PREVALENCE, MOLECULAR PATHOTYPES, CLINICAL OUTCOMES AND ANTIBIOTIC PROFILES OF *ESCHERICHIA COLI, SALMONELLA* AND *SHIGELLA* FROM DIARRHOEA INPATIENTS AGED BELOW FIVE YEARS AT MOI TEACHING AND REFERRAL HOSPITAL

BY

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DECLARATION

Declaration by the Candidate

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DEDICATION

This thesis is dedicated to my family who supported and encouraged me in every step of the way.

ABSTRACT

Diarrhoea remains a major cause of mortality and morbidity worldwide. Diarrhoea in low income settings is attributed to bacterial enteropathogens but viruses and parasites may also be involved. Children infected with HIV are known to experience severe, persistent and recurrent diarrhoea requiring frequent and prolonged usage of antibiotics. However, the actual cause diarrhoea in HIV positive and negative children is unknown. This was a cross-sectional study involving 216 diarrhoea cases aged below five years admitted at Moi Teaching and Referral Hospital. The study determined prevalence, molecular pathotypes, antibiotic profiles of bacterial enteropathogens and clinical outcomes of in-vivo antibiotic susceptibility in relation to HIV serostatus. Fresh stool was collected and processed using standard microbiological methods and multiplex PCR confirmed pathotypes and virulence genes in bacterial isolates. Antibiotic susceptibility to ampicillin, amikacin, ceftriaxone, cefuroxime, ceftazidime, gentamicin, cotrimoxazole, cefipime, ciprofloxacin and imipenem was evaluated using Kirby-Bauer disc-diffusion. Chi-square (χ^2) was used to test for associations between independent and dependent variables while Kruskal-Wallis test compared means between groups at $p \leq 0.05$. Prevalence of diarrhoea was 15.6% while 118(54.6%) of stool samples yielded bacteria. Frequency of bacterial diarrhoea was lower among HIV positive 41(34.7%) compared to HIV negative cases 77(65.3%) with highest frequency among cases aged 0-24 months. The main bacterial pathogens were *Escherichia coli 105*(88.9%)*, Shigella 6(*5.1%) and *Salmonella 5*(4.2%), while pathotypes were EAEC 21(58.3%), EPEC 6(16.7%), EHEC 4(11.1%), EIEC 3(8.3%), ETEC 2(5.6%), *Shigella flexneri 3*(50%), *S. dysentriae 3*(50%), *S. typhimurium 4*(80%) and *S. typhi 1*(20%). Virulence genes detected included EHEC (*stx1, stx_{2}),* ETEC (*elt, est)* EPEC *bfp, EAEC aatA,* EIEC *ipaH, Salmonella invA,* and *Shigella ipaH* and *vir F* genes. Most virulence genes were associated with acute diarrhoea while *Shigella ipaH*, EPEC *bfp,* EAEC *aatA, Shigella virF* and *Salmonella invA* were linked to antibiotic resistance. *Escherichia coli*, *Salmonella* and *Shigella strains* in HIV negative cases were highly susceptible to cefuroxime, ceftazidime, cefepime and amikacin respectively compared to HIV positive cases, with high overall resistance to ampicillin and cotrimoxazole. Diarrhoea persisted in 5(4.3%) of cases, 18(15.5%) died and 93(80.3%) improved and were discharged with medication with better outcomes in HIV negative cases. Cefuroxime, ceftazidime, ciprofloxacin, cefipime and amikacin are effective and may be incorporated into treatment regimens. The study recommends definitive diagnosis and determination of antibiotic profiles for effective management of diarrhoea and prevention of multi-drug resistance. The findings shall contribute to critical development of interventions to monitor, prevent and control childhood diarrhoea.

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ABBREVIATIONS

AAF	Aggregative Adherence Fimbriae
AIDS	Acquired Immune Deficiency Syndrome
AD	Acute Diarrhea
ANC	Antenatal Clinic
AMPATH	Academic Model for Prevention and Treatment of HIV/AIDS
AMP	Ampicillin
AMK	Amikacin
AMR	Antimicrobial Resistance
ARC	AIDS Related Complex
ART	Antiretroviral Therapy
ATC	American Type Culture Collection
AST	Antimicrobial Susceptibility Testing
BD	Bacterial Diarrhea
BDIS	Becton Dickinson Immunocytometry Systems
BFP	Bundle Forming Pilus
cAMP	Cyclic Amino-Phosphate
CBS	Central Bureau of Statistics
CDC	Center for Disease Control
CD_4	Cluster of differrentiation 4
CDM	Ceftazidime
CFs	Colonization Factors

CI	Confidence Interval
CIP	Ciprofloxacin
CPM	Cefuroxime
CTX	Cotrimoxazole
CXM	Cefuroxime
CXR	Ceftriaxone
DNA	Deoxyribonucleic Acid
EAEC	Enteroaggregative *Escherichia coli*
EDTA	Ethylenediamine Tetraacetic Acid
EHEC	Enterohemorrhagic *Escherichia Coli*
EIA	Enzyme Immunoassay
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended Spectrum Beta Lactamases
ETEC	Enterotoxigenic *Escherichia coli*
DNA	Deoxyribonucleic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Flow Automated Cytometry System
GEN	Gentamicin
GARP	Global Antibiotic Resistance Patnership
GMP	Guano-monophosphate
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
H_2O	Water

HUS	Haemolytic Uremic Syndrome
IMViC	Indole -Methyl Red- Voges Proskaeur- Citrate utilization test
IMCI	Integrated Management of Childhood Illness
IMP	Imipenem
ipaH	Invasive Plasmid Adhesin
IREC	Institutional Research and Ethics Committee
KDHS	Kenya Demographic and Health Survey
KNH	Kenyatta National Hospital
LCR	Ligase Chain Reaction
LT	Heat Labile Toxin
LF	Lactose Fermentors
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
MHA	Muller Hinton Agar
$MgCl_2$	Magnesium Chloride
MR	Methyl Red
MTRH	Moi Teaching and Referral Hospital
MOH	Ministry of Health
NASCOP	National AIDS Control Council
NBD	Non Bacterial Diarrhoea
NCCLS	National Committee for Clinical Laboratory Standards
NLF	Non Lactose Fermentors
NRTI	Nucleoside Transcriptase Inhibitors

NTS	Non Typhoid *Salmonella*
NNRTI	Non-Nucleoside Transcriptase Inhibitors
ORS	Oral Rehydration Salts
ORT	Oral Rehydration Therapy
MDR	Multi Drug Resistance
PCR	Polymerase Chain Reaction
pH	Hydrogen Ion Concentration
ProAD	Prolonged Acute diarrhea
PD	Persistent diarrhoea
RNA	Ribonucleic Acid
RFLP	Restricted Fragment Length Polymorphism
SGS	School of Graduate Studies
SOP	Standard Operating Procedure
Stx_1	*shigatoxin* $_1$
Stx_2	*shigatoxin* $_2$
Taq	*Thermus aquaticus*
TBE	Tris Boric Acid EDTA
TSB	Tryptic Soy Broth
TSI	Triple Sugar Iron
UNAIDS	United Nations Programme on HIV AIDS
UNICEF	United Nations Children's Fund
USA	United States of America
USAID	United States Agency for International Development

USAMRU	United States Army Medical Research Unit
UV	Ultra Violet
VCT	Voluntary Counselling and Testing
VP	Voges Proskaeur
WHO	World Health Organization

DEFINITION OF TERMS

Acute diarrhea Passage of 3-4 loose watery stool per day that lasts between 1-6 days with without visible blood.

Antibiotic Any antibacterial agent that destroys or inhibits the growth of other micro-organisms: examples are penicillin, cephalosporin, amino-glycosides, streptomycin, and tetracycline.

AIDS Acquired Immune Deficiency Syndrome (AIDS) is the clinical manifestation associated with advanced Human Immunodeficiency Virus infection of infection.

Aminoglycosides A group of antibiotics usually reserved for use in patients with severe infections. They are effective against a wide range of bacteria including some gram positive and many gram-negative organisms. Aminoglycosides used against these organisms include Amikacin and Gentamicin.

Amikacin One of the Aminoglycosides, amikacin is a semi-synthetic derivative of Kanamycin, which is used to treat infections caused by microorganisms resistant to Gentamicin and Tobramycin.

Antibiotic Resistance Failure of certain bacteria to respond to commonly used antibiotics

Bloody Bloody diarrhea As liquid stools with the presence of blood visible either by the naked

CD4 counts The number of CD4+ cells in a cubic millimeter of blood. A normal CD4 count is from 500 to 1,500 cells per cubic millimeter of blood.

Chronic diarrhoea Passage of 3-4 watery stools per day for not less than 14 days and may be the result of a serious intestinal disorder or of more general disease.

Diarrhoea Abnormally frequent discharge of semisolid or fluid faecal matter from the bowel.

Enteropathogen An organism capable of producing disease in the intestinal tract.

Gastroenteritis A transient disorder due to enteric infection and characterized by the sudden onset of diarrhoea with, or without vomiting.

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CHAPTER ONE: INTRODUCTION

1.1 Background Information

Diarrhoea is a major public health concern worldwide with over 2.5 million deaths occurring annually, mainly in children below five years in developing countries (Wardlaw *et al*., 2010). Diarrhoeal diseases account for 1/5 of all child deaths worldwide, 78% of which are concentrated in Africa and South East Asia (Boschi-Pinto *et al.*, 2008; UN, 2008), particularly in areas of poverty, characterized by unsanitary conditions and usafe water (Kosek *et al.,* 2003). Diarrhoea is the passage of three or more loose or liquid stools per day, recognized by four clinical features, each reflecting the basic underlying pathology and altered physiology which includes acute watery diarrhoea, acute bloody diarrhoea, persistent and diarrhoea with severe malnutrition. It is often accompanied by abdominal pain and may progress to severe dehydration, a serious and potentially life-threatening condition (WHO and UNICEF, 2009).

Children are the most vulnerable worldwide because they are prone to common illnesses and are likely to get repeated infections due to their weak immune systems. The substantial degree of morbidity and mortality due to diarrhea in developing countries, particularly in Africa, is compounded by HIV/AIDS epidemic (Prasad and Nag, 2000). Diarrhoea remains a major clinical problem for patients infected with HIV, especially those with life-threatening AIDS (Wardlaw *et al.,* 2010). It is believed that bacterial pathogens in AIDS patients may manifest differently from infections in immunocompetent hosts, (Navaneethan and Giannella, 2008) prompting a need to continually study the trend of infectious diseases in this group of patients.

The causes of infectious diarrhoea include a wide array of viruses, bacteria, and parasites (Steiner *et al*., 2000). However, persistent diarrhea in resource poor settings characterised by

poor sanitation and unsafe water is largely attributed to bacterial enteropathogens such as *E coli, Salmonella* and *Shigella* (Abu-Elamreen *et al.*, 2008). Recent studies have described a high incidence of pathogenic organisms, especially in children in the Sub-Saharan Africa compared to the rest of the world (Kosek *et al.*, 2003; Fletcher *et al.,* 2011). Bacterial gastroenteritis generally produces more severe symptoms than viral infection, including more frequent and bloody stools, severe cramping (Samie *et al.*, 2012) and usually manifests with symptoms of vomiting, diarrhoea, and abdominal discomfort (WHO and UNICEF, 2009). While immuno-compromised persons are more susceptible to diarrhoeal illness, they are exposed to the same range of pathogens as immunocompetent persons but with severe opportunistic parasitic infections (Rossit *et al.*, 2007). Prevention strategies such as improved water, sanitation and basic hygiene practices do not require knowledge of diarrhoeal aetiology, but others such as vaccination, surveillance and epidemic control would benefit greatly from a comprehensive understanding of the overall burden of pathogen-specific diarrhoeal disease (Fischer *et al.,* 2010).

Bacteria are known to contain virulence genes that encode potent cytotoxins that stimulate acute watery diarrhoea, adherence or invasion factors that induce inflammation and spread to susceptible tissues. The ability to horizontally acquire genetic elements appears to favour the establishment of new virulence properties, causing a broad spectrum of diseases and evolution of new pathotypes (Croxen and Finlay, 2010). The fatality of infections due to these enteric pathogens depends on their serotypes, size of the inocula and status of the host (Ghenghesh *et al.*, 2005). *Shigella* species are perfectly adapted to colonize the host intestine subverting the host's defenses in their favor (Sansonetti, 2006). *Salmonella* have the ability to invade and penetrate intestinal epithelial cells and can remain confined as the intestinal form or progresses

to systemic involvement (Kaper *et al.,* 2004). Diarrheagenic *Escherichia coli* (DEC) has six known pathogenic strains with with different virulence mechanisms (Weintraub, 2007) and are known to cause 30% to 40% of acute diarrhoea and chronic episodes in children in developing countries (O'Ryan *et al.,* 2005) as well as the developed world (Cohen et al., 2005; Nataro *et al*., 2006). In all age groups, severe diarrhoea can lead to hospitalization, serious sequelae such as haemolytic uraemic syndrome and in some cases death. Although most diarrhoeal episodes are self-limiting, it is important to understand pathotypes of diarrheagenic agents and their virulence mechanisms in order to guide in management and prevention of future outbreaks or complications.

Most cases of diarrhea resolve within a short period, however, in infants, the elderly and immunosupressed, antibiotic therapy may result in a dramatic decrease in stool output and decreased length of illness (Petti *et al.*, 2006). Fluid and electrolyte replacement by oral hydration or intravenous fluid therapy is usually the treatment of choice for diarrhoeal disease. However, HIV infection compromises the immune system exposing the infected persons to a wide range of opportunistic infections (OIs) which require prolonged antibiotic use (Cohen *et al.,* 2005). When commensal bacteria are frequently exposed to antibiotics, resistance genes can develop and can be transferred to pathogenic microorganisms inducing antibiotic resistant infections (Okeke *et al.,* 2007; Emacar *et al.,* 2010). Inaccurate antibiotic therapy is associated with increases in adverse patient outcomes prompting the need for evaluation of antibiotic susceptibility of enteric pathogens in order to prevent evolution of resistance (Ibrahim *et al.,* 2000).

Interventions to delay morbidity and mortality from diarrhoea can make huge contributions to the long-term survival of HIV-infected, exposed or uninfected infants and children (Wardlaw *et al.,* 2010). On a daily basis, clinicians are forced to employ empiric treatment on a patient with symptoms and signs of a serious infection before identification of the bacteria and before susceptibility test results are available (Siniravin and Garner, 2006). Premature initiation of antimicrobial therapy in these circumstances can suppress bacterial growth and preclude the opportunity to establish a microbiological diagnosis, which is critical in the management of diarrhea cases. Habitual use of the same antibiotic regimen for all patients with suspected significant bacterial infection may lead to increased resistance and / or poor prognosis. It is critical to isolate the specific pathogens and determine antibiotic susceptibility in life-threatening infections that are likely to require prolonged therapy to improve clinical outcomes.

1.2 Statement of the Problem

Diarrhoea is the third leading cause of mortality and morbidity among children at Moi Teaching and Referral Hospital (MTRH), after malaria and pneumonia. However, the recent roll-out of pneumococcal vaccine by World Health Organization has seen a steady decline in pneumonia rates across the globe, leaving diarrhoea as a major threat. The commonn causes of childhood diarrhoea are parasites, viruses and bacteria. In resource poor settings characteristic of many Kenyan settlements, acute and persistent diarrhoea has been attributed to bacterial enteropathogens. However, due to limited diagnostic facilities, the prevalence of diarrhoea and specific bacterial etiologies in children aged below five years at MTRH in relation to patient HIV status remains unknown. This makes it difficult to quantify comorbidity and its contribution to mortality in the region.

Some studies have demonstrated evidence of shared genetic strategies for pathogenicity in enteric bacteria including different combinations of virulence genes in pathogenic strains but such information is not available in many healthcare settings in Kenya. Although MTRH handles many referral cases from Western Kenya and entire Rift Valley region, there is currently no data to link genotypic diversity and various virulence genes encoding cytotoxins, adherence or invasion factors that promote long-term colonization and predispose to acute or chronic disease in relation to HIV patient HIV serostatus.

Diarrhoea is a common manifestation in HIV-infected individuals and usually a marker of rapid progression to AIDS. Infants and children with enteric bacterial infections are likely to suffer diarrhoea episodes that are severe, prolonged and recurrent requiring frequent and prolonged usage of antibiotics which is thought to induce antibiotic resistance. Although antibiotic resistance is an issue of great concern, antibiotic profiles of enteropathogens to commonly used antibiotics especially in children under five years in relation to HIV status is unknown, prompting the need to investigate and save millions of vulnerable children.

Many bacteria have unpredictable susceptibilities to antimicrobial agents which can be measured *in-vitro* to help guide the selection of the most suitable antimicrobial agent. Some studies have shown that inadequate therapy for infections in critically ill, hospitalized patients is associated with poor outcomes, including greater morbidity and mortality as well as increased length of hospitalization. Whereas definitive diagnosis is the ideal method of selecting the best antimicrobial agent, this has not been fully implemented in most health facilities in Kenya due to

inadequate diagnostic facilities. Consequently, therapy is often empiric and guided by physical signs and symptoms leading to variations in clinical outcomes. There is currently no data to evaluate clinical outcomes in relation to *in-vivo* antibiotic susceptibility among HIV infected and uninfected hospitalized children with diarrhoea at MTRH.

1.3 Objectives

1.3.1 General objective

To determine prevalence, molecular pathotypes, clinical outcomes and antibiotic profiles of *Escherichia coli, Salmonella* and *Shigella* associated with diarrhoea among HIV positive and negative inpatients aged below five years at Moi Teaching and Referral Hospital.

1.3.2 Specific objectives

1. To determine the prevalence of diarrhoea among inpatients aged below five years at MTRH in relation to HIV status.
2. To identify molecular pathotypes of *E coli, Salmonella* and *Shigella* associated with diarrhoea among HIV positive and negative children aged below five years admitted at MTRH.
3. To determine the antibiotic susceptibility profiles of the *E coli, Salmonella* and *Shigella* isolates associated with diarrhoea in children aged below five years admitted at MTRH.
4. To determine clinical outcomes in relation to *in-vivo* antibiotic susceptibility among HIV positive and negative diarrhoea inpatients aged below five years at MTRH.

1.4 Research Questions

1. What is the prevalence of diarrhoea among HIV positive and negative children aged below five years admitted at MTRH?
2. What are the molecular pathotypes of *Esherichia coli, Salmonella* and *Shigella* associated with diarrhoea among HIV positive and negative children aged below five years admitted at MTRH?
3. How is the antibiotic susceptibility of *E coli, Salmonella* and *Shigella Pathotypes* associated with diarrhoea among HIV positive and negative children aged below five years at MTRH?
4. What are the clinical outcomes of *in-vivo* antibiotic susceptibility among HIV positive and negative children admitted with diarrhoea at MTRH?

1.5 Significance of the Study

Moi Teaching and Referral Hospital is the second largest referral Hospital in Kenya that serves the Western region of Kenya with a catchment population of about 15 million. Majority of patients come from rural areas and low income settlements in urban areas characterized by poor infrastructure, lack of potable water and sanitation. The Western Kenya region is also plagued with common diseases such as malaria, pneumonia and diarrhoea frequently occurring among children aged below five years. Although diarrheagenic organisms have been studied in different parts of the African continent, most research has targeted specific organisms and their role in the production of diarrhoea with little consideration on the presence of other organisms and their role in production of inflammation, which might be a considerable part of their pathogenesis (Croxen and Finlay, 2010). Current information on the prevalence of childhood diarrhoea as

well etiology characteristics will guide in the planning for appropriate interventions to reduce child mortbidity and mortality. Profiling the expression of virulence genes in *E. coli*, *Salmonella and Shigella* will promote the understanding of the mechanisms by which enteric bacterial pathogens colonize, spread and at times persist in hosts. Moreover, knowledge about susceptibility profiles of bacteria in different geographical could inform guidelines for empirical therapy and thereby preserve the utility of available antibiotics by limiting the development of antimicrobial resistance. Optimization of therapy against these organisms starts with empirical antibiotic choice. This strategy of "trying to keep one step ahead" implicates the continual development and testing of new antibiotics, which inevitably is more expensive (Obi *et al.*, 2004), but is hoped to be cost effective in disease management and improvement of clinical outcomes especially among critically ill, hospitalized patients.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

Diarrheal diseases remain a leading cause of preventable death, especially among children under five in developing countries (Farthing *et al*., 2013). The normal intestinal tract regulates the absorption and secretion of electrolytes and water to meet the body's physiological needs. More than 98 percent of the 10 liters per day of fluid entering the adult intestines are reabsorbed but the remaining stool water, related primarily to the indigestible fiber content, determines the consistency of normal stool (Keusch, 2001). Although young nursing infants tend to have five or more motions per day, mothers know when the stooling pattern changes and when their children have diarrhea (Black and Lanata, 2002). Acute and persistent diarrhoea in infants and children contributes to substantial mortality and morbidity. In HIV infected children, the resultant gastro- enteric complications from infections can contribute to nutritional deficiency with consequent aggravation of the immunologic conditions (Gassama *et al*., 2001).

2.1.1 Global Situation of Childhood Diarrhoea

Diarrhea remains a leading cause of mortality and morbidity worldwide with the greatest number of deaths recorded in children under 5 years in developing countries (Pop *et al.*, 2014). The proportion of deaths aged below 5 years is 15% worldwide (Black, 1993) and around 25% in Africa and 31% in South East Asia (Fischer *et al.,* 2013; Po *et al.,* 2014), with an estimated 1.7 billion episodes of diarrhoea and approximately 2.9 episodes per child per year (CDC, 2011). Diarrheal diseases account for 1 in 9 child deaths worldwide, kills 2,195 children daily and 801,000 deaths annually which is far greater than AIDS, malaria, and measles combined

(CDC, 2012). For children with HIV, death rate is 11 times higher than the rate for children without HIV (Farthing *et al*., 2013; Fischer *et al.*, 2013).

Despite declines in child mortality between 2000 and 2010 globally, these diseases remain major causes of avoidable deaths accounting for about 30% of all child deaths worldwide. Children and young adults are the most affected, particularly in regions with limited resources and where hygienic practices are not strictly followed (Guerrant *et al.*, 2005). These regions are also burdened with childhood HIV infections characterized by various opportunistic infections (O'Ryan *et al.,* 2005). Children with diarrhoea are at risk of dehydration and therefore early and appropriate fluid replacement is a main intervention to prevent death (Wardlaw *et al.*, 2010). Globally, the current focus targets the recently launched Sustainable Development Goals (SDGs), which aims to reduce child mortality to 20 deaths or fewer per 1000 live births by 2035 which necessitates substantial decreases in mortality from the two illnesses (Fischer *et al.*, 2013).

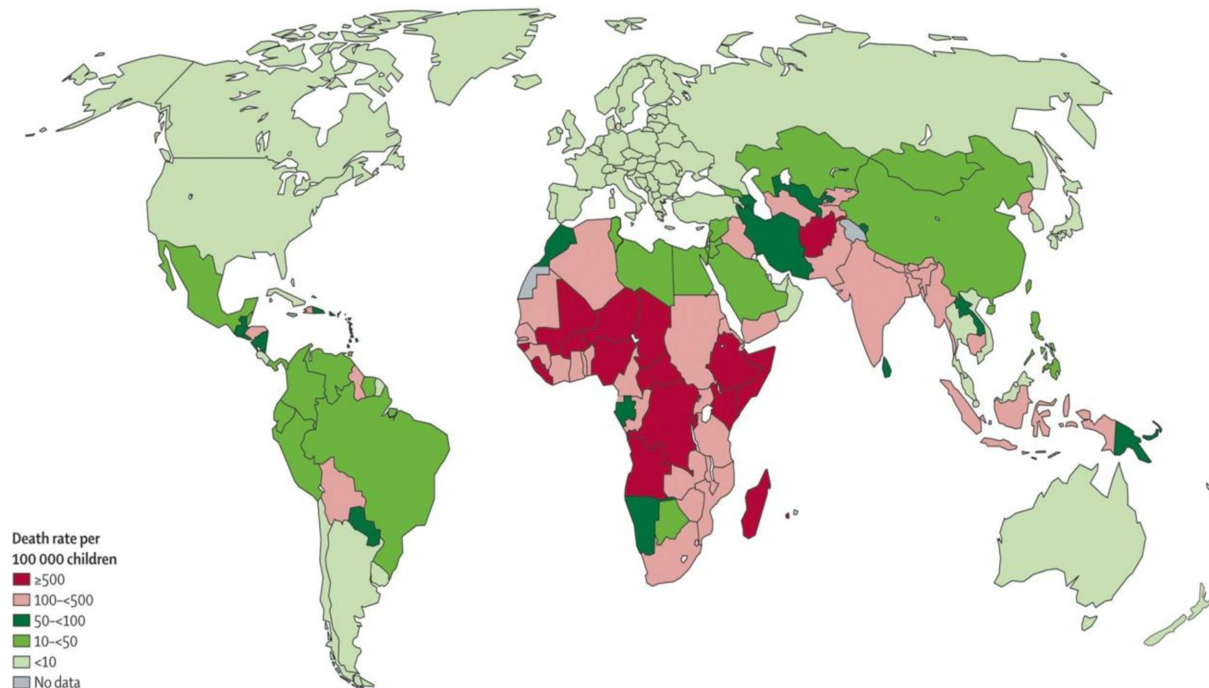


Figure 1: Deaths due to diarrhea per 100,000 children younger than 5 years

Source: WHO, 2014: Global Burden of Diarrhoea

2.1.2 Status of Childhood Diarrhoea in Sub-Saharan Africa

Recent studies have described a high incidence of pathogenic organisms, especially in children in Sub-saharan Africa (SSA) region when compared with the rest of the world (Kosek *et al.*, 2003; Boschi-Pinto *et al.*, 2008; Fletcher *et al.,* 2011). Several studies have shown a high burden of gastrointestinal infections (GI) in the African continent particularly in areas characterized by poverty, overcrowded settlements with poor hygiene, water, and sanitation. Underlying conditions, such as malnutrition, which modify the risk of contracting diarrhea, are also common. These factors combine to facilitate the spread of enteropathogens resulting in epidemics in such settings (Gascon *et al*., 2000). Infectious organisms are transmitted through a

variety of routes and the epidemiology of GI illness varies from one geographical region to another as well as between developing and developed countries (Fletcher *et al.*, 2011)

2.1.3 Status of Childhood Childhood Diarrhoea in Kenya

Kenya is ranked among the top fifteen countries with the highest number of deaths attributed to diarrhoea (Wardlaw *et al.*, 2010) Diarrhoea is the third leading cause of under five mortality in Kenya after malaria and pneumonia accounting for about 9% of under five mortality accounting for 86 child deaths daily. HIV prevalence in Kenya currently stands at 6.6 per cent with estimated 70,000-100,000 infants exposed to HIV annually (Boschi-Pinto *et al.*, 2008; Wardlaw *et al.,* 2010). One third of total paediatric admissions are due to diarrheal diseases and 16% of all deaths in paediatric inpatients are diarrhea related. Available data indicates that mortality rate in children below five in Kenya reduced by 36% from 115 deaths per 1000 live births in 2003, to 74 deaths per 1000 live births in 2008 (Wardlaw *et al.*, 2010). The Millennium Development Goal target (MDG) of 32 deaths per 1000 live births expected by 2015 has not been attained and therefore efforts to scale up interventions against the leading causes of under five mortality, is of prime concern we move into sustainable development goals in 2015. WHO and UNICEF through Integrated Management of Childhood Illnesses (IMCI) strategy recommend prevention, early detection and treatment of the main causes of morbidity and mortality in children below five years (WHO Report, 2004).

2.1.4 Status of Childhood Diarrhoea at Moi Teaching and Referral Hospital

Childhood diarrhoea is the third leading cause of pediatric admissions at Moi Teaching and Referral Hospital (MTRH), Western Kenya (WHO Report, 2004)). A large proportion of

Kenya's population has limited access to affordable and adequate healthcare services. However, with the support from the United States Agency for International Development (USAID)-Academic Model Providing Access to Healthcare (AMPATH) partnership, MTRH is able to provide HIV infection and treatment care of adults and children within 23 public sector clinics in Western Kenya (Einterz *et al.,* 2007). All USAID-AMPATH clinics refer patients for admission to nearby sub-district or district hospitals and more complex cases that require more advanced laboratory and specialist care are referred MTRH in Eldoret town which currently serves a population of over 15 million. Despite the support given by USAID – AMPATH in HIV diagnosis, treatment and care, facilities to handle infectious and other diseases are limited.

2.2 Types of Diarrhoea

Diarrhoea is usually defined in epidemiological studies as the passage of three or more loose or watery stools in a 24-hour period, regardless of other gastrointestinal symptoms or two or more loose stools associated with at least one other symptom of gastrointestinal infection (abdominal pain, cramping, nausea, vomiting, and fever); or either passage of a single loose stool with grossly evident blood and/or mucous (Wardlaw *et al.*, 2010). Exclusively breast-fed infants normally pass several soft, semi-liquid stools each day and it is practical to define diarrhea in this group as an increase in stool frequency or liquidity (Wardlaw *et al.*, 2010). Three clinical syndromes of diarrhoea include acute, dysentery and persistent diarrhoea each reflecting a different pathogenesis and requiring different approaches to treatment.

2.2.1 Acute Diarrhoea

This term refers to frequent episodes of diarrhea that lasts between 1-6 days and involves the passage of frequent loose or watery stools without visible blood (Wardlaw *et al*., 2010). Vomiting and fever may also be present and leads dehydration when fluid and electrolyte uptake is reduced and may also contribute to malnutrition due to loss of digested food. Most deaths occur due to severe dehydration. The most important causes of acute watery diarrhoea in young children in developing countries are rotavirus, Enterotoxigenic *Escherichia coli, Shigella, Campylobacter jejuni,* and *cryptosporidia*. In some areas, *Vibrio cholerae 01, Salmonella* and *enteropathogenic E. coli* are also important agents (Wardlaw *et al.*, 2010).

2.2.2 Dysentery

The term dysentery refers to passage of watery stool with visible blood in the faeces. Important effects of dysentery include anorexia, rapid weight loss and damage to the intestinal mucosa by the invasive bacteria. A number of other complications may also occur and the most important causes of acute dysentery are *Shigella*; others include *Campylobacter jejuni* and infrequently, *enteroinvasive E. coli* or *Salmonella. Entamoeba histolytica* can also cause serious dysentery in young adults but is rarely a cause of dysentery in young children (Alam *et al*., 2013).

2.2.3 Persistent Diarrhoea (PD)

This term refers to diarrhea that begins acutely but is of unusually long duration, at least 14 days or more often leading to malnutrition and weight loss (Wardlaw *et al.,* 2010). The episode may begin either as watery diarrhoea or as dysentery. Marked weight loss is frequent and diarrheal stool volume may also be great with a risk of dehydration. There is no single microbial cause for

persistent diarrhoea, however, *enteroadherent E. coli* and *cryptosporidium* may play a greater role among other agents (Alam *et al.*, 2013).

2.3 Epidemiology of Diarrhoea

2.3.1 Transmission of agents that cause diarrhoea

The etiological agents that cause diarrhea are usually spread by the faecal-oral route, which includes the ingestion of faecally contaminated water or food, person-to-person spread and direct contact with infected faeces. Some common habits that promote spread of enteric pathogens include unhygienic handling and preparation of food or allowing an infant to crawl, or play in an area where human or animal faeces are present (Alam *et al.*, 2013). Good sanitation is much more difficult in under-resourced, over-crowded communities and many of these infections are spread when food or water contaminated with faecal matter is ingested by the child. Diarrhoea may be common and severe when civil conflict or natural disasters force displaced populations to live in temporary, overcrowded shelters where it is difficult to achieve adequate sanitation and practice good hygiene (Goma Epidemiology Group, 1994). Such situations have been experienced in Kenyan internally displaced persons (IDP) and refugee camps.

2.3.2 Behaviors that increase the Risk of Diarrhoea in Children

A number of specific behaviors promote spread of enteric pathogens and thus increase the risk of diarrhea. These include failure to breast-feed exclusively for the first 4-6 months of life. The risk of developing severe diarrhea has been found to be far greater in non-breastfed infants than in infants who are exclusively breastfed (WHO, 2004). Moreover, the risk of death from diarrhoea is also substantially increased. The failure to continue breast-feeding for at least one year of age

is also a risk factor. Prolonged breast-feeding reduces the incidence or severity of certain types of diarrheal disease, such as shigellosis and cholera (Wardlaw *et al.,* 2010). Use of infant feeding bottles that are not properly sterilized also a predisposes to infant diarrhoea as they easily become contaminated with faecal bacteria and when milk is added to such bottles and not consumed immediately, further bacterial growth occurs. The storage of cooked food at room temperature is another risk factor especially when food is cooked and then saved to be used later, it may easily be contaminated, with surfaces or containers. When such food is kept for several hours at room temperature, bacteria in it can multiply several times (Alam *et al*., 2013).

2.4 Host Risk Factors to Diarrheal Diseases

Several host factors are associated with increased incidence, severity or duration of diarrhoea. They include malnutrition, current or recent measles infection and immunodeficiency (WHO, 2014). The frequency, severity, duration, and risk of death from diarrhoea are increased in undernourished children. Diarrhoea and dysentery are more frequent or severe in children with measles or who have had measles in the previous four weeks. This presumably results from immunological impairment caused by the current illness, immunodeficiency as in persons with the acquired immunodeficiency syndrome (Madigan and Martinko, 2005). In rare cases, overuse of antibiotics can lead to overgrowth of the normally harmless intestinal bacteria *Clostridium difficile* which releases a toxin that causes diarrhea that is quite difficult to treat. Similarly, some of the antiretroviral medications, particularly the protease inhibitors, commonly cause diarrhoea as a side-effect (Steuerwald *et al.,* 1997). Children who become constipated may also appear to have 'diarrhoea' when watery stool overflows around a hard impaction (WHO, 2002). Irritable bowel syndrome or lactose intolerance can cause chronic diarrhea, while

ano-rectal irritation caused by infections or overgrowth of candida can also lead to incontinence in a child (WHO, 2009).

Most enteric infections tend to be accelerated by carrier state. During such asymptomatic infections, which may last for several days or weeks, stools contain infectious viruses, bacteria, or protozoal cysts. Such asymptomatic carriers play an important role in the spread of many enteric pathogens, especially as they are unaware of their infection and take no special hygienic precautions as they move from one place to another (Tenover, 2006). In a study of non-faecal *Salmonella* isolates at the Massachusetts General Hospital, the most common risk factors were found to be corticosteroid use, malignancy, diabetes, HIV infection, prior antimicrobial therapy, and immunosuppressive therapy (Huang *et al.,* 2006).

2.5 Etiology of Diarrhoea

Transmission of enteric pathogens often occurs via faecal oral transmission. Currently, using new techniques, experienced laboratories can identify pathogens in about 75% of cases and up to 50% of milder cases detected in the community (Koplan, 1999). The organisms most frequently associated with acute diarrhoea in young children in developing countries are varied including; rotavirus, enterotoxogenic *Escherichia coli, Shigella, Salmonella species* and *Campylobacter jejuni* (Steiner *et al.*, 2000). Others of importance under special circumstances include *V. cholerae 01* in endemic areas and during epidemics, *non-typhoid Salmonella* in areas where commercially processed foods are widely used and enteropathogenic *E. coli* in hospitalized infants (Guerrant *et al.*, 2005). Mixed infections involving two or more enteropathogens occur

in 5-20% of cases seen at health facilities (Boschi-Pinto *et al.*, 2008). A number of other agents have been identified although their role is not well defined or minimal.

2.5.1 *Escherichia coli*

Escherichia coli are Gram-negative, facultative anaerobic and non-sporing bacteria. Cells are typically rod-shaped and about 2µm long and 0.5 µm in diameter, with a cell volume of 0.6- 0.7 μm^3 (Murray *et al.*, 2003). Strains are motile by means of flagella with peritrichous arrangement. *E. coli* live on a wide variety of substrates and use mixed acid fermentation in anaerobic conditions, producing lactate, succinate, ethanol, acetate and carbon dioxide. Optimal growth of *E. coli* occurs at 37°C but some laboratory strains can multiply at temperatures of up to 49°C (Nataro and Kaper, 1998). Growth can be driven by aerobic or anaerobic respiration, using a large variety of redox pairs, including the oxidation of pyruvic acid, formic acid, hydrogen and amino acids, and the reduction of substrates such as oxygen, nitrate, dimethyl sulfoxide and trimethylamine N-oxide (Huang *et al.,* 2006). Common routes of transmission of *E coli* include unhygienic food preparation, farm contamination due to manure fertilization, irrigation of crops with contaminated grey water or raw sewage pigs on cropland, or direct consumption of sewage-contaminated water. Dairy and beef cattle are primary reservoirs of *E. coli* O157:H7 (WHO, 2004) and carry it asymptomatically and shed it in their faeces. Food products associated with *E. coli* outbreaks include raw ground beef, raw seed sprouts or spinach raw milk, unpasteurized juice, unpasteurized cheese and foods contaminated by infected food workers via fecal-oral route (Paterson *et al.*, 2005).

Escherichia coli are best known for their ability to cause intestinal diseases. Five pathotypes of *E. coli* that cause diarrheal diseases are now recognized which include enterotoxogenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC). Each group falls within a serological subgroup and manifests distinct features in pathogenesis. ETEC is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. ETEC are acquired by ingestion of contaminated food and water but adults in endemic areas evidently develop immunity (Murray *et al.*, 2003). The disease requires colonization and elaboration of one or more enterotoxins with both traits being plasmid-encoded. ETEC may produce a heat-labile enterotoxin (LT) that is similar in molecular size, sequence, antigenicity and function to the cholera toxin. The heat stable (ST) causes an increase in cyclic Guano Monophosphate (cGMP) in host cell cytoplasm leading to the same effects as an increase in cyclic aminophosphate (cAMP). The heat stable toxin is known to act by binding to a guanylate cyclase that is located on the apical membranes of host cells, thereby activating the enzyme. This leads to secretion of fluid and electrolytes resulting in diarrhoea (Obi *et al.*, 2004a)**.**

EIEC closely resemble *Shigella* in their pathogenic mechanisms and nature of clinical illness. They penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes a dysentery-like diarrhea with fever. EIEC apparently lack fimbrial adhesins but do possess a specific adhesin that is thought to be an outer membrane protein. Also, like *Shigella*, EIEC are invasive organisms. They do not produce heat labile (LT) or heat stable (ST) toxin. EPEC induce a profuse watery, sometimes bloody, diarrhea. They are a leading cause of infantile diarrhoea in

developing countries. Outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products. Pathogenesis of EPEC involves a plasmid-encoded protein referred to as EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells and a non fimbrial adhesin, which is an outer membrane protein that mediates the final stages of adherence but do not produce ST or LT toxins (Banajeh *et al.*, 2001).

2.5.2 *Shigella*

Shigella species are small, non-capsulated, non-motile Gram negative rods. There are four species of *Shigella* classified on the basis of biochemical and serological differences namely; S. *dysenteriae* (Group A), *S. flexneri* (Group B), *S. boydii* (Group C), and *S. sonnei* (Group D). The geographical distribution and pathogenicity of the four species of *Shigella* are different though the reasons for this are still unclear (Obi and Bessong, 2002). *S. flexneri* is the most commonly isolated species in the developing world and the most frequent cause of morbidity and mortality. *S. dysenteriae* infections also occur in less developed countries, often in epidemics, with periodic pandemic outbreaks. In industrialized countries with good water and sanitation, the predominant species is *S. sonnei*. In general, illness caused by *S. sonnei* is less severe but individual cases of infection with any of the *Shigella* species can be severe (Huang *et al.*, 2007). Infection with *Shigella* protects against subsequent infection with the same serotype; however, because there are multiple serotypes, individuals may become infected several times.

Shigella is spread mostly from person-to-person by the faecal-oral route, via food or water. Contaminated hands often contaminate food that is then served to others but proper handwashing decreases rates of transmission. In some regions, disease rates increase somewhat during the

monsoon season, perhaps due to increased faecal contamination of drinking-water (Hossain and Albert, 1991). There is also evidence that flies, particularly *Musca domestica*, the common housefly, may serve as vectors for the transmission of shigellosis owing to the low inoculum needed to cause disease. Several features of *Shigella* contribute to its invasiveness and pathogenicity. The bacteria are able to invade enterocytes in colonic and rectal epithelia and lyse intracellular phagocytic vacuoles, thus escaping into the cytoplasm where they multiply and then invade adjacent cells. The ability of *Shigella* to colonize the epithelial layer protects the bacteria from exposure to the extracellular environment. Intracellular growth and multiplication ultimately result in the death of epithelial cells, with resultant ulceration and mucosal inflammation (Keusch and Bennish, 1998).

The physical signs and symptoms of shigellosis include abdominal cramps, fever and chills, malaise, diarrhea and / or dysentery, and abdominal tenderness (WHO, 2004). Examination of the rectal mucosa shows it to be inflamed and friable, with ulcers present in severe cases. *Shigella* are the most important cause of dysentery, being found in about 60% of all episodes, and in nearly all severe episodes; watery diarrhea may also occur. *S. flexneri is the mos*t common serogroup in developing countries, but *S. dysenteriae type 1*, which occurs in regional epidemics, causes the most severe disease. Tissue destruction and possibly watery diarrhea are caused in part by the extremely potent *shiga* toxin, produced in relatively large amounts by *S. dysenteriae type 1* (Villamor *et al*., 2004).

2.5.3 *Salmonella*

The bacteria belonging to genus *Salmonella* are gram-negative, non acid fast, non capsulated and non-sporing bacilli, which measure approximately 2-4mm x 0.6 mm. Some species of *Salmonella* are motile whereas others are non-motile (Hossain and Albert, 1991). The organisms grow rapidly on ordinary media and optimum temperature of growth is 37^0C. The *Salmonella* spp. other than *S. typhi* and *S. paratyphi* are reported as opportunistic bacterial infections in HIV/AIDS patients (Oni *et al.*, 1991). The genus *Salmonella* are potential enteric pathogens and a leading cause of bacterial foodborne illness. In addition, *Salmonella* species have been implicated in a spectrum of other diseases, including enteric or typhoid fever (primarily *Salmonella typhi* and *Salmonella paratyphi*), bacteremia, endovascular infections, focal infections such as osteomyelitis and enterocolitis, (typically *Salmonella typhimurium* and *Salmonella enteritidis* (Urio *et al.,* 2001).

The transmission of salmonellae to a susceptible host usually occurs via consumption of contaminated foods and drink. The most common sources of *salmonellae* include beef, poultry, and eggs (Kariuki *et al.,* 2006). Infection with salmonellae is characterized by attachment of the bacteria pili to cells lining the intestinal lumen. *Salmonellae* selectively attach to specialized epithelial cells (M cells) of the Peyer patches where the bacteria are then internalized by receptor-mediated endocytosis and transported within phagosomes to the lamina propria, where they are released. Once there, *salmonellae* induce an influx of macrophages (typhoidal strains) or neutrophils in non typhoidal strains

(Abu-Elamreen *et al.,* 2008). The severity of illness in individuals with salmonellosis is determined not only by the virulence factors of the infecting strain but also by host properties (Kariuki *et al*., 2006).

2.6 Pathogenic Mechanisms in Enteropathogens

2.6.1 Mucosal adhesion.

Bacteria that multiply within the small intestine must first adhere to the mucosa to avoid being swept away. Adhesion is caused by superficial hair-like antigens, termed pili or fimbriae that bind to receptors on the intestinal surface. This occurs, for example, with enterotoxigenic *E. coli* and *V. cholerae 01*. In some instances, mucosal adherence causes changes in the gut epithelium that may reduce its absorptive capacity or cause fluid secretion (Paton and Paton, 1998). Enterotoxigenic *E. coli, V. cholerae 01* and possibly other bacteria such as *Salmonella*, use intestinal secretion by producing toxins that alter epithelial cell function; these toxins reduce the absorption of sodium by the villi and may increase the secretion of chloride in the crypts, resulting in net secretion of water and electrolytes. Recovery occurs when the intoxicated cells are replaced by healthy ones after 2-4 days (Viswanathan *et al.*, 2009).

2.6.2 Toxin Production

Enterotoxigenic *E. coli, V. cholerae 01* and possibly other bacteria such as *Salmonella*, cause intestinal secretion by producing toxins that alter epithelial cell function; these toxins reduce the absorption of sodium by the villi and may increase the secretion of chloride in the crypts, resulting in net secretion of water and electrolytes. Recovery occurs when the intoxicated cells are replaced by healthy ones after 2-4 days (Viswanathan *et al.*, 2009).

2.6.3 Mucosal invasion

Shigella, C. jejuni and enteroinvasive *E. coli* cause bloody diarrhea by invading and destroying mucosal epithelial cells. This occurs mostly in the colon and the distal part of the ileum. Invasion is followed by the formation of micro abscesses and superficial ulcers indicated by the presence of red and white blood cells, or occult blood in the stool. Toxins produced by these organisms cause tissue damage and possibly also mucosal secretion of water and electrolytes (Huang *et al.*, 2007).

2.7 Prevalence and Etiology of Childhood Diarrhoea in relation to Age and Sex

Diarrhoea related mortality rates in developed countries have declined considerably in recent years due to provision of good quality water, improved sanitation and advances in healthcare (Prester *et al.*, 2003). However, in Sub-saharan Africa and developing countries with high poverty index, the burden of HIV and underlying conditions such as malnutrition has increased the risk of contracting acute and persistent diarrhoea (Fletcher *et al.*, 2011). The movement of persons within the same region and from one country to another increases the chance of transmission, thus requiring comprehensive and first-hand information on peculiar situations in each locality. In a quest to unearth the unanswered questions surrounding the burden and etiology of childhood diarrhea in developing countries, the Bill and Melinda Gates Foundation through the Global Enteric Multicenter Study (GEMS) recently reported that pathogens responsible for cases of moderate-to-severe diarrhea in young children from low-income countries are responsible for large-scale alterations in intestinal microbiota composition and that overall genus-level microbiota composition exhibits a shift from high to low levels of *Escherichia* and *Shigella* in younger than older children (Pop *et al.*, 2014). The study reported

diarrheagenic *E. coli* (30%), ETEC (15.4%), Shigella (10.5%), *Salmonella* (8.4%) and *Campylobacter spp.* (8.3%), as the most common bacterial pathogens and confirmed high rates of isolation of pathogens from diarrhoea cases with age related differences.

Some studies have reported variations in etiology of enteric pathogens as well as age related differences in Kenya and other developing countries. In a clinic-based sentinel surveillance for bacterial diarrheal illness undertaken in Asembo Bay, rural community bordering Lake Victoria, Kenya (Brooks *et al*., 2006), a region with high infant and child mortality rates established that the main enteric pathogens from diarrheic stools of children aged <5 years were *Campylobacter* (42%) and diarrheagenic *E. coli* (34%), whereas *Shigella* (65%) was common among children above years. Another study in Busia, Kenya, (Onyango and Angienda, 2010), evaluated the epidemiology of waterborne diarrheal illness among children aged 6-36 months and demonstrated a prevalence of 16.7% with higher frequencies in children aged 6-17 and 36 months. Elsewhere in Bondo District, Nyanza, Kenya, (Tornheim *et al*., 2010), reviewed the epidemiology of hospitalizations of children with diarrhea at all inpatient facilities and established that diarrhea was responsible for 11.2% of hospitalizations with incidence being highest in infants and children. Peak diarrhea incidence was also reported one to two months after heavy rains with notable co-diagnosis with malaria, pneumonia, HIV and tuberculosis.

A study on bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya (Central, Eastern, Nairobi and North Eastern), demonstrated diarrhoea prevalence of 17.7%, with the main pathotypes being EPEC *shigatoxigenic E coli* (0.5%), *Salmonella* (3.5%), *Shigella* (2%) and *Vibrio cholera* O1 (0.7%), (Sang *et al.*, 2012). In another study in Nairobi,

Kenya, Makobe *et al*., (2012), demonstrated that the most predominant diarrheagenic *E. coli* (DEC) was EPEC (19.3%), ETEC (7.2%), EAEC (3.9%), STEC (0.96%) and EIEC (0.48%) with 50.7% of the isolates being from male subjects while 49.3% were from female subjects.

In Tanzania, Moyo *et al*., (2011), examined age-specific etiologic agents of diarrhoea in children aged less than five years in Tanzania, and established that diarrhoea was predominant in ages 0- 12 months with bacteria and viruses accounting for 33.2% and 32.2% of all diarrhoea cases respectively, while parasites were detected in 19.2% patients. Diarrheagenic *Escherichia coli* (DEC) were the most common enteric pathogens (22.9%), followed by *Cryptosporidium parvum* (18.9%), rotavirus (18.1%) and norovirus (13.7%). The study observed a seasonality pattern in relation to diarrhoea. A similar study in Kathmandu Nepal, (Ansari *et al.*, 2012), showed that bacterial infection was highest (69.9%) in the age group of 6-24 months, 19.2% in 0- 6 months and 2.7% was in 49-60 months. Among the total enrolled cases, the prevalence of *Shigella* was 24 (4.6%) followed by *Escherichia coli* 12 (2.3%) and *Salmonella* 10 (1.9%) with higher rates in boys (64.2%) than girls (35.8%).

Variations in diarrhoea etiology in various geographical regions have also been recorded. A study in Southeast Nigeria, (Nweze, 2010), observed the etiology of diarrhoea among patients and healthy subjects and confirmed that several bacterial pathogens, such as *E. coli,* play an important role in the etiology of acute diarrhoea. Other studies from North Africa (Tripoli, Libya) and Middle Eastern (Saudi Arabia), reported prevalence rates of enteropathogens between 46% and 61% (El-Sheikh *et al*., 2001; Ali *et al*., 2005), while a study in a small semi-urban city of Libya, demonstrated that causative agents of acute diarrhea, in order of decreasing

frequency were bacteria, viruses, and protozoa Ali *et al*., (2005). Rotavirus was the most common enteropathogen associated with childhood diarrhea in Libya being detected in nearly 27% of cases. Previous studies from other two major cities in Libya reported higher rates of rotavirus in (31.9%) in Tripoli and (21%) in Benghazi while non-typhoidal *Salmonella* were documented as the major bacterial causes of diarrhoea in children in Tripoli.

Variations in distribution of pathotypes have also been demonstrated in various regions. A similar study in Botswana (Urio *et al*., 2001), reported the isolation of *Salmonella* from 3% of children with diarrhea with only 2 serotypes, *Salmonella typhimurium* and *Salmonella paratyphi* while in Calcutta India, *Salmonella* associated childhood diarrhea was reported in 4 different serotypes with majority (70%) being *Salmonella typhimurium* (Guerrant *et al.,* 2005). Pathotypes of DEC were EPEC (66.6%), followed by ETEC and EHEC (16.7%) each while *Shigella* strains included; *Shigella boydi* (50.0%), *Shigella sonnei* (25%), *Shigella flexneri* (20.8%) and *Shigella dysenteriae* 1 (4.2%), *Salmonella typhi* (20.0%) and S. *paratyphi* 1 (10.0%) were reported among *Salmonella* isolates.

These studies demonstrate the high burden of childhood diarrhoea in developing countries. Isolation rates of principal pathogens varied considerably with age with diarrheagenic *E. coli*, *Shigella, Salmonella* and *Campylobacter* as the main agents. The differences in the number and types of serotypes may be attributed to geographical, social and life-style differences between different communities. There were differences in enteropathogens investigated by these studies which may explain the differences in detection rates with most studies, investigating only one pathogen, a limitation that does not give a true reflection of the overall burden of infectious

pathogens. Rotavirus was the most common virus isolated while *E. coli* predominated among the bacteria. While the etiological agents and their mechanisms of pathogenesis have been elucidated, information on the prevalence of these agents in various regions of Kenya is largely unknown.

Current research indicates that the magnitude of bacterial diarrhea in developing countries is largely unknown and published studies regarding childhood diarrhea are limited to Nairobi and Nyanza, which have well established research centers where laboratory capacity, trained health care and resources are concentrated. Furthermore, despite the growing concern of childhood diarrhoea in Kenya, no study has established prevalence, etiology and related characteristics of infectious agents with regard to HIV status at MTRH. It is therefore important to understand the prevalence and etiology of pathogens responsible for diarrhoea in Kenya and more specifically MTRH, serving the Western Kenya region where infant morbidity and mortality due to diarrhoea is reportedly high.

2.7.1 Prevalence of Diarrhoea versus HIV status

The human intestinal tract is home to a complex community of microbial species which serve as markers of gastro-intestinal health (Zhang *et al.*, 2005). HIV/AIDS disease progression is predicted by viral RNA levels and CD4+ T lymphocyte counts which decline with increase in viral load apparently due to destruction of infected T-cells (Hammer et *al.*, 2006). HIV RNA level is an independent predictor associated with risk of opportunistic infections (OIs). HIV infection increases the incidence and severity of all childhood diseases, including diarrhoea with persistent / chronic diarrhoea being one of its presenting features (Nte and Eneh, 2008). The

prevalence and distribution of bacterial pathogens causing diarrhoea in HIV positive cases has been shown in various studies to include bacteria, parasites, and fungi.

A study to determine HIV seropositivity in children admitted with diarrhoea at Eldoret District Hospital, Kenya (Buku and Esamai, 1994), demonstrated that out 57 children who participated in the study, 50.9% cases were HIV positive with no gender difference. A higher proportion of HIV positive (70.8%) had persistent diarrhoea of over 14 days duration compared to HIV negative (36.7%) cases with diarrhoea of shorter duration. HIV positivity was relatively higher (80%) in patients who presented with pneumonia and malnutrition in addition to diarrhoea compared to children who presented with diarrhoea and vomiting alone. In a nested cohort study in Kisumu, Kenya (van Eijka *et al.*, 2010), compared the frequency and etiology of diarrhea in children aged below 2 years with known HIV status and showed that stool cultures were less likely to yield a bacterial pathogen in HIV positive compared to HIV negative children, while *Campylobacter* was the most frequent bacterial pathogen (20.8%) of stool cultures, followed by *Shigella* (5.4%) and *Salmonella* (3.5%). A similar study to establish the relationship between diarrhea, CD4+ T-cell counts and enteric infections in a community based cohort of HIV infected adults in Uganda, (Brinck *et al*., 2002), demonstrated that diarrhea was common and strongly correlated to low CD4 counts with bacteria being frequently isolated in 49% of diarrheal stools and 39% of stools from asymptomatic individuals.

A study to establish the etiologies of acute, persistent, and dysenteric diarrhea in adults in Bangui, Central African Republic in relation to HIV serostatus revealed that *Shigella* spp., *Campylobacter* spp., and *Entamoeba histolytica* were found in HIV-1 and HIV-2 dysenteric

patients (Gassama *et al.,* 2001). A similar study on the prevalence of intestinal parasitic and bacterial pathogens in diarrhoeal and non-diarroeal human stools from Vhembe District, South Africa showed that bacterial organisms, such as *Campylobacter*, *Salmonella*, *Shigella* and different groups of enteropathogenic *E. coli* were common causes of gastrointestinal diseases (Guerra *et al*., 2009). Studies conducted before the widespread use of anti-retroviral therapy, reported diarrhoea as one of the most common complications of HIV disease among young children even in the United States, where sanitation and water safety are good (Mathews *et al*., 2000). In parts of the world where sanitation and water safety are poorer, diarrhoea is much more common and studies have suggested that diarrhoea is even more likely in children with HIV and the leading cause of death among HIV-infected infants (Pavia *et al*., 1992; Thea *et al*., 1993; van Eijka *et al*., 2010). A hospital–based cohort of HIV-infected patients around Varanasi, India (Suresh *et al.*, 1993), demonstrated that the positivity of finding a pathogen from watery and formed stools was 40% and 24% respectively and was likely to be associated with inflammation. However, the same was not true with the acute diarrhea where risk of harboring the opportunistic infections remained the same. Diarrhea accounted for 13% of all deaths in Indian children younger than 5 years with significant morbidity. Similarly, Khan *et al*, (2013), indicated that *Vibrio cholerae, Shigell*a and *Salmonella* spp. were commonly associated with neonatal diarrhoea in Bangladesh.

The studies indicate a dynamic variability of etiologic agents by geographic region, patient age, immune status and socioeconomic conditions and that persistent diarrhoea related morbidity and mortality is significantly increased by HIV seropositivity. Despite high burden of HIV infection in Sub-Saharan Africa, there is very limited data to compare prevalence of childhood diarrhoea

and other factors among HIV positive and negative children. It is therefore necessary to scale-up surveillance, control and prevention programmes for both HIV and diarrhoea in order to improve case management in the region.

2.8 Molecular Pathotypes of *E coli, Salmonella* and *Shigella*

Molecular epidemiological techniques have led to enhanced detection of outbreaks worldwide and appear to give a better picture of epidemiology of infectious diseases. Studies have demonstrated the sensitivity of molecular-based methods to be greater compared to current conventional methods of analysis (Bisi-Johnson *et al.,* 2011). Gastrointestinal infections due to pathogenic *Enterobacteriaceae* in particularly *Escherichia* and *Salmonella and Shigella* are significant causes of morbidity and mortality worldwide (Okeke *et al*., 2007). These infections are usually self-limiting and may be fatal in hosts with debilitating immune systems (Sack *et al*., 2007). The fatality of infections due to these enteric pathogens depends on their serotypes, size of the inocula and status of the host (Thong *et al.*, 2005). The major distinguishing features between pathogenic and non-pathogenic strains is the presence of virulence genes which code for the various known strategies for pathogenecity such as the ability to adherence, colonization, and invasion factors (Bisi- Johnson *et al*., 2011). The virulence determinants give each pathotype the capacity to cause a clinical syndrome with distinctive epidemiologic and pathologic characteristics, (Brown *et al.,* 2004) and are ideal targets for the determination of the pathogenic potential of any given isolate (Bekal *et al.*, 2003).

A review of studies done in developing countries demonstrates some understanding of pathotypes of *Escherichia coli*, *Salmonella* and *Shigella* associated with diarrhoea. A study on

virulence properties of diarrhoeagenic *Escherichia coli* among patients and healthy subjects in South Eastern Nigeria (Nweze, 2010), showed that 50 (49%) were EPEC, 22(21.57%), ETEC and 7.84% EAEC, with no gender differences. EAEC was found in significant proportions (4.5%) among cases than controls, emerging as the most common bacterial cause of diarrhea although not associated with foreign travel or immunodeficiency. A similar study in Maputo, Mozambique on genetic determinants of pathogenicity of *Escherichia coli* isolated from children with acute diarrhea (Sumbana *et al.*, 2015), demonstrated the complexity of the etiology of diarrhea caused by pathogenic *E. coli* and the risk of the emergence of new pathogenic variants due to the horizontal transmission of pathogenicity factors with EIEC (21%) being the most prevalent DEC with 19% being EPEC, 15% EAEC (15%), 13% ETEC (13%), 5% DAEC (5%) and 1% hybrids. However, studies in Brazil and Mexico (Huang *et al.*, 2007; Contreras and Antonios., 2011), showed that 30%-40% of childhood diarrhea was attributed to EPEC with most strains isolated being typical (*eae*+, *bfpA*+) while only two were atypical (*eae*+, *bfpA*-).

A study on molecular characterization of virulence factors in diarrheagenic *Escherichia coli* isolates from children In Nairobi, Kenya, (Makobe *et al.,* 2012), demonstrated using conventional PCR assay, the predominance of EPEC (19.3%), ETEC (7.2%), EAEC (3.86%), STEC (0.96%) and EIEC (0.48%). These findings differed slightly with a similar study in Igembe District Hospital, Kenya, (Karambu *et al.,* 2013), which identified the main pathotypes as ETEC 9.1%, EPEC 6.8% and EAEC 12.3%, *Salmonella paratyphi* (10.4%), *Shigella flexneri* (1.9%) and *Shigella dysentriae* (0.9%). In Romania, Codruja-Romanita, (2009), also identified *Escherichia coli* pathotypes associated with diarrhea in children under five years as EAEC (29 isolates), atypical EPEC (22 isolates), ETEC *coli* (8 isolates), and VTEC (1 isolate), with only one isolate being categorized as unconventional DEC. Other studies inTanzania (Moyo *et al.*,

2011), and South America (Melo *et al.*, 2008), confirmed that although only a single isolate was investigated for each subject, the prevalence of *E. coli* isolates carrying virulence-associated genes was surprisingly high. Similarly, in Brazil, Araujo *et al*., (2007), further established that typical EAEC and atypical EPEC are the most prevalent diarrhea-associated pathotypes among children.

2.8.1 Virulence Genes in *E coli, Salmonella* and *Shigella* and their role in Diarrhoea

Escherichia and *Salmonella* species are reported to have diverged from a common ancestor based on the evolutionary rate estimates from 5S and 16S rRNA sequence analyses while *Shigella* spp. are considered clonal lineages of *Escherichia coli* (Thong *et al.*, 2005). The major distinguishing factor between pathogenic and non-pathogenic strains of *E. coli* is the occurrence of virulence genes, which code for the various known strategies for pathogenecity. Diarrheagenic *Escherichia coli* (DEC) as a group is known to be responsible for 30% to 40% of acute diarrhoea and chronic episodes in children in developing countries (O'Ryan *et al*., 2005) and developed world (Nataro *et al*., 2006; Cohen *et al.,* 2005). Six categories of DEC have been recognized in various studies including; Enterohaemorrhagic (EHEC), *shigatoxin* producing (EPEC), enteroinvasive (EIEC), Enteroaggregative (EAEC or EAggEC), Enterotoxigenic (ETEC) and diffusely adherent *E. coli* (DAEC), (Nataro and Kaper, 1998; Huang *et al*., 2006). These categories have different virulence mechanisms (Weintraub *et al*., 2007) *which* are genetically coded for by chromosomal, plasmid and bacteriophage DNAs (Huang *et al.,* 2007) and include heat-labile toxin (*lt*) and heat-stable toxin (*st*) in ETEC enteroaggregative mechanisms (Eaegg) in (EAEC) and enteroinvasive (EIEC) mechanisms (Nataro and Kaper, 1998).

EHEC is a highly infectious pathogen that colonizes the distal ileum and large bowel in humans and is often the causative agent of outbreaks of severe gastroenteritis in developed countries. Its main virulence factor is the phage-encoded shiga toxin (stx_1 and stx_1), a defining characteristic of the shiga toxin-producing EHEC O157:H7. ETEC is a common cause of travellers' diarrhoea and is known to have fatal consequences for children below 5 years of age (Croxen and Brett, 2010). ETEC is anchored to enterocytes of the small bowel through colonization factors (CFs) encoding *eae* and *bfp* genes and secretes two toxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) which lead to increased intracellular levels of cyclic Amino-phosphate (cAMP) and cyclic Guano-monophosphate (GMP) resulting in impaired absorption of Na+ and H_2O influx into the lumen resulting in copious quantities of watery stool (Turner *et al.*, 2006).

EAEC is recognized as a cause of endemic and epidemic diarrhoea worldwide that is often watery but may be accompanied by mucus or blood. The characteristic phenotype of EAEC is aggregative adhesion, which involves the formation of a stacked-brick pattern of HEp-2 cells mediated by the genes encoded on virulence plasmids (Guerrant *et al.,* 2005; Nataro *et al*., 2006). EAEC colonization occurs in the mucosa of both the small and large bowels leading to mild inflammation in the colon through secretion of cytotoxins (Kaper *et al*., 2004). It is known to attach to enterocytes in the bowels through aggregative adherence fimbriae (AAF) that stimulate a strong interleukin-8 (IL-8) response allowing biofilms to form on the surface of cells. EPEC is a major cause of potentially fatal diarrhea in infants in developing countries (Kaper *et al*., 2004). This pathotype belongs to a forms attaching and effacing (A/E) lesions on intestinal epithelial cells. The initial attachment of EPEC to enterocytes in the small bowel is thought to involve the bundle-forming pilus (*bfp)* and *eae* gene on EPEC adherence factor (EAF) plasmid

encoding the adhesin intimin responsible for the intimate attachment of the bacteria to the epithelial cells (Gassama *et al*., 2005). EIEC has similar pathogenic mechanisms as *Shigella* and is associated with bloody diarrhea similar to shigellosis (Kaper *et al*., 2004). Virulence is largely due to a plasmid such as *ipaH* that encodes genes required for invasion, cell survival and apoptosis of macrophages (Ogawa *et al.*, 2008; Schroeder and Hilbi, 2008). The virulence genes in DEC are often located on transmissible genetic elements that can be transferred to *E. coli* recipient strains.

Salmonella species possess virulent chromosomal genes such as *invA* (Malorny *et al*., 2003; *iroB* (Soumet *et al*., 1998), *invE* and *slyA* (Contreras and Antonios, 2011). They may also contain fimbriae genes such as *fimy* (Yeh *et al.,* 2002) and *sefA,* (unique sequences such as *Sdf I* (Agron *et al*., 2001) and shiga toxin gene, ST (Dobrindt *et al*., 2003) and finally plasmid genes such as *spv* (Soumet *et al.*, 1999). The *invA* gene of *Salmonella* contains sequences unique to this genus and has been proved to be a suitable PCR target with potential diagnostic application (Jamshidi *et al*., 2008). This gene is recognized as an international standard for detection of *Salmonella* genus (Malorny *et al*., 2003). The gene is essential for full virulence in *Salmonella* and is thought to trigger the internalization required for invasion of deeper tissue (Petti *et al*., 2006). The *sefA* gene,on the other hand, is a good candidate for specific detection of *S. enteritidis* (Woodward and Kirwan, 1996). Strains of *S. enteric* serovar enteritidis often carry serovar-associated plasmids which encode a virulence operon consisting of five genes *spvR,* h*spvA, spvB, spvC* and *spvD* (Aabo *et al*., 2000; Petti *et al.*, 2006). The *spv* genes play a role in the virulence of the host strain (Chiu *et al*., 2006). It is possible that virulence plasmid is sequentially or independently formed by recombination and hybridization (Seas *et al*., 2000;

Malorny *et al*., 2003). These genes can be horizontally transferred and mobilized by an F or F-like conjugative plasmid between the *Salmonella* strains and species (Chiu *et al*., 2006). One main function of the *spv* operon is to potentiate the systemic spread of the pathogen (Seas *et al*., 2000). This potential associated with multidrug-resistance of *spv* operon, *spvR, spvA, spvB, spvC, spvD* virulence plasmids. The integration of resistance genes and additional replicons into a *Salmonella* virulence plasmid constitutes a new and interesting example of plasmid evaluation posing a serious threat to public health.

Salmonella enteritidis (60 kb) present in a few serovars of subspecies of *S. enterica* are responsible for the systemic infection and multidrug resistance in both humans and animals (Chiu *et al*., 2006; Gebreyes *et al*., 2009). They are also responsible for the induction of intracellular bacterial proliferation and apoptosis of infected macrophages (Heithoff *et al*., 2008). The carriage of *spv* gene may increase the propensity of *Salmonella* strains to be of major clinical relevance (Gebreyes *et al*., 2009). Heithof *et al*., (2008), showed that *Salmonella* serovar *typhimurium* isolates driven from human gastroenteritis patients often lose the *spv* gene and accordingly, lack the capacity to cause systemic disease in mice. In a study on molecular detection of *invA* and *spv* virulence genes in *Salmonella enteritidis* isolated from human and animals in Iran, Kumars (2010), found that the presence of spvA, *spvB,* and *spvC* genes in *S. enteritidis* from human source was 90%. *Spv*A, *spvB,* and *spvC* genes were present in 100% of the bovine source isolates. In the case of *S. enteritidis* in poultry source, the presence of *spvA*, *spvB,* and *spvC* was 88.6%. Regarding the presence of virulent plasmid genes in *S. enteritidis,* another study reported lack of *spvC* gene in 7.2% of the samples from human, pig and poultry

sources (Chiu *et al*., 2006) and while analyzing 38 isolates from *Salmonella* serovars with two primers *invA and spvC,* reported the presence of *invA* in all strains (100%).

The genus *Shigella* comprises four subgroups namely; *S. flexneri, S. boydii, S. sonnei,* and *S. dysenteriae* (Sansonetti, 2001). Depending on the virulence potential of the strain and the nutritional status of the individual, shigellosis can progress to severe disease when accompanied by rectal tenesmus, with neurological symptoms such as headache and lethargy (Thong *et al*., 2005). Members of *S. flexneri*, *S. dysenteriae*, *S. sonnei* and *S. boydii* continue to be responsible for mortality and / or morbidity in high risk populations such as children under five years of age (Thong *et al*., 2008). Virulence genes responsible for the pathogenesis of shigellosis may be located on the chromosome or on the *inv* plasmid borne by the organism. They are often multifactorial and coordinately regulated while the genes tend to be clustered in the genome. Previously reported PCR-based detection methods concentrated mainly on the *ipaH* gene alone (Gaudio *et al.*, 1997; Dutta, 2001), or on *ipaH* and *ial* genes in two separate PCR assays (Sethabutr, 1993). The *ial gene* is found on the large *inv* plasmid which is prone to loss or deletions while *ipaH*, on the other hand, is present on both the *Shigella* chromosome and on a large plasmid and is a more stable gene to detect. However, the sole presence of *ipaH* is not an absolute indicator of virulence as loss or deletion of the plasmid renders the bacterium non-invasive and therefore avirulent. *Set1A* and *set1B* are chromosomal genes encoding *Shigella* enterotoxin 1 (*ShET1*), which cause the watery phase of diarrhea in shigellosis (Hale, 1991; Fasano, 1995; Chiu, 1996). *Ial* and *ipaH genes* are responsible for directing epithelial cell penetration by the bacterium and for the modification of host response to infection, respectively (Van Asten and Dijk, 2005).

A study on the detection of virulence genes in Malaysian *Shigella* species by multiplex PCR assay (Thong *et al*., 2005), revealed the presence of chromosomal and plasmid-encoded virulence genes (*set1A*, *set1B*, *ial* and *ipaH*) in *Shigella.* The *ipaH* sequence was present in all the strains, while each of the *set1A*, *set1B* and *ial* genes was present in 40% of the strains tested. Reproducibility of the m-PCR assay was 100% and none of the non-*Shigella* pathogens tested in this study was amplified. Atypical EPEC strains could be classified in two main virulence groups based on their content of OI-122, *lpfA*, and *yjaA* genes. Among children with diarrhea, atypical EPEC isolates belonging to virulence group I (OI-122) and *lpfA* positive, *yjaA* negative were the most common, while the majority of isolates from healthy children were classified as virulence group II strains (OI-122 negative, *lpfA* and *yjaA* positive (Vieira, 2001).

Arising from the reviewed studies, molecular epidemiological techniques have led to enhanced detection of outbreaks worldwide and appear to give a better picture of epidemiology of infectious diseases. The studies show evidence of shared genetic strategies for pathogenicity in enteric bacilli and that various combinations of virulence genes are required for a successful emergence of a pathogen. Most virulence factors of pathogenic *E. coli*, *Shigella* and *Salmonella* strains are plasmid-borne; however, one or more of the essential virulence determinants are extrachromosomal elements and may extert different virulence mechanisms. These findings confirm the great adaptability of certain types of *E. coli* based on the genomic plasticity of the species. In addition, the molecular methods targeting chromosomal or mobile genetic elements coding for virulence traits seem to be the most reliable for diagnosis of enteropathogens, however lack of appropriate infrastructure for diagnosis in most public health institutions in Kenya and other developing countries is restricted to basic conventional methods.

There is therefore need to determine molecular pathotypes of enteric pathogens to enhance understanding of the mechanisms by which enteric bacterial pathogens colonize, spread and at times persist in the hosts especially in HIV cases with a view to improve the surveillance of childhood diarrhoea in the region.

2.9 Treatment of Diarrhoea

2.9.1 Rehydration and Supplementation

Treatment of acute diarrhea is dependent on the severity of clinical signs and symptoms. Dehydration is managed according to WHO guidelines which recommend administration of oral rehydration salts (ORS) and zinc as well as continued feeding (WHO, 2008; WHO and UNICEF, 2009). ORS is the 'gold standard' oral rehydration therapy that enhances fluid replacement while Zinc reduces the duration and severity of diarrhea episodes, stool volume and the need for advanced medical care (Boschi-Pinto *et al.*, 2008). Food intake supports fluid absorption from the gut into the bloodstream to prevent dehydration and helps maintain nutritional status and immunity (WHO, 2004; WHO, 2014). Though fluid and electrolyte replacement is the treatment of choice for acute diarrhea, antibiotics are also indicated for treatment of suspected shigellosis, invasive salmonellosis and diarrheagenic *E coli* infections (Huang *et al*., 2007).

At Moi Teaching and Referral Hospital, ReSoMal (Nutriset, Malaurny, France) is provided for oral rehydration initially observed by nursing staff. For children too weak or unable to rehydrate orally, a nasogastric tube is inserted for rehydration and feeding and additional ReSoMal is given for every loose motion. Intravenous rehydration is reserved for children with impaired

consciousness, persistent vomiting, and shock (defined by WHO in severe malnutrition as altered conscious level, a weak and fast pulse and a temperature gradient (cool hands and /or feet). Half strength Darrow's solution with 5% dextrose or Ringers Lactate with 5% dextrose is prescribed and given according to WHO treatment guidelines (WHO, 2014). Depending on the severity of clinical symptoms, antibiotics may be administered but in less severe cases, stool is recommended for culture and sensitivity to determine the most suitable antimicrobial agent. The antibiotics in common use include ampicillin, cotrimoxazole, benzylpenecillin, amikacin, gentamicin, ciprofloxacin, ceftriaxone, imipenem, cefuroxime, ceftazidime and cefipime.

2.9.2 Antibiotic Susceptibility of *E coli, Salmonella* and *Shigella*

Antibiotics play a key role in treating diseases of bacterial origin, a major cause of morbidity and mortality in the developing world. Early administration of antibiotic treatment is highly effective in eliminating infections but indiscriminate use of antibiotics has led to the emergence of multidrug-resistant strains. Antimicrobial drug resistance is a large and growing problem among organisms that cause diarrheal disease. Although most diarrheal diseases are self-resolving and should not be treated with antimicrobial agents, invasive or protracted infections require chemotherapy and are typically managed empirically (Okeke *et al*., 2007). Recent data from Gabon, Nigeria, and Tanzania (Gascon *et al*., 2000; Nweze, 2010 ; Moyo *et al*., 2011), suggest that resistance among causative organisms of these infections, such as enterotoxigenic, enteropathogenic, and enteroaggregative *Escherichia coli,* appears to be rising. Although oral rehydration therapy has drastically reduced deaths from the disease, prolonged infectious bouts of diarrhea have long-term consequences for physical and cognitive development.

Notable drug-resistant Enteropathogenic *E. coli* outbreaks and sporadic cases have been reported from several African countries, including Kenya and Tanzania (Moyo *et al*., 2011; Makobe *et al*., 2012). The more recently defined enteroaggregative *E. coli* are typically multidrug-resistant and are one of the most common causes of childhood diarrhea, particularly persistent infections (Sang *et al*., 2012). Antimicrobial drug–resistant diarrheagenic *E. coli* pathotypes, including enteroaggregative *E. coli*, are also emerging as important diarrheal pathogens in AIDS patients (Obi and Bessong., 2002).

Widespread antibiotic usage exerts a selective pressure that acts as a driving force in the development of antibiotic resistance. The association between increased rates of antimicrobial use and resistance has been documented for nosocomial infections as well as for resistant community acquired infections. As resistance develops to "first-line" antibiotics, therapy with new, broader spectrum, more expensive antibiotics increases, but is followed by development of resistance to the new class of drugs. Resistance factors, particularly those carried on mobile elements, can spread rapidly within human and animal populations. Multidrug-resistant pathogens vary not only locally but also globally, with newly introduced pathogens spreading rapidly in susceptible hosts (Tenover., 2006).

A study on bacterial aetiology of diarrhoeal diseases and antimicrobial resistance in Dhaka, Bangladesh between 2005–2008 (Ahmed *et al*., 2012), showed varying degree of resistance of *Shigella* to cotrimoxazole, nalidixic acid and ampicillin, and a sharp increase in ciprofloxacin resistance for *S. flexneri* isolates. *Salmonella* showed resistance to nalidixic acid, ampicillin, cotrimoxazole and chloramphenicol, while similar studies in Indonesia and Tanzania reported

increasing resistance of *Shigella* to chloramphenicol, ampicillin (Navia *et al*., 1999; Urassa *et al*, 2000; Ahmed *et al*., 2012)   In Uruguay, (Fernández-Cruz *et al*., 2010), examined antibiotic susceptibility in cases of bloody diarrhoea and demonstrated that Trimethoprim-sulphamethoxazole (TPM-STX) and Ampicillin (AMP) are inappropriate for the empiric treatment of bacterial diarrhoea (BD) cases. In addition, *Shigella* strains showed some degree of cross-resistance to ciprofloxacin (CIP) and nalidixic acid (NAL) and the use of fluoroquinolones was recommended for treating *Campylobacter*, *Salmonella* and *Shigella* associated gastroenteritis and ciprofloxacin for children with bacterial diarrhoea (BD) and for severe cases only.

In Kenya, Sang *et al.,* (2012), identified that the antibiotic with the highest prevalence of antimicrobial resistance was ampicillin followed by TPM-STX and tetracycline. Though still at low levels, the major concern was the emerging resistance of enteric pathogens to quinolones (ciprofloxacin, nalidixic acid, norfloxacin) and gentamycin. Elsewhere in rural Western Kenya, a study on antimicrobial resistant bacteria (Shapiro *et al*., 2001), established that 51% of the isolates were not susceptible to antimicrobial treatment and recommended re-evaluation of empiric treatment strategies for diarrheal disease in western Kenya to improve clinical care. Furthermore, another study among adults in Kenya demonstrated that the prevalence of multi-drug resistant non-typhoidal *Salmonella* (MDR NTS) had risen from 31% in 1993 to 42% in 2003, with high resistance to extended-spectrum cephalosporins and flouroquinolones (Kariuki *et al*., 2001). Infection with MDR NTS was associated with an increased rate and duration of hospitalization, a twofold increased risk of death during a two-year period after infection and an increased rate of invasive infection. However, most MDR NTS isolates were susceptible to

cefotaxime and ciprofloxacin which are expensive drugs that are not easily accessible to the general population (Kariuki *et al*., 2006). Multidrug resistant NTS was believed to be an important cause of invasive disease and death among young children and HIV infection was found to be a significant risk factor for NTS bacteria (Kariuki *et al*., 2004; Kariuki *et al*., 2006; Okeke *et al*., 2007).

A study on antibiotic resistance patterns of pathogenic *Escherichia coli* from HIV positive adults at Mbagathi District Hospital, Nairobi, Kenya showed that 87% of persons living with HIV/AIDS (PLWHA) having diarrhea were resistant to TPM-STX, tetracycline and ampicillin respectively (Emacar *et al*., 2009). The antibiotic resistance for isolates from PLWHA and taking antibiotics (HIV+ A+) was significantly higher compared to isolates from HIV persons not taking antibiotics (HIV+ A-). Onyuka *et al.,* (2011), in a study on multidrug resistance of *Salmonella enterica* serovars *Typhi* and *Typhimurium* isolated from clinical samples at two rural hospitals in Western Kenya, observed resistance in patients to Chloramphenicol, Cotrimoxazole, and Streptomycin with *S. typhimurium* showing higher resistance than *S. typhi.* The same resistance pattern was observed for *S. typhimurium;* however both the isolates were sensitive to Ciprofloxacin. A study to compare prevalence of antibiotic-resistant *E. coli* from healthy adult volunteers in both urban areas (Kenya, Mexico, Peru and the Philippines) and rural areas (Venezuela, Ghana and Zimbabwe), showed higher rates of resistance to Ampicillin (89 %) and tetracycline (92 %) in the urban areas of Kenya than in the rural areas of Ghana and Zimbabwe (Nys *et al.,* 2004).

The occurrence of multi-drug resistance has been reported in several studies done globally. A study in India, on antibiotic sensitivity and plasmid profiles of *Escherichia coli* isolated from pediatric diarrhea (Uma *et al.*, 2009), showed high resistance of *E. coli* to ampicillin, chloramphenicol, cotrimoxazole, imipenem, nalidixic acid and norfloxacin. In Hanoi, Vietnam, a study to compare antibiotic resistance of four groups of *E. coli* and demonstrated that EAEC were significantly more susceptible to CIP than EPEC and ETEC while EAEC were more resistant to CHL (Nguyen *et al.*, 2005). EAEC also showed higher resistance to AMP and SXT compared to ETEC. Among *Shigella* isolates only one was strain *S. sonnei* was susceptible to all antibiotics tested while all others (*S. boydi* and *S. dysenteriae)* showed multi resistance. This agreed with findings in Kenya that described four cases of diarrhea caused by multi-antibiotic-resistant EAEC (Sang *et al.,* 2012). A study on bacterial etiology of acute diarrhea in under five year old children in Kathmandu, Nepal (Ansari *et al.*, 2012), showed that chloramphenicol, tetracycline and gentamicin were effictive against *Salmonella, Shigella* and *Escherichia coli* whereas isolates of *Salmonella* were resistant to ampicillin *in-vitro*.

Codruja-Romanita *et al.*, (2009), in Tripoli, demonstrated through phenotypic testing that the susceptibility of the *E. coli* isolates carrying virulence-associated genes, and showed that 79% were resistant to at least one antibiotic with highest resistance exhibited against ampicillin (42 isolates), streptomycin (34 isolates), TPM-STX (28 isolates), and tetracycline (23 isolates) but different patterns existed among different *E. coli* pathogenic groups, confirming at least seven multidrug-resistant isolates as ESBL producers belonging to ETEC atypical EPEC and EAEC. Increased frequency of drug-resistant strains is of great concern, since resistance to first-line drugs requires more expensive drugs for effective treatment. It is for this reason that CDC

remarks (2015), "Almost every type of bacteria has become stronger and less responsive to antibiotic treatment when it is really needed. These antibiotic-resistant bacteria can quickly spread to family members, schoolmates, and co-workers- threatening the community with a new strain of infectious disease that is more difficult to cure and more expensive to treat." For this reason, antibiotic resistance is among CDC's top concerns.

Overuse of antimicrobials may also expose patients unnecessarily to potential toxicities, prolong illness or increase risk of death (Legros *et al*., 1999). In most of the reviewed studies, antibiotic susceptibility of only one pathogen was studied with *E. coli* being the most commonly studied pathogens in humans, followed by *Salmonella, Shigella* and *Vibrio* species. However, *E. coli*, *Salmonella* and *Shigella* sp. isolates were most commonly tested for resistance to ampicillin, chloramphenicol, ciprofloxacin, cotrimoxazole, gentamycin and tetracycline among others. The major limitation in these studies is that most present findings of antimicrobial resistance surveillance conducted only at referral and private hospitals. The resistance to ampicillin and imipenem may be due to production of beta-lactamases enzymes while the most common mechanism for resistance to cotrimoxazole is acquisition of plasmid-encoded folate reductase enzymes.

Few reports have examined the epidemiology of diarrheal pathogens or looked at drug resistance in rural settings. The increased prevalence of strains exhibiting multi resistance to commonly available, less costly oral antibiotics represents a growing obstacle to the simple, cost-effective therapy of invasive childhood diarrhea in Sub-Saharan Africa. The alarming increase in rates of resistance of many diarrheal pathogens may relate to the frequent use and abuse of antibiotics.

Access to current antimicrobial susceptibility data is of importance to clinicians and is of particular significance to physicians treating hospitalized patients. Knowledge about susceptibility patterns of bacteria in different geographical could inform guidelines for empirical therapy and thereby preserve the utility of available medicines by limiting the development of antimicrobial resistance.

2.9.3 Mechanisms of Antimicrobial Resistance

Bacterial resistance is a matter of great concern to physicians as can result in treatment failure which has serious consequences especially in critically ill patients (Tenover, 2006). Bacteria may manifest resistance to antibacterial drugs through a variety of mechanisms. Some species acquire resistance to antibacterial agents, proliferate and spread under the selective pressure of use of that agent. This may be achieved by acquisition of genes encoding enzymes such as beta lactamases that destroy the antibacterial agent. Secondly, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach the target site and exert its effect.

Thirdly, bacteria may undergo mutation and selection to produce altered bacterial cell wall that no longer contains the binding site for the antimicrobial agent or may acquire mutations that limit access of antimicrobial agents to intercellular targets via down regulation of porin genes a process termed vertical evolution (Islam *et al*., 2015). Resistance genes may also occur through several genetic exchange mechanisms including transformation, conjugation or transduction and is termed horizontal evolution. This occurs between strains of the same species or between different bacterial genera (Guillemot, 1999). In each case, transposons may facilitate transfer and incorporation of acquired resistance genes into host genome or into plasmids. During

conjugation, a gram negative bacteria transfers plasmid containing resistance genes into adjacent bacteria via sex pilus joining the two organisms (Tenover, 2006). During transduction, resistance genes are transferred from one bacterium to another via a bacteriophage, a rare phenomenon. Transformation is a process where bacteria acquire and incorporate DNA fragments from other bacteria that have released their DNA into the environment after cell lysis and move resistance genes into previously susceptible strains. Mutation, selection and genetic exchange mechanisms enable species to adapt quickly to introduction of antibacterial agents to their environment (Tenover, 2006; WHO, 2014).

E. coli and related bacteria also possess the ability to transfer DNA via bacterial conjugation, transduction or transformation, which allows genetic material to spread horizontally through an existing population. This process has led to the spread of the gene encoding *shigatoxin* from *Shigella* to E. coli O157:H7, carried by a bacteriophage (Wise and Andrews, 1998). The resistance to ampicillin and imipenem may be due to production of beta-lactamases enzymes while the most common mechanism for resistance to cotrimoxazole is acquisition of plasmid-encoded folate reductase enzymes.

2.10 Clinical outcomes of *in-vivo* Antibiotic Susceptibility Testing

Interventions to delay morbidity and mortality from diseases such as diarrhoea and pneumonia can make a huge contribution to the long-term survival of HIV-infected, exposed or uninfected infants and children (WHO, 2010). Although the existing interventions such as safe water, ORS and appropriate case management have been responsible for reduction in mortality of children worldwide, the coverage remains low, particularly in resource-poor settings. It is critical to

isolate the specific pathogen in life-threatening infections, especially in situations that are likely to require prolonged therapy (Siniravin and Garner, 2006). The timing of initial therapy is usually guided by the urgency of the situation. In critically ill patients, such as those in septic shock, febrile neutropenic patients and patients with bacterial meningitis, empiric therapy should be initiated immediately after or concurrently with collection of diagnostic specimens (Leekha *et al.,* 2011). Premature initiation of antimicrobial therapy in these circumstances can suppress bacterial growth and preclude the opportunity to establish a microbiological diagnosis, which is critical in the management of diarrhea cases.

Antibiotic resistance surveillance programs in most parts of the world have shown that *E. coli* has a substantial likelihood of being resistant to ampicillin or amoxicillin and that amoxicillin would be a poor empirical choice for treatment of an infection in which *E. coli* is a probable cause (Marteyn *et al.*, 2012). Increasingly, clinical data suggest that many infections with gram-negative bacilli require a shorter duration of therapy than has historically been thought necessary (Chastre *et al*., 2003). When identification and susceptibility testing results are available to the clinician, antibiotic regimens can be fine tuned. Even with the best efforts at optimizing initial empirical regimens, surprises sometimes occur, necessitating intensification of antibiotic therapy. Close liaison with clinical microbiologists may allow clinical use of preliminary identification data. An important role of the microbiology laboratory is to alert the clinician on the presence of new virulent strains not routinely covered by standard empiric therapy or preliminary diagnosis of an infectious agent (Paterson, 2008). This should allow coverage of this organism with trimethoprim/ sulfamethoxazole if a clinically significant infection is apparent (Paterson and Doi, 2007).

Despite these not being final identification or susceptibilities, a 24-h “head start” on the initiation of therapy with antibiotics not typically used as empirical therapy, such as colistin or trimethoprim/sulfamethoxazole, may provide some clinical benefit.

A study to evaluate clinical outcomes (Fernández-Cruz *et al*., 2010), showed that patients in the group with rapid reporting of susceptibility testing had fewer days of fever, decreased antibiotic consumption, decreased rates of *C. difficile* infection, and fewer days of receiving mechanical ventilation.

Antibiotics administration has been found to shorten the duration of diarrhoea and prevent serious complications associated with the infection (Sirinavin and Garner, 2009). In some settings where bacteria (*Salmonella or Shigella*) are known to be causes of diarrhea, antibiotics are given presumptively to all patients with clinically diagnosed infectious diarrhea whilst awaiting culture results. In other settings, antibiotics are given following culture results (definitive treatment). Whereas stool cultures and antimicrobial testing of the isolates is the ideal way to select the most adequate antimicrobial regimen, culture and sensitivity testing is not automated in most public hospitals in Kenya and results are usually available in not less than 48 hours. Consequently, therapy is often empiric and guided by the clinical presentation which may lead to variations in clinical outcomes. However with increased automation, closer liaison between a clinician and microbiologist will greatly facilitate the decision-making process and must be prepared to alter the dose and or infusion rates for certain patients, relying on evidence from small clinical trials to support such actions until they are confirmed in larger clinical trials.

2.11 Diagnosis of Bacterial Enteropathogens

Molecular studies provide a clear understanding of the genomic make-up of bacteria which generally consist of stable regions and variable regions, the flexible part that is composed of bacteriophages, plasmids, transposons as well as unstable large regions, called genomic islands (Bansal, 2008). The magnitude of bacterial diarrhea in developing countries is largely unknown since affordable detection methods are not available in all healthcare settings. Most epidemiological data on diarrheal pathogens from developing countries is scattered since infrastructure for approved testing system for the identification of the strains is limited (Priyadarshan, 2011). The only methods available in few district hospitals are the conventional methods culture, biochemical testing and serology although limited antisera may be available. This method requires a minimum of 48 h for identification of isolates. A review of of efficient, rapid, and simple methods for detection of enteric pathogens is critical for selection of best choice for applicatioHn in resource poor settings for effective control and prevention of childhood diarrhoea (Ogawa *et al.*, 2008).

2.11.1 Culture and Biochemical Tests

Conventional methods used in detection of bacteria and viruses mainly depend on the culture and biochemical identification of bacterial genera (Tebbs *et al.,* 2012). These methods are sensitive and inexpensive, but are both time and material-consuming and can delay the proper diagnosis and treatment regime, resulting in longer hospital stays (Scallan *et al.,* 2011). Culture is used to describe the growth of viable and cultivatable bacteria using commercially prepared growth media. Traditional methods for diagnosis of disease are achieved by culturing bacteria on agar plates followed by its phenotypic and serological properties or histological examination

(Bukar *et al*., 2005). These techniques have some disadvantages such as need for previous isolation of the pathogen and inability to detect low levels of pathogen due to low sensitivity (Imen *et al.,* 2007).

2.11.2 Serological Tests

Immunological-based methods have become a broadly used method for enteric bacteria because they permit sensitive and specific detection. Immunological assay based on antibodies is a technology employed for the detection of bacterial cells, spores, viruses and toxins. Methods based on antigen–antibody interaction have been applied for the detection of food-borne and enteric pathogens in various studies (Araujo *et al.*, 2007; Gómez-Duarte *et al.*, 2010). Polyclonal and monoclonal antibodies are used in these methods. Although, the immunological detection methods are not as specific and sensitive as nucleic acid-based detection, they are faster, more powerful and have the ability to detect both contaminating organisms and their toxins that may not be expressed in the organism's genome.

The detection of the presence of *Salmonella* O and H-antigens were tested by slide agglutination with the commercially available antisera. One loop of appropriate antisera was dropped onto a cleaned glass slide. One loop of overnight culture grown on agar was dispersed in the drop to obtain a homogeneous and turbid suspension. The slide was rocked gently for 30 s and clumping was monitored by a magnifying glass. Serotyping is easy to perform and standardized antisera are commercially available. However, it only allows the assignment of *a* strain such as *Salmonella typhi* to a specific serotype and no further differentiation between strains of the same serotype. Serological analysis usually remains the first step in an epidemiological study and

may be sufficient for investigations associated with uncommon serotypes (Ridha *et al*., 2007). However, smaller labs often do not have access to the pools of serum required for this analysis and may need to rely on other techniques to analyze isolates (Imen *et al.*, 2007).

2.11.3 Analytical Profile Index

The analytical Profile Index (API) system is a version of conventional methods developed for quick identification of the family Enterobacteriaceae and other Gram-negative bacteria. This system consists of a plastic strip with 20 small reaction tubes, containing the separated compartments. The API test system is manufactured by bioMerieux Corp., Marcy Etoile, France. This assay is considered the "gold standard" with an overall sensitivity of 79%. In this technique, a reaction occurs within 24 hours. This system has been useful for identifying pathogenic *Yersinia* isolates, E coli, and other enteric pathogens with the highest sensitivity both at the genus and at the species level (Ohud, 2012). However, the kit and reagents for this diagnostic procedure are costly and are used only in reference and research laboratories.

2.11.4 Phage typing

Individual isolates of many serotypes such as *E. coli*, *Salmonella or Shigella* vary in their susceptibility to lysis by different bacteriophages and this has led to a typing scheme based on reactivity to a panel of bacteriophage. Therefore, a *Salmonella* strain is subjected to a specified set of typing phages and the lytic pattern obtained commonly allows the assignment to a specific phage type. Phage typing is mostly performed for serotypes such as *S. Typhimurium*, *S. enteritidis*, *S. Typhi* or *S. paratyphi*, although phage typing systems are also available for a number of additional serotypes. Phage typing has proven to be an important tool for strain

characterisation and the results obtained have been used since the mid-90s in surveillance, source attribution and outbreak investigations (Hald *et al*., 2007). In general, phage phage typing is only performed by the National Reference Centers, since only these institutions have access to the defined sets of typing phages. The interpretation of the results requires considerable experience (Riley, 2004).

2.11.5 Plasmid profile analysis

Plasmid profile analysis is one of the earliest DNA-based subtyping schemes. It is particularly important, since most of the plasmids harbour virulence and antimicrobial resistance properties. Plasmid content of the host within the same serotype reveals the differentiation according to the profile (the number and molecular sizes of plasmids) obtained. The different plasmid profiles within a serotype points to the lateral transfer by gaining or loosing the plasmid(s). The plasmids found in *Salmonella* differ in size with different functionalities (Imen *et al*., 2007). The detection method is based on the isolation of plasmids followed by agarose gel electrophoresis. To view the plasmid pattern, agarose gel must be stained with ethidium bromide solution and then visualized under UV light. Plasmid analysis has several limitations. Plasmids can rapidly be acquired or lost and single predominant plasmids have become endemic within various serotypes (Gómez-Duarte *et al.,* 2009).

2.11.6 Polymerase Chain Reaction

2.11.6.1 Single PCR

Monoplex PCR can detect a single copy of a target DNA sequence and amplifies a desired region of genome into billions of copies among a complex mixture of heterogeneous sequences

(Rahimi *et al*., 2012). PCR is used for the detection of the pathogenic microorganisms in food or human samples by utilizing nucleic acid for detection. This assay has advantages over culture and other methods for the detection of microbial pathogens such as specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample (Farthing *et al*., 2013; Brooks *et al*., 2006). PCR based methods are used for the detection of a broad range of pathogens like *Staphylococcus aureus, Listeria monocytogenes,* (*Salmonella* (Ali *et al*., 2005). The different forms of PCR based on their methods are real-time PCR, multiplex PCR, and reverse transcriptase, RT- PCR (Ogawa *et al*., 2008).

2.11.6.2 Real Time PCR

RT-PCR is also described as multiplex RT-PCR (Amani *et al*., 2015). Real-Time PCR provides an opportunity for rapid detection of pathogens in food (Amani *et al*., 2015). Real-time PCR combines PCR chemistry with fluorescent probe detection of the amplified product. This method is simpler to carry out compared to conventional PCR method and its test result is fast (Espy *et al.,* 2006). Two kinds of chemical agents are available for real-time PCR products: fluorescent probes that bind specifically to definite DNA sequences and fluorescent dyes that intercalate into any dsDNA (Amani *et al.*, 2015). The simplest and most cost-effective methods employed are sequence independent DNA-binding dyes such as SYBR Green and SYBR Gold, which bound to dsDNA. The advantage of this method is the high sensitivity and specificity, low contamination risk, ease of performance and speed. These attributes have made real-time PCR assay an appealing alternative to conventional culture-based or immunoassay-based testing methods (Espy *et al.,* 2006). TaqMan PCR (Fluorescent probe based real-time PCR) can

amplify target nucleic acid sequences from selected microbes in the samples collected from complex biological environments.

2.11.6.3 Multiplex PCR

Multiplex PCR refers to simultaneous amplification of multiple gene targets using two or more primer pairs directed at pathogen-specific unique sequences within a single reaction. It allows for the simultaneous amplification of more than one target sequence by using multiple sets of oligonucleotides to amplify two or more targets of interest (Amani *et al.*, 2015). The method is applied for the simultaneous detection of several foodborne pathogens. For example, simultaneous detection of *E. coli* O157:H7, *Salmonella* spp. and *E. coli.* Advantages of multiplex PCR include multiple targets that are amplified significantly without extra time, cost, or sample volume; however, there have been reports that multiplexing can reduce sensitivity compared with single reactions because of competition. The disadvantage of multiplex PCR is the competition between oligonucleotide pairs that can reduce PCR sensitivity (Alikhani *et al*., 2013). The optimization of multiplex PCRs can pose several difficulties, including poor sensitivity or specificity and/or preferential amplification of certain specific targets.

The presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers (Brown *et al*., 2004). These nonspecific products may be amplified more efficiently than the desired target, consuming reaction components and producing impaired rates of annealing and extension. Thus, the optimization of multiplex PCR should aim to minimize or reduce such non-specific interactions (Bukar *et al.,* 2005). Nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. Although this adaptation is undoubtedly effective in most cases, it also considerably complicates

the practical application of PCR. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation. Multiplex PCR has been recognized as a rapid, specific, and cost-effective molecular method that has demonstrated its efficient discrimination in serotyping of the most common and food isolates of *S. enterica* subsp. *enterica* (Imen *et al.*, 2007). This technique can be used as an alternative method of standard serotyping in many clinical laboratories.

2.11.7 Pulsed Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) is useful and a gold standard for detection of food-borne zoonotic bacteria that the most important of them are *S. enterica*, *Campylobacter* spp., *E .coli*, *Shigella* spp., *Vibrio cholera*, and *L. monocytogenes* (Demissie *et al*., 2014). This technique is based on molecular assays, culture, and isolation of the bacterial strain from the samples. Using this method, it is possible to validate a full genome; however, genes with small size such as plasmids are not visible on PFGE. Therefore microbiological culture and isolation is needed for detection before the PFGE assay (Melo *et al*., 2008). By cutting the bacterial DNA with rare-cutting restriction endonucleases and running with special electrophoresis separation techniques which use pulsed currents that change polarity at defined intervals, it separates the large fragments of DNA up to 12000 kb and yields strain specific patterns. PFGE has been used to determine whether molecular subtyping was effective in detecting unsuspected clusters or outbreaks of *S. typhimurium* (Bender *et al*., 2008).

PFGE is characterized by a high degree of reproducibility both within and between laboratories (Imen *et al*., 2007). The recent introduction of computerized gel-based data collection and

analysis systems allows better standardization between laboratories thus creating the ability to rapidly compare restriction fragment patterns from isolates analyzed from remote locations (Rahimi *et al*., 2012). PFGE, however, is not always successful. Some serotypes, especially those with certain distinct phage types, can be so genetically homogeneous that multiple genotypic techniques fail to discriminate outbreak from non-outbreak strains. A study in Canada, evaluated the ability of PFGE to differentiate *S. enteritidis* strains that developed during an outbreak of gastroenteritis that was eventually traced to contaminated cheese (Ahmed *et al.,* 2013). Successful discrimination was only achieved with a combination of intensive epidemiological, genotypic and phenotypic methods.

2.11.8 Determination of Prevalence of Diarrhoea and Molecular Pathotypes of Enteric pathogens.

Bacterial pathogens and their toxins can cause illnesses such as diarrhea and spread through population causing more and more outbreaks of disease every year. Children are the most vulnerable and require urgent attention to prevent dehydration, other serious sequelae or in worse cases, death. Therefore, rapid and reliable detection methods are needed for detection, control of infections and maintenance of a research database to guide in planning for treatment interventions as this is lacking in most hospitals in Kenya and more so at Moi Teaching and Referral Hospital. Conventional methods for the detection of enteric pathogen bacteria are sensitive and provide more accurate results. However, traditional standard culture methods require long turnaround time for enrichment and confirmation of presumptive isolates and may require several days to
obtain results.

Molecular methods are based on immunochemical and nucleic acid technologies and are alternatives for conventional methods, because these methods can provide results within hours. Unfortunately, molecular techniques require multiple molecular-grade reagents, equipment and technical expertise most of which are simply not available in many geographical settings in the developing world. As a result of inadequate laboratory facilities in most district and county referral hospitals in Kenya, limited research has been carried out to evaluate trends in prevalence and pathotypes of pathogenic strains agents. There is therefore a need to strike a balance within laboratories and use cost-effective and standard conventional methods for pathogen detection. Hence the choice of diagnostic methods used in this study was meant to achieve intended results by leveraging available diagnostic facilities.

2.12 Antimicrobial Susceptibility testing Method (Kirby Baeur)

The responsibility of the microbiology laboratory includes not only microbial detection and isolation but also the determination of microbial susceptibility to antimicrobial agents. Many bacteria, in particular, have unpredictable susceptibilities to antimicrobial agents, and their susceptibilities can be measured *in-vitro* to help guide the selection of the most appropriate antimicrobial agent. Antimicrobial susceptibility tests are performed by either disk diffusion or a dilution method. In the former, a standardized suspension of a particular microorganism is inoculated onto an agar surface to which paper disks containing various antimicrobial agents are applied. Following overnight incubation, any zone diameters of inhibition around the discs are measured and the results are reported as either; sensitive, intermediate or resistant to each antimicrobial agent tested.

An alternative method is to dilute on a log scale each antimicrobial agent in broth to provide a range of concentrations and to inoculate each tube or, if a microplate is used, each well containing the antimicrobial agent in broth with a standardized suspension of the microorganism to be tested. The lowest concentration of antimicrobial agent that inhibits the growth of the microorganism is the minimal inhibitory concentration (MIC). The MIC and the zone diameter of inhibition are inversely correlated. In other words, the more susceptible the microorganism is to the antimicrobial agent, the lower the MIC and the larger the zone of inhibition.

Continued evaluation and revision of the disc diffusion procedure has been performed by a committee of the Cinical Laboratories Susceptibility Index (NCCLS, 2007). Designating a national standard test for disk diffusion has not only permitted more exacting quality control, but has also allowed valid comparison of results among different laboratories using the procedure. The disk diffusion method of antimicrobial susceptibility testing involves the placement of antibiotic impregnated disks onto Mueller-Hinton agar plates. As soon as the disk comes in contact with the moist agar surface, water is absorbed into the filter paper and the antibiotic diffuses into the surrounding medium. The rate of extraction of the antibiotic out of the disk is greater than its outward diffusion into the medium, so that the concentration immediately adjacent to the disk may exceed that in the disk itself. As the distance from the disk increases, however, there is a logarithmic reduction in the antibiotic concentration. If the plate has been previously inoculated with a bacterial suspension, simultaneous growth of bacteria occurs on the surface of the agar.

When a critical cell mass of bacteria is reached, the inhibitory activity of the antibiotic is overcome and bacterial growth occurs. The time required to reach the critical cell mass is characteristic of each species but is influenced by the composition of the medium and temperature of incubation (Alikhani *et al.*, 2013). The points at which the critical cell mass is reached appear as a sharply marginated circle of bacterial growth, with the middle of the disk forming the center of the circle. The concentration of diffused antibiotic at this interface of growing and inhibited bacteria is known as the critical concentration and approximates the MIC. The zone diameters that are generated by the test are meaningless without reference to the minimum inhibitory concentration (MIC) correlates and interpretative guidelines published by the CLSI.

Surveillance data and hospital or unit antibiograms may inform this decision, although individualization of the initial regimen on the basis of prior antibiotic use and prior isolation of resistant pathogens may be more important (Paterson, 2008). Combinations of antibiotics are often required empirically, and "combination antibiograms" may need to be developed for this purpose. Preliminary data suggest that extending the time over which a dose of antipseudomonal b-lactam antibiotics is infused may improve clinical outcomes; however, this idea remains to be confirmed in randomized trials . The role of direct susceptibility testing in aiding more-rapid initiation of appropriate antibiotic therapy is also being studied (Kaul *et al.*, 2007). When identification and susceptibility testing is complete, the antibiotic regimen for infections due to gram-negative pathogens can be "fine tuned."

The choice of Kirby Baeur disc diffusion method to determine antibiotic susceptibility profiles in this study was based on the fact that most hospitals in Kenya prescribe antibiotics based on availability of the drug rather than the effectiveness of the drug to treat existing infections. There is usually a common "out of stock" scenario that forces patients to take what is available or buy their own drugs, which in most cases are cheap generic drugs that are ineffective and likely to fuel antibiotic resistance.

2.13 Determination of Clinical outcomes of Diarrhoea cases

Serious infections with gram-negative pathogens continue to be associated with considerable mortality (Paterson, 2008). Increasing antibiotic resistance in organisms such as *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Klebsiella pneumoniae* is contributing to difficulties with choosing antibiotics to prescribe for these infections. Optimization of therapy against these organisms starts with the initial empirical antibiotic choice. The lack of new antibiotic options against gram negative pathogens underscores the need for optimization of current therapies and prevention of the spread of these organisms. Antibiograms (in vitro laboratory tests for testing bacterial sensitivity to antibiotics) are often taken into account to define a rational selection of an empirical antimicrobial therapy for treating patients with enteric infections. However, they are not always reliable.

Recent studies have indicated that an '*in vivo- in vitro* paradox' does exist and microbiological resistance determinations in vitro are not always predictive of treatment outcomes *in vivo* (Bishai, 2002). It is seemingly possible that pathogen virulence changes with time and sensitivity to antibiotics also change (Kaul *et al*., 2007) resulting in variations in clinical outcomes.

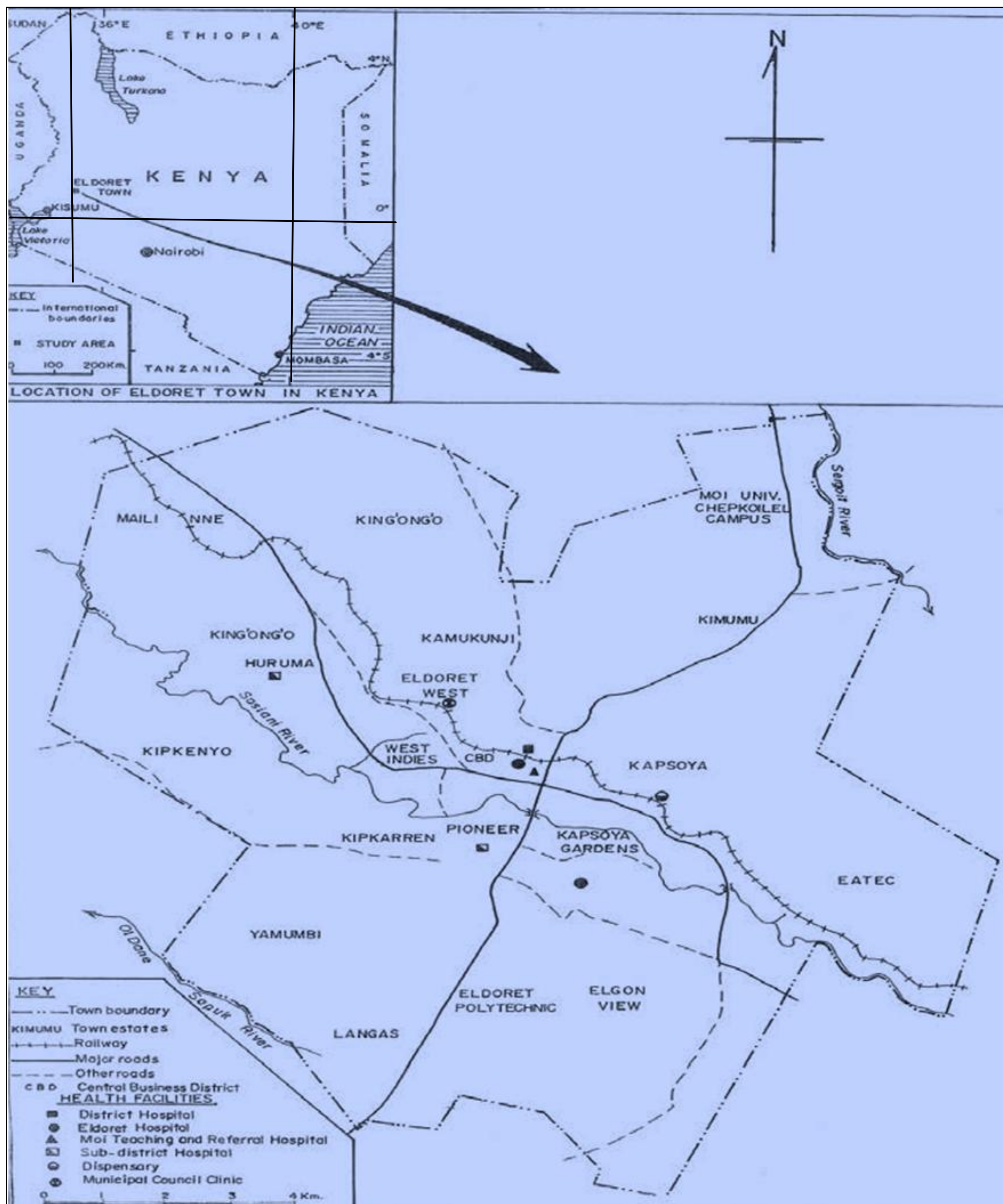
Although most hospitals have records that can be be used to evaluate clinical outcomes. Limited studies in Kenya have analysed and documented this information or related *in-vitro* and *in-vivo* relations in disease. Restrospective evidence can used to determine clinical outcomes of *in-vivo* antibiotic susceptibility from cases undergoing treatment for bacterial diarrhoea or other infectious diseases handled in hospitals.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Site of Study

The study was conducted at Moi Teaching and Referral Hospital (MTRH) in Eldoret, Uasin-Gishu County located about 320 km North West of Nairobi between latitude 0° 31' 54" N and longitude 35° 15' 58" E and lies at an altitude of 2,103 metres above sea level. This is the second largest Referral Hospital in Kenya serving the Western Kenya region including the North and South Rift Valley, Nyanza, Western Province, parts of Eastern Uganda and Southern Sudan. Kenya is a developing country located in sub-Saharan Africa, bordered by Tanzania, Somalia, Uganda, Sudan, and Ethiopia (**Figure 2**). 63% of households in Uasin Gishu County have access to improved drinking water sources, with large discrepancies among urban and rural residents while 75% of households do not have access to an improved sanitation facility predisposing to gastrointestinal infections (KNBS, 2010).

Moi Teaching and Referral Hospital currently has a bed capacity of 750. It also has an antenatal clinic, several medical wards serving various categories of patients including paediatric ward, a Voluntary Counseling and Testing (VCT) centre and diagnostic laboratories. This study was conducted in Tumaini and Upendo Paediatric wards. The two wards have a total bed capacity of 100 but exceeded this at certain peak seasons. Admission in the two wards was done on alternate days, with an average of 25 admissions per day. The average duration of stay in the ward was three days but varied from one individual to another depending on the severity and type of illness. The USAID–Academic Model for Prevention and Treatment of HIV/AIDS (AMPATH) Partnership's clinics and laboratories located within the hospital provide free diagnosis, monitoring and treatment for HIV/AIDS patients.



Source: Uasin Gishu County Ministry of Lands

Figure 2: Map showing Location of MTRH and Health facilities in Western Kenya

3.2 Research Design

This was both a hospital based cross sectional prospective and retrospective study design.

Study participants were children below five years admitted at the paediatric wards with acute or persistent diarrhoea as reported by clinicians. Study participants were placed into two categories, HIV positive and HIV negative cases as confirmed by rapid HIV test paerformed at baseline on admission. Three levels of data was targeted for collection including primary data on baseline demographic characteristics recorded at admission and laboratory diagnostic tests and secondary data on determination of clinical outcomes.

3.3 Study Population

The study involved children aged below five years admitted at MTRH between July 2011 and April 2012 who were either from within Eldoret or referred from other health centres or district hospitals in neighbouring counties. The target group was children admitted following severe diarrhea, characterized by three or more loose stools within a 24-hour period with signs of dehydration indicated by the presence of feeble or impalpable pulse, unrecordable blood pressure, plus one of the following signs; sunken eyes, hoarse voice, protracted skin pinch retraction, and washerwoman's hands. A total of 1382 children were admitted to the hospital during the study period out of which 216 were diarrhea cases from HIV positive and negative groups. HIV serostatus was routinely determined on admission using HIV screening rapid methods; Determine (Abbott, Tokyo, Japan) and Uni-Gold_ (Trinity Biotech, Ireland) while HIV positive cases were confirmed by Western blot assay.

HIV status was categorized as positive, negative or sero-exposed. Based on the criteria laid down by the Center of Disease Control and Prevention, Atlanta (CDC, 2008), a HIV antibody positive case aged 0-18 months, born to a HIV positive mother was considered HIV-sero-exposed while a child above 18 months was considered HIV positive if found to be antibody positive and confirmed positive by Western blot assay. Children who were aged above 18 months and were neither HIV infected nor sero-exposed (antibody negative) were considered HIV negative. However, for purposes of this study, only HIV positive and negative diarrhoea cases were investigated.

3.4 Sample Size Determination

According to Tornheim *et al.* (2010), diarrhoea accounted for 11.2% of hospitalizations in all inpatient facilities in in Bondo District, Nyanza, Kenya. This prevalence value was used to calculate sample size using McCrum-Gardner (2007) Formula or G-power, to compare proportions of HIV seronegative and seropositive children with diarrhoea. According to this formula, sample size estimation should be based on the primary outcome measure, but if there is more than one outcome then the largest sample size should be chosen so that all the outcome measures are fully powered. A statistical power of 80% and significance level (α) = 0.05 was used to calculate sample size (N) using the formula;

$$N = \frac{Z^2 \acute{\alpha} \ (p_1+p_2)\ (100-(p_1+p_2)}{(p_1-p_2)^2}$$

N= number of subjects per arm

Z = the standard normal deviate for α (1.96) at α = 0.05

P_1= Proportion of subjects expected to have diarrhoea among the HIV positive group which is unknown and a standard figure of 50% was applied = 0.5

p_2 is the known prevalence of diarrhoea among hospitalized children below five = 11.2%

Substituting in the formula,

$$N = \frac{1.96^2 \times 0.05 \times 0.0.612 \times 99.388}{0.388^2} = 77.6 \text{ or } 78 \text{ subjects per arm}$$

Total sample population in both groups = 78x 2 = 156 subjects.

To minimize bias, an additional 60 subjects were included, to make approximately 30% contingency to make a total sample size of 216.

3.5 Sampling Criteria

Stratified random sampling proportionate to ward size was used to select children aged below five whose parents/guardians consented to the study. Stratification was based on HIV serostatus following detection or non detection of HIV antibodies by rapid HIV tests. Participants were randomly selected from among HIV seropositive and HIV seronegative diarrhoea cases. Randomization was done to mimize bias during sampling. The study was allowed to run until the desired sample size of 216 was attained.

3.5.1 Inclusion criteria

All HIV positive and negative children aged below five years admitted with diarrhoea at MTRH whose parents / guardians consented to the study.

3.5.2 Exclusion criteria

1. Children who met the selection criteria and whose parents /guardians consented to the study but failed to provide complete information and those who provided inadequate sample analysis.
2. Children who had diarrhoea but whose HIV serostatus was not determined.

3.6 Data Collection Procedures

Structured questionnaires were administered to parents/guardians of eligible children to elicit demographic data. Baseline demographic data was obtained using structured questionnaires administered to parents/guardians of eligible children on admission while clinicians recorded medical history (duration of diarrhoea, antibiotic use or other medication prior to admission and sources) and physically examined the patients and based on the severity of their illness referred them for admission. Sunken eyes, hoarse voice, washerwoman's hands, and protracted skin pinch retraction were signs of severe dehydration characteristic of hospitalized cases. A phlebotomist provided instructions to parents / guardians on how to aseptically collect stool samples for laboratory analysis. Diarrhoeic was collected in sterile leak-proof containers and sent to the microbiology laboratories at MTRH for processing with patient code, name and test required entered appropriately in the laboratory request form accompanying the sample. Standard microbiological techniques (culture, biochemical and serological tests) were used in preliminary identification of isolates and antibiotic susceptibility of isolates by Kirby Baeur techniques was undertaken at MTRH. The determination of molecular pathotypes and virulence markers by multiplex PCR was undertaken at a more advanced laboratory, KEMRI Walter- Reed

Project Microbiology. Information on clinical outcomes of *in-vivo* antibiotic susceptibility was obtained retrospectively from hospital records.

3.6.1 Validity and Reliability

Research assistants were recruited and trained prior to commencement of the study to ensure consistency in questionnaire administration and recording of information. A pilot study was undertaken at Eldoret District Hospital where questionnaires were pre-tested on 30 participants who met the inclusion criteria. The questionnaires were crosschecked to ensure completeness and data accuracy. Analysis established a reliability coefficient of 0.78 and demonstated the ability of the instruments to generate accurate information. Any questions that were vague and did not elicit any direct answers were deleted or reframed accordingly.

3.7 Laboratory Experiments

3.7.1 Culture

Stool samples were inoculated onto selective and differential media including deoxycholate citrate agar (DCA) and MacConkey agar (Oxoid Ltd, Basingstoke, UK). After aerobic incubation at 37°C for 18–24 hours, the plates were observed for growth of lactose (LF) and non-lactose fermenting (NLF) colonies characteristic of enteric bacteria. Evidence of deep red colonies was indicative of lactose fermentation on MacConkey and pale yellow for non-lactose fermentation. Non-lactose fermenting colonies appeared pink colonies on DCA medium. Colonial morphology of the bacteria cultures and microcopy (Gram stain) was used to provide presumptive identification of the isolates.

3.7.2 Microscopy

Plain microscope slides were cleaned thoroughly using lens tissue and labeled using a marker pen. A drop of normal saline was then placed on a slide and a sterile wire loop was used to aseptically transfer a small portion of culture onto the slide to make a smear by spreading the suspension evenly and allowing it to air dry. This was then dried by gently passing over a Bunsen flame to fix the smear. Gram staining was performed to ascertain the Gram reaction of the isolate by placing the slide with the fixed smear uppermost on a staining rack over a sink or staining tray and then flooding with crystal violet for 1 minute. The stain was then washed off and further flooded with Iodine. The stained smear was held at a 45° angle over the sink and washed off with 95% (v/v) acetone until the colour turned pale violet. This was then rinsed off with tap water and flooded with safranin solution, 0.5% w/v, for 30 seconds on a staining rack. This was then rinsed off and the smear blot dried and examined at 100X under oil immersion. Appearance of gram negative rods was indicative of enteric bacteria which were further subjected to biochemical tests to ascertain their identity.

3.7.3 Biochemical tests

Colonial morphology of the cultures and microcopy (Gram stain) was used to provide presumptive identification of the isolates. Further identification of the Gram negative bacilli was done using biochemical tests, (Bailey *et al.,* 2002). Portions of NLF colonies were inoculated into triple sugar iron agar (TSI, Oxoid Ltd, Basingstoke, UK) while LF colonies were subjected to indole, methyl red, voges proskaeur and citrate utilization, (IMViC) tests. Positive control strains *Escherichia coli* ATCC ® 25922, *Salmonella enterica* ATCC 35987 and *Shigella sonnei* ATCC 259310 obtained from Kenya Medical Research Institute (KEMRI) were used.

Indole test was used to differentiate enteric bacteria such as *Escherichia coli* that contain the enzyme tryptophanase that hydrolyzes tryptophan to pyruvic acid. To promote tryptophan hydrolysis, a portion of culture of the suspect organism the organism was inoculated in 1% casitone in a screw capped indole tube and incubated for 18-24 hr at 35-37^{0}C. An equal volume of Kovac's reagent (5% para-diethyl-amino-benzaldehyde in 75% amyl alcohol, 25% concentrated hydrochloric acid) was added and mixed well for about 5 minutes. A positive test was indicated by a red color in the alcohol (upper) layer.

IMViC test was used to positively identify *E. coli* as Indole positiveindicated by a red ring (**Figure 3**) and Methyl red positive by a bright red coloration (**Figure 4**) but VP negative (no change-colorless) and citrate negative (IMV iC, + + - -).

A B C

Figure 3: Indole test

Un-inoculated Negative test Positive test

Figure 4: Methyl Red test

Methyl red, a pH-sensitive dye, turned red at low pH, indicating that the organism produced mixed acids as end products of glucose fermentation. In this test, 0.5 ml of culture was added (aseptically) into a 13 x 100 mm tube containing Methyl red/ Voges-Proskaeur (MR/VP) medium containing of 0.7% peptone (a meat digest), 0.5% glucose, and 0.5% anhydrous dibasic potassium phosphate in water. 10-15 drops of methyl red reagent was then added and the appearance of a red color was positive for strong acid production on glucose while yellow colour was negative. The fermentation of glucose by bacteria resulted in end products that varied from species to species depending on metabolic pathways available to them under culture conditions.

Gram negative bacteria usually ferment glucose to produce lactic, acetic, succinic, and formic acids. The MR-VP tests were used to differentiate between any two types of facultative anaerobic LF enteric species based on their pattern of glucose metabolism. All enteric bacteria produce pyruvic acid from glucose metabolism and subsequently turn red when methyl red is added. However, other enterics use butyl glycol pathway to metabolize pyruvic acid to neutral end products that produce a yellow colour on addition of methyl red. The test is based on the production of acetoin (acetylmethylcarbinol), a neutral end product of glucose metabolism which results in a red colour on addition of alpha naphtha indicator in a positive reaction and a pale yellow copper colour in negative cases. The VP test was performed in two reagent steps A and B. To 1 ml culture suspension in a test tube, 15 drops VP reagent A (5% alpha-naphthal in absolute ethanol) was added and mixed by shaking, followed by the addition of 5 drops reagent B (40% KOH). After shaking gently to aerate, the tubes were examined for the appearance of a red color within 20 minutes. A positive result was indicated by the appearance of a brick red color within 20 minutes while a negative result was indicated by no colour change (**Figure 5**).

Citrate utilization test distinguishes between members of the Enterobacteriaceae based on their metabolic byproducts. The test was used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic (NH_4H_2PO4) as the sole fixed nitrogen source resulting in alkaline carbonates and bicarbonates while ammonium hydroxide. A portion of suspect organism was inoculated onto Simmons citrate agar (Oxoid Ltd, Basingstoke, UK) lightly on the slant using the tip of a needle and incubating for 18-24 hours and then observed for the development of blue color (positive reaction), denoting alkalinization (**Figure 6**). The color change of the bromothymol blue indicator from green to blue is attributed to alkali production by the test organism.

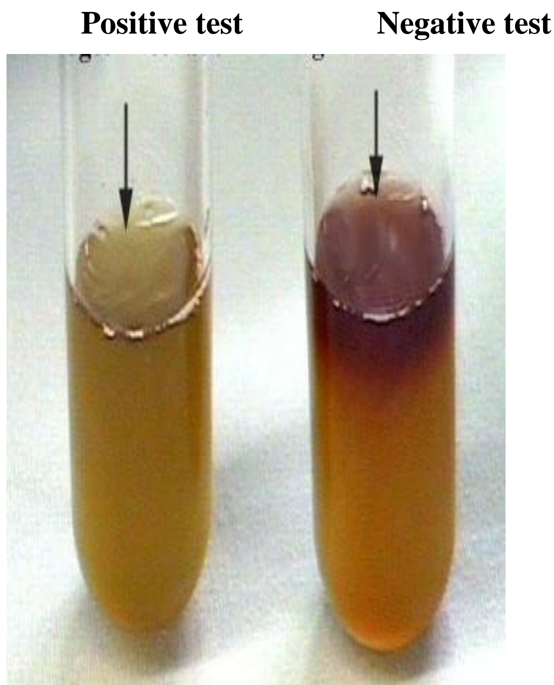


Figure 5: Voges Proskaeur test

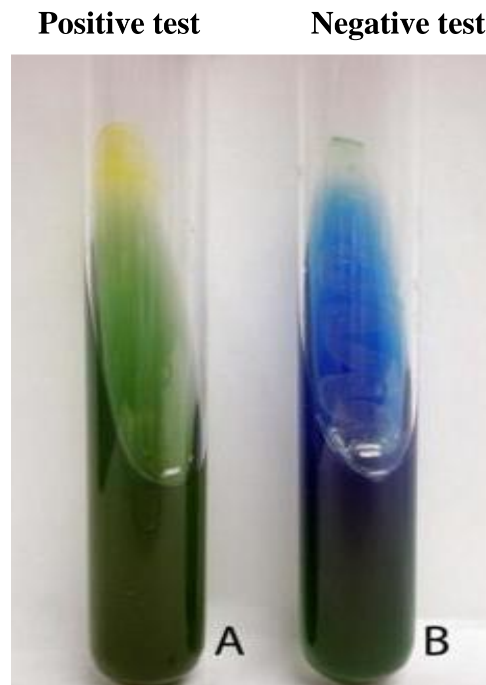
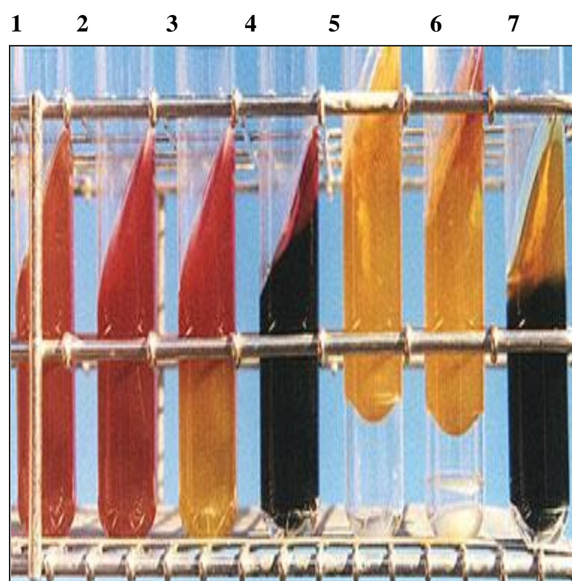


Figure 6: Citrate utilization test

Triple sugar iron test was used to distinguish non- lactose fermenting organisms*,* *Salmonella* and *Shigella* which grow on TSI slant with an acid/alkaline slant and butt with gas or no gas and H_2S -/+ profile respectively. A needle containing the pure culture of suspect organism was stabbed into the medium, up to the butt of the TSI tube and then streaked back and forth along the surface of the slant and incubated at 37^oc for 18 to 24 hours. Varied reactions as indicated aided in identification of isolates (**Figure 7, tubes 1-8**). An Acid slant (yellow) and acid butt (yellow) with or without gas production was indicative of lactose or sucrose fermentation, which served as substrates for continued fermentative activities with maintenance of an acid reaction in both the slant and the butt. An alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt was indicative of no carbohydrate fermentation but instead peptones were catabolized under anaerobic and /or aerobic conditions resulting in alkaline reaction in both slant and butt due to production of ammonia (**tube 1, 2**).

Some isolates however, utilized thiosulfate anion as a terminal electron acceptor, reducing it to sulfide (**tube 4**). Where this occurs, the newly-formed hydrogen sulfide (H_2S) reacted with ferrous sulfate in the medium to form ferrous sulfide, visible as a black precipitate observed in the butt (bottom) of the medium (**tube 7**). Some of the isolates like *Salmonella* produce carbon dioxide (CO_2) recognized as bubbles of gas between the agar and the wall of the tube or within the agar itself producing sufficient split in the agar into two or more sections (**tube 5, 6**). Motility test was also performed to distinguish between suspect *Salmonella* and *Shigella species*. The isolates were inoculated onto Motility Indole urea (MIU) semisolid medium. Any observation of clouding was a positive motility test for *Salmonella* species while non-motile species such *Shigella* remained along the line of inoculation (**Figure 8**).



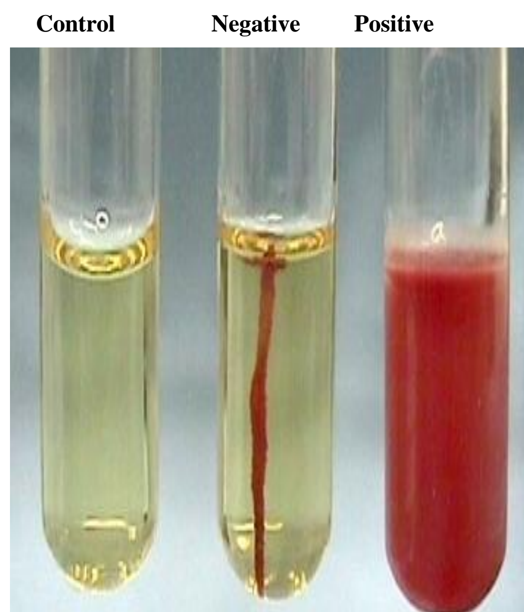


Figure 7: Triple Sugar Iron Test

Figure 8: Motility Indole Urea test

3.7. 4 Serological tests

Serological tests were performed to confirm the specific strains of the isolates presumptively identified by culture and biochemical tests. Serotyping was performed by slide agglutination with polyvalent and monovalent antisera (Welcome Diagnostics) to target specific antigens on *E coli*, *Salmonella* and *Shigella* isolates. The specific antisera used for each isolate were; EPEC: O55, O26, O111, O119, O125, O126, O127, O129, O128, O142; ETEC: serotype O6, O20, O15, O148, O25, O128, O153, O159; EIEC: O28, O29, O124, O136; EHEC: O26, O111, O138, and VTEC O157: H7; EAEC antisera: O94, 095, O97 and O98. The specific antisera for detection of *Salmonella* serotypes were; *S. typhi, S.typhimurium* and *S.enterica* while *S.sonnei, S. boydi, flexneri* and *S.dysenteriae* were used to detect *Shigella* serotypes (**Appendix 6**).

This test is dependent on the clumping of cells or particles when antigen was reacts with specific antiserum. A drop of normal saline was placed on two clean glass slides and two circles drawn heavily with a red wax marker and placed on a white tile. Using a Pasteur pipette, a drop of the cell suspension was placed within each circle and mixed evenly to produce a clear appearance. A drop of specific anti-sera was added to each of the suspensions of isolates and mixed gently using by rocking the slides. After several minutes, the petri-dish was held up and the slide observed from the bottom. Where there was a reaction between antibodies in the antiserum and their homologous antigens on the cell wall of the bacteria, the cells agglutinated and the drop appeared to contain many small clumps indicated by a cloudy appearance. Where there was no agglutination, the cell suspension maintained their original, evenly clear appearance.

3.7.5 DNA Extraction

A three-sample, multiplex PCR was designed for identification of the 9 bacterial strains associated with diarrhea in a single PCR program run. The first set of samples included the six *E.coli* strains (EHEC, EPEC, EAEC, ETEC, DAEC, and EIEC. The second set comprised two *Shigella strains (S.boydi* and *S.dysenteriae) while third set of samples included* two strains of *Salmonella* species (*S. typhi and S.enterica.)* The frozen isolates *E coli, Salmonella and Shigella* previously obtained from stool samples and stored in tryptic soy broth (TSB) at -20^0 C. were allowed to thaw at room temperature in preparation for DNA extraction (**Figure 9**).

Figure 9: Thawing of Samples for DNA extraction

To revive the organisms, each sample was inoculated onto sorbitol MacConkey agar and incubated for 18-24 hours at 35-37^{0}C. A loopful of bacterial colony from each sample was suspended in 200µl sterile deionized water in 1.5ml eppendorf tubes and adjusted to 0.5 McFarland. These were vortexed, boiled in a water bath at 95-100°C for 10 minutes and then cooled to room temperature (RT). The suspension was then centrifuged at 10,000 rpm for 1min at RT and supernatant DNA extract pipetted into 1.5ml microfuge tube and stored at -20^{0}C. To confirm EHEC, ETEC, EIEC, EAEC and EPEC strains, Shigella dysenteriae, S.boydi, Salmonella typhi and S.enterica, multiplex PCR assays were performed as per AFRIMS Enteric

Department protocol, SOP ETR-AD-000-F1, 2011. The list of primers, oligonucleotide sequences and expected amplicon sizes in base pairs are indicated below (**Table 1**).

Table 1: Multiplex PCR Primers, Expected Products and Control Strains

Target virulence factor / gene	Primer	Oligonucleotide sequence (5' -3')	Product size (bp)	Controls
Heat labile toxin, elt	ETEC 508F ETEC 508R	CACACGGAGCTCCTCCAGTC CCCCCAGCCTAGCTTAGTTT	508	ATCC 35401
Heat-stable toxin, est	ETEC 147 F ETEC 147R	GCTAAACCAGTAGGTCT CCCGGTACARGCAGGATTACAACA	147	PDAS 101
shigatoxin -1, stx_1	EHEC 348 F EHEC 348 R	CAGTTAATGTGGTGGCGAAGG CACCAGACAATGTAACCGCTG	348	ATCC 933J
shigatoxin -2, stx_2	EHEC 584 F EHEC 584 R	ATCCTATTCCCGGAGTTTACG GCGTCATCGTATACAGGAGC	584	ATCC 933W
Bundle forming pilus, bfp	EPEC 300F EPEC 300R	GGAAGTCAAATTCATGGGGGTAT GGAATCAGACGCAGACTGGTAGT	300	ATCC 43887
Invasive plasmid adhesin, *IpaH*	EIEC 423F EIEC 423R	TGGAAAAACTCAGTGCCTCT CCAGTCCGTAAATTCATTCT	423	ATCC 43893
Aggregative attachment adhesin, aatA	EAEC 650F EAEC 650R	CTGGCGAAAGACTGTATCAT CAATGTATAGAAATCCGCTGTT	650	MHK 00238-1
virulence invasion factor (*virF*)	POG 396 F POG 396R	AGCTCAGGAATGAATGAAACTTGA C GGCTTGATATTCCGATAAGTCATAG	618	ATCC 81466
invasion plasmid antigen *H(ipaH)*	POG 396 F POG 396 R	CTCGGCACGTTTAATAGTCTGGCTC C TGGAGAGCTGAAGTTTCTCTCTGC	933	ATCC 84358
Invasive plasmid adhesion (invA)	POG 414 F POG 414 R	TATGCCCCATCGTGTGTAGTCTGG TCCGCTGGATCACCATATACCTCCT	312	ATCC 84359

Source: US Army Medical Research Unit (USAMRU) protocol 2011

Positive control DNA standards and primers; ATCC 35401, PDAS 101, ATCC 933J, ATCC 933W, ATCC 43887 and MHK 00238 obtained from Kenya Medical Research Institute and negative controls without virulence genes were used. A three-sample, multiplex PCR was designed for identification of the 9 bacterial strains associated with diarrhea in a single PCR program run. These included the six *E.coli* strains included ETEC, *heat stable toxin* (*st*), *heat*

labile toxin (*lt*), *shiga toxin$_1$* (*stx_1*) and *shigatoxin $_2$* (*stx_2*), EIEC *invasive plasmid adhesin* (*ipaH*), EAEC (*aatA*), EPEC *bundle forming pilus* (*bfp*), *S.boydi, S.dysenteriae S.typhi* and *S.typhimurium*.

3.7.6 Preparation of PCR mixture and Amplification

PCR mixture was prepared by thawing the DNA samples at room temperature, vortexing and then centrifuging for 10 seconds in 0.5ml micro-centrifuge tubes. Dilution of DNA template was done according to manufacturers' instructions (Bioserve Biotechnologies, Laurel, MD, USA) on sample tubes aligned in an 80-well format rack on an ice block. 20µl aliquots of PCR master mix containing 2 ml of 2.5 mM dNTP mixture (dNTP, dCTP, dTTP, dGTP), 2.5µl $MgCl_2$), 2.5µl 10X reaction buffer, 0.3 µl of each primer (reverse and forward), 5µl each of DNA template and *taq* polymerase (Applied Biosystems, Roche Molecular, Inc, Branchburg, New Jersey, USA) were dispensed in polypropylene reaction tubes.

The assay required the addition of 1 µl of DNA template to the 24-µl premade solution mix. PCR samples 1 to 3 (M1, M2, and M3) had all reagents except DNA templates. M1 mix contained primers for amplification of seven gene targets including: *shigatoxins* (*$stx_{1,}$ stx_2*) 1 and 2 from EHEC, heat labile and heat stable toxin (*lt, st*) from ETEC and EPEC, bundle-forming pilus structural subunit (*bfpA*) from EPEC and regulatory gene *aggR* from EAEC. M2 mix contained primers for amplification for the invasion plasmid antigen H (*ipaH*) from EIEC or *Shigella*, virulence invasion factor (*virF*) genes from *Shigella*. M3 contained primers for amplification of *Salmonella invasion plasmid antigen* H (*invA*), and *virulence invasion factor* (*virF*) from *Shigella* spp. (or EIEC). Three nucleotides were added at the 5-prime end of each forward and reverse primer to make the melting time temperature similar to the remaining

primers, as indicated in Table 1. Each reaction had a total volume of 25 µl. The reaction tubes were then sealed, vortexed and then centrifuged for 30 seconds. The reaction mixture was then loaded onto a DNA Gene Amp PCR system 9700 thermal cycler (Applied Biosystems) for amplification of the PCR end products. Amplification was set at 20 cycles of denaturation at 94 ^{0}c for 60 sec, annealing at 64^{0}C for 90 sec, extension at 72^{0}C for 90 sec and post-extension at 72^{0}C for 10 min.

3.7.7 Gel Electrophoresis of PCR products

Agarose gel electrophoresis was performed in 2% agarose gel containing 1.6g of agarose powder in 80ml 0.5 X Ethidium bromide in TBE (44.5Mm Tris, 44.5 mM Boric acid and 1mM EDTA) buffer. 0.5µg/ml 6X orange DNA loading dye (Thermo scientific, USA) was added to 10 µl of amplified PCR product on a parafilm and mixed briefly before loading onto agarose gel. 5-10 µl of 100-bp DNA ladder (Promega, Madison, Wisconsin, USA) was loaded onto the side lanes of gel for amplicon size estimation of PCR products. Electrophoresis was performed at 120 volts until the bands were fully formed. The gel was then rinsed with deionized water and amplified DNA bands visualized by exposure to UV light using gel documentation system (Alpha Imager HP, 250v).

The gel picture was produced from a digital monochrome printer (Mitsubishi Electric, 100-240v) and virulence genes identified based on amplicon sizes of the PCR end products. All the strains were tested with the three-sample multiplex PCR and each strain was recognized based on the DNA banding pattern on the agarose gel as *E. coli* enteropathogens, *Shigella* spp, *Salmonella* spp. EIEC and *Shigella* spp. strains in this study were differentiated by the lactose fermenting

phenotype present in all *E. coli* and absent in all *Shigella* spp. strains. Negative control strains, including normal flora *E. coli* strains, were also tested with the three-sample multiplex PCR. No amplified DNA bands where observed from these negative controls. Furthermore, there was no cross-reactivity observed and among the *E. coli* or non *E coli* templates indicating that the assay was specific. The primers tested in this assay were validated independently for *E. coli* pathotypes, *Salmonella* and *Shigella* species.

3.7.8 Determination of Antibiotic Susceptibility

Antibiotic profiles *Escherichia coli* (105)*, Salmonella* (5) and *Shigella* (6) were evaluated against ten commonly used antibiotics using Kirby-Bauer disc-diffusion method (NCCLS, 2006). The antibiotics comprised ampicillin (AMP, 10 µg), a second generation cephalosporin cefuroxime (CXM 30µg), third generation cephalosporins ceftazidime (CDM 30µg) and ceftriaxone (CXN 30 µg), a fourth generation cephalosporin cefepime (CPM) 10 µg), a second generation flouroquinolone, ciprofloxacin (CIP 30 µg), aminoglycosides, amikacin (AMK 30µg) and gentamicin (GEN 10 µg), a sulfonamide cotrimoxazole (CTX 20 µg) and imipenem (IMP 15 µg), a β lactam. Two pure colonies of each bacterial strain were inoculated into 2 ml sterile Mueller Hinton (MH) broth in Bijou bottles and incubated at 37°C for 6 hours. Turbidity was adjusted to 0.5 McFarland standards and a sterile cotton swab dipped into the suspension and squeezed to remove excess inocula then swabbed on the surface of each of two Mueller-Hinton agar (MHA) plates (**Appendix 7**). Five antibiotic sensitivity discs were dispensed onto the surface of each MHA plate one at a time using sterile forceps and incubated at 35-37°C for 18-24 hours (**Figure 10**). The diameter of the zones of inhibition was measured using a vernier

caliper and interpreted according to NCCLS guidelines for *Enterobacteriaceae* as Sensitive (S), Intermediate (I) and Resistant (R) (NCCLS, 2006), (**Appendix 9**).

Figure 10: Antibiotic Susceptibility testing by Disc Diffusion Method

3.7.9 Determination of clinical outcomes of *in-vivo* Antibiotic Susceptibility

During hospitalization of diarrhoea cases, some of the critically ill patients were put on rehydration therapy and antibiotics (empiric). However, the less critical were immediately started on rehydration and stool samples sent to the laboratory for antibiotic susceptibility testing. Information obtained from hospital records included the nature of response to treatment which was specified in three categories; recovered fully and was discharged, discharged with medication or died while undergoing treatment **(Appendix 8)**. The nature of antibiotic therapy was also clearly indicated, whether empiric or definitive.

3.8 Data Analysis

Data was coded and analyzed using STATA 10 and descriptive statistics such as mean, median, standard deviation (SD) and interquartile range (IQR) were used to analyze for continuous variables categorical variables (age, duration of diarrhoea) while frequency listings was applied for discrete variables such as sex, household income and education level of parent / guardian. Chi square (X^2) test was used to test for associations between independent variables such as age, sex, socio-eeconomic characteristics of households, symptoms and etiology of diarrhoea. Kruskal-Wallis non-parametric was test to compare means between molecular pathotypes and antibiotic profiles of *E.coli, Salmonella, Shigella* and clinical outcomes of *in-vivo* antibiotic susceptibility. In all cases, a p-value of 0.05 was considered statistically significant**.**

3.9 Ethical Considerations

Ethical approval to conduct research was obtained from the Institutional Research and Ethics Committee (IREC) of Moi University / Moi Teaching and Referral Hospital, IREC/2011/120: Approval No. 0007111 (**Appendix 11**). Maseno University School of Graduate Studies approved the proposal to undertake the study. Written informed consent to participate in the study was obtained from the parents/guardians of patients who met the selection criteria, after being fully informed about the study and assured that the information provided would be treated with utmost confidentiality. Participants were informed that participation in the study was voluntary and that they were free to withdraw at any time without compromising their access to treatment at the hospital. The rights of all participants and their dignity was respected and protected.

CHAPTER FOUR: RESULTS

4.1 Baseline Characteristics associated with Diarrhoea

4.1.1 Socio-Demographic Profile of Study Participants

The results in **Table 2**, summarises baseline characteristics of hospitalized patients undergoing treatment for diarrhoea at MTRH. Diarrhoea was reported in both HIV positive and negative cases but with a higher mean age and standard deviation (STD) 24.1 (15.6) vs 11.8(7.9), median and interquartile range (IQR), 22(10, 36) vs 10(6, 16) in HIV positive than negative cases. The minimum age at which diarrhoea was reported was 3 vs 4 months and maximum 60 vs 25 months, respectively, among HIV positive and negative cases. These differences were statistically significant with diarrhoea affecting a wider range of children with regard to age in HIV positive than negative cases, (Chi square test: p=0.001).

Majority of diarrhoea cases were from low income households 186 (86%) earning < 20,000 Kenya shillings per month and living in in mud-walled to semi-permanent houses 135(62.5%), that were commensurate to their income. Most parents/ guardians of diarrhea cases 73(33.8) had either no education or primary level 83(38.4%) an above secondary level. The most common source of drinking water was well / borehole use 78(33.8%) and 73(33.8%) used treated while 19(8.8%) used river water. These characteristics are likely to have increased the risk of diarrhoea in these study groups with no differences among HIV negative cases.

Table 2: Demographic Characteristics of Households

Variable	HIV Positive N= 109	HIV Negative N=107	Total N= 216
Age of child (months)			
Mean (std)	24.11 (15.6)	11.77 (7.88)	
Median (IQR)	22 (10, 36)	10 (6,16)	< 000[1]
Minimum	3	4	
Maximum	60	25	
Sex			
Female	34 (44.2)	43 (55.8)	0.164[1]
Male	75 (54)	64 (46)	
Monthly household Income in Ksh			
Below 10,000	65(54.6)	54 (45.3)	
10,000-19,000	30(44.8)	37 (55.2)	
20,000-29,000	12 (50)	12 (50)	0.683[1]
30,000-40,000	1 (25)	3 (75)	
Above 40,000	1 (50)	1(50)	
Type of residential house			
Mud-walled	39 (50.6)	38 (49.4)	
Semi-permanent	30 (50.9)	29 (49.1)	0.921[1]
Stone/brick walled	40 (50)	40 (50)	
Education level of mother/caregiver			
None	36 (49.3)	37 (50.7)	
Primary	47(56.6)	36 (43.4)	
Secondary	16(40)	24 (60)	0.276[1]
Post secondary	10(50)	10 (50)	
Types of Drinking Water			
Piped treated water	32 (43.8)	41 (56.2)	0.318[1]
River	10 (52.6)	9 (47.3)	
Well/borehole	67 (54)	57(46)	

[1]Chi square test: IQR- interquartile range; SD- standard deviation; Level of Significance, $p \leq 0.05$

4.1.2 Clinical Characteristics of Diarrhoea cases

Results in **Table 3**, show that diarrhoea in HIV positive cases lasted for a much longer period than HIV negative cases as indicated by a higher mean (STD) 8.95(6.77) vs 4.23 (2.89), median (IQR) 7(4, 14) vs 3(3, 5), respectively. The major symptoms experienced among all cases included vomiting, diarrhoea, abdominal pain and fever with varying frequencies but with no significant differences with regard to HIV status, (p= 0.079). There was evidence of multiple infections among HIV positive cases who presented with other illnesses including tuberculosis 4(100% vs 0) and meningitis 8(100% vs. 0) whereas pneumonia 21(71.4 % vs 28.6), oral thrush 13 (76.9% vs 23.1%), malaria 19 (57.9% vs 42.1%) and protein energy malnutrition (PEM) were recorded in both groups. The occurrence of multiple infections in HIV positive cases may be related to immunodefiency among HIV positive cases that predisposes individuals to opportunistic infections.

Table 3: Clinical Characteristics of children admitted with Diarrhoea

Variable	HIV Positive N= 109	HIV Negative N=107	Total N= 216	p value
Duration of Diarrhoea	n=109	n=107		
Mean (std)	8.95 (6.77)	4.23(2.89)		< 0.001[1]
Median (IQR)	7 (4,14)	3 (3,5)		
Symptoms				
Diarrhea only	11 (31.4)	24 (68.6)	35 (16.2)	0.079[1]
Diarrhea and vomiting	24 (44.4)	30 (55.6)	54 (25)	
Diarrhea and vomiting and fever	28 (44.4)	35 (55.6)	63 (29.2)	
Other associated illness				
Meningitis	8 (100)	0	8 (3.70)	< 0.000[1]
Pneumonia	5 (71.4)	2 (28.6)	7 (3.2)	
Protein Energy Malnutrition	8 (61.5)	5 (38.5)	13 (6.0)	
Malaria	11 (57.9)	8 (42.1)	19 (8.8)	
Oral thrush	10 (76.9)	3 (23.1)	13 (6.0)	
TB	4 (100)	0	4 (1.9)	
Number of Diarrhea episodes per day				
Five times or more	76 (52.8)	68 (47.2)	144 (66.7)	0.077[1]
Four times	27 (48.2)	29 (51.8)	56 (26.0)	
Three times or less	6 (40)	10 (60)	16 (7.4)	
Stool culture Result				
Positive bacterial cultures	41 (34.7)	77 (65.3)	118 (54.6)	< 0.000[1]
Negative Bacterial cultures	68 (69.4)	30 (30.6)	98 (45.4)	

[1]Chi square test; IQR- interquartile range; SD- standard deviation; Level of Significance $p \le 0.05$

4.1.3 Prevalence of Diarrhea among Hospitalized Children at Moi Teaching and Referral Hospital

During the study period, a total of 1382 children were admitted in the paediatric wards of MTRH. Prevalence of diarrhoea among among HIV positive and negative children (**Figure 11**), was 15.6% (216/1382). Among these, 139 (64.4 %) were males and 77 (35.6 %) females with no gender difference with regard to HIV status, (Chi square: $p < 0.05$). Out of 216 stool samples, 118(54.6%) yielded bacterial pathogens referred to as bacterial diarrhoea (BD), while 98(45.4%) were non-bacterial (BD), which may either be due to viruses, parasites or fungi that were not investigated in this study. The proportion of bacterial diarrhoea, was lower 41(34.7 %) in HIV positive cases compared to 77(65.3%) in HIV negative cases cases. This means that the high number of acute diarrhoea cases in HIV positive was due to bacterial pathogens while the more prolonged diarrhoea in HIV positive cases was due to non- bacterial agents (Chi square test: $p < 0.000$).

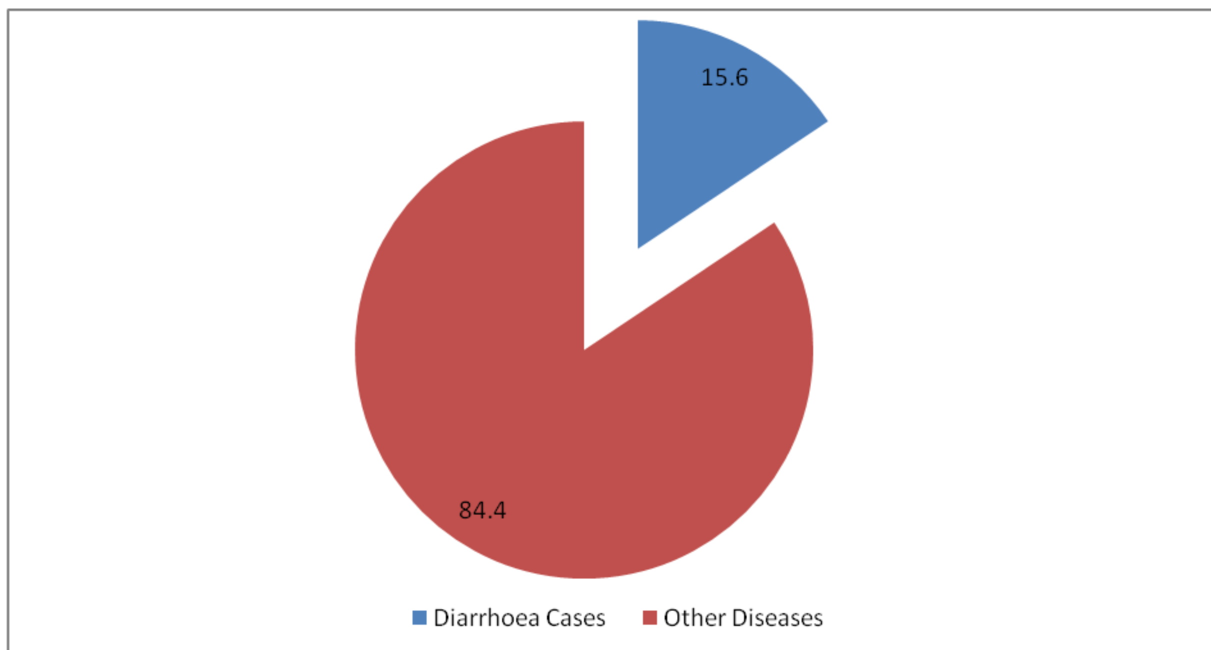


Figure 11: Prevalence of Diarrhea at MTRH

4.1.4 Characteristics of Bacterial Diarrhoea in relation to Age, Sex and HIV status

Results in **Figure 12**, demonstrate a possible link between age, sex and HIV status in attempt to define host susceptibility to infection. There was an overall high number of diarrhoea cases among children aged 0-12 months in both HIV positive and negative groups 54.2% (19, 45), although it persisted among HIV positive through ages 13-24 months 25.4% (7, 23), 25-36 months 13.5% (10, 6), 37-48 months 5.1% (6, 0) and 49-60 months 1.7% (2, 0). BD cases among HIV negative patients were recorded more frequently between the ages 0-24 months 22% (26) cohort but declined with increasing age to nil at 36 months. Frequency of diarrhoea appeared to be higher in males across the ages in both groups except at ages 49-60 months which had equal rating in both males and females among HIV positive cases. This means that HIV status of an individual has a significant effect on diarrhoea which occurs as an opportunistic infection in AIDS.

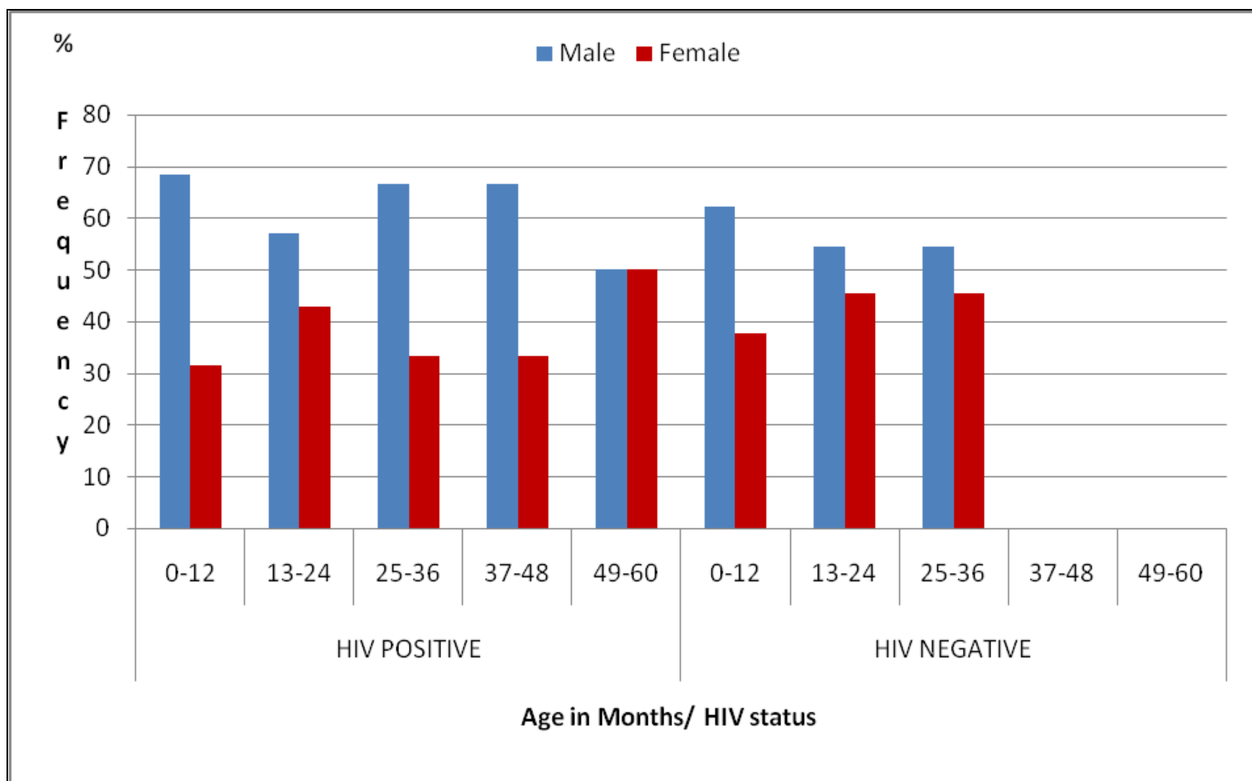


Figure 12: Prevalence of Diarrhoea in relation to Age, Sex and HIV serostatus

4.1.5 Bacterial Enteropathogens detected using Biochemical and Serological tests

The main etiological agents identified using gold standard methods of culture and biochemical tests were *E coli* 105(36.2% vs 63.8%), being of highest frequency followed by *Shigella species 1*6% (16.7% vs 83.3%), *Salmonella species* 5% (0 vs 100%), *Campylobacter* and *Citobacter* 1(100% vs 0), respectively among HIV positive and negative cases, (**Table 4**). There was significant difference in isolation rates of all species of enteric pathogens in relation to HIV status. The three species each recorded lower frequencies in HIV positive compared to HIV negative cases, (Chi square test:

$p<0.05$). *Escherichia coli* species were the major bacterial agents associated with diarrhoea in the study while *Shigella* and *Salmonella* were less frequent. Serotypic analysis identified in *S.typhimurium* and *S. typhi in Salmonella while* S. *dyenteriae* and *S. flexneri* were the notable serotypes in *Shigella.* All the *Salmonella* isolates and 83.3 % of *Shigella* were from HIV negative cases.

Table 4: Bacterial Enteropathogens isolated from Stool samples

Bacterial Species	Strain	HIV Positive No. (%)	HIV Negative No. (%)	Total No. (%)	p value
Escherichia coli	EHEC	0	2 (100)	4 (11.1)	
	ETEC	0	2 (100)	2 (5.6)	
	EAEC	15 (71.4)	6 (28.6)	21 (58.3)	0.002[1]
	EIEC	2 (66.7)	1 (33.3)	3 (8.3)	
	EPEC	2 (33.3)	4 (66.7)	6 (16.7)	
		19 (52.8)	**17 (47.2)**	36 (30.5)	
	Other DEC	19 (26.7)	50 (73.2)	69 (58.4)	
Sub total		**36 (34.2)**	**69 (65.7)**	**105 (88.9)**	
Salmonella	*S. typhimurium*	0	1 (100)	1 (20)	< 0.001[1]
	S. typhi	0	4 (100)	4 (80)	
Sub total		**0**	**5 (100)**	**5 (4.23)**	
Shigella	*S dyenteriae*	0	3 (100)	3 (50)	
	S flexneri	1(33.3)	2 (66.7)	3 (50)	0.002[1]
Sub total		**1 (16.7)**	**5 (83.3)**	**6 (5.1)**	
Campylobacter	*C. jejuni*	1 (100)	0	1 (50)	< 0.000[1]
Citrobacter	*Citrobacter sp*	1 (100)	0	1(50)	
Grand Total		**41 (34.7)**	**77 (65.2)**	**118 (100)**	

[1]Chi square test; Level of significance p≤0.05; Confidence Interval: 95%

EAEC- *Enteroaggregative E. coli*; EHEC- *Enterohaemorrhagic E. coli;* ETEC- *Enterotoxigenic E. coli* EIEC- *Enteroinvasive E. coli;* EPEC- *Enteropathogenic E. coli*

4.2 Molecular Pathotypes of *E coli, Salmonella* and *Shigella*

4.2.1 Detection of Pathotypes and Virulence genes in *E coli, Salmonella and Shigella*

A total of 47 pathotypes of E coli, Salmonella and Shigella were confirmed by multiplex PCR. These were 34.2% (36/105) of *E coli* with the most prevalent category being EAEC 21 (58.3%) followed by EPEC 6(16.7%), EHEC 4(11.1%), EIEC 3 (8.3%) and ETEC 2(5.6%). No EHEC and ETEC strains were detected from isolates of HIV positive cases. EAEC (71.4% vs 28.6%), EIEC (66.7% vs 33.3%) and EPEC (33.3% vs 66.7%) detected in both categories with higher

frequencies among positive cases (**Table 5).** No EHEC and ETEC strains were detected from isolates of HIV positive cases. Pathotypes confirmed in *Salmonella* and *Shigella* species were *S.typhimurium* 1(0 vs 100%), *S.typhi* 4 (0 versus 100%), S. *dyenteriae* 3 (0 vs 100%) and *S. flexneri* 3 (33.3% vs 66.7%), respectively in both categories. There was considerable heterogeneity of the *E coli* isolates with respect to virulence gene content.

A total of seven virulence genes were identified including EHEC shigatoxin genes, *stx1* (0 vs 100%) and stx_2 (0 vs 100%), ETEC heat labile *elt* (0 vs 100%), heat stable toxin *est* (0 vs 100%), EPEC *bfp (*33.3% vs 66.7%), *EAEC aatA* (71.4% vs 28.6%) and EIEC *ipaH* (66.7% vs 33.3%) among isolates from HIV positive and negative cases respectively. One type of virulence gene, the invasive plasmid antigen *invA* 3 (100) was confirmed in *Salmonella* among HIV negative and two among HIV positive cases. Two types of virulence genes *ipaH* and *vir F* were detected in in both groups of *Shigella* species. There were statistically significant differences in distribution of pathotypes and virulence markers among HIV positive and negative diarrhea cases, (Kruskal Wallis test: P<0.05). These differences may be responsible for changes in virulence and severity of illness and may be attributed to individual hosts factors.

Table 5: Pathotypes and virulence genes in *E coli, Salmonella* and *Shigella*

E coli species	**No.**	**Virulence gene**	**HIV Positive**		**HIV Negative**		**Total**		**p value**
			No.	**%**	**No.**	**%**	**No.**	**%**	
EHEC	4	*stx1*	0		2	50	2	5.5	
		stx2	0		2	50	2	5.5	<0.000[1]
ETEC	2	*elt*	0		1	100	1	2.8	
		est	0		1	100	1	2.8	
EAEC	21	*aatA*	15	71.4	6	28.6	21	58.3	
EIEC	3	*ipaH*	2	66.7	1	33.3	3	8.3	
EPEC	6	*bfp*	2	33.3	4	66.7	6	16.7	
	36		**19**	**52.8**	**17**	**47.2**	**36**	**31.0**	
Other *E coli*	0	_	19	27.5	50	72.5	69	59.5	
Sub-Total	**36**			**38**	**67**	**57.8**	**105**	**90.5**	
S. typhi	1	*invA*	0	0	1	100	1	20	
S.typhimurium	4	*invA*	0	0	4	100	4	80	<0.000[1]
				0	**5**	**100**	**5**	**4.3**	
S. dysenteriae	3	*virF*	0	0	3	100	3	50	< 0.000[1]
S. flexneri	3	*ipaH*	1	33.3	2	66.7	3	50	
Sub-Total	**6**		**1**	**16.7**	**5**	**83.3**	**6**	**5.2**	
Grand Total			**39**	**33.6**	**77**	**66.4**	**116**	**100**	

Chi square test; Level of significance: p ≤0.05; Confidence Interval: 95%

EAEC-Enteroaggregative E. coli; EHEC- Enterohaemorrhagic E. coli; ETEC- Enterotoxigenic E. coli; EIEC- Enteroinvasive E. coli EPEC- Enteropathogenic E. coli; elt- heat labile toxin gene; e*st-* heat stable toxin gene; stx_1 – *shigatoxin* factor $_1$; stx_2 – *shigatoxin* factor $_2$; *bfp -bundle forming pilus; ipaH- invasive plasmid antigen* H; *aatA* – aggregative *attachment adhesion* A; *inv*A– *invasive plasmid antigen* A ; *vir F- virulence resistance factor*

4.2.2 Virulence Genes in Diarrheagenic *E coli. Salmonella and Shigella* versus type of Diarrhea

Four virulence genes from two pathotypes of DEC including EHEC *shigatoxigenic* genes *stx1* and *stx2*, 2(100%) and ETEC *elt* and *est* 2 (0 vs 100%) were predominant in HIV negative cases only and were associated with acute diarrhoea (**Table 6**). Moreover, EAEC *aatA* which was the most common was found in both acute 3(14.2 %) vs 12(57.1%) and persistent diarrhoea cases 2(9.5%) vs 4(19.0%) of both groups. EIEC *ipaH* was linked to persistent diarrhoea (66.7% versus 33.3%) in HIV positive and negative cases respectively. All except one pathotype of *Salmonella* and *Shigella* were associated with acute diarrhoea in HIV negative cases and none in HIV positive cases. There were significant differences in distribution of virulence markers and in relation to diarrhoea among HIV positive and negative cases, (Kruskal Wallis test: H= -133.8; p=0.005).

Table 6: Pathotypes and virulence genes in E coli, Salmonella and Shigella versus Type of Diarrhea

DEC Strain	No. %	Virulence Genes	HIV Positive Acute Diarrhea No. (%)	HIV Positive ProAD No. (%)	HIV Positive Sub-Total No. (%)	HIV Negative Acute Diarrhea No. (%)	HIV Negative ProAD No. (%)	HIV Negative Sub-total No. (%)	p value
EHEC	4 (11.1)	*stx1*	0	0	0	2 (100)	0	**2 (100)**	**0.005**[2]
		stx2	0	0	0	2 (100)	0	**2 (100)**	
ETEC	2 (5.6)	*elt*	0	0	0	1 (100	0	**1 (100)**	
		est	0	0	0	1 (100)	0	**1 (100)**	
EAEC	21 (58.3)	*aatA*	3 (14.3)	12 (57.1)	**15 (71.4)**	2 (9.5)	4 (19.0)	**6 (28.6)**	
EIEC	3 (8.3)	*ipaH*	0	2 (66.7)	**2 (66.7)**	1 (33.3)	0	**1 (33.3)**	
EPEC	6 (16.7)	*bfp*	1 (16.6)	1 (16.6)	**2 (33.3)**	4 (66.7)	0	**6 (66.7)**	
Sub-total	**36 (74.6)**		**4 (11.1)**	**15 (41.7)**	**19 (52.8)**	**13 (36.1)**	**4 (11.1)**	**17 (47.2)**	
S.typhi	1 (20)	*invA*	0	0	**0**	1 (100)	0	1 (2.1)	**0.003**[2]
S.typhimuruium	4 (80)	*invA*	0	0	**0**	4 (100)	0	4 (8.5)	
Sub-total	**5 (10.6)**		**0**	**0**	**0**	**5 (100)**	**0**	**5 (10.6)**	
S.dysenteriae	3 (50)	*vir F*	0	0	**0**	3 (100)	0	3 (6.4)	**< 0.000**[2]
s. flexneri	3 (50)	*ipaH*	1 (33.3)	0	**1 (33.3)**	2 (66.7)	0	2 (4.2)	
Sub-total	**6 (12.8)**		**1**	**0**	**0**	**5 (100)**	**0**	**5 (12.8)**	
Grand Total	**47 (100)**		5 (10.6)	15 (31.9)	**20 (42.6)**	23 (48.9)	4 (8.5)	**27 (57.4)**	

[2]Kruskal Wallis test: Level of significance, p≤ 0.05, Confidence Interval: 95%

4.2.3 Pathotypes and virulence genes in *E. coli, Salmonella and Shigella* versus Age / HIV Status

Analysis of DEC virulence genes versus age and HIV status (**Table 7)** showed that at least 80% of DEC virulence markers in EPEC, EIEC, EHEC and ETEC were detected in patients aged 0-24 months. EAEC was detected in all cohorts (0-60) months among HIV positive individuals but only within 0-24 months' cohort in HIV negative cases. EAEC *aatA* was common in all cohorts in HIV positive cases but limited to 0-24 months'cohort in HIV negative cases, There were significant differences in expression of virulence genes with regard to age and HIV status, (Kruskal- Wallis, H = -155; degrees of freedom (df) = 13; p= 0.005; p< 0.05). Pathotypes of *Salmonella* were expressed highly among HIV negative groups (83.3%) compared to the HIV positive 1(16.7%) with most of the cases recorded between ages 0-24 months. The same scenario was observed in the distribution of pathotypes of *Shigella* within the various cohorts. (Kruskal – Wallis test, H = -68; degrees of freedom (df) = 5; p= 0.036). These results indicate that distribution of virulence genes is directly related to the frequency of etiological agents and may be determined by host factors.

Table 7: Pathotypes and virulence genes in E coli, Salmonella and Shigella versus Age / HIV Status

Pathotype	No. %	Genes	0-12 months		13-24 months		25-36 months		37-48months		49-60months		Total		
			HIV+	HIV−	HIV+	HIV−	HIV+	HIV−	HIV+	HIV−	HIV+	HIV−	HIV+	HIV-	p -value
			No.	No.	No.	No.	No.	No.		No.	No.	No.	No.	No.	
ETEC	2 (5.6)	*elt*	0	0	0	1(100)	0	0	0	0	0	**0**	**0**	**1**	**0.005[2]**
		est	0	0	0	1(100)	0	0	0	0	0	**0**	**0**	**1**	
EHEC	4 (11.1)	stx_1	0	1(50)	0	1 (50)	0	0	0	0	0	**0**	**0**	**2**	
		stx_2	0	2(100)	0	0	0	0	0	0	0	**0**	**0**	**2**	
EAEC	21(58.3)	*aatA*	3(14.2)	4(19.0)	3(14.2)	2 (9.5)	3(14.2)	**0**	4(19.0)	0	2 (9.5)	**0**	**15 (41.6)**	**6 (16.7)**	
EIEC	3 (8.3)	*IpaH*	0	1(33.3)	2(66.7)	0	**0**	**0**	0	0	0		**2 (5.6)**	**1 (2.7)**	
EPEC	6(16.7)	*bfp*	0	4(66.7)	1(16.7)	0	1(16.7)	**0**	**0**	**0**	0	**0**	**2 (5.6)**	**4 (11.1)**	
Sub-total	**36 (74.6)**		**3(8.3)**	**12(33.3)**	**6(16.7)**	**5 (13.9)**	**4(11.1)**	**0**	**4(11.1)**	**0**	**2 (5.6)**	**0**	**19 (40.4))**	**17 (36.2)**	
S.typhi	1(20)	*invA*	0	1(100)	0	0	0	0	0	0	0	0	**0**	1 (20)	**0.008[2]**
S.typhimuruium	4(80)	*InvA*	0	0	0	3	0	1	0	0	0	0	0	4 (80)	
Sub-total	**5 (10.6)**		**0**	**1(20)**	**0**	**3(60)**	**0**	**1(20)**	**0**	**0**	**0**	**0**	**0**	**5 (10.6)**	
S.dysenteriae	3 (50)	*vir F*	0	1(33.3	0	2(66.7)	0	0	0	0	0	0	1(16.7.7)	2 (33.3)	**).036[2]**
s. flexneri	3 (50)	*IpaH*	0	1(33.3)	1(33.3)	1(33.3)	0	0	0	0	0	0	1(16.7)	2 (33.3)	
Sub-total	**6 (12.8)**		**0**	**2(33.3)**	**1(16.7)**	**3(50)**	**0**	**0**	**0**	**0**	**0**	**0**	**2 (4.3)**	**4 (8.5)**	
Grand Total	**47 (100)**		**3 (6.4)**	**15 (31.9)**	**7(14.9)**	**11(23.4)**	**4(8.5)**	**1(2.1)**	**4(8.5)**	**0**	**2 (4.3)**	**0**	**21 (44.7)**	**26 (55.3)**	

[2]Kruskal Wallis test: Level of significance, p≤.05; Confidence Interval: 95%

EAEC-Enteroaggregative E. coli; EHEC- Enterohaemorrhagic E. coli; ETEC- Enterotoxigenic E. coli; EIEC- Enteroinvasive E. coli EPEC- Enteropathogenic E. coli; elt- heat labile toxin gene; e*st-* heat stable toxin gene; stx_1 – *shigatoxin* factor $_1$; stx_2 – *shigatoxin* factor $_2$; *bfp -bundle forming pilus; ipaH- invasive plasmid antigen* H; *aatA*–aggregative *attachment adhesion* A; *inv*A–*invasive plasmid antigen* A; *vir F-virulence resistance factor*

4.3 Antibiotic Susceptibility profiles of Isolates

4.3.1 Comparison of Antibiotic Profiles of *E coli, Salmonella* and *Shigella* isolates from Diarrhoea cases

Analysis of antibiotic susceptibility profiles of *Escherichia coli*, *Salmonella* and *Shigella species* (**Table 8**), revealed that enteric species in HIV negative cases were highly susceptible to cefuroxime (74.6%, 20%, 40%), ceftazidime (89.6%, 60% 40%), cefepime (97%, 60%, 60%) and Amikacin (46.3%, 60%, 80%) respectively compared to isolates from HIV positive cases ([1]Chi square test: p=0.000). The isolates in both HIV negative and positive cases exhibited resistance to Ampicillin (44.7%, 100%) vs (49.2%, 40%, 60%) and Cotrimoxazole (78.9%, 100%) vs (40.3%, 80%, 80%). Chi square test for association revealed statistically significant differences in antibiotic profiles between HIV positive and negative cases to all antibiotics except the Ampicillin and Amikacin, ($p \leq 0.05$). Comparison of antibiotic profiles of *E coli, Salmonella* and *Shigella* species exhibited over 45% resistance to Ampicillin and Cotrimoxazole but moderately resistant to Ceftriaxone, Amikacin and Imipenem but with intermediate susceptibility to ceftazidime, Cefepime and Ciprofloxacin which implies the need to seek alternative options or combine more than one antibiotic for increased efficacy.

Table 8: Comparison of Antibiotic Profiles of E coli, Salmonella and Shigella isolates from HIV Positive and Negative cases

		HIV Positive N= 39				HIV Negative N=77				
		E coli sp. n=38	*Salmonella sp.* n= 0	*Shigella sp.* n= 1	**Sub-Total** N= 39	*E coli sp.* n= 67	*Salmonella sp.* n= 5	*Shigella sp* n= 5	**Sub-Total** N=77	
Antibiotic	**Sensitivity**	No. %	No. %	No. %	No. %	No. %	No. %	No. %	No. %	**P-value**
AMP	Intermediate	18 (47.4)		0	**18 (34.6)**	30 (44.8)	2 (40)	2 (40)	**34 (65.4)**	**0.996[2]**
	Sensitive	3 (7.9)	0	0	**3 (37.5)**	4 (6.0)	1 (20)	0 0	**5 (62.5**	
	Resistant	17 (44.7)		1 (100)	**18 (32.1)**	33 (49.2)	2 (40)	3 (60)	**38 (67.9)**	
AMK	Intermediate	23 (60.5)		0	**23 (37.7)**	31 (46.3)	3 (60)	4 (80)	**38 (62.3)**	**0.078[2]**
	Sensitive	4 (10.5)	0	0	**4 (15.4)**	21 (31.3)	1 (20)	0	**22 (84.6)**	
	Resistant	11 (29.0)		1 (100)	**12 (41.4)**	15 (22.4)	1 (20)	1 (20)	**17 (58.6)**	
CXN	Intermediate	27 (71.1)	0	0 (100)	**27 (37)**	43 (64.2)	1 (20)	2 (40)	**46 (63)**	**0.013[2]**
	Sensitive	7 (18.4)		1	**7 (21.9)**	20 (29.8)	3 (60)	2 (40)	**25 (78.1)**	
	Resistant	4 (10.5)		0	**4 (40)**	4 (6.0)	1 (20)	1 (20)	**6 (60)**	
CXM	Intermediate	28 (73.7)		1 (100)	**29 (56.9)**	17 (25.4)	3 (60)	3 (60)	**22 (43.1)**	**< 0.000[2]**
	Sensitive	9 (23.7)	0	0	**9 (14.3)**	50 (74.6)	1 (20)	2 (40)	**54 (85.7)**	
	Resistant	1 (2.6)		0	**1 (50)**	0	1 (20)	0	**1 (50)**	
CDM	Intermediate	17 (44.7)		0	**17 (60.7)**	7 (10.4)	1 (20)	3 (60)	**11 (39.3)**	**< 0.000[2]**
	Sensitive	19 (50.0)	0	1 (100)	**19 (22.6)**	60 (89.6)	3 (60)	2 (40)	**65 (77.4)**	
	Resistant	2 (5.3)		0	**3 (75)**	0	1 (20)	0 0	**1 (25)**	
GEN	Intermediate	10 (26.3)		1 (100)	**11 (18.6)**	45 (67.2)	1 (20)	1 (20)	**48 (81.4)**	
	Sensitive	12 (31.6)	0	0	**12 (37.5)**	14 (20.8)	3 (60)	3 (60)	**20 (62.5)**	**< 0.000[2]**
	Resistant	16 (42.1)		0	**16 (64)**	8 (12.0)	1 (20)	1 (20)	**(36)**	
CTX	Intermediate	8 (21.1)		0	**8 (17.4)**	36 (53.7)	1 (20)	1 (20)	**38 (82.6)**	**0.003[2]**
	Sensitive	0	0	0	**0**	4 (6.0))	0 0	0	**4 100)**	
	Resistant	30 (78.9)		1 (100)	**31 (47)**	27 (40.3)	4 (80)	4 (80)	**35 (53.0)**	
CPM	Intermediate	21 (55.3)	0	0	**21 (84)**	2 (3.0)	1 (20)	1 (20)	**4 (16)**	**< 0.000[2]**
	Sensitive	16 (42.1)	0	0	**16 (18.3)**	65 (97.0)	3 (60)	3 (60)	**71 (81.7)**	
	Resistant	1 (2.6)	0	1 (100)	**2 (50)**	0 0	1 (20)	1 (20)	**2 (50)**	
CIP	Intermediate	19 (50)	0	0	**19 (30.6)**	40 (59.7)	1 (20)	2 (40)	**43 (69.4)**	**< 0.000[2]**
	Sensitive	1 (2.6)	0	0	**1 (3.1)**	27 (40.3)	3 (60)	1 (20)	**31 (96.9)**	
	Resistant	18 (47.4)	0	1 (100)	**19 (86.4)**	0	1 (20)	2 (40)	**3 (13.6)**	
IMP	Intermediate	20 (52.6)	0	0	**20 (34.5)**	36 (53.7)	1 (20)	1 (20)	**38 (65.5)**	
	Sensitive	10 (26.3)	0	1 (100)	**11 (23.9)**	27 (40.3)	4 (80)	4 (80)	**35 (76.1)**	**0.024[2]**
	Resistant	8 (21.1)	0	0	**8 (66.7)**	4 (6.0)	0	0	**4 (33.3)**	

[2] **Kruskal-Wallis test: Level of significance: $p \leq 0.05$; Confidence interval = 95%;** AMP- Ampicillin; AMK- Amikacin, CXN-Ceftriaxone, CDM- Ceftazidime, CPM-Cefipime , CXM- Cefuroxime, CIP- Ciprofloxacin, GEN- Gentamicin, CTX- Cotrimoxazole, IMP- Imipenem

4.3.2 Pathotypes and virulence genes in *E coli, Salmonella and Shigella* versus Type of Diarrhea

Pathotypes and virulence genes in *E coli, Salmonella* and Shigella exhibited significant differences in susceptibility to various antibiotics (**Table 9**), (Kruskal- Wallis test: $p<0.05$). Majority of the pathotypes showed resistance to cotrimoxazole and ampicillin with the greatest resistance displayed by EAEC *aatA,* EPEC *bfp* and EIEC *ipaH*. This means that although the virulence markers are known to aid in invasion and spread of infectious agents in the host mucosal membranes, they may also trigger antibiotic resistance.

Table 9: Pathotypes and Virulence Genes in E coli, Salmonella and Shigella versus Antibiotic Resistance

Pathotype	Gene	AMP	AMK	CXN	CXM	CDM	GEN	CTX	CPM	CIP	IMP	p value
ETEC	*elt*	1 (50)	0	0	0	0	0	1 (50)	0	0	0	**0.002[2]**
	est	1 (33.3)	0	0	0	0	0	1 (33.3)	0	1 (33.3)	0	
EHEC	*stx_1*	1 (50)	0	0	0	0	0	1 (50)	0	0	0	
	stx_2	1 (33.3)	0	0	0	0	0	1 (33.3)	0	1 (33.3)	0	
EAEC	*aatA*	21 (23.9)	15 (17.0)	3 (3.4)	0	1 (1.0)	13 (14.7)	15 (17.0)	1(1.0)	12 (13.6)	7 (7.9)	
EIEC	*ipaH*	3 (18.8)	1 (6.3)	1 (6.3)	0	2 (12.5)	2 (12.5)	3 (18.8)	0	3 (18.8)	1 (6.3)	
EPEC	*bfp*	6 (28.6)	5 (23.8)	2 (9.5)	0	**0**	3 (14.2)	3 (14.2)	**0**	3 (14.2)	**0**	
Sub-total		**34 (25)**	**21 (15.4)**	**6 (4.41)**	**0**	**3 (2.2)**	**18 (13.2)**	**25 (18.3)**	**1 (0.01)**	**20 (14.7)**	**8 (5.9)**	
S.typhimuruium	*invA*	3 (30)	1(10)	1 (10)	0	0	1 (10)	4 (40)	0	0	0	**0.028[2]**
S.typhi	*invA*	0	1(10)	0	3 (30)	0	1 (10)	5 (50)	0	0	0	
Sub-total		**3 (15)**	**2 (10)**	**1(5.0)**	**3 (15)**	**0**	**2 (10)**	**9 (45)**	**0**	**0**	**0**	
S.dysenteriae	*vir F*	2 (25)	1(12.5)	0	0	0	2 (25)	2 (25)	0	1 (12.5)	0	**0.004[2]**
s. flexneri	*ipaH*	1 (12.5)	1 (12.5)	1 (12.5)	0	0	2 (25)	2 (25)	0	1 (12.5)	0	
Sub-total		**3 (25)**	**2 (16.7)**	**1 (8.3)**	**0**	**0**	**4 (33.3)**	**0**	**0**	**2 (16.7)**	**0**	
Grand Total		**40 (23.5)**	**24 (14.1)**	**8 (4.7)**	**3 (1.8)**	**3 (1.8)**	**24 (14.1)**	**37 (21.8)**	**1 (0.01)**	**22 (12.9)**	**8 (4.7)**	

[2]Kruskal Wallis, Level of significance, p≤0.05 Confidence Interval 95%

EAEC-Enteroaggregative E. coli; EHEC- Enterohaemorrhagic E. coli; ETEC- Enterotoxigenic E. coli; EIEC- Enteroinvasive E. coli EPEC- Enteropathogenic E. coli; elt- heat labile toxin gene; e*st-* heat stable toxin gene; *stx_1 – shigatoxin* factor $_1$; *stx_2 – shigatoxin* factor $_2$; *bfp -bundle forming pilus; ipaH- invasive plasmid antigen* H; *aatA* – aggregative *attachment adhesion* A ; *inv*A– *invasive plasmid antigen* A ; *vir F- virulence resistance factor;* AMP- Ampicillin; AMK- Amikacin, CXN- Ceftriaxone, CDM- Ceftazidime, CPM- Cefipime , CXM- Cefuroxime CIP- Ciprofloxacin, GEN- Gentamicin, CTX- Cotrimoxazole, IMP- Imipenem

4.3.3 Risk Factors to Antibiotic Resistance

Using questionnaires, the study sought to establish any predisposing factors to antibiotic resistance by establishing whether the subjects had any other medication at home prior to admission and indeed 25 (21.6%) responded in the affirmative (**Table 10**). Further probing showed differences in prior medications taken which cited included; herbal remedies (42.9% vs 52.1%) and multivitamins / supplements (64.3%, vs 35.7%) between HIV positive and negative cases while both groups reported having used anti-malarial drugs, antibiotics and ORS. Some of the HIV positive cases were on anti-tuberculosis (anti-TB) and ARV (antiretroviral) drugs. Some of the sources of medication cited included health facility; (75% vs 25%), left over drugs from previous illness (60% vs 40%), community health workers (50% vs 50%), purchased from chemist without prescription (20% vs 80%) while those given by friends and relatives (25% vs 75 %) respectively among HIV positive and negative cases.

The study showed significant differences in drug use and type of medication taken prior to admission between HIV positive and negative cases, (Chi square test: $p < 0.05$). The results show that HIV positive cases have a higher likelihood being on prolonged medication either as prophylaxis against opportunistic infections or treatment for other underlying illness unlike HIV negative cases. Such prolonged antibiotic use may induce antibiotic resistance of gut flora.

Table 10: Drug use and Sources versus HIV status

Variable	HIV Positive No. (%)	HIV Negative No. (%)	Total No. (%)	p value
Any other medication prior to admission?				
YES	10 (40)	15 (60)	25 (21.6)	0.005[1]
NO	31 (33.3)	62 (66.7)	93 (78.4)	
Sub-total	**41 (34.8)**	**77 (65.2)**	**116 (100)**	
Type of medication used				
ORS	2 (50)	2 (50)	4 (8)	0.005[1]
Anti-TB drugs	6 (100)	0	6 (12)	
Multivitamins /Supplements	9 (64.3)	5 (35.7)	14 (28)	
Herbal Remedies	3 (42.9)	4 57.1)	7 (14)	
Antimalarial Drugs	2 (50)	2 (50)	4 (8)	
Antibiotics	3 (50)	3 (50)	6 (12)	
ARVs	9 (100)	0	9 (18)	
Sub-total	**34 (68)**	**16 (32)**	**50 (100)**	
Source of drugs		3(14.2)	21 (43.8)	0.010[1]
Health facility	18(85.7)			
Left over drugs from previous illness	4 (40)	6 (60)	10 (20.8)	
Community Health workers	3 (50)	3 (50)	6 (12.5)	
Purchased from a chemist without prescription	1(16.7)	5 (83.3	6 (12.5)	
Sub –Total	**27 (56.3**	**21 (43.7)**	**48 (100)**	

1 Chi square test, Confidence interval = 95%; $p \leq 0.05$; ORS- oral rehydration salt; ARV-anti-retroviral therapy; TB-tuberculosis

4.4 Clinical outcomes of *in-vivo* Antibiotic Susceptibility

4.4.1 Clinical outcomes versus Type of Diarrhoea and Etiological Agent

Results in **Table 11**, indicate that out of 116 cases under antibiotic therapy, diarrhoea persisted in 5(4.3%) of the cases, 18(15.5%) died while undergoing treatment and 93(80.3%) improved and were discharged with medication. Persistence of diarrhoea despite treatment was recorded among 5(100%) of HIV seropositive cases only. Deaths were recorded in 11(61.1 %) vs 7(38.9%), of HIV positive and negative cases respectively and were mainly associated with acute (AD) and persistent diarrhoea 5(27.8%) each while in HIV negative

cases most deaths were due to acute diarrhea 5(27.8%) and dysentery 2(11.1%). Good prognosis was rated 70(75.3%) compared to 23(24.7%) among HIV negative cases. The differences in clinical outcomes among HIV negative and positive cases were statistically significant, Kruskall Wallis: ($p<0.05$).

Evaluation of clinical outcomes versus etiological agents indicate that only 5(100%) of acute diarrhea cases persisted despite treatment and were attributed to *Escherichia coli.* The deaths which occurred in 10(55.6%) vs 8 (44.4%) in HIV positive and negative cases were related to diarrheagenic *E coli 9*(50%) vs 5(27.8%) and *Shigella* 1(5.6%) vs 3(37.5%) respectively. Following antibiotic treatment, 24(25.8%) vs 62(66.7%) of the cases, improved and were discharged with medication among HIV positive and negative cases respectively. *E coli* appeared to be more susceptible to antibiotics as indicated by a good response rate compared to *Shigella* and *Salmonella* cases. HIV negative cases showed better response to treatment compared to HIV positive cases. Differences in virulence mechanisms among enteric pathogens and indidividual host factors may be responsible for this occurence.

Table 11: Clinical outcomes versus Type of Diarrhea and Etiological Agents

	HIV Positive				HIV Negative				
Types of Diarrhea	***AD***	**PD**	***Dysentery***	**ubtotal**	**AD**	**PD**	**Dysentery**	**Subtotal**	**p-value**
Diarrhea persisted despite treatment	0	5 (100)	**0**	**5 (100)**	**0**	**0**	**0**	**5 (100)**	
Died	5 (27.8)	5 (27.8)	1 (5.6)	**11 (61.1**	5 (27.8)	0	2 (11.1)	**7 (38.9)**	**< 0.000²**
Improved and was discharged with medication	9 (20.5)	5 (11.4)	0	**14 (31.9**	24 (54.5)	4 (9.1)	2 (4.5)	**30 (68.1)**	
Recovered fully and discharged	5 (9.8)	6 (11.8)	0	**11 (21.6)**	38 (74.5)	1 (0.02)	1 (0.02)	**40 (78.4)**	
Total	**19 (46.3)**	**21 (51.2)**	**1 (2.4)**	**41 (34.7)**	**67 (87)**	**5 (6.5)**	**5 (6.5)**	**77 (65.3)**	
Etiological Agent	***E coli***	***Salmonella***	***Shigella***	***Subtotal***	***E coli***	***Salmonella***	***Shigella***	***Subtotal***	
Diarrhea persisted despite treatment	5 (100)	0	0	**(100)**	0	0	0	**0**	**0.002²**
Died	4 (50)	0	1 (5.6)	**(55.6)**	5 (27.8)	0	3 (37.5	**8(44.4)**	
Improved and was discharged with medication	4 (25.8)	0	0	**(25.8)**	62 (66.7)	5 (5.4)	2 (2.6)	**69(74.2)**	
Total	**8 (32.8)**	**0**	**1 (0.9)**	**(33.7)**	**67 (56.8)**	**5 (3.4)**	**5 (5.2)**	**77 (65.3)**	

²Kruskal Wallis, Level of significance, $p \leq 0.05$ Confidence Interval 95% ; AD– Acute Diarrhea, PD – Persistent Diarrhea

4.4.2 Clinical outcomes of Diarrhoea cases versus Age and HIV status

Persistence of diarrhea occurred in all cohorts with the highest rating above 24 months in HIV positive cases who may be progressing to AIDS condition compared to HIV negative cases (**Figure 13**). Similarly, a slightly higher number of deaths occurred in ages 0-12 months in 11(61.9%) vs 4(42.9%) in HIV positive compared to HIV negative cases. However, among ages 13-24 months, a group that is highly active, more deaths occurred in HIV negative cases than the latter. There was better prognosis among infants and children aged 0-12 months who also enjoy protection from maternal antibodies with no significant differences between HIV positive 19 (37.3 %) and HIV negative cases 6 (11.8 %).

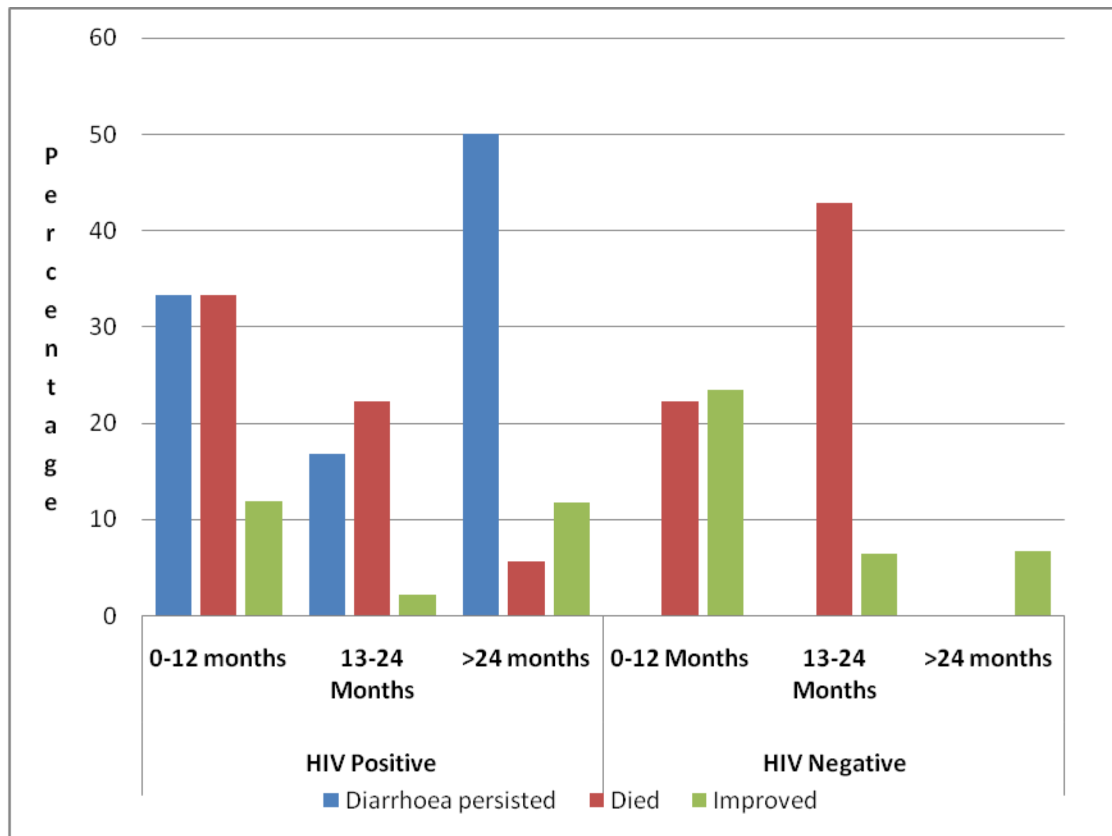


Figure 13: Clinical outcomes versus Age and HIV status

4.4.3 Clinical outcomes between Empiric versus Definitive Treatment

The results yielded significant differences in clinical outcomes between empiric and definitive treatment, (Kruskal-Wallis test: H= 23.7; p value = 0.001). Diarrhea persisted in 3(60%) vs 2(40%) of those treated empirically requiring a shift to other antibiotics following antibiotic susceptibility results (**Table 12**). The study established that 6 (33.3%) of the patients died within a 24 hour period, in HIV positive cases compared to 4(22.2%) in their HIV negative counterparts. Prognosis was better following definitive than empiric treatment 63(90.0%) vs 7(7.5%) and 14(15%) vs 9(9.5%), in HIV positive and negative cases respectively. This means that antibiotic susceptibility testing to determine the best drug of choice for treatment of infections leads to better clinical outcomes.

Table 9: Comparison of Clinical outcomes following Empiric versus Definitive Antibiotic Therapy

Clinical outcomes	HIV Positive			HIV Negative				
	Empiric	**Definitive**	**Subtotal**	**Empiric**	**Definitive**	***Subtotal***	**Total**	**p-value**
Diarrhea persisted despite treatment	3 (60)	2 (40)	**5 (100)**	**0**	**0**	**0**	**5 (4.3)**	**0.001**[2]
Died	6 (33.3)	5 (27.8)	**11 (61.1)**	4 (22.2)	3 (16.7)	**7 (38.9)**	**18 (15.5)**	
Improved and was discharged with medication	9 (9.6)	14 (15.0)	**23 (24.7)**	7 (7.5)	63 (90.0)	**70 (75.3)**	**93 (80.2)**	
Total	**18 (43.9)**	**21 (56.1)**	**39 (34.7)**	**11 (9.5)**	**66 (56.9)**	**(65.3)**	**116 (100)**	

[2]**Kruskal-Wallis test, H= 23.7; Degrees of freedom (df) = 3; p value ≤ 0.05**

CHAPTER FIVE: DISCUSSION

5.1. Prevalence of Bacterial Enteropathogens Associated with Diarrhoea

The present study established 15.6% prevalence of diarrhoea among HIV positive and negative children aged below five years admitted at MTRH (**Figure 11**). This prevalence value was slightly lower but consistent with the national prevalence of diarrhoea in Kenya which stands at 17% according to Kenya National Bureau of Statistics (Macro and ICF, 2010). The national prevalence figure takes into account both outpatient and inpatients but this study focused only on on inpatient children which is generally smaller due to limited capacity in most public hospitals. A similar study in in Nairobi, Kenya, demonstrated prevalence of 17.7% (Sang *et al.,* 2012) and 16.7% in Busia, (Onyango and Angienda, 2010), while 11.2% prevalence was obtained from hospitalized children in Bondo (Tornheim *et al*., 2010). The socio-demographic factors of households in these studies were similar, characterized by low income, poor housing /sanitation and lack of safe drinking water which are risk factors for diarrhoea. Although there was a high number of male than female admissions during the period, there was no significant difference in gender distribution among HIV positive and negative cases (**Table 3**). This concurred with other studies in Kenya (Esamai and Buku, 1994; Makobe *et al*., 2012) and Tanzania (Moyo *et al.*, 2011).

Although the number of male admissions was higher, there was no significant differences wth regard to HIV status. This means that there is no gender bias with regard to health- care seeking behavior for sick children in Kenya, unlike other countries such as India and Pakistan who give preferential treatment to males while girls are neglected (Ansari *et al*., 2012). A higher frequency of bacterial than non bacterial diarrhoea was recorded among HIV negative than positive cases. Although this study did not analyze for viruses and other non-

bacterial agents from culture negative stools, some studies have implicated parasites and viruses as etiological agents of diarrhea in HIV positive patients (Guerrant *et al*., 2005; Obi *et al*, 2012a).

For instance, a study among HIV infected children in Nairobi, Kenya implicated rotavirus as the main etiological agent (Musoke *et al*, 2009), while a different study identified *Cryptosporidium parvum* as an opportunistic pathogen associated with diarrhea in HIV patients (Nyamwange *et al*., 2012). Other studies in Tanzania (Carlson *et al.*, 1999; Moyo *et al*., 2011), have also established a positive correlation between *C. parvum, I. belli* and *E. histolytica* parasitic infections and HIV. The absence of a regular detection of particular bacterial species in HIV-seropositive children, may also be attributed to a fluctuation in the gastrointestinal microbiota or pathogenic agent's due to multidrug chemoprophylaxis (sulfamethoxazole -trimethoprim) and /or other antibiotic treatment to which this group is constantly exposed (Rossit *et al.*, 2007).

There was a significant relationship between age and frequency of diarrhoea with most episodes recorded between 0-24 months of age but extending up to five years in HIV positive cases probably due to their immunocompromised status (**Figure 12**). These findings concurred with previous studies which established that most diarrheal episodes occurred during the first two years of life with incidence being highest in the age group 6-11 months, when weaning often occurs (Janoff and Smith, 1994). This pattern has been noted to reflect combined effects of declining levels of maternally-acquired antibodies, the lack of active immunity in the infant, the introduction of food that may be contaminated with faecal bacteria and direct contact with dirt and other infectious particles as the infant begins to explore (Pavia *et al*., 1992). The decline in diarrhoea cases beyond two years relates to

acquisition of protective immunity (Villamor *et al*., 2004). These findings however differed with those of related studies in Kenya (Makobe *et al*., 2012), probably because the studies concentrated on the general population but did not take into consideration patient HIV status.

E coli species was identified as the main etiological agent of diarrhoea with significantly higher rates of isolation among HIV positive than negative cases while *Shigella* and *Salmonella species* had lower frequencies (**Table 5**). The same trend has also been demonstrated in other studies in Kenya although in varying proportions (Sang *et al*., 2012; Makobe *et al*., 2012; Karambu *et al*., 2013). Slight deviations but following a similar pattern have been seen studies in most countries in Sub-Saharan Africa and other developing countries, Nigeria (Nweze, 2010), *Ethiopia (Demissie et al*., 2014) and Iran (Alikhani *et al*., 2013). Unlike developed countries with good health facilities, safe water and sanitation, developing countries including Kenya have not kept pace. This is why both children and adults still suffer frequent gastrointestinal disorders including diarrhoea, that are characterized by unsafe water and unsanitary conditions associated with poverty characteristic of majority of households from which our cases were drawn. Childhood diarrhea remains an important health concern in the study area. Occurrence of diarrhea could be decreased by interventions aimed at improving sanitation and hygiene, prompt diagnosis and treatment of infection.

5.2 Molecular Pathotypes of *E. coli, Salmonella* and *Shigella*

The study confirmed a total of 47 pathotypes diarrheagenic agents; 36 (34.3%) of *Escherichia coli,* 5(100) *Salmonela* and 6 (100) *Shigella* from both HIV positive and negative cases while 69 (65.7%) of *E coli* were not identified using the selected range of primers which target the most common virulent strains associated with diarrhoea in children

(**Table 5**). The pathotypes identified showed varied expression of virulence markers with the highest being EAEC followed by EPEC, EHEC, EIEC and ETEC and a greater proportion expressed among HIV positive 19 (52.8%) compared to HIV negative cases 17 (47.2%) which may be attributed to superimposed infection due to defective immunity (Rossit *et al*, 2007). Other studies have showed variations in pathotypes with being the most prevalent in Nairobi, Kenya (Makobe *et al*., 2012), South East Nigeria, (Nweze, 2010) and even farther way in in Brazil and Mexico (Huang *et al*., 2007; Contreras and Antonios., 2011). Although no study in Kenya has compared prevalence of DEC pathotypes in relation to HIV serostatus in childhood diarrhea, these findings agree with a study in Tanzania, which also identified DEC as the major agent associated with childhood diarrhoea with 64.1% EAEC, 20.3% EPEC and 15.6% ETEC (Moyo *et al*., 2011).

Similarly, a study to compare virulence genes in diarrheagenic E *coli* of isolates from various parts of Kenya and Japan also identified EAEC as the most frequent pathotype in (36.6% vs 17%) diarrhoea cases, respectively, (Bii *et al*., 2005). However, a study on shigatoxigenic *Escherichia coli* from in Maasailand, Kenya, revealed a different scenario with ETEC (29.8%), EHEC (24%), EAEC (14.2%) and EPEC (3.5%) being identified (Sang *et al.,* 1997). In Nairobi, Kenya, (Makobe *et al*., 2012), identified EPEC (19.3%) as the most prevalent followed by ETEC (7.25%) and EAEC (3.86%) (Makobe *et al*, 2012). In Maputo, Mozambique (Sumbana *et al*., 2015), demonstrated EIEC as the most prevalent, followed by EPEC. With regard to HIV status, these results concurred with studies in South Africa (Germani *et al*., 1998; Gassama *et al*., 2001; Samie *et al*, 2012) which recorded a higher infection rate with EAEC among HIV positive patients. A similar study in Zambia also showed that EAEC genes were detected in both HIV cases and controls with no evidence of long-term carriage (Kelly *et al*., 2003).

There was a low rate of detection of *Salmonella* and *Shigella* in this study with minimal cases among HIV positive group. Other studies in Sub-Saharan Africa, have also reported lower rates of *Salmonella* and Shigella in childhood diarrhoea (Gassama *et al*., 2001; Nweze, 2010). A Study done different parts of Ethiopia, recorded *Salmonella* was reported in 5 (4.2%) cases with Harar (11.5%) and Jimma (15.4%), (Demissie *et al*., 2014). This was much lower than findings reported in Tanzania (Temu *et al*., 2008). However, prevalence rates in India were within the reported range of 1-5% of gastroenteritis cases in most developing countries (Ansari *et al*., 2012). Although *Shigella dysentriae* and *S. flexneri* were the only *Shigella* pathotypes detected in equal proportions in 6 (5.1%) of cases, higher detection rates of *Shigella* have been reported in other studies with *S. flexneri* rating 64.7% in Ethiopia (Demissie *et al*., 2014), Addis Ababa 54.0% (Asrat, 2008), Tanzania 90% (Temu *et al*., 2008), Indonesia 63.2% (Herwana *et al.*, 2010), with rates of *S. dysentriae* being much lower and ranging from 8-15 % in these studies.

The general decline in isolation rates of *Salmonella* anand *Shigella* in Kenya and other developing countries may be due to increased awareness of about personal and environmental hygiene through the continuous health education on the prevention of shigellosis and salmonellosis. It may also be attributed to frequent use of broad spectrum antibiotics to treat opportunistic infections which also kill or inhibit any infectious agents of *Salmonella* or *Shigella* origin. These variations in distribution of causative agents demonstrate the complexity of the etiology of diarrhea caused by enteric pathogens and indicate risk of emergence of new pathogenic variants due to the horizontal transmission of pathogenicity factors among enteric species. From this study, it was evident that virulence genes in DEC occur in both HIV positive and negative patients and may manifest differently depending on host immunity.

Evaluation of pathotypes and virulence markers of and type of diarrhoea in both HIV positive and negative groups, revealed that both groups harboured *E coli, Salmonella* and *Shigella* virulence genes responsible for varied clinical presentations of diarrhea ranging from acute to persistent diarrhoea E coli Pathotypes and virulence markers being predominant. *E. coli* with typical virulence gene profiles have been associated with severe outbreaks of diarrhoea worldwide (Crump *et al.*, 2011). Acute diarrhea in HIV positive cases was associated with EAEC *aatA* and EPEC *bfp* while EIEC ipaH, EHEC $stx_{1,}$ stx_2 and ETEC *elt* and *est* was evident in HIV negative cases, (**Table 6**).

The main virulence factors reported for *Salmonella was invA* linked to invasion of the gastrointestinal mucosa, an essential step in the pathogenesis of infection due to these organisms while *vir F* resistance and invasion factor *ipaH* were amplified in equal proportions in *Shigella*. EAEC *aatA* was responsible for a high proportion of persistent diarrhea in both cases. Several studies have indicated that *E. coli* pathotypes require multiple genes to be fully / highly virulent (Obi *et al.*, 2004b**)**. For instance ETEC with heat-labile toxin *elt*, heat-stable toxin *est* and colonization factor antigens (CFAs); EPEC with *bfp* and *eae* gene and Shiga-toxin-producing *E. coli* (STEC or EHEC) with shiga-like toxin *stx* and *eaeA*, are considered the most virulent (Turner *et al.,* 2006). Although this study did not detect multiple genes in individual isolates, the dual existence of multiple virulence genes as viz-a-viz single gene in other studies raises questions as to whether or not these genes act in synergy to induce acute disease.

EAEC have been found to be genetically heterogeneous containing various virulence genes including *aggR* (Ballal and Ramamurthy, 2007). However, the presence of multiple genes has not been demonstrated in EAEC pathogenesis. This may explain why EAEC is

predominantly associated with persistent diarrhea while strains with multiple genes such as EPEC, ETEC and EHEC are significantly linked to acute diarrhea. Atypical EPEC has also been found to be a heterogeneous group comprising members differing in their virulence potential with particular lineages being closely related to VTEC, sharing certain virulence determinants. Although several studies have demonstrated heterogeneity in virulence mechanisms of EAEC isolates, there have been no clear associations between EAEC and diarrhea (Bouckenooghe *et al.,* 2000). Some studies have described different markers of pathogenesis in EAEC infections including fecal cytokines such as IL-8 and IL-1R, elevated levels of lactoferrin and occult blood in diarrhoea (Greenberg *et al.,* 2002; Obi *et al*., 2004). Whether this is true or not remains unknown and may require further investigation.

Analysis of pathotypes and virulence markers of enteropathogens in relation to age of patients revealed a skewed distribution towards the younger age groups (0-24 months) in both categories except EAEC which was registered in all cohorts among HIV positive cases and this difference was statistically significant. HIV infected children are known to experience diarrhoea episodes that are more severe, prolonged and recurrent than the HIV negative whose decline in diarrhoea incidence beyond two years relates to acquisition of protective immunity (Nweze, 2010).

5. 3 Antibiotic Profiles of *E coli, Salmonella* and *Shigella*

Significant differences in antibiotic susceptibility of *E coli*, *Salmonella* and *Shigella* were demonstrated among HIV positive and negative cases to the antibiotics Ampicillin, Amikacin and Ceftriaxone, Cefepime and Imipenem respectively with greater resistance in the latter. These agreed with findings of a study in Senegal in which enteropathogens from HIV positive cases displayed high resistance to most antibiotics used in treatment of diarrhoea

including Ampicillin, tetracycline, and Cotrimoxazole but were susceptible to Amikacin, gentamicin, and Norfloxacin (Gassama *et al*., 2001). E *coli*, *Salmonella* and *Shigella* isolates were all highly resistant to the conventional antibiotics Ampicillin and Cotrimoxazole (**Table 8**). Previous studies in Kenya have also demonstrated resistance of enteropathogenic bacteria to commonly prescribed antibiotics (Shapiro *et al*., 2001; Okeke *et al*., 2007; Onyango and Angienda, 2010; Sang *et al*., 2012). The high antibiotic resistance noted among HIV positive cases may be attributed to their prolonged use as prophylaxis or treatment of opportunistic infections and poor adherence to treatment regimens among other causes (Emacar *et al*., 2010).

Cotrimoxazole (trimethoprim-suphamethoxazole) is one of the first-line drugs recommended for the empiric treatment of diarrhea (Wardlaw *et al*., 2010). However a high level of resistance was noted in all the three species with a greater resistance among HIV positive cases. Diarrheagenic *E coli* strains EHEC, ETEC, EAEC, EIEC and EPEC were susceptible to Ceftriaxone, Cefuroxime, Ceftazidime, Ciprofloxacin and Amikacin (**Table 9**). Although cephalosporins are not usually used in treatment of diarrhea, the sensitivity of most isolates to second (cefuroxime), third (ceftazidime and Ceftriaxone) and fourth generation cephalosporins (cefepime) and intermediate susceptibility to Amikacin, Imipenem and Gentamicin signifies the relevance of these drugs in treatment of acute and persistent diarrhea in addition to other management strategies. However, these drugs are not affordable to most Kenyans (Kariuki *et al*., 2006).

Virulence factors in EIEC ipaH, EPEC bfp and EAEC aatA appeared to be linked to antibiotic resistance with the latter exhibiting greatest resistance in both cases. Similarly, all the pathotypes and virulence markers in Shigella and Salmonella species were significantly

linked to resistance. Previous studies in Kenya (Bii *et al*., 2005; Sang *et al*., 2012), and in South Africa (Gassama *et al*., 2001; Obi *et al*., 2004b), have established resistance to commonly used antibiotics and emerging multi- resistance of different categories of DEC strains in developing countries (Bisi-Johnson *et al*., 2011). A study in Kenya documented resistant EAEC strains containing aggR gene and exhibiting classical stacked-brick-like aggregative adherence pattern on Hep-2 cells (Bii *et al*., 2005). Similarly, in Central America, EAEC strains from diarrheic stool had increased resistance than ETEC (Ochoa *et al.,* 2004; Nguyen *et al.,* 2005; Bisi-Johnson *et al*., 2011). There is fear that carriers of EAEC strains could risk a treatment failure (Trung *et al*., 2005). A previous study linked multidrug resistant species of non-typhoidal *Salmonella* (MDR NTS) with increased rate and duration of hospitalization and a twofold increased risk of death and invasive infection (Kariuki *et al*., 2006).

Increased resistance has been attributed to extended-spectrum beta-lactamase (ESBL) enzymes produced by the specific pathogenic strains has been cited as one of the causes of increased resistance in *E coli*, *Salmonella* and *Shigella,* (Moyo *et al*., 2011) and the evolution of microbial populations of *Salmonella* via exposure to selective forces has been linked to widespread use of antibiotics in poultry farming (Oni *et al.,* 1991). The virulence genes *invA in Salmonella* and *vir F* resistance may have played a significant role in resistance. *Shigella* and *Salmonella* antibiotic resistance has been noted to be upregulated in HIV owing to frequent exposure to antibiotics following treatment for opportunistic infections or through acquisition of resistant strains from the hospital environment.

On history of medication among subjects, both HIV positive and negative patients were noted to have used some form of medication prior to admission but there were significant

differences among those on ORS, antibiotics and anti-malarial drugs (**Table 10**). Multi-vitamins and herbal remedies were applied in different proportions among HIV positive and negative cases. A previous study in sub-Saharan Africa also reported the use of antibiotics, local herbs and ORS in the treatment of diarrhea at home (Oni *et al*., 1991). There is a possibility that herbal remedies commonly used in rural settings in developing countries may interfere with effective antibiotic therapy, although their exact mechanism remains unclear (Obi *et al*., 2004b). Interestingly, parents/guardians of HIV positive patients were however, careful to obtain medication from health facilities rather than friends and relatives or purchased without prescription as was common in HIV negative cases, a risk factor predisposing to antibiotic resistance. This may be attributed to proper advice provided by clinicians for those on ARV therapy. Furthermore, HIV positive patients had access to free care and treatment for opportunistic infections at the hospital. This also explains why some of them had left over drugs from previous illnesses.

As suggested in previous studies, the overuse and misuse of antibiotics in the treatment of diarrhea could lead to increased resistance (Kakai and Wamola, 2002; Trung *et al*., 2005; WHO, 2014). Evidence of patients obtaining drugs from retail pharmacies in Kenya without prescription is a common phenomenon. These retailers, often operating without a license are preferred because they are more accessible to patients as they are located within the community and most often do not charge consultation fees while at the same time providing quick and negotiable treatment services that meet the financial needs of clients (Okeke *et al.*, 2007).

5.4 Clinical outcomes of *in-vivo* Antibiotic Susceptibility

Acute and persistent diarrheas as well as dysentery is frequently a life threatening condition characterized by signs of severe dehydration, toxemia, marked leucocytosis, high-grade fever, severe tenesmus, gross fecal blood loss and dissemination of infection (Melo *et al.*, 2008). Supportive rehydration therapy and adequate nutritional support, is the cornerstone therapy, regardless of etiology or severity of the process and its prompt adoption is associated with a favorable outcome (Goodman and Segreti, 1999). Antibiotics are known to shorten the duration of diarrhea and prevent serious complications associated with the infection as well reduce the severity of associated symptoms, such as fever, abdominal pain and vomiting (Oketcho *et al.*, 2012).

Furthermore, antimicrobial therapy protects against secondary infections by reducing person-to-person spread of most pathogens, (Melo *et al.*, 2008). Prompt adoption of empirical antimicrobial therapy is also useful in treatment of febrile acute diarrhea in young children as recommended by the World Health Organization (WHO, 2010). The decision to start antimicrobial therapy for acute diarrhea is based on clinical presentation and often made empirically using the narrowest antimicrobial spectrum possible that covers the most likely pathogens in each case. As soon as the results of the stool culture are available, therapy may be altered according to the antimicrobial susceptibility pattern, favoring the use of a narrower-spectrum, cheaper and /or safer drugs if necessary (Diniz-Santos *et al*, 2006).

The study established that out of all patients treated for bacterial diarrhea, 80.3% improved and were discharged with medication while diarrhoea persisted in 4.3% and 15.5%, died while undergoing treatment. A recent study revealed that in low-income settings, acutely ill children are at highest risk of death in the first 48 hours in hospital (Gathara *et al.*, 2013). For

instance, at Kenyatta National Hospital the leading Referral Hospital in Kenya, pneumonia and dehydration contribute to 55% of the admissions and 45% of the deaths (Maina, 2006). Persistent diarrhea in HIV positive cases showed slow response to antibiotic therapy compared to HIV negative cases. This may be attributed to immunosuppression and resistance due to prolonged antibiotic use. This concurred with earlier studies in which children with HIV/AIDS had more frequent diarrhea and had worse outcomes than their HIV-uninfected counterparts (Thea *et al.*, 1993; van Eijka, 2010). In a Swiss cohort study, diarrhea was found to be an independent predictor of poor survival amongst HIV and AIDS patients (Humphreys *et al.,* 2010).

Diarrheagenic *Escherichia coli* were significantly associated with a higher number of deaths in HIV positive than HIV negative cases. However, response to antibiotic therapy was better in diarrhea associated with *E. coli* than that due to *Shigella* and *Salmonella* etiology. Salmonella has been cited as an important cause of opportunistic infections in HIV/AIDS and responsible for severe morbidity in these patients. Studies done in Nyanza, Kenya have shown that *E. coli* have contributed to higher mortalities with varying responses to antibiotic therapy (Shapiro *et al.*, 2001) There were significant differences in clinical outcomes of in-vitro antibiotic susceptibility in relation to age among HIV negative individuals with the younger infants and children responding better than older cases. However, HIV positive cases showed slow response to treatment with increasing age. Previous studies have established that age strongly determines child health outcomes with better outcomes in younger children than older children (Taffa and Chepngeno, 2005). These studies noted that mothers / caretakers tend to pay more attention and are more concerned about the health status of younger children than of older ones. Patients treated definitively following results of specific antibiograms yielded better prognosis than empiric treatment. The fact that diarrhoea

persisted despite empiric treatment in some cases is an indication that empiric therapy is not always effective. There are several arguments against empirical use of antibiotics for acute infectious diarrhoea. The most compelling of them is the fact that acute infectious diarrhea is typically a self-limiting disease, regardless of etiology, with most cases resolving in less than three days (Melo *et al.*, 2008). A few cases, however, require antimicrobial therapy, due to severity of the clinical picture or complications such as dissemination of disease, sepsis or disseminated intravascular coagulation (Diniz-Santos *et al*, 2006).

5.5 Limitations

1. The study isolated 118 bacterial enteropahogens from diarrhoeic stool samples, out of which 47 pathotypes were fully identified with their respective virulence markers using multi-plex PCR. Although all *Salmonella* and *Shigella* strains were identified, total of 69 *E coli* strains identified previously detected as diarrheagenic *E coli* using the serological methods failed to amplify using the selected range of primers in the multiplex PCR reaction suggesting a need to expand the range to target all other possible DEC as well any emerging pathogenic strains.
2. The study investigated bacterial diarrhoea which accounted for 54.6% of diarrhoea cases. However, although non bacterial diarrhoea (45.4%) which may be attributed to other agents such as viruses, parasites and protozoa was a significant proportion that warranted investigation, this was not done as it was beyond the scope of this study.
3. Although, the study determined antibiotic profiles of *E. coli, Salmonella* and *Shigella* against ten selected antibiotics commonly used at MTRH using commercially available sensitivity discs with specified concentrations of antibiotics, the minimum inhibitory concentration (MIC) of antibiotics tested was not determined in this study. This

information would probably guide clinicians in determination of dosages of antibiotics for which the isolates were susceptible.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of Findings

The study documented of 15.6% prevalence diarrhoea of among hospitalized HIV positive and HIV negative children aged below five years at MTRH. A total of 41(34.7%) and 77(65.3%) of bacterial diarrhea cases were HIV positive and negative respectively with higher frequencies occurring in males than females but with no gender differences with regard to HIV serostatus. There was an overall high number of diarrhoea cases among children aged 0-12 months 54.2% (19 vs 45) in both HIV positive and negative groups but but declined with increasing age to nil at 36 months as opposed to persistence across the ages in HIV negative cases. Some of the risk factors found to significantly influenceprevalence of diarrhoea were age, type of residence, source of drinking water, education level of mother or guardian and seasonal variations.

The main enteric pathogens and strains identified in this study were *Escherichia coli* 105(88.9%)*, Shigella 6*(5.1%) and *Salmonella* (4.2%) while the pathotypes detected in order of frequency were EAEC 21(58.3%) followed by EPEC 6 (16.7%), EHEC 4 (11.1%), EIEC 3 (8.3%) and ETEC 2(5.6%), *Shigella flexneri 3* (50%), *S. dysentriae 3* (50%), *S.typhimurium 4* (80%) and *Salmonella typhi 1* (20%). Acute diarrhea was associated with EAEC *aatA* and EPEC *bfp,* EIEC ipaH, EHEC $stx_{1,}$ stx_2 and ETEC *elt* and *est. virF* genes in *Shigella* and i*nvA* genes in *Salmonella* were detected with differences existing with regard to host HIV staus. Significant differences in antibiotic susceptibility of *E coli*, *Salmonella* and *Shigella* were demonstrated among HIV positive and negative cases to the antibiotics ampicillin, amikacin and ceftriaxone, cefepime and imipenem respectively with greater resistance in ampicillin and cotrtrimoxazole. Virulence factors in EIEC *ipaH*, EPEC *bfp* and EAEC *aatA* as well as

ipaH and *virF* genes in *Shigella* and i*nvA* genes in *Salmonella* appeared to be linked to antibiotic resistance with the greatest resistance in EAEC *aatA* but with similar resitance rates in *Salmonella and Shigella* in both cases.

Analysis of clinical outcomes of *in-vivo* antibiotic susceptibility showed that out of all patients (118), treated for bacterial diarrhea, 93(80.3%) improved and were discharged with medication, diarrhea persisted in 5(4.3%) and 18(15.5%) died while undergoing treatment. There were significant differences in clinical outcomes of *in-vivo* antibiotic susceptibility in relation to age and HIV status. Younger infants and children responded better to treatment than older cases and with worse clinical outcomes among HIV positive than negative cases.

6.2 Conclusions

1. The study documented 15.6% prevalence of diarrhoea among children aged below five years admitted at MTRH with higher frequency of bacterial diarrhoea in HIV negative (54.6%) than positive cases (45.4%) and in ages 0-24 months with no gender differences among the groups.
2. The main bacterial enteropathogens detected were *E coli* species 105(88.9%), *Shigella species 6*(5.1%) and *Salmonella species* 5(4.2%) and with the main pathotypes and virulence markers being EAEC *aatA*, followed by EPEC *bfp*, EIEC *ipaH,* EHEC st_{x1} *vs* st_{x2} and ETEC *elt* vs *est in E coli, virF* and ipaH genes in *Shigella* and *invA* genes in *Salmonella.*
3. The antibiotics cefuroxime, ceftazidime, ciprofloxacin, cefipime and amikacin were effective in treatment of bacterial diarrhea while majority of the isolates were resistant to cotrimoxazole and ampicillin.

4. There were significant differences in clinical outcomes of *in-vivo* antibiotic susceptibility with better outcomes among HIV negative than HIV positive cases.

6.3 Recommendations

1. Based on laboratory results, the antibiotics cefuroxime, ceftazidime, ciprofloxacin, cefipime and amikacin are effective and may be incorporated into treatment regimens of childhood diarrhea in Kenya while cotrimoxazole and ampicillin are ineffective and may be excluded from treatment regimens.
2. Continuous surveillance of antibiotic susceptibility profiles of various agents to commonly used antibiotics is necessary to guide in treatment and prevent emergence of multi-drug resistance.
3. The study recommends definitive diagnosis of diarrhea etiology and determination of HIV serostatus for effective management of childhood diarrhea where possible.
4. Determination of Minimum inhibitory concentrations of selected antibiotics may be necessary to determine the actual dosages that are effective in treating specific infections and improve clinical outcomes.

6.4 Suggestions for Future Research

1. Investigate *E coli* as an emerging threat to child health in relation to increased virulence in diarrhoea.
2. Determine viral, parasitic and fungal etiologies of diarrhoea in HIV positive and negative children at MTRH.
3. Develop novel efficient rapid and affordable laboratory diagnostic techniques for use in detection of diarrhoea in lower level hospitals.

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APPENDICES

Appendix 1: Consent Form (English Version)

Title of Study: ***Escherichia coli, Salmonella and Shigella*** **Pathotypes and Antibiotic profiles among HIV Seropositive and Seronegative Diarrhea inpatients aged below 5 years in Western Kenya**

Introduction

This form provides information that you need to know about the study. You are free to ask questions and to freely accept or decline to participate in this study. The researchers are interested in identifying the germs responsible for diarrhea in children aged below five years in Kenya and determine the most effective drugs to treat diarrhea.

Who is eligible to participate in this study?

You can participate in this study if your child has been admitted with diarhea at Moi Teaching and Referral Hospital.

How will you as a participant benefit from this study?

If you agree to participate, you will have tests done on your child to determine the cause of the diarrhoea and to identify the best treatment to be given. However, if you decline to participate in the study, you will continue to receive medical services for your child as usual.

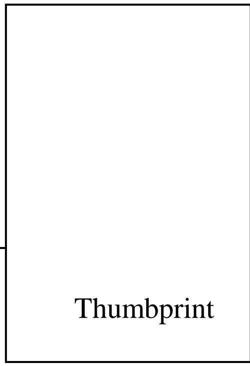
Confidentiality

All the information provided in this study will be kept strictly confidential. However, the researchers have permission to look at your records.

Consent

I have carefully read and understood this Consent Form. I have had an opportunity to ask questions about the study and i am satisfied with all the explanations given to me.

I agree to participate in this study. (Tick whichever is appropriate).



Yes ☐ No ☐

Signature of Parent/Guardian _______________ Date ___________

Signature of subject: ____________________Date _______________

Name of Staff Explaining consent _______________________________

Signature _______________ Date __________________________

Appendix 2: Consent Form (Kiswahili Version)

Seropositive and Seronegative Diarrhoea inpatients aged below 5 years in Western Kenya

Mada

Escherichia coli, Salmonella and Shigella Pathotypes and Antibiotic profiles among HIV

Utangulizi

Fomu hii, inatoa habari unayohitaji kujua kuhusu utafiti huu. Uko huru kuuliza maswali na kukubali au kukataa kushiriki katika utafiti huu. Watafiti wana nia ya kutambua viini vinavyosababisha kuhaisha miongoni mwa watoto wenye umri chini ya miaka mitano nchini Kenya na kuamua dawa bora zaidi ya kutumia kutibu ugonjwa huo wa kuharisha.

Nani, anaweza kushiriki katika utafiti huu?

Unaweza kushiriki katika utafiti huu ikiwa motto wako amelazwa katika hospitali ya rufaa na Mafunzo ya Moi akiwa na ugonjwa wa kuharisha.

Utafaidika vipi na utafiti huu?

Ukikubali kushiriki, mtoto wako atapimwa ili kujua chanzo cha ugonjwa wa kuharisha na kuamua bora zaidi ya kumpa. Hata hivyo ukikataa kushiriki katika utafiti huu, mtoto wako ataendelea kupokea matibabu kama kawaida.

Usiri

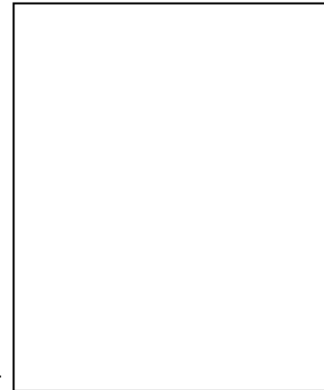
Habari zote zitakazotolewa katika utafiti huu zitakuwa za siri kabisa.Hata hivyo, watafiti wana ruhusa ya kuangalia kumbukumbu zako.

Idhini

Nimesoma kwa makini na kuelewa fomu hii ya idhini. Nimekubaliwa kuuliza maswali kuhusu utafiti huu na nimetosheka na maelezo nilizopewa.

Nakubali kushiriki katika utafiti

huu na nimetosheka na maelezo nilizopewa. Tia alama kwa yoyote iliyo sahihi.



Ndio

Sahihi la mzazi ____________________ Tarehe __________

Sahihi ya mhusika: __________________Date ________________

Jina la mwenye kueleza idhini ______________________________

Sahihi______________Tarehe ________________________

Appendix 3: Research Questionnaire

A. Demographic Data

Fill in the spaces provided and tick (√) where appropriate.

Patient (Hospital) Code______________________ Age in Months_______________

Sex: Male ☐ Female ☐

02. Occupation of Mother/Guardian: Mother____________________

03. Gross monthly income of parents /Guardian in Kenya shillings (√Tick where appropriate).

- ☐ Above 40,000
- ☐ 30,000 – 39,000
- ☐ 20,000 – 29,000
- ☐ 10,000 – 19,000
- ☐ Below 10,000

04. Type of house the patient resides in:

- ☐ Stone /Brick walled
- ☐ Semi-permanent
- ☐ Mud-walled
- ☐ Tin walled

05. Level of education of Mother / Guardian

- ☐ None
- ☐ Primary
- ☐ Post Secondary
- ☐ Tertiary

06. Source of drinking water

- ☐ Piped treated water
- ☐ Well/ Borehole
- ☐ River

Other source

(Specify)________________________________ ______________

B. MEDICAL HISTORY OF PATIENT

07. How long has the child had diarrhea? Specify the number of days.

____

08. What were the main symptoms?

- [ ] Diarrhea, fever, vomiting
- [ ] Diarrhea, vomiting
- [ ] Diarrhea only
- [ ] Other symptoms (Specify)

____

09. What was the frequency of diarrhea episodes per day?

- [ ] Once a day
- [ ] Three times a day
- [ ] Four times a day
- [ ] Five times a day or more

10. Was the child having any other illness apart from diarrhoea during admission?

Yes [ ] No [ ]

If yes, please specify______________________________

11. Has the child been given any medication to treat diarrhea?

- [ ] Yes
- [ ] No

If yes, please specify the medication ______________________

12. When was the medication administered? Specify ________________

____

13. What was the source of the medication for the condition in Question 11 above?

- [ ] Prescribed at a health facility
- [ ] Left over drugs from previous illness
- [ ] Community Health workers
- [ ] Purchased from a chemist without prescription
- [ ] Given by Friends / Relatives

Other (Specify) ______________________________________

14. Is the child on any other medication for any other condition apart from this diarrhea?

☐ Yes

☐ No

If yes, please specify____

Name of Interviewer: ____________________ Designation_________________________

Signature______________________________Date _________________________

Appendix 4: HIV Test Result

HIV TEST (Use a tick (√) where appropriate)   CONFIDENTIAL

Patient (Hospital) code Number ________________Laboratory ID: ____________

Date:_ _ / _ _ / _ _   Age ____________ Sex: __________________________

Laboratory Report: HIV Test (Use a tick (√) where appropriate)

Test Kit	**Result**	
1. Determine TM HIV 1/2	☐ REACTIVE	☐ NON REACTIVE
2. Uni Gold TM HIV 1/2	☐ REACTIVE	☐ NON REACTIVE
3. SD Bioline HIV ½	☐ REACTIVE	☐ NON REACTIVE

Final Results: (To be reported to client)

☐ POSITIVE ☐ NEGATIVE

Client Test Result _____________________

Authorized Signature __________________________________

Designation____

Site Name ____

Appendix 5: Stool Culture

Patient (Hospital) code Number________________ Laboratory ID: ____________

Date:_ _ / _ _ / _ _ Age ____________ Sex: __________________________

STOOL CULTURE WORKSHEET

Sample ID ________Date Received: ____/____/____ Date Inoculated: ____/____/____

Media	**Direct Plating 24 Hour Incubation at 37C**	**Selenite Enrichment 24 Hour Incubation at 37C**
MacConkey Agar (MAC)		
DCA		
Biochemical Test	**Positive (+)**	**Negative (-)**
TSI		
Oxidase		
Urease		
Indole		
Methyl Red		
Voges Proskaeur		
Citrate Utilization		

Appendix 6: Serology

Strain	Specific Antisera	Positive (+)	Negative (-)	Total
EPEC	O55			
	O26			
	O111			
	O119			
	O125			
	O126			
	O127			
	O128			
	O142			
ETEC	O16			
	O15			
	O148			
	O25			
	O128			
	O153			
	O159			
EIEC	O28			
	O29			
	0124			
	O136			
EHEC	O26			
	O111			
	O138			
	O157:H7			
EAEC	O95			
	O97			
	O98			
	O96			

Appendix 7: Antimicrobial Sensitivity Testing (Disc Diffusion Method)

Antibiotic	Result (Zone diameter)	Remarks Sensitive / Intermediate / Resistant
Ampicillin (AMP)		
Amikacin (AMK)		
Ceftriaxone (CXR)		
Cefuroxime (CXM)		
Ceftazidime (CDM)		
Gentamicin (GEN)		
Cotrimoxazole (CTX)		
Cefipime (CPM)		
Ciprofloxacin (CIP)		
Imipenem (IMP)		

Appendix 8: Clinical Outcomes of *in-vivo* Antibiotic Susceptibility

1. How did the child respond to the treatment administered?

☐ Improved and was discharged with medication

☐ Diarrhea persisted despite treatment

☐ Died

2. What medication was administered to the patient?

☐ Antibiotics (Specify) ______________________________________

☐ Rehydration

☐ Other (specify) ____

Appendix 9: Standard Antibiogram Interpretation for Susceptibility Testing

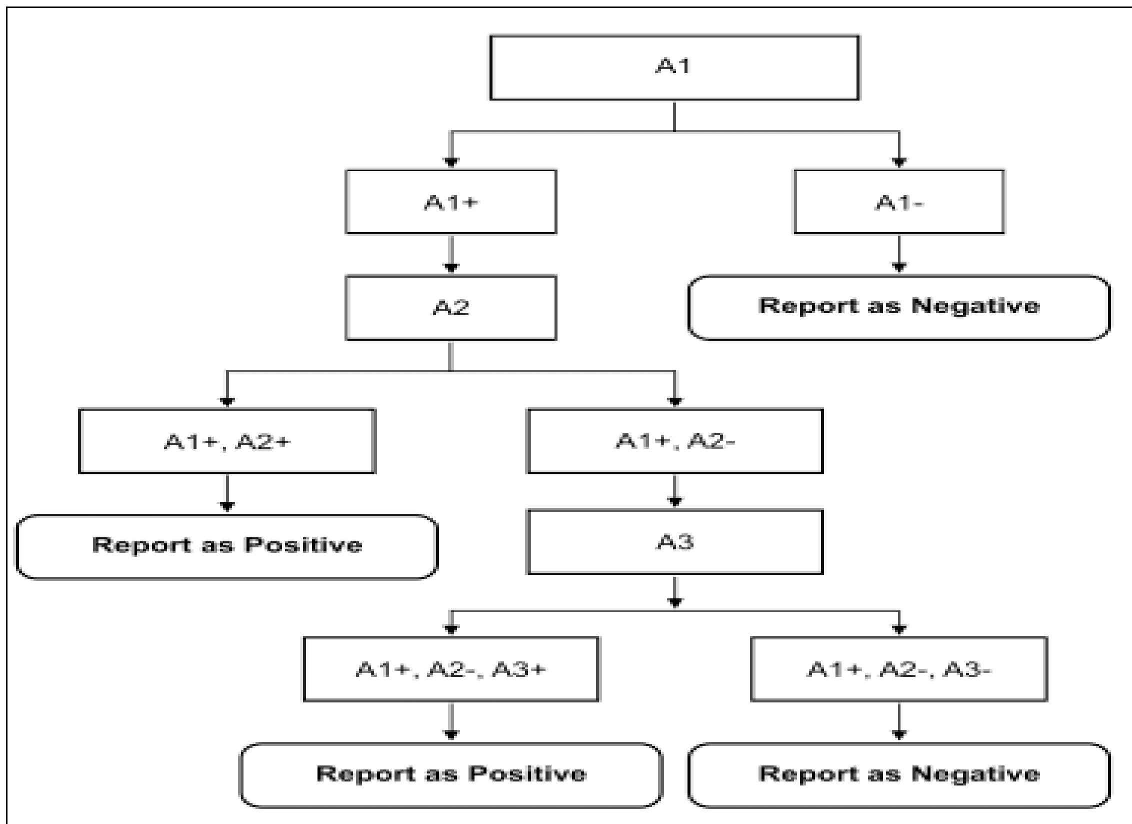
S/No	Antibiotic	Abbreviation	Disc Content	Zone Diameter (mm)		
				Sensitive	Intermediate	Resistant
1	Ampicillin	AMP	30 µg	≥ 17	16	≤ 15
2	Amikacin	AK	10 µg	≥ 17	15-16	≤ 14
3	Ceftriaxone	CRO	30 µg	≥21	14-20	≤ 13
4	Cefuroxime	CXM	30 µg	≥18	15-17	≤ 14
5	Ceftazidime	CDM	30 µg	≥17	14-16	≤13
6	Gentamicin	GN	10 µg	≥15	13-4	≤ 12
7	Cotrimoxazole	CXT	30µg	≥16	11-15	≤ 10
8	Cefipime	Cpm	10 µg	≥20	17-19	≤16
9	Augmentin	AUG	30 µg	≥14	11-13	≤10
10	Imipenem	IPM	10 µg	≥20	17-19	≤16

Appendix 10: Suggested Antibiotic Dilution Ranges for MIC Testing

Antimicrobial agent	**Concentration range(µg/ml)**
Penicillin G	0.015-32
Ampicillin(Gram Negative)	0.13-256
Cephalosporin I generation (Gram Negative)	0.13-256
Cephalosporin II and III generation (Gram Negative)	0.03-64
Cephalosporin III generation	0.13-256
Amikacin	0.06-128
Gentamicin	0.03-64
Cotrimoxazole	0.03-64
Sulphamethoxazole	0.6-1216

Source: A guide to Sensitivity Testing: Report of working party on antibiotic sensitivity testing of the British society for Antimicrobial Chemotherapy. *Journal of Antimicrobial Chemotherapy* 1991; (27): Supplement D, 1-50.

Appendix 11: Diagrammatic Representation of Rapid HIV Testing Algorithm



Key

A1 (First test): Determine HIV 1/2

A2 (Second test): Uni-Gold HIV

A3 (Third test): SD Bioline HIV 1/2

\+ Reactive

_ Non-reactive

Appendix 12: Ethical Appproval

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET
Tel: 33471/2/3

Reference: IREC/2011/120
Approval Number: 000711

16th September, 2011

Dr. Ms. Salina Rono
P.O. Box 5407 – 30100,
ELDORET, KENYA.

Dear Ms. Rono,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee has reviewed your research proposal titled:

"Escherichia Coli, Salmonella and Shigella Pathotypes and Antibiotic Susceptibility among HIV Seropositive and Seronegative Diarrhoea in Patients below Five in Western Kenya"

Your proposal has been granted a Formal Approval Number: **FAN: IREC 000711** on 16th September, 2011. You are therefore permitted to start your study.

Note that this approval is for 1 year; it will thus expire on 15th September, 2012. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Yours Sincerely,

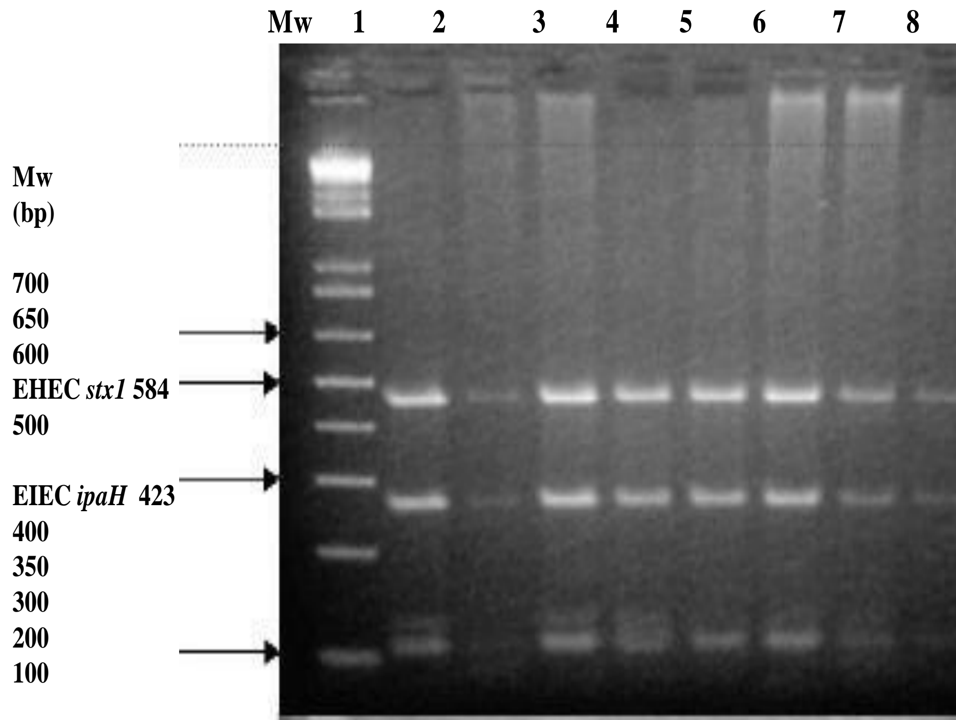
19/09/2011

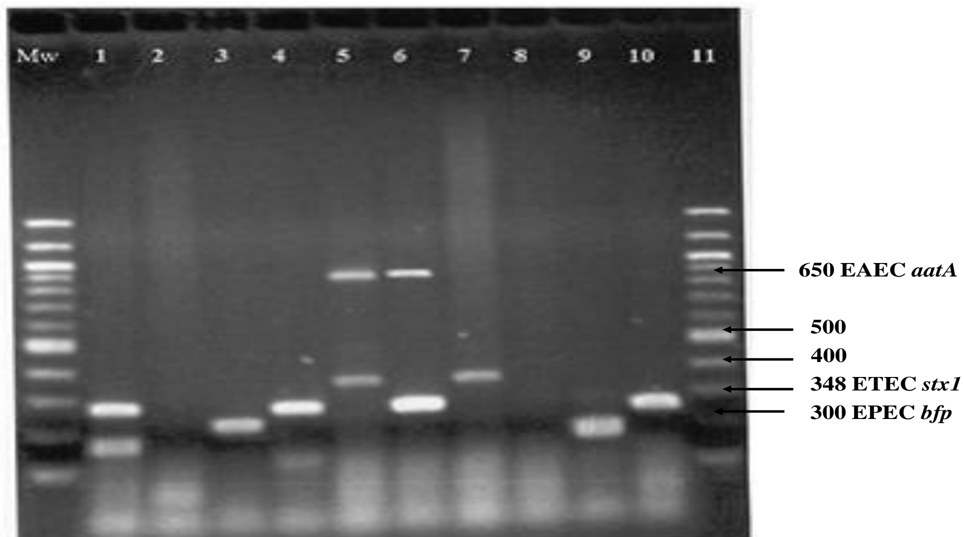
DR. W. ARUASA
AG. CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

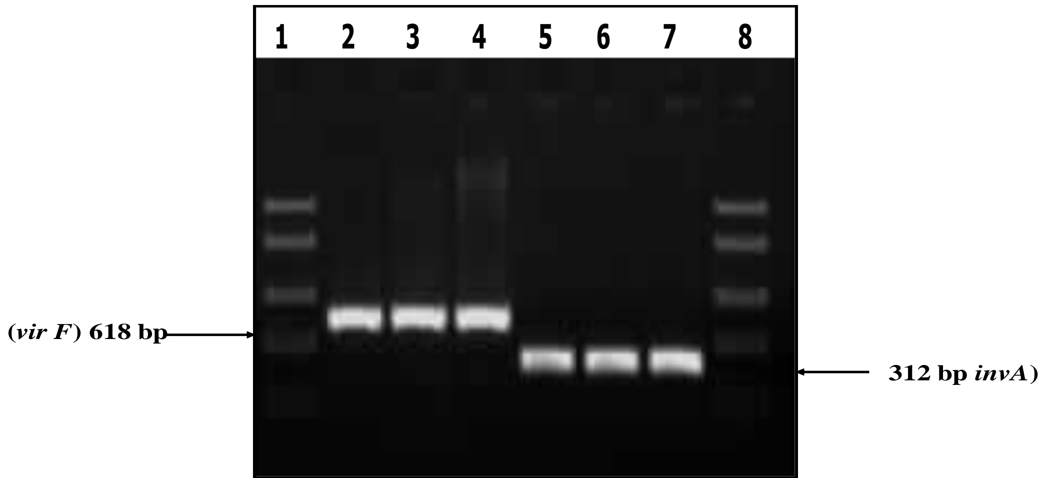
cc: Director - MTRH
Dean - SOM
Dean - SPH
Dean - SOD

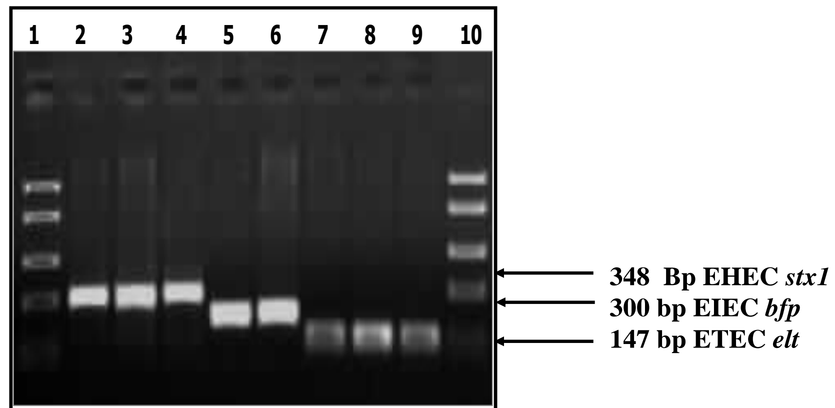
Appendix 13: Serological testing of isolates

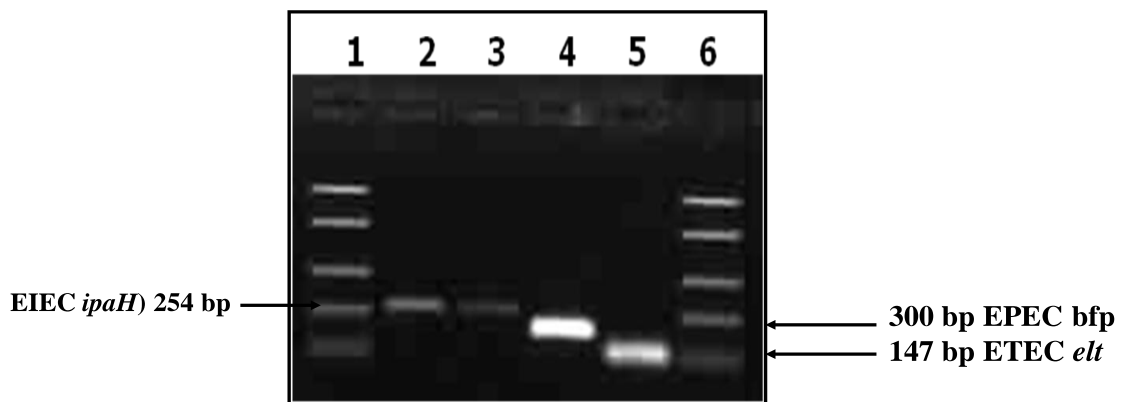
Appendix 13: Agarose Gel Electrophoresis of PCR products of Diarrheagenic *E coli, Salmonella* and *Shigella*

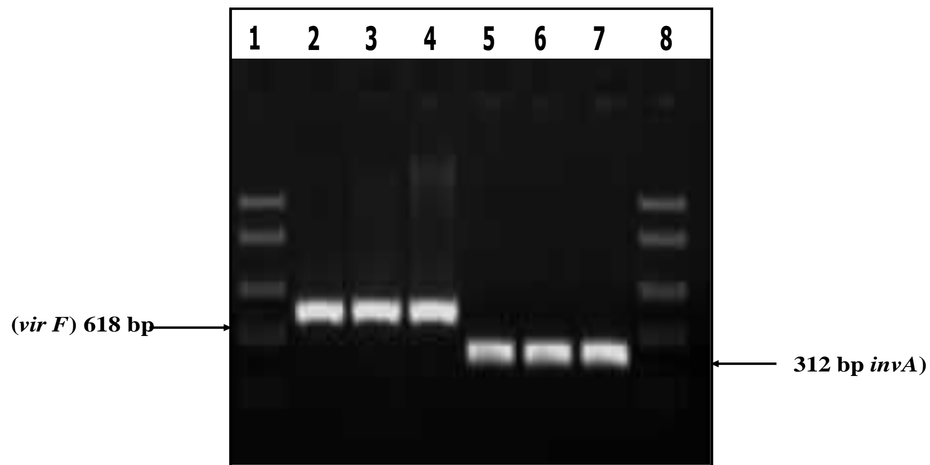


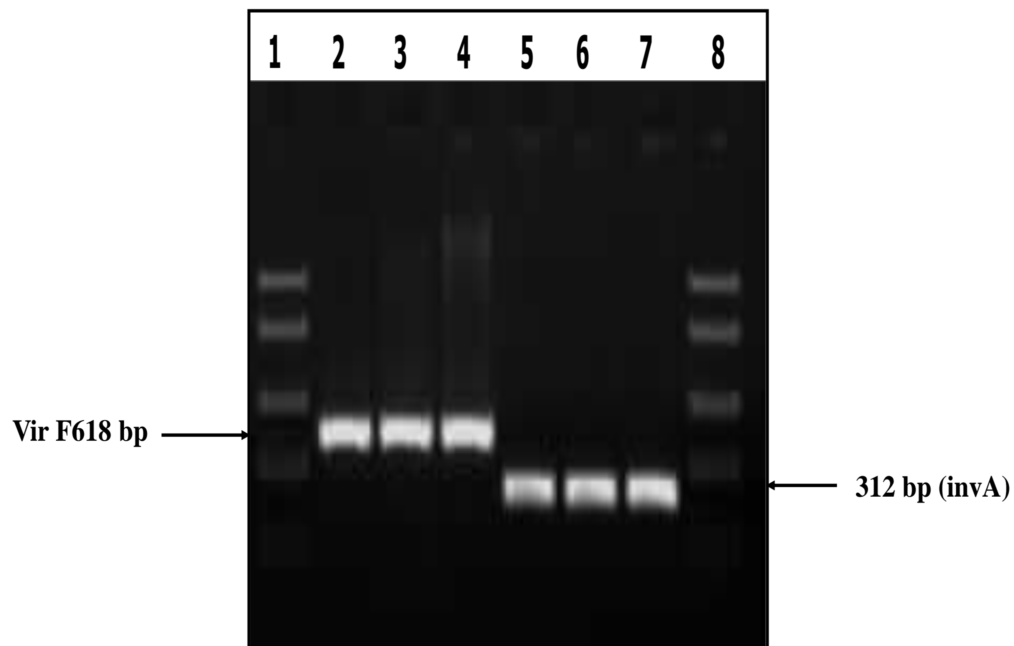



1 2 3 4 5 6 7 8
(*vir F*) 618 bp
312 bp *invA*)


1 2 3 4 5 6 7 8 9 10
348 Bp EHEC *stx1*
300 bp EIEC *bfp*
147 bp ETEC *elt*


1 2 3 4 5 6
EIEC *ipaH*) 254 bp
300 bp EPEC bfp
147 bp ETEC *elt*


1 2 3 4 5 6 7 8
(*vir F*) 618 bp
312 bp *invA*)


1 2 3 4 5 6 7 8
Vir F618 bp
312 bp (invA)