PREVALENCE, VIRULENCE FACTORS AND DRUG RESISTANCE OF ENTEROAGGREGATIVE *Escherichia coli* ISOLATED FROM CHILDREN UNDER FIVE YEARS OLD IN KIAMBU COUNTY HOSPITAL

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MAY, 2018

DECLARATION

I confirm that this thesis is my original work and has not been presented in any other university/institution for certification. The thesis has been complemented by referenced works duly acknowledged. Where text, data, graphics, pictures or tables have been borrowed from other works- including the internet, the sources are specifically accredited through referencing in accordance with anti-plagiarism regulations.

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DEDICATION

To my family for the support you accorded me throughout the duration of my studies and research.

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ABBREVIATIONS & ACRONYMS

AAF	Aggregative adherence fimbriae
aafA	AAF/II fimbrial subunit
aafC	Usher, AAF/II assembly unit
aap	Dispersin, antiaggregation protein
aatA	Dispersin transporter protein
agg3/4C	Usher, AAF/III-IV assembly unit
agg3A	AAF/III fimbrial subunit
agg4A	AAF/IV fimbrial subunit
aggA	AAF/I fimbrial subunit
aggR	A transcriptional activator of EAEC virulence related genes
astA	*E. coli* heat-stable enterotoxin 1
ATCC	American Type Culture Collection
BTB	Bromothymol Blue
CLSI	Clinical and Laboratory Standards Institute
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
DHL	Deoxycholate Hydrogen Sulphide Lactose
DNA	Deoxyribonucleic acid
dTTP	Deoxythyamidine triphosphate
DXT	Doxycycline
E. coli	*Escherichia coli*
EAEC	Enteroaggregative*Escherichia coli*
EAST1	Enteroaggregative*Escherichia coli* heat stable enterotoxin 1
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterhemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
eilA	Salmonella HilA homolog
EPEC	Enteropathogeinc *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
h	Hours
HC	hemorrhagic colitis
HCL	Hydrochloric acid
HEp-2 cells	Human epithelial type 2 cells
HIV	Human Immunodeficiency Virus
HUS	hemolytic uremic syndrome
KCH	Kiambu County Hospital
KEMRI	Kenya Medical Research Institute
KNBS	Kenya National Bureau of Statistics
LIM	Lysine Indole Motility
M	Molar
mg	Milligrams
min	Minutes

ml	Millilitre
mM	Millimolar
MR/VP	Methyl Red/Vogues- Proskauer
NA	Nutrient Agar
NaCl	Sodium Chloride
NUITM	Nagasaki University Institute of Tropical Medicine
OD	Optical Density
OR	Odds ratio
orf61	Plasmid-encoded hemolysin
PCR	Polymerase Chain Reaction
Pet	Plasmid-encoded toxin
SC	Simmons Citrate
sepA	Shigella extracellular protease
Stx	Shiga-toxin
SXT	Sulfamethoxazole-trimethoprim
TSB	Tryptic Soy Broth
TSI	Triple sugar iron
VRGs	Virulence Related Genes
µl	Microlitre

ABSTRACT

Enteroaggregative *Escherichia coli* (EAEC) is recognized as the leading bacterial pathogen in children hospitalized for acute diarrheal illness reflecting a significant cost in health resources. However, asymptomatic carriage of EAEC is also high making it difficult to distinguish the pathogenic and the non-pathogenic strains of EAEC. Establishing a marker which corresponds with diarrheal illness would be of significant value in routine detection of pathogenic EAEC and its differentiation from non-pathogenic EAEC. The aim of this study was to determine the prevalence and identify the putative virulence factors of EAEC between children admitted with diarrhea and in age-matched asymptomatic children under five years old. In this study, 354 fecal samples from diarrheal patients and 354 from asymptomatic controls collected during a case-control study in Kiambu County, Kenya, were analyzed. Microbial culture and biochemical analysis were carried out for confirmatory identification of *Escherichia coli* (*E. coli*). At least five different colonies from each sample identified as *E. coli* were then examined by Multiplex-PCR for determination of EAEC and for the detection of the various putative virulence genes. Enteroaggregative *Escherichia coli* was detected in 60/354 (16.95%) diarrheal patients and 60/354 (16.95%) asymptomatic controls. Genes that encode a transcriptional regulator and *E. coli* heat-stable enterotoxin 1, *aggR* and *astA*, respectively, were detected significantly more in patients than in controls (P=0.0271 and 0.0443, respectively). Usher, aggregative adherence fimbriae (AAF) III/IV assembly unit, *agg3/4C*, was not detected in any EAEC strains from patients while 93.2% of the EAEC strains from asymptomatic controls were found positive for the gene. The most frequent AAF pilin gene was that of AAF/I encoded by *aggA* (10% and 20% in patients and asymptomatic controls respectively) followed by those of AAF/IV (*agg4A*), AAF/II (*aafA*) and AAF III (*agg3A*) respectively. Other virulence associated genes including *pet, orf61, sepA* and *eilA* were not detected in any of the EAEC from patients while their prevalence in asymptomatic controls was 3.3%, 59.3%, 17% and 86%, respectively. In typical EAEC *astA, aatA, agg4A* and *aggA* were detected more in patients than in asymptomatic controls. However, no statistical difference was observed in their distribution between patients and asymptomatic controls on typical EAEC. In atypical EAEC, *astA* was detected significantly more in patients than in asymptomatic controls (P=0.0059, OR =4.17). Several different combinations of the virulence markers were found among the EAEC isolates in this study. High resistance rate was observed in amoxicillin, trimethoprim/sulfamethoxazole and tetracycline. Among the penicillin, sulfonamide and tetracycline groups, most of the EAEC pathogens showed multi-drug resistant patterns. Data from this study showed a high prevalence of EAEC in diarrheal patients in the study area. However asymptomatic carriage of EAEC is equally high. The study also reveals a high degree of variability of genotypic markers in EAEC isolated from Kiambu County. These results underscore the potential usefulness of the *aggR* and *astA* genes as possible virulence markers for detection and differentiation of virulent strains of EAEC from avirulent strains. This study also indicates that a certain subset of EAEC is unrelated to diarrhea, for which *agg3/4C* may be a marker. Overall, the markers reported here can find great application in routine detection of EAEC.

CHAPTER ONE

INTRODUCTION

1.1 Background

Diarrheal diseases account for one in nine child deaths worldwide, making it the second leading cause of death among children under the age of five (Liu *et al.,* 2012). In Kenya, diarrhea has been reported to be the second cause of death among children below five years of age (KDHS 2008). EAEC is recognized as the leading bacterial pathogen in children hospitalized for acute diarrheal illness (Croxen *et al.,* 2013). In Kenya, EAEC has also been detected as the leading bacterial pathogen in children hospitalized with diarrhea (Oundo *et al.,* 2008; Sang *et al.,* 2012) reflecting a significant cost in health resources.

The gold standard diagnostic test for EAEC is the Human epithelial type 2 (HEp-2) cells adherence assay. In order to perform the test, laboratories require facilities for tissue, as well as bacterial culture. However, the requirement for tissue culture facilities and expertise does not permit many laboratories to use the gold standard test for identifying EAEC. Moreover, the current definition of EAEC based on adherence pattern includes pathogenic as well as non-pathogenic strains. EAEC are commonly isolated from asymptomatic individuals (Jiang *et al.,* 2003; Nataro *et al.,* 2006; Cennimo *et al.,* 2009). The genes encoding for numerous adhesins and toxins associated with virulence are also highly variable among strains (Estrada-Garcia and Navarro-Garcia, 2012), as such, there is no clear definition of EAEC-virulence genes associated with diarrheal illness.

A number of studies have recently sought in-depth characterization of EAEC as an etiologic agent of diarrhea (Okeke *et al*., 2000; Dow *et al.,* 2006; Zavari *et al.,* 2010; Boisen *et al.,* 2012; Lima *et al.,* 2013). Despite these studies, a globally applicable marker(s) which corresponds to illness has not been identified possibly reflecting geographical variations and heterogeneity of EAEC. In this case control study, the prevalence of EAEC in Kiambu County between 2011 and 2013 was determined between symptomatic and asymptomatic children under five years old. The putative virulence factors of the detected EAEC were also identified with an aim of enhancing the ability to distinguish the pathogenic and non-pathogenic EAEC.

Antimicrobials are rarely required in the treatment of diarrheal illness and should only be used in severe cases. However, the widespread misuse of antibiotics poses a serious problem of antimicrobial resistance and diminishes the efficacy of affordable and of locally available drugs. Resistance has been observed in more potent including last line antibiotics and a number of epidemics caused by multi-drug organisms have also been reported (Brooks *et al*., 2001; Brooks *et al*., 2006; Shapiro *et al.*, 2001). The sensitivity of the detected EAEC pathotype to the commonly used antibiotics was examined in this study.

1.2 Statement of the problem

Enteroaggregative *E. coli* has been detected as the leading bacterial pathogen in children hospitalized with diarrhea in Kenya (Oundo *et al.,* 2008; Sang *et al.,* 2012) reflecting a significant cost in health resources. However, asymptomatic carriage of EAEC is also

high in several studies (Nataro *et al.*, 2006; Cennimo *et al.*, 2009; Jiang *et al.*, 2003) making it difficult to distinguish the pathogenic and non-pathogenic strains of EAEC.

The lack of well-defined virulence markers for differentiation of virulent strains of Enteroaggregative *Escherichia coli* from avirulent strains further compounds the challenge of diagnosing EAEC gastroenteritis.

1.3 Justification of the study

It was anticipated that this study would help determine the prevalence of EAEC in Kiambu County Hospital. The study also identified the prevalence and distribution of virulence factors of EAEC in children with diarrheal disease and those without diarrheal disease. This was carried out with the aim of coming up with a marker which can differentiate the virulent strains of EAEC and the avirulent strains of thus aiding in diagnosis of pathogenic EAEC. Establishing a marker which corresponds with diarrheal illness is of significant value in routine detection of pathogenic EAEC (Okeke *et al.*, 1999).

1.4 Null Hypotheses

i) There is no association between EAEC and diarrhea in children from Kiambu County Hospital.
ii) There is no difference in distribution of the putative virulence factors between EAEC strains detected in children with diarrhea and in age-matched asymptomatic children.
iii) Enteroaggregative *E. coli* are not multidrug resistant.

1.5 Objectives

1.5.1 General objective

To determine the prevalence, antimicrobial sensitivity and putative virulence factors of EAEC in a case control study in Kiambu County Hospital.

1.5.2 Specific Objectives

i) To determine the association between EAEC and diarrhea in children from Kiambu County Hospital.
ii) To determine the difference(s) in the putative virulence factors between the EAEC strains detected in children with diarrhea and in age-matched asymptomatic children.
iii) To determine drug susceptibility pattern(s) of the detected Enteroaggregative *E. coli* pathotype.

1.6 Significance of the study

The results of this study underscore the potential usefulness of the *aggR* and *astA* genes as possible virulence markers for detection and differentiation of virulent strains of EAEC from avirulent strains. These markers can therefore be recruited in the national health program for routine detection of EAEC in order to enhance management of EAEC infections. In view of the current antimicrobial resistance pattern found in the study area, ceftriaxone, ciprofloxacin, ofloxacin and gentamicin may be used to manage severe cases of diarrheal disease caused by EAEC.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Many pathogens including bacteria, viruses, and parasites have the ability to cause diarrheal disease. Enteropathogenic *Escherichia coli* is one of the most important etiological agents of childhood diarrhea and represent a major public health problem in developing countries (Nataro *et al.,* 1998). Those strains causing intestinal infections can be divided into five major pathotypes: Enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC) and Enteroaggregative *E. coli* (EAEC). The pathotype to which a particular strain belongs is defined by the clinical manifestation of disease, the repertoire of virulence factors, epidemiology and phylogenetic profiles (Croxen *et al.,* 2013).

2.1.1 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* is recognized as a diarrheagenic pathotype of *E. coli* and is defined by its ability to produce either heat-stable enterotoxin (ST) or heat-labile enterotoxin (LT), or both ST and LT enterotoxins (Levine, 1987). A number of studies have been conducted which showed that ETEC is an important cause of diarrhea (Rodas *et al.,* 2011; Shah *et al.,* 2016; Svennerholm *et al.,* 2008). In a study carried out in Kenya from 2009 to 2014, ETEC was the second most isolated bacterial agent of diarrhea in children under the age of five years admitted with moderate to severe diarrhea (Shah *et al.,* 2016).

Enterotoxigenic *E. coli* is also recognized as the most common etiology of travelers' diarrhea responsible for ten to sixty percent of the total infections (Black, 1990).

2.1.2 Enteropathogenic *E. coli* (EPEC)

Enteropathogenic *E. coli* is defined by its production of a characteristic histopathology known as attaching and-effacing and its inability to produce Shiga toxins (Ochoa *et al.*, 2008). It was the first recognized diarrheagenic pathotype of *E. coli* and was first accepted as the etiology of diarrheagenic outbreaks in the developed world (Bray, 1945). The prevalence of EPEC among children with diarrheal disease in the developing world has been previously estimated to range from six to fifty four percent (Ochoa *et al.*, 2008). Shah *et al.*, detected EPEC in nearly ten percent of diarrheal patients in children under the age of five years in Central Kenya (Shah *et al.*, 2016).

2.1.3 Enterohemorrhagic *E. coli* (EHEC)

Enterohemorrhagic *E. coli* are Shiga toxin producing *E. coli.* Shiga toxin produced by EHEC are identical at the protein and genetic levels to the Shiga-toxin (Stx-toxin) produced by *Shigella dysentriae* (Croxen *et al.*, 2013). They are recognized as the primary cause of hemorrhagic colitis (HC) or bloody diarrhea, which can progress to the potentially fatal hemolytic uremic syndrome (HUS). There are many serotypes of Stx-producing *E. coli*, but only those that have been clinically associated with HC are designated as EHEC. Among the serotypes that are clinically significant, O157:H7 is the prototypic EHEC and most often implicated in illness worldwide.

2.1.4 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* are often biochemically atypical, unlike the other diarrheagenic pathotypes, and are difficult to differentiate from *Shigella* spp. The clinical features of EIEC infection are often characterized by fever, abdominal pain, malaise and watery diarrhea or dysentriae with blood (Hong *et al.,* 2007). This is often difficult to differentiate from a clinical illness caused by *Shigella* spp. Enteroinvasive *E. coli* are able invade the epithelium of the colon mediated by a plasmid and chromosomal loci (Cohen *et al.,* 2005).

2.1.5 Enteroaggregative *E. coli* (EAEC)

2.2 Epidemiology

Enteroaggregative *E. coli* has been identified as the etiology of endemic childhood diarrhea in developing countries (Ochoa *et al.,* 2011) and industrialized countries (Tompkins *et al*., 1999), diarrhea in adults including traveler´s diarrhea (Adachi *et al*., 2001; Schultsz *et al*., 2000), as well as persistent diarrhea in HIV-infected patients (Mathewson *et al*., 1998; Mwachari *et al*., 1998). A meta-analysis by Huang *et al*. (2006) also showed that EAEC is a cause of acute diarrheal disease globally. In Kenya, EAEC has been detected as the leading bacterial pathogen in children hospitalized with diarrhea (Oundo *et al.,* 2008; Sang *et al.,* 2012).

Diarrheal disease outbreaks caused by EAEC have been reported in several countries including: Kenya, France, Japan, Britain, Serbia and India (Cobeljić *et al*., 1996; Boudailliez *et al.,* 1997; Itoh *et al*., 1997; Pai *et al*., 1997; Smith *et al*., 1997; Czeczulin *et al*., 1999; Ochi *et al.,* 2016).

The mode of transmission of EAEC is also not well understood and EAEC has only rarely been isolated from a non-human source; from milk in infant feeding bottles (Morais *et al*., 1997) and from stool specimen obtained from cattle (Ochi *et al*., 2016). The most likely mode of transmission is however believed to be fecal-oral route and EAEC outbreaks have been epidemiologically linked to contaminated water and food (Itoh *et al*., 1997; Pai *et al*., 1997). Risk factors for EAEC infection include ingestion of contaminated food and water, poor hygiene, host susceptibility and possibly immunosuppression (Huang *et al.*, 2006).

2.3 Pathogenesis

Three major features of EAEC pathogenesis have been proposed:

1. Abundant adherence to the intestinal mucosa
2. Biofilm formation over enterocyte surface,
3. Production of enterotoxins and cytotoxins, intestinal secretion and damage as reviewed herein.

2.3.1 Adherence and biofilm formation

The defining feature of EAEC strains is their ability to produce the stacked-brick pattern (Nataro *et al*., 1987). The first fimbrial structures described in EAEC were the aggregative adhesion fimbriae (AAF). At least four variants of the AAF major structural subunit have been described so far: *AggA* (AAF/I), *AafA* (AAF/II), *Agg3A* (AAF/III), and *Agg4A* (AAF/IV), all regulated by the transcriptional activator *AggR*, situated on the EAEC virulence plasmid pAA (Nataro *et al*., 1992; Czeczulin *et al*., 1997; Bernier *et al*., 2002; Boisen *et al.*, 2008).

Another molecule involved in EAEC pathogenesis is dispersin, an anti-aggregative secreted protein encoded by the *aap* gene. In this study, the identified EAEC strains were investigated for the presence of these previously described AAF genes I to IV, *AggR* as well as the *aap* gene.

2.3.2 Production of enterotoxins and cytotoxins

Some of the toxins produced by EAEC are the: Enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) that activates chloride channels; plasmid-encoded toxin (*pet*), a cytoskeleton-altering protein; *Shigella* enterotoxin 1 (SHET1) that also induces intestinal secretion; as well as the protein involved in intestinal colonization (*pic*). Furthermore, the gene encoding for the *Shigella* extracellular protease (*sepA*), with IgA endopeptidase activity, originally described in *S. flexneri* by Henderson *et al.* (2004) has also been identified among EAEC strains and was strongly associated with diarrhea (Boisen *et al.,* 2012). Of particular interest is the EAST1; recently, *Escherichia coli* serogroup O-nontypable strain that had a coding gene for EAST1 was identified as the etiologic agent of diarrhea outbreak in Mandera County, Kenya (Ochi *et al.,* 2016). However, the role of EAST1 in sporadic cases of diarrhea in Kenya remains to be established.

2.3.2.1 EAEC heat-stable enterotoxin 1 (EAST1)

Enteroaggregative heat-stable enterotoxin 1 was originally found as an enterotoxin of EAEC (Savarino *et al.,* 1991). However, the role of EAST1 in human disease is still controversial. It has been reported that EAST1 is produced by approximately half of the EAEC (Savarino *et al.,* 1993; Savarino *et al.,* 1996). Consequently, it is not clear if

production of this toxin was relevant to the manifestation of diarrhea due to EAEC. It has also been reported that the EAST1 gene (*astA*), or its variants, were present not only in EAEC but in other diarrheagenic *E. coli* including some EPEC and ETEC (Savarino *et al.*, 1996; Hedberg *et al.*, 1997). In this study, the identified EAEC strains was investigated for the presence of EAST1 in-order to establish their role in sporadic cases of diarrhea in Kiambu County Hospital.

2.3.2.1 Other EAEC Toxins

Eslava *et al*. (1993) first reported an approximately 108 kDa toxin referred to as plasmid-encoded toxin (Pet) able to induce exfoliation of enterocytes in the rat ileal loop model to be associated with an outbreak of diarrhea in Mexico. Plasmid encoded toxin, *pet*, may play an important role as a virulence factor in EAEC in sporadic cases of diarrhea as well (Eslava *et al.*, 1998). Other EAEC candidate Virulence Related Genes (VRGs) include the regulator *eilA* (EAEC HilA homologue) and hypothetical *orf61* (plasmid-encoded hemolysin) (Sheikh *et al.*, 2006; Boisen *et al.*, 2012). The roles of *orf61, eilA* and *pet* in sporadic cases of diarrhea was investigated in this study.

2.4 Clinical symptoms and treatment of EAEC infection

The clinical manifestations of EAEC infections vary considerably among individuals due to complex host-pathogen interactions, including factors like EAEC strain heterogeneity, different amounts of ingested bacteria, host immune responses and host susceptibility (Okeke and Nataro, 2001; Jiang *et al.*, 2003).

Although not all EAEC infections result in symptomatic illness (Adachi *et al.,* 2001), most studies show that EAEC infection results in gastrointestinal disease. Clinical symptoms associated with EAEC gastroenteritis include watery diarrhea, nausea, low grade-fever and anorexia with both cases of acute and persistent diarrhea having been reported (Karch *et al.,* 1997; Jensen *et al.,* 2014). The incubation period of EAEC diarrheal illness ranges from 8 to 18 hours (Huang *et al.,* 2004). A previous study by Steiner *et al.* (1998) also reported an association of EAEC colonization with growth retardation. In many cases, EAEC diarrhea is inflammatory leading to bloody diarrhea in up to a third of patients (Cravioto *et al.,* 1991; Nataro *et al.,* 1996; Steiner *et al.,* 1998).

Antibiotics rarely indicated and should be prescribed on the basis of culture results and only in severe cases. Antimicrobial resistance in *E. coli* has been reported globally and increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. Indeed, *E. coli* is one of the pathogens included in the World Health Organization priority pathogen list resistant to antibiotic drugs. Occurrence and susceptibility profiles of *E. coli* show substantial geographic variations as well as significant differences in various populations and environment (Erb *et al.,* 2007).

In Kenya, a number of studies have been done on the prevalence and antimicrobial resistance patterns of *E. coli* from various geographical areas (Oundo *et al.,* 2008; Sang *et al.,* 2012). This study determined the antimicrobial sensitivity profiles of the detected EAEC isolates from both the patients and the asymptomatic controls in Kiambu County.

2.8 Diagnosis of EAEC infection

Enteroaggregative *E. coli* pathotype has a unique "stacked-brick" aggregative pattern of adherence and the HEp-2 cell adherence assay remains the gold standard for diagnosing EAEC infection (Nataro *et al.*, 2006). However, Limitations of the HEp-2 cell assay include limited availability and time requirements in most laboratories particularly in developing countries. This had led to the search for other diagnostic methods, including polymerase chain reaction (PCR) assays. Multiple genes have been investigated, including *aggR*, *aatA* (CVD432), *aggA*, *aafA*, and *astA* (Tsai *et al*., 2003; Nataro *et al.*, 2006). PCR detection of *aggR* is informative because it can identify typical EAEC, which is postulated to have a more pathogenic role than EAEC lacking *aggR* (Harrington *et al.*, 2005). Another diagnostic tool is a DNA probe to the pCVD432 (*aatA*) gene. Although this test is specific (99%), its sensitivity is variable (15% to 89%) (Cennimo *et al.*, 2009). Multiplex PCR assays have helped overcome the varied genetic composition of EAEC strains with improved sensitivity (Cennimo *et al.*, 2009). In this study, a set of multiplex PCR reactions were carried out to identify the various putative virulence genes in the EAEC isolates.

2.9 Antimicrobial resistance in *E. coli*

Antimicrobial resistance in pathogenic bacteria, including pathogenic *E. coli,* has been reported globally. Increasing rates of resistance among *E. coli* is a growing concern in both developed and developing countries. A rise in bacterial resistance to antibiotics narrows treatment options for infections caused by these bacteria.

In a previous report, up to 95% of cases with severe symptoms are treated without bacteriological investigation (Dromigny *et al.,* 2005). Moreover, antibiotics are among the most commonly prescribed drugs in hospitals (Shankar *et al.,* 2003). In Africa, about 90.1% individuals seek care outside the home, of these, 94.7% take medicines and 36.2% receive antibiotics. Of all those who receive antibiotics, 31.7% do not receive a prescription from a doctor and about 26.4% obtain antibiotics from an informal dispenser (Vialle-Valentin *et al.,* 2011).

In previous research carried out in rural Western Kenya, among patients empirically treated with an antimicrobial agent and whose stool specimens yielded isolates on which antimicrobial resistance testing was done, half were not susceptible to their antibiotic treatment (Brook *et al.,* 2001). Evidently, antibiotics are widely and inappropriately used in Africa resulting to an increase in antibiotic resistance among the pathogenic bacteria. High levels of antibiotic resistance in *E. coli* isolates from Kenya have been observed for ampicillin and trimethoprim/sulfamethoxazole at 95% resistance and 81% resistance to tetracycline (Sang *et al.,* 2012).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Location

The stool samples which were analyzed in this study were obtained from Kiambu County, Kenya. Kiambu County Hospital (KCH) has 316-bed general wards and 67 cots and serves populations from Kiambu Sub-county and its environs that include Nairobi. The county is adjacent to the northern border of Nairobi County and has a population of 1,623,282 according to the 2009 Kenya Population and Housing Census (KNBS, 2010).

3.2 Study Design

The stool samples were obtained in the course of a prospective case-control study of moderate to severe diarrhea among children less than five years old by the Nagasaki University Institute of Tropical Medicine in collaboration with the Kenya Medical Research Institute (NUITM-KEMRI) between 2012 and 2016. This prospective case-control study was aimed at identifying and characterizing rotavirus and all bacterial causes of diarrhea in children under five years old in selected hospitals across the country, Kenya. Children less than five years old admitted with severe diarrhea at KCH were considered eligible. Patients were enrolled upon the parent's consent which was documented (Appendix III). Diarrhea was defined as passage of three or more watery loose stools in the previous 24 h, as noted by the mother or the caretaker.

3.2.1 Inclusion criteria

Children under five years of age who were admitted with diarrheal disease at KCH and had not taken any antimicrobial agents in the week preceding admission were enrolled in the study as patients.

An age-matched asymptomatic control from the same neighborhood as the children with diarrheal illness were also enrolled for each case provided that they had not had an episode of diarrheal illness or taken any antimicrobial agent in the week preceding sampling.

3.2.2 Exclusion criteria

Children admitted with diarrhea who had already taken an antimicrobial agent in the week preceding sampling or those who were over five years old were excluded from the study. An age-matched asymptomatic control was excluded from the study if he had recorded an episode of diarrheal illness or had taken an antimicrobial agent in the week preceding sampling.

3.3 Sample size calculation

The sample size was calculated based on the prevalence of EAEC, 27.7%, in asymptomatic control subjects (Okeke *et al*., 2000), using the formulae provided by Kelsey *et al.* (1996):

$$n = \left(\frac{r+1}{r}\right)\frac{(\bar{p})(1-\bar{p})(Z_{\beta} + Z_{\alpha/2})^2}{(p_1 - p_2)^2}$$

Where (r) is the ratio of asymptomatic controls to patients, ($\bar{P}$) is a measure of variability; to detect an odds ratio of 2.0 or greater, ($Z\beta$) is the desired power (typically

0.84 for 80% power), ($p1 - p2$) is the effect Size (the difference in proportions) and ($Z\alpha$) the desired level of statistical significance (typically 1.96) so that the necessary sample size was estimated to be at least 294 (147 sample size per group).

3.4 Ethical Considerations

The study was approved by the KEMRI-Ethical Review Committee under KEMRI/SSC/1323. Patient names were not used; instead, unique identification codes were used in order to ensure confidentiality. Written consent sought from parents/guardians of the participants prior to sample collection was ascertained in consent forms shown in appendix III. Information obtained from the patients was strictly confined to academic use only.

3.5 Sample collection and Transportation

The stool samples were collected by swabs, placed in Amies transport medium (Oxoid, Basingstoke Hampshire, UK) and transported to the NUITM-KEMRI laboratories in an ice-box container kept at 4 ºC within 24 h of collection. Each specimen collected was attached with a detailed pathological form containing the patient and sample pathological data (Appendix II).

The conditions of the samples to be transported were checked and were not accepted if; the specimen was leaking; the tube was broken and if the samples had no pathological form attached. Counter checking of the name and code on the sample with the one on the pathological form was done in the laboratory upon arrival.

All the samples were received within a biosafety cabinet and the surfaces of the sample containers were disinfected with 70% ethanol. The samples together with the request forms were assigned a code after their acceptance: KDH/sample number for samples collected from symptomatic children and KDH/CF/sample number for those collected from asymptomatic children.

3.6 Identification of *E. coli*

Preliminary identification of *E. coli* by method of stool culture was carried out on two basic types of media; Deoxycholate Hydrogen Sulphide Lactose (DHL) and Bromothymol Blue (BTB) (Eiken Chemical Company Ltd. Japan). The plates were incubated at 37 ˚C overnight. The plate colonies were read after the periods of incubation; 12-24 h of incubation. The following parameters were recorded: size, color, morphology and fermentation. DHL media contains sodium deoxycholate, sodium thiosulphate ammonium iron citrate and sodium citrate which inhibit growth of gram positive bacteria.

Following primary plating, sub culturing was done using the following identification media; Triple sugar iron (TSI), Lysine Indole Motility (LIM), Simmons Citrate (SC), Methyl Red/Vogues- Proskauer (MR/VP) and Urea (Eiken Chemical Company Ltd. Japan). Using a straight wire loop a single colony was picked from the previously incubated plates. The colony was then used to inoculate the biochemical media in the following order: the butt of the TSI agar was stabbed once and the slant was streaked, the LIM media was stabbed once, the SC slant was streaked, and the loop was then immersed several times in MR/VP and a stab was finally made in urea agar.

The tubes were then incubated overnight at 37 ˚C. Colonies were identified based on their biochemical reactions. *E. coli* from the study were stored in Nutrient agar (NA) and 15% glycerol Tryptic Soy Broth (TSB). The 15% glycerol media was stored in -80 ˚C while NA was stored at room temperature.

3.7 Antimicrobial sensitivity testing

E-test was carried out to determine whether or not the identified EAEC are susceptible to the action of the specific antibiotics. The susceptibility panel was chosen on the basis of treatment of infections due to Gram negative bacteria (CLSI, 2014). The strips were applied on freshly prepared and quality-controlled Mueller Hinton agar in which 0.5 McFarland EAEC were uniformly spread. The plates were incubated at 37 ˚C for 18-24 h. The results were read the following day and interpreted according to the manufacturer's instructions (bioMérieux, Marcy-l'Étoile, France) to provide the minimum inhibitory concentrations of the drugs and the results recorded (Appendix I).

3.8 Extraction of E. coli DNA

At least five different colonies from each sample identified as *E. coli* were examined by PCR for the presence of genes encoding different putative virulence factors. DNA was extracted from the identified *E. coli* by the method described by Yokoyama (1993). DNA was extracted from the *E. coli* colonies that had been grown overnight in DHL media. *E. coli* cells were emulsified in 250 µl lysis buffer (0.1 M NaCl, 50 mM disodium EDTA, 0.1 M Tris-HCl, pH 8) containing 0.5% (w/v) sodium dodecyl sulfate 0.5% (w/v), proteinase K (0.5 mg/ml), and RNase (0.8 mg/ml). After emulsification, the tube was placed in a water bath at 60 ℃ for 5 min.

Two hundred microliters of the supernatant were placed in a new eppendorf tube and overlayed with 30 µl of 6 M NaCl and 2 volumes of room temperature 70% ethanol. The tube was thereafter kept at room temperature for five minutes before centrifugation at 1000 X g for 3 min.

The supernatant was discarded, and the crude DNA rinsed with 70% ethanol and dried out by leaving the eppendorf tubes open for a few minutes in the clean bench. Finally, the DNA was suspended in 200 µl of sterile water. The DNA was characterized by evaluating its concentration (an OD of 1 at 260 nm = 50 µg/ml of DNA) and its purity (OD at 260 nm/OD at 280 nm = 1.7-2.0).

Two hundred microlitre (200µl) tubes containing PuReTaq ready-to-go PCR beads (GE Healthcare UK limited, UK) was used to set the polymerase chain reaction. Each reaction contained 2.5 units of PuReTaq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 200 µM dATP, dCTP, dGTP and dTTP, stabilizers, BSA, 1µl each of forward and reverse primers (6 pmol/µl), 2µl of template DNA, and Milli-Q water, added to a final volume of 25µl. The PCR amplifications of the target regions was carried out in a thermal cycler (Bio-Rad, USA).

3.9 Multiplex PCR for detection of EAEC and the EAEC VRGs

A single multiplex reaction was set for the detection of EAEC. Primers and conditions for detecting sequences encoding genes for detection of EAEC are described in Table 3.1. The presence of *aap* and/or *aggR* and/or *aatA* indicated the presence of EAEC.

Table 3.1: Oligonucleotide primers employed for the detection of EAEC pathotype

Primers, Genes and Sequence (5'- 3')	**Amplicon size (b.p)**	**PCR Conditions**	**Reference**
*aggR, aggR*ks1:	254	95°C, 1 min	Ratchtrachenc
GTATACACAAAAGAAGGAAGC		54°C, 1 min	hai *et al*.,
*aggR*ks2: ACAGAATCGTCAGCATCAGC		72°C, 1 min	1997
aatA, (*Pcvd432*)	194	95°C, 1 min	Pass *et al*.,
Eaggfp: AGACTCTGGCGAAAGACTGTATC		54°C, 1 min	2000
Eaggbp: ATGGCTGTCTGTAATAGATGAGAAC		72°C, 1 min	
aap,	282	95°C, 1 min	Toma *et al.*,
aspU-3: GCCTTTGCGGGTGGTAGCGG		54°C, 1 min	2003
aspU-2: AACCCATTCGGTTAGAGCAC		72°C, 1 min	

Primers and conditions for detecting sequences encoding various putative virulence genes are described in Table 3.2. Two multiplex reactions were set for detection of putative toxins; *pet*, *orf61*, *sepA*, *eilA*, *astA* and adhesins*; aggA*, *aafA*, *agg3A*, *agg3/4C*, *aafC*, *agg4A*, respectively. The PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide (2 μg/ml in 1% TBE buffer), visualized under UV light and recorded with the aid of a gel documentation system.

Table 3.2: Primers for amplification of the virulence related genes in EAEC

Gene Target: Primer Sequence (5'- 3')	Description of Target	Amplicon size (b.p)	Reference
Multiplex 1 (Toxins)			
Pet: GGCACAGAATAAAGGGGTGTTT CCTCTTGTTTCCACGACATAC	Plasmid-encoded toxin	302	Restieri *et al.*, 2007
orf61: AGCTCTGGAAACTGGCCTCT AACCGTCCTGATTTCTGCTT	Plasmid-encoded hemolysin	108	Boisen *et al.*, 2012
sepA: GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	Shigella extracellular protease	794	Restieri *et al.*, 2007
eilA: AGGTCTGGAGCGCGAGTGTT GTAAAACGGTATCCACGACC	Salmonella HilA homolog	248	Boisen *et al.*, 2012
EAST1, *astA* gene CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	*E. coli* heat-stable enterotoxin 1	111	Yamamoto *et al.*, 1996
Multiplex II (Adhesins)			
aggA: TCTATCTRGGGGGGCTAACGCT ACCTGTTCCCCATAACCAGACC	AAF/I fimbrial subunit	220	Boisen *et al.*, 2012
aafA: CTACTTTATTATCAAGTGGAGCCGCT A GGAGAGGCCAGAGTGAATCCTG	AAF/II fimbrial subunit	289	Boisen *et al.*, 2012
aafC: ACAGCCTGCGGTCAAAAGC GCTTACGGGTACGAGTTTTACGG	Usher, AAF/II assembly unit	491	Boisen *et al.*, 2012
agg3A: CCAGTTATTACAGGGTAACAAGGGA A TTGGTCTGGAATAACAACTTGAACG	AAF/III fimbrial subunit	370	Boisen *et al.*, 2012
	AAF/IV fimbrial subunit	169	Boisen *et al.*, 2012

agg4A: TGAGTTGTGGGGCTAYCTGGA CACCATAAGCCGCCAAATAAGC TTCTCAGTTAACTGGACACGCAAT TTAATTGGTTACGCAATCGCAAT TCTGACCAAATGTTATATCCTTCAYT ATG	409	Boisen *et al.*, 2012

3.8.3 Data management and analysis

Results were reported in form of Figures and Tables. Data was recorded in the laboratory notebook, entered in the Microsoft Excel Spreadsheet and exported to STATA Version 12.0 (Texas, USA) for analysis. The difference in prevalence of EAEC, virulence related genes and antimicrobial resistance between the symptomatic and asymptomatic group was analyzed by Chi-square test or Fisher's exact test to determine significance of the data. A *P* value less than 0.05 was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 Demographic characteristics of the study population

A total of 354 samples from patients and 354 from asymptomatic controls were analyzed for the identification of EAEC. The mean ages of the patients and asymptomatic controls were 12.5 ± 10.3 (standard deviation) and 11.2 ± 6.5 (standard deviation), respectively, with no statistical difference between the two groups (*P*= 0.3474). Of the 354 patients, 141/354 (39.8%) were females while 213/354 (60.2%) were males. Amongst the asymptomatic controls, 148/354 (41.8%) were females while males were 206/354 (58.2%).

4.2 Confirmatory identification of *E. coli*

E. coli were identified as lactose fermenters, producing pink and yellow colonies on DHL and BTB agar respectively. In DHL media, *E. coli* produced colonies which were medium in size, flat and pink (Figure 4.2). In BTB media, *E. coli* produced colonies which were yellow, medium in size and flat. *E. coli* were identified as TSI positive, both the slant and butt of the TSI agar turned yellow indicating glucose, lactose and sucrose fermentation and production of pyruvic, formic, and lactic acid. There was gas production as well indicated by the appearance of bubbles and/or cracks in the TSI media. There was however no hydrogen sulphide gas production. A negative reaction was observed in SC media evidenced by no growth with no change in color of the SC media (media remained dark green after incubation).

A red broth observed in MR media indicated positive reactions when methyl red was added to the media. *E. coli* bacteria were motile in LIM agar. A red ring appearing at the interface between the top of the LIM agar and the ρ-dimethylaminobenzaldehyde showed that the *E. coli* bacteria were indole positive. A yellow slant and butt of TSI agar with gas bubbles, a dark green SC media indicating a negative reaction, a red broth in MR, positive motility and a red ring at the brim of the LIM media were observed in *E. coli* (Figure 4.3).

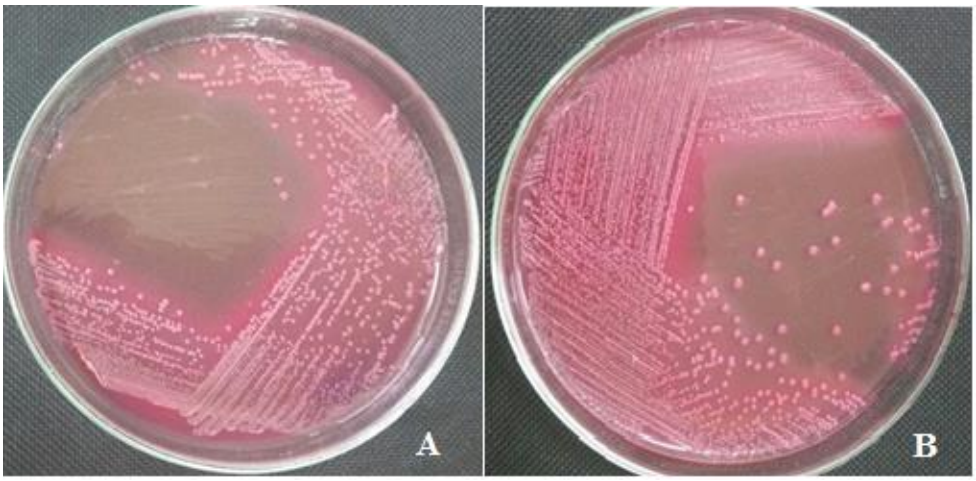

Figure 4.1: Morphological growth of *E. coli* on DHL media.

A: Medium sized, flat and pink (lactose fermenters) colonies of *E. coli* ATCC 25922 on DHL media. B: Medium sized, flat and pink (lactose fermenters) colonies of *E. coli* KDH/CF/552 isolated from an asymptomatic child, on DHL media.

Figure 4.2: Biochemical characteristics of *E. coli* identified by culture.

A yellow slant and butt of TSI agar with gas bubbles, a dark green SC media indicating a negative reaction, a red broth in MR, positive motility and a red ring in LIM media. The urea agar remained pale yellow.

4.3 Prevalence of EAEC and different virulence-related genes

A multiplex reaction consisting of three different diagnostic gene targets; *aap*, *aggR* and *aatA*, was used for the identification of EAEC (Figure 4.4). EAEC was detected in 60/354 (16.95%) in patients as well as in asymptomatic controls. Three different patterns of diagnostic genes were observed in patients: (i) only positive for *aspU*, (ii) positive for both *aap* and *aggR* and (iii) positive for *aap*, *aggR* and *aatA*. The most common pattern observed was *aap* 27/60 (45.0%) followed by 19/60 (31.7%) for *aap*/*aggR* and 14/60 (23.3%) for *aap/aggR/aatA*. Five different patterns of diagnostic genes were observed in asymptomatic controls: (i) only positive for *aap*, (ii) positive for both *aap* and *aggR* and

(iii) positive for *aap*, *aggR* and *aatA*, (iv) positive for *aap*/*aatA* and (v) only positive for *aatA*.

The most common pattern observed in asymptomatic controls was *aap* 34/60 (56.7%) followed by 20/60 (33.3%) for *aap/aggR* and 4/60 (6.7%) for *aap/aggR/aatA*. *aap/aatA* was 1.7% while *aatA* only was 5%.

The detected EAEC strains, 60 from patients and 60 from asymptomatic controls, were further evaluated for detection of 11 different virulence-related genes in two different multiplex-PCR reactions (Figure 4.5 and Figure 4.6). *AggR*, *aafA* and *astA* genes were detected more frequently in patients than in asymptomatic controls. *AggR* and *astA* genes were detected significantly more in patients than in asymptomatic controls (P=0.0271 and 0.0443, respectively). Usher, AAF III/IV assembly unit, *agg3/4C*, was not detected in any EAEC strain from patients while 55/60 (93.2%) of the EAEC strains from asymptomatic controls were found positive for the gene.

The most frequent AAF pilin gene was that of AAF/I encoded by *aggA* (10% and 20% in patients and asymptomatic controls respectively) followed by those of AAF/IV (*agg4A*), AAF/II (*aafA*) and AAF/III (*agg3A*) respectively. Usher, AAF/II assembly unit (*aafC*) was not detected in EAEC from patients while it was detected in 10% of the EAEC from asymptomatic controls. *Pet*, *orf61*, *sepA* and *eilA* were not detected in any of the EAEC from patients while their prevalence in EAEC detected from asymptomatic children was 3.3%, 59.3%, 17% and 86%, respectively (Table 4.1).

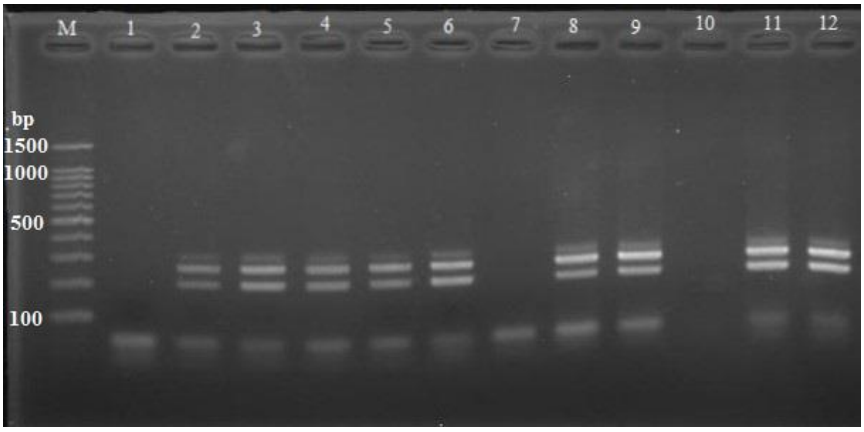

Figure 4.3: A multiplex reaction consisting of three different diagnostic genes.

DNA bands corresponding to the *aap*, *aggR* and *aatA* genes as detected on 2% agarose gels with expected band sizes of 282, 254 and 194, respectively. Lane M: 100 b.p DNA Marker (Gel pilot 100 b.p plus ladder, Hilden, Germany), Lane 1: Negative control (EHEC, KDH/1323). Lanes 2, 3, 4, 5, 6, 8, 9, 11 and 12 shows amplified *aap*, *aggR* and *aatA* genes. No detected genes in lanes 7 and 10.

Table 4.1: Prevalence of the EAEC virulence related genes.

A significantly higher prevalence of *aggR* and *astA* genes in symptomatic children than in asymptomatic children.

Gene Target	**Patients**	**Asymptomatic controls**	***P* value**
Aap	60 (100.0)	57 (95.0)	-
aggR	32 (53.3)	20 (33.3)	0.0271[a]
aatA	14 (23.3)	9 (15.3)	0.2462
Pet	0 (0)	2 (3.3)	-
orf61	0 (0)	35 (59.3)	-
sepA	0 (0)	9 (17.0)	-
EilA	0 (0)	49 (86.0)	-
astA	34 (56.7)	23 (38.3)	0.0443*
aggA	6 (10.0)	12 (20)	0.1250
AafA	2 (3.3.)	0 (0)	-
AafC	0 (0)	6 (10)	-
agg3A	0 (0)	2 (3.3)	-
agg4A	4 (6.7)	7 (11.7)	0.3397
agg3/4C	0 (0)	55 (93.2)	-

[a] Pearson's chi-squared test (Odds ratio =0.4374 and X^2= 4.89), * Pearson's chi-squared test (Odds ratio =2.104 and X^2= 4.04)

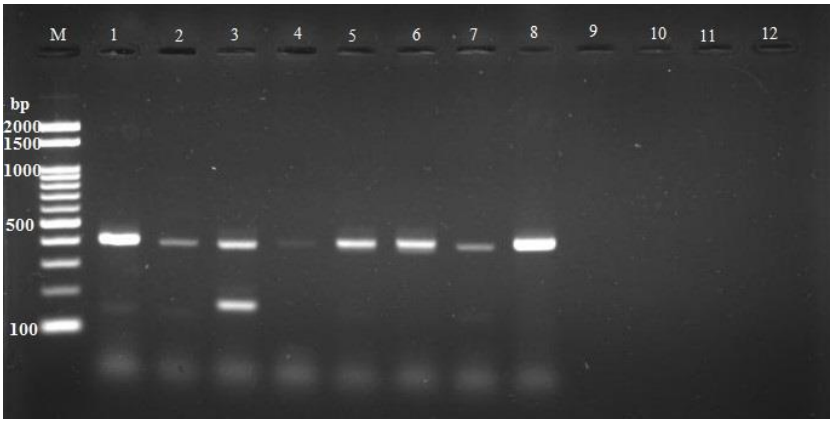

Figure 4.4: Multiplex reaction for the detection of virulence-related genes.

The *agg3/4C* and *agg4A* genes as detected on 2% agarose gels with expected band sizes of 409 and 169, respectively. Lane M: 100 b.p DNA Marker (Axygen Biosciences, New York, USA). Lanes 1, 2, 4, 5, 6, 7 and 8 show amplified *agg3/4C* gene (base size of 409). Lane 3 shows amplification for both the *agg3/4C* and *agg4A* (band sizes of 409 and 169, respectively). Lane 9: Negative control (EHEC KDH/1323). No detected genes in lanes 10, 11 and 12.

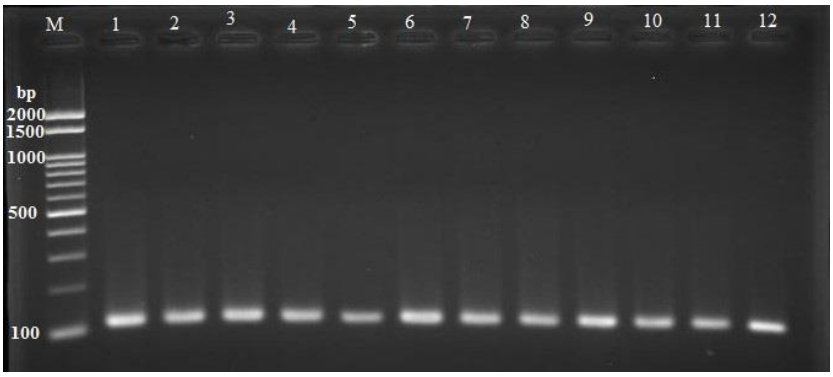

Figure 4.5: DNA bands corresponding to the EAST 1 toxin producing gene.

The *astA* gene as detected on 2% agarose gels with an expected band size of 111 b.p. Lane M: 100 b.p DNA Marker (Axygen Biosciences, New York, USA), Lanes 1-12: Amplified *astA* gene.

4.4 Characteristics of typical and atypical EAEC

To further characterize the identified EAEC strains, EAEC was categorized into typical (*aggR*-positive) and atypical (*aggR*-negative) and analyzed for the presence of different virulence associated genes. *Pet, orf61* and *agg3A* were detected more frequently in typical EAEC than in atypical EAEC (Table 4.2). In typical EAEC *astA, aatA, agg4A* and *aggA* were detected more in patients than in asymptomatic controls. However, no statistical difference was observed in their distribution between patients and asymptomatic controls on typical EAEC. In atypical EAEC, *astA* was detected significantly more in patients than in asymptomatic controls (P=0.0059, OR =4.17).

Table 4.2: Distribution of VRGs among typical and atypical EAEC isolates.

A significantly higher detection of *astA* in atypical EAEC from symptomatic children than asymptomatic children.

Virulence factors	Typical EAEC			Atypical EAEC		
	Patients	**Controls**	***P* value**	**Patients**	**Controls**	***P* value**
Aap	32/32 (100)	20/20 (100)	-	0/28 (0)	37/40 (92.5)	-
aatA	14/32 (43.8)	5/20 (25.0)	0.1719	0/28 (0)	4/39 (10.3)	-
Pet	0/32 (0)	2/20 (10)	-	0/28 (0)	0/40 (0)	-
orf61	0/32 (0)	15/20 (75.0)	-	0/28 (0)	20/40 (50.0)	-
sepA	0/32 (0)	1/19 (5.0)	-	0/28 (0)	8/40 (20.0)	-
eilA	0/32 (0)	12/18 (66.7)	-	0/28 (0)	37/39 (94.9)	-
astA	14/32 (43.75)	8/20 (40.0)	0.8000	20/28 (71.4)	15/40 (37.5)	0.0059[a]
aggA	4/32 (12.5)	3/20 (15)	0.5535*	2/28 (7.1)	9/40 (22.5)	0.0905
aafA	2/32 (6.3)	0/20 (0.0)	-	0/28 (0)	0/40 (0)	-
aafC	0/32 (0)	2/20 (10.0)	-	0/28 (0)	4/40 (10.0)	-
agg3A	0/32 (0)	2/20 (10.0)	-	0/28 (0)	0/40 (0)	-
agg4A	2/32 (6.3)	1/20 (5.0)	0.6733*	2/28 (7.1)	6/40 (15.0)	0.3026*
agg3/4C	0/32 (0)	16/19 (84.2)	-	0/28 (0)	39/40 (97.5)	-

*1-sided Fischer's exact, [a] Pearson's chi-squared test (Odds ratio =4.17 and X^2=7.59)

4.5 Combinations of virulence-related genes

Twelve different combinations of the virulence markers were found among the studied EAEC isolates from symptomatic children while thirty-three different combinations of the virulence markers were found among the studied EAEC isolates from asymptomatic children (Table 4.3 and Table 4.4). The most prevalent gene combinations in patients were; *aap/astA* 18/60 (30%) and *aap/aggR* 7/60 (11.67%). Four different virulence-related genes were detected in each isolate in ten different patients. The most prevalent gene combinations in asymptomatic controls was *agg4A/agg3/4C/sepA/eilA/aap* 6/60 (10.0%). Seven different virulence-related genes were detected together in each isolate in four different asymptomatic controls. More virulence-related gene combinations were observed in asymptomatic controls than in patients (33 versus 12, respectively).

Table 4.3: Distribution of EAEC VRGs from symptomatic children.

Twelve different combinations of the virulence markers among the sixty EAEC isolates from symptomatic children.

Genes	**Frequency**	**Percentage**
aap/aggR/aatA/astA	6	10.00
aggA/aap/aggR/astA	2	3.33
agg4A/aap/aggR/aatA	2	3.33
aap/aggR/astA	6	10.00
aap/aggR/aatA	6	10.00
aggA/aap/aggR	2	3.33
agg4A/aap/astA	2	3.33
aafA/aap/aggR	2	3.33
aap/astA	18	30.00
aap/aggR	7	11.67
aggA/aap	2	3.33
Aap	5	8.33

Table 4.4: Distribution of EAEC VRGs from asymptomatic children.

Thirty-three different combinations of the virulence markers among the sixty EAEC isolates from symptomatic children.

Genes	**Frequency**	**Percentage**
agg3A/agg3/4C/pet/eilA/orf61/aap/aggR	2	3.33
agg3/4C/eilA/orf61/aap/aggR/aatA/astA	1	1.67
aggA/eilA/orf61/aap/aggR/aatA/astA	1	1.67
agg3/4C/eilA/orf61/aap/aggR/astA	2	3.33
agg3/4C/eilA/orf61/aap/aggR/aatA	1	1.67
agg3/4C/agg4A/sepA/eilA/aap	1	1.67
agg4A/agg3/4C/sepA/eilA/aap	6	10.00
agg3/4C/eilA/orf61/aap/aggR	4	6.67
agg3/4C/sepA/eilA/orf61/aap	2	3.33
agg3/4C/eilA/orf61//aap/astA	1	1.67
agg3/4C/eilA/orf61/aap/astA	3	5.00
aafC/agg3/4C/eilA/aap/astA	2	3.33
aggA/eilaA/orf61/aap/aggR	1	1.67
aggA/eilA/orf61/aap/astA	5	8.33
agg3/4C/orf61/aap/aggR	1	1.67
agg3/4C/sepA/aap/aggR	1	1.67
agg3/4C/eilA/orf61/aap	4	6.67
agg3/4C/eilA/aap/aggR	1	1.67
agg3/4C/eilA/aap/astA	2	3.33
aggA/orf61/aap/aggR	2	3.33
agg3/4C/eilA/orf61/aatA	1	1.67
aggA/eilA/orf61/aap	1	1.67
aafC/eilaA/orf61/aap	1	1.67
aafC/orf61/aap/aatA	1	1.67
aafC/eilA/aap/astA	1	1.67
agg3/4C/aap/astA	1	1.67
agg3/4C/eilA/aap	3	5.00
agg4A/aap/aggR	1	1.67
orf61/aap/aggR	1	1.67
agg3/4C/eilA/aatA	2	3.33
aap/aggR/astA	2	3.33
aggA/eilA/aap	1	1.67
Aap	1	1.67

4.6 Antimicrobial susceptibility patterns

E-test strips were used to determine the susceptibility of the detected EAEC (Figure 4.7). Low resistance rate was observed in quinolones: ofloxacin (1.7% versus 0% in patients and controls, respectively) and ciprofloxacin (3.3% versus 0% in patients and controls, respectively) followed by cephalosporin: ceftriaxone (18.3% versus 0% in patients and controls, respectively). Amongst the aminoglycosides, low rate of resistance was observed in kanamycin (1.7% versus 0% in patients and controls, respectively) followed by gentamycin (16.7% versus 0% in patients and controls, respectively). A high resistance rate in patients was however observed in streptomycin (76.7%) while none of the EAEC isolates detected in controls was resistant to the antibiotic (Table 4.5 and Figure 4.8).

High resistance rate was observed in penicillin group: amoxicillin (90.0% versus 100% in patients and controls, respectively), sulfonamides group: trimethoprim/sulfamethoxazole (85.0% versus 100% in patients and controls, respectively) while in tetracycline resistance was significantly higher in patients than controls, 58.3% versus16.7%, respectively (P=0.0443). Among the penicillin, sulfonamide and tetracycline groups, most of the bacterial EAEC pathogens showed multi-drug resistant patterns. Erythromycin showed the highest rate of intermediate susceptibility in patients, 83.3%, while all the controls tested were resistant (Figure 4.9). Ampicillin, erythromycin, streptomycin and SXT showed higher levels of resistance in the control group than in patients. DXT also showed a higher resistance in controls than in patients (Figure 4.10). Chloramphenicol showed a significantly higher resistance in patients than in controls (P=0.0002).

Table 4.5: Antimicrobial susceptibility patterns of the isolated EAEC.

Antibiotic class	**Antibiotic tested**	**Patients**						**Controls**						***P* value**
		Sensitive		Intermediate		Resistant		Sensitive		Intermediate		Resistant		
		N	%	N	%	N	%	N	%	N	%	N	%	
Penicillins	Ampicillin	6	10	0	0	54	90	0	0	0	0	60	100	-
Cephalosporin	Ceftriaxone	49	81.7	0	0	11	18.3	60	100	0	0	0	0	-
Macrolides	Erythromycin	7	11.7	50	83.3	3	5.0	0	0	0	0	60	100	-
Tetracyclines	Tetracycline	25	41.7	9	15.0	26	58.3	11	18.3	39	65	10	16.7	0.0443
	DXT	32	53.3	22	36.7	6	10.0	20	33.3	11	18.3	29	48.4	0.1906
Quinolone	Ciprofloxacin	58	96.7	0	0	2	3.3	60	100	0	0	0	0	-
	Ofloxacin	59	98.3	0	0	1	1.7	60	100	0	0	0	0	-
Chloramphenicol	Chloramphenicol	43	71.7	0	0	17	28.3	42	70.0	16	26.7	2	3.3	0.0002
Aminoglycosides	Gentamicin	50	83.3	0	0	10	16.7	60	100	0	0	0	0	-
	Kanamycin	55	91.7	4	6.7	1	1.7	60	100	0	0	0	0	-
	Streptomicin	14	23.3	0	0	46	76.7	9	15.0	0	0	51	85	0.7057
Sulfonamides	SXT	9	15	0	0	51	85.0	0	0	0	0	60	100	-

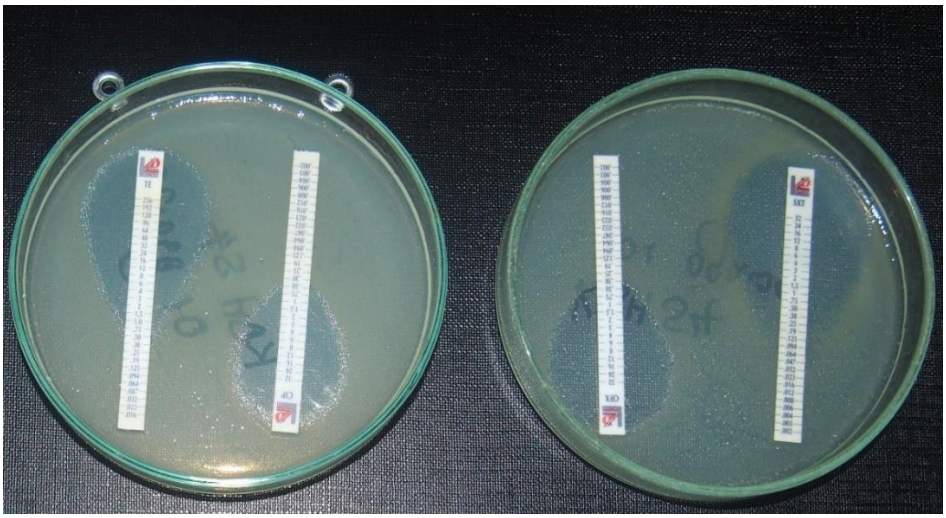

Figure 4.6: Antimicrobial susceptibility test performed by the E-test method.

E-test results indicating susceptibility to tetracycline, ofloxacin, ciprofloxacin and SXT antibiotics

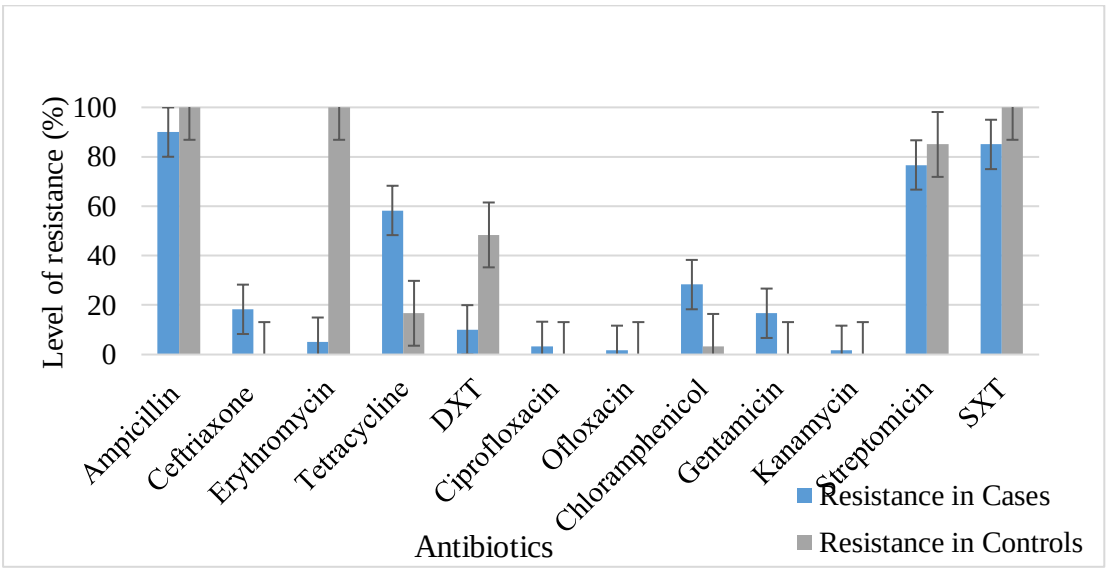

Figure 4.7: Antimicrobial Susceptibility patterns of EAEC.

Levels of resistance and standard error in EAEC detected from symptomatic and asymptomatic children showing low resistance in quinolones and high resistance in penicillin and SXT antibiotics.

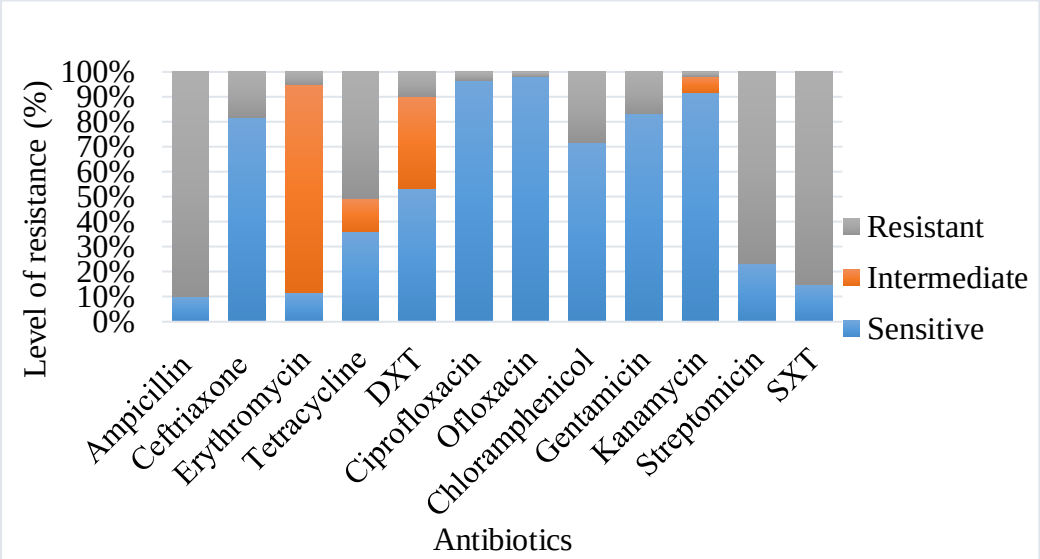

Figure 4.8: Antimicrobial resistance pattern of EAEC from symptomatic children. High resistance in ampicillin and SXT, high susceptibility among the quinolones and a high rate of intermediate susceptibility in erythromycin.

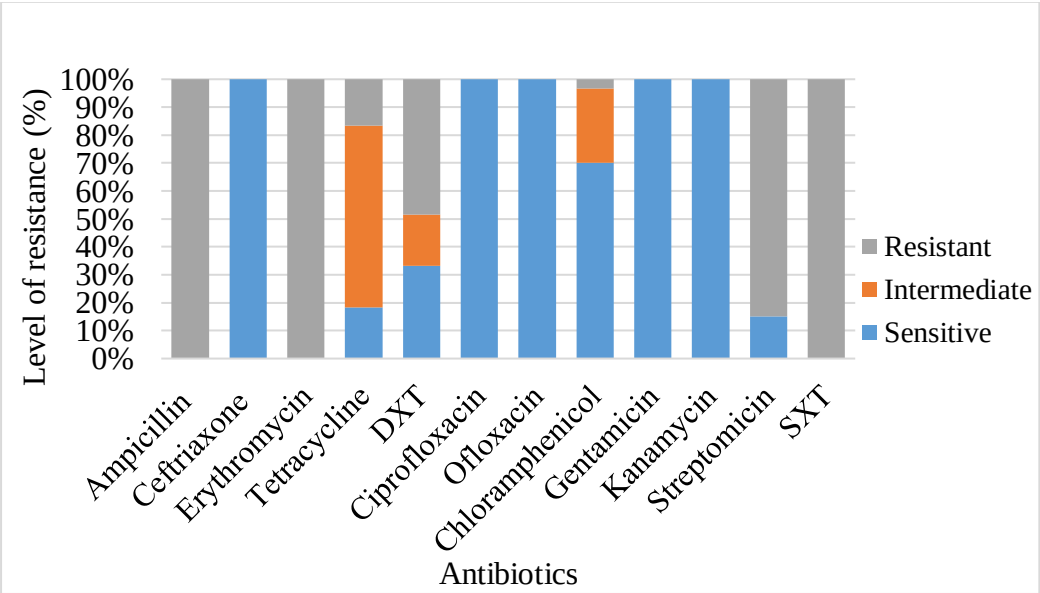

Figure 4.9: Antimicrobial resistance pattern of EAEC from asymptomatic children. High resistance in ampicillin, SXT and erythromycin, high susceptibility among the quinolones and aminoglycosides and a high rate of intermediate susceptibility in tetracycline.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Enteroaggregative *E. coli* (EAEC) is recognized as an important enteric pathogen group. In the past decade, a number of studies have sought in-depth characterization of EAEC as an etiologic agent of diarrhea (Regua-Mangia *et al.,* 2004; Dow *et al.,* 2006; Zavari *et al.,* 2010; Boisen *et al.,* 2012; Lima *et al.,* 2013). Despite these studies, a globally applicable marker(s) which corresponds to illness has not been identified possibly reflecting geographical variations and heterogeneity of EAEC. In this study, the prevalence of EAEC and the role of different virulence-related genes was investigated in a case-control study with an aim of enhancing the ability to distinguish the pathogenic and non-pathogenic EAEC.

EAEC was detected in 60/354 (16.95%) of diarrheal patients from Kiambu County Hospital and 60/363 (16.53%) asymptomatic controls with no significant difference between the two groups (P= 0.8987). This new rate is higher than previously described by the GEMS study (Kotloff *et al*., 2012). It is however similar to studies previously carried out in other regions: In Rio de Janeiro, Brazil, EAEC was the most frequent diarrheagenic *E. coli*, accounting for 14.6% of isolates in children with diarrhea and 11.1% in children without diarrhea and in Nigeria, where EAEC was found in 10.3% of the persons in a study carried out in small-town and rural primary health care centers (Okeke *et al.,* 2000; Regua-Mangia *et al.,* 2004). In Hanoi, Vietnam, 11.5% of children less than five years of age were infected with EAEC (Nguyen *et al.,* 2005). In South Africa 16.5% of children less than five years of age were infected with EAEC (Samie *et al.,* 2007).

In Switzerland EAEC isolates were found in specimens of 19 (10.2%) of 187 children with diarrhea and in 3(2.2%) of 137 children without diarrhea (*P* 0.006) and were the most frequently detected bacteria associated with diarrhea (Pabst *et al.,* 2003). This study confirms the high prevalence of EAEC in this region. However, asymptomatic carriage of EAEC was also high in the present study.

There was no difference in the prevalence of EAEC between the symptomatic and asymptomatic groups. Similar findings have been reported in other studies (Bueris *et al.*, 2007; Lima *et al.*, 2013). In a study carried in Ghana by Opintan *et al.* (2010), EPEC, ETEC and EAEC which were the most frequently isolated *E. coli* pathotypes from infants with diarrheal disease, were also frequently recovered from asymptomatic adults which may place the younger individuals at a risk of infection. Genes encoding *astA* and *aggR* were associated with diarrheal disease. The *aggR* gene is a transcriptional activator gene required for the expression of the anti-aggregation protein gene *aap* and an anti-aggregation protein transporter gene *aatA* which have been commonly used for the detection of EAEC (Toma *et al.,* 2003). Furthermore, pathogenic studies have suggested that *aggR* gene controls the virulence functions in EAEC (Harrington *et al*., 2005). The EAEC strains expressing the *aggR* regulon have been therefore been denoted typical EAEC (Nataro *et al*., 2006).

In previous epidemiological studies on EAEC, typical EAEC strain has been mainly targeted because many investigators have found a strong association with diarrhea (Sheikh *et al.,* 2004; Moyo *et al.,* 2007). In this study, *aggR* gene was detected at a significantly higher prevalence in symptomatic than in asymptomatic children. However, the lack of *aggR* in 45% of EAEC-positive samples from symptomatic children show that this gene may not be a good marker to diagnose EAEC.

In the present study, *aap* gene was detected in all EAEC strains from patients and 95% of EAEC from controls. It therefore may be an appropriate marker for diagnosis of EAEC but would not be preferred for differentiation of the virulent EAEC subpopulations. *AatA* gene was detected in less than a third of EAEC from either symptomatic or asymptomatic children and may therefore not be a suitable marker for diagnosis of EAEC.

To further characterize the identified EAEC strains, they were categorized into typical (*aggR*-positive) and atypical (*aggR*-negative) and analyzed for the presence of different virulence associated genes. In atypical EAEC, *astA* was detected significantly more in patients than in controls (P=0.0059, OR =4.17) further emphasizing the role of *astA* gene in atypical EAEC. Since its first description as an enterotoxin of EAEC in 1991, the role of *astA* in human disease remains controversial (Savarino *et al.,* 1991). It has been reported to be produced by approximately half of the EAEC (Savarino *et al.,* 1993; Savarino *et al.,* 1996). Consequently, it is not clear if production of this toxin was relevant to the manifestation of diarrhea due to EAEC.

Recently, *Escherichia coli* serogroup O-untypable strain that had a coding gene for enteroaggregative *E. coli* heat-stable enterotoxin 1 (EASTEC) was identified as the etiologic agent of a diarrhea outbreak in Mandera County, Kenya (Ochi *et al.,* 2015). Previously, the results of Vila *et al*. (1998) had suggested an association between EAST1-positive strains and diarrhea in children. In addition, Zhou *et al*. (2002) reported on a gastroenteritis outbreak caused by a strain of EASTEC, strain O166:H15, in Osaka, Japan.

In the present study, we found an association between *astA* and sporadic patients of diarrheal disease. However, the prevalence of *astA* in EAEC isolates from asymptomatic children was also very high (56.7% in patients versus 38.3% in controls).

Genes encoding either a fimbrial subunit or an usher assembly unit was found in almost all the controls. Usher, AAF III/IV assembly unit, agg3/4C, was not detected in any EAEC strain from patients while 55/60 (93.2%) of the EAEC strains from controls were found positive for the gene. However, the *aggR* gene was present in several patients in the absence of the fimbrial subunit genes in patients showing the possibility of uncharacterized AAFs or other fimbriae not investigated in the present study. Recently, a novel Aggregative Adherence Fimbriae (AAF/V) was described by Jønsson *et al*. (2105). The presence of this aggregative fimbriae was however not investigated in the present study. A diverse combination of virulence-related genes similar to other studies carried out previously was detected (Nataro *et al.,* 1995; Regua-Mangia *et al.,* 2004; Blanco *et al.,* 2006; Boisen *et al.,* 2012; Lima *et al.,* 2013). However, more virulence-related gene combinations were found in asymptomatic children than in in symptomatic children.

In this study, twelve antibiotics were tested against the detected EAEC. Most tested isolates showed more than ninety percentages sensitivity to ciprofloxacin, ofloxacin and kanamycin, probably due to their seldom use (Shapiro *et al.,* 2001). On the contrary, a high percentage of ampicillin, erythromycin, streptomycin, trimethoprim-sulfamethoxazole, and tetracycline resistance were observed. The high rate of resistance towards these antibiotics may be due to their indiscriminate use because they are readily available over the counter (Shapiro *et al.,* 2001).

Furthermore, high levels of antimicrobial resistance among pathogenic *E. coli* to antibiotics; ampicillin, trimethoprim/sulfamethoxazole (95% resistance) and tetracycline (81% resistance) have been observed in previous studies in Kenya (Sang *et al.,* 1997; Sang *et al.,* 2002;). Also, the majority of the EAEC isolates in this study displayed resistance to trimethoprim/sulfamethoxazole, ampicillin, tetracycline and streptomycin. High carriage rates of antimicrobial resistance among the asymptomatic individuals to trimethoprim/sulfamethoxazole, ampicillin, erythromycin and DXT was found in this study.

5.2 Conclusion

A prevalence of sixteen percent of children admitted with diarrhea in the study area was due to EAEC reflecting a significant cost in health resources. However, an equally high asymptomatic carriage of EAEC among children was also found.

This study reveals a high degree of variability of genotypic markers in EAEC isolated from Kiambu County.

This study also underscores the potential usefulness of the *aggR* and *astA* genes as possible virulence markers for detection and differentiation of virulent strains of EAEC from avirulent strains. This study also indicates that a certain subset of EAEC is unrelated to diarrhea, for which *agg3/4C* may be a marker.

High rates of antimicrobial resistance in ampicillin, erythromycin, trimethoprim-sulfamethoxazole and tetracycline was observed. High carriage rates of antimicrobial resistance among the asymptomatic individuals to trimethoprim/sulfamethoxazole, ampicillin, erythromycin and DXT was also high.

The high level of antimicrobial resistance observed in this study raises a broader discussion about the misuse of antibiotic therapy in children of a very young age in the study area. However, the high prevalence of enteroaggregative *E. coli* resistant to tetracycline show the acquisition of the resistant bacteria as opposed to resistance induced through antimicrobial usage as tetracycline is not usually prescribed for children under the age of five years.

5.3 Recommendations

5.3.1 Recommendations from the study

Based on the results obtained during this study, the following recommendations are suggested:

i) Two virulence associated genes, *aggR* and *astA*, are suitable for routine detection and differentiation of virulent EAEC strains from avirulent strains.

ii) Usher, AAF/III-IV assembly unit (*agg3/4C* gene) is suitable for the detection of EAEC strains which are unrelated to diarrheal disease.

iii) In view of the current antimicrobial resistance pattern found in the study area, ceftriaxone, ciprofloxacin, ofloxacin, gentamicin and kanamycin are appropriate in managing severe diarrheal disease in the study area.

iv) Ampicillin, SXT and streptomycin antibiotics are not appropriate in the management of severe patients of diarrheal disease caused by EAEC in the study area.

5.3.2 Recommendations for future study

EAEC contributes to a substantial burden of diarrheal disease in the study area and further research focusing on exhaustive isolation and characterization of EAEC as a diarrheagenic agent in the study area is necessary. Research focusing on other aspects such as the role of host immune factors may help in understanding the pathogenicity of EAEC as a diarrheagenic agent.

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APPENDICES

APPENDIX I. **Interpretative criteria and quality control of antibiotics**

Antimicrobial agent	Strip antibiotic concentration (µg/mL)	S≤	I	R≥	*E. coli* 25922 MIC µg/mL
Amoxicillin	0.016-256	2	4	8	
Ciprofloxacin	0.002-32	1	2	4	
Ceftriaxone	0.016-256	8	16-32	64	0.032-0.125
Chloramphenicol	0.016-256	8	16	32	
Erythromycin	0.016-256	0.5	1-4	8	
Nalidixic acid	0.016-256	16		32	1-4
Ofloxacin	0.002-32	2	4	8	0.016-0.125
Streptomycin	0.064-1024			1000	2-8
Tetracycline	0.016-256	4	8	16	0.5-2
Trimethoprim/sulfamethoxazole	0.002-32	2		4	0.064-0.25

Ampicillin (AMP), Ciprofloxacin (CIP), Chloramphenicol (CHL), Ceftriaxone (CRO), Erythromycin (E), Nalidixic acid (NAL), Ofloxacin (OFX), Streptomycin (STR), Trimethoprim/sulfamethoxazole (SXT), Tetracycline (TET)

APPENDIX II. **Pathological Investigation Form**

NUITM-KEMRI Project, Nairobi Station
NAGASAKI UNIVERSITY INSTITUTE OF TROPICAL MEDICINE
Centre for Microbiology Research, KEMRI

P.O. Box 19464-00202, Next to KNH Post Office, Nairobi, Kenya TEL 020-272-5120 FAX 020-272-5144

PATHOLOGICAL INVESTIGATION FORM

Patient Ref. No.: _____Date of admission: ___/___/____
(day/month/year) **Patient information**

Name: _____

Date of birth: ___/___/_____ (Day/Month/Year)

Age (months):_____ Sex (M/F):_____ Address: Location_____

Sub-location _____Village_____ Landmark (e.g. nearby school, church etc.) _____ Referred from: _____to_____

Parent/Guardian information

Name: _____ Occupation: _____

Rotavirus Immunization information

Patient immunized against rotavirus? _____ (Yes/No)
If "Yes", type of vaccine: Rotarix or RotaTeq (circle one); No. of doses received: _____
Date of dose 1: ___/___/_____; dose 2:__/__/____; dose 3:__/__/____
(Day/Month/Year)

Clinical information

Temperature: _____ °C. Abdominal pain: _____ (Yes/No)

Date of onset: ___/___/____

Vomiting: _____ (Yes/No) No. of episodes/24hr: _____

Date of onset: ___/___/_____

Diarrhea: _____ (Yes/No) No. of episodes/24hr: _____

Date of onset: ___/___/_____

Other signs & symptoms:
_____

Persons with similar symptoms: Parent/Guardian ☐ Siblings ☐
Others_____

History of medication:
_____

Laboratory information
Date stool specimen collected: ___/___/_____ (day/month/year)

Nature of stool specimen: Watery☐ Bloody ☐ Mucoid ☐
Others_____
Other pathogens identified in stool:
_____

Clinical Diagnosis:
_____

Treatment:
_____

Outcome: Date of discharge/death: ___/___/_____ (day/month/year)

Clinician's name: _____Signature:_____

Date: _____

Specimen coding number: _____ Signature

(NUITM):_____Date:_____

APPENDIX III. **Consent Form.**

FOMU YA KIBALI CHA KUPATA CHOO NA DAMU KUTOKA KWA WAGONJWA WA KUHARISHA

MADA YA UTAFITI: utafiti kuhusu viini vinavyosababisha magonjwa ya kuhara katika maeneo ya Nairobi na magharibi mwa Kenya

Mtafiti mkuu: Yoshio Ichinose[1]

Watafiti wasaidizi: Karama Mohamed[4],Sayaduki Ochi[6],carolyne N. wahome[1],Guyo Huka Sora[1&2],Hillary Limo[5]Haruki Uemura[3],Satoshi Kaneko[1], Samuel Kariuki[2], Njeri Wamae[2],Takao Tsuji[6],Koichi Morita[7],Maasaki Shimada[1]

Taasisi shirikishi

1) Kenya research station, institute of tropical medicine, Nagasaki University, Nagasaki, japan. 2) centre for microbiology research, Kenya medical research institute, Nairobi, Kenya, 3) department of protozoology, institute of tropical medicine, Nagasaki university, Nagasaki Japan, 4) centre for public health research, Kenya medical research institute, 5) division of disease surveillance and response, ministry of health, 6) department of microbiology, fujita health university, school of medicine, 7) department of virology, institute of tropical medicine, Nagasaki university.

Sisi,chuo kikuu cha Nagasaki,taasisi ya magonjwa tukishirikiana na CMR(KEMRI),tumepewa idhini ya kuchunguza masuala ya afya yanayowakumba wakenya na lengo la kuimarisha kiwango cha afya yao.Tumejitolea kufanya utafiti huu unaolenga magonjwa ya kuharisha na viini kwenye damu hasa kwa watoto,na kusababisha maafa.Matokeo ya utafiti huu yataenezwa kwa washikadau wengi ,ikiwepo wizara ya afya,kemri na taasisi husika.matokeo haya pia yatakuwa ya umuhimu kwa wahusika wakuu katika wizara ya afya wenye jukumu la kuimarisha afya ya umma.utafiti huu umeidhinishwa na KEMRI/National Ethical Review Committee.

TARATIBU ZAUTAFITI: Tunachunguza chanzo cha magonjwa ya kuharisha na lengo la kujua kiwango cha magonjwa hayo nchini Kenya.Takriban,sampuli 500 zitahitajika kutoka kwa wanaoharisha na wasioharisha na lengo la kulinganisha kiwango cha maambukizi.Uchunguzi wa damu pia utafanywa ili kubainisha chanzo cha viini kwenye damu vinavyosababisha magonjwa ya kuhara. Sampuli zitaendelea kukusanywa kwa kipindi cha miaka miwili.Ukibubali kushiriki kwa taaluma hii, utaombwa kutoa sampuli ya choo na damu ambayo itafanywa uchunguzi kwa maabara ya NUITM-KEMRI, Nairobi.Tukipata viini husika kwenye sampuli yako au ya mtoto wako,tutafanya uchunguzi zaidi wa kubainisha ni dawa zipi zitakazotumika ili kuleta nafuu.Maelezo utakayotoa yatatusaidia ili tuweze kukupa mawaidha kuhusu njia za keupukana na magonjwa ya kuharisha katika siku za usoni.Matokeo yatakuwa ya umuhimu kwa mradi huu. Sampuli (choo na damu) zitachukuliwa mara moja au mbili kulingana na mahitaji ya utafiti.matokeo haya yatapewa daktari anayekutibu wewe na mwanao.kama umepatikana na viini (bakteria)vinayosababisha maradhi,utafanyiwa uchunguzi zaidi.ikiwa kutahitajika taratibu zaidi tusizoweza kutekeleza kwa

maabara yetu ya Nairobi,sampuli zitapelekwa kwa uchunguzi zaidi katika taasisi yetu ya chuo kikuu cha Nagasaki na pia kwa idara ya somo la microbiologia,katika chuo kikuu cha fujita, shule ya matibabu,Japan

FAIDA/HATARI

Hakuna hatari kutoa sampuli ya choo,bali utahisi woga na uchungu kidogo taratibu ya kutoa damu ikitekelezwa.Tutahitaji mililita kumi ya damu kutoka kwa watu wazima ilhali watoto wa miaka tano na chini watatolewa mililita mbili kwa kutumia njia taratibu na safi zaidi.Damu itawekwa kwenye vyombo safi vinavyotumika mara moja tuu.Wahusika watashiriki kwa kauli yao wenyewe.Ripoti ya utafiti huu haitatakuwa ya umuhimu tu kwa matibabu ya wagonjwa wahusika, bali itakuwa ya manufaa kwa jamii maana itatawezesha washikadau kupendekeza njia mwafaka za kupunguza kuenea kwa maradhi ya kuharisha.Wahusika hawatakuwa na gharama zozote kwani tutagharamia vifaa vya kuchukua sampuli.Jeraha likitokea katika harakati za kuchukua sampuli,tutachukua wajibu.

KUKATAA / KUJIONDOA KWA MUHUSIKA

Si lazima kuhusika katika utafiti huu, haki yako ya kukata au kujiondoa utafiti unapoendelea itaheshimiwa, na haitasababisha kutohudumiwa ki mawaidha na daktari.uhusika wako waweza kusimamishwa na watafiti.

USIRI

Tutafanya lote tuwezalo kuhakikisha usiri wako katika ripoti zozote tutakazo chapisha kuhusu utafiti huu.Kuhakikisha usiri huu, hatutachapisha majina ya wahusika wowote katika ripoti zetu,na katika vyombo vya kuchukulia sampuli. Wahusika watapewa nambari ya kujitambulisha na nambari hizo ndizo zitakazotumika kwa ripoti na katika vyombo.

ANWANI

Ukiwa na swali sasa au siku za usoni kuhusu atafiti huu,tafadhali uliza afisa wahusika wa utafiti huu au wasiliana na mtafiti mkuu Yoshio Ichinose, mradi wa NUITM-KEMRI,nambari ya simu + 254-20-272-0794 ext 324.Kuhusu haki ya kushiriki kwa utafiti huu wasiliana na katibu,KEMRI/National Ethical Review Committee,P.O Box 54840-00200,Nairobi;namnbari ya simu 020-272-2541,0722-205901,0733-400003.

TAMKO

Nimeelewa yaliomo kwa fomu hii ya idhini ,na yote yaliyosemwa kuhusu utafiti na wajibu wangu kama muhusika,pia nimeelewa kuwa nina uhuru wa kujihusisha au kutojihusisha na utafiti huu,pia naweza kujiondoa wakati wowote katika utafiti huu.

Nimekubali kuhusika katika utafiti huu,bila shinikizo zozote

Majina ya muhusik	Sahihi	Tarehe
Jina la shahidi..	Sahihi	Tarehe

KENYATTA UNIVERSITY
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Tel. 8710901 Ext. 57530

Our Ref: I56/CTY/PT/27009/2014

DATE: 29th July 2016

Director General,
National Commission for Science, Technology
& Innovation
P.O Box 36023-00100
NAIROBI

Dear Sir/Madam,

RE: RESEARCH AUTHORIZATION FOR ODOYO ERICK OMONDI– REG. NO. I56/CTY/PT/27009 /2014.

I write to introduce Mr. Odoyo Erick Omondi who is a Postgraduate Student of this University. He is registered for M.Sc degree programme in the Department of Biochemistry & Biotechnology.

Mr. Odoyo intends to conduct research for a M.Sc. Proposal entitled, "Prevalence and Virulence Factors of Enteroaggregative Escherichia Coli Isolated from Children Under Five Years Old in Kiambu County".

Any assistance given will be highly appreciated.

Yours faithfully,

MRS. LUCY N. MBAABU
FOR: DEAN, GRADUATE SCHOOL

HI/nn

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **March 29, 2016**

TO: **PROF. YOSHIO ICHINOSE**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CMR**
NAIROBI

Dear Sir,

RE: **SSC NO: 1323 (*REQUEST FOR ANNUAL RENEWAL*): STUDY OF DIARRHEAGIC AGENTS AS INTESTINAL AND INVASIVE INFECTIONS IN NAIROBI AND WESTERN KENYA**

Thank you for the continuing review report for the period **16th April, 2015** to **2nd March, 2016.**

This is to inform that during the 249th C meeting of the KEMRI Scientific and Ethics Review Unit (SERU) held on 24th March 2016, the Committee **conducted the annual review and approved** the above referenced application for another year.

This approval is valid from **April 16, 2016** through to **April 15, 2017**. Please note that authorization to conduct this study will automatically expire on **April 15, 2017**. If you plan to continue with data collection or analysis beyond this date please submit an application for continuing approval to the **KEMRI/SERU** by **March 04, 2017.**

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

Yours faithfully,

For:

DR. EVANS AMUKOYE,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT