PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF DIARRHOEAGENIC *Escherichia coli* ISOLATED FROM CHILDREN IN MUKURU INFORMAL SETTLEMENT, NAIROBI COUNTY, KENYA

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DECLARATION

I declare that this thesis is my original work and it has not been presented in any other University for award of degree.

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DEDICATION

I dedicate this piece of work to my husband Simon Musawa, my parents Mr. Patrick Kinankau and Mrs. Agnes Kinankau, my siblings Felix Murithi and Denis Muthaura for their love, support, patience and encouragement in pursuit of academic excellence.

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ABBREVIATIONS AND ACRONYMS

AAF	Aggregative-adherence fimbriae
agg	Aggregative gene
AID	Acute Infectious Diseases
AIDS	Acquired Immune Deficiency Syndrome
AMC	Amoxicilin/clavulanic acid
Amp	Ampicilin
C	Chloramphenical
Caz	Ceftazidine
Cip	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
DEC	Diarrhoeagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
eaeA	Attaching and effacing gene
EAEC	Enteroaggregative *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
Fep	Cefepime
Gen	Gentamicin
HIV	Human Immune Virus

KEMRI- CMR	Kenya Medical Research Institute- Centre for Microbiology Research
MCH	Maternal and Child Health
MDG	Millenium Development Goals
MM	Mukuru Kwa Njenga
MR	Mukuru Kwa Reuben
Na	Nalidixic acid
PCR	Polymerase Chain Reaction
PLWHA	People living with HIV/AIDS
S	Streptomycin
STEC	Shiga toxin-producing *Escherichia coli*
sxt 1	Shiga toxin 1 gene
sxt 2	Shiga toxin 2 gene
SXT	Sulphamethaxazole/trimethoprim
Te	Tetracycline
UNICEF	United Nations Children's Fund
WHO	World Health Organization

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ABSTRACT

Diarrhoeal diseases in Kenya are among the five main causes of mortality in children younger than five years. Bacterial diarrhoea has been reported to account for up to 30 % of all cases of infantile diarrhoea. Among children of age five years and below, diarrhoeagenic *E. coli* (DEC) such as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC) are the most important enteric pathogens and are responsible for 30 to 40 % of all the diarrhoeal episodes in developing countries. The circulation of different pathogenic *E. coli* is an important problem in developing countries enhanced by many factors, for example, climatic adversities, poor sanitation, malnutrition and AIDS related immunosuppression among others. Additionally, antibiotic susceptibility profiles vary from time to time. This makes it necessary to carry out susceptibility testing frequently to ensure that the right medication is given. The objectives of this study were to characterize at molecular level the different strains of *E. coli* isolated from diarrhoea children under the age of 5 years in Mukuru Kwa Njenga and Mukuru Kwa Reuben slums in Nairobi, Kenya. Bacteria isolated between May, 2013 and July, 2013 and archived in KEMRI-CMR were used in this study. Biochemical tests were used to confirm identity of the revived samples. The study also evaluated the resistance of the identified strains to different antibiotics (ciprofloxacin 5µg, nalidixic acid 30 µg, tetracycline 30 µg, streptomycin 10 µg, chloramphenicol 30 µg, gentamicin 10 µg , ceftazidine 30 µg, amoxicillin clavulanic acid 20/10µg, sulfamethoxazole trimethoprim1.25/23.75µg , Ampicillin 10µg and cefepime 30µg) using Kirby Bauer technique. Multiplex PCR was used to identify DEC pathotypes through detection of various virulence genes. Data was entered using Excel (Microsoft) and checked for integrity and consistency. Statistical analysis was performed with statistical package for social sciences (SPSS) version 21.0. Categorical variables were analyzed using frequency distributions. The study found out that the isolates registered high resistance against SXT (62.18%) followed by tetracycline (47.44%), ampicilin (46.15%), AMC (18.6%), Streptomycin (14.7 %), nalidixic acid (13.5%) and ciprofloxacin (10.35%). Isolates were highly susceptible to ceftazidine (96.2 %), Cefepime (96.8 %), gentamicin (93.6%) and Chloramphenicol (92.9%). Multi drug resistance was also evident in that 43.59 % of isolates were resistant to at least three antibiotics. The results of one way ANOVA showed that there was no statistical significant difference between antibiotic susceptibility profiles of *E. coli* isolates from male and female children. However, isolates from male children showed higher resistance to cefepime, ceftazidine and streptomycinwhereas isolates from female children showed high resistance to the other eight antibiotics. The findings of the study also revealed that isolates from children in Mukuru kwa Reuben (MR) showed high rate of resistance against most of the antibiotics. However, the differences between susceptibility profiles of isolates from the two locations were not statistically significant. Antibiotic susceptibility profiles did not vary significantly among isolates from children of different ages for most antibiotics apart from SXT and tetracycline ($P=0.03$ and $P=0.05$ at $P<0.05$, respectively). The findings of the study showed that reserve antibiotics are the best treatment of childhood diarrhoea for children in Mukuru slums. The study also recommended that Sulphamethaxazole/Trimethoprim, ampicilin and tetracycline use should be minimized since they are not effective in diarrhoea treatment.

CHAPTER ONE

INTRODUCTION

1.1Background of the study

Diarrhoea is an intestinal condition that is characterized by passage of loose or watery stools in large volumes at abnormally frequent rates.It is also defined as the high frequency of bowel movement relative to the usual pattern for any particular person (WHO, 2013). It is a common symptom of gastrointestinal infections due to ingestion of many bacteria, viruses or parasites that may be transmitted by water, food, utensils, hands and flies. There are three clinical syndromes of diarrhoea: (i) acute watery diarrhoea, lasting less than 14 days; (ii) bloody diarrhoea; and (iii) persistent diarrhoea, lasting at least 14 days. Diarrhoea results in electrolyte loss, dehydration, shock and sometimes death (UNICEF/WHO, 2009).

Globally, diarrhoea is the second leading cause of morbidity and mortality among children under five years in the developing world (UNICEF, 2014; UNICEF, 2012; WHO 2012; Lopez *et al*., 2006). It is second to pneumonia and together they account for almost 29 % of children deaths below five years of age(WHO, 2013). Diarrhoea is said to kill more children than a combination of malaria, AIDS and measles. On average children under the age of 3 years in developing countries experience about three episodes of diarrhoea every year (WHO, 2013). Whereas in developed countries diarrhoea is viewed as “a little more than an inconvenience” (UNICEF/WHO, 2009), in developing countries it causes a lot of deaths and it cannot be ignored. Reports from the World Health Organization and UNICEF show that the burden of childhood diarrhoea varies from one developing region to another, with the greatest burden experienced in sub Saharan-Africa

and South Asia. Diarrhoea has been causing a lot deaths despite the decrease from 2000 to 2013 of more than 50 percent from over 1.2 million to lower than 0.6 million (UNICEF, 2014). This is due to low percentage of children being given oral rehydration, exclusive breastfeeding, levels of water cleanliness and sanitation in general. Most of these are only available for children from wealthy families including exclusive breastfeeding whereby most women from needy backgrounds leave their babies very early after birth to go in search of basic needs unlike wealthy people who take maternity leaves from their respective work places. These conditions always leave the children in low socioeconomic backgrounds being in need (UNICEF, 2014).

Although mortality rates among these children have declined globally from approximately 3.3 million annually in the 1980s (Aruji, 2004) to 79 per 1,000 in 2003 (David *et al*., 2005) the situation in Africa is strikingly different compared with other regions of the world. African region shows the smallest reductions in mortality rates and the most marked slowing down trend.Lack of water, hygiene and sanitation are still a big problem in these countries with most children born in homes with no access to sanitation (UNDP, 2006; UNICEF, 2006). Currently, only 36 percent and 39 percent (in Africa and Asia respectively) of children suffering from diarrhoea in low-income countries are administered with the recommended therapy (UNICEF, 2014). This little progress in can be attributed to reduction of attention from fighting diarrhoea to HIV/AIDS which has led to a reversal of the progress made towards eradication of diarrhoea.

Diarrhoeal diseases cause 13 % of deaths among children below five years in Kenya and are second only to pneumonia (Makobe *et al*., 2012). Millions of dollars are spent on treatment of diarrhoea annually(UNICEF/WHO, 2009). Diarrhoea is more prevalent in

the developing world dueto lack of safe drinking water, sanitation and hygiene, and poorer overall health and nutritional status (UNICEF, 2014). Efforts to define the underlying biological mechanisms have identified nutritional, microbiological and immunological factors to be associated with specific patterns of diarrhoea morbidity and mortality(Makobe *et al*., 2012). The causes of diarrhoea include a wide range of viruses, bacteria and parasites(Moyo, 2007). These causative agents are found in high amounts in developing countries compared to the developed countries. Additionally bacterial causes of diarrhoea are prevalent in developing countries whereas viruses are the most common cause of diarrhoea in developed countries.

The bacterial pathogen most commonly associated with childhood diarrhoea is *E. coli* and at least six categories have been described: enteropathogenic *E. coli* (EPEC); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enterohemorrhagic *E. coli* (EHEC), also known as shigatoxigenic *E. coli* (STEC); diffusely adherent *E. coli* (DAEC); and enteroaggregative *E. coli* (EAEC). Diarrhoeagenic *E. coli* (DEC) disease is prevalent in infants and children and causes diarrhoea outbreaks in many countries(Makobe *et al*., 2012). Currently,DEC also stands as a chief causal factor for diarrhoea among travelers (Rawan, 2012).Identification of DEC strains requires that these organisms be differentiated from non-pathogenic members of the normal flora.Diarrhoeagenic *E. coli* strains are known to cause gastrointestinal illness in humans and other animals (Kobayashi, 2002; Aranda, 2004). Informal settlements in cities such as Nairobi present multiple opportunities for infection with such agents and represent a particular epidemiological setting for transmission of these pathogens.

1.2 Problem statement and justification

Urbanization in sub-Saharan Africahas been increasing at a very fast rate (UN, 2013).However the rate of urban economic growth has not been increasing in the same proportion (UN, 2013).This unsustainable urbanization has led to the growth of slums. Over the last several decades, much has been learnt about human health in Urban and Peri-Urban environments, with an appreciation of the fact that urbanization tends, in poorer groups at least; to lead to lower health and the realization that improving the health of these populations requires multi-sectorial inputs (Harpham, 2009). The circulation of different pathogenic *E.coli* is an important problem in developing countries enhanced by many factors, for example, climatic adversities, poor sanitation, malnutrition and AIDS related immunosuppression among others. These high risk socio-economic parameters lead to favourable environmental condition for emergence of new enteropathogenic organisms especially in childhood (Clarke, 2001). Environmental influences play a significant role in pathogenesis when virulent organisms come into contact with a suitable host (Beceiro *et al*., 2013) In general, regulation and expression of bacterial virulence genes can be influenced by environmental stimuli such as pH, temperature and the availability of certain nutrients (Michael *et al*., 2012).

Additionally, antibiotic resistance in pathogenic bacterial strains has rapidly increased in the last 30 years with the alarming recent appearance of multiple resistant strains, commonly known as superbugs (Nordmann *et al*., 2007).The epidemiological significance of each *E. coli* category in childhood diarrhoea varies with the geographical area and depends on the socioeconomic/sanitary conditions achieved. It has become clear that there are important regional differences in the prevalence of the different categories

of DEC. Monitoring and characterizing *E.coli* strains, relating to clinical symptoms is crucial for epidemiological control, in order to understand genetic rearrangement of virulence factors and track emergence of new pathogenic strains. In Kenya, antimicrobial resistance surveillance has been conducted only at institutional levels, with limited sharing of information and analysis of data. As a result, the actual scale of regional or national antimicrobial drug is not well defined (Sang *et al*., 2012).

Enteric diseases, particularly those caused by bacteria, are readily transmitted in the developing world due to the prevalence of overcrowding of individuals in poorly constructed shelters, lack of piped water and sanitation and widespread faecal contamination of the environment (Levine and Levine, 1994).Diarrhoeagenic *E. coli* are an emerging bacterial threat to human health in Africa and the world (World Health Organisation, 2003; Clarke, 2001). Results from a World Health Organisation report shown that increased levels of microbes of faecal origin in containers, over time, can be associated with increased cases of diarrhoea (Sobsey, 2002). The emergence of new waterborne pathogens, including that of diarrhoeagenic *E. coli*, can be attributed to genetic alteration and adaptation of micro-organisms (rendering them more virulent) to changes in the immuno-competence of the host (rendering them more susceptible to infection) and to socio-economic and environmental changes (increasing the risk of exposure to the pathogen (World Health Organisation, 2003).

1.3 Research questions

i. Are there any significant differences in the antibiotic susceptibility profiles among different *E.coli* isolates?

ii. Are there any effects of gender, age and source of the *E. coli* isolates on antibiotic susceptibility profiles?

iii. Which is the most prevalent *E. coli* pathotype from all the isolates collected?

iv. Are there any virulence genes present in the purposively selected samples of the highly resistant isolates?

1.4 Hypotheses

i. There is no significant difference in the susceptibility profiles of the different *E.coli* isolates.

ii. There are no significant differences in gender, age and source on antibiotic susceptibility profiles of different *E.coli* isolates.

iii. There is no significant difference in the *E. coli* pathotype identified from the different isolates collected?

iv. There is no significant difference in the virulence genes present in the isolates that are highly resistant to antibiotics.

1.5 Objectives

1.5.1 General objective

To determine the phenotypes and genotypes of *E. coli* isolates obtained from diarrhoea children under the age of 5 years living in Mukuru Kwa Njenga and Mukuru Kwa Reuben informal settlements in Nairobi.

1.5.2 Specific objectives

i. To confirm the identity of archived *E. coli* isolates obtained from KEMRI-CMR.

ii. To determine the antibiotic susceptibility profiles of the *E.coli* isolates to antibiotics used to manage diarrhoea in children.

iii. To determine the effects of gender, age and source of *E. coli* isolates on antibiotic susceptibility profiles.

iv. To determine the presence of virulence genes in a purposively selected sample of *E. coli* isolates that are highly resistant to the used antibiotics.

1.6 Significance of the study

The results obtained through this study will be made available to policy makers, scientific community, members of the media, those who contributed information, the community of Mukuru Kwa Njenga and Mukuru Kwa Reuben informal settlements. It is realistic to expect that targeted interventions resulting from recommendations developed in this study would result in a reduction in the incidence of human disease. Additionally, knowledge of the dominant pathotypes in the region will help doctors to prescribe medication that is more specific to the disease causing microorganisms. This will therefore help to guide physicians to choices that are more appropriate when therapy is needed and discourage inappropriate use of the antibiotics.

Surveillance of antibiotic resistance in a local region is very important, because it shows the local pattern of resistance, helps choose useful empirical therapy and most efficacious antibiotic therapy, and can modify antibiotic therapy accordingly.

CHAPTER TWO

LITERATURE REVIEW

2.1 Acute infectious diarrhoea (AID) – A worldwide problem

Acute infectious diarrhoea (AID) is a disease of both children and adults although majorly experienced by children below five years of age. It is and has been a major cause of morbidity and mortality for decades globally as shown by several researchers (UNICEF,2013;2012).Diarrhoea remains a major public health challenge especially in developing countries where it is a leading cause of death second to Pneumonia (UNICEF,2013; UNICEF, 2012; UNICEF/WHO, 2009). According to the latest statistics by UNICEF (2014) diarrhoea is directly associated with 9 per cent of deaths of children below five years globally. Most of these children are from developing countries with Africa and South East Asia contributing approximately 78 per cent of those deaths (Farthing, 2012). Industrialized countries have very few cases of diarrhoea (UNICEF, 2012); however diarrhoea continues to be associated with very high health care costs. Huge chunks of money are directed towards management of health in industrialized countries- vaccination, hygiene and clean water provision.

Developing countries have food security as their number one priority since hunger is their biggest problem with most individuals not able to access a meal a day. This has resulted in most of these countries concentrating on ways to feed the people. Although health is also among the top five problems in these countries they remain incapacitated to give it the attention it deserves as they lack enough funds and therefore have to rely on loans and grants from developed countries. This situation has led to developed countries not devoting a lot of money to health and health related issues hence the current condition of

high rate of diseases in these countries of which diarrhoea is not an exemption. The mean number of episodes of diarrhoeaper year in children under 5 years of age from a developing region is 3 with peak taking place at approximately 3.2 years (Gupta, 2014).The highestincidence (4.8 episodes) takes place during the first year of the children life and these decreasesprogressively to1.4 episodes per year at 4 years of age (Kosek *et al*., 2003). These episodes are responsible for worsening the nutritional status of the bodies of children which leads to poor growth and development as well as malnutrition which is an underlying cause of diarrhoea and vice-versa (Bryce *et al*., 2003 ;WHO, 2013).

Globally diarrhoea is caused as a result of similar factors. Individuals that are exposed to poor living conditions, for example, poor sanitation, improper waste disposal, lack of clean drinking water and bad drainage systems among other factors are more prone to diarrhoea than those living in good environments.Improvements in sanitation, nutrition, education and early access to oral rehydration therapyamong other measures have lowered the lethality of severe AID from 4.6 million in 1982 toan estimated less than 0.6 million in 2014 (UNICEF, 2014).

Several recognized microorganisms such as bacteria, viruses and parasites are associated with severe AID in children (Al-Gallas *et al.*, 2007; DuPont, 2009; O'Ryan *et al.*, 2010; UNICEF 2013). Etiological information that seeks to outline all the causative agents of severe AID in children is scarce. However, many studies performed in different countries have reported diarrhoeagenic *Escherichia coli* (DEC) pathotypes as being the leading bacterial causative agents of AID in children from developing countries. Research by Black *et al*.(2010) and O'Ryan *et al.* (2010) has reported that the frequencies of these

pathogens vary with geographic region and depend on the socioeconomic/sanitary conditions achieved. All these conditions represent 30-40% of the cases (Black *et al*., 2010).

2.2 The burden of childhood diarrhoea in Kenya

The situation of Kenya is similar to that of many developing countries.The number of people moving to urban centers keeps increasing by the day.Kenyan urban population is at present 40 percent of the total population (UN-HABITAT, 2014).The capital city, Nairobi which has a population of approximately 3.363 million people (Kenya demographics profile, 2014) does not have the capacity to sustain those individuals. There is no direct proportion of economic growth to the influx of individuals in the city. This situation has consequently led to development of informal settlements to take care of the large population that is not in a position to affordgood settlements. These informal settlements have been growing at a very fast rate of approximately 5 percent per annum (UNDP, 2007). The Kenyan government had ignored the slums and termed them as illegal for many years until recently (Mutisya, 2011). This situation led to slums from being excluded from city authority planning and budgeting processes (Mutisya, 2011). The individuals in the slums therefore do not benefit from most government contributions to the nation building. They live in low quality houses that are very small and close together. Many individuals live in these small houses that rarely have any toilets. Sanitation facilities are inadequate with human wastes, garbage and dust among other pollutants littered everywhere in the ground (Hodson and Marvin, 2009). The human and animal wastes condition is made even worse by the fact that the sewages lines are open and overflowing. Drainage systems are also in a pathetic state (Mutisya, 2011;Hodson ;

Marvin, 2009).The complex situation facing the slum dwellers continue to escalate without anyone to fully address them.

It is these deplorable living conditions that predispose individuals to many diseases.There are many health related issues within these slums and most common diseases include Malaria, typhoid, dysentery, tuberculosis and AIDS. Malnutrition is visible among the children. This is primarily related to high cost of food in relation to low family income. Children are not exempted from these diseases, as a matter of fact they are the most affected since their immunity is low.In Kenya, like in other developing countries, diarrhoea is a major cause of child mortality and morbidity and comes third after neonatal causes and pneumonia, respectively.

Every Kenyan child under the age of five experiences an average of three bouts of diarrhoea every year, according to the 2008-09 Kenya Demographic and Health Survey (KDHS, 2009). Figures from the KDHS 2008 also show that the prevalence of diarrhoea is highest in children aged between 1 and 2 years. The prevalence rate then falls as children reach the age of two years three, four and five years respectively. This may indicate a number of possibilities such as the failure of the Kenyan Government to put up aggressive measures to curb the illness or under-utilization of treatment options by mothers and other caregivers of children (KDHS 2009). A lot of progress has been made in reducing under-five (U5) mortality in Kenya.However,the current rate of 74 per 1000 live births is a clear sign that a lot of work still needs to be done.Diarrhoea is one of the diseases that cause this high rate of U5 deaths. A lot of attention has not been given to it compared to other diseasessuch as malaria and programs such as immunization, therefore

diarrhoea, which is easy and inexpensive to treat, continues to cut short the lives of dozens of Kenyan children every day.

Data from the KDHS shows that few children suffering from diarrhoea receive the recommended treatments and a good number receive no treatment at all. Additionally, many mothers lack knowledge on the basic rules of diarrhoea management at home which include: increasing the intake of fluids, continued feeding, provision of zinc supplements, and taking the child to as health facility if dehydration persists. Using data from the Kenya Demographic and Health Survey, 2009; this study seeks to examine the child, maternal and household factors that influence mothers' choice of childhood diarrhoea treatment in Kenya.

2.3 Causes of Diarrhoea

Diarrhoea usually occurs when fluid cannot be absorbed from your bowel contents, or when extra fluid is secreted into the bowel, causing watery stools. It is caused by; infection, malnutrition and other causes, for example, poor personal hygiene.

2.3.1 Infection

Diarrhoea is a symptom of infections caused by a host of bacterial, viral and parasitic organisms, most of which are spread by fecal-contaminated water (Adebola *et al*., 2014). Infection is more common when there is a shortage of adequate sanitation and hygiene and safe water for drinking, cooking and cleaning. Viral diarrhoea can present as a sporadic or epidemic illness and can be attributable to a number of different viruses, including rotavirus (commonly seen in babies and small children) and small round structured virus, such as Norwalk agent. Such gastrointestinal infections are usually

associated with vomiting (Albert *et al*., 1999). Bacterial diarrhoea can be caused by a number of organisms, including *Salmonella*, *Shigella*, *Campylobacter* and toxin-producing strains of *E. coli*. Acquisition of these organisms is generally associated with food.

2.3.2 Malnutrition

Children who die from diarrhoea often suffer from underlying malnutrition, which makes them more vulnerable to diarrhoea. Each diarrhoeal episode, in turn, makes their malnutrition even worse. Diarrhoea is a leading cause of malnutrition in children under five years old.

2.3.3 Other causes

Diarrhoeal disease can also spread from person-to-person, aggravated by poor personal hygiene (Mutisya, 2011). Water contaminated with human faeces, for example, from sewage, septic tanks and latrines, is of particular concern. Animal faeces also contain microorganisms that can cause diarrhoea. Food is another major cause of diarrhoea when it is prepared or stored in unhygienic conditions (UNICEF, 2014). Water can contaminate food during irrigation. Fish and seafood from polluted water may also contribute to the disease.Medications such as antibiotics can disturb the natural balance of intestinal bacteria. Antacids and drugs for cancer and blood pressure can also cause diarrhoea, according to the National Institutes of Health. Chronic (i.e. > 2 weeks duration) diarrhoea can be caused by certain intestinal disorders, which include Irritable Bowel Syndrome, Inflammatory Bowel Diseases, including ulcerative colitis and Crohn's disease, chronic pancreatitis, coeliac disease, colon cancers and certain tumours of the small intestine (Campos *et al*., 2004).

2.4 General characteristics of *Escherichia coli*

The scientific history of *Escherichia coli* (*E. coli*) started with its first description in 1885 by Theodor von Escherich (Escherich, 1988). The species *Escherichia coli* comprises Gram-negative, rod-shaped, non-spore forming, motile bacteria which are about 2 μm long and 0.6 μm in diameter, with a cell volume of 0.6-0.7 μm^3 (Darnton *et al.*, 2007).

They are facultative anaerobes, oxidase-negative, glucose, lactose and sucrose fermenting, with an optimum growth pH of 6.0-7.0 and temperature of 37°C. However, some laboratory strains can multiply at temperatures up to 49°C (Fotadar *et al.*, 2005). Taxonomically, *E. coli* belongs to the family *Enterobacteriaceae*, and it is a commensal bacterium residing as the most common and predominant inhabitant in the intestinal microflora of human and other mammals (Nataro and Karper, 1998). However, the establishment of the intestinal *E. coli* flora, is rather a complex process influenced by microbial and host interactions, or by internal and external factors that can have a substantial influence on the prevalence and density of *E. coli*, for example; delivery mode, feeding habits, life-style, environment and immunological status (Adlerberth, 2008; Adlerberth and Wold, 2009; Penders *et al.*, 2006). *Escherichia coli* normally colonizes an infant's gastrointestinal tract within 40 hours of birth where it adheres to the mucus of the large intestine. Without genetic elements that encode for virulence factors they remain benign commensals

2.5 Acquisition of *Escherichia coli*

The gastrointestinal tract of the newborn is considered sterile at birth. However, its colonization begins as soon as the newborn is exposed to a microflora, which occurs after the rupture of the fetal membranes. *E. coli* and other enterobacterial species are among

the first colonizers of the newborn infant's intestine, within a few hours after birth (Adlerberth *et al.*, 1991; Fanaro *et al.*, 2003; Hooper and Gordon, 2001), and thereafter *E. coli* and the host derive mutual benefits for decades (Kaper *et al.*, 2004).

The initial *E. coli* strains colonizing the newborn intestine may have originated from the maternal fecal microflora during delivery (vertical transfer), or be transferred between infants via the nursing staff (horizontal transfer) (Fanaro *et al.*, 2003). Another possible route of exposure isingestion of contaminated food and water (Duriez *et al.*, 2001; Hammerum and Heuer, 2009).Some studies in developed countries have shown that 42-49% of the newborn infants werecolonized by *E. coli* strains, with a mean of 1.6–2.1 strains at day 3 in life (Nowrouzian *et al.*, 2003). Interestingly, this flora was found to be stable and the corresponding carriage rate among infants was only 61–64% at 3-6 months of age, indicating an environment in the modern society in developed countries almost free of contaminating fecal bacteria. This should be compared to the rapid and high turnover of strains in developing countries, since 8.5 *E. coli* strains were found per child during a one year period in a comparable group of infants (Adlerberth *et al.*, 1998a).

Later in life, a newly introduced *E. coli* strain will not necessarily replace already existing strains in the intestines (Sears *et al.*, 1956). In all humans there are some *E. coli* strains that will persist in the intestine of an individual for several weeks, monthsand years in succession (resident strains). Whereas others disappear within a few days or weeks (transient strains), and they may be found in the microflora on a single occasion, or on a few occasions closely spaced in time (Sears *et al.*, 1956). Resident *E. coli* strains have an increased capacity to adhere to colonic epithelial cells by presenting different

colonization characteristics, and are also more likely to belong to specific or particular "pathogenic" clonal groups (Ishii *et al.*, 2007; Kaper *et al.*, 2004; Müller *et al.*, 2007).

2.6 Important roles of *Escherichia coli*

2.6.1 Indicator for fecal contamination

Public and environmental health protection requires safe drinking water, which means that it must be free of pathogenic bacteria. Determining the source of fecal contamination in aquatic environments is essential for estimating the health risk associated with pollution, and for facilitating measures to remediate polluted waterways (Blanch *et al.*, 2006). *E. coli* constitute a part of the intestinal microflora of human and warm-blooded animals, and survive long enough in the different aquatic environments, and are easily isolated, enumerated and identified. It has been used as indicator of fecal contamination (McQuaig *et al.*, 2006; Whitlock *et al.*, 2002), and also to determine the quality and safety of water for consumption worldwide.

2.6.2 Indicator for antibiotic resistance

Microbial resistance to antibiotics is an increasing public health problem worldwide; since administration of antimicrobial agents causes disturbances in the ecological balance between host and microorganisms (Pallecchi *et al.*, 2007; Paterson and Bonomo, 2005; Sullivan *et al.*, 2001), and may promote the emergence of antibiotic-resistant strains that increase in numbers, which may lead to more severe infections. *E. coli* and other membersof the family *Enterobacteriaceae* are well known to develop or acquire resistance to a varietyof antibiotics by different mechanisms. However, production of β-lactamases is the mostcommon and clinically significant mechanism of resistance among this bacterial group (Suarez *et al.*, 2005; Torres *et al.*, 2007; Woodford *et al.*, 2007).

Accordingly, the intestinal *E. coli* microflora may provide an important reservoir for antibiotic-resistant bacteria, and resistance genes, which may be transmitted further to potentially pathogenic bacteria (Pallecchi *et al.*, 2007). Thus, the ecological impact of different antimicrobial agents, as well as the development of antimicrobial resistance before it appears in pathogenic strains and in clinical infections, could be studied in the intestinal *E. coli* flora

2.6.3 Emerging pathogen with potential to spread virulence

Although *E. coli* strains are termed commensals and part of the normal intestinal microflora of human and warm-blooded animals, maintaining a healthy intestinal ecosystem, under certain circumstances they may cause diseases (Kaper *et al.*, 2004). Diseases caused by any *E. coli* strains are either a result of specific or non-specific infections. Unspecific infections may occur where the non-pathogenic, commensal *E. coli* strain become harmful, because of the fact that the host immune system is weak, for example, in preterm-newborn infants, elderly, malnourished and immunocompromised individuals (Kaper *et al.*, 2004). Specific infections are caused by some subsets of *E. coli* strains that represent a versatile and diverse group of microorganisms with several highly adapted clones. These strains have acquired specific virulence factors, which confer on them the ability to adapt to new environments and make them capable of causing a broad range of infections in healthy individuals (Kaper *et al.*, 2004).

2.7Diarrhoeagenic *Escherichia coli* pathotypes

Most*E. coli* are harmless commensals of the human and animal intestine, however, certain specific *E. coli* strains are capable of causing a variety of diseases. Infections due to pathogenic *E. coli* may be limited to colonization of a mucosal surface or can

disseminate throughout the body and have been implicated in urinary tract infection, sepsis/meningitis and gastrointestinal infections (Nataro and Kaper, 1998). The pathogenic *E. coli* species comprise a very versatile group with numerous virulence determinants (virulence factors) including adhesins, invasins, toxins and secretion systems that allow them act as causative agents of diarrhoea in both human and veterinary medicine (Kaper *et al.,* 2004; Kuhnert *et al.,* 2000; Nataro and Karper, 1998). In humans, these pathogens are responsible for three main types of clinical infections: (i) enteric or diarrhoeal diseases, (ii) urinary tract infections, and (iii) meningitis/septicemia. Based on their distinct virulence properties and clinical symptoms of the host, pathogenic *E. coli* strains are divided into numerous categories or pathotypes

2.7.1 Enterotoxigenic *Escherichia coli* (ETEC)

This is the mostrecognized diarrhoeagenic *E. coli*.It is the most commonbacterial cause of diarrhoea in children below five years in developing countries.Although not so common in developed countries, ETEC is the most common pathotype isolated from children having diarrhoea cases in developed countries (WHO, 2009). It was first recognized in piglets as a cause of diarrhoeal disease (Rawan, 2012).The pathotype is closely associated with individuals living in areas with poor sanitary conditions.

ETEC produce toxins which are heat-labile (LT) and/or heat-stable (STa and STb) that are also causing diarrhoea.The genes ST1and LT1 may be carried by ETEC strains on either plasmids or transposons that can be inserted into plasmids or the bacterial chromosome (Kaper *et al*., 2004). LT2, a variant of LT1, has been isolated from animal ETEC strains, but there is no evidence of an LT2 association with diarrhoeal disease in humans or animals (Nataro and Kaper, 1998).It colonizes the surface of the small bowel

mucosa and produces toxins, which give rise to intestinal secretion. Colonization is mediated by one or more proteinaceous fimbrial colonization factors (CFs), which are designated by CFA (colonization factor antigen), CSA (coli surface antigen) or PCT (putative colonization factor) followed by a number.

ETEC causes watery diarrhoea, which can range from a mild, self-limiting disease to a severe, purging one. It is self-regulating and can last from 1- 5 days. Worldwide, ETEC causes approximately 210 million diarrhoea episodes and about 380 000 deaths annually (Rawan, 2012). In endemic areas of ETEC-mediated diarrhoea, infants and children under the age of 5 are the most commonly affected. In these endemic areas ETEC is one of the most common causes of traveler's diarrhoea. It is responsible for 50-60 % of traveler's diseases (Rawan, 2012).

2.7.2 Enteropathogenic *Escherichia coli* (EPEC)

These organisms are a significant cause of infant chronic and persistentdiarrhoea in developing nations. Infection occurs through contaminated hands andweaning foods. Little children and adult carriers act as reservoirs of infection. Children, especially those under 1 year are more prone to EPEC infection which is very rare in adult humans (Rawan, 2012).

EPEC was historically recognized on the basis of serotypes such as O55:H6 and O127:H6. It is an established etiological agent of human infantile diarrhoea.The pathogen subverts intestinal epithelial cell function to produce distinctive "attaching and effacing" (A/E) lesions. These lesions are characterized by localized destruction (effacement) of brush border microvilli, intimate bacterial attachment to the host-cell membrane and

formation of an actin-rich cytoskeletal structure beneath intimately attached bacteria. The pathogenesis of EPEC is in some way unique for enteric bacterial pathogens since it is essentially noninvasive and produces no toxins. The attachment of EPEC to the epithelial cell, described as localized adherence, results in a so-called attaching and effacing lesion (A/E) (Celli *et al*., 2000).The most fascinating aspect of EPEC pathogenesis is that it inserts, through the type III secretion system, its own receptor into the host cell.

EPEC disease is generally the result of growth of EPEC in the small intestine. EPEC cause a watery diarrhoea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are also associated. The symptoms may last for several days, although instances of long, chronic EPEC disease have been noted. There are two main types of EPEC namely typical and atypical EPEC.A-EPEC (atypical enteropathogenic *E. coli*) is EPEC that have lost the EAF (EPEC adherence factor) plasmid.Recent attention has focused on greater understanding of atypical EPEC strains (Trabulsi *et al*., 2002). These strains more commonly cause diarrhoea in industrialized nations than the typical EPEC strains. In addition the atypical EPEC strains have animal and human reservoirs, whereas the typical isolates are almost always associated with human fecal contamination. The atypical isolates have the ability to cause A/E lesions but lack the EAF plasmids. They often have additional virulence factors not seen among the typical strains.

2.7.3 Enteroinvasive *Escherichia coli* (EIEC)

EIEC causes broad spectrum of human diseases. EIEC are transmitted through fecal-oral route. They are biochemically, genetically and pathogenically closely related to *Shigella* species. Patients of all ages get shigellosis like symptoms when infected (Rawan, 2012).

EIEC are non-motile, lactose negative and lysine decarboxylase negative (Rawan, 2012), a characteristic that makes it differ from other *E. coli* strains. EIEC causes invasive inflammatory colitis, but may also elicit a watery diarrhoea syndrome similar to that caused by other *E. coli* pathotypes. The pathogenesis of EIEC infectioninvolves cellular invasion and spread which are plasmid mediated actions (Nataro and Kaper, 1998).

The clinical presentations of EIEC infections are blood and mucus-filled stools, fever and severe cramps. These are very similar to those of diseases caused by *Shigella*. However, they are different in that EIEC requires a large infection dose to produce disease hence low rate of person to person transmission which is not the case in *shigella* (Rawan, 2012). EIEC/*Shigella* rarely invade the blood stream but they invade the intestinal epithelium, specifically (large intestine). They lyse the phagocytic vesicles and replicate freely in the cytoplasm of the host cell and cause disease (Rawan, 2012).

2.7.4 Enteroaggregative *Escherichia coli* (EAEC)

It was originally called enteroadherent- enteroaggregative *E.coli*. It is responsible for causing acute and persistent diarrhoeain children and adults. However, a study by Vu Nguyen *et al*. (2006) shows that EAEC is frequently associated with children less than 2 years of age. Recently, the pathotype has been associated with outbreaks of diarrhoea as well as persistent diarrhoea in immunocompromised individuals, example, HIV patients (Sousa, 2005).Several studies show that it is highly prevalent in developing countries (Makobe *et al*., 2012; Sang *et al*., 2012). Studies have also shown that it is also present in developed countries (Schultsz *et al*., 2000; Knutton *et al*., 2001). A study carried out by Adachi *et al*. (2001) show that EAEC is an important cause of travelers'diarrhoea.

EAEC strains are defined by their distinctive pattern in Hep-2 cell culture (Nataro and Kaper, 1998). EAEC do not secrete the heat stable or heat labile toxins of Enterotoxigenic *Escherichia coli*. They manifest a characteristic aggregative pattern of adherence to HEp-2 cells in culture. This organism can display a wide array of virulence factors some located on the plasmid and other outer membrane adhesins (Sousa and Dubreil, 2001).

2.7.5 Enterohaemorrhagic *Escherichia coli* (EHEC)

Infections caused by EHEC are characterized by watery diarrhoea that progresses to bloody diarrhoea that is accompanied by abdominal cramps (Rawan, 2012). They affect people of all ages; although they are more severe in children and the elderly people. EHEC diseases are mainly caused as a result of eating undercooked or uncooked meat and milk. Cattle are a natural reservoir of EHEC. Most of these raw or undercooked cattle products may be contaminated with faeces hence EHEC strains introduced to the body. EHEC strains do not cause disease in cattle because they are members of the intestinal microflora in cattle. They also have very low infection dose of 10-100 organisms.

Enterohaemorrhagic*E.coli* (EHEC) belongs to a group of *E.coli* pathotypes that produce toxins. Shiga- toxins (*Stx1* and *Stx2)* are key factors that cause pathogenicity of EHEC, *eae* genes are also responsible for virulence of EHEC. These three genes are greatly used as target genes in molecular detection of EHEC pathotype (Ina, 2013).In humans, EHEC colonizes the large intestine (Coombs *et al*., 2011). The shiga toxins cross the intestinal wall a condition that can cause haemolytic colitis (HC) and haemolytic uremic syndrome (HUS) which produces lesions in epithelial cells (Levine *et al*., 1985). EHEC denotes a

subset of STEC and includes a clinical connotation that is not implied with STEC (Nataro and Kaper, 1998).

STEC serotypes, O157:H7 is mainly associated with both outbreaks and sporadic cases of severe disease (Coombs *et al*., 2011). Unlike other DEC pathotypes, it does not ferment sorbitol hence it appears colourless when cultured on MacConkey agar; this is used as its test (Rawan, 2012). Currently, there is no sure treatment of EHEC infection (Goldwater and Bettelheim, 2012); most of the conventional antibiotics promote development of shiga toxin cytotoxicity. They also lead to increased development of HUS (Tarr *et al*., 2005).

2.7.6 Diffusely Adherent *E. coli (*DAEC)

Diffusely Adherent *E. coli*is a heterogeneous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells.It is associated with the watery diarrhoea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Croxen and Finlay, 2010).Diarrhoea associated with DAEC increases with age of children from 18 months to 5 years. The intestinal carriage of these strains is widespread in older children and adults (Nataro and Kaper, 1998).

DAEC rely on *dae* genes to colonize intestinal cells of the gut (Rajendran *et al*., 2010). Most DAEC strains express a surface fimbria designated F1845 and decay- accelerating factor (DAF) that are used in binding to parts of plasma membrane of the intestinal cells. The pathogenesis of DAEC seems to be predominantly mediated through Afa/Dr adhesin interactions with host cells. Additionally, a secreted autotransporter toxin (Sat) has also been implicated in pathogenesis, but nevertheless, the implication of Afa/Dr DAEC

strains in diarrhoea remains controversial. Phenotypic detection of DEAC is based on the mannose-resistant diffuse adhesion of these strains to cultured epithelial HEp-2 or HeLa cells (Nataro and Kaper, 1998).

2.8 Virulence Factors

The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence- related plasmids and chromosomal pathogenicity islands. Diarrhoeagenic *E.coli* carry at least one virulence- related property upon a plasmid. EIEC, EHEC, EAEC and EPEC strains typically harbor highly conserved plasmid families, each encoding multiple virulence factors. Chromosomal virulence genes of EPEC and EHEC are organized as a cluster referred to as a pathogenicity island. Such islands have been described for uropathogenic *E.coli* strains and systemic *E.coli* strains (Boschi-Pinto *et al.,* 2008) as well and may represent a common way in which the genomes of pathogenic and non-pathogenic *E. coli* strains diverge genetically.

VTEC/STEC strains produce verocytotoxins VT1 and/or VT2, encoded by the *vtx 1* and *vtx 2* genes, respectively.VTEC strains account for the most severe clinical manifestations among the DEC strains, including gastroenteritis and bloody diarrhoea, sometimes leading to haemolytic uraemic syndrome (Paton and Paton, 2002). In addition, a subset of STEC strains considered to be highly virulent for humans has the capacity to produce attaching and effacing lesions on intestinal mucosa, a property encoded on a pathogenicityisland termed locus for enterocyte effacement (LEE). LEE carries *eae*, which encodes an outer membrane protein.

EPEC strains cause characteristic attaching and effacing lesions in the small intestine, which are associated with the virulence factor intimin, encoded by the *eae* gene. The *eae* gene may also be present in VTEC strains, and is always present in attaching and effacing *E. coli*, which are less virulent or non-virulent. ETEC strains cause diarrhoea because they produce one or two enterotoxins,heat-stable enterotoxin and heat-labile enterotoxin, encoded by the *est* and *elt* genes, respectively. EIEC strains are related closely to *Shigella* spp. With respect to both phylogeny and pathogenesis. Characteristic of the invasive phenotype of both EIEC strains and *Shigella* spp. is the *ipaH* gene, which is present in several copies on both the chromosome and the invasive plasmid (plnv).

EAEC strains are probably a frequent cause of diarrhoea in both the industrialized and the developing world and several putative virulence factors have been found in EAEC strains. However, all these virulence factors are also encountered in the other *E.coli* groups and none occur in all EAEC strains. Therefore, EAEC strains cannot be identified simply by detection of specific virulence factors or genes. Cell adherence assays comprise the reference standard method which is the only true method for identifying the strains. A large, diverse group of *E.coli* strains, characterized by their diffuse adherence to cells, has also been described. The pathogenic potential of this group still needs to be confirmed (Persson *et al*., 2007)

2.9 *Escherichia coli*typing

2.9.1 Serotyping

Typing is the process of identifying different types of organisms within a species. Traditionally, this process was based on phenotype of the organism. Serotyping analysis was the predominant means by which pathogenic strains were identified. Among

diarrhoeagenic *E.coli,* usually a specific combination of O and H antigens defines the serotype of the strain. *Escherichia coli* of specific serogroups can be associated with certain clinical syndromes (Nataro and Kaper, 1998). Conventional *E. coli* O: Hserotyping by agglutination of somatic and flagellar antigens by the use of anti-*E. coli* polyclonal antiserum is time consuming, expensive and available only in small number of reference laboratories. Traditional serotyping requires the use of large panel of antisera; moreover, it is subjective and cross-reactive. However, for proper diagnosis and treatment, to maximize an isolate's usefulness for surveillance and to determine overall disease trends PCR assays based on O-serotype specific genes have been proposed for molecular tying of many *E.coli* (Li *et al*., 2006).

2.9.2 Molecular typing

The choice of the appropriate molecular typing method to use depends on the problem being solved and the epidemiological context in which the method will be used. The typing method must have the capacity to differentiate all the species in the given isolates if it is to be considered effective (Van *et al*., 2013). Additionally, the method ought to be rapid, inexpensive, easy to perform and interpret and highly reproducible (Van *et al*., 2013). These molecular methods are many and they have different action modes. They keep evolving with time to serve the changing demands in the research field. Apparently, despite the many typing methods none of them is ideal and each of them has their advantages and disadvantages.

Escherichia coli are recovered from clinical specimens on general or selective media at 37 ^{0}C under aerobic conditions for 24 hours. *Escherichia coli* in stools are most often recovered on MacConkey or Eosin methylene blue agar, which selectively allow the

growth of members of the Enterobacteriaceae and permit differentiation of enteric organisms on the basis of their morphology.Identification of diarrhoeagenic *E.coli* requires that these organisms be differentiated from non-pathogenic members of normal flora.

A widely used method in typing *E. coli*is Polymerase Chain Reaction (PCR). It has been a major advance in molecular diagnostics of pathogenic microorganisms of which*E. coli* is not excluded. In PCR, a pair of primers (20-40) bases is used for selective amplification and detection of certain DNA sequences in a target organism. PCR primers have successfully been developed for all categories of diarrhoeagenic *E. coli*. PCR can be used for both diagnosing and typing *E. coli* strains. PCR is beneficial in diagnosing diarrhoegenic *E.coli* because of its high sensitivity, specificity and appropriate rapidity in the detection of target DNA templates. Recently, it was concluded that PCR detects significantly more ETEC infections than does the standard probe based hybridization method (Keskimaki, 2001)

In diagnosis, PCR is commonly used for detecting different virulence associated genes of *E. coli*, such as toxin and adherence associated genes. PCR is also widely used in subtyping by doing virulence genes profiles for different diarrhoeagenic *E. coli* strains. However, conventional PCRs are now being used to detect multiple genes within the same reaction by use of multiple primers. This is called a multiplex PCR which is important in reducing both time and money for each experimental procedure. Several virulent genes are amplified at ago using various combined primers. The PCR products are analyzed by gel electrophoresis with 2.0 % (w/v) agarose gels. The DNA bands are then visualized and photographed under UV light after staining the gel with ethidium

bromide. The specificity of the multiplex PCR assays is determined by using standard reference strains of DEC which are subjected to multiplex PCR and the results compared with those obtained from monoplex PCR assays.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was carried out in Mukuru slums which are located 7 km southeast of Nairobi City Centre at geographical coordinates: 1°18'17"S 36°53'28"E. It belongs to Embakasi Constituency in industrial area. The slum was established about 35 years ago. It is one of the largest slums in the city with a population of around 700,000 people. 'Mukuru' means damping site in Kiswahili. The place was an old quarry where most stones that built the factories were excavated. Mukuru Kwa Njenga has seven villages that have informal structures. The seven settlements are Moto moto, Wape wape, Riara, Sisal, Vietnam, Milimani and M.C.C. Mukuru Kwa Reuben land is approximately 256 km^2 and it is divided into two basic settlements ; old and new Reuben which are further subdivided into Gatoto, Mombasa, Bins, Feed the Children, Kosovo, Gateway, Railway and Rone.

3.2 Study design

This was a cross-sectional retrospective study that was laboratory based. *Escherichia coli*isolates wereobtained from children treated fordiarrhoea at Mukuru Kwa Njenga and Mukuru Kwa Reuben informal settlements, Nairobi

3.3 Strains used in the study

The *E.coli* isolates were donated by KEMRI CMR project entitled "Association of *E.coli* and other microbial causes among children in two informal settlements in Nairobi." Theisolates were collected between May 2013 and July 2013, from children treated at the Maternal and Child Health (MCH) clinics in Mukuru Kwa Njenga and Mukuru Kwa

Reuben slums, Nairobi and archived at KEMRI-CMR. The clinics serve mainly a population of medium to low socio-economic status. The *E. coli* pathogens were isolated from faeces of children aged five years and belowwho had cases ofdiarrhea at admission before being treated with antibiotics. The demographic details of the patients were obtained from KEMRI CMR laboratory records.

3.4 Sample size

The prevalence of diarrhoea in children of 5 years and below in Kenya has been hypothesized to be approximately 12 % (Makobe *et al*., 2012). To obtain the same prevalence with an error of 1% and 95% confidence level the minimum estimated sample size was162 using the formula of Fisher*et al* (1992) shown below.

$$n = \frac{Z^2_{1-\alpha/2}\, P\,(1-P)}{d^2}$$

Where; n = minimum sample size

$Z^2_{1-\alpha/2=}$ 1.96

α= level of significance (0.05)

p=prevalence of a previous study (12%)

d=Absolute precision (0.05)

$n= (1.96)^2 \times 0.12\,(1-0.12)/\,(0.05)^2$

= 162

3.5 Sample selection and biosafety measures

Quota sampling was used to obtain equal isolates from Mukuru Kwa Njenga (MM) and Mukuru Kwa Reuben (MR) slums. Random sampling was further applied for the selection of the required calculated sample size of 162 from the stocked 500*E.coli*strains

grouped into MM and MR. Standard biosafety measures for level 11 pathogens was adhered to during the processing of the specimens.

3.6 Re-identification of bacterial isolates

Using a sterile loop a small portion of the isolates was picked from the vials and inoculated on Brain Heart Infusion Broth. The broth tubes were incubated at 37 ^{o}C for 24 hours in tube racks. Growth of the bacteriawas used to establish the viability of the stocked isolates. The Brain Heart Infusion Broth culture was sub-cultured onto Lactose MacConkey and the plate was incubated at 37^{o}C for 24 hours. Morphological characteristics on these media were used to confirm the earlier laboratory identified and recorded *E.coli* characteristics. Pink colonies with a darker center from lactose MacConkey confirmed the identity of *E.coli*. The *E. coli* colonies were then sub-cultured on Nutrient agar and incubated at 37^{o}Cfor 24 hours. Colonies that were taken from the media were subjected to biochemical reactions.

3.6. 1 Procedures for biochemical tests

The following biochemical tests were carried out on the isolates for identification and characterization (Quinn, *et al*., 2002).

3.6.1.1 Triple sugar iron (TSI) test

The TSI slants were prepared according to the manufacturer's instructions (Appendix II c). The slants were inoculated by aseptically streaking the surface of the slant agar with colonies isolated from the MacConkey agar plate.The agar slants werestabbed from top to bottom. The tube was incubated at 37 ^{o}C for 24 hours in ambient air with caps slightly loosened. They were inspected for color change and the observations were recorded.

3.6.1.2 Citrate utilization test

Simmon's citrate agar slants which had been prepared according to the manufacturers' instructions (Appendix II a) were inoculated by aseptically streaking the slanted region with an isolated colony from MacConkey agar plate. The tubes were incubated at 37^{o}C for 24 hours. The slants were inspected and the observations recorded.

3.6.1.3 Motility (SIM) test

Motility medium which had been prepared according to the manufacturers' instructions (Appendix 1Ib) was inoculated with an isolated colony from MacConkey agar. This was by stabbing in the semi-solid agar with the inoculating sterile needle straight down to about an inch below the surface. It was incubated at 37^{o}C with the caps loosely capped for 24 hours. Observations were made and recorded.

3.6.1.4 Indole production test

Using a sterile inoculating needle, an isolated colony from MacConkey agar plate was inoculated into the tryptone water. The tubes were incubated at 37^{o}C for 24 hours with caps slightly loosened. Kovac's reagent was added (2-3 drops) to the 24 hour culture tubes. Observations were made and recorded.

3.7 Antimicrobial Susceptibility

Antimicrobial resistance was tested using 11 antimicrobial agents (Ciprofloxacin 5µg, Nalidixic acid 30 µg, Tetracycline 30 µg, Streptomycin 10 µg, Chloramphenicol 30 µg, Gentamicin 10 µg , Ceftazidine 30 µg, Amoxicillin clavulonic acid 20/10µg, sulfamethoxazole trimethoprime 1.25/23.75µg and Ampicillin 10µg, on each positive culture as per Clinical Laboratory Standards Institute, (CLSI,2008).*Escherichia*

*coli*ATCC 25922 wereused as quality control strains. The Kirby Bauer disk diffusion susceptibility method was used. Bacterial inoculum of approximately 2×10^8CFU/Ml was applied to the surface of a large (150 mm diameter) Mueller-Hinton agar plate. The commercially-prepared, fixed concentration, paper antibiotic disks were placed on the inoculated agar surface. Plates were then incubated for 16–24 hours at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter using a ruler. The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug were interpreted using the criteria published by the Clinical and Laboratory Standards Institute as sensitive, intermediate or resistant (CLSI, 2008).

3.8 Molecular analysis

3.8.1 DNA extraction

DNA template for PCR was obtained by culturing isolated single colony and a sweep of five colonies in Brain Heart Infusion Broth was incubated overnight at 37^{o}C with shaking. One millilitre of the cell suspension was dispensed into eppendorf tube and centrifuged at 14,000 rpm for 10 minutes and the supernatant discarded. The cell deposit was re-suspended in 100 µl of sterile double distilled water and then boiled in a water bath for 15 min and spun again for 5 min. The supernatant was removed to a fresh, sterile tube as the test DNA template and stored at -20^{o}C for later use in PCR experiments.

3.8.2 Multiplex PCR

Multiplex PCR for categorization of diarrhogenic *E. coli* (DEC) into EAEC, ETEC, EPEC, STEC and EIEC wascarried out using primers (0.2 µmol, HPSF purification) for

identification of the genes (Table 3.1) The specificity of each primer set was confirmed by monoplex PCR and then multiplex PCR was carried out using reference strains (Pass *et al*., 2000; Toma, 2003)**.** Briefly, the optimized PCR protocol was carried out with a 50 µl mixture containing 10X PCR buffer, 50 mM MgCl2, 2.5 mM dNTP 1.0 U of *Taq* DNA polymerase, 10 pmol concentrations of each primer set and 5 µl for DNA template. The PCR amplifications were carried out in a PTC-200 thermal cycler (MJ Research Inc, Watertown, Massachusetts,USA) using a program of initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95^{o}C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min; and final extension at 72°C for 10 minutes.

3.8.3 Electrophoresis analysis

The amplified PCR products were then separated by electrophoresis on a 2.0% agarose gel (AmpliSize; Bio-Rad Laboratories) stained with ethidium bromide in TBE buffer at 100V for ninety minutes. The DNA gel was then visualized by UV transilluminator (Hedge *et al*., 2012, Vidal, R.,2004) and photographed under ultraviolet light using an instant Polaroid camera. Molecular size marker (100-bp DA ladder; Promega, Madison, Wisconsin,USA) was included in each agarose gel run.

Table 3.1: Primer sequences used for the detection of DEC

Target gene	**Name and sequence (5' to 3')**	**Amplicon size (bp)**	**Concentration of primers (µM)**
stx1	Stx1F: AGTTAATGTGGTGGCGAA Stx1R: GACTCTTCCATCTGCCGG	817	25
stx2	Stx2F: TTCGGTATCCTATTCCCG Stx2R: TCTCTGGTCATTGTATTA	474	25
EaeA	eaeF: AAACAGGTGAAACTGTTGCC eaeR: CTCTGCAGATTAACCTCTGC	454	25
InvE	invF: ATATCTCTATTTCCAATCGCGT invR: GATGGCGAGAAATTATATCCCG	382	25
AggR	aggF: GTATACACAAAAGAAGGAAGC aggR: ACAGAATCGTCAGCATCAGC	254	25
ST gene	stF: TTTATTTCTGTATTGTCTTT stR: ATTACAACACAGTTCACAG	171	50
STp gene	stpF: TCTGTATTATCTTTCCCCTC stpR: ATAACATCCAGCACAGGC	186	50
STh gene	sthF: CCCTCAGGATGCTAAACCAG sthR: TTAATAGCACCCGGTACAAGC	166	25
LT gene	ltF: AGCAGGTTTCCCACCGGATCACCA ltR: GTGCTCAGATTCTGGGTCTC	130	25
AstA	astF: GCCATCAACACAGTATATCC astR: GAGTGACGGCTTTGTAGTCC	106	25

Key: F - forward primer, R - reverse primer

3.9 Data analysis

Data on socio-demographic characteristics was summarized using descriptive statistics; frequencies and percentages. Data on antibiotic susceptibility profileswas presented in line and bar graphs.Data collected in this study on impact of age, gender and sex on antibiotic susceptibility profiles wasanalysed using one-way analysis of variance (ANOVA) with SPSS computer software version 21 and represented in tables. Differences at $P \leq 0.05$ were considered to be statistically significant.

CHAPTER FOUR: RESULTS

4.1Distribution and demographic characteristics of study isolates

A total of 162 isolates isolated and stored in a deep freezer were selected randomly. They were re-identified as *E. coli* by growing in Muller Hinton agar followed by lactose MacConkey agar. One hundred and fifty six isolates (96.29%) were identified as pure *E. coli* isolates.The isolates were distributed between 1 and 5 years with 1 being the minimum age and 5 being the maximum age. The average age of the subjects was 3.39 years while the standard deviation was 1.52.Most of the study isolates (40.38 %) were obtained from children aged 5 years whereas isolates obtained from children aged 4 years (13.46%) accounted for the least percent.Most of the isolates (55.8 %) were obtained from Mukuru kwa Njenga whereas Mukuru kwa Reuben contributed (44.2 %) of isolates towards the study (Table 4.1).

Table 4.1:Distribution frequency of the study isolates in relation to source, age and sex of the participants

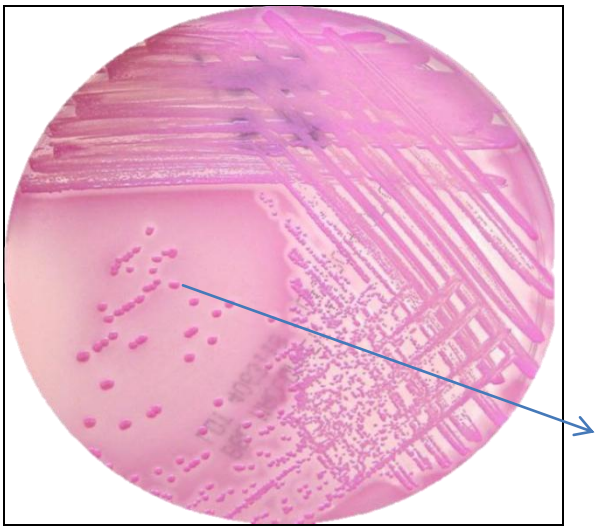
Study participants N (156)	**n**	%
Frequency by source		
MM	87	55.8
MR	69	44.2
Frequency by age (years)		
1	25	16.03
2	25	16.03
3	22	14.1
4	21	13.46
5	63	40.38
Frequency by sex		
Males	73	46.8
Females	83	53.2

Key: MM-Mukuru kwa Njenga; MR-Mukuru kwa Reuben

In terms of frequency of study isolates by sex; the study statistics revealed that females formed the most (53.2 %) of the study subjects compared to males (46.8 %) (Table 4.1).

4.2Isolation of *Escherichiacoli*

Escherichiacoli grew with characteristic pink colonies with a smell of sour milk on MacConkey agar and they haddistinct morphological characteristics (Plate 4.1).The colonies were circular in shape with entire margins. Their surface appeared smooth and shiny. They were slightly raised from the surface of the media and they were small in size (Plate 4.1).



Escherichia coli distinct colony on MacConkey agar

Plate 4.1: Discrete colonies of *Escherichia coli* isolates

4.3 Biochemical results

The identified*Escherichia coli* isolateswere TSI positive.There was gas production which pushed the agar up the tube. Both the slant and the butt turned yellow in colour. On Simmon citrate agar the bacteria was citrate negative. This result is interpreted negative because there was no change (colour remained green) observed on the agar after the incubation period. The bacterium was interpreted as indole positive since there was dark

pink ring that formed on the surface of the broth on adding of Kovac's reagent after incubation. Motility results showed that the bacterium was motile after the incubation period because there was growth in the entire broth hence appeared turbid (Plate 4.2).

Plate 4.2: Biochemical test results of bacterial isolates from Mukuru KwaNjenga and Mukuru Kwa Reuben**.**

4.4 Antibiotic susceptibility results

This sample of results oftested *E. coli* samples collected from Mukuru kwa Njenga (MM) shows a representative of antibiotic susceptibility testing (zones of inhibition) carried out among the eleven antibiotics (Plate 4.3).The isolate in the Plate 4.3 showed that the isolate was highly susceptible to CAZ, FEP and CIP when checked against the controls.

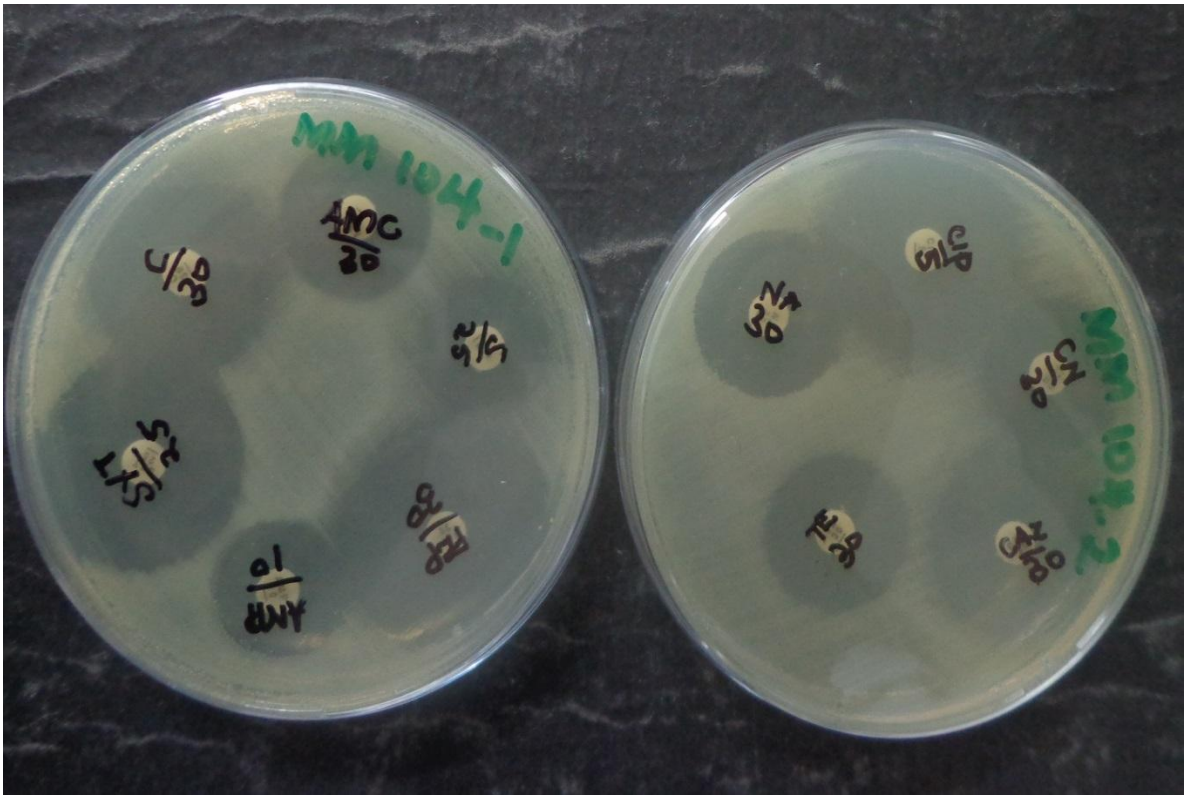


Plate4.3: Zones of inhibition of the eleven antibiotics on a sample obtained from Mukuru kwa Njenga (MM).

4.4.1Overall antibiotic resistance profiles of the *E. coli* isolates

*Escherichia coli*isolates showed a percentage resistance of less than 50 % to ten antibiotics tested. Percentage resistance ranged from 1.28 % to 62.18 %. Generally, resistance was lower in*E. coli*against reserve antibiotics compared to first panel antibiotics. Isolates exhibited lowest resistance against ceftazidine (1.28 %) followed by cefepime (2.56 %). Nalidixic acid (13.46 %)was the most ineffective reserve antibiotic.The highest prevalence of antimicrobial resistance was to SXT followed by Tetracycline and Ampicillin (62.18%, 47.44% and 46.15% respectively).Among the first panel antibioticsisolates showed least resistance Gentamicin (5.13 %)(Figure 4.1).

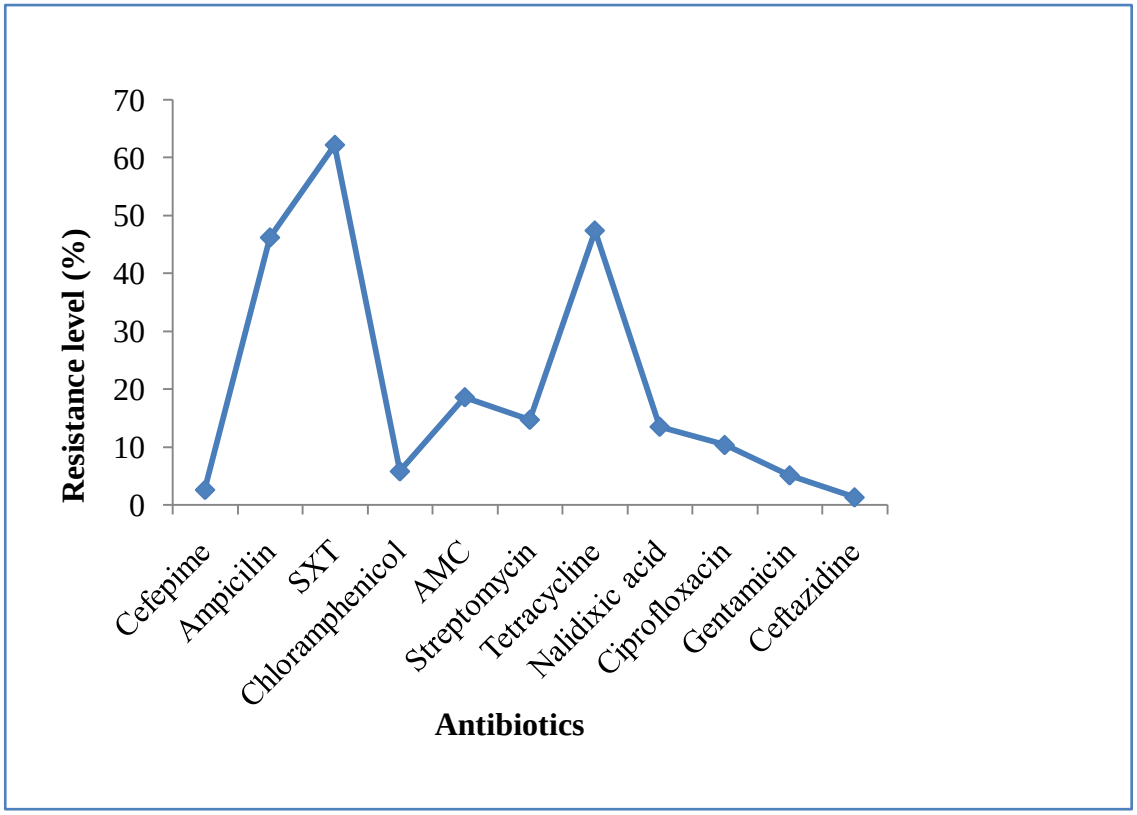


Figure 4.1: Summary of antibiotic resistance pattern of all *E. coli* isolates

4.4.2 Overall antibiotic susceptibility profiles for *E. coli* isolates

The antibiotic susceptibility profiles of the *E. coli* isolates showed that the reserve antibiotics (cefepime, ceftazidine and nalidixic acid)were more effective against microorganismscompared to first panel antibiotics (ampicilin, SXT, chloramphenicol, AMC, streptomycin, tetracycline, ciprofloxacin and gentamicin). Isolates were more susceptible to the reserve antibiotics compared to first panel antibiotics(Figure 4.2). Susceptibility varied within antibiotics from 33 % to 97 %. The isolateswere highly sensitive to ceftazidine (96.2 %), cefepime (96.8 %), gentamicin (93.6%) and Chloramphenicol (92.9%).

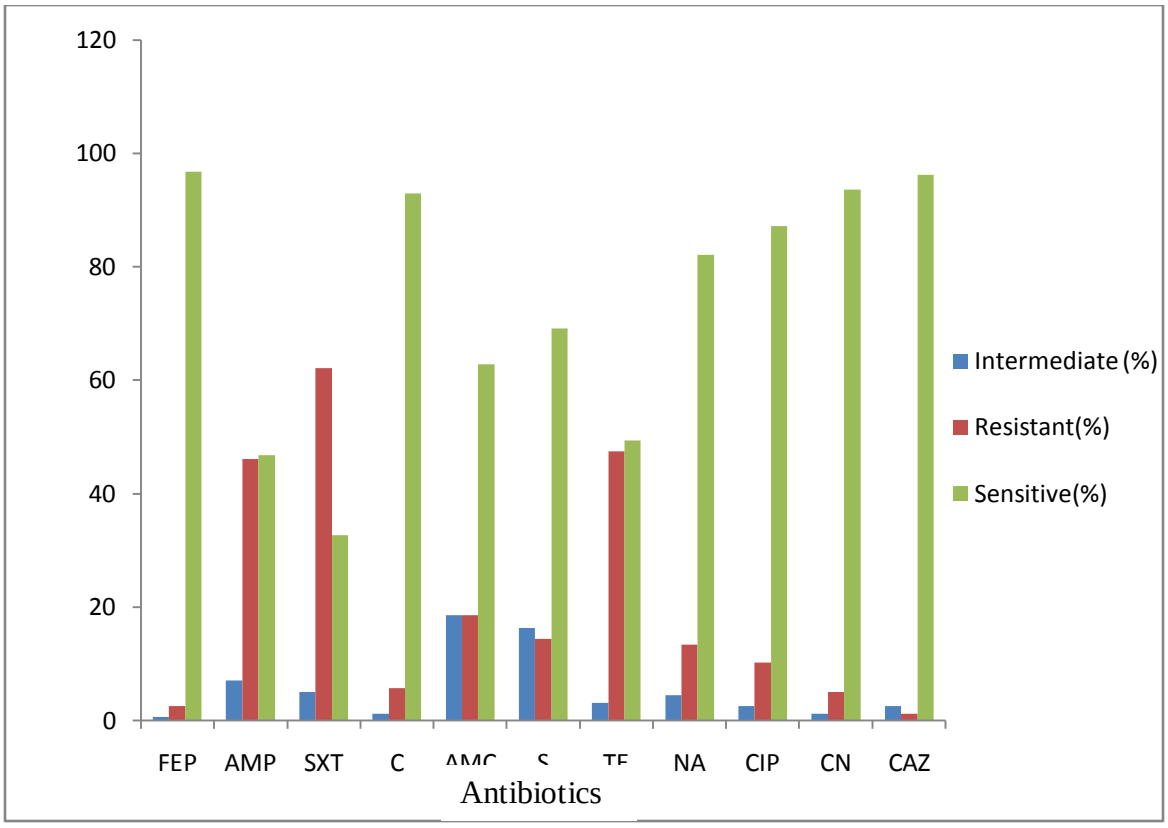


Figure 4.2: Overall susceptibility profileof*E. coli* isolates against the tested antibiotics

4.4.3 Effect of gender on antibiotic susceptibility profiles of *E. coli* isolates

Isolates obtained from females showed high levels of resistance against the eleven antibiotics tested:Sxt (63.9%), chloramphenicol (6.0%), ampicilin (19.3%), nalidixic acid (18.1%), gentamicin (7.2%), Amc (47.0%), tetracycline (48.2%), ciprofloxacin (13.3%).(Table 4.2).

Table 4.2: Percentage susceptibility of different antibiotics within gender

Antibiotic	Gender	N	Susceptible n(%)	Intermediate n(%)	Resistance n (%)	P-value
	F	83	80(96.4)	1(1.2)	2(2.4)	
Fep	M	73	71(97.3)	0(0.0)	2(2.7)	**0.36**
	F	83	37(44.6)	7(8.4)	39(47.0)	
Amp	M	73	36(49.3)	4(5.5)	33(45.2)	**0.15**
	F	83	28(33.7)	2(2.4)	53(63.9)	
Sxt	M	73	23(31.5)	6(8.2)	44(60.3)	**0.48**
	F	83	77(92.8)	1(1.2)	5(6.0)	
C	M	73	68(93.2)	1(1.4)	4(5.5)	**0.36**
	F	83	53(63.86)	14(16.86)	16(19.28)	
Amc	M	73	45(61.6)	15(20.5)	13(17.8)	**0.33**
	F	83	53(63.9)	18(21.7)	12(14.5)	
S	M	73	54(74)	8(11.0)	11(15.1)	**0.43**
	F	83	41(49.4)	2(2.4)	40(48.2)	
Te	M	73	36(49.3)	3(4.1)	34(46.6)	**0.27**
	F	83	66(79.5)	2(2.4)	15(18.1)	
Na	M	73	62(84.9)	5(6.8)	6(8.2)	**0.44**
	F	83	70(84.3)	2(2.4)	11(13.3)	
Cip	M	73	66(90.4)	2(2.7)	5(6.8)	**0.2**
	F	83	76(91.6)	1(1.2)	6(7.2)	
Gen	M	73	70(95.9)	1(1.4)	2(2.7)	**0.2**
	F	83	80(96.4)	2(2.4)	1(1.2)	
Caz	M	73	70(95.9)	2(2.7)	1(1.4)	**0.42**

Fep: cefepime; Amp: ampicilin; Sxt: sulphamethaxazole; C: chloramphenical; Amc: amoxicilin-clavulanic acid; S: steptomycin; Te: tetracycline; Na: nalidixic acid; Cip: ciprofloxacin; Gen: gentamicin; Caz: ceftazidine. MM-Mukuru kwa Njenga; MR-Mukuru kwa Reuben; N-total number of male and females

Isolates from males showed higher resistance to cefepime (2.7%), ceftazidine (1.4%) and streptomycin (15.1%) compared to isolates from femalescefepime (2.4 %), ceftazidine (1.2 %) and streptomycin (14.5 %). However, a one way ANOVA done did not reveal any statistical significant variations between susceptibility profiles of *E. coli* isolates in males and females among the antibiotics tested;cefepime (p=0.36), ceftazidine (p=0.42) and streptomycin (p=0.43) (Table 4.2).

4.4.4 Effect of source of *E. coli*isolate on antibiotic susceptibility profiles

There were no statistical significant differences on the susceptibility profiles of isolates obtained from Mukuru Kwa Njenga and Mukuru Kwa Reuben slums (Table 4.3). The P-values were greater than 0.05 in all the antibiotics tested;cefepime,ampicillin, sulphamethaxazole, chloramphenicol, amoxicilin-clavulanic acid, streptomycin, tetracycline, nalidixic acid, ciprofloxacin, gentamicin and ceftazidine: (0.39), (0.4), (0.38), (0.55), (0.23), (0.25), (0.33), (0.48), (0.43), (0.36) and (0.42) respectively.

Highlevels of resistance were recorded against Sxt, Amp and tetracycline for isolates from both MM and MR while rates of resistance were lower against ceftazidine, chloramphenicol, gentamicin, cefepime and ciprofloxacin.However, *E. coli* isolates from MR recorded high rates of resistance against most of antibiotics (Sxt, 65.2%; chloramphenicol, 8.7%; ampicilin, 53.6%; nalidixic acid, 15.9%; ceftazidine, 1.4%; Amc, 18.8%; tetracycline, 50.7%; ciprofloxacin, 13.0%) although the differences were not statistically significant from those of MM. Isolates from MM showed high resistance to ceftazidine (3.4%), gentamicin (5.7%) and streptomycin (16.1%).

Table 4.3: Percentage susceptibility of the source among different antibiotics

Antibiotic	Source	N	Susceptible n(%)	Intermediate n (%)	Resistant n(%)	P-value
Cef	MM	87	84(96.6)	0(0.0)	3(3.4)	**0.39**
	MR	69	67(97.1)	1(1.4)	1(1.4)	
Sxt	MM	87	33(37.9)	2(2.3)	52(59.8)	**0.38**
	MR	69	18(26.1)	6(8.7)	45(65.2)	
C	MM	87	84(96.6)	0(0.0)	3(3.4)	**0.55**
	MR	69	61(88.4)	2(2.9)	6(8.7)	
Amp	MM	87	45(51.7)	7(8.0)	35(40.2)	**0.4**
	MR	69	28(40.6)	4(5.8)	37(53.6)	
Na	MM	87	74(85.1)	3(3.4)	10(11.5)	**0.48**
	MR	69	54(78.3)	4(5.8)	11(15.94)	
Gen	MM	87	81(93.1)	1(1.15)	5(5.7)	**0.36**
	MR	69	65(94.2)	1(1.4)	3(4.3)	
Caz	MM	87	84(96.6)	2(2.3)	1(1.1)	**0.42**
	MR	69	66(95.7)	2(2.9)	1(1.4)	
Amc	MM	87	56(64.4)	15(17.2)	16(18.4)	**0.23**
	MR	69	43(62.3)	13(18.8)	13(18.8)	
S	MM	87	60(69.0)	13(14.9)	14(16.1)	**0.25**
	MR	69	47(68.1)	13(18.8)	9(13.0)	
Tet	MM	87	46(52.9)	2(2.3)	39(44.8)	**0.33**
	MR	69	31(44.9)	3(4.3)	35(50.7)	
Cip	MM	87	77(88.5)	3(3.4)	7(8.0)	**0.43**
	MR	69	59(85.5)	1(1.4)	9(13.0)	

Key: Fep: cefepime; Amp: ampicilin; Sxt: sulphamethaxazole; C: chloramphenical; Amc: amoxicilin-clavulanic acid; S: steptomycin; Te: tetracycline; Na: nalidixic acid; Cip: ciprofloxacin; Gen: gentamicin; Caz: ceftazidine. MM-Mukuru kwa Njenga; MR-Mukuru kwa Reuben; N-total number of isolates from MM and MR

4.4.5 Effect of age of children on antibiotic susceptibility profiles

There were no statistical significant differencesbetween susceptibility profiles of isolates from children of different ages(Table 4.4).

Table 4.4: Percentage susceptibility of different antibiotics within various ages

		Age(years)					
Antibiotic		**1**	**2**	**3**	**4**	**5**	**P-value**
	I	0(0)	0 (0)	0(0)	0(0)	1(1.6)	
	R	0(0)	1(4)	1(4.8)	2(9.1)	0(0)	
Fep	S	25(100)	24(96)	20(95.3)	20(90.9)	62(98.4)	**0.98**
	I	3(12)	1(4)	2(9.5)	1(4.6)	4(6.4)	
	R	13(52)	13(52)	10(47.6)	13(59.1)	23(36.5)	
Amp	S	9(36)	11(44)	9(42.9)	8(36.4)	36(57.1)	**0.07**
	I	0(0)	4(16)	1(4.8)	0(0)	3(4.8)	
	R	17(68)	16(64)	13(61.9)	16(72.7)	35(55.6)	
Sxt	S	8(32)	5(20)	7(33.3)	6(27.3)	25(39.7)	**0.029***
	I	0(0)	0(0)	0(0)	1(4.6)	1(1.6)	
	R	1(4)	3(12)	0(0)	2(9.1)	3(4.8)	
C	S	24(96)	22(88)	21(100)	19(86.4)	59(93.7)	**0.388**
	I	7(28)	4(16)	6(28.6)	4(18.2)	8(12.7)	
	R	5(20)	49(16)	5(23.8)	5(22.7)	10(15.9)	
Amc	S	13(52)	17(68)	10(47.6)	13(59.1)	45(71.4)	**0.19**
	I	5(20)	7(28)	5(23.8)	5(22.7)	4(6.4)	
	R	4(16)	3(12)	5(23.8)	3(13.6)	8(12.7)	
S	S	16(64)	15(60)	11(52.4)	14(63.6)	51(80.9)	**0.37**
	I	0(0)	1(4)	1(4.8)	1(4.6)	2(3.2)	
	R	10(40)	13(52)	12(57.1)	12(54.5)	27(42.9)	
Te	S	15(60)	11(44)	8(38.1)	9(40.9)	34(53.9)	**0.05***
	I	0(0)	0(0)	2(9.5)	1(4.6)	4(6.4)	
	R	2(8)	4(16)	3(14.3)	5(22.7)	7(11.1)	
Na	S	23(92)	21(84)	16(76.2)	16(72.7)	52(82.5)	**0.24**
	I	0(0)	1(4)	0(0)	0(0)	3(4.7)	
	R	1(4)	4(16)	2(9.5)	4(18.2)	5(7.9)	
Cip	S	24(96)	20(80)	19(90.5)	18(81.8)	55(87.3)	**0.29**
	I	1(4)	0(0)	0(0)	0(0)	1(1.6)	
	R	0(0)	2(8)	1(4.8)	2(9.1)	3(4.8)	
Gen	S	24(96)	23(92)	20(95.2)	20(90.9)	59(93.7)	**0.38**
	I	1(4)	1(4)	1(4.8)	1(4.6)	0(0)	
	R	0(0)	0(0)	0(0)	1(4.6)	1(1.6)	
Caz	S	24(96)	24(96)	20(95.2)	20(90.9)	62(98.4)	**0.47**

Key: Fep: cefepime; Amp: ampicilin; Sxt: sulphamethaxazole; C: chloramphenical; Amc: amoxicilin-clavulanic acid; S: steptomycin; Te: tetracycline; Na: nalidixic acid; Cip: ciprofloxacin; Gen: gentamicin; Caz: ceftazidine.* Statistically significant $p<0.05$

However, a one way ANOVA showed that Sulphamethaxazole/Trimethoprim and tetracycline susceptibility profiles differed significantly with age ($p=0.03$ and 0.05 at $p< 0.05$) respectively. Isolates obtained from children aged 5 years were highly susceptible to Sxt (39.7%) compared to isolates from others ages; those obtained from children aged 2 years were least susceptible (20%). Additionally isolates from children aged 5 years were least resistant to Sxt (55.6 %) while those obtained from children aged 4 years were highly resistant to Sxt(72.7 %).Isolates from children aged 3 years exhibited high resistance to Tet (57.1%) while those from children aged 1 year were highly susceptible and least resistance (60% and 40%) respectively. The trend of antibiotics resistance was also seen to be increasing between ages 1-3 then declining between 4 and 5 years.

4.5Multiple drug resistance

Multi drug resistance levels were also observed among the tested isolates. Results showed that 68 isolates (43.59%) were resistant to at least three antibiotics while only 3 (1.92 %) and 2 (1.28 %) isolates were resistant to 8 and 9 antibiotics respectively (Table 4.5a).

Table 4.5a: Distribution of multiple drug resistant *E.coli*isolates

No. of antibiotics resistant to	No. of isolates	%
3	**68**	**43.59**
4	**38**	**24.36**
5	**24**	**15.38**
6	**11**	**7.051**
7	**5**	**3.21**
8	**3**	**1.92**
9	**2**	**1.28**

Sulphamethaxazole/Trimethoprim, ampicilin and tetracycline were the greatest contributors of MDR; SXT 64(94.12 %), Amp 58(85.29 %) and tet 56(82.35 %) while ceftazidine and cefepime contributed only 2.94 % and 5.55 % to MDR respectively (Table 4.5 b).

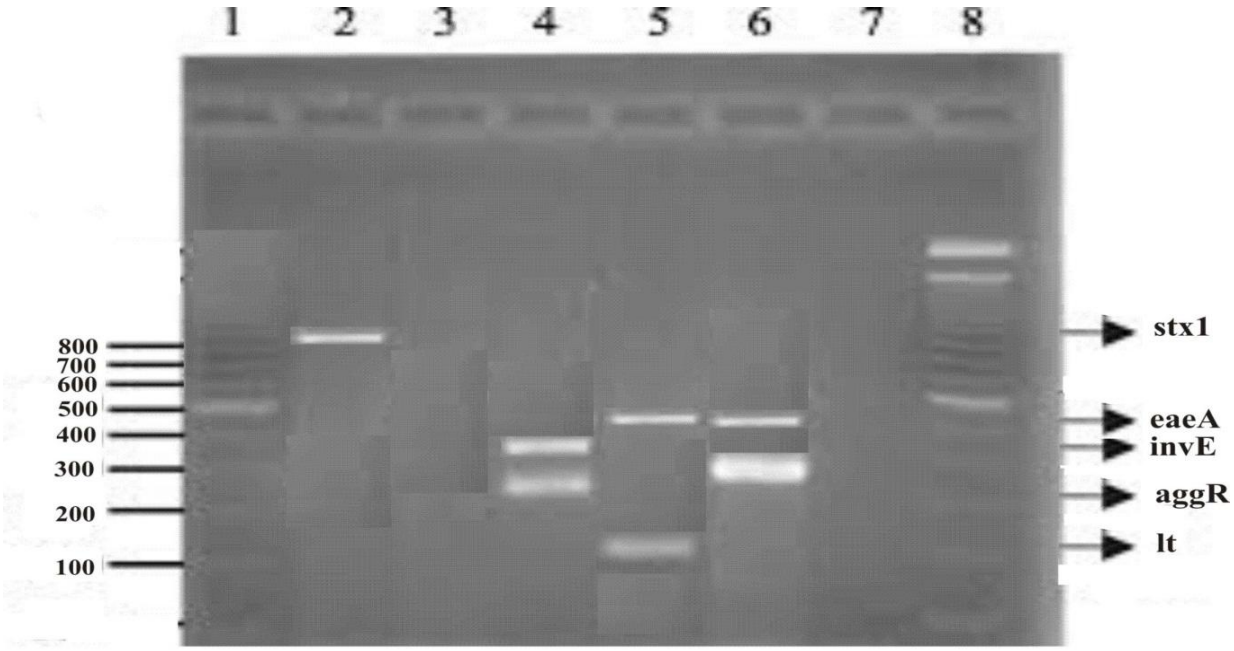
Table 4.5 b: Percentage of individual antibiotics contributing to MDR

Antibiotics	n	%	N=68
Fep	4	5.88	
Amp	58	85.29	
Sxt	64	94.12	
C	9	13.24	
Amc	25	36.76	
S	20	29.41	
Te	56	82.35	
Na	21	30.88	
Cip	16	23.53	
Gen	8	11.76	
Caz	2	2.94	

Fep: cefepime; Amp: ampicilin; Sxt: sulphamethaxazole; C: chloramphenical; Amc: amoxicilin-clavulanic acid; S: steptomycin; Te: tetracycline; Na: nalidixic acid; Cip: ciprofloxacin; gen: gentamicin; Caz: ceftazidine; N- total number of isolates showing MDR

4.6Diarrhoeagenic*E. coli* (DEC) pathotypes

Nine out of eighty four isolates (10.7%) that were tested for pathogenicity were positive for various virulence genes. These were shiga toxin (s*tx 1* and *stx 2*), attaching and effacing gene (*eaeA*), enteroaggregative gene (*agg*), enteroinvasive gene (*inv*) and heat stable genes (*st*) (Table 4.6).



Lane 1 and 8-1000 bp ladder: Lane 2-stx1; Lane 4-invE; Lane 5-eaeA and lt; Lane 6-eaeA; Lane 7-negative control.

Plate 4.4 : DNA products of *E. coli* pathotypes virulent genes in agarose gel electrophoresis.

Table 4.6: Results of multiplex PCR

DEC Pathotype	Virulence gene	Number of isolates	n (%)	N=84
EPEC	*eaeA*	3	3(3.57)	
ETEC	*lt*	2	2(2.38)	
	st	0		
EAEC	*agg*	1	1(1.15)	
EHEC	*stx 1*	1	2(2.38)	
	stx 2	1		
EIEC	*inv*	1	1(1.15)	

eaeA-attaching and effacing gene; lt-heat labile gene; stx 1- shiga toxin 1 gene; Eagg-enteroaggregative gene; Einv- enteroinvasive gene; n-number of isolates positive for the gene; N-total number

EPEC was the most predominant DEC pathotype (3.57%) followed by ETEC and EHEC (2.38%) which were followed by EAEC and EIEC (1.15%).The most common virulence

gene was *eaeA* (Table 4.6). Heat labile (*lt*) gene was the dominant gene responsible for ETEC pathotype while heat stable (*st*) gene was not isolated at all.

Table 4.7: Antibiotic susceptibility patterns of *E.coli* isolates positive for virulence genes

LAB NO.	AGE	GENDER	FEP	AMP	SXT	C	AMC	S	TE	NA	CIP	GEN	CAZ	V.Gene
MM103	4	M	R	R	R	S	I	R	R	R	R	R	I	*eaeA*
MR76	3	F	R	R	R	S	R	R	R	R	R	R	I	*eaeA*
MR143	2	F	S	R	R	S	S	I	R	R	R	S	S	*lt*
MR70	3	F	S	R	R	S	I	I	R	R	R	S	S	*lt*
MM46	4	F	S	R	R	S	I	R	R	R	S	S	S	*stx 1*
MM69	4	F	S	R	R	S	S	I	R	R	R	R	S	*stx 1*
MR183	4	M	S	R	R	I	S	S	R	R	R	S	S	*Eagg*
MR203	1	F	S	R	R	S	R	R	R	S	S	S	S	*Einv*
MR202	5	F	S	S	R	R	R	I	R	S	S	S	S	*eaeA*

eaeA-attaching and effacing gene; lt-heat labile gene; stx 1- shiga toxin 1 gene; agg-enteroaggregative gene; inv- enteroinvasive gene; S-susceptible; I-intermediate; R-resistant; MM-Mukuru kwa Njenga; MR-Mukuru kwa Reuben.

Results showed that 66.67 % of the tested isolates were positive for DEC pathotypes. These were obtained from MR while only 3 (33.33 %) of the isolates were from MM (Table 4.7). Seven isolates (77.78 %) wereobtained from females while only 2 (22.22 %) were obtained from male children.The table also shows that all the isolates that tested positive for virulence genes were resistant to Sxt and tetracycline. Eight isolates were resistant to Ampicilin while seven isolates were resistant to nalidixic acid and ciprofloxacin. Eight isolates were susceptible to chloramphenicol, only one isolate positive for eaeA gene being resistant. Additionally, seven isolates showed high susceptibility to cefepime and ceftazidine. The two isolates that were resistant to cefepime and ceftazidine were noted to contain *eaeA* virulence gene meaning that they were positive for EPEC. The two isolates were also seen to be resistant to gentamicin which was effective against six of the isolates positive for virulence (Table 4.7).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

5.1.1 Reaction of the isolates on biochemical agars

The presumed *E. coli* isolates were TSI positive with production of acid and gas. A yellow slant indicated that the organism was able to ferment sucrose and/or lactose; a yellow butt indicated that the organism was also able to ferment glucose. During this fermentation, acids were produced that made the indicator phenol red which is a component of the TSI to turn yellow. There was also production of gas which pushed the agar up from the bottom of the tube.

Escherichia coli isolateswere citrate negative that is, they were not able to utilize citrate as the main carbon source so the colour remained green. *Esherichia coli* produced tryptophanase enzyme which cleaved tryptophan in the medium producing indole which on adding Kovac's reagent that reacted with the indole forming a dark pink coloured ring on the surface of the medium hence termed as indole positive. LIA was used to differentiate microorganisms on the basis of lysine decarboxylase and H_2S.*Escherichia coli* were LIA positive. A cloudy precipitate with H_2S was observed.

5.1.2 Antibiotic resistance

In general, 75 % of the isolates were resistant to at least one antibiotic whileover 43 % of the isolates exhibited resistance to at least three antibiotics with first panel antibiotics showing higher resistance than reserve antibiotics. Highest resistance was detected against Sxt (62.2%) followed bytetracycline (47.4%) and ampicilin (46.2%) (Figure 4.2). The findings could be attributed to the fact that these antibiotics arerelatively cheap and

can be illegallyacquired over the counter without prescription (Emacalar *et al*., 2011). These factors can therefore contribute to a misuse of SXT and hence the high antibiotic resistance trends. SXT is the most commonly used antibiotic since the advent of HIV/AIDS (Maartens, 2002). The selective pressure generated by overuse explains the relatively high prevalence of resistance in *E. coli* isolates. SXT is currently the most used antibiotic as a prophylactic drug and for treatment of most bacterial infections (Emacalar*et al*., 2011). In Kenya, SXT is retailed for as little as Kenya shillings 60 per dose and is even sold without prescription (Emacalar*et al*., 2011).These results indicated that the bacteria may have been exposed to these antibiotics previously hence acting as a selective force for resistance. High resistance realized againstthese antibiotics is in line with earlier studies carried out in Kenya and Khartoum (Sang *et al*., 2012; Emacalar *et al*., 2011).A study carried out by Sang *et al*., 2012 reported high levels of resistance to ampicilin (87.6%), SXT (68%) and tetracycline. These findings concur with the current study although the levels of resistance to ampicillin (87.6 %) were very high compared to that of present study (46.2 %). Additionally a study by Emacalar*et al*. (2011) at Mbagathi district hospital reported high incidences of SXT, tetracycline (83.3% and 45.83% respectively).These findings indicate that SXT, ampicillin and tetracycline may have been overused and misused hence the high levels of resistance with different research findings over different times. Additionally, tetracycline and ampicillin are very old antibiotics that are widely used. They are also given as a first line treatment for most bacterial infections (Chopra and Roberts, 2001).

Resistance to fluoroquinolones in *E. coli* is mainly associated with mutation in chromosomal genes for DNA gyrase (gyrA) or topoisomers IV (topo IV), and these are

usually targets of action by quinolone class (Hopper, 2000; Villa *et al.*, 2000). Chloramphenical is a potent inhibitor of microbial protein synthesis, its resistance could have occurred as a result of mutations that are less permeable to the drug or production of chloramphenical acetytransferase plasmid encoded enzyme by the bacteria that inactivates the drug. Resistance to chloramphenical featured in most of the MDR strains of *E.coli* isolated in Kenya by Sang.(2012).

The isolates were highly susceptible to ceftazidine, cefepime and gentamicin. These are expensive fourth generation cephalosporins that are not easily available in any common shops and chemists. They are mostly given under doctor's prescription. This explains why these antibiotics are not easily misused hence the low resistance by isolates. These drugs are therefore considered to be very effective in diarrhoea treatment. Fourth generation cephalosporins have a wide spectrum due to high affinity to proteins bonding penicillin hence the drug can easily penetrate into the bacterium.

Approximately 43.59 % of the isolates used in the study were resistant to more than three antibiotics. Currently, SXT, tetracycline and ampicillin are being used in most of the developing countries to treat diarrhoea because of their low cost and availability (Nguyen *et al.*, 2005). Kenya is not exempted in this list and this has led to these drugs contributing a large percentage to multi drug resistance as shown in Table 4.5b.This finding is comparable to previously reported studies on children from Peru and Bolivia, Vietnam, Tanzania, Mexico, Argentina and Mozambique that reported a high prevalence of multi drug resistance in*E. coli* isolates (Nweze, 2010).

5.1.3 Effect of age, source and gender on antibiotic resistance profiles

There were no statistical differences on susceptibility profiles from samples obtained within the same geographical regions (MM and MR).The results of the study were comparable to that of Sang *et al*., 2011 which reported no significant differences among samples collected in four Kenyan provinces, although this study was done on samples from different geographical locations.However, out of the panel of the eleven antibiotics used in this study, isolates from Mukuru Kwa Reuben (MR) recorded a higher resistance against eight antibiotics compared to three antibiotics of which isolates from Mukuru Kwa Njenga (MM) exhibited higher resistance (Table 4.3). This can be attributed to the fact that children in MR have easier access to antibiotics compared to those in MM. There were many chemists, pharmacies and shops in MR which sold the antibiotics to individuals over the counter even without doctor's prescription. Mukuru kwa Njenga has fewer chemists hence individuals here do not have easy access to most of the antibiotics (except those sold in shops) compared to those from MM. It was also notable that most individuals in those areas had below average education hence very little knowledge of the antibiotics if any. The antibiotics were also inexpensive and affordable to most individuals. All these factors put together contribute to the drugs being misused and abused hence the isolates become highly resistant to them compared to those who cannot access them at wish.

According to the study, age did not significantly affect susceptibility profiles of most of the antibiotics. It was only seen to significantly affect the profiles of SXT and tetracycline (P=0.029 and p=0.05 respectively) as shown in Table 4.4. There are no statistical differences between susceptibility profiles among isolates children of different

ages because children are exposed to antibiotics at very tender ages; as early as 1 year through breastfeeding which is a universal practice for mothers. However, resistance against different antibiotics increasesup to three years followed by a decline between four and five years. At age 2 and 3 the children are learning to move around and they crawl and touch everywhere to try and support themselves. This exposes them to bacteria on the surface from running water, sewage and poorly disposed waste among others. These children thereafter suffer bouts of diarrhoea and have to be treated from time to time with these antibiotics. It is this act that leads to development of multi resistance in isolates from children of these ages. At the age of four and five years, they are still exposed to the bacteria in their surrounding since they are playful; however the level is lower than previously since they can at least move with support. This is partly one of the major reasons why resistance is lower as the children do not suffer diarrhoea as often hence do not take drugs as often. These results are similar to those of Makobe *et al*., 2011 who reported no significant differences in susceptibility profiles among children below five years in Mbagathi district hospital.

Results also showed that there are no significant differences between susceptibility profiles of isolates from males and females for different antibiotics (Table 4.2). However, isolates from females show higher resistance for eight of the eleven antibiotics tested.There has been uncertainty over the effect of age on resistance profiles of antibiotics (Sahoo, 2012). Some studies have reported isolates from males to be highly resistant to antibiotics (Bartoloni, 2006; Sahoo, 2012); while others show isolates from females as the most resistant (Vatopoulos*et al*.,1998; Zaoutis, 2005). Anatomical and hormonal differences between male and female children can influence the disease

profile(Institute of Medicine, 2001). The immune system response is slower in female children and this makes them highly susceptible to diseases (Institute of Medicine, 2001). Additionally, the time between the onset of symptoms of diarrhoea is higher in girls than boys (Mitra *et al*., 2000). These factors may contribute to the disease attacking female children more frequently, a situation that leads to them taking drugs more hence the high resistance.

5.1.4 Detection of virulence genes

The prevalence of the pathogenic *E. coli* in this study was found to be 10.7% which was slightly lower than the findings of Sang. (2012) who reported the prevalence of pathogenic *E. coli* in four regions of Kenya to be 11.2%. The findings of the study indicate that females account for the biggest percentage (77.7 %) of individuals attacked by virulent diarrhoeagenic *E.coli*compared to males (22.2 %).However, the findings are in agreement with many studies (Nweze, 2010; Rappelli *et al*., 2006 and Oundo *et al*., 2007). The study findings also indicated that individuals from MR were more susceptible to virulent diarrhoeagenic *E. coli* compared to those individuals from MM. Additionally, most of the DEC strains were seen to be multidrug resistant. Multidrug resistant DEC strains have been reported in previous studies (Alizadeh *et al*., 2007; Yang *et al*., 2009; Kalantar *et al*., 2011). Another study by EI Metwally *et al*. (2007), reported that 56% of DEC isolates were multidrug resistant (simultaneous resistance against SXT, ampicillin and tetracycline). Most studies indicate that multidrug resistant *E. coli* are widespread among the DEC strains and occurrence of resistant DEC could be because of environmental conditions, including transmission of resistant isolates from adults to children, or from animals to humans (Jafari *et al*., 2009). Also, Souza *et al*. (2009), found

a high rate of antimicrobial drug-resistance (65%) among DEC strains isolated from children.

Among the pathogenic *E.coli* isolates studied EPEC was the predominant DEC pathotype followed by ETEC and EHEC and finally EIEC and EAEC. However, majority of the studies have found ETEC to be the most predominant DEC pathotype, in other developing countries such as Bangladesh, Mozambique, Mexico, Egypt, Tanzania followed by EPEC,EAEC,EHEC and EIEC (Paniagua *et al*., 2007;Shaheen *et al*., 2004; Rapelli *et al*.,2006; Qadri *et al*., 2005). EPEC has been reported responsible for a high mortality and morbidity among children, especially in developing countries where poor sanitary conditions prevail (Nataro and Kaper, 1998). This simply explains the high prevalence of EPEC in the present study. However, the prevalence of EPEC (3.57%) reported in the current study is lower compared to 19.3% reported by Makobe *et al*. (2012), in a study carried out in Mbagathi district hospital. Majority of current prevalence reports show further reduction of EPEC prevalence in diarrhoeal episodes in children (Paniagua *et al*., 2007; Rapelli *et al*., 2006; Vu nguyen *et al*., 2006). However, EPEC still remains the most prevalent aetiological agent leading to diarrhoea. EPEC possessing*eaeA* gene aids in colonization of GIT by attachment and effacement of epithelial cell microvilli and develop intimate contact with the cell membrane (Eklund *et al*., 2002).

ETEC which is considered an important pathogen in children, especially during the first six months of life, was second most isolated diarrhoeagenic *E. coli* strain in this study. The isolation rate 2.38% was below the lower limit of the isolation rate range 10 to 30% described by Nataro and Kaper(1998) in Brazil, Bangladesh and Chile countries.It was also lower than that of Makobe *et al*., 2011 (7.25%) in Kenya although the findings agree

with the findings of Makobe in that the most prevalent gene coding for ETEC was *lt* gene.These findings also agree with reports from studies done in Sweden (Svenungsson *et al*., 2000) and Vietnam (Vu Nguyen *et al*., 2006) where *lt* was the most predominant ETEC virulence gene. In this study no proportion of ETEC was found expressing st genes.

EAEC was also found to be 1.15% prevalent in the study. These findingsdid not agree with those of Makobe *et al*. (2012) whose prevalence of EAEC was 3.86%. In EAEC virulence is mediated by aggregative-adherence fimbriae (AAF), which aids in colonization of the intestinal mucosa and colon, followed by secretion of enterotoxins and cytotoxins. There are large number of studies reporting a decreasing prevalence, such as Adachi *et al*. (2001) 25%, Rodrigues *et al*. (2002), Vu Nguyen *et al*. (2006) 11.6% and the rising proportion of diarrhoeal cases in which EAEC are implicated suggest that EAEC may be an important agent of pediatricdiarrhoea.The isolation rate in this study was lower than the reported EAEC isolation rates.However its proportion compared to the other DEC pathotypes shows that it is becoming an important contributor to diarrhoeal cases.

EHEC strains were prevalent with an occurrence rate of 1.15%.The two isolates obtained possessed *Stx1* in one isolate and *Stx2* genes for the second isolate. This prevalence was higher than that of Makobe *et al*., 2011 whose prevalence was reported as 0.97%.Of the different shiga toxins *Stx 2* has been found to be related to high virulence and is significantly associated with STEC strains for bloody diarrhoea and HUS patients (Friedrich *et al*., 2002).EIEC had a prevalence rate of 1.15%.This was higher than the

prevalence rate reported by Makobe *et al*.(2012) (0.48%). This pathotype is associated with bacillary dysentery-like diarrhoea resembling *shigella*species (Muller *et al*., 2007).

5.2 Conclusion

1. Antibiotic resistance was registered to be highest against Sxt (62.2%) followed by tetracycline (47.4%), ampicilin (46.2%), AMC (18.6%), Streptomycin (14.7 %), nalidixic acid (13.5%) and ciprofloxacin (10.35%). *Escherichia coli* were highly susceptible to some antibiotics with low resistance rates: ceftazidine (1.3%), Cefepime (2.6%), gentamicin (5.1%), and Chloramphenicol (5.8%).

2. Widespread use of antimicrobial agents in treatment of diarrhoea and other infections has led to serious problems of antimicrobial resistance which has led to a high percentage (43. 59%) of multi drug resistance among diarrhoeagenic pediatrics.

3. EPEC DEC pathotype is the major contributor of diarrhoea to children followed closely by EHEC and ETEC which have the same prevalent rates.

4. Although EAEC and EIEC have low prevalence rates their isolation suggests that they may be important emerging agents of pediatricdiarrhoea.

5. Virulence genes found in DNA extracted from samples within the same geographic region, in this case Mukuru slums are not significantly different.

6. Age and gender had no impact/influence on antibiotic susceptibility profiles among samples collected within the different geographical locations in the same geographical region.

5.3 Recommendations

Based on the findings of the study, the following recommendations were made.

a) Reserve antibiotics (cefepime, ceftazidine and nalidixic acid) are the best treatment for childhood diarrhoea for children in Mukuru slums. They are very effective against *E. coli*compared to first panel antibiotics. Sulphamethaxazole/Trimethoprim, ampicilin and tetracycline are not effective in diarrhoea treatment hence their use should be minimized. Gentamicin is the most effective first panel antibiotic and can be recommended for treatment in cases where reserve antibiotics are missing or are unaffordable.

b) EPEC is the most common pathotype of *E.coli* in Mukuru slums and there is need for specific treatment.

c) DEC pathotypes as evidenced by the detection of a wide variety of virulence genes are clinically significant in causing diarrhoea in children at Mukuru slums creating a need for continued investigations to institute appropriate management for such infections

5.4 Future research

Recommendations to Ministry of Health,Research institutions and Hospitals;

a) Research concerning the possibility of transfer of resistance factors to among the bacteria by carrying out conjugation protocol and plasmid extraction.
b) Research targeting genes responsible for antimicrobial resistance and pathogenicity islands in the DEC pathotypes.

c) More research is needed to establish the sources and extent of DEC infections in Kenya to institute appropriate management for such infections.

d) Continued research on DEC pathotypes to establish the extent of mobility of virulence genes in the *E.coli* strains to establish potential risks for DEC strains.

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APPENDICES

APPENDIX I

Age, gender, source and susceptibility profiles of all the isolates

LAB NO.	SOURCE	AGE	GEN-DER	FEP	AMP	SXT	C	AMC	S	TE	NA	CIP	GEN	CAZ
110	MM	1	F	S	I	S	S	S	S	S	S	S	S	S
28	MR	1	F	S	R	R	R	R	S	R	R	S	S	S
230	MR	1	M	S	S	R	S	I	S	S	S	S	S	S
195	MR	1	F	S	S	S	S	S	S	R	S	S	S	S
15	MR	1	F	S	R	R	S	S	S	S	S	S	S	S
49	MR	1	F	S	R	R	S	R	I	R	S	S	S	S
23	MM	1	M	S	I	R	S	S	S	S	S	S	S	S
149	MM	1	M	S	R	R	S	S	S	S	S	S	S	S
229	MR	1	F	S	R	S	S	I	I	R	R	R	I	S
117	MM	1	F	S	R	R	S	S	R	R	S	S	S	S
1	MM	1	M	S	S	S	S	S	S	S	S	S	S	S
144	MR	1	M	S	R	R	S	S	I	R	S	S	S	I
92	MM	1	M	S	S	S	S	I	S	S	S	S	S	S
57	MR	1	F	S	R	R	S	I	S	S	S	S	S	S
203	MR	1	F	S	R	R	S	R	R	R	S	S	S	S
217	MR	1	M	S	R	S	S	R	I	R	S	S	S	S
83	MR	1	F	S	S	S	S	S	S	S	S	S	S	S
35	MR	1	M	S	R	R	S	I	S	R	S	S	S	S
255	MM	1	M	S	R	R	S	R	S	S	S	S	S	S
15	MM	1	M	S	S	S	S	S	S	S	S	S	S	S
14	MR	1	F	S	R	R	S	S	S	S	S	S	S	S
232	MR	1	F	S	S	R	S	S	R	S	S	S	S	S
182	MM	1	F	S	S	R	S	I	R	R	S	S	S	S
47	MR	1	F	S	I	R	S	I	I	S	S	S	S	S
228	MR	1	F	S	S	R	S	S	S	S	S	S	S	S
25	MR	2	M	S	S	I	S	I	S	R	S	S	S	S
25	MM	2	F	S	S	R	R	R	I	R	S	S	S	S
11	MR	2	M	S	S	R	R	S	S	S	S	S	S	S
90	MR	2	M	S	R	R	S	S	S	R	S	S	S	S
141	MM	2	F	S	R	R	S	S	S	S	S	S	S	S
150	MR	2	F	S	R	R	S	S	I	R	S	S	S	S
4	MM	2	M	S	R	S	S	I	I	R	S	S	S	S
75	MR	2	F	S	R	R	S	S	S	S	S	S	R	S
40	MM	2	F	R	R	R	S	I	I	R	R	R	R	I
250	MM	2	F	S	S	S	S	S	S	R	S	S	S	S

152	MM	2	F	S	S	S	S	S	S	S	S	S	S	S
22	MR	2	M	S	R	R	S	S	S	S	S	I	S	S
95	MM	2	M	S	R	R	S	S	I	S	S	S	S	S
143	MR	2	F	S	R	R	S	S	I	R	R	R	S	S
231	MR	2	M	S	S	I	S	S	S	R	S	S	S	S
258	MM	2	M	S	I	S	S	S	S	S	S	S	S	S
194	MR	2	F	S	S	I	S	S	S	S	S	S	S	S
94	MR	2	F	S	R	R	S	S	R	R	R	R	S	S
211	MR	2	F	S	R	R	R	R	S	S	S	S	S	S
5	MM	2	F	S	R	R	S	R	I	S	S	S	S	S
24	MR	2	F	S	S	I	S	I	S	R	S	S	S	S
122	MM	2	F	S	S	R	S	S	R	R	R	R	S	S
91	MM	2	M	S	S	R	S	S	S	R	S	S	S	S
228	MR	2	M	S	R	R	S	R	R	I	S	S	S	S
110	MM	2	F	S	S	S	S	S	S	S	S	S	S	S
235	MR	3	M	S	S	R	S	R	S	S	S	S	S	S
121	MR	3	F	S	R	R	S	S	S	R	S	S	S	S
91	MM	3	M	S	R	R	S	I	S	S	S	S	S	S
27	MR	3	M	S	S	R	S	S	S	S	S	S	S	S
49	MM	3	F	S	S	S	S	S	S	S	S	S	S	S
142	MM	3	M	S	R	R	S	S	I	R	S	S	S	S
75	MR	3	F	R	R	R	S	R	R	R	R	R	R	I
70	MR	3	F	S	R	R	S	I	I	R	R	R	S	S
55	MR	3	F	S	S	S	S	S	S	S	S	S	S	S
48	MM	3	M	S	S	S	S	S	S	S	S	S	S	S
234	MR	3	F	S	R	R	S	S	I	I	S	S	S	S
12	MM	3	F	S	I	R	S	I	I	S	S	S	S	S
85	MM	3	F	S	R	R	S	S	R	R	S	S	S	S
115	MM	3	M	S	R	I	S	R	R	R	S	S	S	S
135	MM	3	M	S	R	R	S	I	R	R	S	S	S	S
38	MR	3	F	S	R	R	S	R	I	R	R	S	S	S
11	MM	3	M	S	S	S	S	R	S	R	S	S	S	S
249	MM	3	F	S	S	R	S	I	R	R	I	S	S	S
175	MM	3	M	S	S	S	S	S	S	R	I	S	S	S
153	MM	3	F	S	I	S	S	S	S	S	S	S	S	S
155	MR	3	M	S	S	S	S	I	S	R	S	S	S	S
11	MM	4	F	S	S	R	S	S	S	S	S	S	S	S
150	MM	4	F	S	S	S	S	R	S	S	S	S	S	S
88	MR	4	F	S	R	S	S	S	S	R	S	S	S	S
99	MM	4	M	S	R	R	S	R	S	R	R	R	S	S
115	MM	4	M	S	R	R	S	R	S	R	S	S	S	S
72	MM	4	M	S	S	R	S	S	S	S	S	S	S	S
93	MM	4	M	S	R	R	S	S	I	S	S	S	S	S

103	MM	4	M	R	R	R	S	I	R	R	R	R	R	I
32	MR	4	M	S	R	R	S	S	I	R	S	S	S	S
45	MM	4	F	S	R	R	S	I	R	R	R	S	S	S
101	MM	4	M	R	R	R	S	I	R	R	S	S	S	R
59	MM	4	F	S	R	R	S	S	I	R	R	R	R	S
147	MM	4	F	S	R	R	S	I	I	R	S	S	S	S
183	MR	4	M	S	R	R	I	S	S	R	R	R	S	S
237	MM	4	F	S	R	R	R	R	S	R	S	S	S	S
27	MM	4	M	S	S	S	S	S	S	S	S	S	S	S
53	MR	4	F	S	S	S	S	S	S	S	S	S	S	S
12	MM	4	F	S	R	R	S	S	I	R	S	S	S	S
193	MR	4	M	S	I	R	R	S	S	I	I	S	S	S
158	MM	4	F	S	S	R	S	S	S	S	S	S	S	S
109	MM	4	M	S	S	S	S	R	S	S	S	S	S	S
138	MM	4	F	S	S	S	S	S	S	S	S	S	S	S
221	MR	5	M	S	S	R	S	S	S	S	S	S	S	S
57	MM	5	M	S	S	R	S	S	S	R	S	S	S	S
117	MR	5	F	S	S	S	S	S	S	S	S	S	S	S
32	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
20	MM	5	F	S	S	S	S	S	S	S	I	S	S	S
75	MM	5	F	S	S	S	S	I	S	R	S	S	S	S
150	MM	5	M	S	R	R	R	R	S	R	R	I	R	S
45	MR	5	F	S	R	S	S	1	S	R	S	S	S	S
209	MR	5	F	S	S	S	S	S	S	S	S	S	S	S
178	MR	5	M	S	R	R	S	S	S	R	S	S	S	S
135	MM	5	F	S	R	R	S	R	S	R	R	I	R	S
94	MM	5	F	S	R	R	S	R	S	R	S	S	S	S
201	MM	5	F	S	I	R	S	S	S	S	S	I	S	S
45	MR	5	M	S	R	R	S	I	S	S	S	S	S	S
154	MM	5	M	S	R	R	S	R	S	R	S	S	S	S
90	MM	5	F	S	R	S	S	R	S	S	S	S	S	S
45	MM	5	M	S	S	S	S	S	S	S	S	S	S	S
210	MR	5	M	S	R	R	S	S	I	R	I	S	S	S
84	MR	5	M	S	S	S	S	S	S	R	S	S	S	S
252	MM	5	F	S	S	S	S	S	S	R	S	S	S	S
89	MM	5	M	S	R	S	S	S	S	S	S	S	S	S
45	MM	5	M	S	R	R	S	I	R	R	R	R	I	S
140	MR	5	F	S	I	S	S	S	S	S	S	S	S	S
245	MM	5	M	S	S	R	S	S	S	S	S	S	S	S
179	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
104	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
250	MM	5	M	S	R	R	S	S	R	R	S	S	S	S
139	MR	5	M	S	S	R	S	S	R	R	I	S	S	S

19	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
87	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
179	MM	5	F	S	S	R	S	S	S	R	S	S	S	S
120	MM	5	F	S	S	R	S	S	S	S	S	S	S	S
181	MM	5	F	S	R	R	S	S	I	R	S	S	S	S
88	MM	5	F	S	R	R	S	S	R	R	S	S	S	S
119	MR	5	M	S	S	S	S	S	S	S	S	S	S	S
77	MR	5	M	S	S	S	S	S	S	S	S	S	S	S
13	MR	5	F	S	S	R	S	S	S	R	S	S	S	S
123	MR	5	M	S	R	R	S	S	R	R	R	R	S	S
7	MM	5	F	S	R	R	S	S	S	R	S	S	S	S
9	MR	5	M	S	S	S	S	S	S	S	S	S	S	S
47	MM	5	M	S	S	S	S	S	S	S	S	S	S	S
17	MM	5	F	S	S	S	S	S	S	S	S	S	S	S
195	MR	5	M	S	R	R	R	R	R	S	S	S	S	S
39	MR	5	F	S	R	R	S	S	S	S	S	S	S	S
1	MR	5	F	I	R	R	S	R	R	R	R	R	R	R
259	MM	5	F	S	I	R	S	S	S	S	S	S	S	S
10	MR	5	M	S	S	I	S	S	S	S	S	S	S	S
112	MM	5	M	S	S	S	S	S	S	S	S	S	S	S
257	MM	5	M	S	S	I	S	S	S	S	S	S	S	S
200	MR	5	F	S	R	R	I	S	S	R	R	R	S	S
12	MR	5	M	S	R	R	S	R	S	R	S	S	S	S
220	MM	5	F	S	S	R	S	S	S	S	S	S	S	S
31	MM	5	M	S	S	S	S	I	S	R	S	S	S	S
247	MM	5	M	S	S	R	S	S	S	S	S	S	S	S
159	MM	5	F	S	S	R	S	R	I	I	R	R	S	S
152	MM	5	M	S	R	R	S	S	R	I	S	S	S	S
115	MR	5	F	S	R	R	S	I	S	S	S	S	S	S
8	MM	5	M	S	S	R	S	S	S	R	S	S	S	S
148	MR	5	M	S	S	S	S	S	S	S	I	S	S	S
149	MR	5	M	S	S	I	S	I	S	R	S	S	S	S
202	MR	5	F	S	S	R	R	R	I	R	S	S	S	S
42	MM	5	M	S	I	S	S	I	S	S	S	S	S	S
122	MM	5	M	S	S	R	S	S	S	S	S	S	S	S

APPENDIX 11

Biochemical tests

a) Citrate utilization test

Simmons citrate agar is used for this test. The basis of this test is based on whether or not citrate is utilised as the sole source of carbon.

Ingredients	**gm/litre**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15

Preparation

23g of Simmons citrate agar was dissolved in 1000ml distilled water and left to soak for 15 minutes. It was heated to boiling to dissolve the ingredients. It was distributed in 3ml amounts in bijoun bottles and autoclaved at 121 ^{o}C for 15 minutes. When cool enough to handle, it was removed from the autoclave and allowed to set in the slope position.

b) Sulphur Indole Motility Agar (SIM Agar)

A motility-indole medium has been found to be helpful in the identification of the Enterobacteriaceae.SIM Medium is therefore designed to determine three characteristics: hydrogen sulphide production, indole production and motility. SIM Medium can be used in conjunction with Triple Sugar Iron Agar to assess the ability of the culture to ferment lactose, sucrose and glucose.

Ingredients of SIM medium

Typical Formula *	gm/litre
Tryptone	20.0
Peptone	6.1
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5
pH 7.3 ± 0.2 @ 25°C	

Procedure

30g of SIM medium was suspended in 1 litre of distilled water and boil to dissolve the medium completely. Dispense into final containers and sterilise by autoclaving for 15 minutes at 121°C.

c) Triple sugar iron (TSI) Agar.

TSI is a composite medium for the differentiation of Enterobacteriaceae by three sugar fermentations (lactose, glucose and sucrose).The media is adjusted to PH 7.4 ± 0.2 @ 25°C.

Ingredients of TSI

Typical Formula*	gm/litre
`Lab-Lemco' powder	3.0
Yeast extract	3.0
Peptone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Glucose	1.0
Ferric citrate	0.3
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Procedure:

Suspend 65g in 1 litre of distilled water. Bring to the boil to dissolve completely. Mix well and distribute. Sterilize by autoclaving at 121°C for 15 minutes. Allow the medium to set in sloped form with a butt about 1 in deep.

d) Tryptone Water

This is a liquid medium for the production of indole by micro-organisms. It has a high content of tryptophan and it is more reliable than peptone water.

Ingredients of Tryptone

Typical Formula*	**gm/litre**
Tryptone	10.0
Sodium chloride	5.0
pH 7.5 ± 0.2 @ 25°C	

Preparation

15g of media was dissolved in 1 litre of distilled water and distribute into final containers. It was then sterilized by autoclaving at 121°C for 15 minutes and poured in tubes.

e) Kovacs reagent

Components' of Kovac's

Reagent:

paradimethylaminobenzaldehyde	5 grams
amyl alcohol	75ml
concentrated hydrochloric acid	25ml

This reagent is used in conjunction with tryptone water when performing indole test. A dark red colour in the amyl alcohol surface layer constitutes a positive indole test; no change in the original colour of the reagent constitutes a negative test.

APPENDIX III

Media

a) MacConkey agar

This is a medium for isolation of coliforms in biological specimens.

Preparation

52gm was dissolved in 1000ml distilled water. It was heated to boiling to dissolve the medium completely. It was then sterilized by autoclaving at (121°C) for 15 minutes and placed in a water bath set at 50°C. It was then mixed well before pouring in petri dishes.

Ingredients

Typical Formula*	gm/litre
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0
pH 7.4 ± 0.2	

b) Mueller Hinton agar

The major use of Mueller-Hinton Agar is for Antimicrobial Susceptibility Testing.

Preparation

38gm was dissolved in 1000ml of distilled water. It was heated to boiling to dissolve the medium completely. It was sterilized by autoclaving at (121°C) for 15 minutes and placed in a water bath set at 50°C.

Ingredients

Typical Formula*	**gm/litre**
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar	17.0
pH 7.3 ± 0.1 @ 25°C	

APPENDIX IV

Solutions

Preparation

1. 10 M NaOH

Dissolve 80g sodium hydroxide pellets in 200ml de ionized water.

2. 0.5M EDTA, pH 8.0

Add 37.22 g $Na_2EDTA.2H_2O$ to 160ml de ionized water. Adjust the pH to 8.0 with ≤ 4 g sodium hydroxide pellets, fill up to 200 ml with deionized water. Sterilize by autoclaving.

3. 1MTris. HCl, pH 8.0.

Dissolve 121.1 g Tris base in 800 ml deionized water. Adjust the pH to 8.0 with HCl, fill up to1 litre with deionized water. Sterilize by autoclaving.

4. 20 X TE buffer (1 X is 10mM Tris, 1mM EDTA).

Dissolve 24.11 g Tris base and 7.45 g $Na_2EDTA.\ 2H_2O$ in 800 ml deionized water. Adjust the pH to 8.0 with HCl, fill up to 1 litre with deionized water.

5. 5 X TBE electrophoresis buffer.

(1 X is 89mM boric acid and 2.8mM EDTA)

Dissolve 53.89 g Tris base, 27.51 g boric acid and 5.21 g $Na_2EDTA\ .\ 2H_2O$ in 1 litre deionized water.

6. 10 mg/ml Ethidium bromide.

Dissolve 0.5 g ethidium bromide in 50 ml deionized water in dark bottle. Store at 4 ^oC.

7. 70% Ethanol.

Mix 140 ml ethanol and 60 ml sterilized deionized water in the sterilized bottle.