river through soil leaching and runoffs from agricultural lands. High occurrence of bacteria in river might pose serious risk to consumers as other farmers use irrigation water from surface water resources for the irrigation of fresh produce. The high bacterial loads also pose risks to the users who still depend on the rivers for their domestic purposes. Also the study revealed raw vegetables contain high loads of pathogenic bacteria, this contamination might be the result of lack of hygiene and sanitation precautions from agricultural farms and in stores. From the findings of the study, it has been observed that vegetable contamination by pathogens occurs in farms than in shelves in the shops. The occurrence of *Klebsiella* spp. was low in vegetables as compared to other analysed samples while *Citrobacter* was the dominant organisms in vegetables. Also high bacterial loads were observed in hospital effluents samples, these pathogens might have come from infected patients and they gained entrance to the hospital sewage system through patient's excretions. This high occurrence of these pathogens in the analysed hospital effluents might pose health risk as these hospitals discharge their effluents untreated to the municipal sewage systems and the other discharge its effluent to the river.

The findings of this study revealed high resistance against tetracyclines, sulphonamides, penicillins and aminoglycosides. This means these antimicrobials have lost their spectrum activity and therefore fail to treat infections. The extensive use of antimicrobials in agriculture might have be the reason that caused these pathogens to develop resistance. The MARP evaluated on this study revealed that all the target organisms were resistant to more than one antibiotic. For all the MARI values that are greater than 0.2 obtained in this study indicates that

the isolates were exposed to antibiotic pressure and this pressure might have resulted from the extensive usage of antimicrobials. Therefore, without measures established to decrease the extensive usage of antimicrobials, the field of medicine will run out of effective broad spectrum antimicrobials. The findings in this study highlights the need for urgent development of alternative antimicrobials and the need to implement rules and regulations for limiting the use of carbapenems as to try and conserve their broad spectrum activity.

RECOMMENDATIONS

- It is unsafe to consume uncooked vegetables and therefore vegetables should be thoroughly cooked at all times.
- Consumers should be conscious when preparing foods such as ready ready-to-eat salads as raw vegetables can cause significant health implications when contaminated with pathogenic bacteria.
- Fields such as agricultural fields should conduct continuous monitoring of their soils, fertilizers and irrigation water to evaluate occurrence of microorganisms and workers should keep hygienic precautions at all times to decrease the chances of contamination.
- Another factor that drives to contamination of fresh produce by microorganisms is the use of contaminated vehicles, and hence agricultural farmers should clean their transporting vehicles.
- Hospitals should always treat their primary effluent before discharging it to rivers or municipal wastewater systems.

- There should be regulations enforced on agricultural facilities regarding the use of antimicrobial agents for non-therapeutic purposes.
- Agricultural facilities should also treat their primary effluents before discharging it to rivers in order to reduce the chance of contamination of surface waters.
- There should be enforced rules and regulations on each antimicrobial agent prescribed in order to reduce the rate of AMR.

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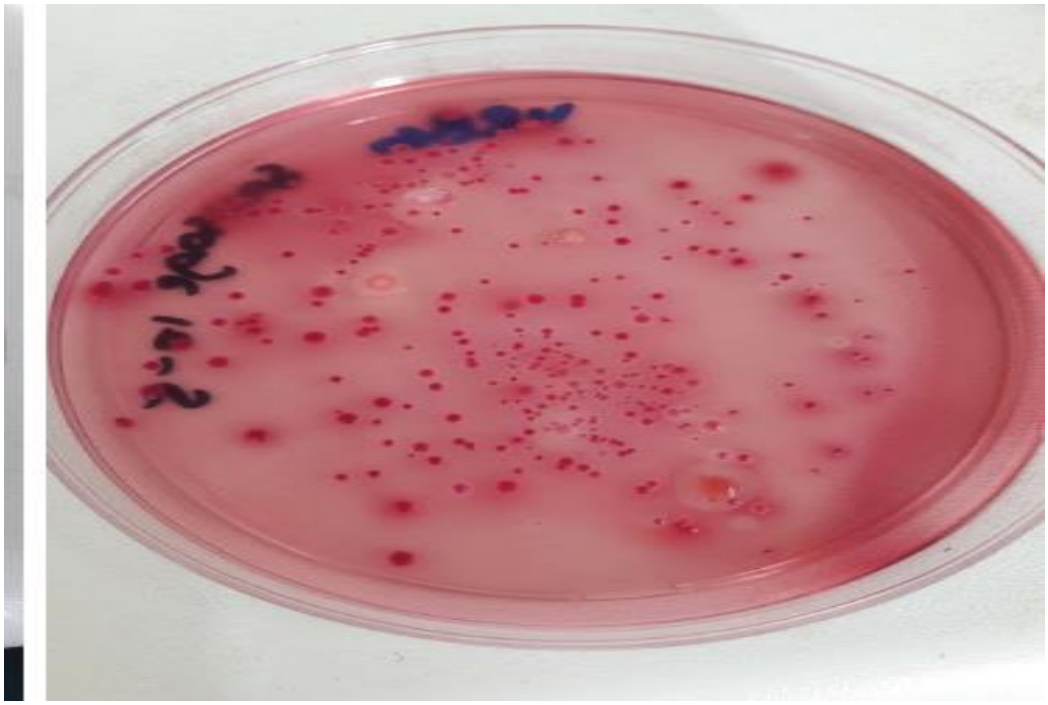
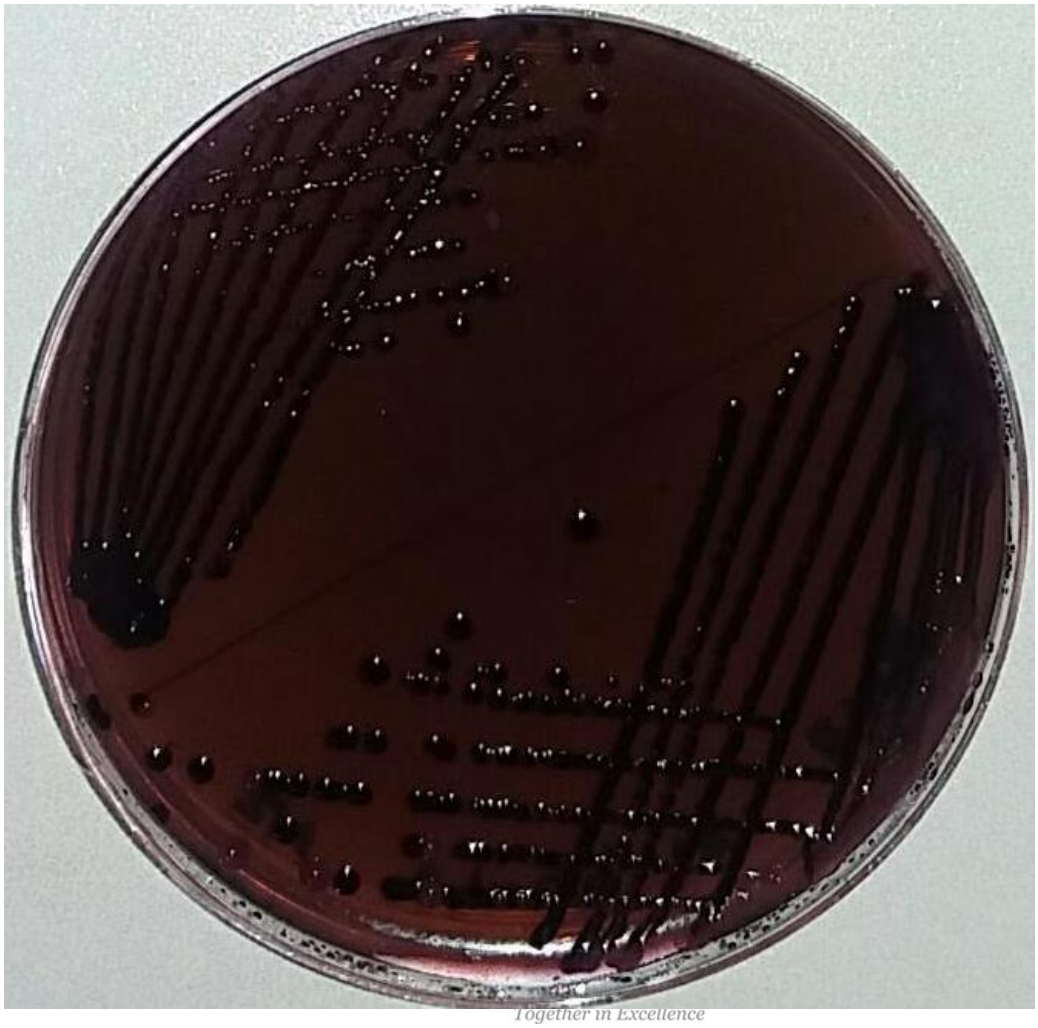
APPENDICES

Appendix 1: Photographs taken during the course of work

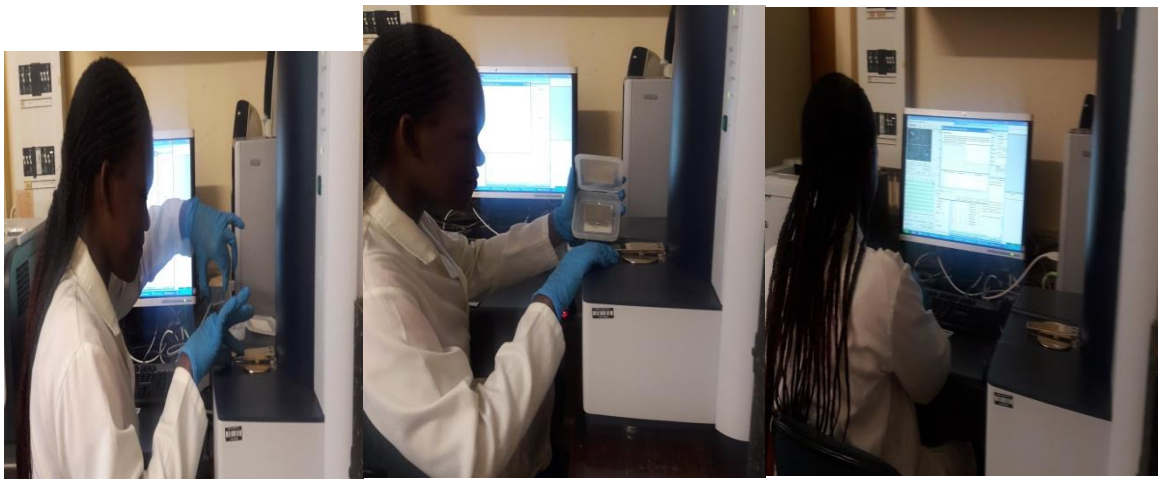
Appendix 1a: photograph of one of the selected sampling sites.



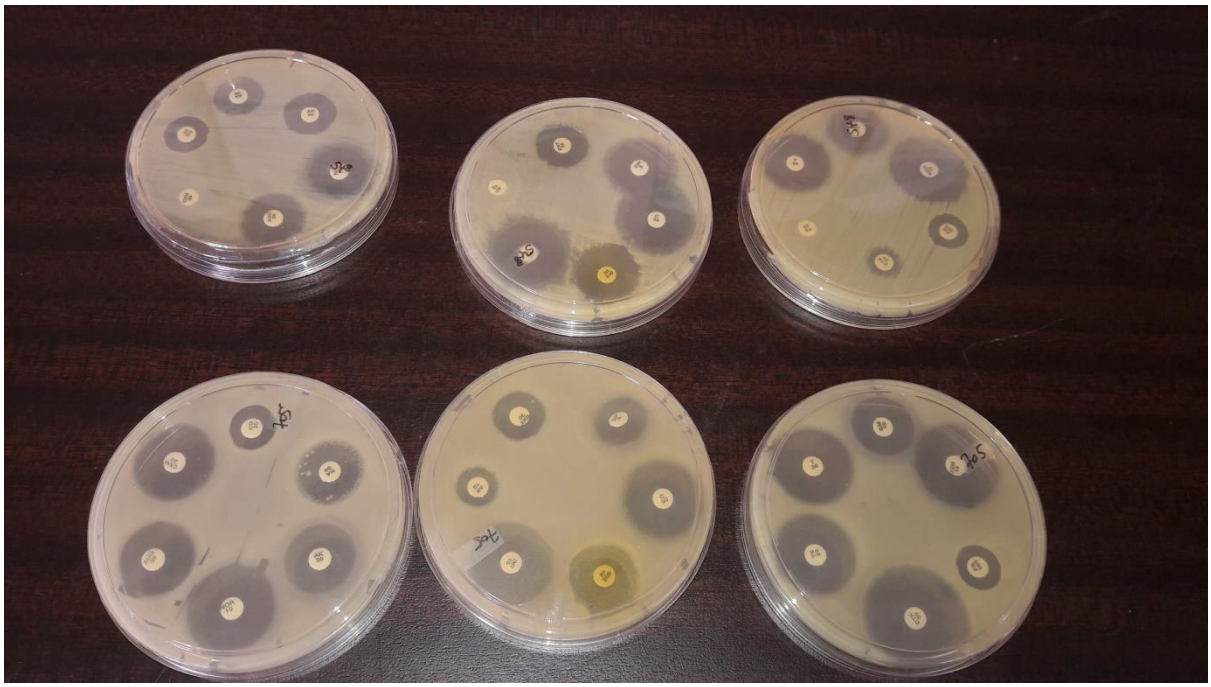
Appendix 1b: photograph of Violet Red Bile Glucose agar, for the enumeration of Enterobacteriaceae.



Appendix 1c: photograph of Eosin Methylene Blue agar during isolation of the targeted organisms.

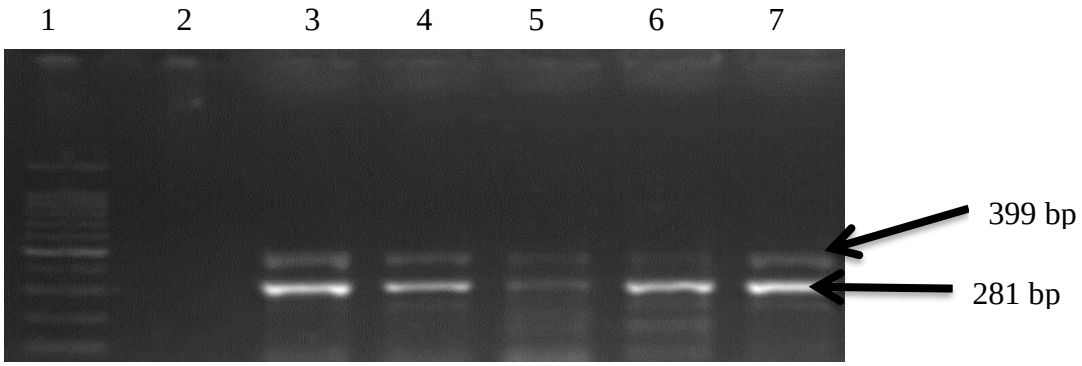


Appendix 1d: photographs taken during MALDI-TOF analysis.

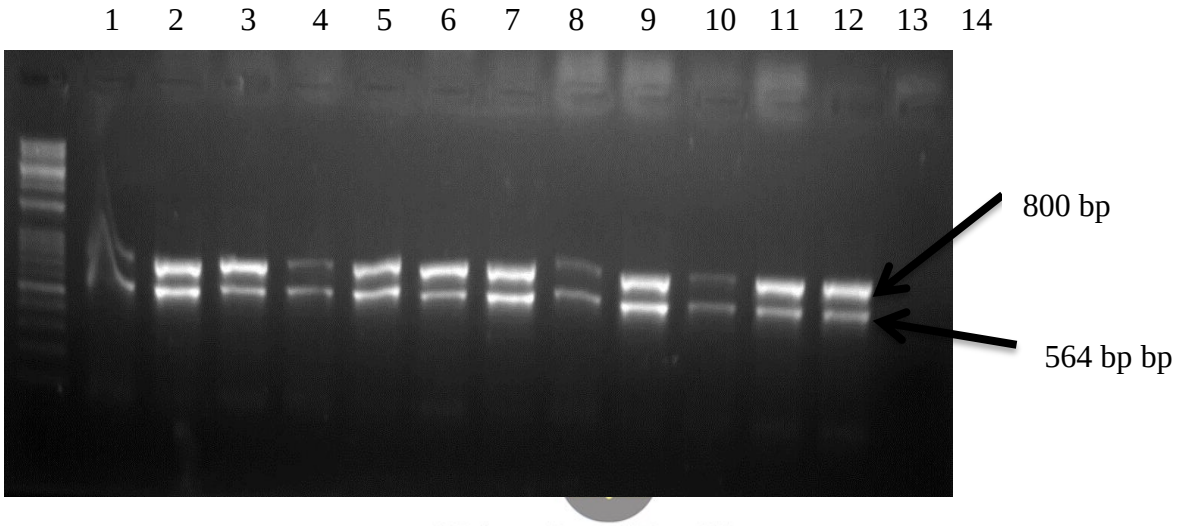


Appendix 1e: photographs of plates during susceptibily test.

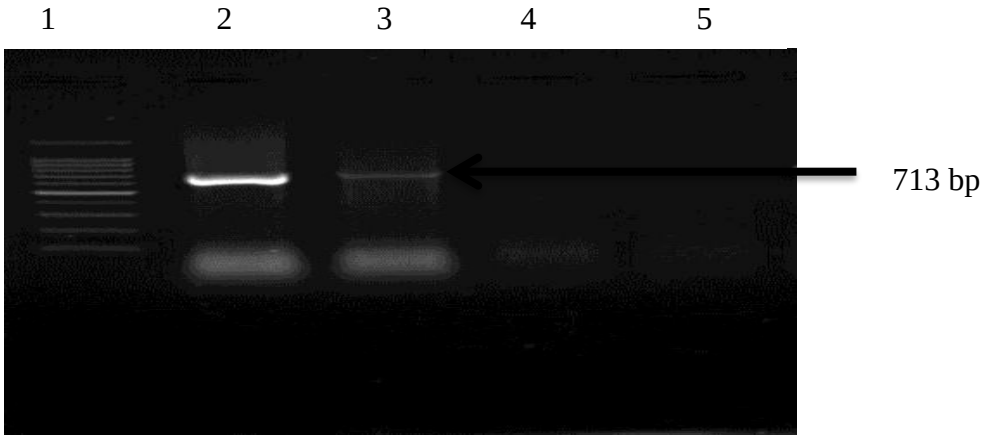
Appendix 2: List of other Gel Electrophoresis pictures of the detected resistant genes



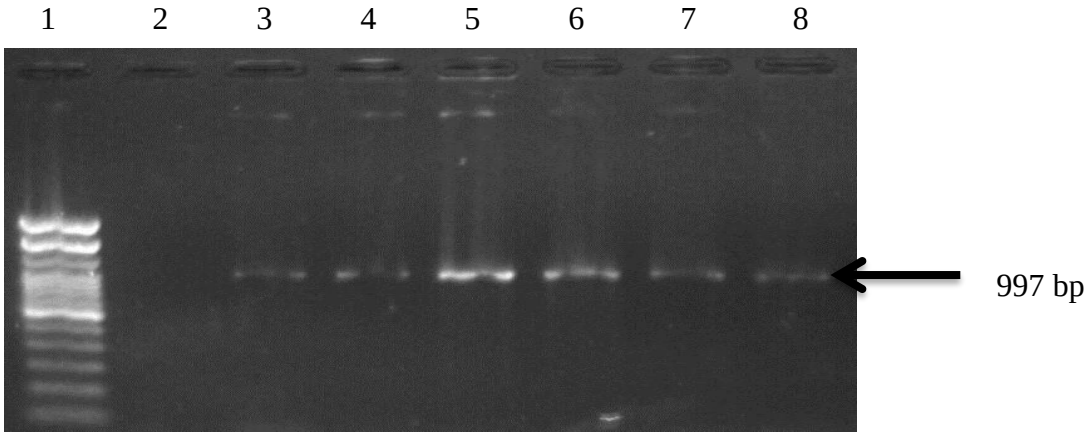
Appendix 2a: Gel picture representing molecular detection of *bla*$_{GES}$ (399 bp) and *bla*$_{OXA-48}$ (281 bp) = genes. Lane 1: DNA Ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), lane 3 – 7: Isolates exhibiting resistance genes.



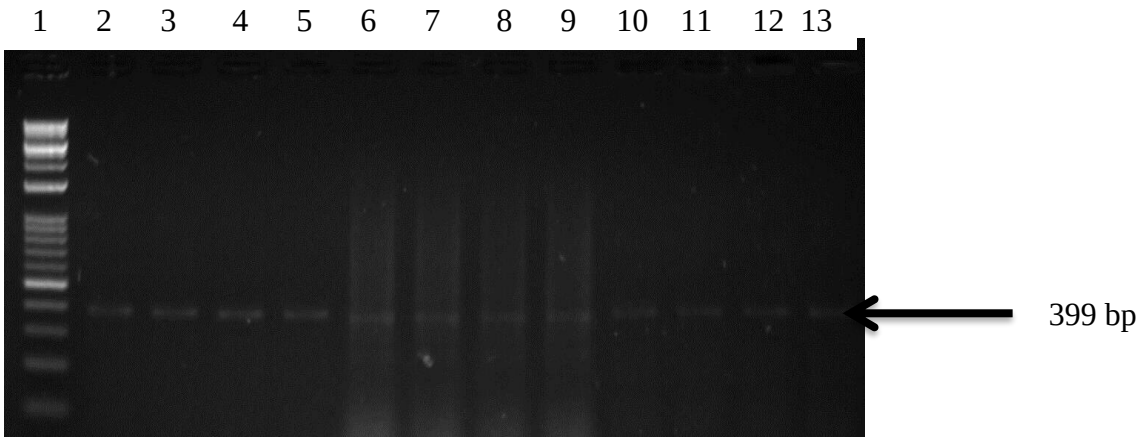
Appendix 2b: Gel picture representing molecular detection of *bla*$_{TEM}$ *(*800 bp*) and bla*$_{OXA}$ *(*564 bp)resistance genes. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 14: isolates exhibiting resistance genes.



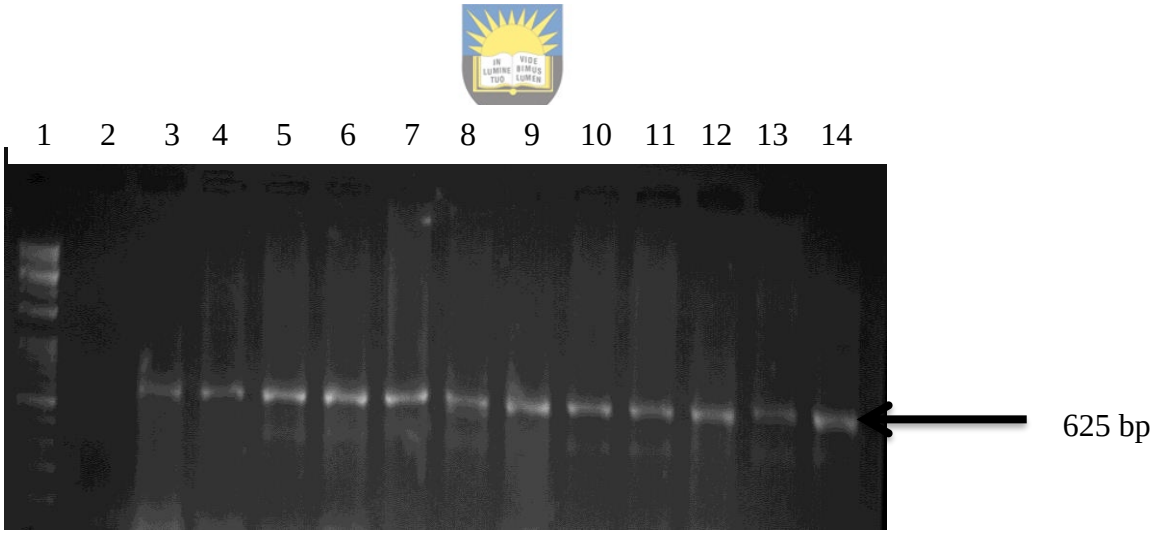
Appendix 2c: Gel picture representing molecular detection of *bla*$_{SHV}$ (713 bp) resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2 & 3: isolates exhibiting resistance genes, Lane 4: negative control (water + all PRC components).



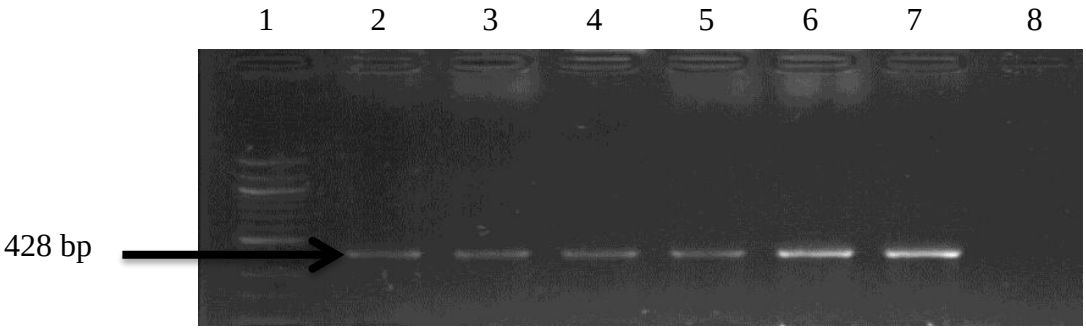
Appendix 2d: Gel picture representing molecular detection of *bla*$_{DHA}$ *(*997 bp*)* resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 8: isolates exhibiting resistance genes.



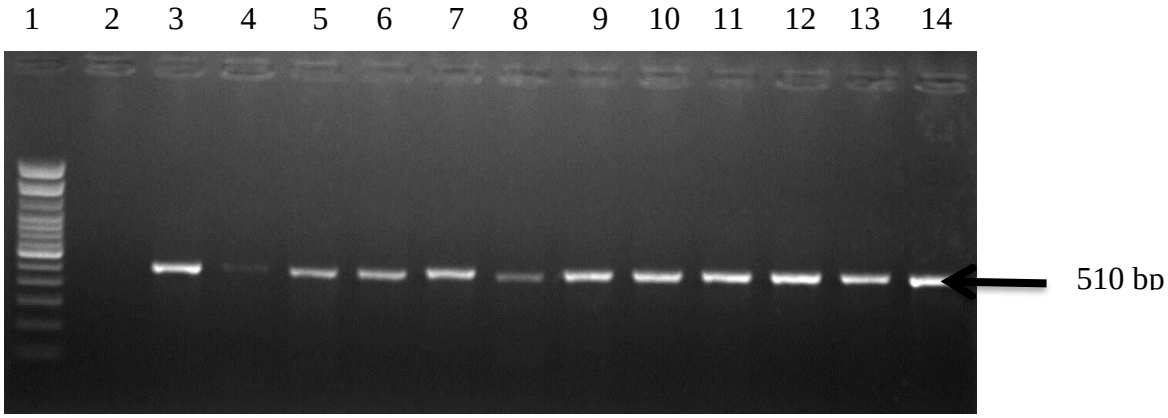
Appendix 2e: Gel picture representing molecular detection of *bla*$_{GES}$ (399 bp) resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 13: isolates exhibiting resistance genes.



Appendix 2f: Gel picture representing molecular detection of *sul2* (625 bp) resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 14: isolates exhibiting resistance genes.



Appendix 2g: Gel picture representing molecular detection of *aac2A* (428 bp) resistance gene. Lane 1: ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), Lane 3 – 14: isolates exhibiting resistance genes.



Appendix 2h: Gel picture representing molecular detection of *aphA2* (510 bp). Lane 1: DNA Ladder (Molecular Marker Thermo-Scientific, 100 bp), Lane 2: negative control (water + all PRC components), lane 3 – 14: isolates exhibiting resistance genes.