PREVALENCE OF ENTEROTOXIGENIC *ESCHERICHIA COLI* AMONG CHILDREN UNDER FIVE YEARS IN SIAYA COUNTY, WESTERN KENYA

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other university or institution for a degree or any other award.

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Sign…….. Date……

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DEDICATION

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ABSTRACT

One of the most common causes of moderate to severe diarrhea among children and adults in developing countries is enterotoxigenic *Escherichia coli* (ETEC). It causes an estimated 400 million diarrheal episodes and 380,000 deaths globally in children less than 5 years of age annually in developing countries. Diarrhea in under-fives due to ETEC in Latin America is 34%, in Africa is 31% and in South Asia (Indian Subcontinent) is 31%. In Kenya, ETEC prevalence ranges from 1.5% to 10% yet there are insufficient data on ETEC and its enterotoxins associated with diarrhea in children in a rural setting as most previous studies have focused on the prevalence of ETEC in all age groups in urban settings. A recent study on diarrhea in Siaya County reported etiologies of diarrhea to be caused by nontyphoidal *Salmonella*, *Campylobacter*, *Shigella*, and rotavirus.The prevalence of ETEC was not reported.This was a case-control study carried out in Asembo, Karemo, and Gem sites in Siaya County, Kenya. The cases were children with diarrhea while controlswere those without diarrhea. This study aimed to investigate the prevalence of ETEC in children underfive years, characterize the ETECenterotoxins, and determine the enterotoxin associated with diarrhea in children underfive years in Siaya County. Three hundred and eighty-three (383)childrenunder five years of age who presented with moderate-severe diarrhea (cases) were enrolled at health facilities while 535 matched controls were enrolled at home within two weeks of case enrolment. Immediately after enrolment, stool swabs were collected from both cases and controls and transported to the laboratory within 18 hours. The stools were cultured on MacConkey for isolation and identification of *E. coli* colonies by morphologic and biochemical tests.Confirmed *E. coli* colonies were picked and tested by polymerase chain reaction (PCR) to identify ETEC heat-stable (ST) and heat-labile (LT) enterotoxins. Out of the 383 cases enrolled, 169 (44.1%) were females, 214 (55.9%) males. Out of 535 controls, females were 242 (45.2%) and males 293 (54.8%). The participants were categorized by age as 0-11 months 154 (40.2%) cases and 168 (34.6%) controls, 12-23 months were 131 (34.2%) cases and 175 (36%) controls, and 24-59 months were 98 (25.6%) cases and 143 (29.4%) controls. The median age was 14 months for cases and 16 months for controls. The overall prevalence of ETEC was 11%, 13.6% in cases, and9.2% in controls. The difference in prevalence among cases and controls was statistically significant$p$ = 0.035. ETEC infection was higher in children less than 23 months than those above 24 months 38(13.3%) and 14(14.3%),respectively, in cases and 38(9.9%) and 11(22.6%) respectively in controls. ETEC enterotoxin distributions were as follows: LT only 19(5%), 27 (7.1%) ST only and 6 (1.67%) LT/ST in cases while LT only 17(3.2%), 23 (4.3%) ST only and 9 (1.7%) LT/ST in controls. There was no ETEC enterotoxin associated with diarrhea, however, ST-only enterotoxin was the most detected in both children with and those without diarrhea. The majority of the diarrheal children produced non-bloody, mucoid thick liquid stool without pus. The results from this study havesignificancein providing a better view of the prevalence of ETEC variants and their toxins distribution. This study has also indicated that ETEC should be included in routine laboratory testswhen determining the etiologies of diarrhea in children under five years. The high ETEC prevalence from this study points to higher contamination of the environment with ETEC and its enterotoxins hence, have significance in providing a better view of the prevalence of ETEC variants and their toxins and mapping out future studies in the country using modern molecular techniques. The controls should be studied further to find out if they developed diarrhea. More research on ETEC related diarrheal studies need to be conducted in children with and without diarrhea to give more insight on ETEC related diarrhea in Kenya for policy formation.

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ABBREVIATIONS AND ACRONYMS

ADP - Adenosine diphosphate

AFs - Attributable fractions

AMP - Adenosine 5'- monophosphate

API - Analytical profile index

BD - Becton Dickinson

CDC - Centre for disease control

CFA - colonization factor antigen

CFU - Colony forming unit

CFTR - cystic fibrosis transmembrane conductance regulator

CGHR - Centre for global health research

DNA - Deoxyribonucleic acid

DSS - Demographic surveillance system

EPEC - Enteropathogenic *Escherichia coli*

ETEC - Enterotoxigenic *Escherichia coli*

GEMS - The Global Enteric Multicenter Study

GMP - Guanosine 5'-Monophosphate

GSa - GTP binding protein of the alpha subunit

kDa - Kilo Dalton

KEMRI - Kenya medical research institute

LT - heat-labile enterotoxin

LTB - Heat labile enterotoxin binding to the B subunit

MIO - Motility indole ornithine

MSD - Moderate to severe diarrhea

NAD - Nicotinamide adenine dinucleotide

ORs - Odd ratios

PCR - Polymerase chain reaction

pH - Hydrogen potential

SERU - Scientific ethics review unit

SHC - Sentinel health center

SOP - Standard operating procedures

ST - Heat stable enterotoxin

STh - Human heat-stable enterotoxin

STp - Porcine heat-stable enterotoxin

TAC - Taqman array card

TAE - Tris-acetate ethylenediaminetetraacetic acid

TSA - Trypticase soy agar

UMB - University of Maryland

VIDA - Vaccine impact on diarrhea in Africa

WHO - World Health Organisation

DEFINITION OF TERMS

Case- a child of age 0-59 months with ≥ 3 loose stools in 24 hours and one or more of the following: sunken eyes, loss of skin turgor, requiring intravenous rehydration, or hospitalization, who sought care from outpatient or in-patient department of a study sentinel health center (SHC) within 7 days of illness onset.

Control- a child of age 0-59 months without diarrhea and matched to the case by age, gender, and time that the index case presented.

Diarrheal episode- beginning on the first day of a loose stool after at least three consecutive no-diarrheal days and were considered to have ended when followed by 3 days without diarrhea.

Loss of skin turgor- abdominal skin pinch with slow (but ≤2 seconds) or very slow (>2 seconds) recoil.

Moderate-to-severe-diarrhoea (MSD) - is defined by an episode of diarrhea (≥3 loose stools within 24 hours) with onset within the past 7 days and at least 7 days after the end of any previous episode, and at least 1 of the following: sunken eyes, more than normal; loss of skin turgor; intravenous rehydration administered or prescribed; visible blood in stool; or hospitalization with diarrhea.

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CHAPTER ONE
INTRODUCTION

1.1 Background of the Study

Diarrhea is one of the most common health problems, especially in developing countries such as Kenya. Around 2 billion diarrheal diseases occur each year worldwide and are responsible for killing around 1.9 million children aged below 5 years (Rahman *et al.*, 2020). The occurrence rate of diarrheal disease is higher in the developing country due to unimproved sanitation, inadequate hygiene, and poor drinking water access, as well as poorer overall health and nutritional status. Moderate to severe diarrhea is characterized by the production of (≥3 loose stools within 24 hours) with onset within the past 7 days and at least 7 days after the end of any previous episode. The major causative agents of diarrhea are *Escherichia coli*, *Vibrio cholerae*, *Campylobacter* spp., *Shigella* spp., *Clostridium difficile*, rotavirus, and norovirus or parasites, for example, Giardia and *Entamoeba histolytica*. Among these, *E. coli* is the most common and frequently observed causative agent of diarrhea (GDB, 2017). There are two types of *E. coli* strains, pathogenic and nonpathogenic. There are five major groups of pathogenic *E. coli* strains, which are (i) enterotoxigenic *E. coli* (ETEC) that causes infantile and traveler's diarrhea; (ii) enteropathogenic *E. coli* (EPEC) that causes infant diarrhea; (iii) enteroaggregative *E. coli* that is associated with watery diarrhea, but it can be accompanied with blood or mucus and cause persistent diarrhea; (iv) enteroinvasive *E. coli* that causes dysentery; and (v) enterohemorrhagic *E. coli* that causes hemorrhagic colitis and the hemolytic–uremic syndrome (Jafari *et al.*, 2012).

Enterotoxigenic *Escherichia coli* (ETEC) is a leading bacterial cause of diarrhea in children younger than 5 years in developing countries, international travelers, and also neonatal and post-weaning animals (Lamberti *et al.*, 2014; Nagy *et al.*, 2005; Platts-Mills *et al.*, 2015).Each year, ETEC is responsible for an estimated 280 million diarrhea episodes and 300 000–500 000 deaths annually in children under the age of five globally (Duan *et al.*, 2019; Platts-Mills *et al.*, 2015). Diarrhea in under-fives due to ETEC in Latin America is 34%, in Africa is 31% and in South Asia (Indian Subcontinent) is 31%. In 2015, diarrheal diseases caused an estimated 688 million illnesses and 499,000 deaths worldwide among children below five years and are second only to pneumonia as a cause of death in this cohort (Mansour *et al.*, 2019).

Studies done in Kenya have reported ETEC prevalence of 4.3% in Central, 7.25% in Nairobi, 24.1% in Maasailand, 1.5% in Kericho, and 3.8% in Kisumu(Makobe *et al.*, 2012; Mbuthia *et*

al., 2018; Sang *et al.*, 2012; Swierczewski *et al.*, 2013).A study done in Siaya before the introduction of rotavirus showed that most attributable cases of moderate-to-severe diarrhea were due to four pathogens: rotavirus, Cryptosporidium, enterotoxigenic *Escherichia coli* producing heat-stable toxin (ST-ETEC; with or without co-expression of heat-labile enterotoxin), and *Shigella*(Kotloff *et al.*, 2013; Panchalingam *et al.*, 2012). Another study done in Siaya to assess risk factors for death among children less than five years old hospitalized with diarrhea in rural western Kenya reported etiology of diarrhea as 10% Nontyphoidal *Salmonella*, 5% *Campylobacter*, 4%*Shigella*, and 19% rotavirus (O'Reilly *et al.*, 2012). The prevalence of ETEC was not reported in the previous study.

Transmission of ETEC is through contaminated food and water with human waste (Gonzales-Siles *et al.*, 2016). Its high infectious dose makes direct person-to-person spread rare. Mothers are always in direct contact with children in their daily lives and are responsible for many activities including food preparation, wiping children when they pass stool, feeding children, and many others. These activities during child handling can be an important source of contamination of the food consumed by the children if they do not observe proper hygiene practices (Roussel *et al.*, 2019).

To establish an infection, a high dose of ETEC estimated at10^6 to 10^{10} organisms is required for immune stable individuals (Skrede *et al.*, 2014). In young children whose immunity has not fully developed and in the elderly due to low immunity may be susceptible to infection at lower doses (Skrede *et al.*, 2014). Production of enterotoxin and its action on enterocytes must occur. Secretion of watery diarrhea can be caused by either heat-labile (LT) or heat-stable (ST) toxins, both of which stimulate chloride secretion and inhibit chloride absorption in small intestine epithelial cells. The LT is structurally and functionally similar to cholera toxin and it appears as a very small protein that does not appear to elicit an immune response (Yang *et al.*, 2019).

The diarrheal disease caused by ETEC was first recognized to be consisting of a cholera-like illness in both adults and children(Gomes *et al.*, 2016; Kumar *et al.*, 2018). Many studies around the world have since been done in urban areas where the burden of ETEC is lower and they show that ETEC-induced diarrhea may range from very mild to very severe (Al-Gallas *et al.*, 2007; Bueris *et al.*, 2007; Kotloff *et al.*, 2013; Qadri *et al.*, 2005).Diarrhea produced by ETEC is of the secretory type, where the disease begins with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, which lead to

dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool (Duan *et al.*, 2019; Lamberti *et al.*, 2014; Yun *et al.*, 2018). This loss of fluids progressively results in a dry mouth, rapid pulse, lethargy, decreased skin turgor; decreased blood pressure, muscle cramps, and eventually shock in the most advanced forms (Hosangadi *et al.*, 2017; Madhavan *et al.*, 2015).

Dehydration due to ETEC infection is categorized from mild to severe, and this clinical characteristic is important in administering adequate treatment. Diarrhea lasts only 3 to 4 days and is self-limited. The patient survives if hydration is maintained and without any sequelae. In the previous study 'Global Enteric Multicentre Study (GEMS)', conducted in Asembo and Gem sites in Siaya County Kenya, ST-ETEC was a significant pathogen causing diarrhea in infants at all the site before the introduction of the rotavirus vaccine. By contrast, ETEC producing LT alone was not a significant cause of moderate-to-severe diarrhea in infants at any site (Panchalingam *et al.*, 2012).

The ST-only enterotoxin has been reported by most studies to be the most expressed enterotoxin (Kharat *et al.*, 2017; Platts-Mills *et al.*, 2015).A systematic review by Isidean et al. (2011) also reported that in the GEMS sites and Latin America and the Caribbean, the Middle East and North Africa, and South Asia, the prevalent toxin in endemic populations is ST (Isidean *et al.*, 2011). A study on pathogenicity and phenotypic characterization of Enterotoxigenic *Escherichia coli*isolates from a birth cohort of children in rural Egypt reported that among the ETEC-associated diarrheal episodes, 334 ETEC diarrheal episodes (44.2%) were associated with LT-expressing ETEC, 291 (38.5%) were associated with ST-expressing ETEC, and 131 (17.3%) were associated with isolates that produced both enterotoxins. The distribution of ETEC enterotoxins varies with geographical location(Mirhoseini *et al.*, 2018) hence the need to characterize ETEC enterotoxins in children in Siaya County, Western Kenya.

The detection and characterization of clinical ETEC isolates have been accomplished by a variety of phenotypic and genotypic methods. These include phenotypic assays for toxins and CFs based on recognition of monoclonal antibodies (MAbs) (Sjoling *et al.*, 2007)and genotypic methods based on either DNA/DNA hybridization (Steinsland *et al.*, 2003), PCR, or real-time PCR techniques (Vidal *et al.*, 2011).

This study has provided estimates of the burden of diarrheal disease associated with ETEC in children less than five years of age in the rural community of Asembo, Karemo, and Gem in

Siaya County, Western Kenya which was not captured by other studies done in Kenya focusing on urban areas and participants of all age groups (Swierczewski et al., 2013). The high prevalence is an indication of heavy contamination of the environment with ETEC.

1.2 Problem Statement

Information about enterotoxigenic *E. coli* in Kenya is scarce, particularly in Siaya County because ETEC is not routinely tested when children with diarrhea report to various health facilities. Reliable ETEC diagnostic procedures must include detection of both heat-stable (ST) and heat-labile (LT) enterotoxin using molecular assays. Unfortunately, many studies of diarrheal pathogens disregard ETEC because ETEC cannot be differentiated from commensal *E. coli* using conventional culture-based methods. As a result, there are insufficient data on ETEC as a contributing factor to diarrhea. Most studies conducted in Kenya have focused on the burden of ETEC in urban areas largely ignoring rural set-ups. This study employed PCR to detect ETEC enterotoxins and focused on children under five years because infection due to ETEC is common in children under five years than in older populations.Asembo, Karemo, and Gem sites were chosen to represent rural settings in Siaya County. No study has reported the prevalence of ETEC in Siaya County after the rotavirus vaccine was rolled out in Kenya.

1.3. Objectives

1.3.1 Main Objective

To determine the prevalence of enterotoxigenic *E. coli* among children under five years in Siaya County, western Kenya.

1.3.2 Specific Objectives

1. To characterize ETEC enterotoxins in children underfive years in Siaya County, western Kenya.
2. To determine the ETEC enterotoxin associated with diarrhea in children underfive years in Siaya County, estern Kenya.

1.3.3 Research Questions

1. What is the prevalence of enterotoxigenic *E. coli* in children under five years in Siaya County, western Kenya?
2. What ETEC enterotoxinsare associated with diarrhea in children underfive years in Siaya County, western Kenya?

1.4 Significance of the Study

The Enterotoxigenic *E. coli* ETEC is a leading bacterial cause of diarrhea in children younger than 5 years in developing countries, travelers, and neonates. Periodic surveillance is important for the detection, treatment, and management of infection due to ETEC.The ETEC cannot be distinguished from commensal *E. coli* or other pathovars without molecular testing, they often go unrecognized unless there is a recognized cluster of children cases that leads to testing in specialized public health laboratories. This suggests that ETEC may be commonly missed by culture-dependent methodologies in common use in clinical microbiology laboratories. This study was set to detect enterotoxigenic *E. coli* using *E. coli* PCR. The data obtained from this study provide a better view of the prevalence of enterotoxigenic *E. coli*in both children with and those without diarrheaand the association of enterotoxins with diarrhea. This studyhas shown heavy contamination of the environment with ETEC hence,need for proper hygiene and sanitation. Enterotoxigenic *E. coli*should be included during the diagnosis of diarrheal diseases in children. The availability of accurate, up-to-date assessments at the county level becomes even more important to guide strategic planning and resource allocation.

CHAPTER TWO
LITERATURE REVIEW

2.1 Escherichia Coli

The leading facultative anaerobe of the human flora *is Escherichia coli* (*E. coli*). It colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit (Houghteling *et al.*, 2015). *Escherichia coli* remains harmlessly confined to the intestinal lumen; however, in an immunosuppressed host or when gastrointestinal barriers are breached, even normal non-pathogenic strains of *E. coli* can cause infection. Infections due to pathogenic *E. coli* may be restricted to the mucosal surfaces or can spread throughout the body.*E. coli* is the mostfrequently observed causative agent of diarrhea. Three general clinical syndromes result from infection with pathogenic *E. coli* strains: urinary tract infection, sepsis/meningitis, and enteric/ diarrheal disease(Kohler *et al.*, 2011).

Escherichia coli is a Gram-negative, oxidase-negative, rod-shaped bacterium from the family Enterobacteriaceae (Croxen *et al.*, 2013). It can grow both aerobically and anaerobically, preferably at 37°C, and can either be nonmotile or motile, with peritrichous flagella (Croxen *et al.*, 2013). *E. coli* is readily isolated from fecal samples by plating on selective media. The change in pH due to lactose fermentation is used to differentiate between lactose-fermenting and non-lactose-fermenting strains, as lactose-positive *E. coli* colonies will appear red or pink on media such as MacConkey agar (Croxen *et al.*, 2013). Not all *E. coli* strainsferment lactose, so caution must be used when using this diagnostic. While this selective plating can aid in isolating *E. coli* from Gram-positive bacteria and some other Enterobacteriaceae members, further morphological, phenotypic, and genotypic characteristics need to be tested for further identification and verification of pathotypes (Croxen *et al.*, 2013). Traditional culture techniques for pathogenic *E. coli* can be time-consuming and laborious. The adoption of molecular techniques has allowed for more rapid detection and identification of the different pathotypes (Croxen *et al.*, 2013).

2.2 Identification of Diarrheagenic *E. coli*

The detection and characterization of clinical ETEC isolates have been accomplished by a variety of phenotypic and genotypic methods. These include phenotypic assays for toxins and CFs based on recognition of monoclonal antibodies (MAbs) (Sjoling *et al.*, 2007)and genotypic methods based on either DNA/DNA hybridization (Steinsland *et al.*, 2003), PCR, or real-time PCR techniques (Vidal *et al.*, 2011).

Serotypic analysis was the main method by which pathogenic strains were differentiated before the identification of specific virulence factors in diarrheagenic *E.coli* strains(Gomes *et al.*, 2016). According to the modified Kauffman scheme, *E. coli* is serotyped based on their O (somatic), H (flagellar), and K (capsular) surface antigen profiles (Fratamico *et al.*, 2016; Olesen, 2017). Phenotypic assays built on virulence characteristics identification of diarrheagenic *E. coli* strains involve differentiation of these organisms from non-pathogenic members of the normal flora(Jafari *et al.*, 2012).

Detection of diarrheagenic *E. coli* has focused on the identification of characteristics that determine the virulence of these organisms. This may include in vitro phenotypic assays that correlate with the presence of specific virulence traits or detection of the genes encoding these traits. One of the most useful phenotypic assays for the diagnosis of diarrheagenic *E. coli* is the HEp-2 adherence assay(Lozer *et al.*, 2013). Molecular methods remain the most popular and most reliable techniques for differentiating diarrheagenic strains from non-pathogenic members of the fecal flora and distinguishing one category from another(Chukwu *et al.*, 2020; Yun *et al.*, 2018). Substantial progress has been made both in the development of nucleic acid-based probe technologies as well as PCR methods.

Primers for PCR have been developed for several categories of diarrheagenic *E. coli*. The most highly conserved feature of diarrheagenic *E. coli* strains is their ability to colonize the intestinal mucosal surface despite peristalsis and competition for nutrients by the indigenous flora of the gut(Houghteling *et al.*, 2015). The presence of surface adherence fimbriae is a property of nearly all *E. coli* strains, including non-pathogenic varieties(Kallas *et al.*, 2020). However, diarrheagenic *E. coli* strains have specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Pereira *et al.*, 2013).While various techniques have been identified to be useful in the diagnosis of ETEC, it remains to be investigated in Siaya County as ETEC is not included in routine diagnosis of causes of diarrhea in children.

There are two types of *E. coli* strains, pathogenic and non-pathogenic. There are five major groups of pathogenic *E. coli* strains, which are (i) enterotoxigenic *E. coli* (ETEC) that causes infantile and traveler's diarrhea; (ii) enteropathogenic *E. coli* (EPEC) that causes infant diarrhea; (iii) enteroaggregative *E. coli* that is associated with watery diarrhea, but it can be accompanied with blood or mucus and cause persistent diarrhea; (iv) enteroinvasive *E. coli* that causes dysentery; and (v) enterohemorrhagic *E. coli* that cause hemorrhagic colitis and

the hemolytic–uremic syndrome (Canizalez-Roman *et al.*, 2016; Jafari *et al.*, 2012). Among the six recognized categories of diarrheagenic *E. coli*, ETEC is the most common.

The diarrheagenic *E. coli* can be identified using a multiplex PCR assay, in which several PCR primers are combined to detect one of several different diarrheagenic *E. coli* pathotypes in a single reaction(Yang *et al.*, 2019). After multiplex PCR, various reaction products can usually be differentiated by product size, but a second detection step is generally performed to identify the respective PCR products definitively. This study used PCR to detect ETEC enterotoxins.

2.3 Enterotoxigenic *E. coli* (ETEC)

One of the most important causes of bacterial diarrhea in developing countries is ETEC (Nazarian *et al.*, 2014; Zhang *et al.*, 2015). The bacteria cause 15 to 20% of diarrhea in children underfive years of age in less developed countries and it is the major common cause of traveler's diarrhea in persons who travel to Africa, Asia, and Latin America (Lamberti *et al.*, 2014). In many of these countries, a much higher prevalence rate has been reported in infants under 12 months (Lamberti *et al.*, 2014). Almost 10 million traveler's diarrhea cases have been reported worldwide per year (Zhang *et al.*, 2012). One interesting point is that 10 to 14% of people with traveler's diarrhea caused by ETEC show symptoms of Bowel syndrome later (Bourgeois *et al.*, 2016). The incidence of ETEC strains causing diarrhea in persons over 5 years of age is decreased, however, it is seen that older people are also prone to diarrhea caused by ETEC (Bourgeois *et al.*, 2016). The world health organization reports estimated the death toll from diarrhea caused by ETEC is about 157,000 persons a year, roughly equivalent to 9% of deaths from diarrhea (Madhavan *et al.*, 2015). In 2013, an average of 42,000 reported deaths due to diarrhea caused by ETEC in children under 5 years of age has attracted a lot of attention (Bourgeois *et al.*, 2016). Diarrhea caused by ETEC can be mild or severe case with plenty of water disposal, abdominal pain, nausea, and vomiting, and rarely with fever and headache (Svennerholm *et al.*, 2012). The incubation period varies between 1 and 2 days and after the onset of the disease, it may be possible to dispose of 10 L of water daily in the form of loose stools. In this case, patients need hospitalization and intensive care (Fleckenstein *et al.*, 2013). The ETEC can be isolated from both symptomatic and asymptomatic carriers, with significant mortality rates in children.

2.3.1 ETEC Virulence Factors and Pathogenesis

The disease caused by ETEC is spread by swallowing 10^6 to 10^{10} numbers of the bacteria. When the bacteria reach the small intestine, infection is established. Bacteria through surface colonization agents attach to the intestinal epithelium and colonization occurs on the surface of small intestine cells (Fleckenstein *et al.*, 2013). After attachment and colonization of bacteria, enterotoxins produced affect the epithelial cells in the area. Production of heat-labile or heat-stable enterotoxins is the major factor of ETEC virulence. The toxins may cause diarrhea independently of each other. ETEC strains could produce simultaneously only ST, LT, or both types of toxin (Alerasol *et al.*, 2014; Nazarian *et al.*, 2012). The condition that leads to diarrhea caused by bacteria is shown in **Figure 2.1** (Clements *et al.*, 2012). The *Escherichia coli* produces heat-labile type I and II enterotoxins that are differentiated by genetic, biochemical, and immunological properties. The heat-labile enterotoxin, type I (LT-I), is an 84 kDa heterohexamer composed of pentameric B subunit and an A subunit (Joffre *et al.*, 2016). A subunit is made of two domains, which are linked by a sulfide bond. A1 domain is the active portion of the toxin and an A2 domain with a helical shape is placed inside a pentamer B subunit (Joffre *et al.*, 2016). There are two immunotypes of LT; LT-I and LT-II which share the same ganglioside receptor and mode of action, but are antigenically distinct (Hajishengallis *et al.*, 2013; Jobling, 2012; Rodas *et al.*, 2011). The heat-labile enterotoxin induces its toxic effect via binding to ganglioside GM1 at the apical surface of intestinal cells. B subunit link to the ganglioside GM1 at the host cells causing toxin endocytosis.

The A subunit passes through the cell membrane and reacts with ADP ribosylating factor. Avoiding GTPase activity of the Gsα protein leads to continuous activity of adenylate cyclase enzyme and cAMP (3′,5′-cyclic adenosine monophosphate) increasing. The cAMP increase causes activation of cAMP-dependent kinase protein. This enzyme also causes phosphorylation and stimulates chloride channels at the apical membrane. Following these changes, secretion of electrolytes and water into the intestine, resulting in diarrhea (Fleckenstein *et al.*, 2013). Another type of LT toxin, which is found in some E. coli strains, has been named heat-labile toxin II (LT-II). Thetoxin has no immunological cross-reactivity with the cholera toxin. The similarity of the amino acid sequence of this toxin type with the amino acid sequence of the cholera toxin and heat-labile toxin type I reaches less than 14% (Joffre *et al.*, 2016). LT-II toxin does not cause fluid accumulation in the adult rabbit's intestine and does not bind to ganglioside GM1. Moreover, genetic information related to the

toxin is on the bacterial chromosome. The importance of toxin type II in pathogenicity in humans is not well-known(Joffre *et al.*, 2016).

The enterotoxigenic *Escherichia coli*also produces heat-stable enterotoxins, which are cysteine-rich small peptides. The ETEC heat-stable enterotoxins in terms of sequence and three-dimensional structure are very similar to guanylin and uroguanylin. This toxin secretes and binds to the extracellular portion of the guanylyl cyclase enzyme located on the surface of intestinal epithelial cells. After this binding, the functional intracellular portion of guanylyl cyclase protein is activated, which eventually causes the accumulation of cGMP in the cell. The increasing of intracellular cGMP leads to activation of cGMP-dependent Protein Kinase II. Also by phosphorylation the channels' regulator, kinase enzyme causes chloride secretion and inhibition of sodium chloride absorption. In this condition, epithelial cellsdehydrate and diarrhea occurs in a patient (Mirhoseini *et al.*, 2018). Type I and II heat-stable enterotoxin produced by ETEC. Toxin STI (STa) that binds to guanylyl cyclase, is divided into two types (ST-P) ST-Ia and (ST-H) ST-Ib (Mirhoseini *et al.*, 2018).

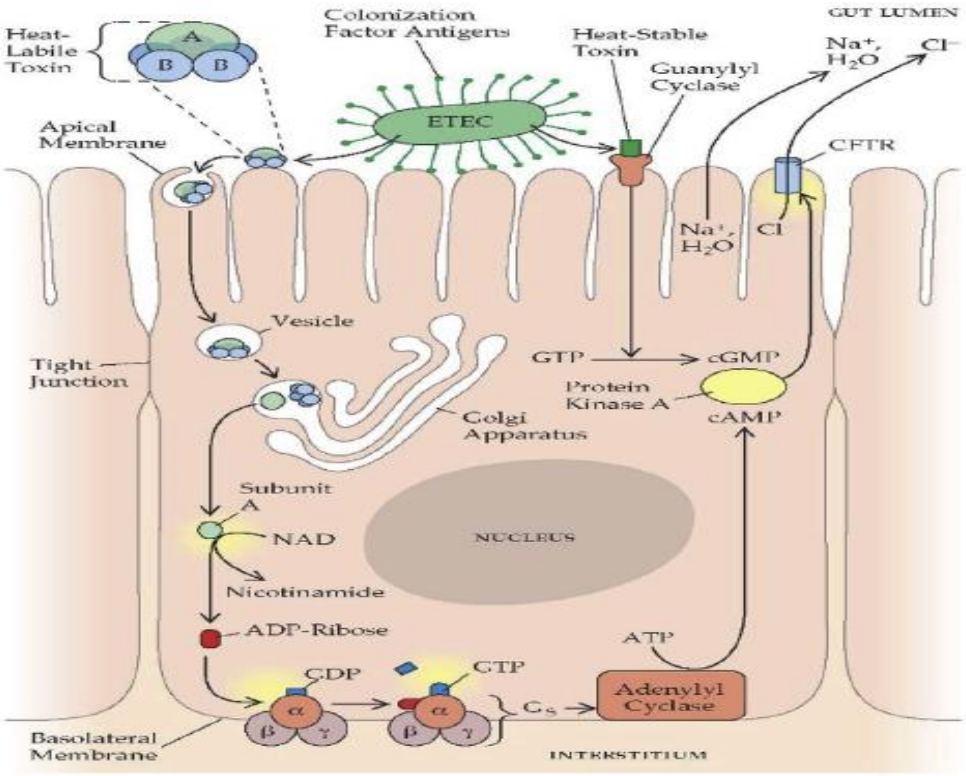

Figure 2. 1: Mechanisms by which enterotoxigenic *E. coli* cause disease

Pathogenesis of ETEC bacteria invasion involves two steps which are intestinal colonization, followed by an elaboration of diarrheagenic enterotoxins. Activation of adenylyl and

guanylate cyclase leads to the formation of cAMP and cGMP, stimulates water and electrolyte secretion by intestinal endothelial cells(Connell, 2007).

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Heat-stable toxins (LTs) are large oligomeric toxins that are closely related in structure and function to the cholera toxin (CT) expressed by *Vibrio cholera* (Yang *et al.*, 2019). The genes encoding LT (*elt* or *ext*) reside on plasmids that may also contain genes encoding ST and/or colonization factor antigens (CFAs) (Duan *et al.*, 2019; Jobling, 2012). In contrast to LTs, the STs are small monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability of these toxins (Yang *et al.*, 2019). Heat-stable enterotoxin is not a single toxin but a family of small toxins that fall into two subgroups; methanol soluble STa (or STI) and methanol insoluble STb (or STII), which differ in nucleotide and amino acid sequences (Yun *et al.*, 2018).

The ETEC Pathovar is defined by the production and effective delivery of heat-stable (ST) and or heat-labile (LT) enterotoxins to epithelial receptors in the small intestines. In the classical ETEC pathogenesis paradigm, plasmid-encoded colonization factors facilitate small intestinal colonization. To cause diarrhea, ETEC strains must first adhere to small bowel enterocytes, an event mediated by surface fimbriae (Fleckenstein *et al.*, 2013; Gonzales-Siles *et al.*, 2016). Enterotoxigenic *E. coli* adheres to epithelial surfaces using CFAs and putative colonization antigens jointly referred to as CFAs. Enterotoxigenic *E.coli* pathogenic to humans have been described, but they are almost always encoded by plasmids also encoding ST and/or LT enterotoxins (Duan *et al.*, 2019; Yang *et al.*, 2019; Yun *et al.*, 2018). The identification of ETEC has long relied on the detection of these enterotoxins. Deoxyribonucleic acids (DNA) probes are used for the detection of LT and ST enterotoxins in ETEC (Waters *et al.*, 2017).

2.3.2 Epidemiology of ETEC

The ETEC is among the most common bacterial causes of diarrhea-associated morbidity and mortality in children younger than age 5 years in developing countries. The peak incidence occurs in children 6 to 36 months of age, where ETEC, rotavirus, and *Shigella* are the most common pathogens (Kotloff *et al.*, 2013). The ETEC is also the number one cause of travelers' diarrhea(Svennerholm *et al.*, 2012)reportedly accounting for 20% to 60% of all travelers' diarrhea globally with at least 20% of affected travelers being bedridden for part of

their trip and 40% changing their itinerary because of diarrhea(Svennerholm *et al.*, 2012). Travelers' diarrhea, primarily caused by ETEC, is also the most common medical problem for military workers from developed countries deployed in less-developed areas of Asia or Africa, with an average incidence of 29% per month and up to 60% per month in hyperendemic regions(Riddle *et al.*, 2006).Previous studies have reported on ETEC infection in all age groups while this study focused on children under five years of age since ETEC infection decreases with an increase in age.

2.3.3 Prevalence of ETEC in Under-five Children

From various parts of the world, ETEC has been reported to be the main etiological agent of diarrhea among children under 5 years of age at 1.86 %- 20.7% (Hosangadi *et al.*, 2017; Kharat *et al.*, 2017; Panchalingam *et al.*, 2012). Data from studies done in Bangladesh, Mexico, Peru, Egypt, Argentina, India, Nicaragua, and Tunisia indicate an ETEC prevalence rate of 18-38 %(Qadri *et al.*, 2007; Rivera *et al.*, 2010) in symptomatic children. These rates are much lower in reports from Vietnam and Brazil (4%, and 3.7 %)(Al-Gallas *et al.*, 2007; Bueris *et al.*, 2007; Kotloff *et al.*, 2013; Qadri *et al.*, 2005). Recent studies from other developing countries reported an ETEC prevalence of 5.2% in children below 2 years in Peru (Qadri *et al.*, 2007; Rivera *et al.*, 2010).

A study done in central Kenya to determine the etiology of diarrhea in children under five years reported ETEC 4.3% (Mbuthia *et al.*, 2018). A study conducted in Mbagathi District hospital on children less than 5 years of age reported a burden of 7.25%(Makobe *et al.*, 2012). A study was done in Maasailand to determine the prevalence and genetic characteristics of Shigatoxigenic Escherichia coli from patients with diarrhea reported ETEC prevalence of 24.1% (Sang *et al.*, 2012). A study done in Kericho (Swierczewski et al., 2013) enrolled patients with diarrhea and without diarrhea at Kericho District hospital and Kisumu District hospital and reported ETEC prevalence of 1.5% in patients with diarrhea and 1% in healthy individuals in Kericho. In Kisumu, the ETEC burden rate reported was 3.8% in patients with diarrhea and 1.7% in healthy individuals(Swierczewski e*t al.*, 2013). A study done in Kajiado and Narok district reported ETEC prevalence of 8.2%, this study was done in patients of all ages. A study done in the central region of Kenya among diarrheal children below five years reported a prevalence of 4.3% (Mbuthia *et al.*, 2018). Several studies have been done in various parts of Kenya to determine ETEC prevalence but none has reported on ETEC in children under five years in a rural set-up in Siaya County.

2.3.4 Distribution of ETEC Enterotoxins

The prevalence of LT, ST, or ST/LT toxin phenotypes remarkably varies between different regions in the world (**Figure 2.2**) (Mirhoseini *et al.*, 2018). Previous studies reported ST dominance in Nigeria (Okeke *et al.*, 2000). A high prevalence of LT- and ST-producing ETEC isolates have been reported by studies on diarrhea among children, including those performed in Egypt, Tunis, and Bolivia (Isidean *et al.*, 2011; Rodas *et al.*, 2011). A study done in Bangladesh reported 43% LT/ST, 27% ST and 30% ST(Yasmin 2014). A study was done in Kenya to determine *Escherichia coli* pathotypes and Shigella serogroups in diarrheic children reported pathogenetic profile of LT 8 (2.3%), 23 (6.5%) LT/ST, and 6 (1.7%) ST enterotoxins(Nyanga *et al.*, 2017).The difference in the distribution of ETEC enterotoxins indicates the need for continuous monitoring of these enterotoxins especially in children leaving in rural areas and whose immunities are still naïve. The discrepancy between findings from various studies may be attributable to variations in geographical factors and target populations, hence the need to determine the prevalence of ETEC in children under five years in rural set-ups like Siaya County.

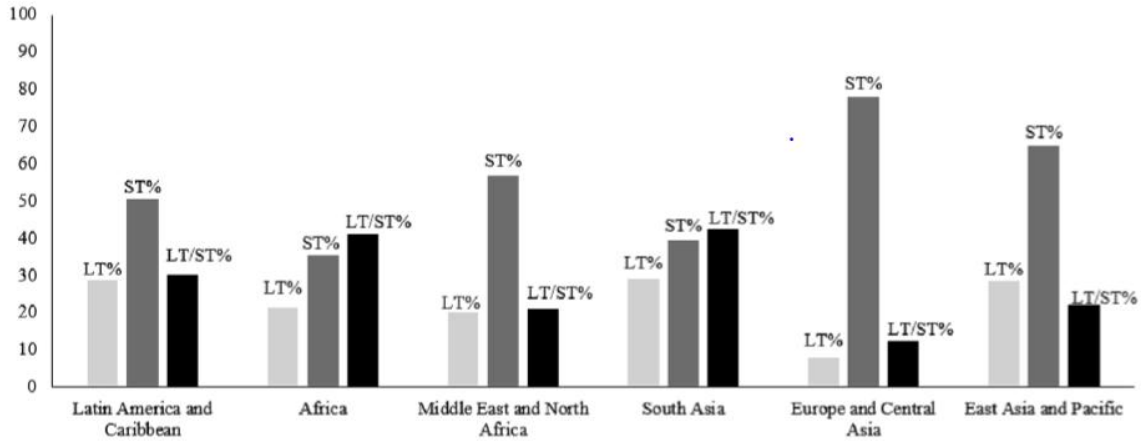

Figure 2.2: The LT and ST enterotoxin prevalence by different regions.

Figure showing LT and ST enterotoxin distribution by different regions. LT is Heat-labile enterotoxins and ST is Heat-stable enterotoxins. (Isidean *et al.*, 2011).

2.3.5 Association of ETEC with Diarrhea in Children below 5 years of Age

Enterotoxigenic *Escherichia coli*is an under-recognized but extremely important cause of diarrhea in the developing world where there is no adequate clean water and poor sanitation (Roussel *et al.*, 2019). They are the most commonly isolated bacterial enteropathogens in children below 5 years of age in developing countries and account for several hundred

million cases of diarrhea and several tens of thousand deaths each year (Platts-Mills *et al.*, 2015). Previous studies have shown that fecal isolation of ETEC is associated with diarrheal symptoms in children less than five years(Fleckenstein *et al.*, 2019; Platts-Mills *et al.*, 2015).

While oral rehydration therapy and other measure have contributed to a decline in deaths due to diarrhoeal illness, ETEC has linked to post-diarrhoeal squalene including malnutrition, growth stunting, and impaired cognitive development greatly compounding to impact of these infections (Anderson *et al.*, 2019). A study done in Siaya to assess etiologies for diarrhea and risk factors for death among children less than five years, reported Nontyphoidal *Salmonella, Shigella,* and rotavirus as the causes of diarrhea(O'Reilly *et al.*, 2012). The association of ETEC with diarrhea in under-fives was not reported. This study has reported on the prevalence of ETEC in diarrheal and non-diarrheal children under five years in Siaya County.

2.3.6 Diarrhoea in Children Caused by ETEC

Food and water are the most common vehicles for ETEC transmission (Roussel *et al.*, 2019). Thus, fecal contamination is the principal reason for the high incidence of ETEC infection throughout the developing world. ETEC infections in areas of endemic infections tend to be clustered around warm, wet months when multiplication of ETEC in food and water is most efficient(Chao *et al.*, 2019; Gonzales-Siles *et al.*, 2016). This study focused on a rural setup where clean water and sanitation are still a challenge.

The sampling of both food and water sources from areas of endemic ETEC have demonstrated high rates of ETEC contamination(Yang *et al.*, 2019) where asymptomatic ETEC excretion is also commonly found. ETEC infections among young children in endemic regions are thought to result in acquired immunity and decreasing incidence of infection with age (Chakraborty *et al.*, 2019). Repeated ETEC infections are common among children in low-income countries because of multiple pathotypes associated with the disease; however, the decrease in the incidence of symptomatic illness with increasing age shows that protective immunity develops, and the incidence of ETEC diarrhea in low-income countries peaks in the first 2 years of life (Bourgeois *et al.*, 2016; Isidean *et al.*, 2011; Kotloff *et al.*, 2013). This study focused on children below 5 years as their immunity is still naïve.

The diarrhoeal disease caused by ETEC that was recognized in the 1970s and 1980s consisted of a cholera-like illness in both adults and children (Lamberti *et al.*, 2014). Since then, other studies around the world have shown that ETEC-induced diarrhea may range from very mild

to very severe (Clermont *et al.*, 2011; J. Liu *et al.*, 2011). There are, however, short-term, asymptomatic carriers of the organisms (Mansour *et al.*, 2014). Diarrhea produced by ETEC is of the secretory type. The disease begins with a sudden onset of watery stool (without blood or inflammatory cells) and often vomiting, which leads to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool (Fleckenstein *et al.*, 2013). The loss of fluids progressively results in a dry mouth, rapid pulse, lethargy, decreased skin turgor; decreased blood pressure, muscle cramps, and eventually shock in the most severe forms. The degree of dehydration is categorized from mild to severe, and this clinical distinction is important in the provision of adequate therapy. The patients are afebrile. Usually, diarrhea lasts only 3 to 4 days and is self-limiting, and if hydration is maintained, the patients survive without any sequelae (Shaheen *et al.*, 2009) Greenwood *et al*., 2008). The pathophysiology of the illness caused by ETEC is essentially the same as that caused by *Vibrio cholera* and the clinical picture is identical, especially in adults. Studies with human volunteers have shown that the infective dose is high for both diseases. For ETEC, the dose is around 10^6 to 10^{10} CFU, with lower doses being less pathogenic (Abu-Elyazeed *et al.*, 1999) The need for a large infectious dose, the proliferation of the bacteria in the small bowel through colonization factors, and the production of enterotoxins, and the watery, secretory type of diarrhea which produces clinical dehydration are comparable in both diseases. Both organisms produce an immunologic protective response, reflecting the observation that the attack rates are higher in children and decrease with age (Lamberti *et al.*, 2014; Meraz *et al.*, 2008).

Studies over the last few years have documented that ETEC is usually a frequent cause of diarrhea in infants younger than 2 years of age (Hosangadi *et al.*, 2017). The susceptibility of infants and young children has also been observed in other settings that have poor public health and hygiene conditions (Bueris *et al.*, 2007). The characteristics of the toxin types and CFs present on ETEC strains isolated from young children vary among countries where ETEC is endemic (Bueris *et al.*, 2007; Kotloff *et al.*, 2013; Rao *et al.*, 2003). Studies to better understand the natural infection pattern of ETEC is being conducted with cohorts of infants to discern the infection and reinfection pattern as well as the age group most at risk for infection. Several studies have reported that ETEC diarrhea and asymptomatic infections are most frequent during warm periods of the year suggesting that travelers to these regions are also more at risk of developing ETEC infections during the warm seasons (Gonzales-Siles *et al.*, 2016; Mansour *et al.*, 2019). In Bangladesh, ETEC follows a very characteristic biannual

seasonality with two separate peaks, one at the beginning of the hot season, that is, the spring, and another peak in the autumn months, just after the monsoons, but it remains endemic all year and such seasonality may be initiated by climatic changes and spread by environmental factors (Harris *et al.*, 2008; Sheikh *et al.*, 2010). As the atmospheric temperature increases when spring sets in after the cooler winter months, there is increased growth of bacteria in the environment and this continues in the summer months. This study focused on a rural setup that is experiencing challenges with getting clean water, poor sanitation, and disposal of excreta which most other studies have not reported on.

2.3.7 Prevention and Treatment of ETEC

Measures to prevent and treat diarrhoeal disease, including illness caused by ETEC, include prevention methods, such as improved sanitation and hygiene, access to safe drinking water, exclusive breastfeeding, optimal nutrition, and vaccines against other pathogens(Lamberti *et al.*, 2011). About 844 million people get water directly from surface water sources or use unprotected wells and springs(WHO,2017). Also, 2.3 billion people lack facilities for excreta disposal and 892 million people practice open development defecation (WHO, 2017). Global access to improved sanitation and clean water is a long-term goal and represents the ideal solution 13 to preventing transmission of ETEC. This will be difficult to achieve and sustain in the near term, given the financial and logistical constraints in low-resource regions. Breastfeeding is also one of the most effective prevention interventions for diarrhoeal diseases and it provides a wide array of proven benefits to infants and young children, (Lamberti *et al.*, 2011) however, breastfeeding during a child's first hour of life, exclusively for six months of age, and two years overall is well short of universal(Victora *et al.*, 2016). The correction and maintenance of hydration are always most important for case management. Adequate nutrition should be provided to children in low resource settings, where all diarrhoeal diseases are frequent. The Zinc treatment can speed recovery time (WHO, 2019). While the available treatment strategies have been increasingly used successfully over the past decades, there are notable limitations and issues with coverage and sustainability. Therefore, vaccination is considered one of the most equitable preventive interventions. As already indicated, the rise of antibiotic-resistant enteric bacteria has made the prevention of infectious diarrhea, and the need for an effective vaccine, an even greater public health priority (LSHTM, 2017).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study site

This study was conducted in health facilities within Asembo, Karemo, and Gem sites in Siaya County,Western Kenya, representing an area of ~500 km^2 and a population of ~135,000 persons (population density ~300 persons/km^2), about 50–65 km west of Kisumu city (Figure 2). The altitude is about 1,100 m, the average monthly temperature is 24.5°C, and annual rainfall is 1,358 mm (O'Reilly *et al.*, 2012). Rainy seasons generally occur in March to May and October to November(Adazu *et al.*, 2005; Blackwelder *et al.*, 2012; Odhiambo *et al.*, 2012). Residents, predominantly of the Luo ethnic group(O'Reilly *et al.*, 2012), earn their living through small-scale business, farming, and fishing(Lindblade *et al.*, 2004). The main source of cooking fuel is firewood(O'Reilly *et al.*, 2012),and the main source for drinking water is Lake Victoria, streams, and rivers(Hawley *et al.*, 2003).

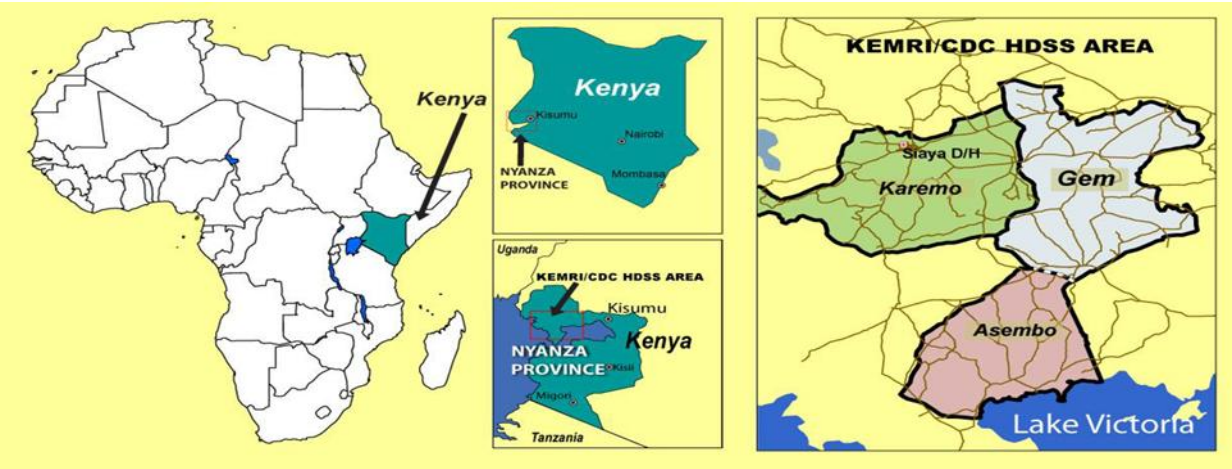

Figure 3.1: Map of Siaya County.

Figure showing the map of the study sites; Gem, Karemo, and Asembo sites which are located in Siaya County. Siaya County lies between Latitude 0^o 26' to 0^o 18' North and Longitude 35^o 58' East and 34^o 33' West(Odhiambo *et al.*, 2012).

This study used stool samples collected from a case-control study – Vaccine Impact on Diarrhea in Africa (VIDA) in children less than 5 years of age. Sentinel health centers (SHCs) were chosen based on the response from the care caregiver on where children under five years of age sought care when they experience moderate to severe diarrhea. The health facilities listed by caregivers were Lwak Mission Hospital, Mahaya, Abidha Health Centre,

Ndori and Ong'ielo Health Centres (Asembo), Ting, Wangi, Kogelo, Siaya County Referral Hospital (Karemo), and Akala Health Centre (Gem).

3.2 Study Design

This study was a case-control study nested within an ongoing study 'Vaccine Impact on Diarrhea in Africa' and enrolled children under 5 years of age seeking care at sentinel health facilities when they experienced moderate to severe diarrhea. Their matched controls were enrolled from the controls' places of residence.

3.3 Study Population

Residents were mainly of the Luo ethnic group who earned their living through subsistence farming and small businesses. Sources of domestic water used by this population include rivers, streams, wells, and boreholes. The residents did their washings by the riverbanks and streams and bathed within the rivers which contributed to diarrheal disease transmission since the same water was used for drinking and cooking. Several residents used pit latrines, a few usedthe ventilated improved toilets, and the majority used bush and open sewer which majorly contributed to diarrheal disease transmission. Study populations whose stool samples were tested were children less than five years of age who presented at health facilities and those children who did not present with diarrhea and lived within the same locality as those who presented at the health facilities with diarrhea and from villages within Asembo, Karemo and Gem sites in Siaya County, Kenya. Case report forms were designed to collect information during child enrollment and capture demographics, birthdate, sex, and stool appearance for both cases and controls. (Appendix 5).

3.4 Case Recruitment

Children under 5 years of age seek care at sentinel health facilities when they experience moderate to severe diarrhea. A study clinician evaluated each child with diarrhea for eligibility. Eligible episodes were new (onset after $\geq$7 diarrhea-free days), acute (onset in the previous 7 days), and fulfilled$\geq$1 of the following criteria for moderate to severe diarrhea: 1) sunken eyes (confirmed by parent/caretaker as more than normal); 2) loss of skin turgor; 3) intravenous hydration administered or prescribed, 4) hospitalized with diarrhea (Kotloff *et al.*, 2012). To ensure even sampling throughout, the sites each enrolled the first ~8-9 eligible cases per age stratum (0-11 months, 12-23 months, and 24-59 months) per fortnight throughout a 3 months enrollment period.

3.4.1 Case Inclusion Criteria

1. A child less than 5 years of age.
2. Resides in the demographic surveillance system (DSS) catchment area.
3. Seeking care at a sentinel health center (SHC) in the regions mentioned above.
4. Diarrhea, defined as 3 or more loose stools within the previous 24 hours.
5. The diarrheal episode began at least 7 days after the last occurrence of diarrhea (O'Reilly *et al.*, 2012).
6. The onset of diarrhea was no more than 7 days before study enrollment.
7. Diarrhea meets at least one of the following criteria for "moderate-to-severe":
    a) Sunken eyes, more than normal.
    b) Loss of skin turgor.
    c) Intravenous rehydration is administered or prescribed.
    d) Dysentery (diarrhea with visible blood in stool).
8. Hospitalized with diarrhea.
9. Parental consent obtained.
10. Rectal swabs were obtained before the administration of antibiotics.

3.4.2 Case Exclusion Criteria

1. No diarrheal episode
2. A rectal swab was not obtained **before** antibiotic use.
3. Parents did not give consent.

3.5 Control Recruitment

For each child with diarrhea enrolled in the study, one to three healthy control children were randomly selected from the community or village in which the case resides. An algorithm to determine the number of controls to enroll: 1:1 case: control matching if 7–9 cases were enrolled; 1:2 matching if 4–6 cases were enrolled, and 1:3 matching if ≤3 cases were enrolled in a week. A total of 535 controls were enrolled. The controls were matched to the cases by age, gender, and time that the index cases presented. At least 4 children who met the matching criteria were randomly selected from the DSS database as potential controls. A field worker visited the home of selected children sequentially and explained all aspects of the study. If the parent/primary caretaker expressed interest and the child met eligibility criteria, informed, written consent was obtained, and arrangements were made to collect a stool sample.

3.5.1Control Inclusion Criteria

1. Has been residingin the demographic surveillance system (DSS) catchment area for not less than three months.
2. No diarrhea within 7 days of enrollment.
3. Age-matched to index case as follows: ±2 months for cases 0-11 months, and ±4 months for cases 12-59 months. The matched control may not exceed the stratum boundaries of the case, e.g., a control for an 11-month-old case must be between the ages of 9 and 11 months, and control for 13 months old must be between the ages of 12 and 17 months.
4. Same-gender as the case.
5. Same or nearby village or community as the case.
6. Time: enrolled within 14 days of presentation of the index case.
7. Parents consented.

Each site followed an algorithm beginning with the case's village/neighborhood, and then proceeding to villages/neighborhoods located at an increasing distance from the case's village/neighborhood until control can be identified.

3.5.2 Control Exclusion Criteria

1. A control was excluded from the study if itresided outside the demographic surveillance system (DSS) catchment area.
2. If the child was on antibiotics.
3. If the child had previously been enrolled as a case or control.

3.6 Sample Size Calculation

3.6.1 Sample Size Calculation for Cases

The sample size was estimated using Yamane's formula(Yamane, 1967)

$$n = \frac{N}{1 + N(e)^2}$$

Where:

N= Population of children who visited health facilities in the study area.

n= sample size

e= level of precision

$$n = \frac{3726}{1 + 3726(0.05)^2}$$

n= 362.22

n= 363±20 = 383 cases.

One to three matched controls per stratum were chosen to provide 80% power (2-sidedα=.05)assuming 20% of the potential controls consented:

3.6.2 Sample Size Calculation for Controls

The sample size for controls was estimated using Neyman's formula (Neyman, 1933)

$$n = \left(\frac{\mathrm{Z}1 - \alpha/2 + Z1 - \beta}{ES}\right)^2$$

Where:

n= Sample size

α = Confidence level, 0.05.

1- α/2= 1- 0.05/2 = 0.975

$Z_{1-\alpha/2}$ = is the value from the standard normal distribution holding 1- α/2 below it i.e 1.960

$Z_{1-\beta}$ =80% Power $Z_{0.80}$ =0.84

ES= Effect size

$$ES = \frac{P1 - PO}{P1(1 - P1)}$$

Where:

P_0 = the proportion under H_0

P_1 = the proportion under H_1

Therefore, $ES = \frac{P1-PO}{\sqrt{p1(1-P1)}} = ES = \frac{0.05}{\sqrt{0.2(1-0.2)}} = ES = \frac{0.05}{\sqrt{0.2*0.8}} = 0.125$

$$n = \left(\frac{\mathrm{Z1} - \alpha/2 + Z1 - \beta}{ES}\right)^2$$

$$n = \left(\frac{1.96 + 0.84}{0.125}\right)^2$$

$n = 502$ ± 7% to cater for dropouts.

$$n = 502 \pm 33$$

$n = 535$ controls

3.7 Collection of Stool Swabs

Each stool swab was moistened by dipping it in the Cary-Blair medium (Becton Dickinson, BD) that was used for transport. The polyester tip of the swab was gently inserted into the child's rectum and rotated 360°. A properly collected rectal swab was stained or covered with fecal material and inserted into a tube of Carry-Blair. The tube of Carry-Blair containing the rectal swabs was inserted into zip lock bags and carefully placed in a cooler box having cold icepacks. The specimen was delivered to the laboratory and plated within 18 hours of processing.

3.8 Laboratory Procedures

3.8.1 Specimen Accession

Upon receipt in the laboratory, the specimen number was entered into a laboratory fecal specimen report form labeled with the subject's study number. The specimens were examined for acceptability: proper labeling, sealed containers (no leaks or cracks), and satisfactory low temperature of the transport container.

3.8.2 Inoculation and Incubation of Primary Media

All media used in this study were obtained from BD-Difco (Sparks, MD, USA). Inoculation of MacConkeymedium was done using a stool swab from Carry-Blair. Inoculation was done

by rolling the stool-containing swab over a one (1) inch area approximately one-half away from one edge of the MacConkey plate. The plates were then streaked for isolation using a newly sterilized loop(Panchalingam *et al.*, 2012). The plates were then incubated in an inverted position, aerobically at 35°C-37 °C for 18-24 hours(Fischer Scientific, Pennsylvania, USA).

3.8.3 Isolation and Identification of Escherichia coli

After overnight incubation of the MacConkey plates, well-isolated lactose fermenting bacterial colony (pink) resembling *E. coli* was aseptically picked and inoculated on motility indole ornithine medium (MIO). A trypticase Soy Agar (TSA) plate was also streaked for pure culture isolation. The inoculated TSA plates and MIO tubes were then incubated at 35°-37°C for 18-24 hours. After overnight incubation, motility, indole,and ornithine production testswere performed. Kovac's reagent (Biomerieux) was used to check for indole production. Indole positive colonies were frozen using trypticase soy broth with 20% glycerol (Panchalingam *et al.,* 2012) awaiting PCR. For lactose fermenting colonies that are indole negative, an analytical profile index (API) (BioMérieux, Marcy-l'Etoile, France) was performed to rule out that they were not *E. coli*. Those lactose fermenting colonies that turn out not to be *E. coli* were discarded(Panchalingam *et al.*, 2012).

3.8.4 DNA Extraction

Frozen Bacterial controls and three putative *E. coli* colonies from each sample were cultured fresh on TSA agar plates for DNA extraction. The DNA was prepared by touching the center of 3 bacterial colonies using sterile inoculating loops and re-suspending in 200ul of sterile molecular biology-grade water (Research Products International). The sample was then vortexed to re-suspend adequately. This was then boiled at 100°C for 10 minutes followed by centrifugation at 10,000 rpm for 5 minutes (Fisher, Pennsylvania, USA). The supernatant was tested immediately by PCR(Panchalingam *et al.*, 2012).

3.8.5 Polymerase Chain Reaction (PCR)

The DNA obtained from the above process was analyzed by PCR amplification to detect the heat-stable, heat-labile toxins (ST, and LT). The gene targets that define ETEC are either *eltB* for LT, *estA* for ST, or both *(eltB* and *estA*).

A master mix was made up of 7.39 µL molecular biology grade water (VWR, Catalogue # 68100-000), 2.5 µL of NEB 10xThermopol PCR buffer with 2mM MgCl (New England Biolabs), 2.0µL of 1.25mM deoxynucleotide triphosphates (dNTPs) (Fermentas), 4.9 µL

primers (CVD-Baltimore) and 0.25 µL NEB Taq polymerase (5U/µL, New England Biolabs). The master mix was completely mixed by tapping the tube and a quick short spin. This master mix cocktail was adequate for one reaction. The components of the master mix were adjusted to suit the number of samples(Panchalingam *et al.*, 2012).

3.8.6 PCR Procedure

The contents of the master mix tube were mixed thoroughly and dispensed 17 µL to each labeled sample and control tube. A DNA Template of 3 µL was then dispensed to each tube with a master mix. The tubes were placed in an EppendorfMastercycler and the program which includes pre-heating at 96°C for 4 minutes, denaturation at 95°C for 20 seconds, annealing at 55°C for 20 seconds, elongation at 72°C for 1 minute, and final extension at 72°C for 7 minutes was started. This was repeated for 35 cycles with the preheating and final elongation occurring just once(Panchalingam *et al.*, 2012).

Table 3.1: Primer Sequences and the Expected Amplicon Sizes for *E. coli* PCR

Pathogen	Primer	Target Gene	Primer sequence (5'-3')	Amplicon
ETEC	LT-F	eltB	CACACGGAGCTCCTCAGTC	508
	LT-R	eltB	CCCCCAGCCTAGCTTAGTTT	
	ST-F	estA	GCTAAACCAGTAG/AGGTCTTCAAAA	147
	ST-R	estA	CCCGGTACAG/AGCAGGATTACAACA	

Table showing primer sequences and expected amplicon sizes for *E. coli* PCR; ETEC is enterotoxigenic *E. coli,* eltB is Heat-labile enterotoxin B chain and estA is Heat-stable enterotoxin A, F is a forward primer and R is a reverse primer(Panchalingam *et al.*, 2012).

3.8.7 Staining and Visualization

The resulting products were resolved on a 2% Agarose (RPI, Catalogue # A20090) gel stained with 10ul SYBR SAFE (Sigma-Aldrich, St. Louis, MO) and visualized on a UV Transilluminator (Spectroline Corporation, Westbury, NY). The 1 kb plus A 100-bp DNA ladder (New England Biolabs) was used as a molecular size marker in the gel. To ensure that the PCR was successful there was the appearance of bands with the correct base pairs. The following were the genotype designations: for heat-stable enterotoxin at 147bp and heat-labile enterotoxin at 508bp.

3.9 Data Analysis

Generated data was captured in an Excel spreadsheet and subjected to accuracy checks. The analysis was done using STATA version 13.1.

Logistic regression was used todetermine the prevalence of ETEC. To characterize ETEC enterotoxins, crosstabulation and logistic regression were used. The Association between moderate to severe diarrhea and ETEC enterotoxin was assessed using conditional logistic regression. Comparisons were drawn using a two-tailed Pearson's Chi-square test. A *P*-value of less than 0.05 was statistically significant.

3.10 Ethical Considerations

Approval for the study was obtained from the Kenya Medical Research Institute-Scientific and Ethics Review Unit (KEMRI/SERU); KEMRI SSC Protocol: 2996 (Appendix 1). Proposal approval for this study was obtained from Maseno University School of Graduate Studies (SGS) (Appendix 2). This current study only collected samples from parents/guardians who provided written informed consent for sample collection and future testings (Appendix 3 and 4). The current study was conducted in compliance with the protocol, good clinical practice (GCP) guidelines, and all applicable regulatory requirements. Participant's confidentiality was ensured by coding and omitting information that identifies them.

CHAPTER FOUR

RESULTS

4.1 Demographic Characteristic of Study Participants

A total of 918 stool samples were collected for isolation and identification of enterotoxigenic *E. coli* of which 383(41.7%) were cases while 535(58.3%) were control (Table 4.1). The females were 411 (44.8%) while males were 507(55.2%). The median age of children with diarrhea (cases) was 14 months (IQR 8-24) and that for children without diarrhea (controls) was 16 months (IQR 9-26). Children with diarrhea were slightly younger than those without diarrhea (median age 14 v 16 months). The female cases were 169(44.1%), female controls 242(45.2%), male cases 214(55.9%) and male controls 293(54.8%). The majority of cases 40.2% were children of age 0-11 months and less than 25.6% were cases of age 24-59 months. The proportion of male cases and control children enrolled were slightly higher (55.9% and 54.8% respectively) and females (44.1% and 45.2%).

Table 4. 1: Demographic characteristics of the study participants

Demographic characteristic	**Children with diarrhea (Cases), n=383**	**Children without diarrhea (controls), n=535**
Age (months)	**n (%)**	**n (%)**
0-11	154 (40.2%)	184(34.4%)
12-23	131(34.2%)	197 (36.8%)
24-59	98 (25.6%)	154 (28.8%)
Median age in months	14 (IQR 8-24)	16 (IQR 9-26)
Gender	n (%)	n (%)
Female	169 (44.1%)	242 (45.2%)
Male	214 (55.9%)	293 (54.8%)

Table showing the summary of demographic characteristics of participants. n is the total number of children. IQR is the interquartile range.

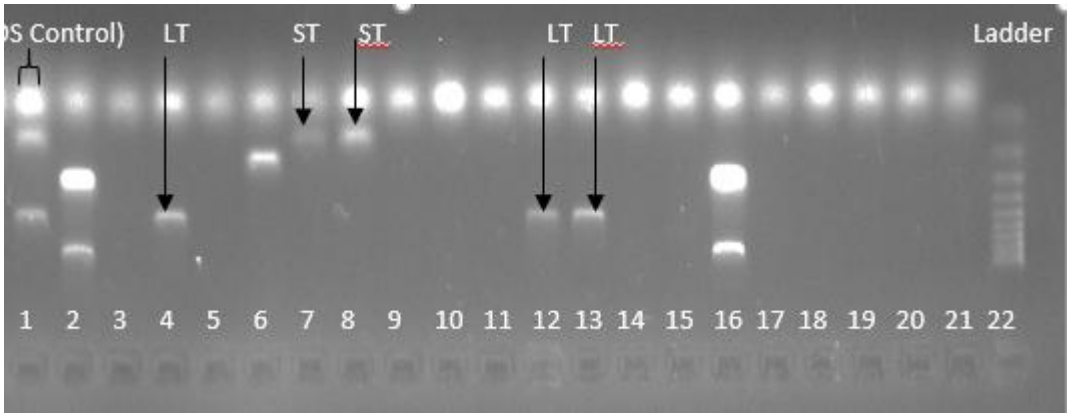

Figure 4. 1:PCR Results

Figure showing PCR detecting ETEC pathotype. Lane 1 Positive control (LT, 508bp, ST, 147 bp).Lane 4, 12, and 13 LT (508bp). Lane 7 and 8 ST(147bp). Lane 22 molecular weight marker.

4.2 Prevalence of ETEC in Children Under Five Years of Age in Siaya County, Western Kenya

Logistic regression was used to access ETEC prevalence. The overall prevalence of ETEC in children under five years in this study was 11% (Table 4.2). The prevalence of ETEC was significantly higher in cases 52 (13.6%) than in controls 49 (9.2%), *P*= 0.035).

Table 4.2: Prevalence of ETEC in children with diarrhea and those without diarrhea

Patient characteristic	**ETEC All positive, n (%)**	**ETEC negative, n (%)**	***P-value***
Children with diarrhea	52 (13.6)	331 (86.4)	
Children without diarrhea	49 (9.2)	486 (90.8)	
Total (N)	101 (11)	817 (89)	0.035

Table showing the prevalence of ETEC in children with and without diarrhea. n is the number of isolates; N is the total number of samples, ETEC is enterotoxigenic *E. coli*and *p*= 0.035 is the *p*-value showing higher ETEC prevalence in cases than controls.[a]Statistical significance determined by the Chi-square test.

4.3 Characterization of ETEC enterotoxins in children under five years in Siaya County, Western Kenya

Of the 383 cases tested, 27 (7.1%) were positive for ETEC-ST and 19 (5 %) for ETEC-LT and 6 (1.6%) for ETEC ST/LT enterotoxins (Figure 4.2). On the other hand, of 535 controls enrolled, 23 (4.3%) were ETEC-ST, 17 (3.2%) were ETEC-LT and 9 (1.7%) were ETEC LT/ST enterotoxins positives. ETEC ST was the most prevalent enterotoxin in both cases and controls 27 (7.1%) and 23 (4.3%), respectively.

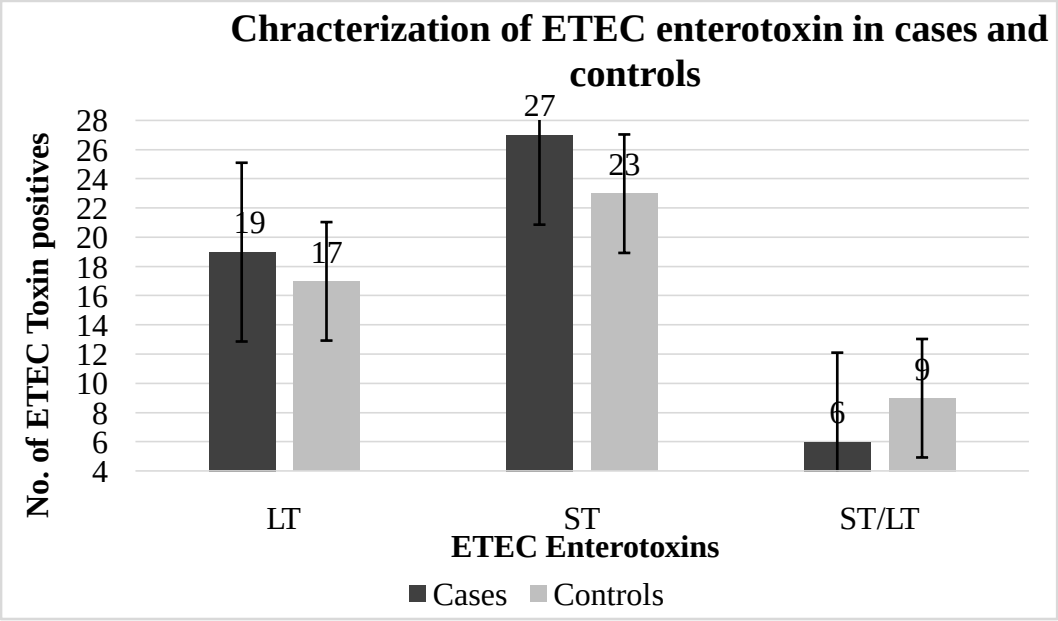

Figure 4.2: Characterization of ETEC toxin in children under five yearsin Siaya County

Figure showing ETEC toxin in children with diarrhea and those without diarrhea. LT is a Heat-labile enterotoxin and ST is a Heat-stable enterotoxin. Cross tabulation and logistic regression were used to characterize ETEC enterotoxin among diarrheal and non-diarrheal children by age and gender. Of the 214 male cases and the 169 female cases, 28 (13.1%) and 24 (14.3%) were positive for ETEC respectively, (Table 4.3). Out of 293 male controls and 242 female controls, ETEC positives were 24(8.2%) and 25(10.3%) respectively. Of the 383 diarrheal children, infection due to LT was higher in children of 0-11 months 7 (4.6%) than those of 24-59 months 6 (6.1%). This was similar to non-diarrheal children 7 (3.8%) in children of 0-11 months and 3 (2%) in children of 24-59 months. Infection due to ST was higher in children of 12-23 months in both diarrheal 14 (10.7%) and non-diarrheal children 13 (6.6%). Infection due to LT/ST was higher in children above 24 months in diarrheal 4(4.1%) and children of 0-11 months 4 (2.1%) in non-diarrheal children.

Table 4. 3:Distribution of ETEC among diarrheal and no-diarrheal children by age and gender

Characteristics	Cases (diarrheal), n=383							Control (Non-diarrheal), n=535						
	Overall n (%)	ALL Positive	OR (95% CI)	p-value	LT Positive	ST Positive	LT/ST Positive	Overall n (%)	ETEC positive	OR (95% CI)	p-value	LT Positive	ST Positive	LT/ST Positive
Age in months														
0 - 11	154 (40.2)	17 (11.0%)	Ref		7 (4.6%)	9 (5.8%)	1 (0.65)	184 (34.4%)	16 (8.7%)	ref		7(3.8%)	5(2.7%)	4 (2.2)
12-23	131 (34.2)	21(16.0%)	1.5 (0.777-3.06)	0.219	6 (4.6%)	14 (10.7%)	1 (0.76)	197 (36.8%)	22 (11.2%)	1.32 (0.67-2.60)	0.422	7(3.6%)	13(6.6%)	2 (1.0)
24 - 59	98 (25.6)	14(14.3%)	1.34 (0.63-2.87)	0.445	6 (6.1%)	4 (4.1%)	4 (4.08)	154 (28.8%)	11 (7.1%)	0.81 (0.36-1.80)	0.601	3 (1.9%)	5(3.3%)	3 (1.9)
Gender														
Males	214 (55.9)	28(13.1%)	Ref		9 (4.2%)	16 (7.5%)	3 (1.40)	293(54.8%)	24 (8.2%)	ref		11(3.8%)	9(3.1%)	4(1.4)
Females	169 (44.1)	24(14.2%)	1.10 (0.61-1.98)	0.751	10 (5.9%)	11 (6.5%)	3 (1.78)	242 (45.2%)	25 (10.3%)	1.29 (0.72-2.33	0.394	6(2.5%)	14(5.8%)	5(2.1)

Table showing the prevalence of ETEC in children with and without diarrhea. n is the number of children enrolled OR is the odds ratio, CI is the confidence interval at 95%; [a]Statistical significance determined by the Chi-square test.

4.4 Clinical characteristics associated with ETEC enterotoxins

Cross tabulation was used to assess the clinical characteristics and outcome of ETEC in cases. Examination of stool produced by the cases showed that 363 (94.8%) were non-bloody, 232 (60.6%) mucoid, 377 (98.4%) without pus, 189 (49.3%) Thick liquid, 178 (46.5%) opaque watery, 14 (3.7%) rice watery, 2 (90.5%) soft and non was formed (Table 4.4). To characterize the cases by the number of loose stools, 52 (13.6%) passed loose stool 0-3 times a day, 221 (57.7%) passed loose stool 4-5 times a day, 103 (26.9%) passed loose stool 6-10 times a day and no case produced above 10 loose stools a day. Out of 363 `non-bloody stools, LT-only positives were 19 (100%), 26 (94.8%) ST-only, and 6 (100%) ST/LT. Of 232 mucoid stools, LT-only were 9 (47.7%),15 (55.6%) ST-only and 1 (16.7%) LT/ST. LT/ST enterotoxins were associated with mucoid stool 95% CI, *P*=0.027. Out of 151 non mucoid stools, LT were 10 (52.6%), 12 (44.4%) ST and 5 (38.7%) ST/LT. ETEC enterotoxins were isolated in cases who passed 4-5 loose stools a day as follows [LT 8(3.6%), ST 18(8.1%) and LT/ST 4(1.%)] in 0-3 loose stools 5 (9.62%) LT and 5 (9.6%) ST, in 6-10 loose stools 6 (5.8%) LT, 4 (3.9%) ST and 2(1.9%) LT/ST and no enterotoxin isolated in more than 10 loose stools. However, these differences were not statistically significant.

Table 4.4: Clinical characteristics associated with ETEC enterotoxins

Variable	All cases, n (%)	LT Positive	LT Negative	*P*-value	ST Positive	ST Negative	*P*-value	LT/ST Positive	LT/ST Negative	*P*-value
Blood										
Yes	20 (5.2)	0	20 (5.5)		1 (3.7)	19 (5.3)	0.713	0	20 (5.3)	0.562
No	363 (94.8)	19 (100)	344 (94.5)	0.294	26 (94.8)	337 (94.7)		6(100)	357 (94.7)	
Mucus										
YES	232 (60.6)	9 (47.4)	223 (61.3)		15 (55.6)	217 (60.96)	0.580	1(16.67)	231 (61.3)	**0.027**
NO	151 (39.4)	10 (52.6)	141 (38.7)	0.86	12 (44.4)	139 (39.04)		5(38.73)	146 (83.3)	
Pus										
YES	6 (1.6)	0	6 (1.7)		1 (3.7)	5 (1.40)	0.354	0	6 (1.6)	0.755
NO	377 (98.4)	19 (100)	358 (98.4)	0.573	26 (96.3)	315 (98.6)		6(100)	371 (98.4)	
Stool Appearance										
Formed	0	0	0		0	0				
Soft	2 (0.5)	0	2 (100)		0	2 (100)		0	2 (100)	
Thick liquid	189 (49.4)	10 (5.3)	179 (94.7)		17 (9.0)	172 (91.01)		4(2.1)	185 (97.9)	
Opaque watery	178 (46.5)	8 (4.5)	170(95.5)	0.724	9 (5.1)	169 (94.94)	0.147	2(1.1)	176 (98.9)	0.461
Rice water	14 (3.7)	1 (7.1)	13 (92.9)	0.769	1 (7.1)	13 (92.86)	0.060	0	14 (100)	
Number of loose stools										
0 - 3 times	52 (13.6)	5 (9.6)	47 (90.4)		5 (9.6)	47 (90.38)		0	52 (100)	
4 - 5 times	221 (57.7)	8 (3.6)	213 (96.4)	0.08	18 (8.1)	203 (91.86)	0.732	4(1.8)	217 (98.1)	0.935
6 - 10 times	103 (26.9)	6 (5.8)	97 (94.2)	0.390	4 (3.9)	99 (96.12)	0.163	2(1.9)	101 (98.1)	
Above 10 times	7 (1.8)	0	7 (100)		0	7 (100)		0	7 (100)	

Table showing clinical characteristics associated with ETEC enterotoxins in children with diarrhea. n is the number of children; [a]Statistical significance determined by the Chi-square test. ST is Heat-labile enterotoxin, LT is Heat-labile enterotoxin.

4.5 Association of ETEC Enterotoxins with Diarrhea in Children under Five Years in Siaya County, Western Kenya

Conditional logistic regression modeling was used to estimate the association between ETEC enterotoxin andmoderate to severe diarrhea. There was no ETEC enterotoxin associated with diarrhea. The most isolated enterotoxin was ST although it was not statistically significant (p= 0.073), (Table 4.5).

Table 4.5: Association of ETEC enterotoxins with diarrhea in children under five in Siaya County, Western Kenya

ETEC toxin profiles	**Children with diarrhea N= 383 n (%)**	**Children without diarrhea N= 585 n (%)**	**Crude Odds Ratio 95% CI**	***P*-value**
LT only	19 (5.0%)	17 (3.2%)	0.63 (0.32-1.22)	0.173
ST only	27 (7.1%)	23 (4.3%)	0.59 (0.33-1.05)	0.073
LT/ST	6 (1.6%)	9 (1.7%)	1.08 (0.379-3.05)	0.892

Table showing association of ETEC enterotoxin with diarrhea in children under five years in Siaya County. N is the total number of children enrolled; n is the total number of positives. [a]Statistical significance determined by the Chi-square test. ST is heat-stable enterotoxin, LT is Heat-labile enterotoxin.

CHAPTER FIVE

DISCUSSION

5.1 Prevalence of Enterotoxigenic in Children under Five Years in Siaya County Western Kenya *E. coli*

Enterotoxigenic *Escherichia coli* (ETEC) bacteria is one of the top five causes of moderate-to-severe diarrhea in children < 5 years in developing countries and remains a major public health problem. It is a pathogenic variant of *E. coli* defined by the production of diarrheagenic heat-labile (LT) and heat-stable (ST) enterotoxins. In this study, the overall prevalence of ETEC in children under five years old was 11%. The prevalence of ETEC in children with diarrhea (cases) was 13.6% while that of children without diarrhea (controls) was 9.2% (Table 4.1). The prevalence in this study was higher compared with a study done in Kenya where the prevalence was reported at 4.3%(Mbuthia *et al.*, 2018). A study done in Kericho – Kenya enrolled patients with diarrhea at Kericho District hospital and Kisumu District hospital and reported an ETEC prevalence rate of 1.5% in patients with diarrhea and 1% in healthy individuals in Kericho (Swierczewski *et a*l., 2013). Similarly, the ETEC prevalence rate reported in Kisumu was 3.8% in patients with diarrhea and 1.7% in healthy individuals(Swierczewski *et al*., 2013). The differences in prevalence may be because the current study focused on children below 5 years of age and larger sample size while the study done by Swierczewski looked at the overall prevalence in children and adults.

Furthermore, there were multiple surveillance sites in Siaya County covered by the current study and in more remote, rural areas in contrast to the single surveillance site in the urban city of Kisumu(Swierczewski *et a*l., 2013). The surveillance site in Kericho also was conducted in urban settings where urban residents have higher proximity to proper sanitation and safe drinking water, as opposed to those from more rural, remote locations. The inclusion of more rural areas with suspect water quality and possibly poorly treated water could account for the significant detection of ETEC in this study.

A study done in Kenya to determine the high prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya showed a prevalence of 16.3% which was higher than the overall prevalence in this study (Iijima *et al.*, 2017). The study done by Iijima collected samples from children under five years in Busia, Kisumu, Nairobi, and Mombasa. The Collection of samples from more than one study site could have contributed to the higher prevalence. Also, Iijima used real-time PCR to detect ETEC enterotoxins while this study used conventional PCR.

A study done in Zambia to determine the prevalence of Enterotoxigenic *Escherichia coli* toxins and colonization factors among Children presenting with moderate to severe diarrhea recorded a higher ETEC prevalence (40%) than the prevalence in this study (Simuyandi M, 2019). The higher prevalence of ETEC in Zambian children could be because the study By Simuyandi used Luminex™ xTag Gastrointestinal Pathogen Panel to detect ETEC while this study used PCR.

The prevalence of ETEC was significantly higher in children with diarrhea than without diarrhea. This data is consistent with that of Kericho and Kisumu district hospitals (Swierczewski *et al.*, 2013) where it was observed that prevalence in cases was higher than in controls. The higher prevalence of ETEC in cases could point towards the widespread contamination of the environment and ETEC as a major cause of diarrhea. Recovery of ETEC in controls suggests that ETEC is rarely encountered in healthy children and those from which ETEC was isolated might be recovering from diarrhea or were in the pre-symptomatic stage of infection. A study on the detection of enterotoxigenic *E. coli* in hospitalized children with and without diarrhea in Blantyre, Malawi reported that the detection of ETEC was significantly higher in children with diarrhea than in those without diarrhea (12.7%) vs (7.3%), respectively(Trainor *et al.*, 2016). This was concurring with the report from this study.

A multi-site study done to re-assess and refine estimates of diarrhea etiology from the etiology, risk factors, and Interactions of Enteric Infections and Malnutrition and the consequences of child Health and Development (MAL-ED) indicated ETEC prevalence of 18.8% (Platts-Mills *et al.*, 2015). This was much higher than what is reported in this study. This could be because the MAL-ED study had several study sites and a large sample size than this study.

Studies conducted previously have shown that children below 24months of age are more susceptible to ETEC infection (Kotloff *et al.*, 2013; Mansour *et al.*, 2013) which agrees with this study (38 ETEC positives in0-23 months and 14 ETEC positives in 24-59 months. Similarly, in a prospective community-based diarrhea study in South America, Africa, and Asia, the Interaction of Malnutrition and Enteric Infections found ETEC to be an important cause of diarrheal illness in the second year of life in these regions (Platts-Mills *et al.*, 2015). In this present study, ETEC toxins were more prevalent in diarrheal children of 24 months and below than in diarrheal children above 24 months of age. Diarrhea could be high among

children below 24 months due to their underdeveloped immune system that can mount an effective immunological response.

Infection due to ETEC decreased with an increase of age above 24 months (Table 4.4). Although this study was a case-control study, the findings were in agreement with the results of a study that was conducted among patients with diarrhea in Muranga (Mbuthia *et al.*, 2018). A study in Zambiaalso reported ETEC to be a childhood disease, to its substantially higher incidence in early childhood than in older age groups(Chiyangi *et al.*, 2017). ETEC toxins decrease after the age of 24 months probably due to environmental and immunological factors. Another factor could be due to acquired immunity among individuals which prevents ETEC infections and immunogenetics and diversity among individuals (Bourgeois *et al.*, 2016). Increased immune responses due to repeated episodes of ETEC infection in age below 24 months prevent ETEC infections (Anderson *et al.*, 2019). The finding of this study provides evidence that ETEC is a common isolate among children below 5 years within Siaya County.

5.2 Characterization of ETEC Enterotoxins in Children Under-five Years with and without Diarrhea in Siaya County, Western Kenya

In this study, ST-producing strains were predominant in both children with diarrhea and those without diarrhea (Fig 4.2). A study done in Nairobi- Kenya (Nyanga *et al.*, 2017) in children less than 5 years with diarrhea detected ETEC strains to produce LT/ST in 23(6.5%), while those expressing LT alone were 8 (2.3%) and ST alone was 6 (1.7%) which is not in agreement with this study as LT and ST were lower than that of this study while the number of LS/ST was higher than that of this study. This could be due to a difference in the study site as this study was conducted in a rural setup where the incidence of ETEC infection is high. Their study did not include children without diarrhea; therefore, did not compare with frequencies in controls to determine the association.

The results in this study, however, concurs with a study done in etiology of diarrhea and global, regional, and national causes of mortality in children< 5 years which showed that heat-stable (ST) toxin was predominant in ETEC (Khalil *et al.*, 2018; Kharat *et al.*, 2017; L. Liu *et al.*, 2015). This distribution also concurs with a previous study by GEMS that showed ETEC producing ST alone or together with LT are major contributors of the burden (Kotloff *et al.*, 2013).

While LT expression was more common in the strains isolated in Mexico, Peru, Argentina, and India, ST expression was more common in the strains isolated in Bangladesh, Egypt, and Nicaragua. In these countries, the rate for simultaneous expression of LT and ST varied between 7% and 49 % (Qadri *et al.*, 2005). In astudyby Ozerol et al, the rate of ST expression in the ETEC strains isolated from children aged 0-5 years with diarrhea was 28%, while for LT expression, the rate was 71% for the same group of children (Ozerol *et al.*, 2005). In this study, 41.8% of the ETEC strains isolated from children with diarrhea expressed ST, whereas 58.3% expressed LT. The higher prevalence of ETEC strains that express ST than those expressing LT is an important finding because the ST expressing ETEC strains are considered to be more pathogenic than those expressing LT.

A study done in Kenya to determine the prevalence of diarrheagenic *Escherichia coli* among children with diarrhea in Kenya reported that LT-only was the most isolated (41/58 (70.7%)(Iijima *et al.*, 2017)while in this study, ST was the most isolated 50 (5.5%).This difference in the distribution of ETEC enterotoxin could because of differences in geographical locations.

An examination of ETEC distribution among cases and controls showed different rates of isolation from children of different age groups (from 1 month to 59 months). The highest proportion was among cases and controls of less than 24 months of age (Table 4.4). This was consistent with the results of a study investigating the burden of ETEC in children under 24 months in Bangladesh (Qadri *et al.*, 2007). The attack rate for ETEC illness appears to be highest during the first 24 months of life in endemic areas with substantial declines thereafter suggesting that protective immunity develops following infection. Infection in children of <24 months could be due to their underdeveloped immune system that is incapable of mounting an effective immunological response. Also, risk of placing contaminated fingers and fomites in the mouth is greatly increased due to physiological phenomenon like teething and crawling which begins at this age.

A study in Nigeria on the etiology of diarrhea and virulence properties of diarrhoeagenic *Escherichia coli* among patients and healthy subjects in Southeast Nigeria reported 22 (21.57%) ETEC strains isolated from 12 (54.55%) males and 10 (45.45%) females (Nweze 2010). The high ETEC infection in males is similar to the findings of this study. Results of previous studies in Ibadan, Ile-Ife, and Lagos (all in southeastern Nigeria)

suggest that the incidence of ETEC is higher among males than among females(Nweze, 2010).

5.3 Association of ETEC Toxin with Diarrhea in Children Under-five Years in Siaya County, Western Kenya

In this study, no ETEC enterotoxin was associated with diarrhea. In the previous study 'Global Enteric Multicentre Study (GEMS)', conducted in Asembo and Gem sites in Siaya County Kenya, ST-ETEC was a significant pathogen causing diarrhea in infants at all the site before the introduction of rotavirus vaccine(Kotloff *et al.*, 2013). ETEC producing LT and LT/ST were not a significant cause of moderate-to-severe diarrhea in infants at any site(Kotloff *et al.*, 2013). The difference in findings from this studycould be because of a smaller sample size compared to that of GEMS.

Several studies have shown thatchildren with diarrhea are more likely to have ST-ETEC than individuals without diarrhea (Qadri *et al.*, 2005; Yang *et al.*, 2019). This agrees with this study where ST only toxin was higher in the cases compared to LT only and ST/LT toxins. This could be because ETEC expressing ST are considered more pathogenic than those expressing LT. Detection of these specific enterotoxins can be important in the development of vaccines targeting ETEC.

The majority of the cases had a mucoid thick-non bloody stool and without pus. This is likethe findings of a study conducted in Murang'a which showed that children who presented with watery stool or mucoid stool remained associated with ETEC(Mbuthia *et al.*, 2018). This could be due to the ability of enterotoxins to bind to the bowel epithelial cells increasing cAMP which stimulates sodium chloride production. When the action of cAMP exceeds the absorptive power of the epithelial cells, a watery diarrhea results(Fleckenstein *et al.*, 2013).

A study done in Iran showed that the most common symptoms of diarrhoeagenic children with ETEC were watery stools (80.95%), (Nazarian et al., 2014). Higher proportions of enterotoxins were isolated in cases that had that produced 4-5 loose stools a day. This could be because once attached to the intestinal epithelium, ETEC elaborate LT and/or ST enterotoxins, which induce the characteristic watery diarrhea.

5.4 Limitations of the Current Study

1. Data fromthis study maynotbe generalized toall children under 5 years in Siaya County as itwas not conducted inall rural sites in Siaya County.
2. Molecular diagnosis of Enterotoxigenic *E. coli* was performed on cultures but not stool samples.

CHAPTER SIX

SUMMARY, CONCLUSION, AND RECOMMENDATION

6.1 Summary

This study was done in children under five years with (cases) and without diarrhea (controls) in Siaya County, Western Kenya. Using bacterial culture and a sensitive molecular diagnostic method, PCR, I have identified ETEC heat-labile (LT) and heat-stable (ST) enterotoxins. The general prevalence of ETEC in children under five years old was 11%. ETEC was detected significantly more often in children with diarrhea (cases) 13.6% than in children without diarrhea (controls) 9.2%. Analysis of the ETEC toxin distribution in this pediatric population identified ST as the most prevalent followed by LT then LT/ST. Heat-stable (ST) toxin was associated with diarrhea. ETEC infection was higher in children less than 24 months and this decreased with an increase in age.

6.2 Conclusions

Due to the differences in the occurrences of ETEC, the implementation of immunoprophylactic measures for the control of ETEC diarrhea should be assessed and variations in the phenotypic, genotypic, and pathogenic properties of the bacterium isolated should be identified.

1. The enterotoxigenic *Escherichia coli* expressing ST only, LT only,and LT/ST were detected in diarrheal and non- diarrheal children. The ST-only enterotoxin was the most isolatedsuggesting its high pathogenicity.
2. No ETEC enterotoxin was associated with diarrhea.

6.3. Recommendations

6.3.1 Recommendations for this Study

1. Further investigations should be done on the controls who expressed ETEC LT, ST, and LT/ST enterotoxins to see if they developed diarrhea or not.
2. Understanding the prevalence of ST-producing ETEC subtypes in this population helps inform potential targets for future vaccine development.

6.3.2. Recommendations for Further Studies

1. Further research on the association of ETEC related diarrhea and stool appearance needs to be conducted to come up with an algorithm for clinical diagnosis of ETEC associated diarrhea.
2. Further investigations on the transmission of ETEC in the community and association with diarrhea should be considered.

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APPENDICES

Appendix 1: KEMRI/SERU Annual Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel:(254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

KEMRI/RES/7/3/1 February 25, 2019

TO: **RICHARD OMORE**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGHR**
KISUMU

4/3/2019

Dear Sir,

RE: **SSC PROTOCOL NO. 2996 (*REQUEST FOR ANNUAL RENEWAL*): VACCINE IMPACT ON DIARRHEA IN AFRICA ASSESSMENT (VIDA) VERSION 6.0 (17TH APRIL 2018)**

Thank you for the continuing review report for period **March 24, 2018** to **January 31, 2019.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **March 24, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **March 23, 2020**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval by **February 09, 2020.**

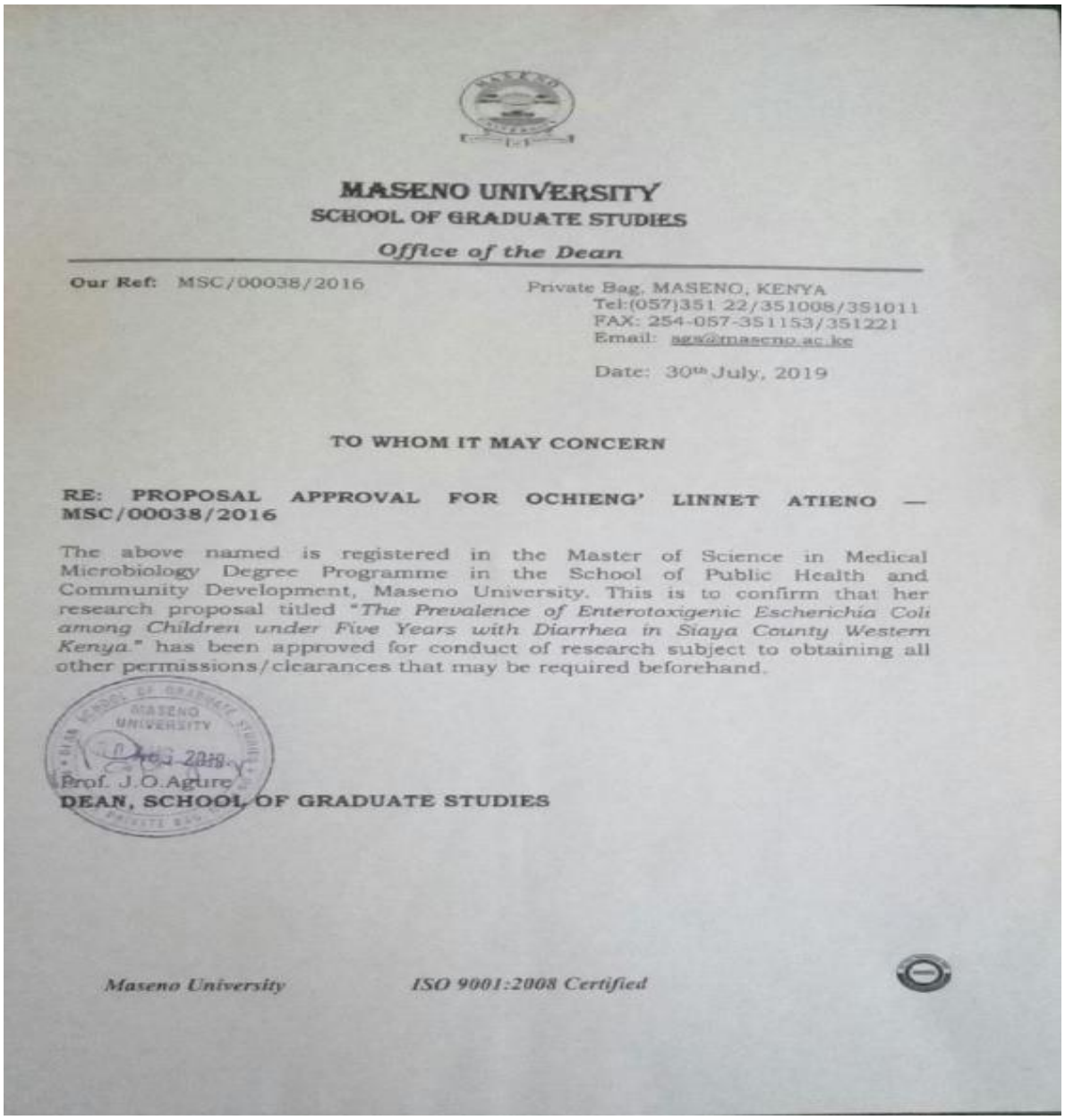
You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

For **ENOCK KEBENEI**
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix 2: Maseno University SGS Approval Letter



MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: MSC/00038/2016

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 30th July, 2019

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR OCHIENG' LINNET ATIENO — MSC/00038/2016

The above named is registered in the Master of Science in Medical Microbiology Degree Programme in the School of Public Health and Community Development, Maseno University. This is to confirm that her research proposal titled *"The Prevalence of Enterotoxigenic Escherichia Coli among Children under Five Years with Diarrhea in Siaya County Western Kenya."* has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

SCHOOL OF GRADUATE STUDIES MASENO UNIVERSITY

Prof. J.O.Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

Appendix 3:Consent for Cases

CONSENT FORM FOR CHILDREN WITH DIARRHEA

(CASE CONSENT FORM-ENGLISH)

Project Title: DISEASE BURDEN OF DIARRHOEA DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI AMONG CHILDREN UNDER FIVE YEARS IN SIAYA COUNTY, KENYA

Investigator: Linnet Atieno Ochien'g

Consent Form: Consent for parent/guardian of children less than five years old attending clinics and hospitals participating in disease burden of diarrhea due to Enterotoxigenic*Escherichia coli* among children under five years in Siaya County Western Kenya

Today's date (dd/mmm/yyyy)	□□/□□□/□□□□
Childs DSS Permanent ID	□□□-□□□□-□□-□□□
Child's study number	
Name of the ill child	

Phone number of contact person: Linnet Atieno +254 707 880 091.

Diarrhea is one of the most common causes of illness and death in young children. Most of the time, diarrhea is caused by germs that are passed to the child in food or water or from other people. Linnet Atieno in collaboration with the Kenya Medical Research Institute (KEMRI) is conducting a research study to learn the disease burden due to Enterotoxigenic E.*coli*, germs that cause diarrhea in children, so that better ways to prevent these illnesses can be found. The study will also tell us the consequences of moderate to severe diarrhea in infants due to enterotoxigenic*E.coli*and ways to prevent diarrheal illnesses in your community. This study will include children who are treated at several health centers in your area for diarrhea.

You have brought your child to the doctor because he/she has diarrhea. We may be able to identify which germ is making your child ill. To do this, we must collect a stool sample that your child passes. If your child does not pass a stool before treatment is started, we will insert two small swabs (thin wooden sticks with a cotton tip) into his/her rectum and collect

some stool. There are no risks to your child by collecting stool but collecting stool with a swab or sharing personal information can be a little uncomfortable for your child or you. Your child's name will be removed from the samples before they are sent off-site.

We will interview you for about one hour to collect general information about your child, your child's illness, and the vaccines your child has received. We will check your child's vaccination card, and sometimes check your child's vaccination records at the health center to be sure we have the correct information We will also ask where you live. We would also like to ask your permission to visit you at home in about 2 months to collect information about your child's health. We will check your child's arm size, weight, and length to see how he or she is growing. The home visit will last about 30 minutes. You may have a DSS number to show you are in this study. This will help us to learn how big of a problem diarrhea is in children who live in Asembo, Gem, Karemo,and nearby areas.

We will give you a card so you can record whether your child had diarrhea each day for the next 14 days. Diarrhea means that your child passed at least 3 loose or watery stools on that day, that you believe are not normal for your child. We will teach you how to complete the card. We will collect this card when we visit you at home in a month, so you must complete it and keep it in a safe place.

The benefit of this study is that the information collected may help your doctor to know the best treatment for your child. By learning the burden due to enterotoxigenic*E. coli* as a germ that causes moderate to severe diarrhea, we can understand the need for prevention with new or improved vaccines or medicines in Kenya and other parts of the world**.** We will try as much as possible to ensure community leaders and Government administration staff in your location are involved in creating an understanding of the study objectives, justification, and methods. Subsequently, the findings from this study will be shared and discussed with the community members at the end of the study.

What we talk about and your child's test results will be kept private. The information collected about your child will be shared with our study collaborators, but it will not contain your child's name. We will protect the information that could contain your child's name by keeping it in locked cabinets and limiting the people who can see it. This information may be reviewed by people who work on the Siaya County study site and the ethics committees at KEMRI (this group is responsible for protecting people who take part in research). However,

in the case of publication of the results of the study, you or your child's name or other facts that might point to you will not appear when we present this study or publish the results.

As we've said before, we would like to get a stool sample from your child at this visit and test it at the KEMRI/CDC laboratory in Kisumu.

The tests will be done for free. You will not be paid to take part in the study. CDC/KEMRI will not cover any costs for treatment or complications of the diarrheal illness itself or other illnesses or injuries not related to the study.

You are free to choose to take part in this study, and you can stop participating at any time. If you choose not to participate in this study, you could have your child's samples tested for germs elsewhere (in some instances this might be at your own expense), or not at all. Should you refuse to take part in the study, or decide to stop participating, you will continue to receive your usual medical care.

Please ask any questions that you may have about this study. If you would like to contact us later, please call Linnet Atieno Tel +254-707-880-091. You may also get information about the study and your rights as a participant by contacting the KEMRI Scientific and Ethics Review Committee through the Acting Head at KEMRI headquarters:Tel (254) (020) 2722541,2713349,0722-205901,0733-400003

___ I have read and understood the information on this form.

___ I have had the information on this form explained to me.

_____

Name of Parent/ Primary Caretaker (print)

_____

Signature or Fingerprint of Parent/ Primary Caretaker Date

_____

Name of Witness (print)

_____ ___

Witness to Consent procedures Date

(Optional unless the subject is illiterate or unable to sign)

_____

Name of Investigator or Authorized
Representative obtaining informed consent (print)

_____ _____ _

Signature of Investigator or Authorized Date
Representative obtaining informed consent (print)

Appendix 4: Consent for Controls

CONSENT FORM FOR CHILDREN WITHOUT DIARRHEA

(CONTROL CONSENT FORM-ENGLISH)

Project Title: DISEASE BURDEN OF DIARRHOEA DUE TO ENTEROTOXIGENIC ESCHERICHIA COLI AMONG CHILDREN UNDER FIVE YEARS IN SIAYA COUNTY, WESTERN KENYA

Investigators: Linnet Atieno Ochien'g

Consent Form: Consent for parent/guardian of children less than five years old in the community who do not have diarrhea and are disease burden of diarrhea due to enterotoxigenic*Escherichia coli* among children under five years in Siaya County, Western Kenya

Today's date (dd/mmm/yyyy)	□□/□□□/□□□□
Childs DSS Permanent ID	□□□-□□□□-□□-□□□
Child's study number	
Name of the child	

Phone number of contact person: Linnet Atieno Tel. +254 707 880 091

Diarrhea is one of the most common causes of illness and death in young children. Most of the time, diarrhea is caused by germs that are passed to the child in food or water or from other people. Linnet Atieno in collaboration with the Kenya Medical Research Institute (KEMRI) is conducting a research study to learn the disease burden due to enterotoxigenic*E.coli,* germs that cause diarrhea in children, so that better ways to prevent these illnesses can be found. The study will also tell us the consequences of moderate to severediarrhea in infants due to enterotoxigenic*E.coli*and ways to prevent diarrheal illnesses in your community. This study will include children who are treated at several health centers in your area for diarrhea.

This study will include children who received medical care for diarrhea at health centers in your community and children of the same age who remain healthy. Your child is invited to participate in this study because he/she is well. By comparing children who are ill with those

who are healthy, we can better understand what is making children ill. To do this, we must collect a stool sample that your child passes. There are no risks to your child by collecting a stool. Your child's name will be removed from the samples before they are sent off-site.

We will interview you for about one hour to collect general information about your child and your child's health. We will check your child's vaccination card, and sometimes check your child's vaccination records at the health center to be sure we have the correct information. We will also check your child's arm size, weight, and length to see how he or she is growing. We would also like to ask your permission to visit you at home in about 1 month to check your child's weight and height and collect information on your child's health and environment one more time. The second visit will last about 30 minutes.

We will give you a card so you can record whether your child had diarrhea each day for the next 14 days. Diarrhea means that your child passed at least 3 loose or watery stools on that day, that you believe are not normal for your child. We will teach you how to complete the card. We will collect this card when we visit you again in a month so you must complete it and keep it in a safe place. We would also like to connect the results from this study about your child's diarrhea and vaccination with the results of another study that you and your child may be in. The other study is the DSS study in Asembo, Gem, and Karemo. You may have a DSS number to show you are in this study. This will help us to learn how big of problem diarrhea is in children who live in Asembo, Gem, Karemo.

There is no direct benefit to your child if you participate. However, by learning the disease burden due to enterotoxigenic*E. coli,* whichis a germ that causes moderate to severe,we can understand the need for prevention with vaccines or medicines in Kenya and other parts of the world.

What we talk about and your child's test results will be kept private. We will protect information that could contain your child's name by keeping it in locked cabinets and limiting the people who can see it. This information may be reviewed by people who work on the Kenya study site(this group is responsible for protecting people who take part in the research. However, in the case of publication of the results of the study, you or your child's name or other facts that might point to you will not appear when we present this study or publish the results.

As we've said before, we would like to get a stool sample from your child at this visit and test it at the KEMRI/CDC laboratory in Kisumu.
The tests will be done for free. You will not be paid to take part in the study. CDC/KEMRI will not cover any costs for treatment or complications of the diarrheal illness itself or other illnesses or injuries not related to the study.

You are free to choose to take part in this study, and you can stop participating at any time. Should you refuse to take part in the study, or decide to stop participating, you will continue to receive your usual medical care.

Please ask any questions that you may have about this study. If you would like to contact us later, please call Linnet Atieno Tel. +254-707-880-091. You may also get information about the study and your rights as a participant by contacting KEMRI Scientific and Ethics Review Committee through the Acting Head at KEMRI headquarters:Tel (254) (020) 2722541,2713349,0722-205901,0733-400003

___ I have read and understood the information on this form.

___ I have had the information on this form explained to me.

_____

Name of Parent/ Primary Caretaker (print)

_____ _

Signature or Fingerprint of Parent/ Primary Caretaker Date

_____

Name of Witness (print)

_____ __

Witness to Consent procedures Date

(Optional unless the subject is illiterate or unable to sign)

_____

Name of Investigator or Authorized Representative obtaining informed consent (print)

_____ _____

Signature of Investigator or Authorized Representative obtaining informed consent (print) Date

Appendix 5: Demographic and clinical characteristic Collection form.

STOOL/ RECTAL SWAB COLLECTION

					2 0
Site	*Centre*	*Child ID*	*Day*	*Month*	*Year*

1. What is the child's date of birth?

Day	*Month*	*Year*

2. What is the child's sex? F M
3. Time and date when whole stool passed/excreted:

a. Date first whole stool passed/excreted:

Day	*Month*	*Year*

b. Time first whole stool passed/excreted: | | | | | *(24-hour clock)*

4. Consistency of whole stool sample: *(select one)*

☐ grade 1 (formed) ☐ grade 2 (soft) ☐ grade 3 (thick liquid)
☐ grade 4 (opaque watery) ☐ grade 5 (rice water-clear watery)

5. Characterization of stool sample (whole stool or rectal swab):

Blood ☐ *No* ☐ *Yes* **Pus** ☐ *No* ☐ *Yes* **Mucus** ☐ *No* ☐ *Yes*

6. If the child is a case, did s/he receive antibiotics after arriving at the health centre but before producing the whole stool specimen? If the child is a control, did s/he receive antibiotic during the 4 hours prior to stool collection?

☐ *No* ☐ *Yes* ☐ *DK*

[If 'Yes', check the appropriate boxes ("X" all that apply). If 'No', go to Question 7.]

☐ Ampicillin
☐ Cotrimoxazole
☐ Selexid/Pivmecillinam
☐ Chloramphenicol/Thiamphenicol
☐ Azithromycin
☐ Penicillin
☐ Amoxycillin
☐ Metronidazole (Flagyl)
☐ Other antibiotic, specify_____

☐ Nalidixic acid
☐ Ciprofloxacin/Norfloxacin/other fluoroquinolone
☐ Gentamycin
☐ Erythromycin
☐ Other macrolides
☐ Ceftriaxone or other 3rd generation cephalosporin
☐ 1st or 2nd generation cephalosporin

7. If antibiotic was given:

a. Date of first antibiotic: | | | | | | | | |

b.Time of antibiotic:

STOOL /RECTAL SWABS COLLECTION

Site *Centre* *Child ID*

6. If the child is a *case* **and** *was given antibiotics* at the health centre **before** the child produced a *whole stool specimen*, were rectal swabs collected from the child before the child received antibiotics?

☐ *No* ☐ *Yes* ***[If 'Yes', continue. If 'No', go to Question 7.]***

a. Date rectal swabs obtained: 2 0 *Day* *Month* *Year*

b. Time rectal swabs obtained: *(24hour clock)*

7. Time and date when whole stool/rectal swab placed in transport media:

a. Date whole stool/rectal swab placed in transport media:

Day *Month* *Year*

b Time whole stool/rectal swab placed in transport media: *(24hour clock)*

☐ ☐

10. Swab (rectal swab/whole stool) in Cary Blair: *No* *Yes*

11. Time and date when sample received by lab personnel:

a. Date sample received by lab personnel: 2 0

Day *Month* *Year*

b. Time sample received by lab personnel: *(24hour clock)*

Interviewer's Name _____ *Staff code*

*Quality Control's Name*_____ *Staff code* *Day* *Month* *Year*