

**BACTERIAL AND PARASITIC CO-INFECTIONS,
ANTIMICROBIAL SUSCEPTIBILITY AND ESBL GENES
CARRIAGE IN BACTERIA ISOLATED FROM CHILDREN
BELOW 5 YEARS WITH DIARRHEA ATTENDING
MUKURU SLUM HEALTH CLINICS**

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**Bacterial and Parasitic Co-Infections, Antimicrobial Susceptibility and
ESBL Genes Carriage in Bacteria Isolated from Children below 5
Years with Diarrhea Attending Mukuru Slum Health Clinics**

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**A Thesis Submitted In Partial Fulfillment for the Degree of Master of
Science in Infectious Diseases and Vaccinology in the Jomo Kenyatta
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DECLARATION

This Thesis is my original work and has not been presented for a degree in any other university.

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This thesis has been submitted to the University for Examination with our approval as supervisors:

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DEDICATION

This project is dedicated to my sister Mary Kamau who tirelessly supported me in many ways since the beginning of my study. May the Almighty God bless you. Dedicated also to my family. Their prayers and moral support have been the driving momentum that has enabled me to achieve the targeted goal. To my husband Warren Moore for his patience and strength, without whom this study wouldn't have been complete. I wish to thank my friend Waihenya Ndirangu who has always supported and encouraged me to do my best in all matters of life. I wish to thank my academic sponsors; Mary Wambui Ng'ang'a, Isaac Waihenya Ndirangu and Warren Moore. I would not have had this opportunity without their generosity. May the Almighty God bless them.

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LIST OF ABBREVIATIONS

AMC	Amoxicillin Clavulanic Acid
AMP	Ampicillin
API 20E	Analytical profile index 20 <i>Enterobacteriaceae</i>
AST	Antimicrobial Sensitivity Testing
ATCC	American type culture collection
BPS	Base pair
CAZ	Ceftazidime
CGA	Clonal group A
CHL	Chloramphenicol
CIP	Ciprofloxacin
CMR	Centre for Medical Microbiology Research
CPD	Cefpodoxime
CRO	Ceftriaxone
CTX	Cefotaxime
CXM	Cefuroxime
EAEC	Enteraggregative <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
ESBL	Extended Spectrum Beta-Lactamase
ESBL	Extended Spectrum Beta-Lactamases

ESCs	extended-spectrum cephalosporins
FEA	formalin-ethyl acetate
GEN	Gentamycin
KAN	Kanamycin
MDR	Multi Drug Resistance
MHA	Mueller-Hinton Agar
NAL	Nalidixic acid
NTS	Non-typhoidal <i>Salmonella</i> NTS
PCR	Polymerase Chain Reaction
SHV	Sulphydryl variable
SMX	Sulphamethoxazole
SPP	Species
SXT	Cotrimoxazole
TCY	Tetracycline
TEM	Temoniera
WHO	World Health Organization

ABSTRACT

Diarrhea comes second as the most frequent cause of deaths in children younger than five years in developing countries. In Kenya, the overall two-week period incidence of diarrhea in children is 17.1%. This study aimed at investigating prevalence of bacterial-parasitic co-infections and antimicrobial resistance in children below 5 years presenting with diarrhea at Mukuru Kwa slum health clinics in Nairobi County, 25 km East of Nairobi city, Kenya. One hundred and seventy-four stool samples of children presenting with diarrhea were collected and analysed for enteric bacterial pathogens, protozoans and helminths. Pathogenic enteric bacteria were isolated using differential media and API20E kits. Antimicrobial susceptibility tests were determined using Kirby-Bauer disk diffusion method. PCR method was used for Pathotyping of *E. coli* isolates and screening for Extended Spectrum Beta Lactamase enzymes. Parasites were detected by direct wet mounts and formal ether method. *E. coli* was the leading bacteria isolated in 98 (47.3%) cases. Thirteen (13.3%) were pathogenic. Two pathotypes of *E. coli* were observed - Enteroaggregative *E. coli* was found in 9 cases and accounted for 9.2%. Prevalence of enteropathogenic *E. coli* was at 4.1% with 4 cases. *Salmonella spp* was found in 12 cases (5.8%) of which 5 cases had *Salmonella typhimurium* at 41.7%, *Salmonella enteritidis* in 4 cases at 33.3%, and *Salmonella typhi* in 3 cases at 25% of total *Salmonella* isolates. Prevalence of *Serratia marcescens* was at 1.4% having been found in 3 cases, *Aeromonas sobria* in 2 cases at 1.0% and *Providencia spp* in only one case at 0.5%. Other enterics isolated include *Proteus spp*, *Klebsiella spp*, *Pseudomonas spp*, *Enterobacter spp*, *Citrobacter spp* and *Morganella morganii*. *Entamoeba coli* and *Entamoeba histolytica* were isolated together in two samples, having a co-infection with *Salmonella typhi* in one of the cases. *Cryptosporidium parvum* was isolated from one sample. ESBL carriage was at 10.1%. The highest proportion of ESBL genes was *bla*^{TEM-1} gene in 8 isolates at 3.9%, *bla*^{oxa-48} gene was second highest at 2.9%. *bla*^{CTX-M-15} gene was at 1.9% and *bla*^{shv-1} gene at 1.5%. At a prevalence of 2.29%, co-infection and co-infestation was not a major factor in cases of childhood diarrhea studied. The major concern in this study is resistance of enteric bacterial pathogens in childhood diarrhea leading to Multi drug resistance (MDR) isolates at 17%. They are an important etiology, making these also a likely cause of recurring childhood diarrhea in this study. Improved public health measures, proper sanitation, clean water supply and interventions with bacterial targeted vaccinations should be included in the mandatory childhood vaccinations. Evidence based prescriptions by AST data should be employed to reduce the rate of AMR in children.

CHAPTER ONE

INTRODUCTION

1.1 Background of study

Diarrhea is defined as having loose or watery stools at least three times per day or more frequently than normal for an individual (Bhan *et al.*, 2005). Diarrhea often lasts for a few days and results in dehydration due to fluid loss. Signs of dehydration often begin with loss of the normal stretchiness of the skin and changes in personality. This can progress to decreased urination, loss of skin colour, a fast heart rate and a decrease in responsiveness as it becomes more severe. Loose but non-watery stools in babies who are breastfed, however, may be normal (Jahansson *et al.*, 2009).

The most common cause of diarrhea is an infection of the intestines by a virus, bacteria, or a parasite; a condition known as gastroenteritis. These infections are often acquired from food or water that has been contaminated with the pathogens or directly from another person who is infected. (El-Shabrawi *et al.*, 2015). Diarrhea may be divided into three types: short duration watery diarrhea, short duration bloody diarrhea and persistent diarrhea if it lasts for more than two weeks. The short duration watery diarrhea may be cholera. If blood is present it is also known as dysentery (Jahansson *et al.*, 2009).

Children are very susceptible to bacteria and parasites infestations due to their under-developed immune systems (Boru *et al.*, 2010). Rotavirus was the leading cause of diarrhea among children under 5 in the early 1990s (El-Shabrawi *et al.*, 2015; Bern *et al.*, 1992). Since introduction of Rotavirus vaccine other viruses, parasites and bacteria cause serious diarrheal diseases often associated with morbidity and mortality and some are not vaccine preventable and if the vaccine is available it is not well distributed in the community (Kosek *et al.*, 2003).

Diarrheal disease may have significant negative impact on both physical fitness and mental development and health. Early childhood diarrhea causes malnutrition resulting to reduced physical fitness and work productivity. This happens even in

adults (Cynthia *et al.*, 2008). It has been shown that, even when controlling for helminth infection and early breastfeeding, children who had experienced severe diarrhea had significantly lower scores on a series of tests of intelligence (Das *et al.*, 2013).

In 2012 the cases of childhood diarrhea worldwide were at 0.76 million or 11% (CDC, 2014). The World Health Organization (WHO) estimated diarrhea deaths among children aged less than five years in Africa in 2004 to be at 17.5% (WHO, 2002). More than 10 million children below five years of age die each year worldwide, with six countries accounting for half of these deaths among them being India, Nigeria and China. Africa has 19 countries that comprise the 20 where mortality due to diarrhea mostly occurs (Cynthia *et al.*, 2008).

Globally, diarrhea comes second as the most frequent cause of death and in developing countries pneumonia is usually the leading cause of death amongst children aged five and below (CDC, 2014). It accounts for more than one-third of the deaths in this age group -more than HIV, malaria and measles combined. Malnutrition plays a major role in these cases (WHO, 2014). An estimated 1 billion episodes of diarrhea occur each year in children below five years, causing 5 million deaths annually, out of which 80% occur in the first two years of life (Carlos *et al.*, 1990).

In Kenya, diarrhea is a leading cause of morbidity, accounting for 17% of childhood illnesses (Boru *et al.*, 2010). Frequent episodes cause malnutrition, stunted growth and poor intellectual development (CDC, 2014). Still, many diarrhea cases recur in 20% of diagnosed and treated cases. This is mostly due to poor diagnostic techniques such as lack of laboratory evidence based microbiological analysis of samples in local clinics, which do not determine co-infections and/or infestation with intestinal parasites (Garrett *et al.*, 2008).

Nontyphoidal *Salmonella*, Enteropathogenic *E. coli* (EPEC) and *Shigella* are the bacterial pathogens commonly associated with mortality among Kenyan children with diarrhea who report to a hospital (Ciara *et al.*, 2012). These organisms are the most prevalent; hence the most treated, thus increasing the possibility of resistance. Studies show that resistance to amoxicillin, co-trimoxazole, chloramphenicol and

cephalosporins has been emerging (Chris *et al.*, 2007). Resistance to fluoroquinolones has also been reported and extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae have been reported (Karambu *et al.*, 2013).

1.2 Problem Statement

Children bear almost seventy percent of diarrhea disease burden in Kenya and this is largely attributed to unsafe drinking water and poor sanitation (WHO, 2005). Diarrhea was the second most common cause of deaths in children younger than five years (0.76 million or 11%) in 2012 (CDC, 2014). Diarrhea continues to be a major cause of morbidity and mortality in children less than five years, yet it is preventable when appropriate measures are put in place. Frequent episodes of diarrhea are also a common cause of malnutrition. Other long-term problems that can result include stunted growth and poor intellectual development (WHO, 2014).

In 80% of the cases, co-etiological factors causing diarrhea to go undetected due to poor diagnostic techniques and lack of routine microbiological analysis of specimen in poor resource clinics (Boru *et al.*, 2010). Children are very susceptible to diarrhea and worm infestations due to their under developed immune systems and many diarrhea cases recur in 20% of diagnosed and treated cases. (Boru *et al.*, 2010; Garrett *et al.*, 2008). Although mortality rates have declined in many countries in recent years, largely because of improved sanitation, supply of clean water and advances in health care and treatment, outbreaks of diarrhea diseases continue to affect many millions of children; The overall two-week period incidence of diarrhea in under-fives in Kenya is 17.1% (Karambu *et al.*, 2013).

1.3 Justification

The extent or the role of co-morbidities of bacterial and parasitic nature, as causal factors in diarrheal illnesses is not well understood. Data on the prevalence and antimicrobial resistance of the main circulating enteropathogenic bacteria remain limited in Kenya. Better knowledge of circulating endemic and epidemic enteric pathogens and their antimicrobial resistance is crucial for effective treatment strategies (John *et al.*, 2006). Lack of awareness, resources and facilities to conduct

Extended Spectrum Beta Lactamases (ESBL) identification, contribute to the spread of multidrug resistance in most Enterobacteriaceae organisms (Ciara *et al.*, 2012).

1.4 Research questions

1. What is the prevalence of enteric bacterial pathogens and parasites in children with diarrhea attending Mukuru slum health clinics?
2. Which are the enteric bacterial pathogens that co-infect together with other intestinal parasites in children with diarrhea attending Mukuru slum health clinics?
3. What are the antimicrobial susceptibility profiles of enteric bacterial pathogens isolated from children with diarrhea in Mukuru slum health clinics?
4. Which ESBL genes are in enteric pathogens isolated from children with diarrhea attending Mukuru slum health clinics?

1.5 Objectives

1.5.1 General objective

To determine enteric bacterial and parasitic co-infections, antibiotic susceptibility profiles and presence of ESBL genes in bacterial pathogens isolated from children less than five years with diarrhea, seeking health care at Mukuru slum health clinics.

1.5.2 Specific objectives

1. To determine prevalence of bacterial and parasitic etiologies in children with diarrhea attending Mukuru kwa Njenga, Municipal City Council and Mukuru kwa Reuben health clinics
2. To determine bacterial and parasitic co-infections in children with diarrhea attending Mukuru Kwa Njenga, Municipal City Council and Mukuru Kwa Reuben health clinics?
3. To determine antimicrobial susceptibility profiles of enteric bacterial pathogens isolated from children with diarrhea attending Mukuru Kwa Njenga, Municipal City Council and Mukuru Kwa Reuben health clinics.

4. To determine ESBL genes present in enteric pathogens isolated from children with diarrhea attending Mukuru Kwa Njenga, Municipal City Council and Mukuru Kwa Reuben health clinics.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diarrhea disease Burden

More than 1 billion cases and 4 million deaths per year are attributed to diarrhea worldwide (Bhan *et al.*, 2005). In developing countries an estimated 100 million episodes occur each year in children below five years, causing 5 million deaths each year out of which 80% occur in the first two years of life (Carlos *et al.*, 1990). The trend of bacterial enteropathogens varies in developed and developing countries. The trends of bacterial enteropathogens causing gastroenteritis varies to the standard of living and environmental hygiene (Bhattacharya *et al.*, 2009). In 2012, diarrhea was the second most common cause of deaths in children younger than five globally, rated at 12%. Frequent episodes of diarrhea are also a common cause of malnutrition (CDC, 2014). Other long-term problems that can result include stunted growth and poor intellectual development. So, the periodic renewal of knowledge about trends of the bacterial enteropathogens is very essential.

For early and specific intervention for diarrhea, it's very important to know the exact pathogen and its antimicrobial sensitivity profile to decrease financial burden involved both for the patient and the healthcare system. Determining how frequently pathogens exist together and which species mostly co-infect together would be a major step in preventing recurrence of diarrhea. This would help in alleviating the burden that is diarrhea and to aid in the proper interventions for controlling the disease.

2.2 Causative agents of mortality and morbidity in children less than five years

The prevalence of life-threatening infections caused by consumption of contaminated food and water has increased worldwide and is becoming an important cause of morbidity and mortality in developing countries (Steiner *et al.*, 2006). A wide array of microorganisms causes diarrhea in children. These include bacteria, parasites and viruses (Brooks *et al.*, 2006).

2.2.1 Diarrheal bacteria

Five pathotypes of *Escherichia coli* are responsible for as much as 25% of the cases. Typical enteropathogenic *Escherichia coli* (EPEC) is associated with a high risk of mortality in 0–11 months aged children (Sousa *et al.*, 2013). Based on their distinct virulence properties and the clinical symptoms of the host, pathogenic *E. coli* strains are divided into numerous categories or pathotypes. The diarrheagenic *E. coli* strains include: enterotoxigenic *E. coli* (ETEC) strains, which are associated with traveller's diarrhea, childhood diarrhea, porcine and bovine diarrhea; enteropathogenic *E. coli* (EPEC) strains, which cause diarrhea in children and animals; enterohemorrhagic *E. coli* (EHEC) strains, which are associated with haemorrhagic colitis and haemolytic-uremic syndrome in humans; enteroaggregative *E. coli* (EAEC) strains, which are associated with persistent diarrhea in humans and enteroinvasive *E. coli* (EIEC) strains, which are involved in invasive intestinal infections, watery diarrhea, and dysentery in humans and animals (Raghavan *et al.*, 2006)

Salmonellosis is spread by faecal oral route and causes a wide range of human diseases like enteric fever, gastroenteritis and bacteraemia (Weber *et al.*, 1999). Nontyphoidal *Salmonella* and *Shigella* are the ones commonly associated with mortality among rural Kenyan children with diarrhea who access a hospital (Ciara *et al.*, 2012). Non-typhoidal *Salmonella* (NTS) is a dominant contributor to invasive bacterial disease with high mortality in Africa (Weber *et al.*, 1999). *Salmonella spp* typically causes an intestinal infection that causes fever, abdominal cramps, and diarrhea lasting well past a week (Hohmann, 2001). Typhoid fever however causes a systemic infection with serious medical implications (Bhan *et al.*, 2005). Typhoid symptoms include headaches and fevers, but diarrhea is absent.

It is estimated that *Salmonella* infections are responsible for 1.3 million illnesses annually worldwide and result in 16,000 hospitalizations and 600 deaths (CDC, 2006). Humans are the only carriers, and infected persons may be asymptomatic. Rose spots on the cheeks are diagnostic factor. In young children and the immunocompromised symptoms can be fatal. They last generally up to a week and

appear 12 to 72 hours after *Salmonella* bacterium ingestion (Black *et al.*, 2003). Serotyping for *Salmonella* isolates is necessary using commercial antisera. This allows for characterization of *S. Paratyphi A*, *S. Paratyphi B*, *S. Paratyphi C*, *S. typhi*, *S. typhimurium* and *S. enteritidis* which are the species mostly in circulation (Esteban *et al.*, 2007).

Shigella species are responsible for 10 to 15% of acute diarrhea in children less than 5 years of age (Sousa *et al.*, 2013). *Shigella* is the second cause of moderate-to-severe diarrhea among children 1 to 5 years old after *EPEC* (Morris *et al.*, 2005). The symptoms of *Shigella* infection range from mild watery diarrhea to severe bacillary dysentery with fever, abdominal pain, blood and mucus in stool samples (Das *et al.*, 2013).

Campylobacter foetus is an opportunistic human pathogen that can cause bacteremia and thrombophlebitis. Though rare, along with *C. coli* it can lead to fatal septicaemia in children and immunocompromised individuals. Infection with *Campylobacter foetus* is only a problem in immunocompromised patients, pregnant mothers, and infants. Other patients usually recover without complications (Mahmud *et al.*, 2009). It is a species of gram negative with s-shaped rod morphology like members of the genus *Vibrio*. *Vibrio cholerae* O1 causes secretory diarrhea, associated with severe outbreaks when hygiene is low cholera outbreaks have been linked to consumption of unsafe food and drinking water, exposed foods and bulk cooking of food at gatherings (Acosta *et al.*, 2001). There are other causative organisms that thrive in different environments (Table 2.1).

Table 2.1: Causative organisms of diarrhea (Vernacchio, 2006).

Organisms that commonly cause food poisoning	
Food History	Organism
Dairy	<i>Campylobacter, Salmonella spp</i>
Eggs	<i>Salmonella spp</i> , especially <i>S. enteritidis</i>
Meats	<i>C. perfringens, Bacillus cereus, Aeromonas, Campylobacter</i> and <i>Salmonella spp</i>
Ground beef	<i>E coli O157:H7</i>
Poultry	<i>Campylobacter spp</i>
Pork	<i>C. perfringens, Yersinia enterocolitica</i>
Seafood	<i>Astrovirus, Aeromonas, Plesiomonas,</i> and <i>Vibrio spp</i> especially <i>V. parahaemolyticus</i>
Vegetables	<i>Aeromonas spp, C. perfringens, E. coli</i>
Mayonnaise containing salads, highly processed foods	<i>Staphylococcus aureus</i>
Rice; starchy foods	<i>Bacillus cereus</i> (vomiting)
Canned foods, honey (children under 1 year of age)	<i>Clostridium botulinum</i>

Vibrio cholerae is endemic in different regions of the world and causes severe outbreaks. Cholera disease is usually caused by *Vibrio cholerae* O1 and is endemic in sub-Saharan Africa (Bhattacharya *et al.*, 2009). Cholera causes massive loss of body fluids by causing watery diarrhea, leading to loss of electrolytes and severe dehydration. If left untreated, death can occur. *Vibrio cholera* inhabits water bodies. Toxigenic strains are causative agents of fatal diarrhea (WHO, 2002). *Campylobacter* infections has highest incidence among children younger than 4 years, though the most elevated rate of hospitalization is in persons more than 50 years in developing countries. Continuous increase in the number of *C. jejuni* has been seen, with

occurrence rates as high as 73 cases for every 100,000-populace reported (Oberhelman *et al.*, 2000). *Campylobacter* gastroenteritis is especially common during the first 5 years of life. Isolation rates in children with acute diarrhea range from 10%-46%. In developed countries, the cases are higher in winter than other seasons. Most patients recover from *C. jejuni* infection within 5 days, after suitable antimicrobial treatment (Yamazaki *et al.*, 2007).

2.2.2 Diarrheal Parasites

Parasitic infections, caused by intestinal helminths and protozoan parasites, are among the most prevalent infections in humans. These infections are most prevalent in the developing world where adequate water and sanitation facilities are lacking. They inhabit the human gut and cause a significant burden of human disease. It is estimated that 3.5 billion people worldwide harbour parasites and 450 million are ill because of the parasites (Garcia *et al.*, 1997). Parasites causing diarrheal are widespread in water supplies. They pose a serious threat that is worsened by limited resources (Hague, 2007).

In developed countries, protozoan parasites more commonly cause gastrointestinal infections compared to helminths. Intestinal parasites cause a significant morbidity and mortality in endemic countries. Helminths include nematodes (roundworms), cestodes (tapeworms), and trematodes (flatworms). Most are soil-transmitted helminths or geohelminths. They include *Ascaris lumbricoides* (roundworm), *Trichuris trichiuria* (whipworm), *Ancylostoma duodenale*, and *Necator americanicus* (hookworms) (Hague, 2007). Intestinal helminths are rarely a cause of mortality except in heavy infestation cases. *Ascaris lumbricoides* has been known to block windpipes and nasal passages as well as intestinal lumen causing great morbidity (Neva *et al.*, 1994). Other species of intestinal helminths are not widely prevalent.

The most commonly isolated protozoans are *Giardia intestinalis* or *G. Lamblia* causing giardiasis, *Entamoeba spp* causing amoebiasis, *Cyclospora cayetanensis* causing cyclosporiasis and *Cryptosporidium spp.* causing cryptosporidiosis. *Giardia spp* is the most prevalent parasitic cause of diarrhoea in both developed and developing countries (Hague, 2007). The World Health Organization (WHO)

estimates that approximately 50 million people worldwide suffer from invasive amoebic infection each year, resulting in 40-100 thousand deaths annually. Amoebiasis is the third leading cause of death from parasitic diseases worldwide (WHO, 1997; Petri *et al.*, 2000). Cryptosporidiosis is becoming most prevalent in both developed and developing countries among children aged less than five years and patients with AIDS. Spread of these protozoan parasites is as a result of poor sewage disposal and poor quality of drinking water. Food and water-borne outbreaks of these protozoan parasites have occurred in developing countries and their spread occurs through faecal contamination. Other species of protozoan parasites can also be found in the human gut, but they are not pathogenic (Hague, 2007).

In parasitological tests, two preparations of each specimen are usually made on each slide: one unstained preparation and another stained preparation (Garcia *et al.*, 1997). The saline wet mount is an unstained preparation made by using physiological saline which demonstrates the motility of trophozoites stage of the protozoan species. However, internal structures are often poorly visible. The method is however fast, simple and provides a quick answer when positive (Alum *et al.*, 2010). The method also provides an approximation of the parasitic burden and it can be used with unpreserved specimens to detect the characteristic motility of trophozoites. Wet mounts can also be used as a safeguard, as some protozoa may at times not concentrate properly because of unknown factors (Neva *et al.*, 1994).

The iodine mount method is used in differentiation and identification of parasites by characteristic morphological features and details of internal structures. The method is simple to perform, quick, and cheap. It allows visualization of parasitic ova and cyst morphology (Figure 2.1) It has a disadvantage in that the preparation dries within a few minutes, rendering it unreadable and unreliable to visualize live nematodal larvae (Missaye *et al.*, 2013). Methylene blue dye and glycerol combination is a simple substitute to the iodine mounts and saline mounts as it lasts longer and provides better visualization and differentiation from vegetable matters and other debris in the stool, Glycerol, by its hygroscopic nature, absorbs water molecules from the environment and prevents drying out of wet mounts (Roka *et al.*, 2013). To identify helminths, concentration techniques are used to increase relative number of parasites for scanty

infections and to reduce faecal debris for better visualization. Two concentration techniques can be used; sedimentation method and floatation method. Sedimentation method aims at detecting heavy eggs like *Ascaris lumbricoides* eggs, operculated eggs of Trematodes, larvae of *Stercoralis* and cysts. Floatation method aims at detecting *Schistosoma* trematode, cestodes and nematodes like hookworm which are light and usually float during concentration techniques (Garcia *et al.*, 1994, Hayes *et al.*, 1985).

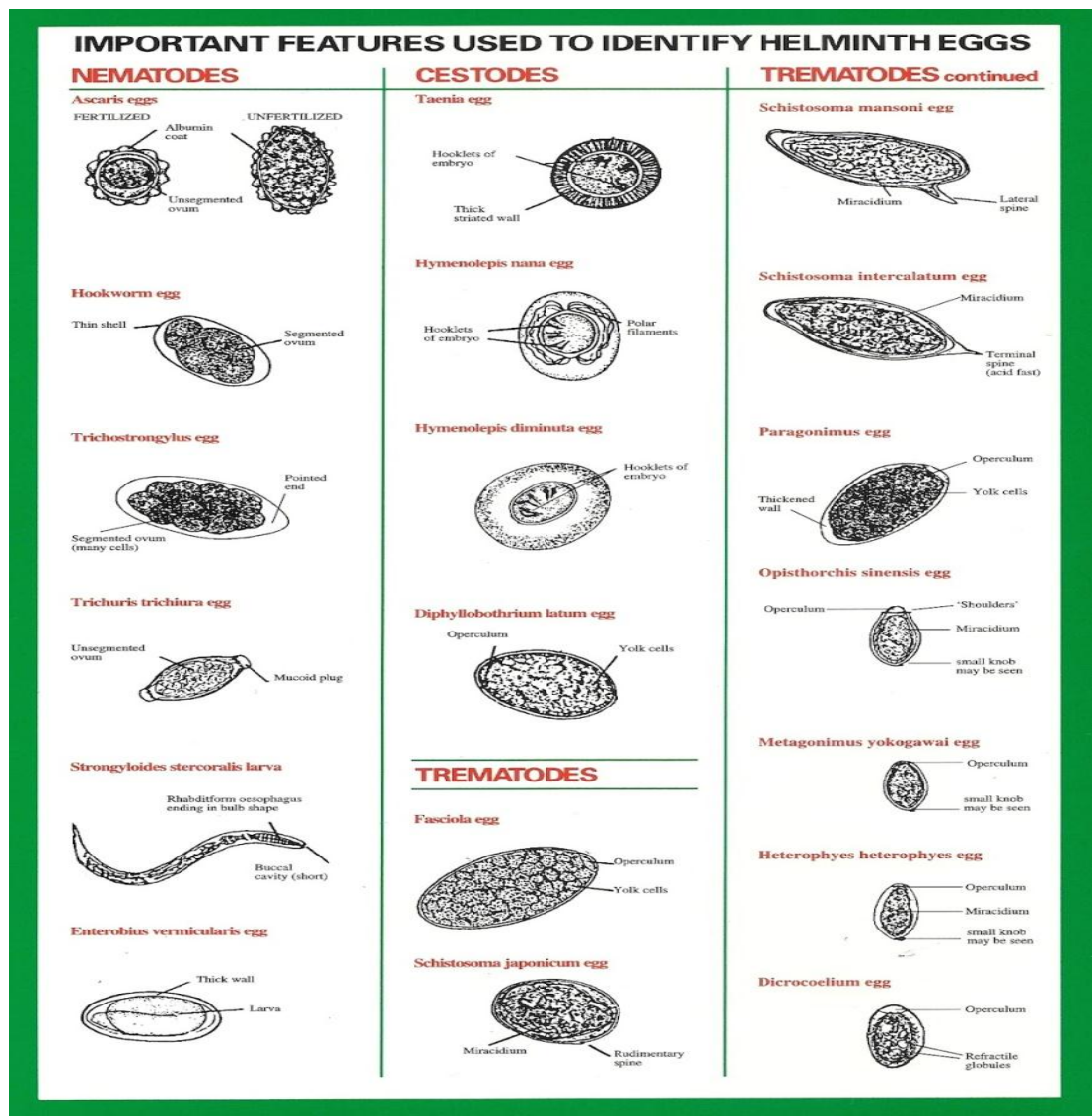


Figure 2.1: Features used to identify helminths (Garcia *et al.*, 1994).

2.3 Transmission of pathogens

Transmission of intestinal bacterial pathogens occurs from person to person through the faecal-oral pathway and ingestion of contaminated food and water (Black *et al.*, 2003). Food borne diseases are transmitted via contamination of drinking water or food with faeces from infected people. Untreated sewage being released into drinking water supply or not washing hands before preparing food also causes transmission of enteric pathogens. The pathogen must be in existence from a source, be in plenty for a sufficient inoculum and to reach a susceptible host to cause disease (Shapiro *et al.*, 2001).

In developing countries most sewage is discharged into the environment or on cropland and even in developed countries there are periodic system failures resulting in a sanitary sewer overflow (Lanata *et al.*, 2013). This is the typical mode of transmission for infectious agents of cholera, Hepatitis A, Polio, Rotavirus, *Salmonella spp*, *Shigella spp* and *Campylobacter jejuni* infections. Street vended foods and consumption of raw or undercooked shellfish exposes one to *E. coli*, *Salmonella*, *Shigella*, *Campylobacter*, *Clostridium* species, *Giardia*, *Entamoeba* and *Ascaris* which can all cause disease (Moyo *et al.*, 2011). If the infection is in the small intestine symptoms include watery diarrhea and vomiting. Infections in the large intestine usually result in dysentery involving small fecal volume, with mucus and blood. Some diseases follow certain predisposing conditions like antibiotic therapy causing pseudomembranous colitis (Lanata *et al.*, 2013).

Children are very susceptible to environmental health related diseases such as diarrhea and worm infestations due to their under developed immune systems. They bear almost seventy per cent of the diarrhea disease burden in Kenya and this is largely attributed to unsafe water and poor sanitation (WHO, 2005). *Shigella spp* is also an endemic pathogen in Central American countries causing severe diarrheal disease that requires hospitalization in children (Black *et al.*, 2003)

A *Campylobacter* bacterium infects the gastrointestinal tract and cause diarrhea, fever, and cramps (Oberhelman *et al.*, 2000). Good hand-washing and food safety habits will help prevent campylobacteriosis. It mostly affects immunosuppressed persons and sometimes it is self-limiting. Babies under 1 year old, teens, and young adults are most commonly affected. Transmission of bacterial pathogens is a four-stage process. It involves pathogen exit from the reservoir, attachment to the new host, entry to the new host and finally exit from the host (Shapiro *et al.*, 2001). Intervention at these four stages should take care of diarrheal diseases and outbreaks.

2.4 Co-relation between stool appearance and diarrheal aetiologies

Knowing the stool characteristics is very useful in narrowing down the causative agent of an intestinal infection. Stool contains bacteria and metabolic waste products such as bacterially altered bilirubin. Such faecal markers can be indicative of various diseases, such as gastro intestinal bleeding. Blood in stool cause black stools and could be indicative of haemolytic bacteria or other intestinal problems that result in digested blood. Stool that is too mucoid could be indicative of Giardia infection (Roka *et al.*, 2013). Possible pathogens that can be assumed after macroscopic examination of stool characteristics are outlined in Table 2.2.

Table 2.2: Correlating stool characteristics with causative agents (Roka *et al.*, 2013).

Stool Characteristics	Small intestines infection	Large intestines infection
Appearance	Watery	Mucousy and/or bloody
Blood	Possibly positive but never gross blood	Possibly grossly bloody
pH	Possibly <5.5	>5.5
WBCs	<5/high power field	>10/high power field
Serum WBCs	Normal	Leucocytosis, bandemia
Organisms	Viral <i>Rotavirus, Adenovirus</i> <i>Calicivirus, Astrovirus</i> <i>Norwalk virus, Noroviruses</i>	Invasive bacteria <i>E. coli, Shigella, Yersinia</i> <i>Salmonella, Campylobacter</i> <i>and Aeromonas spp</i>
	Toxic bacteria <i>E. coli, C. perfringens</i> <i>Vibrio spp, Bacillus cereus</i>	Toxic bacteria <i>Clostridium difficile</i>
	Parasites <i>Giardia spp</i> <i>Cryptosporidium spp</i>	Parasites <i>Entamoeba species</i>

2.5 Co-infections and co-infestations

Diarrhea due to infectious agents and co-infestation with intestinal worms is rated by WHO as the second contributory factor in the mortality and morbidity of children (WHO, 2005). Host genetics are thought to have a significant influence on susceptibility to these infections. Immune status, which can be influenced by

concurrent disease and/or environmental factors complicates the capacity to control these infections. In many parts of the world, exposure to bacterial infections and simultaneous infection with helminths is very common, although the nature of the interaction is still unclear (Actor, 1993).

There is a growing interest in understanding how an existing helminth infection affects the ability of the host to develop an appropriate immune response to other concomitant infections or antigens (Nokes *et al.*, 1991). In studies of host immunity, *H. polygyrus*, a natural murine nematode parasite that resides in the duodenum was shown to induce a vigorous Th2-type immune response, act as a mucosal adjuvant thus altering host immune response to orally administered protein antigen (Shi *et al.*, 1998).

In a study done at Mbagathi hospital in Nairobi Kenya by Mbae *et al.*, (2013), on the etiologies of diarrhea in children using multiplex PCR, out of the 100 stool samples analysed 52% had *Salmonella* and *Shigella* co-infection. Of this 28/52 were females while 24/52 were male children below the age of five. *Salmonella* and *Vibrio* co-infection was at 18% with 11/18 males and 7/18 females. *Salmonella* and *Campylobacter* co-infection was at 4% with males and females equally co-infected at 2/4 or both genders. *Shigella* and *Vibrio* co-infection was 17% with more females; 10/17 co-infected compared to 7/17 males. *Shigella* and *Campylobacter* co-infection was 4%, of this 3/4 were females while 1/4 were males. *Campylobacter* and *vibrio* co infection was 3% with 1/3 females while 2/3 males co infected.

The study however did not look at co-infestation with intestinal helminths. Out of the 100 stool samples analysed however there was triple co-infection with different aetiologies; *Salmonella*, *Shigella* and *Vibrio* infections was at 14%. *Salmonella*, *Shigella*, *Campylobacter* and *Vibrio* infections was at 2% (Mbae *et al.*, 2013).

There is evidence to support the hypothesis that helminth infection causes impaired immune response to concurrent viral, bacterial, and parasite infections and even to vaccinations. This happens by impairing the ability of the host to control an infection and promote bacterium-mediated intestinal injury (Actor, 1993).

2.6 Effects of enteric pathogens in children

Developed countries like Norway, Denmark, Australia, Portugal and the United States also have reported outbreaks of food borne illnesses (Kotloff *et al.*, 2013). In Kenya, diarrhea is a leading cause of morbidity, accounting for 17% of childhood illnesses (Boru *et al.*, 2010). The WHO ranks diarrheal disease as the second most common cause of morbidity and mortality in children in the developing world (Figure 2.2). This is due to the underdeveloped immune system of the children. The Child Health Epidemiology Research Group (CHERG), created by the WHO in 2001, has used various methods to determine specific causes of mortality. It estimated that the syndrome of diarrhea accounted for 18% of all deaths in children under the age of five globally, with malnutrition as a co-morbid condition in 53% of all deaths (WHO, 2005).

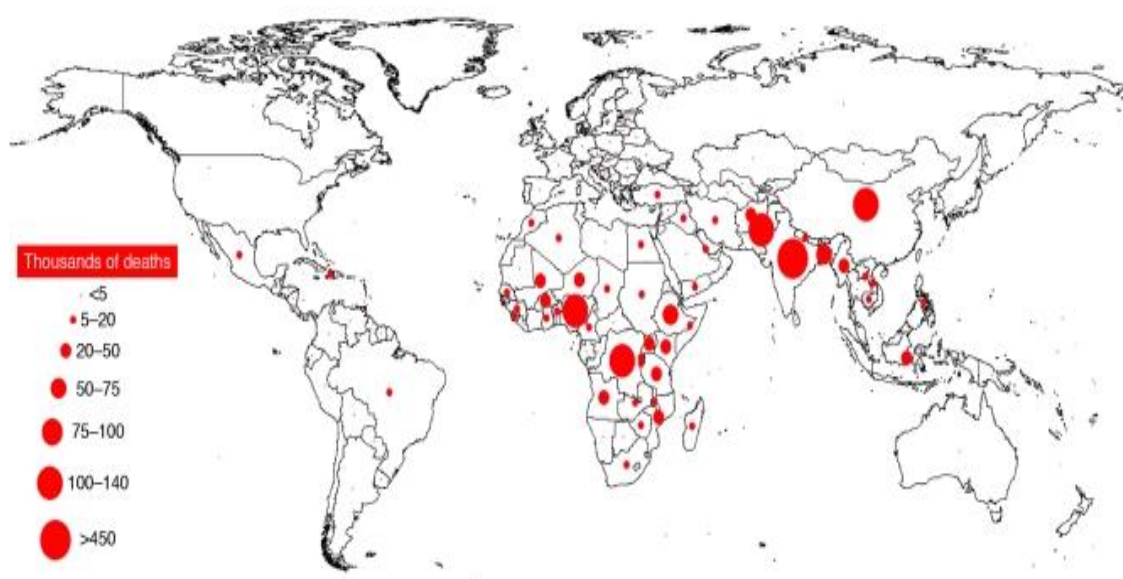


Figure 2.2: Worldwide distribution of deaths caused by diarrhea in children less than five years of age in 2013 (Kotloff *et al.*, 2013).

In the United States, *Salmonella* and *Campylobacter* have been causing food borne disease since 1997 and *Shigella* is reported as the third highest (Lanata *et al.*, 2013). A study by Lee and Puthuchery (2002) on bacterial enteropathogens in childhood diarrhea in a Malaysian urban hospital showed that *Shigella spp* were the third most common bacteria responsible for childhood diarrhea. Members of the genus *Shigella*, namely *S. flexneri*, *S. dysenteriae*, *S. sonnei* and *S. boydii* are responsible for mortality and morbidity in children under five years, senior citizens, toddlers in day-care centres, homosexual men and people from war torn countries. *Shigella flexneri* and *Shigella dysenteriae* type 1 infections are usually characterized by frequent passage of small amounts of stool and mucus or blood. Watery stool followed by typical dysenteric stool maybe present with *S. dysenteriae* type 1 infection. *S. sonnei* and *S. boydii* infections are less severe with watery faeces but little mucus or blood (Steiner *et al.*, 2006). Shigellosis has been estimated to infect 164.7 million and of these, 163.2 million were in developing countries and the remaining in industrialized nations. Every year the mortality rate is approximately 0.7% (Lanata *et al.*, 2013). In Kenya, *Shigella flexneri* is the most commonly isolated (Boru *et al.*, 2010).

Campylobacter infections are among the most common bacterial infections in humans. They cause both diarrheal and systemic illnesses. Enteric *Campylobacter* infections cause inflammatory bloody diarrhea or dysentery syndrome. *Campylobacter jejuni* is usually the most common cause of community-acquired inflammatory enteritis causing watery diarrhea. *Campylobacter* infects over 2 million people each year (Persson *et al.*, 2005). It adheres and produces heat-labile enterotoxins that induce secretory diarrhea It then invades and proliferates within the intestinal epithelium, leading to cell damage and inflammatory response. Translocation of the organism into the intestinal mucosa and proliferation in the lamina propria and mesenteric lymph nodes leads to extra intestinal infections such as meningitis, cholecystitis, urinary tract infection, and mesenteric adenitis. *C. Jejuni* in patients who have predisposing factors causes bacteraemia that can lead to severe complications (Sabrina *et al.*, 2009).

In a study done on food borne infections among refugees in Eastleigh, Nairobi Kenya in 2013, 22% of the samples taken from children who died contained nontyphoidal *Salmonella*, 11% contained *Shigella*, 9% contained rotavirus, and 5% contained *Campylobacter*. Infants under 1 year who died were nearly seven times more likely to have nontyphoidal *Salmonella* in their stools and children under 5 years old who died were five and half times more likely to have nontyphoidal *Salmonella* in their stools. EPEC, *Salmonella* and *Campylobacter spp* were similarly frequent in children with watery diarrhea at 11.1%, 9.2% and 11.4% respectively (Boru *et al.*, 2010).

2.7 Antibiotic Resistance of enteric bacteria

Antibiotic resistance is the ability of a microbe to stop an antimicrobial drug to which it was previously sensitive (WHO, 2014). Drugs use for effective treatment of an ailment fails to work. There are three main ways by which resistance can occur: by natural resistance in certain types of bacteria, by genetic mutation or by one species acquiring resistance from another. Resistance can happen spontaneously owing to random mutations, to a build-up of resistance over time, due to misuse of antibiotics or antimicrobials (WHO, 2014). Studies show that multi-resistance to amoxicillin, co-trimoxazole and chloramphenicol has increased tremendously (Chris *et al.*, 2007). Emerging resistance to fluoroquinolones and extended-spectrum cephalosporins (ESCs) in Enterobacteriaceae has also been reported (Karambu *et al.*, 2013). This resistance is due to presence of resistant genes called Extended Spectrum Beta Lactamases are (ESBLs), which are enzymes produced in some gram-negative bacilli that mediate resistance to extended –spectrum Cephalosporins and aztreonam. These genes are then identified using PCR method. (Chris *et al.*, 2007).

2.8 Extended spectrum β -lactamase enzymes

Extended spectrum β -lactamase enzymes (ESBL) are modified β -lactamase enzymes mainly derived from TEM1/2, SHV-1 and CTX-M plasmid mediated enzymes, which hydrolyze expanded spectrum cephalosporin to varying degrees (Chris *et al.*, 2007). Resistance can develop in Enterobacteriaceae that produce ESBL enzymes which hydrolyze all cephalosporins, penicillin and monobactams and are inhibited by clavulanic acid, sulbactam, and tazobactam. Although ESBL have been detected in

most Gram-negative species, *E. coli* is one of the most frequently reported producers of these enzymes. *Escherichia coli* often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species (Laxminarayan *et al.*, 2013).

Unfortunately, ESBL producing organisms often possess resistance determinants to other important antibiotic groups, such as aminoglycosides and fluoroquinolones, leaving an extremely limited range of effective agents (Kariuki *et al.*, 2007). The production of types of ESBL, like CTX-M are more selective to cefotaxime than to other broad cephalosporins. CTX-M type have been increasingly detected previously in *E. coli* and in *Salmonella typhimurium* (Chris *et al.*, 2007).

Initially these bacteria contained a single ESBL gene, but later multiple ESBL genes are commonly present in a single strain, further complicating the process of detecting them and identifying an appropriate treatment regimen (Boyd *et al.*, 2004). Standard PCRs with amplicon sizing by gel electrophoresis are especially useful for identifying genes which encode antimicrobial resistance (Aranda *et al.*, 2004).

To date, TEM and SHV are the most common variants of ESBLs and more than 90 TEM-type and more than 25 SHV-type β lactamases have been identified. TEM-1 is responsible for up to 90% Ampicillin and penicillin resistance and SHV-1 β -lactamases are responsible for up to 20% of the plasmid-mediated Ampicillin resistance in *Enterobacteriaceae* class of bacteria (Karambu *et al.*, 2013). The emergence of OXA enzymes that can confer resistance to third generation cephalosporins and carbapenems, particularly in *Enterobacteriaceae* enzymes are important in ESBL analysis. These enzymes are numerous, and they mutate continuously in response to the much pressure of antibiotic use, leading to development of ESBL (Karambu *et al.*, 2013).

ESBLs genes of resistance have been detected in a variety of *Enterobacteriaceae*. Bacterial isolates that have a zone of inhibition of 27 mm and below for cefotaxime, a zone of inhibition of 22 mm and below for Ceftazidime and are resistant or intermediate to Ceftriaxone should be tested for production of ESBLs. It has been observed that while antibiotics revolutionized the treatment of infectious diseases in the 20th century, resistance threatens to render these drugs ineffective in the 21st

century. As soon as a new antimicrobial agent is discovered or synthesized, bacteria evolve mechanisms to overcome the effects of the new agent (Komolafe, 2003). Lack of awareness, resources and facilities to conduct ESBL identification contribute to the spread of multidrug resistance in most Enterobacteriaceae organisms (Ciara *et al.*, 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out at Mukuru Kwa Njenga, Municipal City Council and Mukuru Kwa Reuben health clinics, situated at the heart of Mukuru slums, 20 km east of Nairobi City (Figure 3.1).

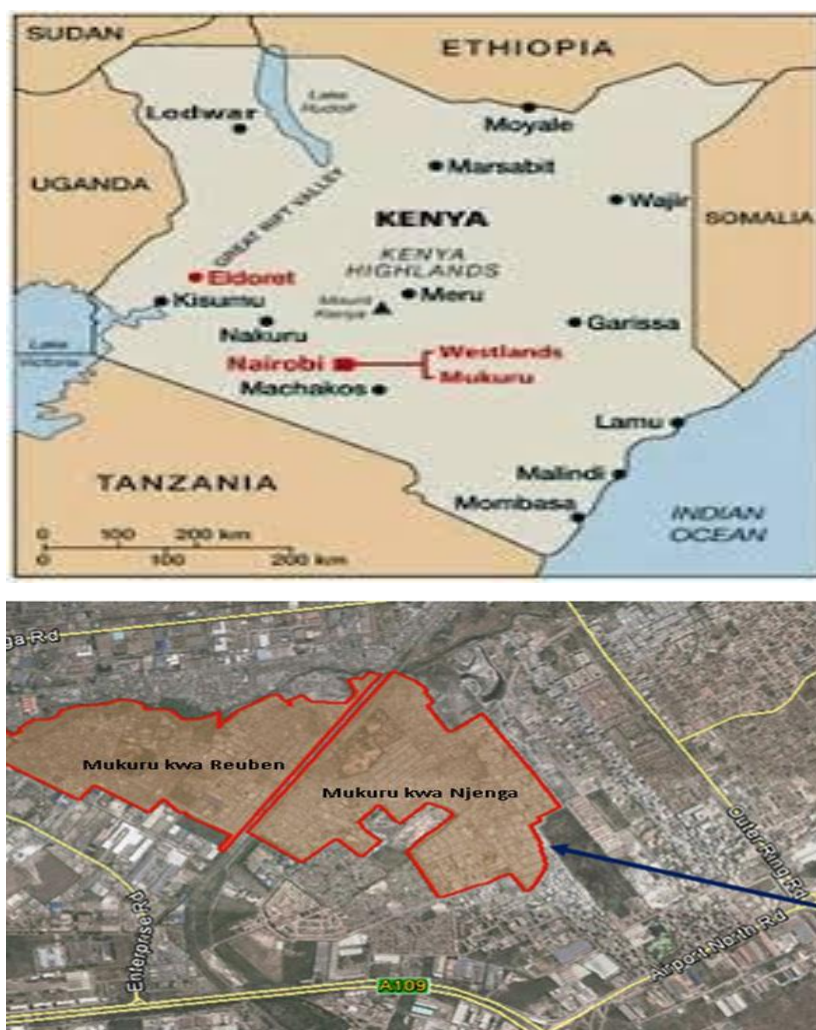


Figure 3.1: Maps showing location and the bird's eye view of Mukuru slum study site. (www.google.com/maps)

Mukuru slum is one of the largest slums with a population that exceeds 100,000. It is sub divided into eight sub villages. It has huge gigantic holes exposed as it was an old quarry. In addition, Mukuru residents are extremely poor and have 2-5 dependants. There is no formal drainage or sewer disposal system leading to many infections due to unhygienic environment. Due to congestion and dampness in structures in the informal settlement, other diseases such as Tuberculosis, malaria and pneumonia are common causes of death. The main health facilities that serve this population are Mukuru Kwa Njenga, Mukuru Kwa Reuben and Municipal City Council health clinics (www.rubencentre.org).

3.2 Study design

This was a lab based cross-sectional study to detect and characterize causative agents of diarrhea in children less than 5 years of age.

3.3 Study Population

Target population was children less than five years presenting with diarrhea at Mukuru Kwa Njenga, Mukuru Kwa Reuben and Municipal City Council health clinics.

3.3.1 Inclusion Criteria

- Children less than five years of age who were passing three or more loose or watery stools in the 24-h period prior to presentation attending Mukuru kwa Njenga, Mukuru kwa Reuben and Municipal City Council health clinics.
- Children whose parents gave consent to participate in the study.

3.3.2 Exclusion criteria

- Children who had already been under medication for the ongoing episode of diarrhea.

3.4 Sample size estimation

According to a study done among rural Maasai children under five years of age in the Maasai region of the Kenyan Rift Valley, it was established that the prevalence of enteropathogens co-infections in children with diarrhea was 13% (Joyce *et al.*, 1996). Therefore, the following formula was used to determine sample size (Charan *et al.*, 2013);

$$n = Z^2 \times p \times q / d^2$$

Where:

n = Desired sample size (if target population is greater than 10,000)

Z = Standard normal deviation at the required confidence interval

$$(1.96)$$

p = proportion in the target population estimated to have measured character (0.13)

$$q = 1 - p$$

d = level of statistical significance at 95% confidence level = 0.05

Therefore;

$$= (1.96)^2 \times 0.13 \times (1-0.13)/0.05^2$$

$$= 3.8416 \times 0.13 \times 0.87/0.0025$$

$$= 3.8416 \times 0.13 \times 348$$

= 173.793984, rounded off 174 samples were used

3.5 Sampling criteria

To achieve the sample size required, the study chose to perform homogenous purposive sampling consecutively from each of the three outpatient clinics. The sampling was performed from 8 am up to 1 pm to allow transportation and processing of samples in due time. The attending physician was requested to refer to the investigator the first 10 patients, who were below 5 years of age and who were passing loose, watery, mucoid or bloody stools in the 24-hr period prior to presentation. The patient should not have been under medication for the ongoing diarrheal episode. Explanation of the study was done, and consent was sought from the guardian of the patient. The guardian was then requested to consent for participation of the child by signing the requisite consent form.

3.6 Sample collection and transportation

For children whose guardian gave consent, patients details were recorded in a notebook and given a unique identifier number, which was given to the guardian in case of any need for follow-up. A fecal container or rectal swabs were labeled with the unique identifier number and fecal containers given to the patients for collection of stool sample. The investigator collected two rectal swabs from young children who couldn't produce a stool sample on request. One set was placed in Cary Blair transport medium for bacteriology analysis. Rectal swabs were also prepared from the stool samples and placed in Cary Blair transport medium. All samples were transported daily in a cooler box to the Kenya Medical Research Institute, (KEMRI) Centre for Microbiology Research (CMR), Nairobi.

3.7 Laboratory processing

3.7.1 Macroscopic examination of stool samples

Diarrheal samples collected from these hospitals were processed at the Kenya Medical Research Institute, (KEMRI) Centre for Microbiology Research (CMR), Nairobi. Samples were macroscopically examined for consistency, colour, presence of blood and mucus and recorded in a notebook.

3.7.2 Microscopy for examination of parasites in stool samples.

For these procedures, 100 fields were examined for each sample, initially by using a low-power (10x) objective and then again by using a high-power (40x) objective of the compound microscope. To confirm internal morphology, 100× oil immersion objective lens was used. Fresh stool specimens were examined directly for vegetative forms of parasites. Normal saline wet mounts and iodine wet mounts were prepared separately. A small volume of stool sample was mixed with a drop of physiological saline on a glass slide. A cover slip was placed over the specimen and examined under a microscope (Alum *et al.*, 2010).

Faecal concentration was done by formalin-ethyl acetate (FEA) technique to allow detection of organisms that may have been missed by using a direct wet smear. First, 10% Formalin was added to the specimens achieving a range of formalin to stool ratio of 3:1. The specimens were stirred and sieved using a gauze into a small beaker. 7 ml of filtrate were transferred into 15ml tube. 3 ml ethyl acetate was added to the mixture. The tubes were capped and shaken for 30 seconds. They were then centrifuged at 500 RPM for 3 minutes. The ether layer, debris layer and formol-saline layers were discarded to leave the sediment. The sediment was re-suspended and disposable pipettes used to draw the sediment from the tube and placed on two slides. On one slide, a drop of lugol's iodine (diluted in 1: 5 distilled water) was added to the wet preparation (Levine *et al.*, 1983). A cover slip was placed on the wet prep and observed under a microscope.

Modified acid-fast staining procedure was used for identification of oocysts of the coccidian species, including those of *Cryptosporidium* in concentrated Stools (Roka *et al.*, 2013). The second slide with concentrated stool was allowed to dry and then fixed with absolute methanol for 30 seconds. It was then stained with Kinyoun's carbol fuchsin for 3 minutes and rinsed briefly with distilled water and drained. Acid alcohol was used to destain the smear for dropwise until purple colour stopped running off the slide and then rinsed with distilled water. The smear was counterstained with malachite green for 1 minute and rinsed briefly with distilled water. The slide was dried, mounted with a coverslip and observed under a

microscope. *Cryptosporidium spp* stained a pinkish-red colour and the background stained uniformly green. Recording was done on a note book, awaiting data processing.

3.7.3 Culture of the samples

Rectal swabs were inoculated in to enrichment selenite F media and incubated at 37⁰C for 24 hours. All media used in processing was prepared ahead of time as per manufacturer's instructions with a mean thickness of the medium in the plate at 4.0 mm. After incubation, all sub-cultures for bacterial isolation were plated on MacConkey media and Xylose Lysine Deoxycholate agar (XLD) (Oxoid, Hampshire, United Kingdom) and incubated at 37⁰C for 24 hours to differentiate lactose fermenters from non- lactose fermenters by cultural characteristics of colour. Cream colonies on MacConkey media were indicative of non- lactose fermenters, characteristic of enteric pathogens (Laupland *et al.*, 2013). *E. coli* are lactose fermenters but were of interest in the study since some of them are pathogenic. All were selected and further processed as they were the most likely cause of diarrhea. *Salmonella* isolates were confirmed on XLD media. *Salmonella* species metabolise thiosulfate to produce hydrogen sulphide hence had black centres. These suspect colonies were purified on Mueller Hinton agar and incubated at 37⁰C for 24 hours (Laupland *et al.*, 2013).

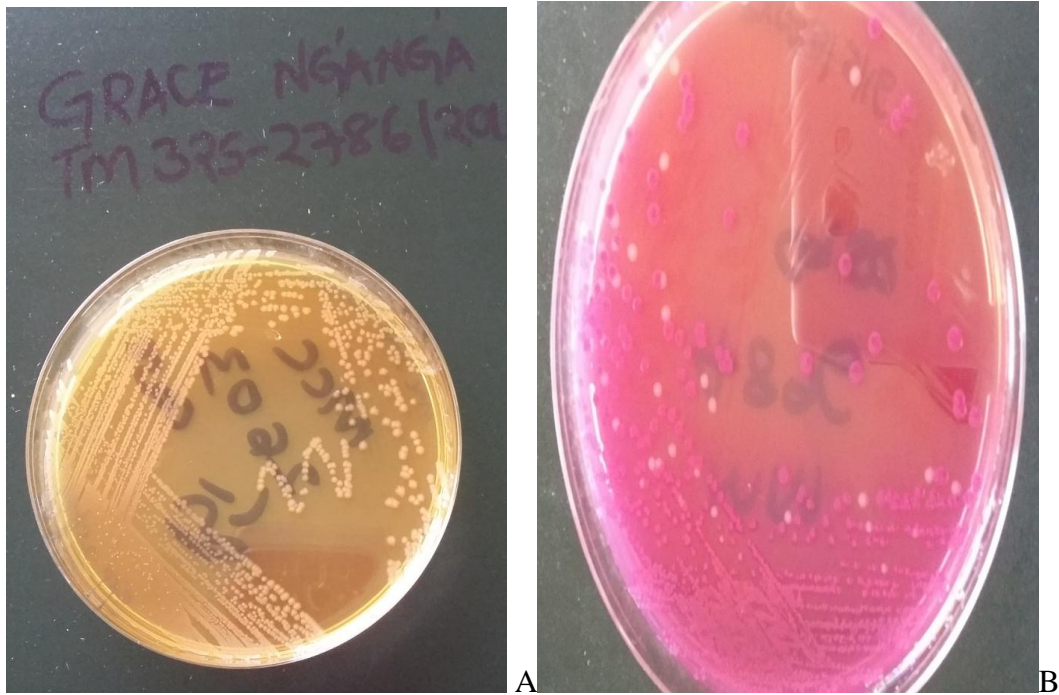


Figure 3.2: Cultures of stool samples on MacConkey media showing non-lactose fermenters in A and in B, a mixture of lactose fermenters (Pink colonies) and non-lactose fermenters (cream colonies).

3.7.4 Biochemical tests

All non-lactose fermenters and selected lactose fermenters were cultured on Simmon's Citrate Agar, Urease media, Triple Sugar Iron (TSI) and Sulphur Indole Motility Media (SIM) and incubated at 37⁰C for 18-24 hours. For isolates not readily identifiable by the first method, biochemical tests were repeated on API20E kits (biomerieux, Boston, USA). These isolates were also purified on Mueller Hinton Media for further processing like pathotyping and serotyping. All samples were incubated at 37⁰c for 24 hours. Recording was done in a notebook. After identification, the isolates were stocked in Tryptose Soy Broth, labelled and frozen under -80⁰C for future analysis.

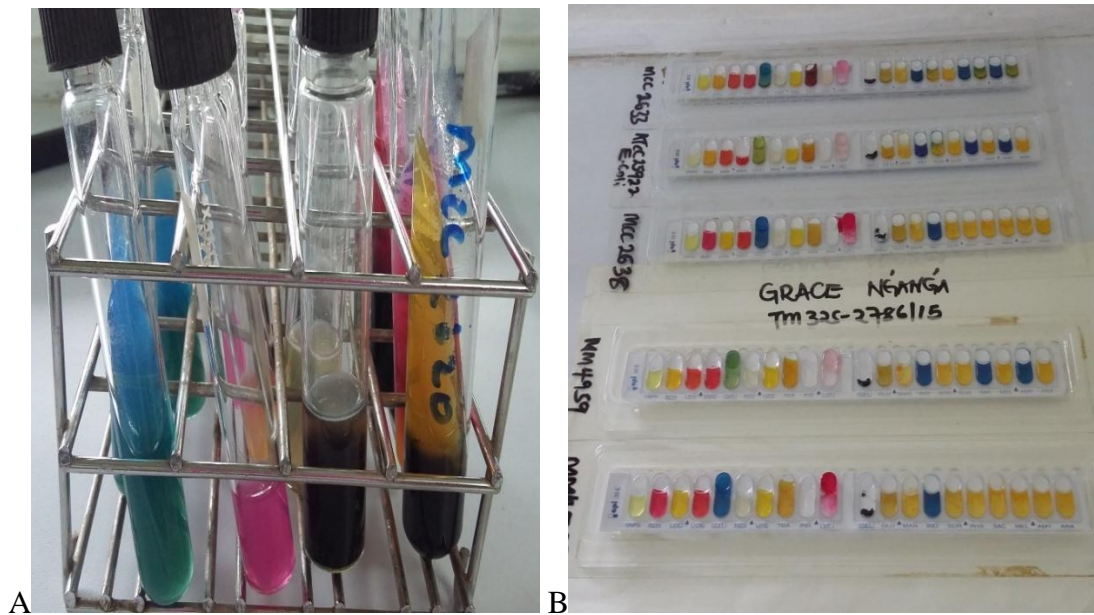


Figure 3.3: Biotyping of isolates using biochemical media in A and API kits in B above.

3.7.5 Serotyping of isolates

Serotyping was done for isolates identified from biochemical testing, using commercial antisera (BD diagnostics, Ontario, Canada). Serotyping was done on isolates not identifiable to species level using biochemical media or API kits. Two drops of the antisera were placed on a microscope slide. A sterile wire loop was used to place each isolate on the antisera and mixed thoroughly. If agglutination occurred the isolate was confirmed as the strain which the antisera is against. Positive isolates agglutinated with antisera used. Recording was done adjacent to previous results to await data processing.



Figure 3.4: Serotyping of isolates showing agglutination in the first well indicating a positive serotype and a negative serotype in the second well without agglutination.

3.7.6 Pathotyping of *E. coli* isolates

A loopful of *E. coli* solid cultures were suspended in 250 μ l of PBS saline solution in a sterile Eppendorf tube. It was dissolved by gently shaking the tube. The solution was boiled for 15 minutes at 90⁰C on a heating block. The tubes were centrifuged for 13 minutes at 13000 rpm. The supernatant containing the DNA was transferred to a clean tube (Aranda *et al.*, 2004). 5 μ l of the DNA was used as a template and probed by use of PCR method which permits amplification of target DNA for pathotypes of *E. coli*. The PCR conditions were 30 cycles of amplification at a denaturation temperature of 95⁰C for 1 min, at an annealing temperature of 55⁰C for 1 min and an extension temperature of 72⁰C for 3 min. This step was followed by a final extension at 72⁰C for 10 min. Specific sequences unique to different biotypes of *E. coli* were used to detect *eae*, *stx*, *eaf*, *ipaH*, *AggR*, *pcvd*, *stp* and *aspu* genes (Table 3.1).

Table 3.1: *Escherichia coli* PCR primer sequences, Standard HPSF Quality at 0.20 $\mu\text{m}/\text{ol}$ scale (Nataro *et al.*, 2008)

Group	Target	Primer Sequence (5' to 3')
EIEC	<i>ipaH</i> 600bp	F: GTT CCT TGA CCG CCT TTC CGA TAC CGT C R: GCC GGT CAG CCA CCC TCT GAG AGT AC (Nataro <i>et al.</i> , 2008).
EPEC	<i>eaf</i>	F: CAG GGT AAA AGA AAG ATG ATA A R: TAT GGG GAC CAT GTA TTA TCA
EPEC	<i>eae</i> 881bp	F: CTG AAC GGC GAT TAC GCG AA R: CCA GAC GAT ACG ATC CAG (Nataro <i>et al.</i> , 2008).
EAEC	<i>pcvd</i> 432bp	F: CTG GCG AAA GAC TGT ATC AT R: CAA TGT ATA GAA ATC CGC TGT T (Nataro <i>et al.</i> , 2008).
	<i>AggR</i> 254bp	F: GCT GGG CAG CAA ACT GAT AAC TCT R: CAT CAA GCT GTT TGT TCG TCC GCC G (Nataro <i>et al.</i> , 2008).
	<i>aspu</i> 282bp	F: CTT TCC CCT CTT TTA GTC AGG R: ATA ACA TCC AGC ACA GGC AGG (Nataro <i>et al.</i> , 2008).
EHEC	<i>stx-1</i> 518bp	F: AGCTGCAAGTGCGGGTCTG R: TACGGGTTATGCCTGCAAGTTCAC (Nataro <i>et al.</i> , 2008).
ETEC	<i>stp</i>	F: TCTGTATTATCTTTCCCTC R: ATAACATCCAGCACAGGC

3.7.7 Antibiotic Susceptibility testing procedure

Antibiotic susceptibility testing was performed using Kirby – Bauer disc diffusion technique (James *et al.*, 2003). The antibiotics used were ampicillin 10 µg, tetracycline 30 µg, gentamicin 10 µg, sulphamethoxazole 100 µg, chloramphenicol 30 µg, cefuroxime sodium 30 µg, ceftazidime 30 µg, ceftriaxone 30 µg, cefotaxime 30 µg, ciprofloxacin 5 µg and nalidixic acid 30 µg, cefpodoxime 10 µg, kanamycin 30 µg. The antibiotics used were chosen based on their use in the management of enteric bacterial infections (Karambu *et al.*, 2013). The inoculums from purity plate for susceptibility testing were emulsified in normal saline and suspensions compared with McFarland 0.5 turbidity standard. *E. coli* ATCC 25922 strain was used as the test standard.

Using swabs, uniform spreading was done on Mueller-Hinton agar plates to form a bacterial lawn then allowed to dry for 3 minutes. An antibiotic disc dispenser was used to dispense discs containing specific antibiotics onto the plate. Flame-sterilized forceps were used to gently press each disc onto the agar surface (Bonev *et al.*, 2008). Plates were then incubated at 37 °C for 24 hours. After incubation, zones of inhibitions were measured, and interpretation of results was according to WHONET data analysis software. All data was recorded in a notebook and Microsoft Excel to await data processing.

3.7.8 Phenotypic confirmatory disk diffusion test for ESBL production

Bacterial suspensions were made by emulsifying bacterial cultures in normal saline and compared with McFarland 0.5 turbidity standard. *E. coli* ATCC 25922 strain was used as the test standard. The suspensions were spread evenly over entire plate surface of MHA agar plates and allowed to dry for 5 mins before applying antibiotic discs. All isolates were evenly inoculated on Mueller Hinton Agar plates (MHA). Negative (*E. coli* ATCC 25922) control strains were inoculated on separate plates. The confirmatory procedure required a Ceftazidime 30 mg disc, a Ceftazidime-Clavulanate disc, a Cefotaxime 30 mg disc and a Cefotaxime-Clavulanate discs which were then placed on the MHA plates. After 18 hours incubation at 37 °C, the zone around each disc was measured. ESBL production was inferred when the zone of

inhibition around the Ceftazidime –Clavulanate or Cefotaxime-Clavulanate discs were expanded by > 5mm compared to the respective Ceftazidime or Cefotaxime discs alone (Rawat & Nair, 2010).

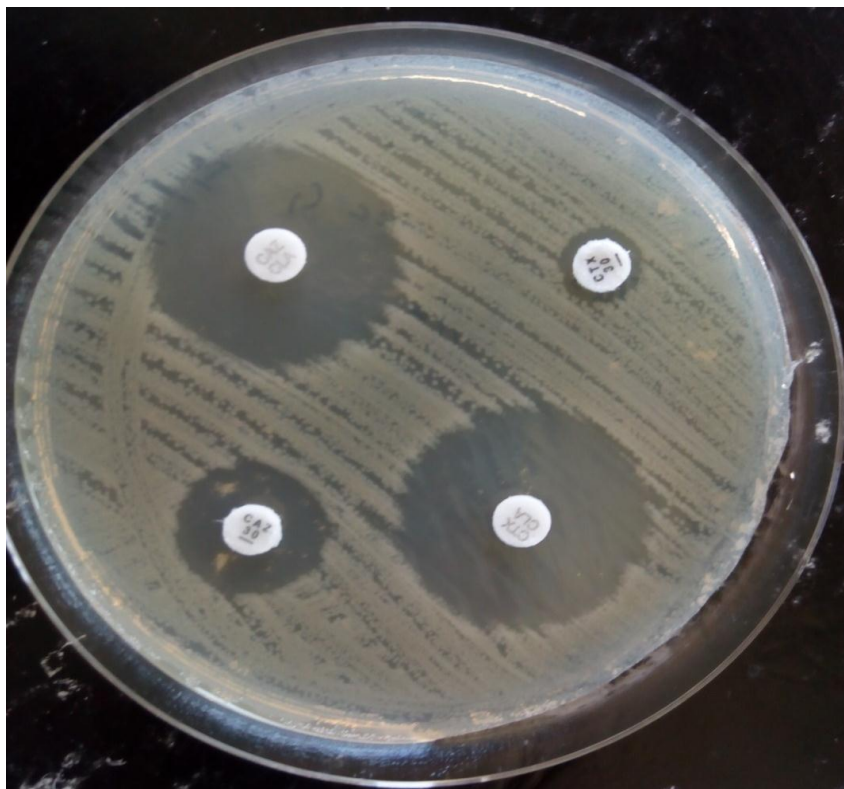


Figure 3.5: Phenotypic confirmatory disk diffusion test for ESBL production from *Escherichia coli* strain.

3.7.9 Determination of ESBL genes in bacteria pathogens isolated from cases of diarrhea

ESBL gene detection was done on all bacterial pathogens isolated. Using PCR method, screening was done for ^{bla}*OXA-48*, ^{bla}*SHV-1*, ^{bla}*CTX-M-15*, and ^{bla}*TEM-1* genes. A loopful of solid cultures were suspended in 250 ul of phosphate-buffered saline solution in a sterile Eppendorf tube and dissolved by gently shaking the tube. The solution was boiled for 15 minutes at 90⁰C on a heating block. The tubes were centrifuged for 13 minutes at 13000 rpm. The supernatant containing the DNA was

transferred to a clean tube (Aranda *et al.*, 2004). Isolated DNA was probed using primer sets (Table 3.3). The PCR conditions were 30 cycles of amplification at a denaturation temperature of 95°C for 1 min, at an annealing temperature of 55°C for 1 min and an extension temperature of 72°C for 3 min. This step was followed by a final extension at 72°C for 10 min (Table 3.2).

Table 3.2: PCR isolation process for enteric bacteria DNA (Kiratisin *et al.*, 2008)

Gene	TEM	SHV gene	CTX-M gene	Cycles
<i>Initial denaturation</i>	95 °C, 5 min	95 °C, 5 min	95 °C, 5 min	1
<i>Denaturation</i>	94 °C, 1 min	94 °C, 1 min	94 °C, 1 min	35-40
<i>Annealing</i>	50 °C, 30 secs	50 °C, 30 secs	60 °C, 30 secs	
<i>Extension</i>	72 °C, 2 min	72 °C, 1.5 min	72 °C, 1.5 min	
<i>Final extension</i>	72 °C, 10 min	72 °C, 10 min	72 °C, 10 min	1
<i>Storage</i>	10 °C, forever	10 °C, forever	10 °C, forever	NA

3.7.10 Gel electrophoresis

PCR products were used as templates and 5µl DNA was used for each analysis. Specific primers were used to amplify genes of antibiotic resistance (Table 3.3). The extracts were mixed with the loading dye and carefully loaded into the wells prepared in 1.5% agarose gel. In each gel a 100 bp ladder was included as a size marker. The amplified PCR products were subjected to electrophoresis on agarose gel in 0.5 X TBE buffer at 100 volts direct current for 1 hour. Thereafter the gel was stained with ethidium bromide. Photography of the gel was done by ultraviolet illumination using a transmitted illumination camera fitted with a polaroid film (Brody & Kern, 2004).

Table 3.3: Nucleotide sequences of PCR primers to be used to amplify genes of antibiotic resistance (Kiratisin *et al.*, 2008)

Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference (s)
<i>bla</i> _{TEM-1}	F=TCCGCTCATGAGACAATAACC R=TTGGTCTGACAGTTACCAATGC	931bp	(Kiratisin <i>et al.</i> ,2008)
<i>bla</i> _{SHV-1}	F= TGGTTATGCGTTATATTCGCC R= GGTTAGCGTTGCCAGTGCT	868bp	(Kiratisin <i>et al.</i> ,2008)
<i>bla</i> _{CTX-M-15}	F=TCTTCCAGAATAAGGAATCCC R=CCGTTTCCGCTATTACAAAC	593bp	(Kiratisin <i>et al.</i> ,2008)
<i>bla</i> _{OXA-48}	F=5' ATGTGCAGYACCAGTAARGTKATGGC 3' R=5' GTGTGTTTAGAATGGTGATCGCATT 3'	920bp	(Kiratisin <i>et al.</i> ,2008)

3.8 Data management and analysis

Data was recorded in lab work books and later entered in Microsoft excel software and saved in a hard disk drive. Data was analyzed using WHONET Inc. (Chicago). The output of the analysis was presented in form of frequency tables, bar charts and gel photographs. Prevalence of resistant strains was calculated from the frequency of isolation using the relative Frequency formula $\% = (f / n) \times 100$ where 'f' is the number of times the phenomenon being tested occurred and 'n' is the total number of tests. Antibiotic susceptibility patterns were presented in histograms. All results were interpreted at 95% CI.

3.9 Dissemination of findings

This study results were presented before a scientific panel in Jomo Kenyatta University of Agriculture and Technology. It was also published in the Imperial Journal of Interdisciplinary Research Vol- **3, Issue-4 (2017)** of IJIR. The following are the URLs for the Issue and published article. **Vol-3, Issue-4: <http://www.onlinejournal.in/v3i42017/> Published Article: <http://www. Online journal. in/IJIRV3I4/052.pdf> ISSN: 2454-1362.**

3.10 Ethical considerations and expected benefits of research

This study was done in accordance with the ethical standards of Kenyatta University Ethical Review committee (KU-ERC) on human experimentation from whom ethical approval for this study was obtained. Consent to participate in the study was sought from parent or guardian of study participants. The data generated from this study has provided a platform for advocacy for evidence-based results which can guide in diagnosis and management of childhood diarrhea. This will reduce Prevalence of Antimicrobial Resistance (AMR) hence reduce cost of hospitalization by ensuring that only effective drugs are prescribed. All study procedures used in this study are cost effective and can easily be emulated in low resource clinics.

CHAPTER FOUR

RESULTS

4.1 Demographic profiles of the study participants

A total number of 89 (51%) males and 85 (49%) females who were all under 5 years of age and suffering from diarrhea were recruited for the study (Figure 4.1). Samples distribution was 76 (44%), 59 (34%) and 39 (22%) from Mukuru Kwa Njenga, Municipal City Council and Mukuru Kwa Reuben health clinics respectively.

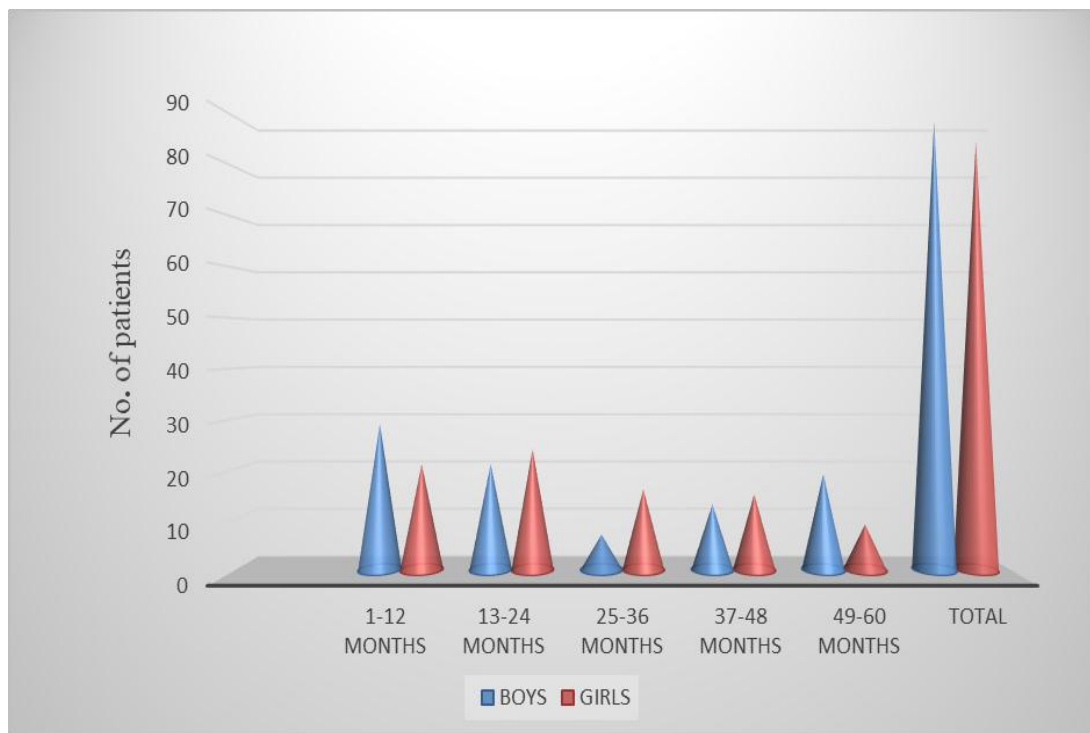


Figure 4.1: Demographic data of children under 5 years reporting with diarrhea at Mukuru kwa Njenga health clinics. Majority of the participants (26%) were under 1 year of age.

4.2 Bacteria species isolated from children under 5 years of age with diarrhea

Of the 174 samples collected, *E. coli* was the most isolated organism in 98 (47.3%) of cases. Other bacterial species were also isolated from the samples (Table 4.1).

Table 4.1: Enteric bacteria isolated from children less than 5 years presenting with diarrhea.

Isolated organism	Number of isolates	Percentage prevalence
<i>Escherichia coli</i>	98	47.3%
<i>Salmonella spp</i>	12	5.8%
<i>Serratia spp</i>	3	1.4%
<i>Aeromonas sobria</i>	2	1.0%
<i>Providencia spp</i>	1	0.5%
<i>Proteus spp</i>	13	6.3%
<i>Klebsiella spp</i>	38	18.4%
<i>Citobacter spp</i>	32	15.5%
<i>Enterobacter spp</i>	6	2.9%
<i>Morganella morganii</i>	1	0.5%
<i>Pseudomonas spp</i>	1	0.5%

4.2.1 Pathotyping and serotyping of isolates

PCR was used to identify pathogenic *E. coli* isolates. Of the 98 *E. coli* (47.3%) isolated, 13 (13.3%) were pathogenic. Enteroaggregative *E. coli* (EAEC) was found in 9 cases and accounted for 9.2 %. Of these, *AggR* gene was found in 5 (5.1%) isolates. *aspu* gene, which also codes for EAEC, was found in 4 (4.1%) isolates (Figure 4.2). *pcvd* gene was not detected. Enteropathogenic *E. coli* (EPEC) - *eae* gene- was found in 4 (4.1%) cases. *eaf* gene also coding for EPEC was not detected. Three *E. coli* isolates from the 3 different clinics were found to have two virulent genes in the same isolate; *AggR* and *aspu* both coding for Enteroaggregative *E. coli* (EAEC).

A total of 12 (5.8%) *Salmonella* isolates were identified. Serotyping of *Salmonella* isolates was done by use of commercial antisera. This identified 4 cases (33.3%) of *Salmonella enteritidis*, 5 cases (41.7%) of *Salmonella typhimurium* and 3 cases (25%) of *Salmonella typhi* (Figure 4.3).

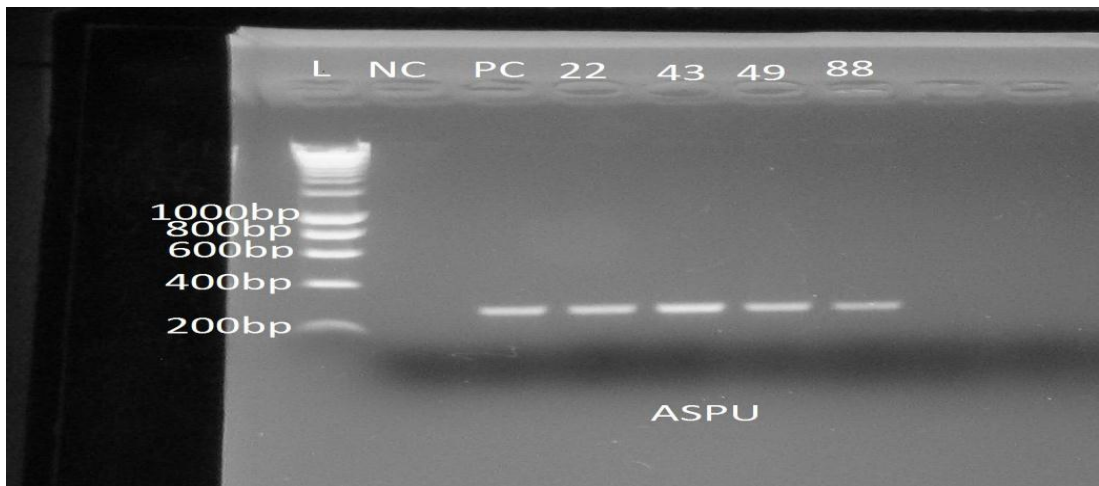


Figure 4.2: Gel electrophoresis showing *aspu* genes which code for EAEC, 280 base pairs. PC: Positive Control, NC: Negative Control, L: Ladder (molecular size marker).

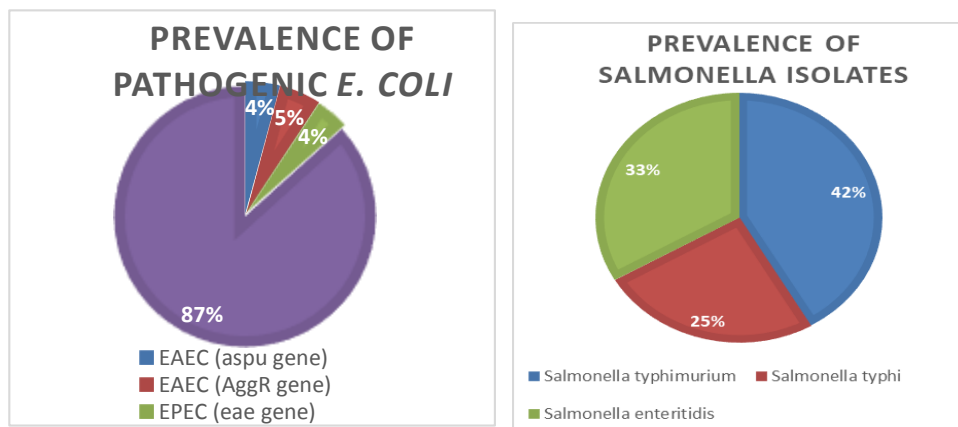


Figure 4.3: Percentage occurrence of pathogenic *E. coli* and *Salmonella* serotypes in children with diarrhea.

4.3 Parasites isolated from children with diarrhea

Entamoeba coli and *Entamoeba histolytica* were isolated together in 2 cases, both in 37-48 months age group (Figure 4.4). *Cryptosporidium parvum* was isolated from one sample. No other ova or cysts were isolated.

4.4 Association of pathogen type with age of children suffering from diarrhea

Salmonella isolates were highest in children less than 12 months having 8 cases accounting for 66.6 % of all *Salmonella* isolates compared to only 4 cases for all other age groups. Pathogenic *E. coli* isolates were found in every age group as opposed to other pathogens, with a peak in the 3rd year of life. All *Entamoeba* were detected in the 4-year-old children and only 1 case of *Cryptosporidium parvum* in a 2-year-old was isolated (Figure 4.4).

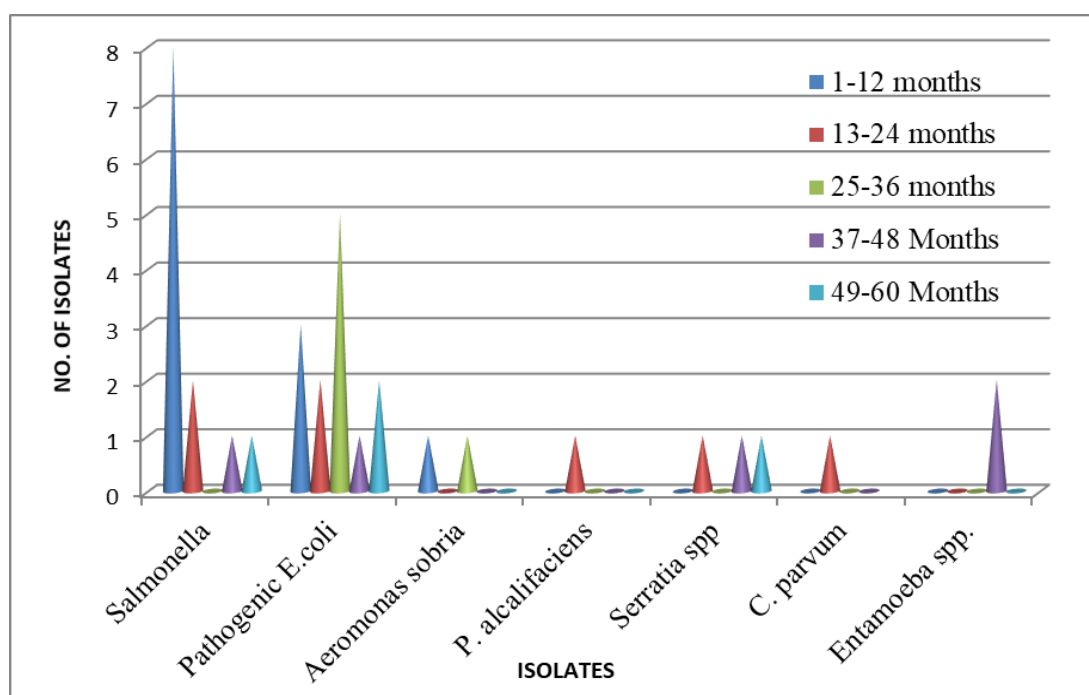


Figure 4.4: Organisms isolated in association with the age group of the patients with diarrhea.

4.5 Rate of co-infection and co-infestation found in children under 5 years with diarrhea

In this study, there were only 4 cases of co-infection and co-infestation making the prevalence 2.29%. Prevalence was calculated using the relative Frequency formula $\% = (f / n) \times 100$. *Salmonella spp* was the common organism in co-infection cases in children less than 5 years of age. In 3 cases of positive Enteroaggregative *E. coli* isolation, the isolates had both *AggR* gene and *aspu* genes in their genomes. One of these isolates also possessed the *bla_{oxa}* gene of resistance. *Entamoeba coli*, *Entamoeba histolytica* and *Salmonella typhi* were seen to co-infect in 1 case.

4.6 Antimicrobial sensitivity profiles of bacterial pathogens isolated from the cases of childhood diarrhea

Prevalence of Multi Drug Resistance (MDR) characterized by presence of ESBL production was at 17% and was a major factor in pathogens associated with diarrhea in children that were studied. There was a high level of resistance to antibiotics, highest resistance being to cefuroxime at 70% and sulphamethoxazole at 64% resistance. For the quinolones, Nalidixic acid resistance was at 28% and Fluoroquinolone (ciprofloxacin) resistance was at 18%. The least resistance was to aminoglycosides (kanamycin (8%) and gentamycin (10%). Prevalence of resistance to other antimicrobials is shown in figure 4.5.

Resistant

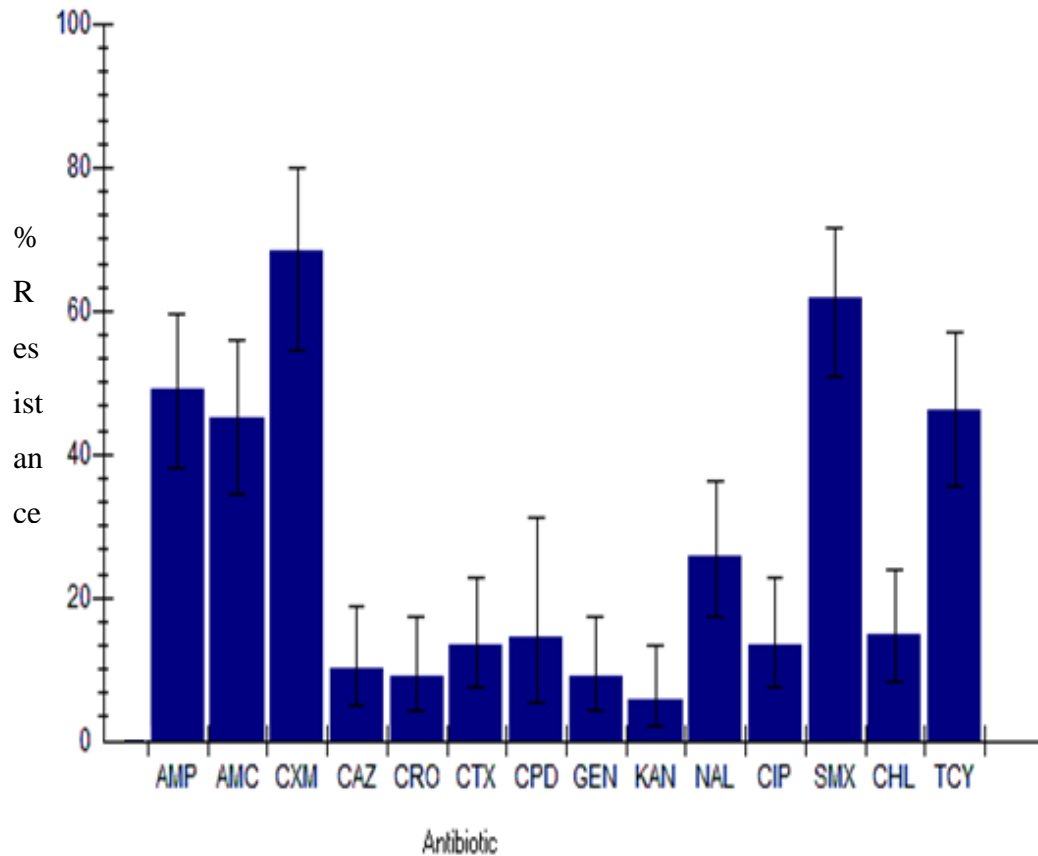


Figure 4.5: Percentage resistance of all isolates recovered from children with diarrhea against given antibiotics.

- AMP-Ampicillin, AMC-Amoxicillin-Clavulanic acid, CXM-Cefuroxime, CAZ-Ceftazidime CRO- Ceftriaxone, CTX-Cefotaxime CPD- Cefpodoxime GEN-Gentamicin, KAN-Kanamycin, NAL-Nalidixic acid, CIP-Ciprofloxacin, SMX-Sulfamethoxazole, CHL-Chloramphenicol, TCY-Tetracycline.

4.7 Extended Spectrum Beta Lactamase (ESBL) genes found in pathogens isolated from children with diarrhea

ESBL was screened for all pathogens identified as ESBL producers from AST data. All were *E. coli* isolates. Overall prevalence of ESBL carriage was at 10.1%. PCR screening showed that the *bla**TEM-1* gene of resistance had the highest prevalence being found in 8 isolates at 3.9%. *bla**OXA-48* was found in 6 isolates accounting for 2.9% of all isolates. *bla**CTX-M-15* gene was found in 4 isolates and accounts for 1.9% of the cases (Figure 4.7). *bla**SHV-1* gene was found in 3 isolates and accounted for 1.4% of all isolates (Figure 4.6).

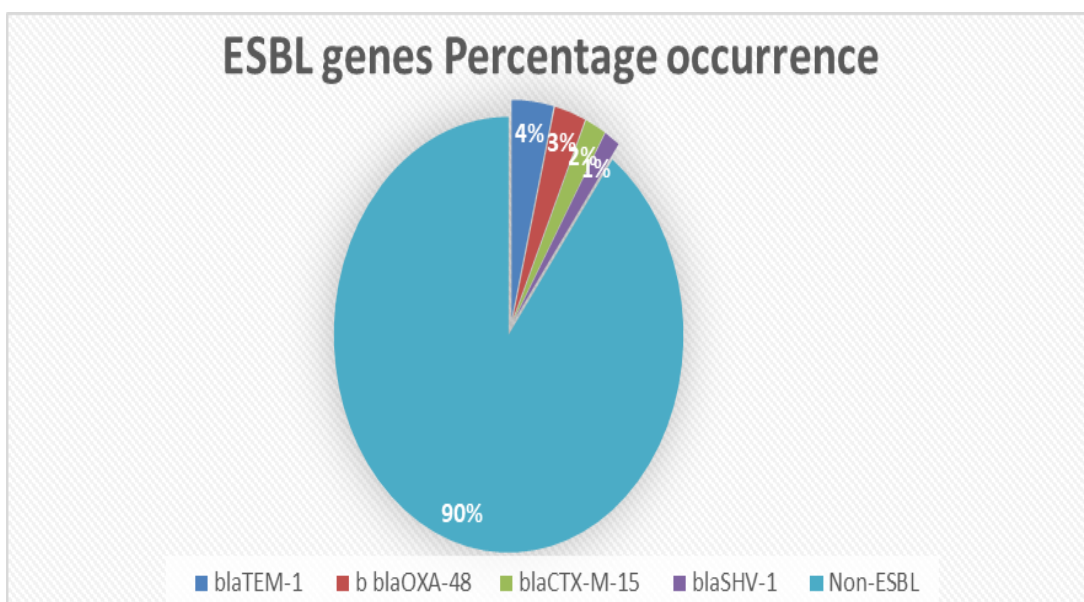


Figure 4.6: Percentage occurrence of resistance genes isolated from pathogens causing diarrhea in children below 5 years.

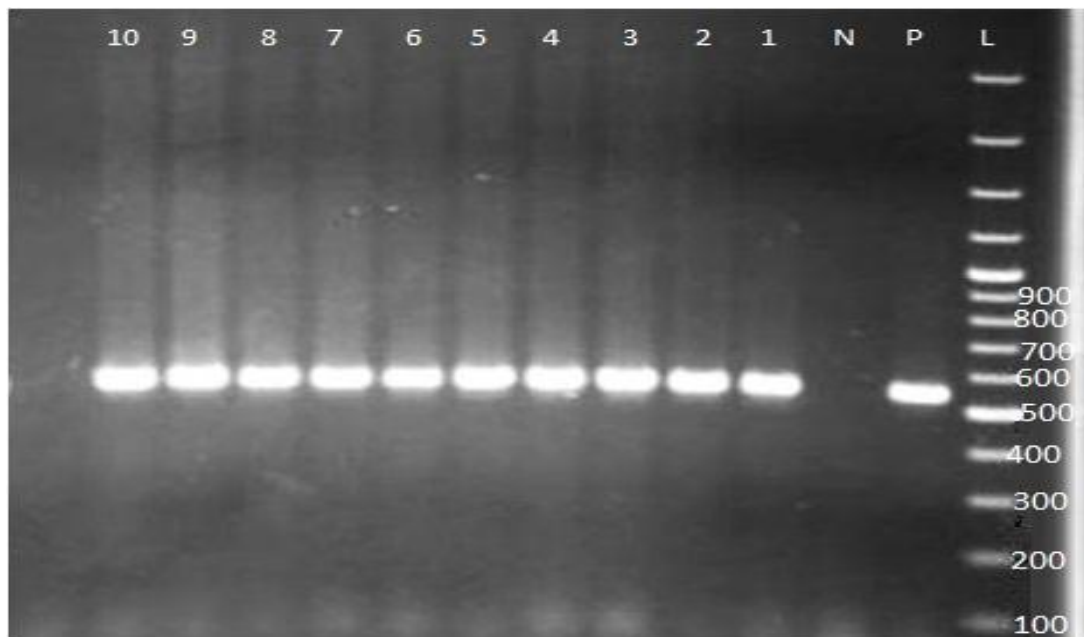


Figure 4.7: Gel electrophoresis showing *bla*_{CTX-M-15} ESBL gene, 593 base pairs. P: Positive Control, N: Negative Control, L: Ladder (molecular size marker).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

In this study, *E. coli*, *Klebsiella spp* and *Citrobacter spp* were the most prevalent isolates. The genera *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, and *Citrobacter* are collectively known as the coliform bacilli. Many species including *Proteus spp* are members of the normal intestinal flora even in children. Coliforms and *Proteus* species rarely cause extraintestinal disease unless host defences are compromised. Disruption of the normal intestinal flora by antibiotic therapy may allow resistant nosocomial strains to colonize or overgrow. They are opportunistic pathogens and cause nosocomial infections in the urinary tract, surgical sites, bloodstream and some pneumonias (Guentzel, 1996).

Pathogenic *E. coli* was the leading pathogen from cases of diarrhea and *Salmonella* species came second. This agrees with a previous etiology and epidemiology of diarrhea study which obtained prevalence data from pooled data worldwide. The study reported that the most common etiologic agents of diarrhea in children in decreasing order of prevalence as: rotavirus, Pathogenic *E. coli*, *shigella*, *campylobacter*, and non-typhoidal *Salmonella* (Carlos *et al.*, 1990). Pathogenic *E. coli* are hard to detect using traditional culture methods let alone in poor resource clinics. These may go undetected except by use of PCR and can be assumed to be a source of recurring diarrheal episodes and transmission of resistant genes. This study found three *E. coli* isolates from the 3 different clinics to have two virulent genes in the same genome; *AggR* and *aspu* genes both coding for Enteroaggregative *E. coli* (EAEC). Initially these bacteria contained a single virulent gene, but later multiple virulent genes are commonly present in a single strain, further complicating the process of detecting them and identifying an appropriate treatment regimen (Boyd *et al.*, 2004). In a previous study by Bekal *et al.*, (2003) on rapid identification of *Escherichia coli* pathotypes by virulence gene detection with DNA microarrays, Virulence factors belonging to two different pathotypes were detected in one human *E. coli* isolate (strain H87-5406). The isolates in that study had sequences that

were closely related to gene sequences of an enterohemorrhagic strain (EDL933), a human enteropathogenic strain (E2348/69), or an animal enteropathogenic (RDEC-1) strain.

Salmonella spp was more prevalent in children less than 1 year compared to other pathogens. For instance, there were 8 cases in 1-year old children compared to only 4 cases in children 2-5-year olds. Moreover, from demographic data, this age group had the highest number of patients visiting these clinics due to diarrhea as opposed to the other age groups. 80% of deaths due to diarrhea occur in the first two years of life (Carlos *et al.*, 1990). These results correlate well with a study done in 2015 in Mukuru study site which concluded that at a younger age of below 5 years, the immune system is still developing and pathogenic bacteria such as *Salmonella spp* will cause more infections than in older age groups (Kariuki *et al.*, 2010). Fewer infectious particles are required to cause infection in this age group.

Three species of parasites were isolated, *Entamoeba coli* and *Entamoeba histolytica* *Cryptosporidium parvum* at age 2. Studies in the United States have documented that cryptosporidiosis is usually low at 4% of stools sent for parasitological examination (White *et al.*, 2015). *Cryptosporidium* can be difficult to diagnose and usually is missed unless specific tests are performed for detection of *Cryptosporidium* species specific antigens. Together, all the parasites accounted for 1.7% of all cases. In a previous study in the Mukuru study site over a period of two years (2010-2011), 25.6% of children below 5 years with diarrhea were positive for at least one intestinal parasite (Mbae *et al.*, 2013). This study collected samples over a 4-month period and this could be a major limitation that can contribute to the variance in isolation rates. Rainy seasons also tend to give a rise to high infection rates than the dry season when the study was done (Patz *et al.*, 2003). However, this study findings agree with Gatei *et al.* (2006) where *Cryptosporidium parvum*, *Entamoeba* species and *Giardia lamblia* were the most common parasites isolated in Kenya. These were the same ones isolated in this study except for *Giardia Lamblia*. However, these numbers likely underrepresent the true number of infections, owing to the poor sensitivity of traditionally used techniques employed in this study (Laupland *et al.*, 2013).

This study found a co-infection and infestation prevalence of 2.29%. *Entamoeba spp* was found in the same cases with *Salmonella spp* and EAEC making co-infection a possible etiology in causing recurrence of diarrhea in this study setting. A previous study by Joyce *et al.* (1996) reported a co- infection of 13% in Kenyan rural Maasai children. The variance in prevalence data could be attributed to use of multiplex PCR to detect presence of enteropathogens in the reference study. PCR method detects DNA of live and dead organisms even from previous infections. This method may show a false higher prevalence of the causative agent of diarrhea; however, it is still highly specific method and most preferable (Soejima *et al.*, 2008). In this study setting, traditional microbiological methods of detection were used, making specificity lower, and the study detected active\live organisms only responsible for the current diarrheal episode. These traditional methods are however useful in that they can be emulated in low resource setting clinics.

In Kenyan hospitals, Augmentin, ceftazidime and ceftriaxone are the most frequently prescribed β -lactams for intraabdominal infections in children because of their availability in oral and intravenous formulations (Boru *et al.*, 2010). Ceftazidime and ceftriaxone showed a resistance rate of 12% and 10% respectively so use of this antibiotic should not be heavily relied upon. The least level of resistance was seen in aminoglycoside (Kanamycin at 8%) compared to all other antibiotics. A study done at Minia university hospital in Egypt advocated use of older antibiotics as valuable choices for treatment of serious infections and supported use of aminoglycosides. Using checkerboard titration method, the most frequently-observed outcome in combinations of aminoglycosides with β -lactams or quinolones was synergism. The most effective combination was amikacin with ciprofloxacin which showed 100% Synergism (Gad *et al.*, 2011). These antibiotics can therefore still be recommended as the mainstay of treatment for children. It's noteworthy that Tetracycline is not recommended in treatment regimen in children. In this study it was used for the purposes of Antimicrobial Resistance assessment as these bacteria cross contaminates in a household set up, and as a measure of resistance level in community age groups. Prescribing antibiotics to children should be done carefully as their intermediate status were high. For example, the lowest resistance level was at 8% against Kanamycin but a high intermediate susceptibility level of 13% was seen. Other

classes of drugs that are a cause for concern in their high intermediacy are ampicillins at 11.5%, third generation cephalosporins (Ceftazidime) at 14% and Cepheids (Cefpodoxime) at 14%. The pathogens showed a high possibility of increase of resistance prevalence towards these antibiotics.

MDR at 17 % was a major factor in pathogens associated with diarrhea in children. This study found multidrug resistance including to newer cephalosporins which poses a threat. In general, all enteric bacterial isolates were highly resistant to Cefuroxime sodium (72%) and tetracycline (56%). This was expected as CXM is a second-generation cephalosporin while Tetracycline is a highly overused drug. Global surveillance studies demonstrate that fluoroquinolone resistance rates have increased in almost all bacterial species in the past years except *S. pneumoniae* and *H. influenzae*, but still 10 to 30% of the isolates had first-step mutations which they conferred as low-level resistance to other bacteria. Fluoroquinolone resistance has increased causing higher levels of intraabdominal infections as well as urinary infections which are passed on to children in a community setup. There is a high increase of 50% in some parts of the world especially in Asia (Axel, 2012). The increasing prevalence of infections caused by antibiotic-resistant bacteria in children is of serious concern since it predisposes risk factors of transferring resistant genes to other species of bacteria (Rasheed *et al.*, 2014).

This is one of the few studies that has evaluated the magnitude of ESBL producing isolates and characterized the genetic profile among *E. coli* isolates obtained from patients. Four ESBL genes were detected; *bla*_{CTX-M-15}, *bla*_{OXA-48}, *bla*_{SHV-1}, and *bla*_{TEM-1} which was the most prevalent gene. The existence of the two dominant genes *bla*_{TEM-1} and *bla*_{OXA-48} presumes that these could have been the major cause of resistance in children with diarrhea. All ESBL genes were found in *E. coli* isolates and none in the other bacterial pathogens that were screened which may indicate better management of intestinal infections but with negative impact on normal flora like *E. coli* which mutate under antibiotic stress. This is similar to a study in Burkina Faso, West Africa in which ESBL producers were more often found in *E. coli* at 67.5 % (Abdoul *et al.*, 2016). This can be attributed to the fact that *E. coli* being a normal flora is highly exposed to antibiotics during treatment in any event of an

infection. This constant exposure makes *E. coli* mutate with time and pass on mutations to other enterics. Overall the prevalence of ESBL producing *E. coli* makes their empirical treatment difficult and outcome unpredictable (Rasheed *et al.*, 2014). ESBL carriage in children poses a major challenge for management of acute and severe diarrhea in this study setting. When ESBL is confirmed, the isolate is considered as resistant to all penicillin and cephalosporins regardless of the initial disc diffusion result for each drug (Rawat & Nair, 2010)

In developing countries like Kenya, detection of ESBL is not commonly carried out in many microbiology units, as well as clinics that serve the community, due to lack of resources and facilities for conducting ESBL identification (Ciara *et al.*, 2012). Despite the rise in the prevalence of ESBL producing *E. coli* in some countries, there are very few reports from Africa and especially from Kenya (Kariuki *et al.*, 2007). Thus, information on infections caused by ESBL producing organisms is limited particularly in this study setting where microbiological culture analysis doesn't take place during clinical visits. For this reason, many clinicians have not fully appreciated the immense significance of microbiological analysis of all samples and ESBL detection (Iroha *et al.*, 2009).

5.2 Limitations of the study

Multiplex polymerase chain reaction (mPCR) was the intended method to assess co-infection in this study. It is a technique which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. However, these multiplex PCR kits are expensive, and a kit is required for each of the different groups of enteropathogens. mPCR has been successfully applied on genetics screening, genotyping and diagnostic analysis of infectious diseases. It is also cost-saving for large scale PCR analysis. However, it is notable that these local sampling sites could not in practical sense manage to use PCR pathogen detection method for every child who visits. PCR method also detects DNA of dead organism even from previous infections.

5.3 Conclusion

The study demonstrated the presence of a wide array of other enteric pathogens that would otherwise be missed with a simple wet preparation as usually employed in low resource clinics. Wet preparations only detect parasites in stool, but microbiological culture methods are required to detect bacterial pathogens. There were cases of co-infection between *Salmonella spp* and pathogenic *E. Coli*. Presence of pathogenic strains of *E. Coli*, which can only be detected using PCR method, indicates that better microbiological methods are needed in Kenyan clinics. Demographic data showed a heavy disease burden on children below 1 year. This age group had the highest number of patients suffering from diarrhea and had the highest *Salmonella* burden.

Multidrug resistant and ESBL producing isolates were present in these clinics making these also a likely cause of childhood diarrhea and recurring episodes in this study setting. Four genes of resistance were found in children under 5 years. Some isolates also had multiple virulent genes which would be hard to detect in poor resource clinics.

Ceftazidime and ceftriaxone are the most frequently prescribed β -lactams for intraabdominal infections in children because of their availability in oral and intravenous formulations (Boru *et al.*, 2010). Ceftazidime and ceftriaxone showed a resistance rate of 12% and 10% respectively so use of this antibiotic should not be heavily relied upon. Many antibiotics had considerable intermediate status and these antibiotics may show resistance in the near future. Aminoglycosides like Kanamycin had the least level of resistance but high intermediate status. Better solutions and practices related to antibiotic prescriptions are needed to reduce presumptive diagnosis and prescription of broad-spectrum antibiotics.

5.4 Recommendations

Traditional methods demonstrated in this study are economical especially on large scale diagnosis. This study recommends that clinical laboratories develop a habit of microbiological analysis of all specimen type. This will determine co-infection status thus making sure each pathogen is treated appropriately, to reduce the rate of MDR in

children. This will reduce the rate of recurrence of diarrheal episodes as well as ease economic burden in the society.

This study advises on improved public health measures including proper sanitation, clean water supply coupled with interventions with bacterial targeted vaccinations. Vaccines against Enterotoxigenic *Escherichia coli* (ETEC), *Salmonella* and rotavirus should be included in the mandatory childhood vaccinations. Other vaccines that should be given include for cholera and shigella infections. Community health education on personal hygiene, treatment of human waste and access to treated water should be enhanced so as to reduce the incidence of diarrhoea among pre-school children.

Invention and development of newer infectious disease management strategies as opposed to antibiotic use should be encouraged and funded. More government resources should be allocated to development of vaccines against these infectious pathogens. For example, phage therapy which is a promising, but an underdeveloped, underfunded field could yield groundbreaking results if emphasis on new treatment methods was placed. Hospitals should have a monitoring and surveillance policy to record prevailing mutant strains in order to institute effective and credible treatment and management of childhood diseases. Monitoring of ESBL carriers and regular surveillance in hospitals will aid to minimize high rate of infections and spread of resistant pathogens among children less than 5 years.

Extended spectrum Beta-lactamase producers should not be treated with the third or fourth generation of cephalosporins, and β lactams but instead use other therapeutic alternatives such as, chloramphenicol and aminoglycosides that are effective against ESBL producers. Evidence based prescription of antibiotics in children is advocated for, as many organisms showed resistance and intermediate resistance to antibiotics.

Antibiotic Susceptibility tests should be mandatory before antibiotic prescription to reduce ESBL carriage and transfer to other bacteria. As previously discussed, Antibiotic Synergism tests have showed 100% efficacy in some studies. As such drug combinations should be considered to come up with more effective ways treat AMR strains.

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APPENDICES

Appendix I: Ethics approval and consent to participate

The procedures followed in this study were in accordance with the ethical standards of the Kenyatta University Ethical Review committee on human experimentation (See attached form). Consent was sought from the guardians of the children with diarrhea and they retained a signed copy of the consent form which has principal investigators and the ethical body, Kenyatta University Ethical Review Committee (KU-ERC) contact information.


KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

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Our Ref: KU/R/COMM/51/737

Date: 23rd May, 2016

Ng'ang'a Grace Wanjiru
Jomo Kenyatta University of Agriculture & Technology,
P.O Box 62000-00100,
Nairobi

Dear Wanjiru,

APPLICATION NUMBER PKU/507/E45- "ANTIMICROBIAL RESISTANCE, BACTERIAL AND PARASITIC CO-INFECTIONS IN CHILDREN BELOW 5 YEARS WITH DIARRHOEA ATTENDING MUKURU KWA NJENGA HEALTH CLINICS."

1. IDENTIFICATION OF PROTOCOL
The application before the committee is with a research topic, "Antimicrobial resistance, Bacterial and Parasitic Co-infections in children below 5 years with Diarrhoea attending Mukuru kwa Njenga Health Clinics." received on 15th April, 2016 and discussed on 17th May, 2016.

2. APPLICANT
Ng'ang'a Grace Wanjiru, College of Health Sciences

3. SITE
Mukuru kwa Njenga and Mukuru kwa Reuben Health Clinics Health Clinics, Kenya

4. DECISION
The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

- (i) Scientific design and conduct of study,
- (ii) Recruitment of research participant,
- (iii) Care and protection of research participants,
- (iv) Protection of research participant's confidentiality,
- (v) Informed consent process,
- (vi) Community considerations.

AND APPROVED and that the research may proceed **ON CONDITION** that you incorporate its advise below.

5. ADVICE/CONDITIONS

With respect to matters of scientific design and conduct of study and recruitment of research participants, the following specific conditions must be fulfilled in writing before an approval can be granted. The manner of fulfilling these should be outlined and submitted to KU-ERC as soon as possible.

1. **Scientific design and conduct of the study**

- a) Clarify the research design.
- b) What value will this study add in the management of diarrhea?
- c) Is the study of any value to the sick children? *(*) antibiotic profiles.*

2. **Care and protection of research participants.**

- a) Specify how care and protection of research participant will be provided.
- b) What will happen to the children who samples will be used in the study?
- c) What about stool positive samples treatment or referral.
- d) How many per clinic and allocation to site.
- e) The sample size using the formula given should be 174 not 100 as erroneously calculated.

3. **Protection of research participant's confidentiality.**

- a) Specify how protection of research participant's confidentiality will be assured.
- b) What about doing intervention and specimen collection?

4. **Informed consent**

- a) Indicate the contacts of principal investigator and KUERC.

5. **Community considerations**

- a) State the community considerations in the research.

When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.



DR. TITUS KAHIGA
CHAIRMAN: KENYATTA UNIVERSITY ETHICS REVIEW COMMITTEE

I GRACE WANJIRAU NGANGA'.....
accept the advice given and will fulfill the conditions therein.

Signature..... GW..... Dated this day 07 of JUNE 2016.

cc. Vice-Chancellor
DVC-Research, Innovation and Outreach

Appendix II: Informed consent form

You are being invited to take part in this research study because your child has presented with diarrhea. Diarrhea is a serious disease in young children. The study aims to identify the germs that are causing diarrhea in children. The germs targeted have names like *Escherichia coli*, *Salmonella* and *Shigella* which you can get from consumption of food or water or from contact with people or animals that are infected with them and they cycle between humans and sometimes between humans and animals.

Please read the following information. If you cannot read, please listen as the investigator explains this information to you very carefully. Ask for explanation for any words, terms or sections that are not clear. Ask also any questions that you have about this research study. If you decide to take part in this research study, you are asked to sign this form. You should keep your copy for your records. It has information, including important names and telephone numbers, which you can use in the future either to ask questions, for clarifications or for results.

Project title:

BACTERIAL AND PARASITIC CO-INFECTIONS, ANTIMICROBIAL SUSCEPTIBILITY AND ESBL GENES CARRIAGE IN CHILDREN BELOW 5 YEARS WITH DIARRHEA ATTENDING MUKURU SLUM HEALTH CLINICS

1. Purpose of the study

This study aims to detect enteric bacterial pathogens in diarrhea stool specimens and their susceptibility to antibiotics as well as intestinal parasites, from children less than five years attending Mukuru Kwa Njenga health clinics.

2. Procedures to be followed and dissemination of findings

This is a non-invasive procedure where there is no pain. In this study, stool sample will be obtained from your child. Once investigation is over the results will be communicated to you through the attending clinician for a period not exceeding one

month upon your request. In case a harmful germ has been detected may return to the hospital with these findings and seek medical intervention for your child.

3. Risks

There are no risks involved. There may be discomfort to your child in case a rectal swab is required. In case you want to withdrawal at any period during the study, you are free to do so without any consequences. No specific data or contact information will be disclosed to any other part except the attending clinician in case of a referral.

4. Benefits

The study will yield results on possible bacterial and parasitic pathogens which may be infecting children leading to diarrhea. Once these organisms are isolated and identified, we will be able to know which medicines best for treatment and this will assist the doctor to manage problem efficiently and proper medication will be given to children for speedy recovery. These results will also aid future diagnosis in children. There is no financial benefit from the study.

5. Confidentiality of records

The results that are related to this study will be maintained in confidence. Your child's stool will be given a unique identifier number and thus the name will not be identified by any person except the principal investigator. No identity of any specific patient in this study will be disclosed in any public reports or publications. The specific results will only be submitted to the clinician and revealed to you upon request by you. The general data is the one which will be used to report on the study and in publications.

6. Obtaining additional information

You as the guardian of the child are encouraged to ask any questions to clarify any issues at any time during the participation in this study. You can also seek to know the outcome of the study or your child's results. Keep a copy of this agreement for your own information. Contacts are:

Grace Wanjiru Ng'ang'a,

Principal investigator

phone number: 0723738499

E-mail: gracenganga10@gmail.com

Kenyatta University Ethics Review Committee

P.O BOX 43844-00100

Nairobi

Tel: 8710901/12

E-mail: secretart.kuerc@ku.ac.ke

7. Name of participant.....

8. Date of Birth.....Age.....Yrs. Sex.....

9. Address..... Telephone.....

10.E-Mail address.....

11. Signature

I have read the above information and have had the opportunity to ask questions and all of my questions have been answered satisfactorily. I consent for my child to participate in the study as has been explained and as I have understood it. I have been given a copy of this consent form for my own records and future reference.

Signature.....Date.....

I have fully explained the relevant details of this study to the person named above.
By virtue of my training on how to conduct research in this field, I'm qualified to perform this role.

Signature.....Name of PI

Date.....

Appendix III: Funding

This work was funded by Prof. Samuel Kariuki of the Centre for Microbiology Research, KEMRI. He is supported by National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health under award number 1R01AI099525 and The Wellcome Trust. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Competing interests

There was nothing competing interests in this work.

Antimicrobial Resistance, Bacterial and Parasitic co-infections in Children Below 5 Years with Diarrhea, Attending Mukuru Kwa Njenga Health Clinics.

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Abstract : Introduction: In Kenya the overall two-week period incidence of diarrhoea is 17.1%. This study aimed to investigate prevalence of bacterial, bacterial-parasitic co-infections, and antimicrobial resistance in children below 5 years presenting with diarrhoea at Mukuru Kwa Njenga health clinics in Nairobi County, 25 km East of Nairobi city, Kenya.

Materials and Methods

Stool samples of children presenting with diarrhoea at the 3 outpatient clinics were obtained and analysed for bacterial pathogens, protozoans and helminths. Routine microbiological procedures were used to isolate pathogenic enteric bacteria using MacConkey media and Xylose Lysine deoxycholate agar. Biochemical testing was done on commercial API20E kits. Antimicrobial susceptibility tests were determined using Kirby-Bauer disk diffusion method on Mueller Hinton agar and commercial antibiotic disks. PCR was used for Pathotyping of *E. coli* isolates and screening for Extended Spectrum Beta Lactamases.

Results

Salmonella spp was found in 6.89 % of the cases with *Salmonella* Enteritidis at 2.29%, *Salmonella* Typhimurium at 2.87% and *Salmonella* Typhi at 1.72%. No *Shigella* species were isolated. Two pathotypes of *E. coli* were observed in 7.47 % of the cases - Enterotoxigenic *E. coli* accounted for 5.17 % of these with prevalence of AggR gene being higher than *stx* gene. *pcvd* gene was absent. Prevalence of enteropathogenic *E. coli* was 2.29%. Prevalence of *Serratia* spp was at 1.72%, *Aeromonas sobria* at 1.15% and *Providencia* spp at 0.57%. Other enterics included *Proteus* spp, *Klebsiella* spp, *Pseudomonas* spp, *Enterobacter* spp, *Citrobacter* spp and *Morganella morganii*. Multi drug resistance (MDR) was found in 15% of bacteria. *Entamoeba coli* and *Entamoeba histolytica* were isolated together in two samples,

having a co-infection with *Salmonella* Typhi in one of the cases. *Cryptosporidium parvum* was isolated from one sample. The highest proportion of ESBL genes was *oxa* at 5.26%. *CTX-M-15* gene was second highest at 4.39% of the cases and *shv* gene at 2.63%.

Conclusion

Pathogenic *E.coli* was the leading pathogen isolated from diarrheal cases, followed by *Salmonella* spp. Co-infection and co-infestation was not a major factor in cases of childhood diarrhea studied. MDR isolates at 15% are an important etiology, making these important likely causes of childhood diarrhea in our study settings. Improved public health measures including proper sanitation, clean water supply coupled with intervention with bacterial targeted vaccinations.

1. Introduction

Pneumonia has been the leading cause of death and diarrhoea comes second as the most frequent cause of death globally amongst children aged five and below [2]. Until the introduction of the rotavirus vaccine, which saw the incidence of the disease decrease drastically, rotavirus was the leading cause of fatal diarrhoea in children less than 5 years of age. In developing countries an estimated 1,000 million episodes of diarrhoea occur each year in children below five years, causing 5 million deaths annually, out of which 80% occur in the first two years of life [1] It accounts for more than one-third of the deaths in this age group -more than HIV, malaria and measles combined. Malnutrition plays a major role in these cases. The World Health Organization (WHO) estimated diarrhoea deaths among children aged less than five years in Africa in 2004 to be 17.5% [4] Children are very susceptible to diarrhoea and worm

infestations due to their under developed immune systems [3].

In Kenya, diarrhoea is a leading cause of morbidity, accounting for 17% of childhood illness [3]. Frequent episodes cause malnutrition, stunted growth and poor intellectual development [2]. Still, many diarrhoea cases recur in 20% of diagnosed and treated cases. This is mostly due to poor diagnostic techniques which do not determine co-infections and/or infestation with intestinal [5].

Early childhood malnutrition resulting from any cause reduces physical fitness and work productivity in adults and diarrhoea causes childhood malnutrition [6]. Diarrheal disease has significant impacts on mental development and health; it has been shown that, even when controlling for helminth infection and early breastfeeding, children who had experienced severe diarrhoea had significantly lower scores on a series of tests of intelligence [7]. Emergence of antimicrobial resistance in bacterial causes of diarrhoea complicates management of infections especially in resource poor settings where effective alternative options are either unavailable or too expensive to be afforded by the population [10].

This study aimed to determine bacterial and parasitic co-morbidities, antibiotic susceptibility of the bacterial pathogens and presence of resistance genes in bacteria from children less than five years with diarrhoea, seeking health care at Mukuru Kwa Njenga and Mukuru Kwa Reuben health clinics in Nairobi County, Kenya.

2. Methods

Study population

This was a population based epidemiological cross-sectional study with laboratory based analysis. The study was carried out at Mukuru Kwa Njenga, Mukuru Kwa Reuben health clinics and Municipal Council Clinic, 20 km east of Nairobi city serving a population of nearly 150,000. The target population was children, less than five years presenting with acute diarrhoea who had not been treated for the current episode of diarrhoea. Consent was sought from the guardians of the children through the attending physician. Sociodemographic data of patients such as age, gender and contact person were recorded. Purposive sampling was done to allow transportation and processing of samples within 4 hours of collection. The study was approved by the Kenyatta University Ethical Review Committee (KU-ERC).

Microbiological procedures

Fresh faecal specimens were collected and transported to CMR-KEMRI for processing. They

were examined in the laboratory directly for vegetative forms of parasites and saline wet mounts prepared for ova and cysts detection. All cultures for bacterial isolation were done on MacConkey media and Xylose Lysine deoxycholate agar (XLD) (Oxoid, Hampshire, United Kingdom) and biochemical tests done on API20E kits (biomerieux, Boston, USA). Serotyping for *Salmonella* spp was done using commercial antisera (BD diagnostics, Ontario, Canada).

Antimicrobial sensitivity testing was done using the Kirby Bauer disk diffusion method. *E.coli* 25922 strain was used as control for disk potency and quality of media. The antibiotics used were ampicillin 10µg, tetracycline 30µg, gentamicin 10µg, sulphamethoxazole 100µg, chloramphenicol 30µg, cefuroxime sodium 30µg, ceftazidime 30µg, ceftriaxone 30µg, cefotaxime 30µg, ciprofloxacin 5µg and nalidixic acid 30 µg, cefpodoxime 10, kanamycin 30. Antimicrobial sensitivity results were read as diameters of the zones of inhibition around each antimicrobial disk and the results were interpreted using CLSI the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012).

DNA from *E.coli* and other enteric bacteria was extracted using the boiling method. *E.coli* pathotyping by PCR method to detect *eae*, *sxt1* and *sxt2*, *eaf*, *ipaH*, *AggR*, *pcvd* and *aspu* genes. ESB gene detection was done on all *E. coli* isolates and bacterial pathogens only, using PCR method and screening for *oxa*, *shv*, *CTX-M-15*, and *tem* genes. All study procedures were done in accordance with the ethical standards of the Kenyatta University Ethical Review committee (KU-ERC) on human experimentation from whom ethical approval for this study was obtained.

Results

Bacteria isolation

Stool samples were collected from 174 children who were less than five years of age. Of these 98 (56.3%) had *E.coli*. Of the 98 *E. coli*, 13 (7.5%) were pathogenic. Enteroaggregative *E. coli* (EAEC) was found in 9 cases and accounted for 5.2%. Of these, AggR gene was found in 5 isolates, the number being higher than *aspu* gene which was found in 4 isolates. *pcvd* gene was not detected. Enteropathogenic *E. coli* (EPEC)-*eae* gene- was found in 4 cases (2.3%). *Salmonella* was isolated from 12 (6.9%) cases with 4 cases (2.3%) of *Salmonella* Enteritidis, 5 cases (2.9%) of *Salmonella* Typhimurium and 3 cases (1.7%) of *Salmonella* Typhi.

Table 1 is a summary of the main bacterial species isolated from the children presenting with diarrhea.

Table 1: Enteric bacterial pathogens isolated from children less than 5 years presenting with diarrhoea.

Isolated organism	Number of isolate	Percentage
<i>Escherichia coli</i>	98	56.3%
<i>Salmonella</i> spp	12	6.9%
<i>Serratia</i> spp	3	1.7%
<i>Aeromonas sobria</i>	2	1.2%
<i>Providencia</i> spp	1	0.6%
<i>Proteus</i> spp	13	7.5%
<i>Klebsiella</i> spp	38	21.8%
<i>Citobacter</i> spp	32	18.4%
<i>Enterobacter</i> spp	6	3.5%
<i>Morganella morganii</i>	1	0.6%
<i>Pseudomonas</i> spp	1	0.6%

Parasitology

Entamoeba coli and *Entamoeba histolytica* were isolated together in 2 cases. In one case the patient also had *Salmonella* Typhi. *Cryptosporidium parvum* was isolated from one sample. No other ova or cysts were isolated.

Association of pathogen type with age of the patients

Pathogenic *E. coli* isolates were found in every age group as opposed to other pathogens with a peak in the 3rd year of life. *Salmonella* isolates were highest in children less than 12 months having 8 cases accounting for 66.6% of all cases of compared to only 4 cases for all other age groups. All *Entamoeba* were detected in the 4 year olds and only 1 case of *Cryptosporidium parvum* in a 2 year old as illustrated in Fig 1.

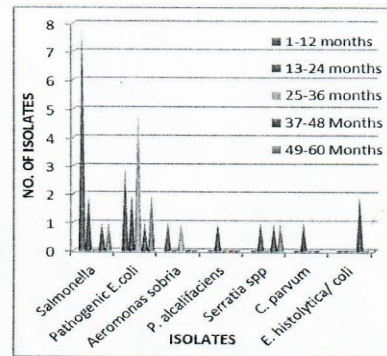


Fig 1: Organisms isolated in association with the age group of the patients.

Rate of co-infection and co-infestation from 174 stool samples collected

There were no bacterial-bacterial coinfections but there was 1 case of bacterial-parasite co-infection. There was also co-infection between organisms with pathogenic genes and ESBL carriers as shown in Table 2.

Antimicrobial sensitivity testing

The highest resistance was to cefuroxime at 70% and sulphamethoxazole at 64% resistance. Nalidixic acid resistance was at 28% and Fluoroquinolone (ciprofloxacin) resistance was at 18%. The least resistance was to aminoglycosides, kanamycin (8%) and gentamycin (10%). Prevalence of resistance to other antimicrobials is shown in Figure 2.

Table 2: Prevalence of co-infections between parasites, pathogenic *E. coli* and ESBL in children less than 5 years of age.

	<i>Salmonella</i> spp	ea ec	eh ec	sh v	CT X-M-15	oxa	<i>Entamoeba histolytica/Coli</i>
<i>Salmonella</i> spp		0.6%	0.6%	0%	0.6%	0.6%	0.6%
EAE C					0.6%	1.2%	0%
EHE C						0%	0%
SHV						1.2%	0%
CTX-M-15						1.2%	0%
OXA							
<i>Entamoeba histolytica/Coli</i>							

Screening for Extended Spectrum Beta Lactamase (ESBL) production

The highest proportion of ESBL genes was found in *E. coli* isolates with 6 isolates having the *oxa* gene, accounting for 5.7% of all isolates that were screened for the gene. CTX-M-15 gene was second highest with 4 isolates and accounts for 4.4% of the cases. SHV gene was found in 3

isolates and accounted for 2.7% of all screened isolates. MDR at 15% was a major factor in pathogens associated with diarrhea in children that were studied.

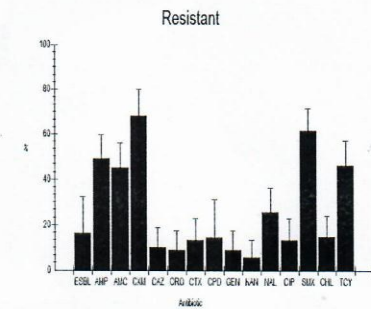


Fig 2: Percentage resistance of all isolates against given antibiotics.

3. Discussion.

In this study, pathogenic *E. coli* was the leading isolate from cases of diarrhea at 7.5% followed by *Salmonella* spp at 6.7%. *Salmonella* species was more prevalent in children less than 1 year compared to other pathogens. Enterotoxigenic *E. coli* (EPEC) cases were highest in year 3 of life. This is in agreement with a previous etiology and epidemiology of diarrhea study which obtained prevalence data from pooled data worldwide; the commonest etiologic agents of diarrhea for all ages in decreasing order of prevalence as: rotavirus, ETEC, *shigella*, *campylobacter*, *Vibrio cholerae*, and non-typhoidal *Salmonella* [1].

Only two types of parasites were isolated, *Entamoeba* spp mostly at age 4 and *Cryptosporidium parvum* at age 2. Together all the parasites accounted for 1.7% of all cases. In a previous study in the Mukuru study site over a period of two years (2010-2011) 25.6% of children below 5 years with diarrhea were positive for at least one intestinal parasite [8]. Our study collected samples over a 4 month period and this could be a major limitation that can contribute to the variance in isolation rates. Rainy seasons also tend to give a rise to high infection rates than the dry season when the study was done.

However, our study findings are in agreement with Gatei et al. [9] where *Cryptosporidium parvum*, *Entamoeba* species and *Giardia lamblia* were the most common parasites isolated in Kenya although no *Giardia Lamblia* trophozoites were isolated in this study.

There were only three cases of pathogenic *E. coli* in 2 year olds, but the cases were higher in the 3 year olds. Younger children were more prone to *Salmonella* infections. For instance there were 8 cases in 1 year olds compared to only 4 cases in children 2-5 year olds. These results correlate well with a study done in 2015 in the same Mukuru study site. At the younger age the immune system is still developing and pathogenic bacteria such as *Salmonella* spp will cause more infections than in older age groups [10]. In our study we found low prevalence (0.6%) of co-infection-infection.

MDR was a major factor in pathogens associated with diarrhea in children. Ciprofloxacin resistance *E. coli* and *Salmonella* was 18%. Global surveillance studies demonstrate that fluoroquinolone resistance rates have increased in almost all bacterial species in the past years except *S. pneumoniae* and *H. influenzae*, but still 10 to 30% of the isolates had first-step mutations which they conferred as low level fluoroquinolone resistance. Indeed fluoroquinolone resistance has increased causing higher levels of intraabdominal infections as well as urinary infections, an increase of 50% in some parts of the world especially in Asia [11].

We found that all the enteric bacterial isolates were highly resistant to Cefuroxime sodium (72%) and tetracycline (56%). This was expected as CXM is a second generation cephalosporin while Tetracycline is a highly overused drug. The least resistance was to aminoglycosides, kanamycin (8%) and gentamycin (10%). It is noteworthy that gentamycin may show effectiveness *in vitro* but be ineffective *in vivo*.

Another unusual occurrence was in *Enterobacter cloacae*, *Serratia* species and *Providencia* species being susceptible to penicillin, cephalosporins and cephamycins. These enterobacteriaceae possess inducible cephalosporinases and hence susceptible strains are rare and when these drugs are used in therapy, though susceptible *in vitro*, they may develop resistance during therapy. Testing of repeat isolates is always advised. Previously on, aminoglycosides, fluoroquinolones and third-generation cephalosporins were the mainstay of treatment against *Serratia* and *Providencia* infections. Now these isolates demonstrate multiple antimicrobial resistance to these agents which is a direct reflection on patterns of antimicrobial use [12, 13].

All ESBL genes were found in *E. coli* isolates and none in the other bacterial pathogens that were screened. The *oxa* gene was most prevalent, being found in the same cases with *Salmonella*, *eaec* and together with other ESBL genes like CTX-M-15 and *shv*. This is similar to a study in Burkina Faso, West Africa in which ESBL producers were more

often found in *E. coli* at 67.5% [14] When ESBL is confirmed, the isolate is considered as resistant to all penicillins and cephalosporins regardless of the initial disc diffusion result for each drug. In a global surveillance report, two-thirds of Enterobacteriaceae producing extended spectrum - lactamases have also become fluoroquinolone resistant in the recent past [11]

4. Conclusion.

In this study pathogenic *E. coli* was the most common (7.7%) isolate from children with acute diarrhoea and *Salmonella* came second at 6.3%. Children under two years are more prone to *Salmonella* infections as opposed to later years. Co- infection and co-infestation did not play a major role in causing childhood diarrhoea, although in all the cases *Entamoeba histolytica* and *Entamoeba coli* always presented together in the cases. ESBL carriers also showed a high level of fluoroquinolone resistance and to other commonly used antimicrobials, posing a major challenge for management of severe diarrhoea. Increasing rates of multidrug resistance including to newer cephalosprins and fluoroquinolones poses a major challenge for management of acute diarrheal disease in our study settings

Abbreviations

ESBL-Extended Spectrum Beta-Lactamases
MDR-Multi Drug Resistance
AST-Antimicrobial Sensitivity Testing
EPEC-Enteropathogenic *E.coli*
EAEC- Enterogaagregative *E. coli*

5. Declarations

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Competing interests

None.

Ethics approval and consent to participate

The procedures followed in this study were in accordance with the ethical standards of the Kenyatta University Ethical Review committee on human experimentation. Consent was sought from the guardians of the children with diarrhoea through the attending physician. The study was explained in a way the guardians of the participants understood. They retained a signed copy of the consent form which has principal investigators and the ethical body, Kenyatta University Ethical Review Committee (KU-ERC) contact information.

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Appendix V: Key identification Characteristics for Enterobacteriaceae

**Key identification characteristics for
Enterobacteriaceae**

GENUS/SPECIES	Fermentation of				Gas	MR	VP	Indole	Citrate	Urease	H ₂
	G	L	S	M							
<i>Escherichia coli</i>	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(-)	(-)
<i>Shigella</i>	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(-/+)	(-)	(-)	(-)
<i>Shigella sonnei</i>	(+)	(+)	(-)	(+)	(-)	(+)	(-)	(-)	(-)	(-)	(-)
<i>Salmonella</i>	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(-)	(+)	(-)	(+)
<i>Klebsiella Pneumo.</i>	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)
<i>Enterobacter</i>	(+)	(-)	(+)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(+)
<i>Serratia</i>	(+)	(+)	(-)	(+)	(+)	(-/+)	(+)	(-)	(+)	(-)	(-)
<i>Proteus</i>	(+)	(-)	(-)	(+)	(-/+)	(+)	(-)	(+)	(-/+)	(+)	(+)
<i>morganella</i>	(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)
<i>Yersinia</i>	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(-/+)	(-)	(-/+)	(-)

G: Glucose, L:Lactose, S:Sucrose, M: Manitol, MR: Methyl Red, VP: Voges Proskauer

Appendix VI: Salmonella Serovars identification guide

SALMONELLA SEROVARS

<u>TYPE</u>	<u>SOMATIC (O)ANTIGEN</u>	<u>FLAGELLAR(H) ANTIGEN</u>	
		<u>PHASE 1</u>	<u>PHASE 2</u>
Paratyphi A	GROUP A FACTOR 2(O:2) [A] 1,2,12	a	1,5
Paratyphi B	GROUP B FACTOR 4(O:4) [B] 1,4,[5],12	b	1,2
S. typhimurium	1,4,[5],12	i	1,2
S. stanley	GROUP B FACTOR 27 1,4,[5],12,27	d	1,2
S. schwarzengrund	1,4,12,27	d	1,7
Paratyphi C	GROUP C1 FACTOR 7 (O:7) [G] 6,7,vi	c	1,5
S. cholerasuis	6,7	c	1,5
S. muenchen	GROUP C2 FACTOR 8(O:8) [C2] 6,8	d	1,2
S. newport	GROUP C3 FACTOR 20 6,8,20	e,h	1,2
S. typhi	GROUP D1 FACTOR 9 (O:9) [D] 9,12 (vi)	d	-
S. enteritidis	1,9,12	gm	-

SHIGELLA

<u>TYPE</u>	<u>POLYVALENT ANTISERA</u>	<u>MONOVALENT ANTISERA</u>	
S. dysenteriae	Group A	1 – 15	(A1,A2,A3...A13)
S. flexneri	Group B	1 – 6, X,Y	(B1,B2,B3,B4,B5,B6)
S. boydii	Group C	1 – 19	(C1,C2,C3.....C18)
S. sonnei	Group D	1	D1

REF:

1. ANTIGEN FORMULAS OF THE SALMONELLA SEROVARS 2001 8th Edition KAUFFMANN-WHITE SCHEME
2. MANUAL FOR IDENTIFICATION AND ANTIMICROBIAL SUSCEPTIBILITY TESTING pg. 130

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Appendix VII: API 20E Interpretation guide

