

**Characterisation of Enterotoxigenic *Escherichia coli* and other
Enteric Pathogenic Bacteria isolated in Machakos District Hospital,
Kenya.**

Bonventure Wachekone Juma

**A Thesis Submitted In Fulfillment for the Award of the Degree of
Doctor of Philosophy in Medical Microbiology in the Jomo Kenyatta
University of Agriculture and Technology**

2013

DECLARATION

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

Signature

Date.....

Bonventure Wachekone Juma

This thesis has been submitted for examination with our approval as University Supervisors.

Signature.....

Date.....

Dr. Samuel Kariuki

KEMRI, Kenya

Signature.....

Date.....

Dr. Peter G. Waiyaki

KEMRI, Kenya

Signature.....

Date.....

Dr. Wallace Bulimo

UON/KEMRI, Kenya

Signature.....

Date.....

Prof. Marion Mutugi

JKUAT, Kenya

DEDICATION

I dedicate this work to my mother Cyril Naliaka who has been my encouragement to achieve my professional goal, and to the love of my wife Esther, daughter Ruth and son Giovanni whose unending love and support make everything I do possible.

My soul doth magnify the Lord, and my spirit hath rejoiced in God my Saviour... For he that is mighty hath done to us great things; and holy is his name (Luke 1: 46-49).

ACKNOWLEDGEMENT

I would like to register my great and heart felt appreciation to my able and experienced supervisors Dr. Samuel Kariuki, Dr. Peter Waiyaki, Dr. Wallace Bulimo and Prof. Marion Mutugi for their guidance, encouragement, patience and great skills in Microbiology, Molecular Biology, Computational Biology, Public Health and Epidemiology which made this thesis a success. My great appreciation also goes to Dr. David Schnabel and Dr. Max Tai Wu from United States Army Medical Research Unit – Kenya for allowing me to pursue this degree in order to realize my professional goals. To Dr. Edwin Oaks from USAMRU-K Microbiology Hub - Kericho, thank you very much for allowing me use of the Micro scan and reagents in your laboratory for confirmation of the Extended – Spectrum Beta Lactamases (ESBL). To Dr. John Klena from US Navy Area Medical Research Unit - 3 Cairo – Egypt (NAMRU-3), thank you very much for providing me with the primers for CFA and CS detection in ETECs. To the MOH Machakos District Hospital where sample collection was done, thank you very much for your encouragement during times of low patient turnover and for allowing me to carry out my study successfully in your hospital. My sincere gratitude also goes to the clinical officers and laboratory technicians of Machakos District Hospital who made this study successful. To my long time academic classmate and colleague Dr. George Ngondi Michuki whom we have shared challenges in this academic journey at the Institute of Tropical Medicine and Infectious Diseases, thank you.

This work was done at the Centre for Microbiology Research, Kenya Medical Research Institute / US Army Medical Research Unit – Kenya (USAMRU-K) collaborative research programs in Nairobi, Kenya and the International Livestock Research Institute (ILRI). The following people assisted me in various aspects to see the success of the project: Ms. Finnley Osuna, Mr. Peter Mbogo, Mr. Erastus Mulinge, Mr. Ayub Ongechi, Ms. Elizabeth Odundo, Mr. Ronald Ng’etich, Mrs. Jane Ochieng, Ms. Agnes Munyalo, Mr. Ronald Kirera and Ms. Janet Ndonge; thank you all. To my beloved wife Esther, daughter Ruth and son Giovanni, thank you for nurturing, loving and supporting me throughout. It is your unending love, encouragement, support and laughter that made everything possible. To all those that I have not mentioned I salute your great assistance.

TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENT	iv
TABLE OF CONTENTS.....	vi
LIST OF TABLES	xiii
LIST OF FIGURES	xiv
LIST OF PLATES	xv
LIST OF APPENDICES.....	xvi
LIST OF ABBREVIATIONS.....	xvii
ABSTRACT	xxi
CHAPTER ONE.....	1
1.0 INTRODUCTION.....	1
1.1 Justification for the study	3

1.1.1 Statement of the problem.....	Error! Bookmark not defined.
1.2 Study questions	4
1.3 Objectives	4
1.3.1 General objective.....	4
1.3.2 Specific objectives.....	5
CHAPTER TWO	6
2.0 LITERATURE REVIEW	6
2.1 Microbial diarrhoea.....	6
2.1.2 Isolation, identification and common themes in virulence of <i>E. coli</i>	7
2.1.3 Detection of <i>Escherichia coli</i> causing Diarrhoea	8
2.1.4 Serotyping.....	11
2.1.5 Genotyping	11
2.1.6 Enterotoxigenic <i>E. coli</i> (ETEC).....	13
2.1.6.1 Heat-stable (ST) and heat-labile (LT) enterotoxin production and other virulence associated factors.....	14
2.1.6.2 Infant diarrhoea and travelers' diarrhoea.....	16
2.1.6.3 The History of ETECs	18
2.1.6.4 Biology of ETECs	19
2.1.6.5 Epidemiology of ETECs.....	23

2.1.6.5.5 Comparison of ETEC Diarrhoea and Cholera in Children and Adults	28
2.1.6.5.6 Presence of ETEC in Food and Water in the Environment	28
2.1.6.5.7 ETEC Infections and Malnutrition	30
2.1.6.5.8 ETEC infections in International Travelers	31
2.1.6.5.9 Clinical features of ETEC.....	32
2.1.6.5.9.1 Disease Severity	32
2.1.6.5.10 Laboratory assays for ETEC	34
2.1.6.5.10 Treatment and Management of ETEC.....	37
2.1.6.5.11 Rehydration	37
2.1.6.5.12 Antimicrobials	38
2.1.6.5.13 Multidrug Resistance in ETEC.....	39
2.1.6.5.14 Nutritional and Micronutrient Therapy.....	40
2.1.6.6 Prevention of ETECs.....	41
2.1.6.6.1 Vaccine Development.....	41
2.1.6.6.2 Purified CFs and Enterotoxoids	42
2.1.6.6.3 Inactivated Whole-Cell Vaccines.....	43
2.1.6.6.4 Live Oral ETEC Vaccines	44
2.2 Shiga toxin producing <i>E. coli</i> (STEC)	45
2.2.1 Nomenclature.....	45
2.2.2 Shiga toxins (Stx) and other factors affecting the pathogenic properties of STEC	46
2.2.3 Sources of STEC infections.....	48

2.3 Enteropathogenic <i>E. coli</i> (EPEC)	48
2.3.1 Attaching-and-effacing (A/E) adherence of EPEC strains	49
2.3.2 Epidemiology of EPEC infections	50
2.4 Enteroaggregative <i>E. coli</i> (EAEC).....	51
2.4.1 Aggregative adhesion (AA) and other proposed virulence characteristics ...	51
2.4.2 Diarrhoea caused by EAEC	52
2.5 Enteroinvasive <i>E. coli</i> (EIEC)	53
2.6 Diffusely adherent <i>E. coli</i> (DAEC).....	54
CHAPTER THREE	56
3.0 MATERIALS AND METHODS	56
3.1 Study Design.....	56
3.2 Study site.	56
3.3 Isolates.....	57
3.4 Study population	58
3.4.1 Inclusion criteria.....	58
3.4.2 Exclusion criteria.....	58
3.5 Sample size.	58
3.6 Specimen collection	59
3.6.1 Stool collection	59

3.7 Laboratory procedures.....	60
3.7.1 Microbial procedures (culture).....	60
3.7.2 Isolation and identification of <i>E. coli</i> pathotypes	61
3.7.3 Characterisation of <i>E. coli</i> pathotypes by mPCR.....	62
3.7.4 Multiplex Chain Reaction amplification for <i>E. coli</i> pathotypes	63
3.7.5 Characterization of ETEC CFAs.....	65
3.7.6 Multiplex PCR for colonization factor antigens detection	67
3.7.7 Multiplex Polymerase Chain Reaction for non <i>E. coli</i>	69
3.7.8 DNA purification for sequencing.....	69
3.7.9 Sequencing of PCR product.....	70
3.8 Isolation and identification of other enteric diarrhoeal pathogens	71
3.8.1 Serology.....	73
3.9 Antibiotic Susceptibility tests (disc diffusion)	73
3.9.1 Testing for Extended - Spectrum β Lactamases (ESBLs)	74
3.9.2 Minimum Inhibitory Concentration (MIC)	76
3.9.3 Plasmid extraction and analysis.	76
3.9.4 Preparation of Bacteria for Conjugation experiments	76
3.9.5 Pulsed Field Gel Electrophoresis (PFGE)	77
CHAPTER FOUR.....	79
4.0 RESULTS.....	79
4.1 Demographic data of study participants.....	79

4.2 Prevalence of ETEC (LT, ST, LTST, CFAs, CSs	84
4.3 Age and clinical data associated with ETEC isolates expressing CFA/I, CS5 plus CS6, CS6, and CS19.....	88
4.4 Prevalence of other <i>E. coli</i> pathotypes	92
4.5 Prevalence of other enteric pathogenic bacteria	94
4.6 Detection of shigella enterotoxin genes by pcr assays.....	97
4.7 Antibiotic susceptibility profiles of ETEC, other <i>E. coli</i> pathotypes and other pathogenic bacterial isolates.....	99
4.8 Extended spectrum beta lactamases.....	104
4.9 Detection of gyrase A, B and Topoisomerase iv genes by PCR and sequencing	105
4.10 Test for invasiveness	110
4.10.1 Plasmid profile analysis.....	110
CHAPTER FIVE	112
5.0 DISCUSSION.....	112
5.1 Prevalence of ETEC (LT, ST, LTST, CFA, CS)	112
5.2 Prevalence of other <i>E. coli</i> pathotypes.....	117
5.3 Prevalence of Enteroaggregative <i>E. coli</i>	120

5.4 Prevalence of other enteric pathogenic bacteria	122
5.5 Susceptibility testing	127
5.5.1 Antibiotic susceptibility profiles of ETEC, other <i>E. coli</i> pathotypes and other pathogenic bacterial isolates	127
5.5.2 Extended - Spectrum Beta Lactamases (ESBLs)	130
5.5.3 Detection of Gyrase A, B and topoisomerase IV genes by PCR and Sequencing	135
5.5.4 Plasmid analysis	137
CHAPTER SIX	141
6.0 CONCLUSIONS AND RECOMMENDATIONS.....	141
6.1 Conclusions	141
6.2 Recommendations	142
REFERENCES	145

LIST OF TABLES

Table 2.1	Characteristics of the diarrhoeagenic <i>e.coli</i>	10
Table 3.1	Primers amplification conditions and positive controls used for mPCR	63
Table 3.2	Primers sequences for amplification of colonization factors	68
Table 3.3	Antibiotic pannel.....	74
Table 4.1	Antibiotic use prior to visiting Hospital.....	83
Table 4.2	Age range of patients infected with ETEC isolate expressing CFA1, CS5 plus CS6, CS6, CS14 and CS 19	87
Table 4.3	Distribution of ETEC colonization factor antigens by Phenotype ...	88
Table 4.4	Clinical data and ETEC colonization factor antigens expression.....	89
Table 4.5	Number of patients from whom bacteria were isolated	94
Table 4.6	Biochemical characterization of non typable <i>Shigella</i> spp and enteroinvasive <i>E. coli</i> (EIEC)	96
Table 4.7	MIC ranges for ETEC, isolates.....	102
Table 4.8	Fluoroquinolone resistance and amino acids substitution in bacteria isolates from Machakos District Hospital.....	109

LIST OF FIGURES

Figure 4.1	Signs and symptoms of 301 patients with diarrhoea at Machakos District Hospital 2006-2008.....	80
Figure 4.2	Study subjects occupation in Machakos District Hospital between 2006-2008	80
Figure 4.3	Patients stool consistency from Machakos District Hospital 2006 to 2008.....	81
Figure 4.4	Study participants age distribution.....	82
Figure 4.5	Seasonal association of ETEC CF and CS expression from September 2006 to March 2008.....	90
Figure 4.6	Frequencies of <i>E. coli</i> pathotypes isolated at Machakos District Hospital in 2006-2008.....	92
Figure 4.7	Drug susceptibility testing on ETEC, other <i>E. coli</i> pathotypes and other enteric bacterial isolates from Machakos District Hospital.....	99
Figure 4.8	Antibiotic susceptibility profiles by age from patients in Machakos District Hospital.	100
Figure 4.9	Fluoroquinolone resistance in bacterial isolates from Machakos ...	101
Figure 4.10	Drug susceptibility of the transconjugant bacteria.....	104
Figure 4.11	ESBL activity for phantom phenomena by Double Disc Diffusion method.....	105
Figure 4.12	Consensus tree of Gyrase A genes and its nearest neighbours.	108

LIST OF PLATES

Plate 4.1	Multiplex PCR amplification of ETEC virulence genes from patients in Machakos District Hospital 2006-2008.	85
Plate 4.2	Amplification of ETEC CFAs and CS by mPCR	86
Plate 4.3	PFGE gel using <i>Xba</i> I digest pattern variations in ETEC isolates from Machakos District Hospital.	91
Plate 4.4	gel picture of mPCR amplification of different <i>E. coli</i> pathotypes toxins.	93
Plate 4.5	Multiplex PCR amplification of Shigellae enterotoxin genes from patients in Machakos District Hospital 2006-2008.....	97
Plate 4.6	PFGE patterns of <i>Xba</i> I digest <i>Shigella spp</i> from diarrhoeal patients in Machakos District Hospital.	98
Plate 4.7	PCR products of the <i>GyrA</i> , <i>B</i> , <i>par C</i> and <i>par E</i> quinolone resistance genes in <i>E. coli</i> and <i>Shigella spp</i>	106
Plate 4.8	Plasmid profiles of multidrug resistance Shigella strains	111

LIST OF APPENDICES

Appendix 1:	Informed consent agreement	185
Appendix 2:	Questionnaire.....	193
Appendix 3:	Ethical Review committee approval	194

LIST OF ABBREVIATIONS

AA	Aggregative adhesion
AE	Attaching-and-effacement
aEPEC	atypical Enteropathogenic <i>Escherichia coli</i>
Asp	Asparagine
Bfp	Bundle forming pile
CDC	Centre for Disease Control and Prevention
CFA	Colonisation Factor Antigen
CMR	Centre for Microbiology Research
CNF1	Cytotoxic necrotizing factor 1
CNF2	Cytotoxic necrotizing factor 2
CS	Coli-Surface antigens
CSC	Center Scientific Committee
CT	Cholera toxin
DEC	Diarrhoeagenic <i>E. coli</i>
DEP	Diarrhoeagenic <i>Escherichia coli</i> pathotypes
DDBJ	DNA Data Bank of Japan
DDD	Double disc diffusion
DNA	Deoxyribonucleic acid
EaeA	Attaching and effacing intimin gene
EAEC	Enteraggregative <i>Escherichia coli</i>
EAF	Enteropathogenic adherence factor plasmid
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>

<i>Ehly</i>	Enterohaemolysin
EIEC	Enteroinvasive <i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
ERC	Ethical Review Committee
ESBL	Extended - Spectrum Beta Lactamase
ETEC	Enterotoxigenic <i>Escherichia coli</i>
GyrA	Gyrase A
<i>GyrB</i>	Gyrase B
H₂S	Hydrogen sulfide
HC	Hemorrhagic colitis
HUS	Hemorrhagic uremic syndrome
ILRI	International Livestock Research Institute
KEMRI	Kenya Medical Research Institute
LEE	Locus of enterocyte effacement
LT	Heat Labile toxin
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MIO	Motility Indol Ornithine
MOH	Ministry of Health
MPCR	Multiplex Polymerase Chain Reaction
NAMRU-3	Naval Medical Research Unit Number 3.
parC	Topoisomerase C
parE	Topoisomerase E

PCR	Polymerase Chain Reaction
Per	Transcriptional activator
PFGE	Pulsed Field Gel Electrophoresis
QRDRs	Quinolone resistant determining regions
rCTB	recombinant cholera toxin B
SC	Simmon's citrate
SEN	Chromosomal gene encoding SHET2 toxin
Ser	Serine
SET	Chromosomal gene encoding SHET1 toxin
SSC	Scientific Steering Committee
ST	Heat stable toxin
STa	Heat stable toxin a
STEC	Shiga toxin producing <i>Escherichia coli</i>
STh	Heat stable toxin from human
STp	Heat stable toxin from porcine
Stx	Shiga toxin
USAMRU-K	United States Army Medical Research Unit-Kenya
tEPEC	typical Enteropathogenic <i>Escherichia coli</i>
TCBS	Thiosulfate Citrate Bile Salts Agar
TD	Travelers diarrhoea
Tir	Translocated intimin receptor
TSI	Triple Sugar Iron
UV	Ultraviolet Light
VT	Verotoxin

WHO

World Health Organization

XLD

Xylose Lysine Desoxycholate

ABSTRACT

Diarrhoeal disease is a worldwide public health problem mostly affecting children, the immunocompromised and elderly in developing countries. Among children in developing countries, Enterotoxigenic *Escherichia coli* (ETEC) alone is responsible for more than 650 million diarrhoeal episodes and 2,000,000 deaths annually. However, in most developing countries including Kenya, there is a paucity of data on prevalence and characteristics of specific bacterial etiologies of diarrhoeal illness. Diarrhoea outbreaks have been common on yearly basis in Kenya. This study was carried out from September 2006 to March 2008 in Machakos District Hospital, a referral hospital serving most of the lower part of the former Eastern Province. Its catchment includes the neighbouring divisional and district hospitals in Kitui, Mwingi and Mbooni. It has a bed capacity of 250. During the study, a total of 301 participants were enrolled following specific inclusion criteria. The participants ranged in age from 4 months to 85 years with a median age of 14 years and 7 months. The study was designed to detect bacterial pathogens associated with diarrhoea and in particular ETEC. Faecal specimens were collected and cultured in selective and differential media and *E. coli* phenotypes identified using standard biochemical methods. A mixture of eight specific primers were used in a single PCR (Multiplex Polymerase Chain Reaction; mPCR) to detect diarrhoeagenic *E. coli* pathotypes. Ninety eight isolates from thirty three out of 300 participants (11%) identified as ETEC positive were subcultured on Colonising Factor Antigen Agar (CFA) and analysed for hydrophobicity as presumptive indication of CFA presence. This gave a presumptive confirmation for CFA and Coli Surface Antigens (CS) which were then analysed using another set of mPCR with specific primers. Other

bacterial agents were biochemically identified. They included *S. dysenteriae* (8/300; 2.7%), *S. flexneri* (13/300; 4.3%), *S. sonnei* (1/300; 0.3%), *S. boydii* (1/300; 0.3%), Untypable *Shigella* (44/300;14.7%) *Salmonella* spp (1/300;0.3%), *Serratia* spp (1/300; 0.3%), *Pseudomonas* spp (1/300; 0.3%), and *Klebsiella* spp (37/300; 12.3%). *Shigella* and *Salmonella* were then serotyped using specific polyvalent and monovalent antisera from Denka Seiken, Japan. Antibiotic susceptibility tests were performed on all isolates using the disc diffusion and minimum inhibitory concentration methods. ETECs as well as other Enteric bacteria were resistant to more than four antibiotics (Chloramphenicol (28%), Cotrimoxazole (78%), Co-amoxiclav (70%) Erythromycin (98%) Ciprofloxacin (5%), Cefotaxime (18%) and Tetracycline (56%). Those found resistant to third generation cephalosporins were tested for Extended Spectrum Beta Lactamases (ESBL) while those resistant to quinolones were tested for Gyrase resistance genes (Gyrase A, B and Topoisomerases). Sequencing was done on all Gyrase and topoisomerase positive isolates for detection of mutations. From the study, ETEC was detected significantly more often in children less than five years old (25/300) than in those aged above six years (8/300) ($X^2 = 4.2$; $P < 0.05$). Among the ETECs isolated, CFAI, CS1, CS2, CS3, CS5, CS6, CS14 and CS19 were detected. Other than ETEC which was the main focus of the study, Atypical enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and enteroraggregative *E. coli* (EAEC) were also detected though with slightly lower frequencies in both children and adults (16.0%, 8% and 36.4%, respectively). Enterohaemorrhagic *E. coli* (EHEC) was only isolated in children and adults with bloody diarrhoea (2.1%). Enteroraggregative *E. coli* was the most prevalent pathotype (83/228 ;36.4%). *Shigella* spp (67/300; 22.3%) were isolated in

patients of all age groups. The drug susceptibility results revealed multidrug resistance in these isolates. ETECs were resistant to Chloramphenicol (28%), Cotrimoxazole (78%), Augmentin (70%) Erythromycin (98%) Ciprofloxacin (5%) and Tetracycline (56%). The quinolone resistance gene (*gyrA*) was detected in 10% of quinolone resistant isolates. Sequence analysis of strains with decreased susceptibilities or total resistance to fluoroquinolones showed a single mutation at either Ser-83 or Asp-87 codon. No mutations were detected in the *gyrB*, and topoisomerase genes. Extended – Spectrum Beta Lactamase (ESBL) was detected in (18/300; 6%). ETEC, other *E. coli* pathotypes and *Shigella* spp were shown to be heterogenous by Pulsed Field Gel Electrophoresis. The main etiologies of diarrhoea found were ETEC and other *E. coli* pathotypes, *Shigella* spp and *Salmonella* spp while the main CFs detected were CFAI (25/98), CS3 (1/98), CS6 (13/98), CS7 (2/98), CS14(12/98) and CS19 (12/98). The establishment of the existence of ETECs carrying varied CFA / CSs and multidrug resistance (MDR) phenotypes in this study is of great public health concern. There is a possibility of a reservoir in the population in the study area and the possibility of the reservoir spreading is real. This may lead to difficulties in the management of diarrhoeal disease. It is possible CS19 was detected for the first time in this country. The CS19 together with CS6 which are persistent adhesins can be utilised in the formulation of a novel vaccine for the prevention of diarrhoea due to ETEC.

CHAPTER ONE

1.0 INTRODUCTION

Escherichia coli are the predominant facultative anaerobic bacteria in the human colonic flora (Blanco *et al.*, 2006). *E. coli* usually remains harmlessly confined to the intestinal lumen; however, in the debilitated or immunosuppressed host, or when gastrointestinal barriers are violated, or when mutations occur due to acquisition of resistance plasmids, *E. coli* strains of normal flora can cause infection (WHO, 2011; Weintraub, 2007; Kaper *et al.*, 2004). Clinical syndromes associated with pathogenic *E. coli* infection include enteric/diarrhoea disease, urinary tract infection and sepsis/meningitis (Nataro *et al.*, 2006).

Contaminated food and water are important vehicles for transmission of various enteric pathogens (Huang *et al.*, 2006; Diamert, 2006; Adachi *et al.*, 2001). The food industry, restaurants and private homes occasionally fail to meet adequate cooling, storage, preparation and other hygiene standards, and food may be contaminated with faecal flora due to improper hygienic practices by the preparers. This makes possible the transmission of enteric pathogens and the spread of diseases caused by *E. coli* pathotypes. Among enteric pathogens, diarrhoeagenic *E. coli*, *Salmonella* and *Shigella* species belong to the most common bacteria causing intestinal infections in both developing and industrialized countries (WHO, 2011; Paredes-Paredes *et al.*, 2011; Oundo *et al.*, 2008; Vu Nguyen *et al.*, 2006).

Diarrhoeagenic *E. coli* have been classified into different categories according to their special characteristics. Enteropathogenic *E. coli* (EPEC) has been linked to

infant diarrhoea in developing countries (Blanco *et al.*, 2006). It was commonly found in industrialized countries and was known as "dyspepsiekoli"(Vu Nguyen *et al.*, 2006; 2005). Enterotoxigenic *E. coli* (ETEC) has been regarded as the most common pathogen causing travelers' diarrhoea (TD), irrespective of destination (WHO, 2009; Steffen *et al.*, 2004). Enteroaggregative *E. coli* (EAEC) and Shiga toxin-producing *E. coli* (STEC) are other key *E. coli* pathogroups (Kaur *et al.*, 2010; Adachi *et al.*, 2001). The former have been implicated as one of the etiological agents of diarrhoea both in developing countries and in outbreaks of gastroenteritis in industrialized countries. The latter strains are well-known causes of bloody diarrhoea and haemorrhagic colitis (HC) in humans (Vu Nguyen *et al.*, 2006; Nataro and Kaper, 1998; Sang *et al.*, 1996; 1997).

Diarrhoeagenic *E. coli* was among the first pathogens for which molecular diagnostic methods were developed (Roy *et al.*, 2010; Nataro and Kaper 1998). Molecular methods, especially Polymerase Chain Reaction (PCR) and Pulsed Field Gel Electrophoresis (PFGE) are currently considered among the most reliable techniques for differentiating *Salmonella*, *Shigella*, and *Vibrio* and diarrhoeagenic *E. coli* strains from nonpathogenic members of the stool flora and for distinguishing one *E. coli* pathogroup from another (Blanco *et al.*, 2006; Kariuki *et al.*, 2002). Advancement in diagnostic techniques have made it possible to re-evaluate the roles of various diarrhoeagenic *E. coli* groups in diarrhoea in humans. Most studies done on enteric pathogens concentrated mainly on the diarrhoeagenic *E. coli* pathotypes but did not include ETEC toxins (CS and CFAs) associated with diarrhoea. Infant and travelers' diarrhoea is associated with these toxins. The toxins (CFAs/CSs) are vital for vaccine

development if well characterized and documented. Machakos County was chosen on the basis of frequent diarrhoea episodes and its close proximity to Nairobi. This study aims at establishing the prevalence of ETEC and ETECs' colonizing factor antigens (CFAs) and coli surface antigens (CSs) circulating in Machakos County, other enteric pathogenic bacteria using current molecular techniques and establishment of drug susceptibility patterns for management of diarrhoea.

1.1 Statement of the problem

Diarrhoea caused by both diarrhoeagenic *E. coli* and other enteric bacterial pathogens in Kenya is an important cause of morbidity and mortality in people of all ages. Machakos District Hospital is one of the regions in Kenya where diarrhoea is prevalent and persistent. Research information on the prevalence of bacteria types and resistance to antimicrobial agents from other regions in Kenya other than Machakos are available and this permits evaluation of the effectiveness of the available drugs. Most studies done in Machakos District indicated diarrhoeal disease as the third leading cause of morbidity and mortality. The studies indicated morbidity and mortality were due to Shigellae, *Vibrio* spp and *Salmonella enterica serova typhi*. These is under contant sueveillance by the Ministry of Health. However, no data is available that implicates ETEC and ETEC CFAs and CS as caused of diarrhoea. The problem may be compounded by lack of current diagnostic and detection techniques for detecting ETEC, ETEC CFA and, CS. Currently they rely on conventional culture methods which may lead to misdiagnosis. Observation for ETEC, ETEC CFAs, CS have not been investigated in machakos District Hospital.

1.2 Justification for the study

In the light of diarrhoeal outbreaks in Machakos District Hospital, periodic surveillance of ETEC, ETEC CFAs, and CS prevalence is important for treatment and management. ETEC CFAs, CS, virulence and antimicrobial susceptibility patterns should be determined for ease of management. Armed with current molecular techniques, the current study was set to detect Enterotoxigenic *E. coli*, CFA, CS, other diarrhoeal bacterial pathogens, their virulence and antibiotic susceptibility profiles using modern techniques (mPCR, PFGE and sequencing). The data obtained from this study together with epidemiological findings will provide a better view on *E. coli* pathotype prevalence, ETEC virulence properties and highlight the emerging threat of multidrug resistance pathogenic bacteria that are increasing in the Machakos District Hospital. In addition, this will also help in mapping out future studies in the country using modern molecular techniques and may also affect treatment approaches and contribute towards vaccine development.

1.2.1 Study questions

- Is Enterotoxigenic *E. coli* a major cause of diarrhoea in patients attending Machakos District Hospital?
- What CFAs/CS are commonly isolated in Machakos District Hospital?

1.3 Objectives

1.3.1 General objective

To Characterise Enterotoxigenic *E. coli* and other diarrhoeagenic enteric bacterial pathogens isolated from patients with diarrhoea in Machakos District Hospital.

1.3.2 Specific objectives

- 1). To determine the prevalence of diarrhoeagenic *E. coli* and other enteric diarrhoeal pathogens from patients with diarrhoea in Machakos District Hospital.
- 2). To determine the nature of colonizing factor antigens from the strains of ETEC isolated from patients with diarrhoea.
- 3). To investigate the genetic basis of antimicrobial resistance in ETEC and other enteric bacteria pathogens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Microbial diarrhoea

Diarrhoea associated with bacterial, viral or parasitic infections is the most common infectious illness experienced by millions of children in developing countries, and also the major cause of disease in international travelers (WHO, 2009; Roy *et al.*, 2010; Mosquito *et al.*, 2011). According to the World Health Organization (WHO, 2009), more than 16 million deaths are due to infectious diseases. Out of these deaths, diarrhoea accounts for over 3 million. The main examples of diarrhoeal diseases associated with bacteria are cholera, dysentery, typhoid and diarrhoea due to ETEC (Bryce *et al.*, 2005). Cholera remains an important cause of illness in many developing countries and has been estimated to result in more than 120, 000 deaths each year. Bacillary dysentery due to either *Shigella dysenteriae* type 1 or Entamoeba contributes to a large degree of bloody diarrhoeal illness based on reports from Kenya, Egypt, Ethiopia and Nicaragua (John *et al.*, 2003; Johnie *et al.*, 2005; Rao *et al.*, 2003; Vilchez *et al.*, 2009). In children in developing countries, ETEC is responsible for more than 650 million diarrhoeal episodes and 2,000,000 deaths annually (Vilchez *et al.*, 2009; Wenneras and Erling., 2004; Cravioto *et al.*, 1998).

The most readily recognized bacterial agents of gastroenteritis in industrialized countries are *Salmonella spp* and *Campylobacter spp* (Al-Gallas *et al.*, 2007; Botelho *et al.*, 2003; Keskimaki *et al.*, 2001). Moreover, acute diarrhoea may be associated with a large number of other bacteria, such as *Yersinia enterocolitica*,

Staphylococcus aureus and *Bacillus*. The various shigellae can cause diarrhoea or the syndrome known as bacillary dysentery. In particular, incompletely prepared or incompletely heated food has been reported as the source of different diarrhoeal pathogens (Gillespie *et al.*, 2006; Murray *et al.*, 1995).

Additionally, other organisms such as parasites and viruses are common causes of diarrhoea around the world (Roy *et al.*, 2010). The protozoans are usually acquired by ingestion of food or water contaminated with faeces (Qadri *et al.*, 2007). Usually no symptoms result from the presence of these parasites in the colon, but some people experience abdominal pain and diarrhoea. Rotaviruses is a worldwide common cause of diarrhoeal disease and is more common in developing world (Cunliffe *et al.*, 2012; Shabir *et al.*, 2010; Vesikari *et al.*, 2007; Kheyami *et al.*, 2006; Parvathi *et al.*, 2011; Li *et al.*, 2004; 2009; Qadri *et al.*, 2007; Ahmed *et al.*, 2006). From these studies it is indicated that it is more common to children and vaccine trials have been successful for its management in addition to improved sanitation (Shabir *et al.*, 2010; Vesikari *et al.*, 2007; Kheyami *et al.*, 2006; Parvathi *et al.*, 2011). On the other hand, Caliciviruses have been shown to cause large outbreaks of diarrhoea in industrialized countries (Bucardo *et al.*, 2008; Nataro and Kaper, 1998).

2.1.2 Isolation, identification and common themes in virulence of *E. coli*.

E. coli is the type species of genus *Escherichia* which contains mostly motile Gram-negative bacilli within the family Enterobacteriaceae and the species *coli*. The most highly conserved feature of diarrhoeagenic *E. coli* strains is their ability to colonise the intestinal mucosal surface despite peristalsis and competition for nutrients

(Amisano *et al.*, 2011; Nishikawa *et al.*, 2002; Nataro and Kaper, 1998). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains. However, diarrhoeagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonizing ability and allow adherence to the small bowel mucosa, a site that is not normally colonized (Levine., 1987; Bryce *et al.*, 2005; Weintraub, 2007). Three general paradigms have been described by which *E. coli* may cause diarrhoea. Namely; enterotoxin production (ETEC and EAEC), invasion (Enteroinvasive *E. coli* (EIEC) and intimate adherence with membrane signalling (EPEC and STEC). However, the interaction of the organisms with the intestinal mucosa is specific for each category (Al- Gallas *et al.*, 2007). The versatility of the *E. coli* genome is conferred mainly by two genetic configurations namely, virulence-related plasmids and chromosomal pathogenicity islands. STEC, EPEC, EAEC and EIEC strains typically harbor highly conserved plasmid families, each encoding multiple virulence factors (Al-Gallas *et al.*, 2002). Chromosomal virulence genes of STEC and EPEC are organized as a cluster referred to as a pathogenicity island (Spano *et al.*, 2008; Shaheen *et al.*, 2004; McDaniel *et al.*, 1995).

2.1.3 Detection of *Escherichia coli* causing Diarrhoea

Escherichia coli can be recovered from clinical specimens on general or selective media incubated at 37°C under aerobic conditions. *E. coli* in stools are most often recovered on MacConkey enriched agar with either lactose or sorbitol sugars- or Eosin methylene blue agar, which selectively grow members of the Enterobacteriaceae and permit differentiation of enteric organisms on the basis of their morphology and colour (Adachi *et al.*, 2002; Murray *et al.*, 1995).

Identification of diarrhoeagenic *E. coli*, *Salmonella*, *Shigella* and *Vibrio* strains requires that these organisms be differentiated from non-pathogenic members of the normal flora.

Substantial progress has been made in the development of nucleic acid-based technologies. The use of DNA probes for detection of heat-labile (LT), heat-stable (ST), in ETEC, SEN and SET enterotoxins in *Shigella* revolutionized the study of these organisms by replacing the costly animal models used in toxin detection (Moseley *et al.*, 1982). Since then, gene probes have been introduced for all *Salmonella*, *Shigella*, *Vibrio* and diarrhoeagenic *E. coli* categories (Al- Gallas *et al.*, 2007).

Other widely used methods are Polymerase Chain Reaction (PCR) and Pulsed Field Gel Electrophoresis (Bischoff *et al.*, 2005; Kariuki *et al.*, 2004; Osek, 2001). They have been a major advance in molecular diagnostics of pathogenic microorganisms, including *Shigella*, *Salmonella*, *Vibrio* and *E.coli*. In PCR, a pair of primers (20-40 bases) is used for selective amplification and detection of a certain DNA sequence in a target organism. PCR primers have successfully been developed for all categories of diarrhoeagenic *E. coli*, *Salmonella*, *Shigella* and *Vibrio*. PCR can be used in both diagnosing and typing *Salmonella*, *Shigella*, *Vibrio* and *E. coli* strains. Advantages of PCR include high sensitivity, specificity and appropriate rapidity in the detection of target DNA templates. However, substances within stools such as heat labile proteins, complex polysaccharides and vegetative materials have been shown to interfere with PCR (Bryce *et al.*, 2005; Thornton and Passen., 2004; Stacy-Phipps *et*

al., 1995). It was concluded that PCR detects significantly more *Vibrio* and ETEC infections than does the standard probe based hybridization method (Caeiro *et al.*, 1999). In diagnostics, PCR is commonly used for detecting different virulence associated genes of *E. coli*, such as toxin and adherence associated genes as shown in (Table 2.1) below.

Table 2.1 Characteristics of the diarrhoeagenic *E. coli*

Category	Characteristic	Virulence determinants	Main targets	Published detection methods
	Serogroups			
	O groups	Toxins		
STEC	05,022,026,055,091, 0103,0111,0113,0117, 0118,012,801,450,157	StxI,Stx2, EAST1,Ehly, A/E,LEE	Stx1,Stx2	Olsvic and Strockbine, 1993 Cocolin <i>et al.</i> , 2003
SPEC	026,055,086,0111,0114, 0119,0125,0126,0128, 0158	A/E,EAF,BfP, LEE	Eae,EAF plasmid, <i>bfpA</i>	Jerse <i>et al.</i> , 1990 Gannon <i>et al.</i> , 1993 Heuvelink <i>et al.</i> , 1995
EAEC	03, 015, 044, 077, 086, 0110,0127 06,08,025,078,0126, 0148,	EAST1, AA	EA plasmid	Klena <i>et al.</i> , 2005
ETEC	0153,0169	LT,ST,EAST1	CFAs,elt,ext	Murray <i>et al.</i> , 1995 Yavzori <i>et al.</i> ,1998

Other methods employed for detecting certain types of *E. coli*, *Salmonella*, *Shigella* and *Vibrio* have been developed, especially for STEC. The latex agglutination test (Verotox-F assay, Denka Saiken, Tokio, Japan) for detection of toxins produced by STEC has been found to be 98% sensitive and 100% specific in comparison with the classical Vero cell assay (Beutin., 2006; Karmali *et al.*, 1999). Immunomagnetic separation with magnetic beads coated with antibody against *E. coli* O157 has been

found more sensitive than direct culture of these strains using sorbital MaCconkey (Chapman and Siddons, 1997).

2.1.4 Serotyping

Prior to the identification of specific virulence factors in *Shigella*, *Salmonella*, *Vibrio* and diarrhoeagenic *E. coli* strains, serotype analysis was the predominant means by which pathogenic strains were differentiated (Nataro and Kaper, 1998). In 1947, a scheme for the serologic classification of *E. coli* was proposed and this is still used in a modified form today (Vaux *et al.*, 2011). According to the modified Kauffmann scheme, *E. coli*, *Salmonella* *Shigella* and *Vibrio* are serotyped on the basis of their O (somatic), H (flagella), and K (capsular) surface antigens. However, only extra intestinal *E. coli* are encapsulated (Jann and Jann, 1992). Thus, among diarrhoeagenic *E. coli* and *Salmonella*, usually a specific combination of O and H antigens defines the serotype of the strain. *E. coli* of specific serogroups can be associated with certain clinical syndromes, but it is not in general the serologic antigens themselves that confer the virulence but the toxins such as heat stable, heat labile and hemolysins (Aslani *et al.*, 2009; Queenan *et al.*, 2007).

2.1.5 Genotyping

In recent years, the use of molecular “fingerprinting” methods has become standard practice in microbiology for evaluating the epidemiology of infectious diseases, investigating suspected outbreaks of bacterial infections, and typing bacteria (Bono, 2009). Pulsed-field gel electrophoresis (PFGE) allows the generation of simplified chromosomal restriction fragment patterns without having to resort to probe

hybridization methods (Boxrud *et al.*, 2007; Kandakai-Olukemi *et al.*, 2009; Hien *et al.*, 2008; Kariuki *et al.*, 2004). In this method, restriction enzymes that infrequently cut DNA are used for generating large fragments of chromosomal DNA, which are then separated by special electrophoresis (Alam *et al.*, 2006; Kariuki *et al.*, 2004). PFGE has been applied to subtyping of several Gram-positive and Gram-negative bacteria (Onyango *et al.*, 2009; Tenover *et al.*, 1997; Okeke *et al.*, 2000; Kariuki *et al.*, 2004; Geornaras *et al.*, 2001; Shaheen *et al.*, 2009). It is now widely used in epidemiological surveillance and common interpretation schemes are in place such as Tenover's method of analysis for fragment difference and binumeric software (Tenover *et al.*, 1997).

The Center for Disease Control and Prevention (CDC) initiated Pulse Net, a national computer network of public health laboratories that employs standard methods to subtype STEC O157:H7 strains (CDC 2001; 2004). Laboratories within the network can transmit PFGE patterns electronically to a databank at the CDC, where they are automatically compared with patterns of other isolates. If the patterns submitted by laboratories in different locations during a defined time period are found to match, the CDC computer will alert Pulse Net participants of a possible multistate outbreak (CDC 2006; Hunter, 2005; Kam *et al.*, 2008).

Ribotyping is a method based on DNA probes that recognize conserved RNA operon genes. Ribotyping is essentially a Southern blot analysis in which strains are characterized for restriction fragment length polymorphism of their individual ribosomal genes (Anjum *et al.*, 2007). Within a species, and particularly within a

strain, the DNA sequences and the restriction digest patterns of genes encoding rRNA are highly conserved and thus serve as a molecular fingerprint for that organism (Lutz *et al.*, 2010; Ballmer *et al.*, 2007). This technique is universally applicable and has also been accepted as a clinical tool for typing *Salmonella*, *Shigella*, *Vibrio* and *E. coli* (Parvathi *et al.*, 2011; Alavandi *et al.*, 2006; Fukui *et al.*, 2007; Bjorn –Arne, 2007; Tarkka *et al.*, 1994). All these molecular typing methods have allowed highly discriminant genotyping, and are useful tools for demonstrating that isolates from different sources are identical, closely related or not related at all (Al- Gallas *et al.*, 2007; Bertin *et al.*, 2004; Mickelsen 1997). Currently, there exist new and quicker techniques on market such as microarray and short gun sequencing that can identify and differentiate microorganisms and show their degree of relatedness in a shorter time (Lutz *et al.*, 2010).

2.1.6 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* strains are an important cause of diarrhoea worldwide in travelers from industrialised countries to less developed countries, and this is thought to be imported to the industrialized countries as shown by DuPont *et al* (1971) who first showed that ETEC strains were capable of causing diarrhoea in adult volunteers. Since then, several studies have been conducted and these showed that ETEC is a major cause of diarrhoea in children, adults and immunologically naïve population (Rodas *et al.*, 2011; WHO, 2009; Svennerholm *et al.*, 2008; Boschi-Pinto *et al.*, 2008; Al-Gallas *et al.*, 2007; Aranda *et al.*, 2007; Rao *et al.*, 2003). In general, ETEC strains are considered to represent a pathogenic prototype: the organism colonise the surface of the small bowel mucosa and express their enterotoxins,

leading to secretion of electrolytes and water (WHO, 2012). Whereas *Shigella*, *Vibrio* and rotavirus can be readily detected by standard assays, ETEC is more difficult to recognize and therefore it is now appreciated as being a cause of either infant diarrhoea or of cholera – like disease in all age groups (Rodas *et al.*, 2011; WHO, 2009; Gomez-Duarte *et al.*, 2010; Onyango *et al.*, 2009; Parvathi *et al.*, 2011). Among the six recognized diarrhoeagenic categories of *E. coli*, ETEC are most common, particularly in the developing world rather than the developed world (Vilchez *et al.*, 2009; Nishikawa *et al.*, 2002). Specific virulence factors such as enterotoxins and colonization factors differentiate ETEC from other categories of diarrhoeagenic *E. coli* (Sjoling *et al.*, 2007). ETEC belong to a heterogeneous lactose-fermenting *E. coli* comprising of a wide variety of O antigenic types, which produce enterotoxins, which may be heat labile and/or heat stable, and colonization factors which allow the organism to readily colonize the small intestine and thus, cause disease (Bolin *et al.*, 2006; Weiping *et al.*, 2006; Gutierrez-Cazarez *et al.*, 2000; Nataro and Kaper 1998).

2.1.6.1 Heat-stable (ST) and heat-labile (LT) enterotoxin production and other virulence associated factors

Enterotoxigenic *E. coli* is defined as *E. coli* strains that produce at least one of the two defined groups of enterotoxins: ST and LT enterotoxins (Roy *et al.*, 2010; Levine, 1987). Thus, identification of ETEC has long relied on detection of the enterotoxins. The use of DNA probes for detection of LT and ST enterotoxins in ETEC revolutionized the study of all diarrhoeal organisms by replacing the old and costly animal models of toxin detection with newer techniques (Gomez-Duarte *et al.*,

2010; Berlanda *et al.*, 2008; Forsberg and Petzold., 2007; Munoz *et al.*, 2006). Heat stable toxins (LTs) are large oligomeric toxins that are closely related in structure and function to the cholera toxin (CT) expressed by *Vibrio cholerae*. The two immunotypes of LT, LT-I and LT-II, share the same ganglioside receptor and mode of action, but are antigenically distinct (Svennerholm., 2011; Estrada-Garcia *et al.*, 2009).

The genes encoding LT (*elt* or *etx*) reside on plasmids that may also contain genes encoding ST and/or colonization factor antigens (CFAs) (Holmgren *et al.*, 2009; Anantha *et al.*, 2004; Heyman 2004; Nataro and Kaper 1998). In contrast to LTs, the STs are small, monomeric toxins that contain multiple cysteine residues, whose disulphide bonds account for the heat stability of these toxins (Nataro and Kaper, 1998). ST 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 (Usein *et al.*, 2009; Turner *et al.*, 2007; Duport *et al.*, 2006; Dietrich *et al.*, 2005; Jolly *et al.*, 2004). Besides the production of LT and/or ST, some ETEC strains may also express EAST1 and colonization factor antigens and coli surface antigens (Wajima *et al.*, 2011; Puiprom *et al.*, 2009; Chakraborty *et al.*, 2001).

ETEC strains have two major virulence determinants, enterotoxins and CFAs. To cause diarrhoea, ETEC strains must first adhere to small bowel enterocytes, an event mediated by surface fimbriae (Vidal *et al.*, 2009; Steinsland *et al.*, 2004; Gaastra and Svennerholm 1996). ETEC adhere to epithelial surfaces by means of CFAs and

putative colonization antigens, jointly referred to as CFs. There exist three major morphologic varieties of CFs: rigid rods; bundle-forming flexible rods; and thin flexible wiry structures (Rodas *et al.*, 2011; Puiprom *et al.*, 2009; Rivera *et al.*, 2010). At least 20 different CFs in ETEC pathogenic to humans have been described, but they are almost always encoded by plasmids also encoding ST and/or LT enterotoxins (Harro *et al.*, 2011; Blackburn *et al.*, 2009; Rao *et al.*, 2005; Rao *et al.*, 2003). ETEC strains cause diarrhoea through the action of these enterotoxins.

2.1.6.2 Infant diarrhoea and travelers' diarrhoea

Food and water are the most common vehicles for ETEC transmission (Huang *et al.*, 2006; Daniels *et al.*, 2000; Yates, 2005; Valentiner., 2003). Thus, faecal 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 (Steffen *et al.*, 2004; Steffen *et al.*, 2003; Levine 1987). Sampling of both food and water sources from areas of endemic ETEC have demonstrated high rates of ETEC contamination (Connor ., 2010; Estrada-Garcia *et al.*, 2009; Ashley *et al.*, 2004; Von wright *et al.*, 1981) where asymptomatic ETEC excretion is also commonly found (Abu-Elyazeed *et al.*, 1999; Valentiner., 2003). In developing countries, children under 2 years of age typically have two to three episodes of diarrhoea per year, with ETEC infections representing more than 25% of all these diarrhoea cases, and contribute significantly to the mortality within group (WHO, 2011; Mandomando *et al.*, 2007; Rappelli *et al.*, 2005; Okeke *et al.*, 2003). Oral rehydration therapy is often lifesaving in infants and children with ETEC

diarrhoea (Khaing-Khaing-Ghi, 2009; Steffen *et al.*, 2003; 2005; Jiang *et al.*, 2000; 2003; Insenbarger *et al.*, 2001; Black *et al.*, 1981;1984).

Although ETEC infection occurs most frequently in infants, immunocompromised adults are also susceptible and in addition, ETEC has been regarded as the most common pathogen in travelers' diarrhoea irrespective of the tourist destination (Javier *et al.*, 2011; Daniels, 2000; Steffen *et al.*, 2005; Adachi *et al.*, 2001). The percentages of ETEC, however, vary from study to study, from nation to nation, and from season to season (Greenwood *et al.*, 2008; Meraz *et al.*, 2008). Studies suggest that 20-60% of travelers visiting areas where ETEC infection is endemic, experience diarrhoea; typically, 20-40% of cases are believed to be due to ETEC (Porter *et al.*, 2010; Shah *et al.*, 2009; Freedman *et al.*, 1998; Steffen *et al.*, 2004; Jiang *et al.*, 2002; Nataro and Kaper, 1998). Many studies have investigated the effect of dietary self-restrictions on the risk of TD. However, the dietary self-restrictions have had none or just limited benefit. An oral cholera vaccine containing killed *V. cholerae* and purified cholera toxin B subunit has been reported to provide some protection against TD due to ETEC infections (Lopez *et al.*, 2005; Connor ., 2010; Gould *et al.*, 2009; WHO, UNICEF, 2009; Torrel *et al.*, 2009; Jeuland *et al.*, 2009; Landry, 2006; Steffen *et al.*, 2004; WHO, 2001). The said protection is presumably due to the structural and antigenic similarity between ETEC's heat labile toxin and Cholera Toxin (Lundkvist *et al.*, 2009; Tarantola *et al.*, 2008; Weinke *et al.*, 2008; Hill *et al.*, 2006; Sanchez *et al.*, 2005; Qadri *et al.*, 2005).

2.1.6.3 The History of ETECs

Escherichia coli was first suspected as being a cause of children's diarrhoea in the 1940s, when nursery epidemics of severe diarrhoea were found to be associated with particular serotypes of *E. coli* (Maity *et al.*, 2010; Qadri *et al.*, 2005; Daniels *et al.*, 2000). These specific serotypes, designated enteropathogenic *E.coli*, were epidemiologically incriminated as the cause of the outbreaks. Studies of rabbit ileal loops with these strains (Rodrigo *et al.*, 2009; Black *et al.*, 1981) showed only a poor correlation of fluid accumulation with the incriminated *E. coli* serotypes and were not definitive. Volunteer experiments confirmed that ingestion of large numbers of these organisms resulted in diarrhoea, and the ingested strains were recovered in the stools (Huang *et al.*, 2006; Qadri *et al.*, 2005; Gibbons *et al.*, 1977).

The history of enterotoxigenic *E. coli* was better understood from the 1950s (Ashley *et al.*, 2004; Steffen *et al.*, 2003) when live *E. coli* strains isolated from children and adults with a cholera-like illness were injected into ileal loops of rabbits, isolated and found that large amounts of fluid accumulated in the loops, similar to that seen with *Vibrio cholera* (Eric *et al.*, 2001; Daniels *et al.*, 2000). These *E. coli* isolates were found to produce a strong cholera-like secretory response in rabbit ileal loops, both as live cultures and as culture filtrates (Jiang *et al.*, 2002). The patients were also found to have antitoxin responses to the heat-labile enterotoxin produced by these organisms (Latife *et al.*, 2011; Qadri *et al.*, 2004; Jiang *et al.*, 2002; Waiyaki *et al.*, 1985; Donta and Smith, 1974). Similar studies have been done with animals that also demonstrated strains of *E. coli* to be responsible for diarrhoeal disease in several

animal species such as pigs, calves, and rabbits (Clermont *et al.*, 2011; Bauchard *et al.*, 2010; Daniels *et al.*, 2000). Studies of these animal enterotoxigenic *E. coli* paralleled and sometimes preceded those done with human strains; these organisms were also found to produce enterotoxins and specific colonization factors (Maity *et al.*, 2010; Daniels *et al.*, 2000; Qadri *et al.*, 2000; Waiyaki *et al.*, 1985).

2.1.6.4 Biology of ETECs

2.1.6.4.1 LT and ST Enterotoxins

Following the initial discovery of ETEC in humans, there was an intensive effort to further characterize its mechanisms of pathogenesis and means of laboratory identification (Huang *et al.*, 2006; Nataro and Kaper, 1998). Over a period of several years, its major virulence mechanisms were identified (Mandomando *et al.*, 2007; Hamond *et al.*, 2004). ETEC produce one or both enterotoxins; heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), that have been fully characterized, cloned, and sequenced, and their genetic control in transmissible plasmids was identified (Hamond *et al.*, 2004; Qadri *et al.*, 2000; Gill *et al.*, 1980). ETEC also produces one or more of many defined colonization factors (pili/fimbrial or nonfimbrial), also under plasmid control (Eitan *et al.*, 2011; Qadri *et al.*, 2000; Gaastra *et al.*, 1982; 1996).

LT was found to be very similar physiologically, structurally, and antigenically to cholera toxin and to have a similar mode of action (Biais *et al.*, 2010; Jiang *et al.*, 2002). The molecular mass 84 kDa (Qadri *et al.*, 2005) and the subunit structure of the two toxins were essentially identical, with an active (A) subunit surrounded by

five identical binding (B) subunits (Qadri *et al.*, 2007; Gill *et al.*, 1980). Following colonization of the small intestine by ETEC and release of the LT, the LTB subunits bind irreversibly to GM1 ganglioside, and the A subunit activates adenylate cyclase, which results in increases in cyclic AMP, which stimulates chloride secretion in the crypt cells and inhibits neutral sodium chloride in the villus tips. When these actions exceed the absorptive capacity of the bowel, purging of watery diarrhoea results (Roy *et al.*, 2010; Qadri *et al.*, 2006; Gill *et al.*, 1980).

2.1.6.4.2 Colonization Factors

More than 22 colonization factors (CFs) have been recognized among human ETEC and many more need to be characterized (Shaheen *et al.*, 2009; Huang *et al.*, 2006; Gaastra *et al.*, 1996). The CFs are mainly fimbrial or fibrillar proteins, although some CFs are not fimbrial in structure (Andersson *et al.*, 2011; Guereña-Burgueno *et al.*, 2002; Gillespie *et al.*, 2006; Evans *et al.*, 1973; Evans *et al.*, 1978). Notable among these is CS6, an antigen increasingly being isolated in recent studies (Isidean *et al.*, 2011; Shaheen *et al.*, 2009; Turner *et al.*, 2001). The CFs allows the organisms to attach and colonize the small bowel, thus allowing expression of either LT or both LT and ST in close proximity to the intestinal epithelium. Studies with humans as well as experimental animals have shown that CF-positive bacteria but not their isogenic CF-negative mutants colonize and induce diarrhoea (Clermont *et al.*, 2011; Qadri *et al.*, 2000).

All CFAs except CFA/I have the CS designation in the present designation. Some of the better-characterized CFs can be subdivided into different families; these include the colonization factor I-like group (including CFA/I, CS1, CS2, CS4, CS14, and

CS17)) and the coli surface 5-like group (with CS5, CS7, CS18, and CS20) and those that are unique (CS3, CS6, and CS10 to CS12) (Isidean *et al.*, 2011; Qadri *et al.*, 2005; Qadri *et al.*, 2004; Mariana *et al.*, 2000; Gaastra *et al.*, 1996; McConnell *et al.*, 1991; Evans *et al.*, 1973). Within each of the families above there are cross-reactive epitopes that have been considered as candidates for vaccine development (Turner *et al.*, 2011; Harro *et al.*, 2011).

Of the wide range of CFs, the most commonly present on diarrhoeagenic strains include CFA/1, CS1, CS2, CS 3, CS4, CS5, CS6, CS7, CS14, CS17, and CS21 (Harro *et al.*, 2011; Rodas *et al.*, 2010; Svennerholm *et al.*, 2004; 2008). These have been found on ETEC strains worldwide in various frequencies. However, CFs have not been detected on all ETEC, and on roughly 40% of strains worldwide have no known CFs. This could be due to the absence of CFs, or loss of CF properties on subculture of strains, or to lack of specific tools for their detection (Anderson *et al.*, 2011; Harro *et al.*, 2011; Shaheen *et al.*, 2009).

2.1.6.4.3 ETEC Serotypes

Besides determination of the toxins and CFs, serotyping, such as determination of O serogroups associated with the cell wall lipopolysaccharides and H serogroups of the flagella, has been used to identify and characterize ETEC (Dupont *et al.*, 2001; Dupont *et al.*, 2003). In early studies in Bangladesh, it was suggested that typing of the most prevalent serotypes might be used to identify ETEC (Shaheen *et al.*, 2009). However, as shown in numerous studies in different countries, clinical ETEC isolates may belong to a large number of serotypes. Furthermore, ETEC serotype profiles may change over time (Dupont *et al.*, 2001; Jiang *et al.*, 2003).

Based on an extensive database analysis of ETEC from a number of different countries all over the world, it was shown that in the ETEC antigen the largest variety was the 'O' antigen (Steinsland *et al.*, 2010; Svennerholm *et al.*, 2008; 2004; Qadri *et al.*, 2000; Darfeuille *et al.*, 1990). Thus, 78 different 'O' groups were detected in the 954 ETEC isolates included in the study (referred to as ETEC database). In addition, there were several rough strains that lacked side chains, thus being nontypeable with regard to 'O' antigen, or strains that had unknown serogroups. The most common 'O' groups in this retrospective study were O6, O78, O8, O128, and O153 that accounted for over half of the ETEC strains (Rodas *et al.*, 2009; Svennerholm *et al.*, 2008; Turner and Feil., 2007).

In Egypt, a large variation of 'O' groups was also recorded with 47 'O' groups being represented among the 100 ETEC strains isolated; however, an entirely different 'O' group pattern was recorded than in the database, the most common 'O' groups in the study being O159 and O43 (Shaheen *et al.*, 2009; Qadri *et al.*, 2000).

2.1.6.4.4 ETEC Strains in Animals

Enterotoxigenic *E. coli* is a major cause of severe diarrhoeal disease in suckling and weanling animals (Liu *et al.*, 2011; Gaastra *et al.*, 1996). Animal ETEC strains are known to produce enterotoxins similar to those of human strains and to possess species-specific CFs. The LT from animal strains, designated LT1, is similar to the LT produced by human ETEC, however, another variety designated LTII is only found in animals and is not associated with clinical disease (Steffen *et al.*, 2005; Steffen *et al.*, 2003; Girardeau *et al.*, 2003).

Animal strains produce two major types of ST, designated STa (STI) and STb (STII). STa, which is a small molecule of ca. 2.0 kDa, was the first of the enterotoxins to be identified in animals (Donta and Smith., 1974). As in humans, both STh (STIb) and STp (STIa) may be produced by animal strains. Animal strains (rarely human strains) can also produce STb, a slightly larger ST (ca. 5 kDa), which does not activate intracellular nucleotide levels and whose mechanism of action is poorly understood (Clermont *et al.*, 2011; Steinsland *et al.*, 2004).

2.1.6.5 Epidemiology of ETECs

2.1.6.5.1 Age-Related Infections in Children and Adults

Studies over the last few years have documented that ETEC is usually a frequent cause of diarrhoea in infants younger than 2 years of age (Estrada-Garcia *et al.*, 2009). In Egypt it was found to be the most common cause of diarrhoea in the study infants, accounting for about 70% of the episodes and the incidence was higher in males than females (Rao *et al.*, 2003). In an investigation in children 0 to 5 years in Mozambique, Brazil and Bangladesh, 90% of cases of ETEC diarrhoea reporting to the hospital were aged between 3 months and 2 years (Latife *et al.*, 2011; Mandomando *et al.*, 2007; Qadri *et al.*, 2000; Rao *et al.*, 2003).

The susceptibility of infants and young children has also been observed in other settings which 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 (Mandomando *et al.*, 2007; Bueris *et al.*, 2007; Rao *et al.*, 2003). Studies to better understand the natural infection pattern of ETEC are being conducted with cohorts of infants to discern the

infection and reinfection pattern as well as the age group most at risk for infection. In studies of infants in East and south Africa, Egypt, and Bangladesh, the rate of ETEC infections in community-based studies increased from about 3 to 6 months of age, similar to the surveillance data of hospitalized patients in a diarrhoeal hospital in Bangladesh (Sabrina *et al.*, 2007; Mandomando *et al.*, 2007; Steinsland *et al.*, 2004; Qadri *et al.*, 2006). The age at which a primary ETEC infection can be documented depends to some extent on the phenotype of ETEC that is infecting the child. It was reported that in the youngest age group, 3 months, ETEC strains producing STh and LT were most common, whereas at 6 to 7 months ETEC strains producing STp, STp LT, and SThLT predominated (Steinsland *et al.*, 2004; Shaheen *et al.*, 2009).

The incidence of ETEC infections in developing countries decreases after 5 years of age with a decrease of infections between the ages of 5 to 15 years. The incidence increases again in those over 15 years of age and about 25% of ETEC illness is seen in adults (Usein *et al.*, 2009). Limited epidemiological information is available for adults, and those available are mostly from Vietnam and Bangladesh (Hien *et al.*, 2008; Qadri *et al.*, 2006). It was in these settings that ETEC was first described extensively and was shown to be a cause of adult diarrhoea resembling cholera in the severity of infection. It thus became obvious that adults with severe dehydrating cholera-like illness attributable to ETEC infections are not uncommon (Harris *et al.*, 2008). In hospitalized patients, adults often present with more severe forms of ETEC diarrhoea than children and infants. Interestingly, further analyses have shown that the elderly are also susceptible to ETEC infections requiring hospitalization (Black *et*

al., 2003). ETEC was found to be the second most frequently isolated (13%) bacterial pathogen after *V. cholerae* O1 20% (Yoder *et al.*, 2006).

2.1.6.5.2 Relation to Presence of LT, ST, and Colonization Factors

Indeed, ETEC expressing LT only have been considered less important as pathogens, especially since they are more frequently isolated (than the other two toxin types) from healthy persons than from patients (Gaastra *et al.*, 1996; Qadri *et al.*, 2000). This could be related to the low prevalence of CFs on the LT-producing ETEC strains (Gaastra *et al.*, 1996; Clemens *et al.*, 2004). Thus, in many epidemiological studies, the CFs have been detected on less than 10% of LT-producing ETEC strains, compared to over 60% of the ST- and LT/ST-expressing ETEC. LT-producing ETEC strains may be highly pathogenic, given that they may have been isolated from patients with severe dehydrating diarrhoea (Clemens *et al.*, 2004).

A comparison of the toxin pattern of the infecting strains in patients hospitalized with diarrhoea in the different age groups shows that the toxin phenotype did not change with age (Clemens *et al.*, 2004). In longitudinal studies with infants, both LT and ST phenotypes of ETEC were found to be associated with diarrhoea (Abu-Elyazeed *et al.*, 1999; Cravioto *et al.*, 1990; Cravioto *et al.*, 1988). This has been shown to be the case also in hospital (Martin *et al.*, 2003; Martin *et al.*, 1999) as well as community-based studies (Shaheen *et al.*, 2009; Abu-Elyazeed *et al.*, 1999). However, in hospital-based studies, ETEC producing both LT and ST or ST alone were found to cause relatively more severe disease than those caused by LT-producing ETEC

strains (Isidean *et al.*, 2011; Shaheen *et al.*, 2009; Qadri *et al.*, 2004; Martin *et al.*, 1995).

Although over 22 CFs have been detected on ETEC, only six to eight are more frequently isolated from diarrhoeal stools (Clemens *et al.*, 2004; Nashimura *et al.*, 2002). Of these, CFA/I and CS1 to CS6 are the predominant types (Shaheen *et al.*, 2009; Gaastra *et al.*, 1996). These CFs are mostly present on ETEC producing ST or both LT and ST. It is believed that immunity to strains that express the nonimmunogenic ST is derived from the anti-CF response to the protein adhesins. Thus, in the development of vaccines, these CFs as well as LT are being included to give a broad-spectrum protection (Svennerholm *et al.*, 2011; Turner *et al.*, 2011; Onyango *et al.*, 2009; Tobias *et al.*, 2010).

2.1.6.5.3 Single versus Mixed Infections

Coinfection with ETEC and other enteric pathogens is common, which may lead to problems in determining if the symptoms are caused by the actual ETEC infection and understanding the actual pathogenesis of the infection (WHO, 2009; Steffen *et al.*, 2004; Clemens *et al.*, 1990). Mixed infections are frequent and may be seen in up to 40% of cases (Black *et al.*, 1993). The presence of enteric pathogens in asymptomatic persons is also known to be high in areas of poor sanitation. The incidence of mixed infections seems to increase with age in studies in Brazil and fewer copathogens were seen in infants than in older children and adults with ETEC diarrhoea (Clemens *et al.*, 2004; Mariana *et al.*, 2000). In cases of mixed infections in children, rotavirus is the most common, followed by other bacterial enteropathogens such as *V. cholerae*, *Campylobacter jejuni*, *Shigella* spp.,

Salmonella spp., and *Cryptosporidium* (Parvathi *et al.*, 2011; Oundo *et al.*, 2008; Ahren *et al.*, 2006; Kariuki *et al.*, 2004; John *et al.*, 2003; Martin *et al.*, 1999; Waiyaki *et al.*, 1985; Ngugi *et al.*, 1985). In travelers' diarrhoea, enteroaggregative *E. coli* and *Campylobacter* spp. have been common pathogens together with ETEC (Oundo *et al.*, 2008; Huang *et al.*, 2006; Adachi, 2002).

2.1.6.5.4 Seasonality of ETEC

Several studies have reported that ETEC diarrhoea 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 (Nweze, 2010; Harris *et al.*, 2008; Huang *et al.*, 2006; WHO, 2006; Qadri *et al.*, 2005; Jiang *et al.*, 2002). 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. Furthermore, with the advent of rains in the monsoon season, there is enhanced contamination of surface water with fecal material and the surface water can thus become heavily contaminated (Mariana *et al.*, 2000; Donta and Smith., 1974). Seasonality for the different toxin phenotypes has also been suggested, with ST-producing ETEC strains being more common in the summer whereas LT-producing ETEC strains are present all year round and do not show any

seasonality (Chowdhury *et al.*, 2010; Harris *et al.*, 2008; Okeke *et al.*, 2003; Abu-Elyazeed, 1999).

2.1.6.5.5 Comparison of ETEC Diarrhoea and Cholera in Children and Adults

In Bangladesh and Tunisia cholera and ETEC diarrhoea are still endemic just as in any developing country (Al-Gallas *et al.*, 2007; Mandomando *et al.*, 2007; Qadri *et al.*, 2005; Gaastra *et al.*, 1982 Clemens *et al.*, 1990). Both diseases share a biannual periodicity, peaking once in the spring and again in the autumn and remaining endemic all year (Mandomando *et al.*, 2007). In the spring ETEC infections appear to be more prevalent than *V. cholerae* O1 infections. In 4-year cholera surveillance study in Bangladesh it was found that ETEC and not *V. cholerae* was often the cause of the diarrhoea in some of the field areas (Faruque *et al.*, 2004; Sack *et al.*, 2000; Bhandari *et al.*, 2002). In Kenya, Cholera out breaks are more common just after on set of rains (Jiang *et al.*, 2002). It is also not surprising to have concomitant outbreaks of both ETEC and *V. cholerae* during peak seasons and during outbreaks in Bangladesh (Faruque *et al.*, 1998; Chakraborty *et al.*, 2001). Active screening for ETEC needs to be carried out in outbreaks and epidemics for both epidemiological and public health purposes.

2.1.6.5.6 Presence of ETEC in Food and Water in the Environment

Diarrhoea due to ETEC, like other diarrhoeal illnesses, may be the result of ingestion of contaminated food and water (Begun *et al.*, 2007; Daniels *et al.*, 2000). In any situation where drinking water and sanitation are inadequate, ETEC is usually a major cause of diarrhoeal disease. Surface waters in developing countries have been found to harbour these organisms and transmission can occur while bathing and/or

using water for food preparation. These forms of transmission are common in areas where it is endemic both in the local populations and in international travelers to these areas (Kiranmayi, 2010; Oksuz *et al.*, 2004; Jo *et al.*, 2004).

Transmission of ETEC by processed food products outside of the developing world is less commonly seen but well documented (Daniels *et al.*, 2000). It was found that of 240 isolates of *E. coli* from foods of animal origin in the United States, 8% were contaminated with ETEC which produced either or both LT and ST (CDC, 2011; Kiranmayi, 2010; Hammerum, 2009). None of these food products were associated with diarrhoeal outbreaks. In studies carried out in the 1970s in Sweden, however, outbreaks of diarrhoea due to food-borne ETEC were reported (Danielson *et al.*, 1979). Similar findings were reported from Brazil where 1.5% of 1,200 *E. coli* strains from processed hamburger or sausage were found to be ETEC (Mariana *et al.*, 2000).

Contaminated food and water sources both contribute to seasonal outbreaks, which affect tourists. Thus, ETEC is a cause of travelers' diarrhoea more often in the warm than in the cool season. Studies done in Kenya and Bolivia has shown that ETEC could be isolated from sewage-contaminated rivers (Leonor *et al.*, 2011; Ahmed *et al.*, 2009; Kwena *et al.*, 2003; Black *et al.*, 2003; Waiyaki *et al.*, 1985). Furthermore, contaminated food and water were found to be the source of ETEC infections in Lima, Peru (Black *et al.*, 1989).

Surface water sources in Bangladesh, in both rural and urban areas, and in rural areas in Kenya are highly contaminated with ETEC (Qadri *et al.*, 2004; Waiyaki *et al.*,

1985). Thus, in these studies in Bangladesh and Kenya in 1981 and 1985 respectively, ETEC strains were obtained from clinical samples as well from ponds, rivers, and lakes around the clinical field sites.

2.1.6.5.7 ETEC Infections and Malnutrition

As in other diarrhoeal diseases, malnutrition is a risk in more severe enteric infections, including those caused by ETEC, possibly due to the immunocompromised nature of the host that also predisposes these individuals to a greater bacterial load on the mucosal surfaces of the gut than the well-nourished child (Brown, 2003).

Diarrhoeal illness including that caused by ETEC was found to be more severe in children with malnutrition (Cooke ., 2010; Leonor *et al.*, 2011; Mondal *et al.*, 2012). Micronutrient deficiency such as vitamin A and zinc is quite common in developing countries and generally increases the morbidity due to diarrhoeal illnesses. Zinc enhances innate immunity (Alaullah *et al.*, 2011; Daniels *et al.*, 2000). It has been estimated that in Mexico over 40% of children younger than 5 years of age may have zinc deficiency (Chakraborty *et al.*, 2001). Supplementation with zinc increases the adaptive immune responses to cholera vaccination in children and adults and in children with shigellosis (Alaullah *et al.*, 2011; Saidi *et al.*, 2010; Martin *et al.*, 2003; Daniels *et al.*, 2000). The effect of micronutrient deficiency on the morbidity and protective immune responses in ETEC diarrhoea is an area that needs more attention for management.

Other factors, such as breast-feeding, may have the capacity to prevent ETEC diarrhoea (Ahren *et al.*, 1986). Factors in milk such as specific secretory immunoglobulin A antibodies, glycoconjugates and receptor analogues as well as innate and anti-inflammatory factors may all contribute to decrease the infection (Schaible *et al.*, 2007). This is supported by the fact that protective antibacterial antibodies in breast milk have been shown to be directed primarily against *V. cholerae* O1 polysaccharide (Keusch *et al.*, 2003; Glass *et al.*, 1983). Hyperimmune bovine colostrum containing high titers of ETEC CF antibodies has been shown to provide temporary protection against ETEC challenge (Mandomando *et al.*, 2007; Freedman *et al.*, 1998). Breast feeding reduces overall diarrhoea and mortality (Léonor *et al.*, 2011). A reduction in diarrhoeal episodes has been seen in infants who had been breastfed for the first 3 days of life, irrespective of other dietary practices, emphasizing the positive effects of colostrum (Cooke *et al.*, 2010; Pelletier and Frongillo, 2003).

2.1.6.5.8 ETEC infections in International Travelers

ETEC remains endemic all year round but is highest during the warm season, reflecting the seasonal difference of ETEC and other bacterial enteropathogens in the country visited suggesting that travelers are more vulnerable to the diarrhoeal illnesses at these times (Javier *et al.*, 2011; Nicholas and Daniels, 2005; Martin *et al.*, 2003). In travelers, the phenotypes of ETEC strains vary from country to country. LT- ETEC was more commonly isolated from visitors to Jamaica (Steffen *et al.*, 2003). LT/ST ETEC was most often seen in visitors to India (Martin *et al.*, 2003) while ST- ETEC in visitors to Kenya (Steffen *et al.*, 2003; Jiang *et al.*, 2002; Daniels

et al., 2000). Strains that are circulating in a particular country, infecting primarily children, and contaminating the water and food sources (as well as the hands of the food handlers) may determine the type of ETEC infecting the travelers. Travelers to such countries do not know the cause of their diarrhoeal illness since it cannot be identified on site. The data available suggest that from 20% to 40% of traveler's diarrhoea cases may be caused by ETEC and the children resident in those countries have rates of 20% of hospitalized diarrhoeal episodes caused by ETEC (Javier *et al.*, 2011; Jiang *et al.*, 2002). Thus, ETEC seems to be the most frequent cause of traveler's diarrhoea in North Americans and Europeans visiting developing countries (Shah *et al.*, 2009; Meraz *et al.*, 2008; Ericsson, 2003).

2.1.6.5.9 Clinical features of ETEC

2.1.6.5.9.1 Disease Severity

The diarrhoeal disease caused by ETEC that was recognized in 1970s and 1980s consisted of a cholera-like illness in both adults and children (Fleckenstein *et al.*, 2006; Black *et al.*, 1989). Since then, other studies around the world have shown that ETEC-induced diarrhoea may range from very mild to very severe (Liu *et al.*, 2011; Clermont *et al.*, 2011). There are, however, short-term, asymptomatic carriers of the organisms (Jiang *et al.*, 2002; Black, 1990). The diarrhoea 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 lead to dehydration from the loss of fluids and electrolytes (sodium, potassium, chloride, and bicarbonate) in the stool (Fleckenstein *et al.*, 2006). 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 the diarrhoea lasts only 3 to 4 days and is self-limiting, and if hydration is maintained, the patients survives 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 cholerae* (Bradley *et al.*, 2011; Ahren *et al.*, 1986; 1998) 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 diarrhoea 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 (Meraz *et al.*, 2008; Black *et al.*, 1981).

In comparison with children, adults with ETEC diarrhoea seem to have more dehydrating illness, requiring longer hospitalization and more intravenous fluid management. This may be because of more delay in reaching a treatment facility or other underlying conditions. In children, rotavirus and ETEC diarrhoea share similar

clinical characteristics but differ from cholera in being less severe (Ahmed *et al.*, 2006).

2.1.6.5.9.2 Mortality from ETEC Diarrhoea

Mortality data due to ETEC infections are difficult to estimate. Similar to cholera, if patients with severe ETEC disease are treated on time, mortality should be low (Javier *et al.*, 2011; Yoder *et al.*, 2006). Although untreated cholera patients may have a high mortality (50%), untreated ETEC patients would be expected to have a lower mortality rate based on the lesser severity of overall illness (Porter *et al.*, 2010; Insenbarger *et al.*, 2001). In a World Health Organization report, it has been suggested that there are 380,000 deaths annually in children less than 5 years of age that are caused by ETEC (WHO, 1999). However, there are no well-documented mortality figures for ETEC-induced diarrhoea, because the microbiologic diagnosis cannot be done easily in many settings, and therefore only rates for cholera, which is cultured easily, can be accurately determined. Most of the time ETEC-related deaths would be counted as diarrhoeal deaths in many countries and it is presumed, however, that there is significant mortality in patients not receiving treatment (Nicholas and Daniels., 2005; Jiang *et al.*, 2002).

2.1.6.5.10 Laboratory assays for ETEC

Since ETEC is recognized by the enterotoxins it produces, diagnosis must depend upon identifying either LT and/or ST. Because the assays necessary are very cumbersome, it is thought that some other marker could be a proxy in identification

(Shaheen *et al.*, 2009). Initially the serogroups of ETEC were identified and found to be relatively few, and therefore it was thought that perhaps serotyping could be used to differentiate ETEC from other *E. coli*, including the enteropathogenic strains whose characteristic serotypes are known (Black *et al.*, 1981). Serotyping use was found limited (Echeverria *et al.*, 1982; 1983; 1984) and when it became clear that a very large number of *E. coli* serotypes could be enterotoxigenic, this was abandoned.

Direct identification of the enterotoxins of ETEC has evolved over the past years. Physiological assays, the rabbit ileal loop model for LT and the infant mouse assay (Qadri *et al.*, 2004) for ST were initially used as the gold standards before other simpler assays could be identified. Since LT was strongly immunogenic whereas ST was not, diagnostic assays developed along different lines. Direct action of LT on two tissue culture cell lines, Y1 adrenal cells (Donta and Smith., 1974) and Chinese hamster ovarian cells (Gill *et al.*, 1980), were found to provide physiological responses that could be detected by morphological changes in tissue culture. These changes were specific for LT and could be neutralized by antitoxin. The two tissue culture assays were widely used for LT recognition until the development of the enzyme-linked immunosorbent assay technology in 1977 (Black, 1990). Other assays such as staphylococcal coagglutination (Chapman and Siddons., 1997), passive latex agglutination (Black, 1993), immunoprecipitation in agar, and the Biken test (Alam *et al.*, 2003) were found to be specific but were not used widely for diagnostic purposes. Enzyme-linked immunosorbent assays became a widely used method for detecting LT, particularly using microtiter GM1 ganglioside methods (Black, 1993). Subsequently, combined GM1 enzyme-linked immunosorbent assays for ST and LT

were developed and have been used in different epidemiological studies (Binsztein *et al.*, 1991).

ST testing in infant mice continued to be used widely and could be enhanced by the use of culture pools, thereby minimizing the numbers of infant mice (Alam *et al.*, 2003). From the 1980s methods using molecular diagnostic techniques began and these are the methods that are being improved on (Moseley *et al.*, 1982; Altboum *et al.*, 2001) and have shown that the genes controlling the enterotoxins could be detected using P-labeled DNA probes derived from plasmids for both LT and ST. This method was shown to be specific and sensitive and could detect as few as 1 to 100 CFU per gram of material (Echeverria *et al.*, 1982). Variations of this technology, including both polynucleotide and oligonucleotide probes with both radioactive and non-radioactive labeling, have been found to be useful in detecting ETEC both in clinical and environmental samples and is widely used (Echeverria *et al.*, 1978;1982; 1984; 1989; Martin *et al.*, 1999).

In 1993, PCR was first used in ETEC diagnosis (Black *et al.*, 1993). It was found to be useful for diagnosis directly on fecal material as well as isolated colonies. It has also been adapted to a multiplex form so that the diagnosis of LT- and ST-producing organisms as well as other diarrhoeagenic *E.coli* can be made simultaneously (Martin *et al.*, 2003). Currently, PCR and sequencing are the commonest and standard methods for detecting ETEC (Steinsland *et al.*, 2003; 2010; Rodas *et al.*, 2009; Turner *et al.*, 2007). In addition, DNA probes, with either radioactive or nonradioactive detections or GM1 enzyme-linked immunosorbent assays using

monoclonal antibodies against ST or LT have been the most widely used methods for detection of ETEC toxins (Clemens *et al.*, 2004).

2.1.6.5.10 Treatment and Management of ETEC

The treatment of diarrhoeal disease due to ETEC is the same as that for cholera or any other acute secretory diarrhoeal disease (WHO, 2011; 2012; 1990; 1999). The correction and maintenance of hydration is always most important and Pharmacological therapy is limited to micronutrient support (Guarino *et al.*, 2008; Elliott *et al.*, 2007). Provision of adequate nutrition is critical in children in the developing world, where most diarrhoeal diseases are frequent (Leonor *et al.*, 2011; Pelletier and Frongillo., 2003; Ahmed *et al.*, 2009). The guidelines for therapy of all diarrhoeas have been widely disseminated by the World Health Organization (WHO, 1993; 2009).

2.1.6.5.11 Rehydration

Rapid rehydration using intravenous fluids (such as Ringer's lactate) is required initially for all patients with severe dehydration. After restoration of blood pressure and reversal of major signs of dehydration, patients can be put on oral rehydration solutions for the remainder of therapy. For all other patients with lesser degrees of dehydration, therapy with oral rehydration solutions alone can be used. In management of acute gastroenteritis in children, thorough history and examination with evaluation of hydration status, nutritional status and comprehensive clinical evaluation for any complications or associated illness must be made. Based on this, a decision is made on method of rehydration, feeding, and if there are any indications

for special investigations (Forsberg and Petzold., 2007; Elliott *et al.*, 2007; Alam *et al.*, 2003).

2.1.6.5.12 Antimicrobials

The use of antimicrobials in the treatment of ETEC diarrhoea is problematic, since an etiologic diagnosis cannot be made rapidly (King *et al.*, 2009). This differs from the treatment of cholera, an epidemic disease, where clinical findings and rapid laboratory tests can readily lead to correct diagnosis. In the treatment of cholera, antimicrobials form an integral part of therapy because they lead to a marked decrease in stool output and shortening the course of disease (Steffen *et al.*, 2003). Childhood diarrhoeas however, are caused not only by ETEC but also by other bacterial and viral agents. Clinical presentations are not sufficient to differentiate them making it difficult to study the effect of antimicrobials in children with ETEC disease. Antimicrobials are therefore not used routinely in treatment of childhood diarrhoea (Cooke., 2010; Forsberg and Petzold., 2007). ETEC diarrhoea treatment using Tetracyclin in adults (determined retrospectively) showed only a minimal effect on the severity or duration of diarrhoea (Leonor *et al.*, 2011; Ouyang-Latimer *et al.*, 2011).

Antimicrobials, however, are of definite value in the treatment of travelers' diarrhoea, a diarrhoeal syndrome in which the clinical symptom is well recognized and ETEC is known to be the most frequent pathogen (Hill *et al.*, 2006; Gill *et al.*, 1980). It should be noted, however, that antimicrobials used for travelers' diarrhoea will treat not only ETEC but also most of the other known causes (enteroaggregative

E. coli, *Shigella*, and *Campylobacter*) of the diarrhoea (Pazhani *et al.*, 2011). Though the management of diarrhoea is oral rehydration, treatment is recommended to reduce the shedding and spreading of resistance strains.

2.1.6.5.13 Multidrug Resistance in ETEC

The antibiotic use has changed since the late 1970s, when doxycycline and trimethoprim-sulfamethoxazole were the drugs of choice. Due to increasing microbial resistance of ETEC, newer drugs have been developed (King *et al.*, 2009; Pacheco *et al.*, 2006). A fluoroquinolone such as ciprofloxacin, levofloxacin, or ofloxacin are currently the drugs of choice, since no significant resistance to these drugs has developed (Kariuki *et al.*, 2004; Ericsson, 2003). A newer non-absorbed drug, rifaximin, has also been shown to be as effective as a fluoroquinolone and was approved for use in the United States and Peru (Bischoff *et al.*, 2002; DuPont *et al.*, 2001). Multidrug resistance is increasing in ETEC due to the widespread use of chemotherapeutic agents in countries where diarrhoea is endemic. Antimicrobial sensitivities, however, have only been studied extensively in international travelers and during common source outbreaks of disease or specific epidemiologic studies in areas where diarrhoea is endemic (Smith *et al.*, 2010; Raghunath *et al.*, 2008). The primary reason for this is the difficulty of recognizing the organisms in some developing countries with limited capacity.

A single conjugative plasmid carried genes for both antibiotic resistance and enterotoxin production, the result of recombination of an R factor with an enterotoxin-carrying plasmid (Cohen *et al.*, 2005; 2010). Studies in different

developing countries reported that antibiotic resistance and the ability to produce enterotoxin were frequently transferred together and suggested that the widespread use of antibiotics could result in an increase in enterotoxigenic strains (Rodas *et al.*, 2011; Nunes *et al.*, 2003; 2011). Plasmids coding for both antibiotic resistance and ST could be transferred *in vitro* to *E. coli* K-12 and *in vivo* in suckling mice, suggesting that antibiotic selective pressure could result in a wider distribution of ETEC (Cohen *et al.*, 2000; 2010; Onyango *et al.*, 2009).

A marked increase in resistance in ETEC was first reported in 1980s, when it was found that during a cruise ship outbreak the epidemic strain O25: NM was resistant to tetracycline and sulfathiazole (Ahren *et al.*, 1986; 1998) and in a hospital outbreak; all isolates of the epidemic strain were also resistant to tetracycline. Resistance has been reported to more than four antibiotics (Onyango *et al.*, 2009; Al-Gallas *et al.*, 2007).

2.1.6.5.14 Nutritional and Micronutrient Therapy

Diarrhoea causes loss of Zinc in the stools, which exacerbates the zinc deficiency in children with acute diarrhoea. Studies have demonstrated that addition of zinc to the therapy of diarrhoea (ORS) in children with acute diarrhoea leads to an estimated reduction in duration of illness by 16% and a decrease in mortality (Muna *et al.*, 2007; Kwena *et al.*, 2003; Bhandari *et al.*, 2002; Golden *et al.*, 1985). The mechanism for the effect of Zinc treatment on the duration of diarrhoea is mainly inhibition of cAMP induced Cl secretion by blocking basolateral membrane K channels and improving absorption of water and electrolytes by the intestines

(Bettger *et al.*, 1981). In addition, Zinc sulphate has an antimicrobial effect on enteric pathogens (Surjawidjaja *et al.*, 2004).

Nutritional therapy for all childhood diarrhoeas, including those due to ETEC, is an integral part of diarrhoea treatment. Episodes of diarrhoea due to any cause, including ETEC, result in decreased nutritional status and thus inhibit growth in children (Alam, 2003). Attention to providing food, particularly breast milk, early in the course of therapy is essential. Additional food during and following the diarrhoeal episode will help in catch-up growth (Qadri *et al.*, 2007; Bhandari *et al.*, 2002; Ahmed *et al.*, 1999).

2.1.6.6 Prevention of ETECs

2.1.6.6.1 Vaccine Development

Prevention of ETEC infection is clearly related to water and sanitation, including food preparation and distribution. In the developing world, such major improvements will be a long time coming. It is estimated that it would take US\$200 billion to make the improvements necessary to prevent fecally spread diseases in developing world (Harro *et al.*, 2011; Eric *et al.*, 2001; Clemens *et al.*, 1990). Other methods on a microscale are presently being done: building safe-water wells, chlorination/filtration/heating of drinking water, and building and improving latrines. These attempts to block transmission are certainly effective if implemented but cannot solve the problem quickly. Therefore, there is much interest in the development of vaccines for prevention of ETEC disease (Harro *et al.*, 2011; Waiyaki *et al.*, 1985).

Based on the great impact of ETEC infections on morbidity and mortality, and probably also on nutritional status (Leonor *et al.*, 2011; Cravioto *et al.*, 1990), particularly of children in areas where they are endemic, an effective ETEC vaccine is highly desirable (WHO, 2006). Such a vaccine is feasible since epidemiologic evidence and results from experimental challenge studies with human volunteers have demonstrated that specific immunity against homologous strains follows ETEC infection (Clemens *et al.*, 2004). Furthermore, multiple infections with antigenically diverse ETEC strains seem to lead to broad-spectrum protection against ETEC diarrhoea (Turner *et al.*, 2011). Experimental studies with animals and indirect evidence from clinical trials suggest that protective immunity against ETEC is mediated by secretory immunoglobulin A antibodies directed against the CFs, other surface antigens, and LT; ST, which is a small peptide, but does not elicit neutralizing antibodies following natural infection (Arne *et al.*, 2010; Svennerholm *et al.*, 2008).

2.1.6.6.2 Purified CFs and Enterotoxoids

Various purified CFs have been tested as oral immunogens but have been considered less suitable since they are expensive to prepare and sensitive to proteolytic degradation (Altboum *et al.*, 2001). To protect the fimbriae from degradation in the stomach, purified CFs have been incorporated into biodegradable microspheres. However, no significant protection was induced by any formulation of purified CFs against subsequent challenge with ETEC expressing the homologous CFs, either when immunizing with high doses of a combination of CS1 and CS3 or recombinantly produced CS6 (Harro *et al.*, 2011; Glenn *et al.*, 2005; 2009). Since

LTB as well as the immunologically cross-reactive cholera toxin B subunit are strongly immunogenic and lack toxicity, they are stable in the gastrointestinal milieu, and are capable of binding to the intestinal epithelium. This makes them suitable candidate antigens to provide anti-LT immunity. The cholera toxin B subunit has also afforded significant protection against ETEC producing LT or LT/ST both in countries where ETEC is endemic and in travelers but it is possible that an LT toxoid might be slightly more effective than cholera toxin B subunit (Turner *et al.*, 2011; Arne *et al.*, 2010).

An alternative administration route that has been considered is to give an ETEC vaccine by the transcutaneous route. Such administration of *E. coli* CS6 together with LT has induced immune responses against CS6 in about half of the volunteers and anti-LT responses in all of the volunteers (Liu *et al.*, 2011; Harro *et al.*, 2011).

2.1.6.6.3 Inactivated Whole-Cell Vaccines

Another approach that has been extensively attempted is to immunize orally with killed ETEC bacteria that express the most important CFs on the bacterial surface together with an appropriate LT toxoid, that is, cholera toxin B subunit or LTB (Richard *et al.*, 2007; Svennerholm *et al.*, 2004). A vaccine that consists of a combination of recombinantly produced cholera toxin B subunit and formalin-inactivated ETEC bacteria expressing CFA/1 and CS1 to CS5 as well as some of the most prevalent O antigens of ETEC has been extensively studied in clinical trials in travelers as well as in children in areas where ETEC is endemic (WHO, 2009; Eric *et al.*, 2001; Daniels *et al.*, 2000). This recombinant cholera toxin B (rCTB)-CF ETEC

vaccine has been shown to be safe and gave rise to significant immunoglobulin A immune responses in the intestine and increased levels of circulating antibody-producing cells in a majority of adult Swedish volunteers (Ahren *et al.*, 1998). The vaccine has also been well tolerated and given rise to mucosal immune responses against the different CFs of the vaccine in 70% to 100% of volunteers of different age groups from 18 months to 45 years in Egypt and Bangladesh (Qadri *et al.*, 2004; Eric *et al.*, 2001). However, due to an increased frequency of vomiting in the youngest children (6 to 18 months), a reduced dose of the vaccine, for example a quarter dose that can be given safely and with retained immunogenicity to infants has been identified (Caeiro, 2003).

2.1.6.6.4 Live Oral ETEC Vaccines

The potential of live ETEC vaccines has been suggested based on previous findings in human volunteers that a live vaccine strain expressing different CSs afforded highly significant protection against challenges with wild-type ETEC expressing the corresponding CS factors (Martin *et al.*, 2003). For example, different live multivalent *Shigella*/ETEC hybrid vaccines have been constructed in which important fimbrial CFs are expressed along with mutated LT (Svennerholm *et al.*, 2004; Sizemore *et al.*, 2004; Alboum *et al.*, 2001). Such vaccine candidates have expressed CS2 and CS3 fimbriae or CFA/I, CS2, CS3, and CS4 as well as a detoxified version of human LT (Sizemore *et al.*, 2004). These candidate vaccine strains are still under evaluation for safety and immunogenicity in different animal models, including macaques (Levin *et al.*, 2001).

2.2 Shiga toxin producing *E. coli* (STEC)

The recognition of STEC as a distinct class of pathogenic *E. coli* resulted from two epidemiological observations (Cheng *et al.*, 2005). Riley, *et al.* (1983) investigated two outbreaks characterised by severe abdominal pain and watery diarrhoea followed by bloody diarrhoea (Iman *et al.*, 2011). This illness, designated as HC, was associated with the ingestion of undercooked hamburgers at a fast food restaurant chain. Stool cultures from these patients yielded a rare *E. coli* serotype O157:H7. The second was by Karmali *et al.* (1983) who reported the association of sporadic cases of Haemolytic Uremic Syndrome (HUS) with faecal cytotoxin and cytotoxin producing *E. coli* in stools. The two clinical microbiological observations, one based on a rare *E. coli* serotype and the other on the production of a specific cytotoxin, led to the recognition of a novel and increasingly important class of enteric pathogens causing intestinal and renal diseases.

2.2.1 Nomenclature

The discovery of STEC along distinct paths of investigation resulted in a parallel nomenclature, a situation that still exists (Cohen *et al.*, 2005). The term Verotoxigenic *E. coli* or Vero cytotoxin producing *E. coli* (VTEC) was derived from the observation that these strains produced a toxin that was toxigenic for Vero cells (Olesen *et al.*, 2005). The term VTEC is still widely used in the United Kingdom and in many European scientific publications. The term enterohaemorrhagic *E. coli* (EHEC) was originally coined to denote strains that cause HC and HUS (Nataro and Kaper, 2006). According to the latest nomenclature (Maity *et al.*, 2010), these strains are called Shiga toxin-producing *E. coli* (formerly Shiga like toxin-producing *E. coli*

(SLTEC), a term that reflects the cytotoxin produced by these strains (Cohen *et al.*, 2005).

2.2.2 Shiga toxins (Stx) and other factors affecting the pathogenic properties of STEC

Shiga toxin producing *E. coli* strains produce Stx toxins, also known as Vero toxins (VT) or Shiga-like toxins (Slt). Stx toxin is essentially identical at the genetic and protein levels to the Stx-toxin produced by *Shigella dysenteriae* 1. The Stx family contains two major, immunologically non-cross-reactive groups called Stx1 and Stx2, encoded by the *stx1* and *stx2* genes (Iman *et al.*, 2011; Nataro and Kaper, 2006). Both toxins are composed of five B subunits (encoded by *stxB*) and a single A subunit (encoded by *stxA*) and both, *stxA* and *stxB*, are located on a temperate bacteriophage inserted into the STEC chromosome. A single STEC strain may express Stx1 only, Stx2 only, or both toxins, and even multiple forms of Stx2. The prototypical Stx1 and Stx2 toxins, respectively, have 55% and 57% sequence identity in A and B subunits. While Stx1 is highly conserved, sequence variation exists in Stx2. Three types of Stx2 have been identified: Stx2, Stx2c and Stx2e (Kilic *et al.*, 2007; Botelho *et al.*, 2003). The subtype Stx2e is classically associated with pig oedema disease rather than human disease, but occasional strains that express only this variant are isolated from patients with HUS (Clermont *et al.*, 2011). The various subtypes are wholly interchangeable between the Stx and VT nomenclatures Stx1=VT1=Slt1, Stx2e=VT2e=Slt2e (Carattoli *et al.*, 2005). Most molecular diagnostic methods for STEC are aimed at the detection of genes encoding Stx (Olsvik and Strockbine 1993; Cocolin *et al.*, 2003). Improved diagnostics have

enabled detection of more STEC types found in patients with HC or some other STEC associated disease, and new clones continue to emerge, for example Stx2-producing STEC O26:H11 found in Germany (Jiang *et al.*, 2002; Cohen *et al.*, 2005).

The only potential STEC adherence factor that has been demonstrated as playing a role in intestinal colonisation is the 94-97 kDa outer membrane protein intimin. It is encoded by the *eae* gene and produces extensive attaching-and-effacing (A/E) lesions in the large intestine, featuring intimate adherence of the bacteria to the epithelial cells (Blanco *et al.*, 2006; Afset *et al.*, 2003; 2004; 2006; Blanco *et al.*, 2003). This *eae* gene is also found in EPEC.

The *eae* is only one of many genes located on the 35 kb pathogenicity island called the locus of enterocyte effacement (LEE), which confers the A/E phenotype for EPEC (Nataro and Kaper, 2006). The STEC LEE contains genes encoding intimin, translocated intimin receptor (Tir), the secreted proteins EspA and EspB, and a type III secretion pathway (Blanco *et al.*, 2006; Blanco *et al.*, 2005; Blanco *et al.*, 2004c). Formation of A/E lesions depends upon interaction between bacterial outer membrane protein (intimin) and bacterially encoded receptor protein Tir, which is exported from the bacterium and translocated into the host cell membrane (Paton *et al.*, 1998; Bischoff *et al.*, 2005). The true roles of EspA and EspB are unknown, but type III secretion systems are responsible for secretion and translocation of critical virulence determinants found in a variety of Gram-negative human, animal and plant pathogens (Smith *et al.*, 2010; Garrido *et al.*, 2006; Kimata *et al.*, 2005; Mora *et al.*, 2004).

2.2.3 Sources of STEC infections

STEC can be found in the faecal flora of a variety of animals including cattle, sheep, goats, pigs, cats and dogs (Clermont *et al.*, 2011; Boerlin *et al.*, 2006; Boerlin *et al.*, 2005; Fairbrother *et al.*, 2005). The most important animal species in terms of human infection is cattle, but the prevalence of STEC O157:H7 in cattle varies significantly from country to country. Surveys of cattle have found STEC O157:H7 in 1.3% of animals in the United Kingdom (Chapman *et al.*, 1997). STEC strains are usually isolated from healthy animals, but may be associated with an initial episode of diarrhoea in young animals followed by asymptomatic colonization (Fairbrother *et al.*, 2005). The STEC O157:H7 isolation rates from animals are much lower than those of non- O157 serotypes. The main route of STEC into the food chain is through contamination of meat by intestinal contents and faeces in the abattoir (Girardeau *et al.*, 2003; Bingen-Bidois *et al.*, 2002; Bouckennooghe *et al.*, 2000). STEC are transmitted to humans by food, water, and from person to person. Most cases are caused by ingestion of contaminated foods, particularly foods of animal origin, beef being a major vehicle of infection (Bauchard *et al.*, 2010; Trabulsi *et al.*, 2002).

2.3 Enteropathogenic *E. coli* (EPEC)

During the 1920s in Germany, it was shown by serologic typing that strains of “dyspepsiekoli” could be implicated in outbreaks of paediatric diarrhoea. In 1955, the term enteropathogenic *E. coli* was first coined referring to those strains of *E. coli* that had been epidemiologically linked with childhood diarrhoea (Cheng *et al.*, 2005). It was defined for decades solely on the basis of ‘O’ and ‘H’ serotypes found typically

in diarrhoeal children younger than two years (Johnson *et al.*, 2006; Kehl *et al.*, 2002). However, this definition has changed drastically in recent years as knowledge of this organism has increased. EPEC is still an important category of diarrhoeagenic *E. coli*, which then was linked to infant diarrhoea in industrialised countries and now in the developing world (Manning *et al.*, 2007; Johnson *et al.*, 2006; Kehl *et al.*, 2002).

2.3.1 Attaching-and-effacing (A/E) adherence of EPEC strains

According to a recent resolution, a diarrhoeagenic *E. coli* that produces a characteristic histopathology known as attaching-and-effacing (A/E) and does not produce Shiga toxins can be called EPEC (Ochoa *et al.*, 2008). Genes involved in EPEC pathogenesis are similar to those implicated for STEC except for the Stx-encoding phage on STEC chromosome and the presence of some other proposed virulence determinants found in STEC (Prere *et al.*, 2006; Nataro and Kaper, 1998). The A/E lesion is characterised by intimate adherence between bacteria and epithelial cells and effacement of intestinal microvilli. A/E can be observed in intestinal biopsy specimens from patients or infected animals (Rodas *et al.*, 2011; Bielaszewska *et al.*, 2007; 2008).

The gene necessary for mediating A/E has been identified and termed *eae*. The *eae* gene codes for a protein called intimin, which is required for full virulence of EPEC (Afset *et al.*, 2006; Brooks., 2005). Similar A/E lesions are seen in animal and culture models of many STEC strains and *Hafnia alvei* isolated from children with diarrhoea (Clermont *et al.*, 2011; Gould *et al.*, 2009). The overall pattern for these

eae sequences from different bacteria shows high conservation in the N-terminal region and variability in the C-terminal region (Feng *et al.*, 2007). The role of intimin in human disease was demonstrated by studies on volunteers, who ingested an isogenic *eae* negative mutant of EPEC E2348/69 (O127:H6). Diarrhoea was seen in all volunteers who ingested the wild-type E2348/69 compared with four of 11 volunteers who ingested the isogenic mutant (Blanco *et al.*, 2006).

2.3.2 Epidemiology of EPEC infections

As with other diarrhoeagenic *E. coli*, transmission of EPEC is fecal-oral, with contaminated hands, contaminated food, or contaminated fomites serving as vehicles. In adult outbreaks, waterborne and foodborne transmission has been reported, but no particular type of food has been implicated as more likely to serve as a source of infection (WHO, 2011; Raffaelli *et al.*, 2007). The most notable feature of the epidemiology of disease due to EPEC is the striking age distribution seen in persons infected with this pathogen (Vu Nguyen *et al.*, 2005). EPEC infection is primarily a disease of infants younger than 2 years (Beutin *et al.*, 2003). The reason for the relative resistance of adults and older children is not known, but loss of specific receptors with age is one possibility (Garrido *et al.*, 2006). However, EPEC can cause diarrhoea in an adult if the bacterial inoculum is high enough. The infectious dose in naturally transmitted infection in infants is not known, but it is presumed to be much lower than adults (Prere *et al.*, 2006; Cheng *et al.*, 2005; Olesen *et al.*, 2005).

Although several outbreaks of diarrhoea due to EPEC have been reported in healthy adults in industrialised countries (Cheng *et al.*, 2005; Banatvala *et al.*, 2001), little is known about the current status of EPEC as a diarrhoeagenic agent in most developing countries, since it is not routinely assayed for from stool samples. In Germany and Australia, EPEC was detected with PCR and Microarrays in 3.6% of children's diarrhoeal samples which exceeded the rates of *Salmonella*, *Shigella* and *Campylobacter* (Beutin *et al.*, 2003).

2.4 Enteroaggregative *E. coli* (EAEC)

EAEC, often also referred to as EaggEC, has been epidemiologically implicated as one of the etiological agents of diarrhoea in industrialised and developing countries (Oundo *et al.*, 2008; Nataro *et al.*, 2006; Huang *et al.*, 2004). The group is heterogenous, comprising a diverse range of serotypes that possess a variety of putative virulence factors (Kaur *et al.*, 2010; Huang *et al.*, 2004; 2006; 2008).

2.4.1 Aggregative adhesion (AA) and other proposed virulence characteristics

EAEC strains are defined as *E. coli* strains that do not secrete enterotoxins LT or ST and that adhere to HEp-2 cells in an aggregative adhesion (AA) pattern (Thomazini *et al.*, 2011; Araujo *et al.*, 2007; Adachi *et al.*, 2001). The pathogenesis and the site of EAEC infection in the human intestine are not well understood. However, a characteristic histopathologic lesion and several candidate virulence factors have been determined (Huang *et al.*, 2008). Virtually all EAEC strains carry a 60 Mda plasmid that contains a gene that confers AA and a gene that encodes EAEC heat-stable enterotoxin-1 (EAST1) (Huang *et al.*, 2006; Berner *et al.*, 2002). However, the

role of EAST1 in diarrhoea has not yet been determined, although EAST1 clones yield net increases in shortcircuit current in the rabbit mucosa when the chamber model is used (Elias *et al.*, 1999; 2002).

2.4.2 Diarrhoea caused by EAEC

Studies have shown the association of EAEC with diarrhoea, most prominently with persistent diarrhoea (>14 days). In most studies conducted, EAEC have been detected either with PCR or with a DNA probe (Scavia *et al.*, 2008; Iwanaga *et al.*, 2002). Although most reports have implicated EAEC in sporadic endemic diarrhoea, an increasing number of reports have described EAEC also in outbreaks. In Brazil, EAEC has been detected in up to 68% of persistent diarrhoea cases (Nashimura *et al.*, 2002). In India, EAEC have been associated with persistent diarrhoea and cases of sporadic diarrhoea detected in household surveillance (Bangar *et al.*, 2008; Bhardwaj *et al.*, 2006). Interestingly, in Brazil, the isolation of EAEC from the stools of infants was associated with a significantly lower height and/or weight of the EAEC carriers than the mean of the populations, irrespective of the presence of diarrhoeal symptoms (Piva *et al.*, 2003; Elias *et al.*, 2002).

In Germany 2% of children with diarrhoea have EAEC in their stools (Christoph *et al.*, 2010). In Austria, EAEC was the most common *E. coli* group found in patients with diarrhoea. In Japan, EAEC O113:H10 caused a large outbreak where 2,697 school children developed severe diarrhoea. In the United Kingdom, four outbreaks of diarrhoea have been reported due to EAEC strains representing several different serotypes (O19, O62, O73, O86, O113, O116, O125, O134). Each of the outbreaks

was associated with consumption of a restaurant meal, but no single source could be implicated (Kaur *et al.*, 2010; Smith *et al.*, 2010; Huang *et al.*, 2008; CDC, 2006). In another British and Vietnamese study, EAEC were the most common diarrhoeagenic *E. coli* group detected in diarrhoeal patients (Vu Nguyen *et al.*, 2006; Torres *et al.*, 2005; 2001). In the United States and Western Nepal, EAEC have been linked with diarrhoea in human immunodeficiency virus-infected patients (Kaur *et al.*, 2010; Wilson *et al.*, 2006).

2.5 Enteroinvasive *E. coli* (EIEC)

EIEC strains are often biochemically atypical and difficult to identify. Antigenically, biochemically and pathogenically these strains resemble *Shigella* so much that the illness caused by EIEC has often been mistaken for shigellosis (Ruiting *et al.*, 2004). Clinical features include fever, abdominal cramps, malaise, toxemia and watery diarrhoea or typical dysentery with blood, mucus and many faecal leukocytes (Hong *et al.*, 2007). Both *Shigella* spp. and EIEC have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci. In addition, both have one or more secretory enterotoxins that may play roles in diarrhoeal pathogenesis (Maurelli *et al.*, 2007; Cohen *et al.*, 2005). True prevalence of EIEC diarrhoea is not known. Endemic sporadic disease occurs in some areas, generally where *Shigella* spp. are also prevalent, but the epidemiologic features may be different from those of *Shigella* spp (Landry *et al.*, 2006). EIEC has also been associated with diarrhoea occurring in travellers and with outbreaks of food poisoning due to ingestion of contaminated food (Kingombe *et al.*, 2005; Steffen *et al.*, 2004).

2.6 Diffusely adherent *E. coli* (DAEC)

The term diffusely adherent *E. coli* (DAEC) was initially used to refer to any Hep-2-adherent *E. coli* strain that did not form EPEC-like microcolonies (Rappelli *et al.*, 2005; Nataro and Kaper 1998). With the discovery of EAEC, most authors now recognise DAEC as an independent category of potentially diarrhoeagenic *E. coli* (Ochoa *et al.*, 2008; Bouguenec and Servin., 2006). Little is known about the pathogenic features of DAEC induced diarrhoea and no PCR assay has been described as identifying DAEC. In Santiago, Chile and India, the relative risk of DAEC in association with diarrhoea increased with age from 1 to 4-5 years and the reason for such an age-related phenomenon is unknown (Rajandran *et al.*, 2010; Spano *et al.*, 2008). Strains of DAEC account for a large proportion of diarrhoea cases among hospitalised patients in France and Brazil who have no other identified enteropathogen (Huang *et al.*, 2006; Lopez *et al.*, 2005; Scaletsky *et al.*, 2002).

The main vehicles of transmission of diarrhoea in the developed world are food from animal origin such as hamburgers and dairy products due to failure in meeting adequate cooling, storage, preparation and hygienic standards. Food may be contaminated by faecal flora due to improper hygienic practices by the preparers (Rodas *et al.*, 2011; Jiang *et al.*, 2002; Kariuki *et al.*, 2004; Jay *et al.*, 2004; Crump., 2002). In many developed countries hamburgers are prepared and stored in close proximity to raw meat in butcheries and that the same cutlery used on raw meat is also used to prepare meat for cooking and this may create chances for cross contamination and transmission (Jay *et al.*, 2004). In the developing countries such as Egypt, Tanzania, Kenya, Ghana, Cameroon, Nigeria, Mali, South Africa and

Zambia, the most common vehicle of transmission is contaminated water (Cooke, 2010; Aibinu *et al.*, 2007; Jiang *et al.*, 2002; Waiyaki *et al.*, 1985). People tend to use untreated water for drinking and domestic chores or even share it with animals. In some Kenyan rural settings people share river water with animals despite the water being contaminated with faecal material of animal origin (Waiyaki *et al.*, 1985).

Diagnostic methods for the rapid detection of contaminating microorganisms have improved over the course of time. The methods evolved from normal culture methods, vero cell assays, animal models, Nucleic acid based technologies, use of DNA probes, PCR and mPCR, microarrays, sequencing as well as the PFGE which allows the generation of simplified chromosomal restriction fragment patterns for epidemiological surveillance. Enteric pathogens vary regionally. Practical variations include detection methods used, CFA, CS and toxin production, antibiotic patterns, plasmid distribution and serotypes. What ultimately counts is that the capability that is developed can be used in the management and control of diarrhoea. A prime candidate will be the development of vaccines.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This was a cross - sectional study in which samples from patients of all age groups presenting with diarrhoea were collected from September 2006-March 2008 to study ETEC, other *E. coli* pathotypes and other enteric bacterial pathogens in Machakos County.

3.2 Study site.

The study was carried out at the Machakos County (1⁰ 31' 0''S, 37⁰ 16' 0'' E), which is situated 65kms South East of Nairobi. The County borders the Nairobi County and Kajiado County to the West, Thika and Maragua to the north, Mbeere and Mwingi to the East, Makueni and Kitui Counties to the south. The main inhabitants of the County are the Akamba people and the main economic activity is subsistence farming in which animal and crop husbandry are practiced. The major animals kept are cattle, sheep, goats and chicken. Several rivers including Athi, Mwanja and Thwake are the main sources of water in the County. For most of the year, the County is dry though it receives short low seasonal rainfall. The major endemic diseases in the district according to the Ministry of Health Plan 2002-2008 are malaria, diarrhoea, acute respiratory infections (ARI) and skin infections.

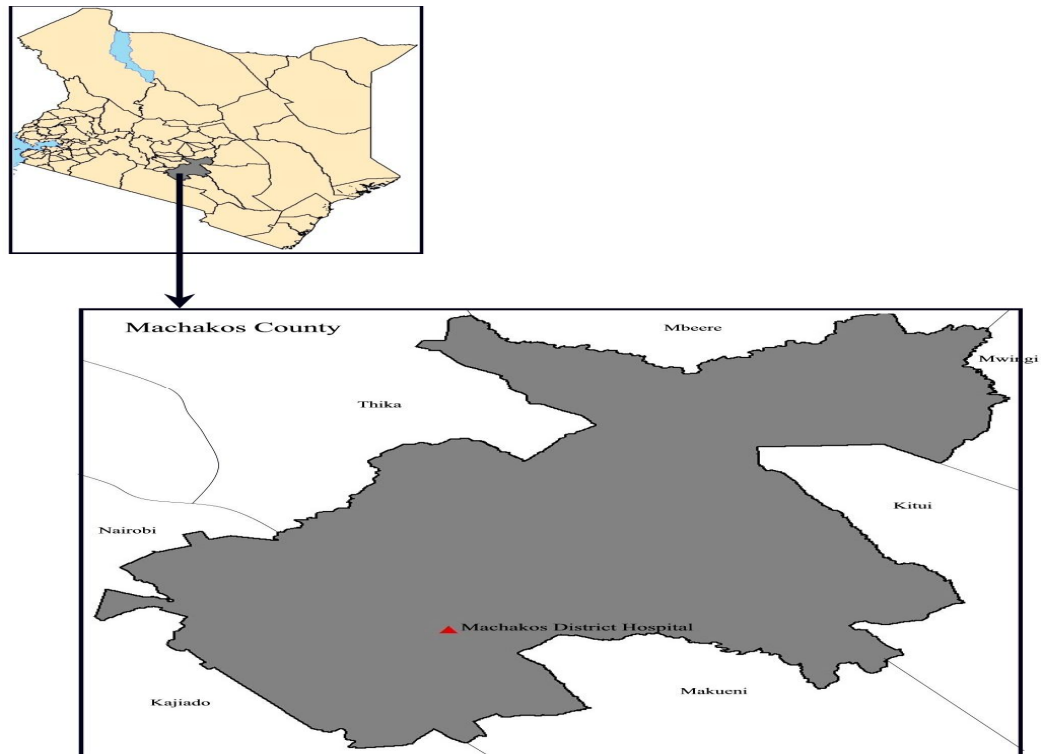


Figure 3.1 Maps of Kenya and Machakos County

3.3 Isolates

E. coli, *Shigella spp* and *Salmonella spp* isolated from stool samples obtained from patients presenting with the symptoms of diarrhoeal disease in Machakos District Hospital during the study period (2006-2008) were analysed. The specimens were collected only after the patient had met the inclusion criteria and given a signed consent to the study clinician. Stool samples were collected in a standard stool cup and transferred immediately to Carry Blair transport medium after macroscopic examination for consistency (loose, mucoid and or bloody). The stool was immediately shipped with documentation to the KEMRI-CMR-USAMRU-K-Nairobi Laboratory for culture and identification. The samples were cultured immediately after they arrived in the Nairobi Laboratory for biochemical identification, serology,

PCR, PFGE and sequencing. All identified and speciated bacterial isolates from the samples were stocked and stored.

3.4 Study population

3.4.1 Inclusion criteria.

Patients aged three months old and above presenting with:

- 1). Three or more loose, watery, mucoid or bloody diarrhoea in the last 24 hours.
- 2). Those consenting to inclusion in the study
- 3). Those presenting with abdominal pain / cramps.
- 4). Those presenting with fever and / or headache with or without vomiting.

3.4.2 Exclusion criteria.

- 1). Those presenting with signs other than the ones described in 3.4.1.
- 2). Those declining consent for participation.
- 3). Those who were below the age of 3 months.

3.5 Sample size.

Assuming a sample size of 29% ETEC isolation (El - Gendy *et al.*, 1999) and degree of freedom at 95% confidence interval, the total number of samples was:

$$N = \frac{Z^2 (1-a) p (1-p)}{D^2} \quad (\text{Harper, 1980})$$

Where:

N= Minimum samples required during the study

Z= 1.96 (Standard error of the mean)

A= Absolute precision at 5%

P= 0.29 prevalence of *E. coli*

D= 0.05 absolute precision

The sample size (N) required will be:

$$N = \frac{1.96^2 (1-0.05) 0.29(1-0.29)}{0.05^2}$$

N= 300.574

N= 301 Samples.

3.6 Specimen collection

After taking demographic information that is; place of birth, place of residence, occupation, type of contact, type of toilet used, source of drinking water, prescription drug use in the past two weeks, pulse and temperature were recorded on the questionnaire (Appendix 2), stool samples that were described as bloody, mucoid, watery or loose were collected from all patients presenting to the hospital and meeting the inclusion criteria.

3.6.1 Stool collection

After the patient or guardian had been thoroughly briefed about the study and given signed consent to participate in the study, a single stool sample was taken in a sterile stool cup. In the case of a minor, the guardian or parent gave the consent. Care was taken during stool collection to avoid urine, soil or water mixing with the stool because these could interfere with the microorganisms of interest. Part of the sample

was transferred to the Carry Blair transport medium (MML Diagnostics Packaging inc.). It was mixed well, filling up to the red fill line indicated. Care was taken to ensure that the cap(s) were tight during mixing. Any blood or mucous present in the stool was recorded, and included as part of the sample. In cases where a patient was unable to produce stool, a rectal swab was taken using a sterile cotton swab (Harwood Products Company, Gilford, Maine, U.S.A). The cotton swab was inserted in the rectum sphincter about 1-2 cms and rotated 360°C twice and removed. The swab was observed physically for the presence of fecal material and placed in the Carry Blair medium. The sample was then labeled with a unique study number and packaged in the plastic zip log packs, and placed in the cool box ready for shipment to the KEMRI-CMR/USAMRU-K Laboratory for processing. The samples were delivered within six hours after collection to avoid loose of some fastidious bacteria.

3.7 Laboratory procedures.

3.7.1 Microbial procedures (culture)

The stool samples upon arrival at the laboratory were macro examined, recorded and plated immediately on different selective, differential, enriched and nonselective media for target pathogens and incubated for 18 to 24 h at 37°C. The aerobic media used included Sorbitol, Lactose MacConkey, Xylose-Lysine Desoxycholate, Selenite F and TCBS. Nutrient and Mueller Hinton agar were used for subcultures and antibiotic susceptibility testing respectively. Representative colonies from each plate were subcultured for purity on nutrient agar and then used for biochemical testing (Heuvelink *et al* 1995) and sterotyping. The colonies were selected as follows: Two

to three colonies (black, medium and small size pink or clear) from XLD for suspected *Salmonella* spp and *Shigella* spp; 5-6 colonies (5-lactose and one non-lactose fermenters) from Lactose MacConkey for different *E. coli* pathotypes including ETEC; both yellow and green colonies from TCBS for *Vibrio cholerae* and *Vibrio parahemolyticus* respectively, and one clear colony from Sorbital MacConkey to detect *E. coli* 0157:H7. Isolates confirmed as *E. coli* using TSI (A/A, -H₂S), Simmon citrate (green), motile, and indole positive were confirmed for different pathotypes including ETEC by use of mPCR. Those confirmed as ETEC were further subcultured on CFA agar before colonization factor antigens(CFAs) and CSs were sought using salting out (Binsztein *et al.*, 1991) and later on identified by another Multiplex PCR using highly specific oligonucleotide primers for CFAs and CS (Klena *et al.*, 2005).

3.7.2 Isolation and identification of *E. coli* pathotypes

Samples were plated immediately upon arrival in the laboratory on freshly prepared and quality controlled Lactose/Sorbital MacConkey agar and incubated at 37°C for 18 - 24 h. Five to six pink colonies (lactose fermenters) were picked based on their morphological characteristics (size, shape) and analysed through the three major biochemical tests [triple sugar iron (TSI), motility, indole and ornithine (MIO), and Simmon's citrate (SC)]. The positive results for positive *E. coli* were read as TSI: A/A, +- Gas and -H₂S. MIO: + motility, +indole, +-ornithine; Simmon's citrate: negative. Those confirmed as *E. coli* were further tested by using multiplex PCR for pathotype detection. This was done using specific primers (Table 3.1). The selected primer sequences matched completely with the genes of corresponding *E. coli*

pathogroup in the gene bank. The pathotypes oligonucleotides were combined as follows: VT1, VT2, VT2e, eae; CNF1, Enteroaggregative; CNF2 and Enteroinvasive. Pairs were chosen for ease of differentiating the band sizes based on the ladder. After amplification, 20 µl of the product was loaded to a 2% agarose and run using electricity. After the DNA has run more than three quarters of the gel, the power was disconnected, and the gel transferred to the alpha imager hood, visualized under ultra violet light and picture taken using alpha imager for documentation.

3.7.3 Characterisation of *E. coli* pathotypes by mPCR.

The DNA from all the isolates identified as *E. coli* was analysed by mPCR using specific primers as indicated in Table 3.1. For DNA extraction, fresh isolates (18-24 h old) were inoculated in 2 mls freshly prepared and quality controlled brain heart infusion broth and incubated at 37°C for 18-24 h and the DNA extracted and stored at -20°C (QIAamp DNA extraction kit handbook).

Table 3.1 Primers, amplification, conditions and positive controls used for mPCR

TARGET	Primer sequence	AMPLICON SIZE(bp)	REFERENCE	REACTION CONDITIONS(°C)	CONTROLS
STEC	CAGTTAATGTGGTGGCGAAG	894	Olsvik and Strockbine 1993	95 , 95 ,62 ,72 ,72	ATCC 43894,
stx STEC	CTGCTAATAGTTCTGCGCATC CTTCGGTATCCTATTCCCGG	478	Olsvik and Strockbine 1993	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	RH 3536 ATCC 43894,
stx EPEC	CGATGCATCTCTGGTCATTG TGCGGCACAACAGCGGCGA	629	Heuvelink <i>et al</i> 1995	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	RH 3536 RH 4283(=EPEC E234869)
eac	CGGTCGCCGCACCAGGATTC CTGGCGAAAGACTGTATCAT	630	Schmidt <i>et al</i> 1995	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	RH 4260(=E.coli 17-2)
EAEC	CAATGTATAGAAATCCGCTGTT TCTCTATATGCACACGGAGC	321	Olsvik and Strockbine 1993	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	ATCC 35401
LT	CCATACTGATTGCCGAAT	186	Frankel <i>et al</i> 1989	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	ATCC43886
EPEC	TCTCTATGTGCATACGGAGC TCTGTATTGCTTTTTTCACC	186	Frankel <i>et al</i> 1989	1', 5', 1', 1', 5', 95 , 95 ,62 ,72 ,72	ATCC 35401
ST	TTAATAGCACCCGGTACAAGC			1', 5', 1', 1', 5',	

The primers with different amplicon sizes for detection of different *E. coli* pathotypes. Abbreviations are as indicated on page xvii.

3.7.4 Multiplex Chain Reaction amplification for *E. coli* pathotypes

The DNA obtained from the above process was analysed by mPCR amplification to detect different *E. coli* toxins. For every toxin a master mix was made up of 15.2 µl distilled water, 2.5 µl of 10X PCR buffer, 1.5 µl , 0.5 µl dNTPs (25mM each nucleotide), primers, 0.3µl taq polymerase and 4 µl template DNA. The mastermix was then completely mixed by tapping the tube and quick short spinning. The master mix cocktail was meant for one reaction. To run 50 samples each component was increased by a factor of 50.

After completion of the master mix, a 21 µl volume of the mastermix was pipetted directly into the bottom of a sterile microeppendorf tube for each reaction and the

tubes labeled using a permanent marker with specific color coding for VT, eaea, Eagg, Einv, CNF1 and CNF2. After transfer of the cocktail, 4 µl of template (DNA) was added in respective tubes and adequate mixing was ensured by quick spinning to collect the reaction mixture at the bottom of the tube. After spinning, the tightly capped tubes were placed in the temperature block (thermal cycler) according to toxins being investigated (based on colour codes) and ensured that each tube was firmly seated by pressing on the tubes individually using a plastic roller. Sterile distilled water and positive control strains of *E. coli* pathotype toxins were used as negative and positive controls, respectively. For amplification, the PCR program was set at 95°C for 1min followed by 72°C for 1 min for 5 cycles; 95°C for 1 min followed by 62°C for 1min and then 72°C for 1 min for 20 cycles; and 72°C for 5 min for final elongation of the amplified DNA product.

The PCR products were then separated by agarose gel electrophoresis by adding 5µl of loading dye containing 0.25% bromophenol blue in 40% sucrose to a 25µl reaction mixture and loaded 10 µl to a 2% agarose gel. The buffer in the electrophoresis chamber and in the agarose gel was 1X tris-borate EDTA which also contained 1 µg/ml ethidium bromide. Eighty volts and 25mA were applied across the gel for migration of the amplified DNA. The DNA was visualized by exposing the gel to ultra violet light and photographed on alpha imager gel documentation machine. Since the Ethidium bromide used is a health hazard, the gel was immediately neutralized by a destainer and safely discarded in a double biohazard bag.

3.7.5 Characterization of ETEC CFAs

3.7.5.1 Salting out.

All isolates identified as *E. coli* by growth on Lactose MacConkey as lactose fermenters and positive by biochemical tests {TSI (A/A, -H₂S, +- gas), MIO (+- motile, +indole) and Simmon's citrate- (blue color)} and having growth on CFA media were tested for hydrophobicity. TSI and Simmon's citrate were performed by touching a fresh isolated colony on MacConkey agar with sterile straight wire, stab the butt and streak the slant and lastly stab the MIO in that order without going back to the primary plate. The CFA agar contained bile salts and samples cultured on it were incubated at 37°C for a period of 20 h. The testing was performed as outlined by Tamatsukuri *et al.*, (1992). The fresh bacterial isolates to be tested were prepared to McFarland turbidity standard 1 (10⁹ CFU). For surface hydrophobicity, this concentration was suspended in 20 µl of 0.002 M Na₂HPO₄ at a pH of 6.8 on a clean grease-free glass slide. An equivalent volume of 20 µl of NH₄SO₄ buffer was added on to the suspension and the slide rocked just as in the serotyping of *Shigella* spp, *Salmonella* spp and *E. coli*. Any form of agglutination was considered a positive result (Honda *et al.*, 1983).

3.7.5.2 Heat stable and Heat labile toxin testing (STI, STII and LT).

The DNA from all the isolates identified as *E. coli* were analysed by PCR using primers with high specificity for heat labile and heat stable toxins that are elaborated by enterotoxigenic *E. coli* pathotype (Table 3.1A). For extraction of DNA, fresh isolates (18-24 h old) were inoculated in 2 mls freshly prepared and quality controlled brain heart infusion broth and incubated at 37°C for 18-24 h. Then, the

DNA was extracted and stored at -20°C (QIAamp DNA extraction kit, USA, handbook).

3.7.5.3 Multiplex Polymerase Chain reaction amplification for ETEC toxins

The DNA obtained from the above process was analysed by PCR amplification to detect the heat stable, heat labile toxins (STI, ST2 and LT {Table 3.1A}). For every virulent gene, a master mix was made up of 15.2 µl distilled water, 2.5 µl of 10x PCR buffer, 1.5 µl , 0.5 µl dNTPs (25mM each nucleotide), primers, 0.3µl taq polymerase and 4 µl template DNA. The mastermix was then completely mixed by tapping the tube and A quick short spin. This master mix cocktail was adequate for one reaction. Running 50 samples required increasing each component by a factor of 50.

After preparation of the master mix, a 21 µl volume was pipetted directly into the bottom of a sterile microeppendorf tube for each reaction and the tubes labeled using a permanent marker with specific color coding for LT, STI and STII. After transfer of the cocktail, 4 µl of template (sample) was added in respective tubes and adequate mixing was ensured by quick spinning to collect the reaction mixture at the bottom of the tube. After spinning, the tightly capped tubes were placed in the temperature block (thermal cycler) according to the toxins being investigated and ensured that each tube was firmly seated by pressing on the tubes individually using a plastic roller. Sterile distilled water and positive control strains of ETEC known to produce ST and LT toxins were used as negative and positive controls, respectively. For amplification, the PCR program was set at 95°C for 1min followed by 72°C for 1

min for 5 cycles; 95°C for 1 min followed by 62°C for 1min and then 72°C for 1 min for 20 cycles; and 72°C for 5 min for final elongation of the amplified DNA product.

The PCR products were then separated by agarose gel electrophoresis by adding 5µl of loading dye containing 0.25% bromophenol blue in 40% sucrose to a 25µl reaction mixture and loaded 10 µl to a 2% agarose gel. The buffer in the electrophoretic chamber and in the agarose gel was 1X tris-borate EDTA which also contained 1 µg/ml ethidium bromide. Eighty volts and 25mA were applied across the gel for migration of the amplified DNA. The DNA was visualized by exposing the gel to Ultra Violet light and photographed using the alpha imager gel documentation machine. Since Ethidium bromide used is a health hazard, the gel was immediately neutralized by a destainer and discarded in a double biohazard bag.

3.7.6 Multiplex PCR for colonization factor antigens detection

DNA was extracted as described for *E. coli* above. Prior to this technique, CFAs were presumably identified by the hydrophobicity method. The mastermix was done in the same way as in the detection of the ETEC toxins. The only difference was the increase in the number of cycles from 25 to 35 cycles and oligonucleotide primer sequences (Table 3.2 as described by Klena *et al.*, 2005). The grouping of the different CFAs and CSs were as follows: **Group 1:** CS4, CS6, CS13, CS14, CS19; CS20. **Group 2:** CS3, CS5, CS7, CS8, CS10, CS12, CS13. **Group 3:** CS1, CS2, CS15, CS17, CS18, CS20. The grouping was based on significant molecular size differences visible on the gel. After the complete run of the thermal cycler was done;

the PCR products were loaded in the 2% agarose gel and visualized by UV light using the alpha imager, photographed and documented for interpretation and record.

Table 3.2 Primers sequences for amplification of colonization factors

CFA type	primer sequence	Amplicon size
CFA/I	ACTATTGGTGCAATGGCTCTGAC CAGGATCCCAAAGTCATTACAAG	497
CS1	GAGAAGACCATTAGCGTTACGG CCCTGATATTGACCAGCTGTTAG	410
CS2	ACTGTAACTGCTAGCGTTGATCC TGCTTCCTGCATTAATAACGAGT	358
CS3	CCCACTCTAACCAAAGAAGTGG CGTATTTCCAGCATTTTTATCCA	300
CS4	ATTGATATTTTGCAAGCTGATGG GTCACATCTGCGGTTGATAGAGT	242
CS5	CAACCGTATCAGGTTCTGTTTTG CAAATGTTACCGGAGCTACAAAG	558
CS6	AAATGTATCCCAGGTAACGGTCT TGTTGATTAGGCGTAACCTCTGT	250
CS7	TGCTCCCGTTACTAAAAATACG GGCATTTCATATCAATAGAAAATATGAGAC	300
CS8	TATGAGCCTKCTGGAAGTYATCAT TATTGTAGTATTATCAGTAGCAGCCA	150
CS12	TTACGTCTCTGATCATGGCTGTTA ATAGTCATTAAGTGCATTTGCATCAAC	562
CS13	TTG ATG TGA TGG TTA TCG CTT AAAATCCAGGGTGGCGATCTG	212
CS 14	AGAGGTGAATAAAACCTTGTTGGA ATAATACCCAGCGTCCATTTTCT	585
CS15	TTCTTTCTGTATTGTCTTTTTACCC TTCTTTCTGTATTGTCTTTTTACCC	412
CS17	ACTCTRTCGCATTAACCTATTCT GTCACCTTCATCGGAATTTGCGAG	169
CS18	TTTGCTGCACTGCCTGCGAA TAACAGTACCAGCTTTAACCTGAC	518
CS19	AGAGTTTGATCCTGGCTCAG AAGGAGGTGATCCAGCCGCA	210
CS20	ATGATTATGCCCTTTTAACTATGG CAA GTTTTTGATCGCTTCCAATA	101

3.7.7 Multiplex Polymerase Chain Reaction for non *E. coli*

3.7.7.1 Multiplex Polymerase chain reaction for *Shigella* toxins

DNA for *Shigella* isolates was extracted following the method described for *E. coli* pathotypes above. The PCR mastermix and thermal cycler conditions were as described in section 3.7.5.3 above. The toxins investigated were: iPaH, SET, SEN and ial. The nucleotide sequences were as described in Nayak *et al.*, (2004).

3.7.8 DNA purification for sequencing

The DNA was excised from agarose resulting from gyrase A, B and topoisomerase PCR product using a clean and sharp scalpel and weighed in colourless tubes. Three volumes of buffer QG were added to 1 volume of gel (100mg: 100µl). This was later incubated at 50°C for 10 min to dissolve the gel. Following dissolution of the gel, a yellow colour was observed. One gel volume of isopropanol was added to the sample and mixed and transferred to the QIAquick spin column into a clean 2 ml collection tube. The sample was applied to the QIAquick column to bind DNA before centrifuging for a minute. A volume of 0.75ml of Buffer PE was added to QIAquick column and centrifuged for a minute at 13,000rpm and the QIAquick column placed into a 1.5 microcentrifuge tube and the DNA eluted by adding 50µl of buffer EB or water to the center of the QIAquick membrane. The column was centrifuged for a minute and the filtrate stored at -80°C for sequencing.

3.7.9 Sequencing of PCR product

The DNA sequencing reactions and processes were performed using the BigDye procedure (Applied Biosystems, 2002). The cocktail which consisted of 100-200 ng template, 2 pmol of primer, 4 μ l of BigDye sequencing mixture and the required volume of distilled water to achieve a final volume of 10 μ l were done in a thin walled 200 μ l tube. The cocktail was run in a thermal cycler for 26 cycles at a denaturation temperature of 96°C for 30 sec, annealing at 50°C for 15 sec and 60°C for 4 min. Following these reactions, the end product was held at 0°C - 4°C for 4 h until further treatment. The excess reaction components were removed prior to sequencing. In removing the excess reaction mix, 10 μ l of water was put on the surface of an edge biosystems gel filtration cartridge contained in a microcentrifuge tube and centrifuged at 1000xg (3000RPM in a Beckman model 12 centrifuge, (Applied Biosystems, USA) for a minute. The column cartridge was then transferred to a new centrifuge tube. A volume of 10 μ l of water was added to the 10 μ l sequencing reaction and mixed. After mixing, the 20 μ l volume of the solution was applied to the top of the column matrix (exactly at the middle of the matrix not at the edges) and centrifuged at 10000xg for 2 min. The solution was vacuum dried and submitted for sequencing.

The sequence data for gyrase and topoisomerases were then analysed and compared to a known database at the following website; ([http:// www.ddbj.nig.ac.jp/](http://www.ddbj.nig.ac.jp/)) DNA Data Bank of Japan (DDBJ). The multiple sequence alignment was done by MRbayes and general time reversible (**GTR**) model softwares for phylogeny. The alignment was run for 1,000,000 reactions. MRbayes software had the capability to

simultaneously run in two parallel directions and give the final consensus phylogeny tree. The consensus tree was analysed by the Fig Tree software to compare the distance between the isolates and the reference sequences that were obtained from the Gene Data Bank and the results recorded and interpreted. The conversion rates of alignment between the purines and pyrimidines were analysed by Tracer for complete or partial conversion. For a successful reaction and alignment the tracer yielded all black ESS values and this was termed successful.

3.8 Isolation and identification of other enteric diarrhoeal pathogens

The other diarrhoeal bacterial pathogens (*Shigella* spp, *Salmonella* spp and *Vibrio* spp) were identified by biochemical methods and serotyped using commercial kits as follows:

DAY 1

The samples were received in the laboratory from the site and recoded in the laboratory note book with the study numbers for identity. The stool samples were double checked macroscopically for consistency before culture. This was to ensure that cultures were done from the stool portions where blood and / or mucus was visible to enhance recovery of the agents responsible for diarrhoea.

After macroscopic examination, the stools were plated on selective, differential and enriched media and incubated at 37°C for 18-24 h. All the media were quality controlled using *E. coli* ATCC 25922, *S. flexneri* ATCC 700930, *V. cholerae* ATCC 14033, *S. typpimurium* ATCC 14028 and checked for sterility by incubating un

inoculated plate at 37⁰C for 18-24 h. The study did not seek to detect *Campylobacter* due to lack of resources involved in processing it.

DAY 2

The culture plates were removed from the incubator and checked for growth of specific microorganisms. For XLD plates, two colonies with different morphologies (one with a black hallow at the center, one clear or pink) were picked and transferred directly to the biochemical tests (TSI, MIO, SC). The inoculated biochemical tests were incubated at 37⁰C for 18-24 h to be read the following day. The black centred colony colouration were suspected as *Salmonella* spp while clear or pick colonies were suspected *Shigella* spp. Green and yellow colonies were to be picked from TCBS as *Vibrio* spp suspects and subcultured on freshly prepared and quality controlled nutrient agar for serotyping into either Inaba, Ogawa, Hikojima or Bengal cholera strains. The Selenite F broth inoculated on the first day was also checked for turbidity on this day and subcultured on XLD for enhancement of *Salmonella* spp or *Shigella* spp recovery. However, all TCBS plates did not grow anything apart from one plate with the positive control strain of *V. cholerae* (ATCC 39315).

DAY3

All the biochemical tests performed on the second day were removed from the incubator, observed, read and interpreted for speciation. If the results from TSI were alkaline and the butt acid, while indole test and Simmon's citrate were negative without H₂S production, *Shigella* spp was suspected. On the other hand, if H₂S was positive, *Salmonella* spp was suspected. A subculture was made directly from TSI to freshly prepared and quality controlled nutrient agar for serotyping the following day using both polyvalent and monovalent antisera from Denka and Seiken, Japan for

Shigellae speciation. For the cultures which came from TCBS to nutrient agar in day two were to be serotyped using *Vibrio* polyvalent and monovalent antisera and results recorded. For any positive serotyping results, a second reading was done for confirmation. For *Salmonella* spp, an O, H and Vi antigen tests were carried out for confirmation. Results from the Selenite F subcultures were inoculated into the three biochemicals, incubated as above for growth of *Salmonella* spp and *Shigella* spp.

DAY4

All the serotypes of *Shigella*, *Salmonella* and *Vibrio* were taken for antibiotic susceptibility testing. The tests were based on the CLSI 2008.

DAY5

The results from the antibiotic sensitivity were read and interpreted in reference to *E. coli* ATCC 25922 and the isolates stocked in beads soaked in glycerol for further experimentation.

3.8.1 Serology

Serotyping of all the targeted etiological agents identified as *Shigella*, *Salmonella* and / or *Vibrio* was done using antisera prepared by Denka Seikan, Japan. Serotyping on *E. coli* isolates was not done.

3.9 Antibiotic Susceptibility tests (disc diffusion)

The antibiotic susceptibility profiles were performed according to the Kirby-Bauer disk diffusion method and interpreted based on the CLSI, (2008). The following antimicrobial agents were tested on all *Shigella* spp, *Salmonella* spp, *Klebsiella* spp and *E. coli* strains (Table 3.3). A concentration of 0.5 McFarland standard

suspensions were prepared using BBL Prompt (Becton Dickenson). The susceptibility panel was chosen on the basis of treatment of infections due to Gram negative bacteria (CLSI, 2008) (Table 3.3).

Table 3.3 Antibiotic panel for gram negative bacteria

Antibiotic	Disc potency
Tetracycline (Te)	30µg
Chloramphenicol (C)	30µg
Ampicillin(AMP)	10µg
Erythromycin(E-)	15µg
Gentamicin (GM)	10µg
Amoxillin / clavulanic acid (AMC)	30µg
Amikacin (AM)	30µg
Ciprofloxacin (CIP)	1µg
Norfloxacin (NOR)	10µg
Trimethoprim/Sulfamethoxazole	23.75µg
Cefuroxime (CXM)	30µg
Nalidixic Acid(NA)	30µg
Fosfomicin(F)	30µg
Cefotaxime (CXT)	30µg
Ceftazidime (CAZ)	30µg

3.9.1 Testing for Extended - Spectrum β Lactamases (ESBLs)

Bacterial isolates: The bacteria were identified and tested for different antimicrobial susceptibility with beta lactams, aminoglycosides, penicillins and quinolones. Those

that were resistant to third generation cephalosporins were tested for the presence of the extended spectrum beta lactamases using double disc diffusion (DDD), E test and confirmed by VITEK.

ESBL detection methods

Vitek system: The Vitek is an automated system for identification and/or susceptibility testing. Vitek susceptibility is interpreted by the in build system.

Double disc-diffusion (DDD) method: This method was used again to reconfirm the strains that were ESBL positive by Etest and/or Vitek GNS-532 cards done using the microscan. The DDD method that was used here employed four discs, one containing ceftazidime (30 µg), cefotaxime (30 µg) and cefuroxime (30 µg), and three with a combination of the same antibiotics (10, 10 and 1 µg, respectively) with the addition of clavulanate (Oxoid). A broth culture of the test organism was adjusted to a 0.5 McFarland standard and inoculated onto freshly made and quality controlled Mueller–Hinton agar (Oxoid). The combination discs and the corresponding standard cephalosporin disc were placed at the recommended distance (30mm) from each other on the plate. The plates were incubated at 37 °C for 18 h aerobically before the zone sizes were recorded. A positive result was indicated by a zone-size difference of ≥ 5 mm diameter between the combination disc and the corresponding standard antibiotic disc, as recommended by the manufacturer.

For all ESBL detection methods, the known ESBL-producing *E. coli* strains SA1636 (TEM-3) and SA1652 (SHV-2) were used as positive controls. *E. coli* NCTC 10418 was used as a negative control.

3.9.2 Minimum Inhibitory Concentration (MIC)

The strips were applied on freshly prepared and quality controlled Mueller Hinton agar in which either 0.5 MacFarland *Shigella*, *Salmonella* or *E. coli* were uniformly spread. The plates were incubated at 37⁰C for 18-24 hours. The results were read the following day and interpreted according to CLSI (2008) to provide the minimum inhibitory concentrations of the drugs and the results recorded.

3.9.3 Plasmid extraction and analysis.

Plasmid extraction and profiling was done on all the multidrug resistant strains of *E. coli* pathotypes, *Shigella* spp and *Salmonella* spp isolated from patients of all age groups who presented with diarrhoea. The method used was as described by Birnboim and Dolly (1979). The double stranded DNA was precipitated with two volumes of ethanol at room temperature and mixed by vortexing. Plasmid DNA was resolved on 1% agarose gel at 100V for four hours. The gels were stained with ethidium bromide and visualized by ultra-violet light and analysed by gel electrophoresis. The molecular size markers used were *E. coli* 861: 147, 63, 43.5 and 6.9 ; V517-53.7, 7.2, 5.6, 3.9, 3.0, 2.7 and 2.1 kb.

3.9.4 Preparation of Bacteria for Conjugation experiments

Conjugation experiments were done after observations that the bacteria were multidrug resistant. These were done to determine if resistance plasmids were transmissible. Experiments were carried out using strains that were resistant to ampicillin and sensitive to nalidixic acid. *E. coli* K 12 was used as the recipient bacterium. The *E. coli* K12 was nalidixic acid resistant. The donors (*Salmonella* spp, *Shigella* spp and *E. coli*) and the recipient (*E. coli* K12 nalidixic acid resistant,

F-) were grown separately in nutrient agar overnight and thereafter subcultured in 3ml broth at 37⁰C for 3h to get into log phase and incubated in a rotating incubator or shaker at 37⁰C. 4.5 ml of warm and freshly made broth was dispensed in sterile tubes to be used for diluting the concentrations of both donor and recipients (0.5: 4.5). The donor and recipient cultures were then mixed in the ratio of 1:1 and incubated. The mixture was centrifuged at 13,000g for one minute using 1.5ml tubes. After centrifugation the pellets were washed with sterile phosphate buffered saline by resuspending cells using vortexer, centrifuged again and washed using sterile phosphate buffer. The mixtures were then subcultured using a sterile loop onto MacConkey agar, one containing nalidixic acid, the other containing ampicillin at 30µg/ml. The nalidixic was included in the agar in order to select the transconjugant. MacConkey agar enriched with ampicillin was meant to select for donors *Salmonella* spp, *Shigella* spp and *E. coli* while MacConkey agar enriched with Nalidixic acid was to select for only *E. coli* K12. The MacConkey agar enriched with both nalidixic acid and ampicillin only grew the transconjugant *E. coli* K12. Thereafter, the *E. coli* K12 was tested for susceptibility against nalidixic acid and ampicillin for proof of conjugation by receiving the plasmid for ampicillin resistance from donors.

3.9.5 Pulsed Field Gel Electrophoresis (PFGE)

Genomic DNA of various strains of *E. coli*, *Shigella* spp and *Salmonella* spp were prepared in agarose plugs as described by Kariuki *et al.* (2000: 2001) from an overnight culture in Luria batani broth. For complete digestion of DNA, the *Xba*I (Life Technologies, Paisley, UK) enzymes were added according to the manufacturer's instructions. PFGE of agarose plug inserts was performed in the

contour clamped homogenous electric field CHEF-DRIII (Bio-Rad Laboratories, Hercules, CA, USA) on a horizontal agarose 1% gel for 22 h at 120V, pulse time of 1 to 40 sec at 14°C. A DNA size standard (lambda ladder, Bio-Rad) consisting of (c. 22 fragments) of increasing size from 48kb -1000kb were used as the DNA size standard makers. The gels were stained with 0.05% ethidium bromide and observed on a gel Doc 2000 (Bio-Rad). Restriction endonuclease digest pattern of all *NotI* and *XbaI* digest genomic DNA were compared and similarities and differences scored basing on Tenovar's method (Tenovar *et al.*, 1997). Using the Dice coefficient method and clustered by the unweighted pair group arithmetic averaging method (molecular Fingerprinting program version 1.4.1, Bio-Rad) that puts the isolates into PFGE clonal groups (Kariuki *et al.*, 2002; Kariuki *et al.*, 2004). Those isolates showing indistinguishable PFGE banding were taken to be part of the outbreak while isolates showing 2-3 band difference in PFGE patterns were said to be closely related and possibly part of the cause of disease. Those with 4-6 band differences were assumed possibly related, and, those with more than 6 band differences were deemed different (Tenovar *et al.*, 1997).

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic data of study participants

4.1.1 Age and sex distribution

This study was conducted from September 2006 to March 2008. During this period, 301 participants with diarrhoea and aged between 3 months and 85 years with a median age of 6 years and 5 months were enrolled. Their stool samples were collected, examined and analysed. This was done after the patients had given consented by signing (Appendix 1). The study was approved by Kenya Medical Research Institute (KEMRI) Scientific Committee (SC) of the Center for Microbiology Research (CMR), the Scientific Steering Committee (SSC No. 989 of 2006) and the National Ethical Review Committee (ERC- appendix 3). The lowest participant age recruited was four months while the highest was 85 years. The male female ratio was 137:164 (1:1.2).

4.1.2 Study participants Clinical presentation.

Diarrhoea, stomach cramps and vomiting were the main presentation by study participants. The most common presentation was loose or watery stool (53%). This was followed by stomach cramps (20%), vomiting and headach (10%) and dehydration(5%). Joint pain was the least presentation at 2% (Figure 4.1).

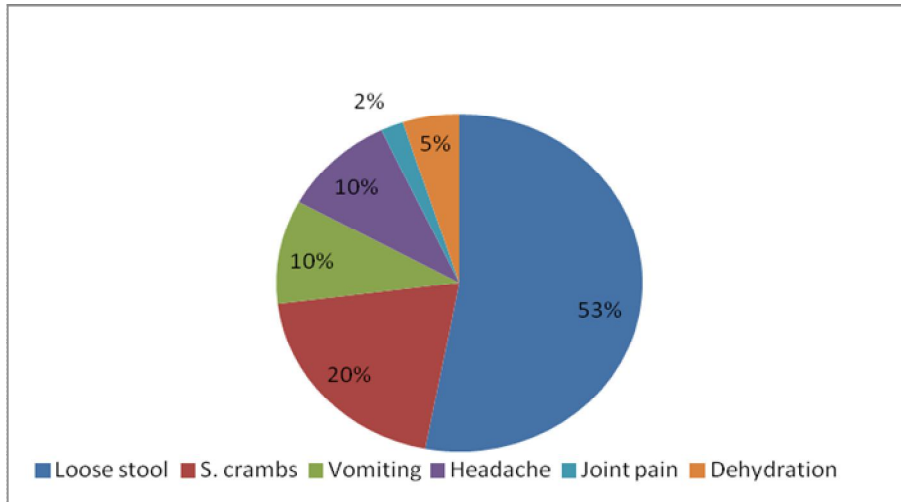


Figure 4.1 Signs and symptoms of 301 patients with diarrhoea at Machakos District Hospital 2006-2008

4.1.3 Study participants occupation

The patients had different types of occupation. They include farmers (58%), house wives (31%), students (7%), Butchers(1%), and plumber(1%). Most of the study subjects were farmers who practiced animal farming and growing of short seasoned crops (Figure 4.2).

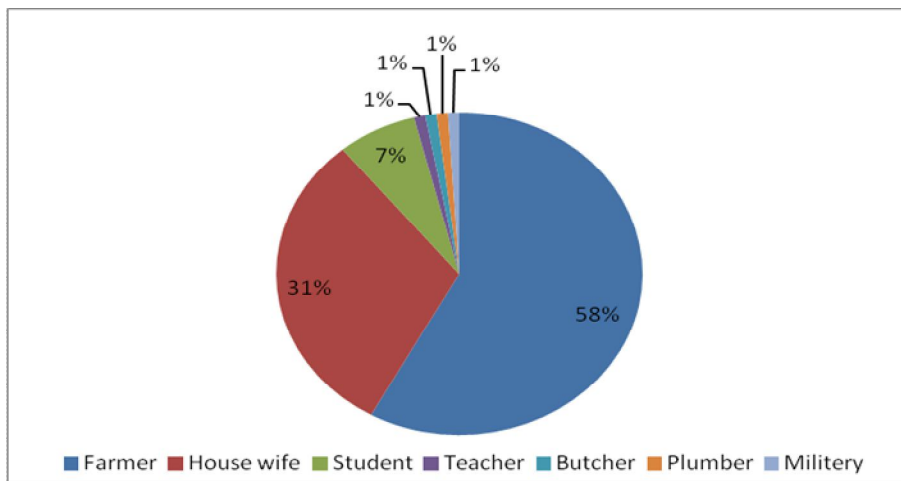


Figure 4.2 Study subjects occupation in Machakos District Hospital between 2006-2008

4.1.4 Study participants stool

Macroscopic examination of stool consistency showed that 6% were bloody, 12% bloody/mucoid, 34% mucoid and 48% watery (Figure 4.3).

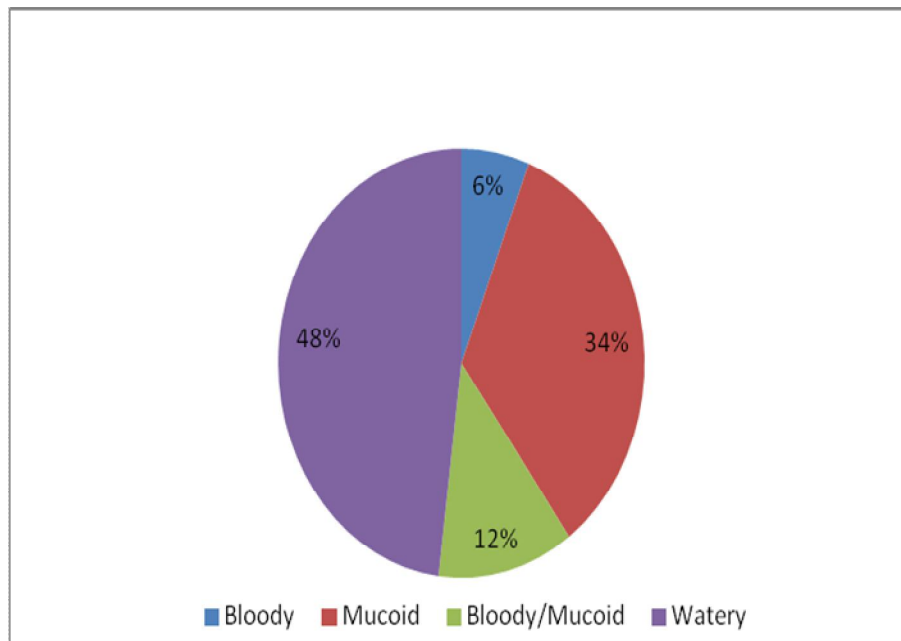


Figure 4.3 Patients stool consistency from Machakos District Hospital 2006 to 2008

4.1.5 Study participants age distribution

The participants recruited below five years were 145 (48.3%), between 6 years to 10 years were 17 (5.7%), 11 to 15 years were 11(3.7%), 16 to 20 years 20(6.7%) 21 to 25 years 36(12%), and 36(12%) above 40 years (Figure 4.3 B). Most of the study

participants were below five years of age. One of the study subjects did not respond to the study questions and was omitted from analysis.

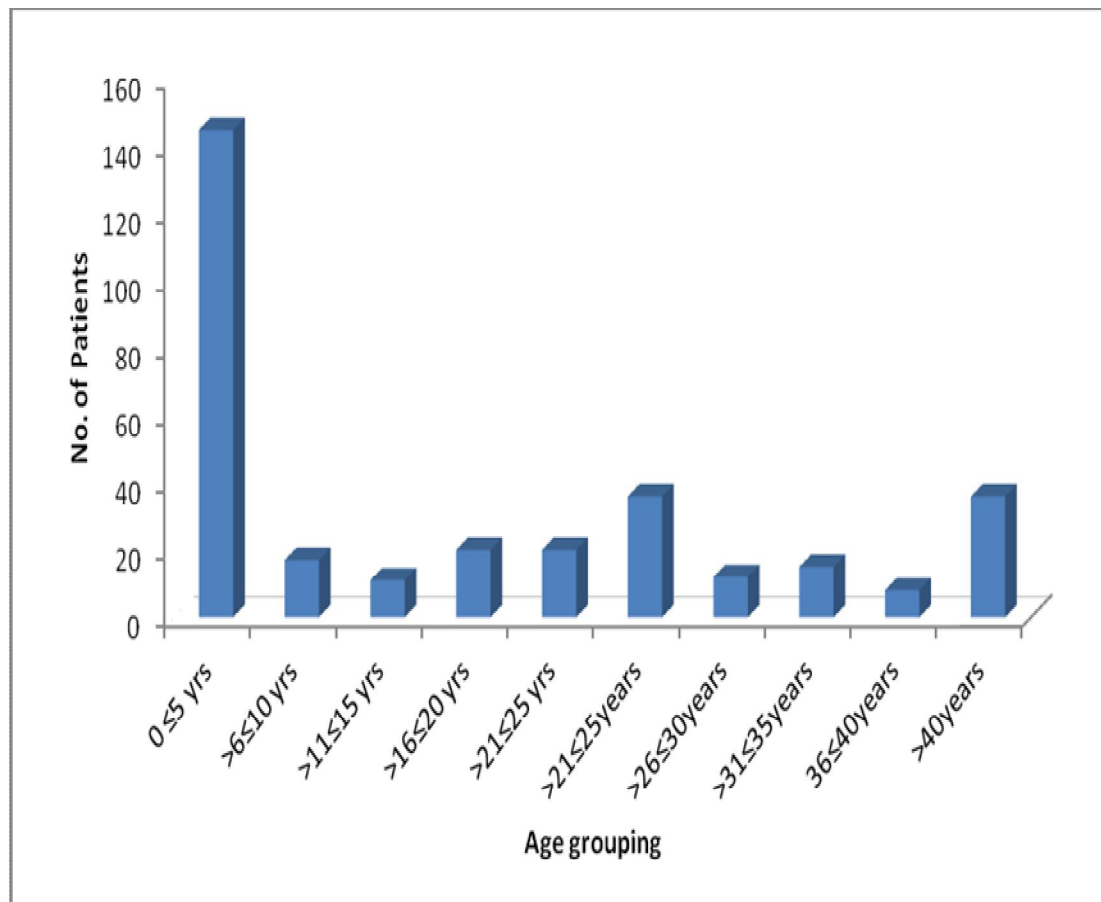


Figure 4.4 Study participants age distribution

4.1.6 Antibiotic use before visiting the Hospital

Out of the 300 study subjects 22 /300 (7.3%) had used antibiotics before presenting to the Hospital (Table 4.1). The most commonly used antibiotics were co-trimoxazole, Chloramphenicol and Streptomycin. There was a significant difference in the numbers and diversity of micro-organisms isolated from subjects who had

used antibiotics and those who did not ($X^2 = 5.34$; $P < 0.05$). The micro-organism diversity was high in numbers in those who did not use any antibiotic before presentation in the hospital for treatment. The antibiotics taken were suspected to have killed or suppressed recovery of most of the microorganisms. The difference was statistically significant ($X^2 = 4.07$; $P < 0.05$).

Table 4.1 Antibiotic use prior to visiting Hospital

Antibiotic	No. of patients
Cotrimoxazole	12
Ampicillin	2
Gentamicin	1
Streptomycin	4
Augmentin	1
Chloromphenical	2
Total	22

The type of toilets used included pit latrine (162/300), flash toilet (54/300), bush (32/300) and those who did not respond on the type of toilet used (52/300). Majority of the people used pit latrine 162/300 (54%). The source of water included , boreholes 82/300(28.7%), river 62/300(20.7%), Dam water50/300(16.7%) and tap water 50/300(16.7%).

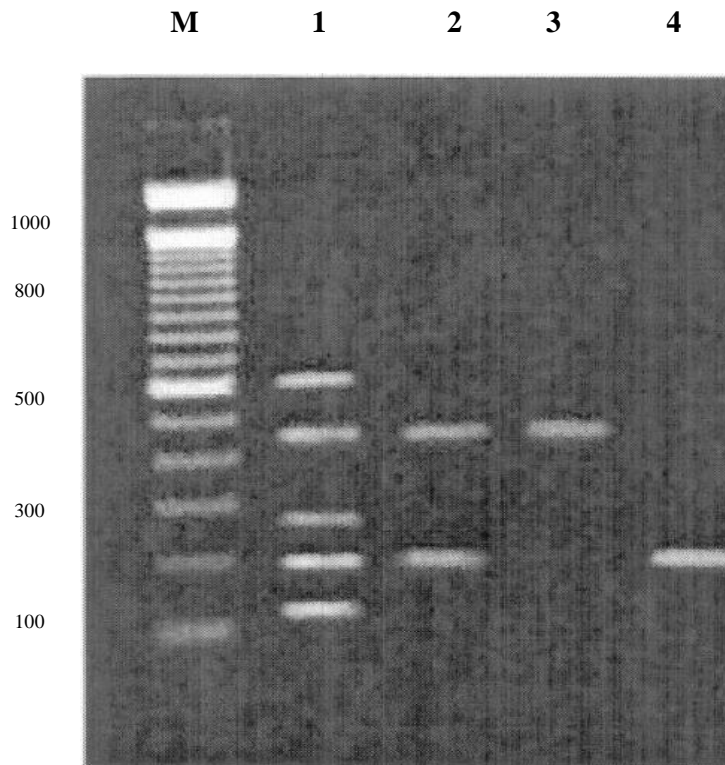
4.2 Prevalence of ETEC (*LT*, *ST*, *LTST*, CFAs, CSs

4.2.1 Prevalence of ETEC *LT* and *ST*.

ETECS *LT* and *ST* were categorized in groups as *LT*, *STI* and *STII* toxins with varied CFAs using oligonucleotides primers designed from highly conserved regions of ETEC and CFs from gene bank (DNA Data Bank of Japan (DDBJ) (Plate 4.1). Out of 300 samples, 33 / 300 (11%) were cultured positive for ETECS. Out of these different genes were amplified: 56/98 ETEC carried *LT*, 37/98 *ST* and 6/98 *ST*. Some samples carried more than one gene (6/98; 6.1%) as indicated in plate 4.1. The gene sizes ranged from 150BP to 500BP.

4.2.1.1 Amplification of ETEC *LT* and *ST*

Out of the 33 positive samples, 98 ETEC isolates were recovered from 13 children under five years of age, 2 children aged between 6 and 10 years and 18 subjects above the age of 16 years. No ETEC were isolated from the ages between 10 and 15 years (Plate 4.1, Table 4.3). Out of the 33/300 study participants, the Multiplex PCR amplifies difference genes responsible for virulence. They include Heat labile, *LT* (56/98), Heat stable, *ST* (36/98) and those that were double (*LT/ST* 96/98).

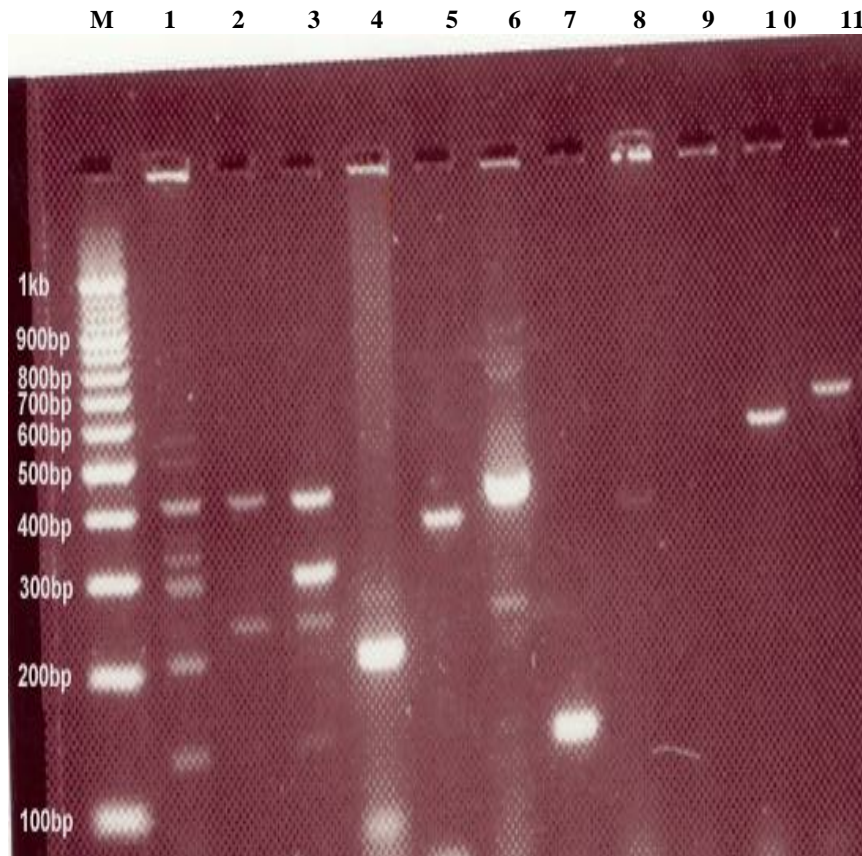


Lanes: M: 100Bp maker, Lane1: Pooled positive control; Lane 2: ST and LT; Lane 3: ST (500bp); Lane 4: LT (200bp)

Plate 4.1 Multiplex PCR amplification of ETEC virulence genes from patients in Machakos District Hospital 2006-2008.

4.2.1.2 Amplification of ETEC CFAs and CS

The 98 ETEC isolates recovered from different age groups yielded varied number of CFAs and CS as indicated in Plate 4.2 and Table 4.2. However the age range between 10 to 15 years did not yield any CFA or CS. The CFAs and CS recovered were distributed as follows: CFA1 (14/98), CS5+CS6 (6/98), CS6 alone (5/98), CS14 (2/98) and CS19 (9/98).



The CFAs and CS of ETEC amplified by use of mPCR using specific primers. Lane M: 100bp ladder, Lane 1: pooled positive control, Lane 2: CS 5 & CS 6, Lane 3: CS 5, CS 7, CS 6, Lane 4: CS 19, CS 20, Lane 5: CS 2, Lane 6: CS 5 & CS 6, Lane 7: CS 8, Lane 8: CS 5, Lane 9: negative control, Lane 10 CS 12, Lane 11 CS 14.

Plate 4.2 Amplification of ETEC CFAs and CS by mPCR

4.2.1.3 Age range of participants with positive CFA and CS

Most of the detectable CFAs and CS were recovered from participants below the age of five years as compared to other age groups (24/36; 66.7% Table 4.2). Majority of the CFA recovered was CFA1. CS19 was detected and reported for the first time. The age range between 10 years and 15 years was unique as it did not yield any CFA or CS.

Table 4.2 Age range of patients infected with ETEC isolate expressing CFA1, CS5 plus CS6, CS6, CS14 and CS 19

CF type	Age			
	3mn-≤5yrs	>5yrs-≤10yrs	>10yrs-≤15yrs	>15yrs
CFAI	8(33.3)	5(20.8)	0	1(4.2)
CS5+CS6	3(12.5)	2(8.3)	0	1(4.2)
CS6	4(16.7)	1(4.2)	0	0
CS14	2(8.3)	0	0	0
CS19	7(29.2)	2(8.3)	0	0

4.2.1.4 Colonization Factor Antigens by phenotype

The ETECs isolated were 56 (57%) *LT*, 6(6.1%) *LT / ST* and 36 (36.6%)*ST* expressing different CFs as indicated in (Table 4.3). There was a range of colonization factor antigens based on ETEC phenotypes. More CFs were recovered in *ST* than *LT* phenotype (Table 4.3).

Table 4.3 Distribution of ETEC colonization factor antigens by Phenotype

CF type	<u>ETEC toxin</u>			
	<i>LT</i> N=56	<i>LT ST</i> n=6	<i>ST</i> n=36	Mixed n=10
CFAI	5(9%)	3(50%)	15(42%)	2(20%)
CSI	0	0	0	0
CS1+CS3	0	0	2(5.6%)	0
CS2+CS3	0	0	0	0
CS3	0	0	1(2.8%)	0
CS4+CS6	0	0	0	0
CS5	0	0	0	0
CS5+CS6	10(18%)	0	3(8.3%)	3(30%)
CS6	4(7%)	1(16.7%)	5(13.9%)	3(30%)
CS7	0	1(16.7)	1(2.8)	0
CS12	0	0	1(2.8%)	0
CS17	0	0	0	0
CS19	3(5%)	0	7(19.4%)	1(10%)
CS20	0	0	0	0
No CFs	34(61%)	1(16.7%)	1(2.7%)	1(10%)

4.3 Age and clinical data associated with ETEC isolates expressing CFA/I, CS5 plus CS6, CS6, and CS19.

The demographic and clinical data for the subjects with ETEC-associated diarrhoea were grouped according to the CF that the isolate expressed. Overall, ETEC isolates expressing a CF were more commonly recovered from children under five years of

age (Table 4.2). In addition, CS6 and CS19-expressing isolates were more frequently identified in the stools of children ranging between one and five years of age ($X^2 = 21.65$; $P < 0.05$, Tables 4.2 and 4.3).

Table 4.4 Clinical data and ETEC colonization factor antigens expression

Clinical data	ETEC-CF expression				Non CF n= 60(%)
	CFA1 n=14(%)	CS5/6 n=6(%)	CS6 n=5(%)	CS19 n=9(%)	
Hospitalisation	5(36)	1(16)	0	2(22)	7(12)
Dehydration	6(43)	0	1(20)	1(11)	20(33)
Vomiting	3(21)	2(33)	4(80)	6(66)	38(63)
Temp>38°C	10(71)	4(66)	3(60)	7(86)	20(33)
Mucus in Stool	5(36)	4(67)	4(80)	8(88)	33(55)
Stomach cramps	3(21)	2(33)	2(40)	6(66)	40(67)

Note: Figures in bracket denotes percentage

In the analyses of the clinical data and CF types, ETEC cases associated with other bacterial (*Shigella* spp, *Salmonella* spp, *Klebsiella* spp), infections were excluded to avoid the analysis of data for subjects with overlapping symptoms due to the presence of other enteric pathogens. The lower percentage of hospitalization among subjects infected with ETEC isolates expressing CS6 and CS19 was not significant compared to the percentage of children infected with ETEC isolates expressing other CF types ($X^2 = 1.21$; $P > 0.05$) (Table 4.3). Among the subjects infected with ETEC isolates expressing CFA/I, the percentage with dehydration (43%) (Table 4.4) noted was higher compared to the percentage among those infected with ETEC isolates

expressing other CF types (Table 4.3). Visible mucus in the stool was reported in 46% of subjects infected with ETEC isolates expressing CFA/I, CS5 plus CS6 and CS 19 (Table 4.4). The ETEC CFs recovery rate was higher in warm seasons than wet seasons as indicated in Figure 4.5.

4.3.1 Seasonality of ETEC CFA and CS

Different CFAs and CS were recovered more in warm than wet seasons all the year round. CFA1 was the major CFA recovered during the study period. CS 19 was detected and reported for the first time in the country.

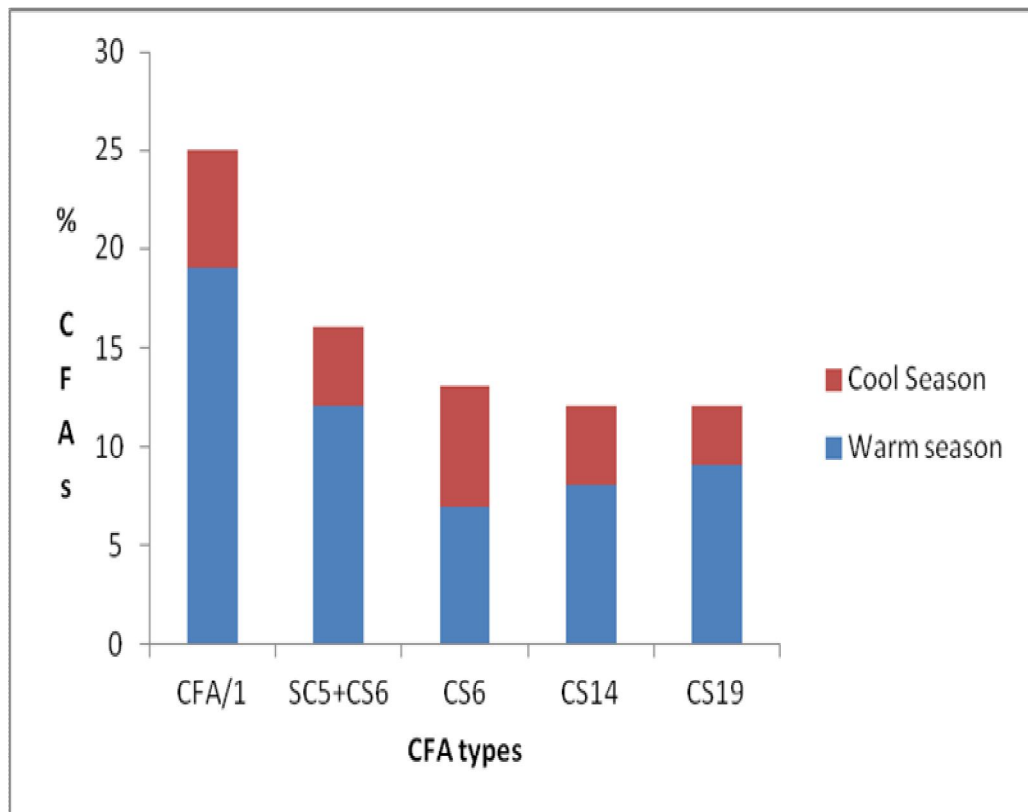
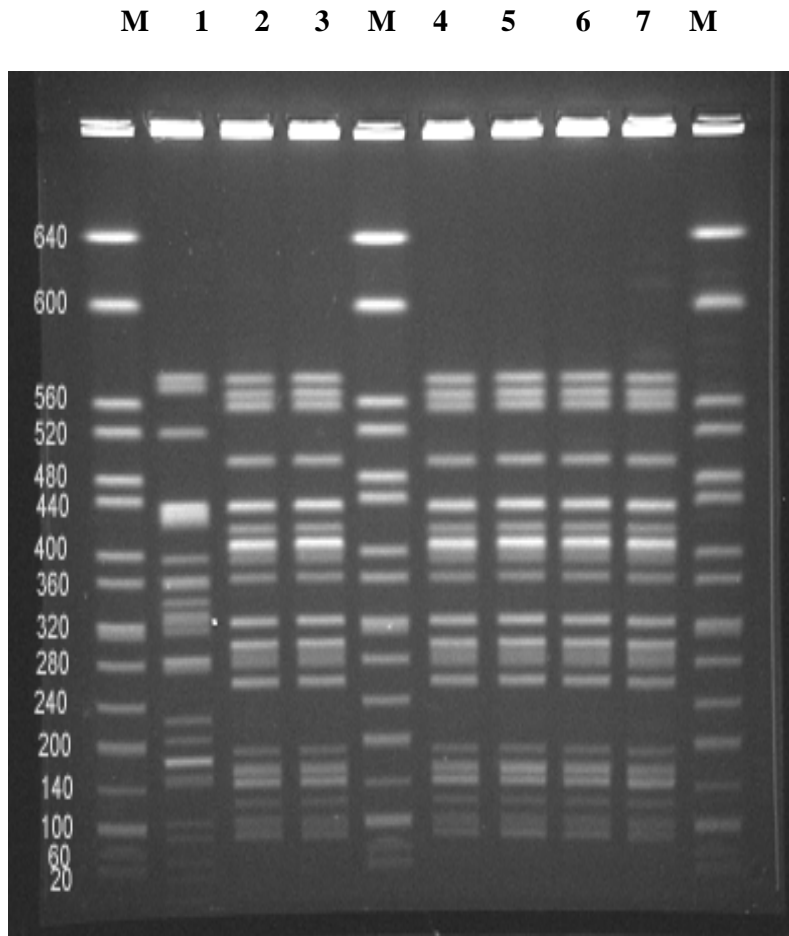


Figure 4.5 Seasonal association of ETEC CF and CS expression from September 2006 to March 2008.

4.3.2 Genetic relatedness of ETEC

The ETEC isolates were shown to be similar as indicated by PFGE bands which were within one to three band variations (Plate 4.3). From all the ETEC isolates (33/300; 11%), 21/33 (63.6%) had 2-3 band difference and 12/33(36.4%) had undistinguishable bands.



Lanes M indicates 40-kb lambda molecular size ladder; lanes 1, 2, 3, 4, 5, 6, and 7 indicates ETEC isolates that were shown to be within two to three bands difference.

Plate 4.3 PFGE gel using *Xba*I digest pattern variations in ETEC isolates from Machakos District Hospital.

4.4 Prevalence of other *E. coli* pathotypes

4.4.1 Other *E. coli* pathotypes

After excluding one sample for which patient data was incomplete, and those samples which were negative by PCR (60 samples), or which gave equivocal results in the PCR (10 samples), 230 (76.6%) samples of the original 300 were available for analysis.

4.4.2 Multiplex PCR Detection of *E. coli* pathotypes

The pathotypes were detected by mPCR and the results indicated EAEC as the most prevalent pathotype (13.7%) followed by enteroinvasive *E. coli* (12.2%), ETEC (11%) and CNF1 was the least prevalent (Figure 4.6, plate 4.4 and Table 4.5)

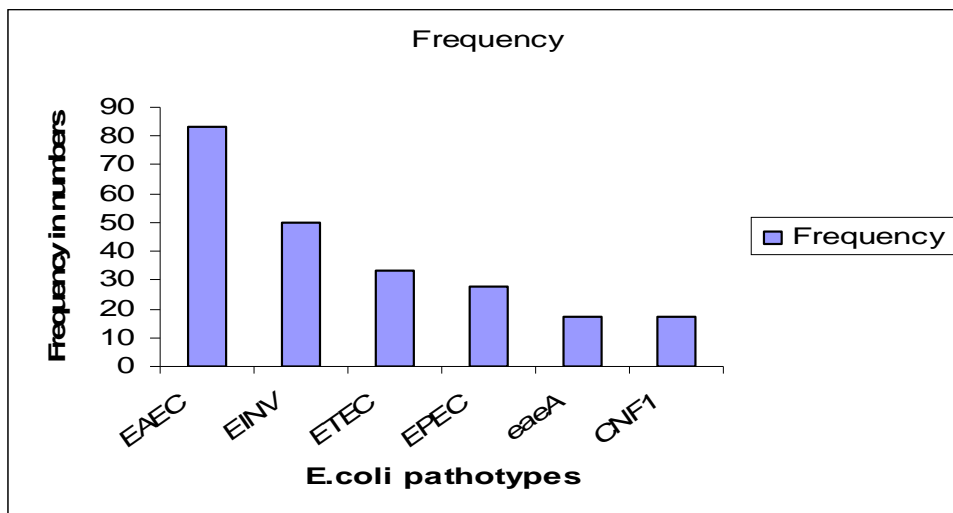
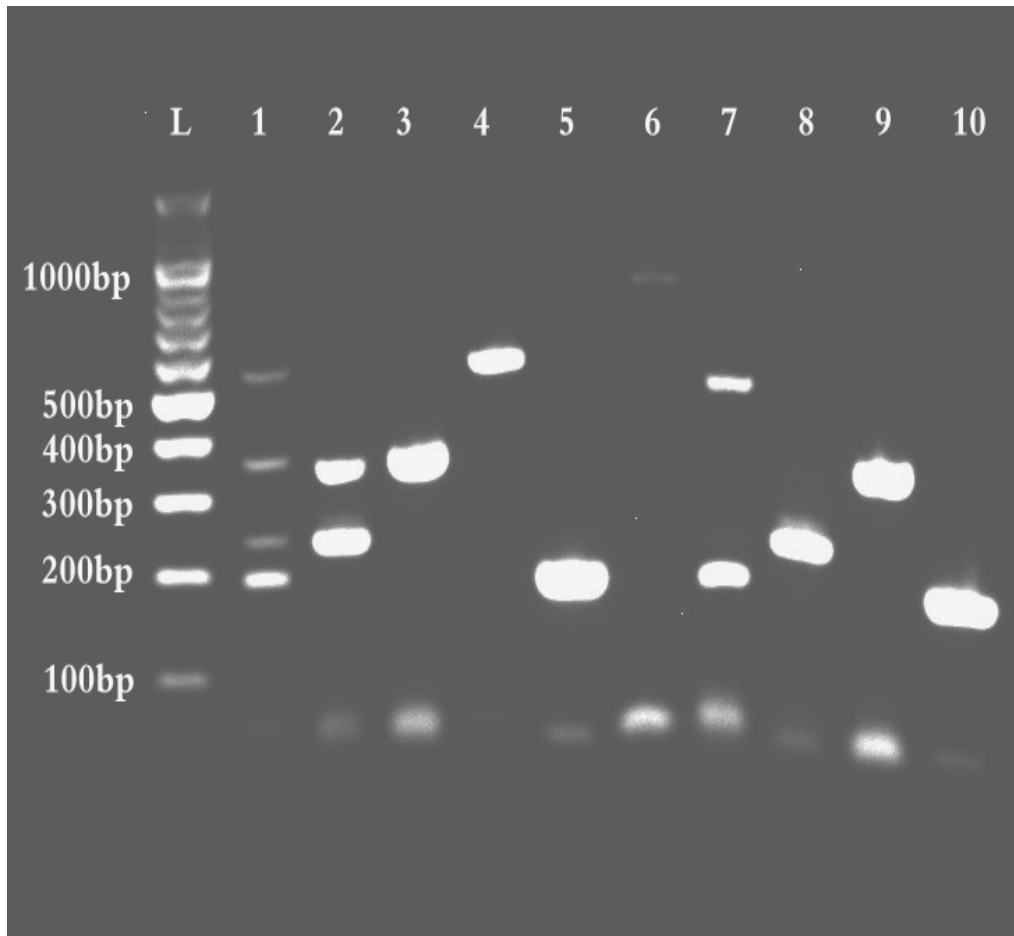


Figure 4.6 Frequencies of *E. coli* pathotypes isolated at Machakos District Hospital in 2006-2008.



Lanes L: 100 BP ladder, Lane 1; Pooled positive control, Lane 2: *ST1* and *ST2*, Lane 3: *LT*, Lane 4: *CNF1*, Lane5: *eaeA*, Lane6: negative control, Lane 7: *ST2* and *ipaH*, Lane8: EAEC, Lane 9: *ST2*, Lane 10: *ST1*.

Plate 4.4 gel picture of mPCR amplification of different *E. coli* pathotypes toxins.

4.4.3 Pathotype distribution

Overall, EAEC was the most prevalent of all *E. coli* pathotypes isolated from patients seen in Machakos District Hospital and *CNF1* was the least prevalent. *CNFII* was not detected (Table 4.5). Bacteria distribution was similar in both male and female participants.

Table 4.5 Number of patients from whom bacteria were isolated

Bacteria	Male	Female	Total
EAEC	22	19	41
EIEC	13	12	25
ETEC	17	16	33
EPEC	7	6	13
Eae	4	4	8
CNF1	4	4	8
CNF2	0	0	0
Non pathogenic <i>E. coli</i>	21	32	53
<i>Shigella</i> spp	35	32	67
<i>Salmonella</i> spp	1	0	1
<i>Klebsiella</i> spp	18	19	37
<i>Serratia</i> spp	0	1	1
<i>Pseudomonas</i> spp	0	1	1
<i>Proteus</i> spp	2	0	2
No growth	3	7	10

4.5 Prevalence of other enteric pathogenic bacteria

All strains that had characteristics for shigella were examined and exhibited biochemical characteristics typical of the genus *Shigella* and of the species *S. flexneri*, (13/67, 19.4%), *S. dysenteriae* (8/67, 11.9%), *S.boydii* (1/67, 1.5%) *S.*

sonnei (1/6, 1.5%) and untypable *Shigella* (44/67, 65.7%). Two different groups of non-typable *Shigella* were found based on mannitol fermentation in which 10/67 (14.9%) were positive and 57/67 (85.1%) were negative (Table 4.6).

4.5.1 Characterization of *Shigella* spp by enzymatic and sugar fermentation

Shigella spp that were untypable were analysed by using different sugars and enzymes as indicated in Table 4.5. From the biochemical test, 85.7% of the untypable *Shigellae* did not ferment mannitol.

Of these, 85.7% of the *Shigella* isolates did not ferment mannitol, produced indole, and were able to utilize sodium acetate, maltose, xylose, mannose, trehalose, sorbitol, and rhamnose. In contrast, 14.3% of the isolates were able to ferment mannitol but unable to utilize sodium acetate, maltose, xylose, mannose, trehalose, sorbitol, and rhamnose and were negative for indole production. These results were used to group the untypable *Shigella* in two groups of mannitol fermenters (14.3%) and non-fermenters (85.7%).

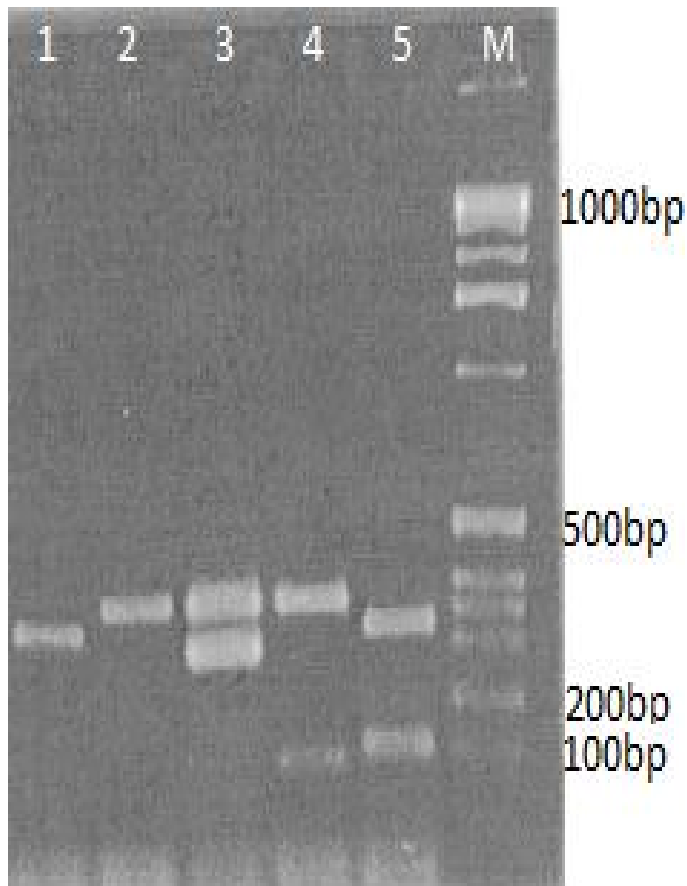
Table 4.6 Biochemical characterization of non typhable *Shigella* spp and enteroinvasive *E. coli* (EIEC)

Biochemical reaction	percentage positive or negative				
	<i>S. dysenteriae</i>	<i>S. flexineri</i>	<i>S. boydii</i>	<i>S. sonnei</i>	EIEC
Adonitol	-	-	-	-	-
α methyl glucosidase	-	-	-	-	-
Arabinose	+	+	+	+	+
Arabitol	-	+	-	-	+-
Arginine	+	+	+	+	+
Cellobiose	-	-	-	+	-
Dulcitol	+	+	+	-	+
Esculin	-	-	-	-	+-
Glucose	+	+	+	+	+
Glucose gas	-	+	-	-	+
Glycerol	+	+	+	+	+
Indol	+	+	+	-	+
Inositol	-	-	-	-	+
Lactose	-	+	+	+	+
Lysin	-	-	-	-	-
Malonate	-	-	-	-	-
Maltose	+	+	+	+	+
Mannitol	-	+-	+-	+-	+-
Mannose	+	+	+	+	+
Melibiose	-	+	+	+	+-
Methyl red	+	+	+	+	+
Motility	-	-	-	-	+-
ONPG	+	+	+	+	+
Ornithine	-	-	+	+	+
Raffinose	-	-	-	-	-
Rhamnose	+	+	+	+	+
Simmon's citrate	-	-	-	-	-
Sodium acetate	-	+	-	-	+
Sorbital	+	+	+	+	+
Sucrose	-	+	-	+	+
Trehalose	+	+	+	+	+
TSI	-	-	-	-	-
Xylose	+	+	+	+	+

Key: + all strains positive (100%); - all strains negative (100%); +- equivocal; TSI triple sugar ion

4.6 Detection of shigella enterotoxin genes by pcr assays.

The *Shigella* enterotoxin genes *SET1*, *ial* and *ipaH* genes were spread across *Shigella* strains. *set1* was detected in 16/67(23.9%) and *ipaH* in 4/67(6%). Two strains of *S. flexneri* harbored both *SET1* and *ipaH* enterotoxins (Plate 4.5).

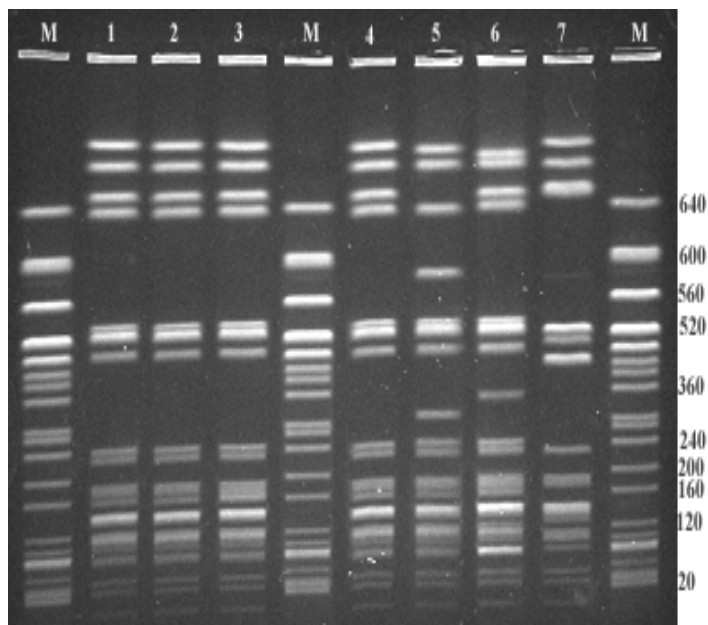


Lanes 1 and 5 *ial* and *ipaH* (350bp), Lanes 2 and 4 *Set1* and *ipaH* (410BP), Lane 3: *Set1* and *ial*(410BP, 350BP) respectively; Lane M: 100Bp molecular maker.

Plate 4.5 Multiplex PCR amplification of *Shigellae* enterotoxin genes from patients in Machakos District Hospital 2006-2008.

4.6.1 Analysis of Shigellae by PFGE

PFGE analysis of *Xba*I-digested chromosomal DNAs of the atypical *S. flexneri*, *S. dysenteriae*, and *S. boydii* strains yielded 16 to 19 reproducible DNA fragments ranging in size from 20 to more than 640 kb (Plate 4.6). From all the Shigellae isolates (38/67; 56.7%), 25/67 (37.3%) had 2-3 band difference and 4/67(6%) had undistinguishable bands.



Lanes M indicates 40-kb lambda molecular size ladder; Lanes 1, 2, 3, and 4 shows indistinguishable digest patterns indicating likelihood of same clone while 5, 6, and 7 indicates Shigella isolates that were shown to be within two to three bands difference.

Plate 4.6 PFGE patterns of *Xba*I digest *Shigella* spp from diarrhoeal patients in Machakos District Hospital.

4.7 Antibiotic susceptibility profiles of ETEC, other *E. coli* pathotypes and other pathogenic bacterial isolates

The isolates analysed included *Shigella* 67/300(22.3%), *E. coli* 500 (52%), *Klebsiella pneumoniae* 37(12.3%), *Salmonella* spp 1/300(0.3%), *Proteus* spp 1/300(0.3%) and *Serratia marcescens* 1/300(0.3%). They were resistant to more than four antibiotics including Ciprofloxacin (5%), Nalidixic acid (8%), Erythromycin (98%), co-amoxiclav(70%) , Ceftriaxone(6%), Cefotaxime(17%), Ampicilin (80%), Cotrimoxazole (78%), and Chloramphenicol (28%) Figures 4.7, 4.8, 4.9, 4.10 and Table 4.7.

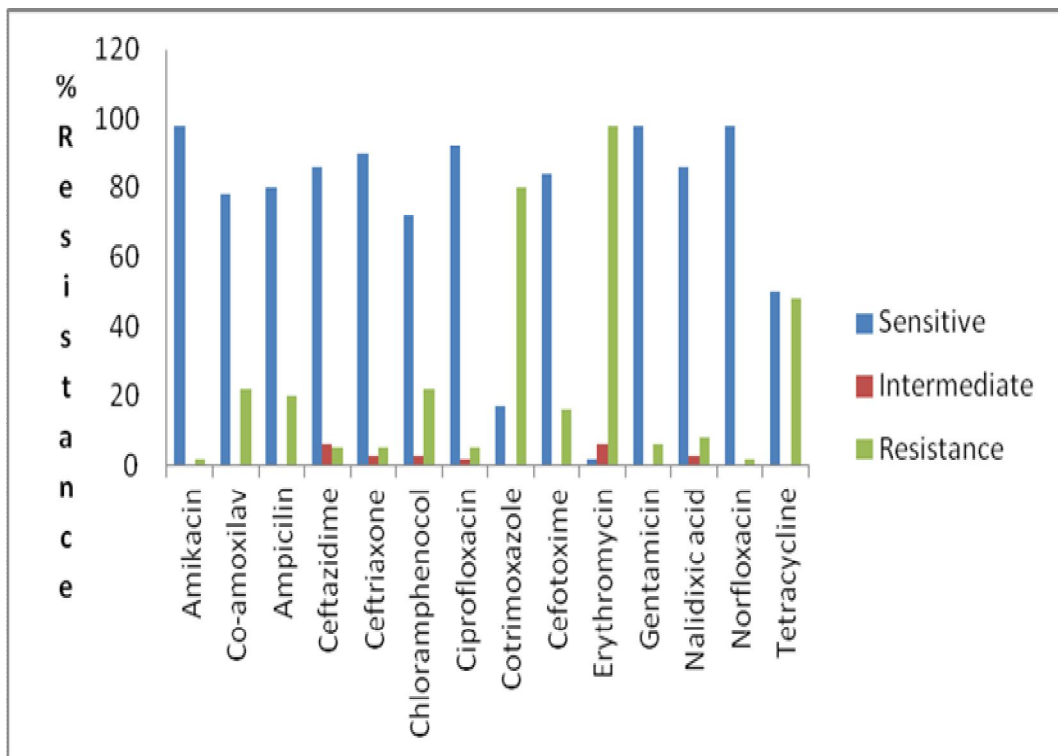


Figure 4.7 Drug susceptibility testing on ETEC, other *E. coli* pathotypes and other enteric bacterial isolates from Machakos District Hospital

4.7.1 Antibiotic susceptibility by age grouping

When analysed based on age grouping, there was high resistance in ages below ten years(42%) and above 16 (23%) years. The resistance was low in ages between 10 and 16 years (18%, Figure 4.8).

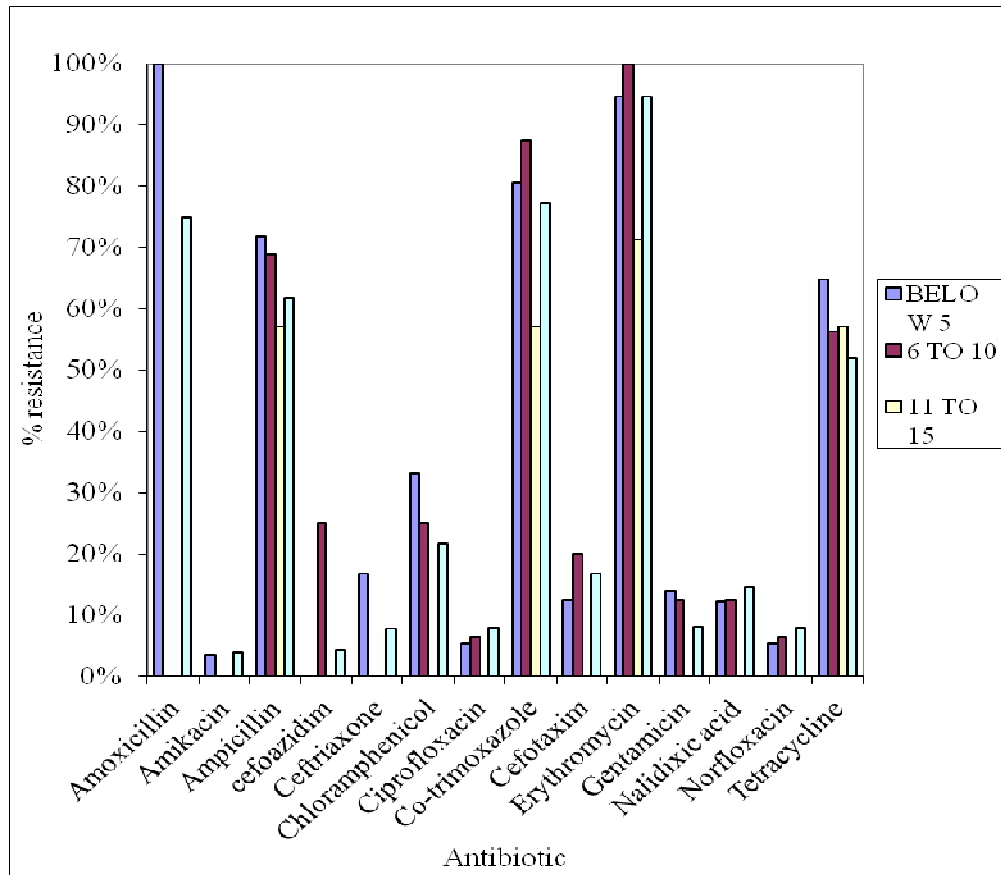


Figure 4.8 Antibiotic susceptibility profiles by age from patients in Machakos Dsitric Hospital.

4.7.2 Fluoroquinolone and Cephalosporins susceptibility testing

There was observed increase in resistance to quinolone(10%) and Cephalosporins (18%) by ETEC, other *E. coli* pathotypes and other enteric pathogenic Bacteria as

shown by both disc diffusion and minimum inhibition concentration methods (Figure 4.9; Table 4.7).

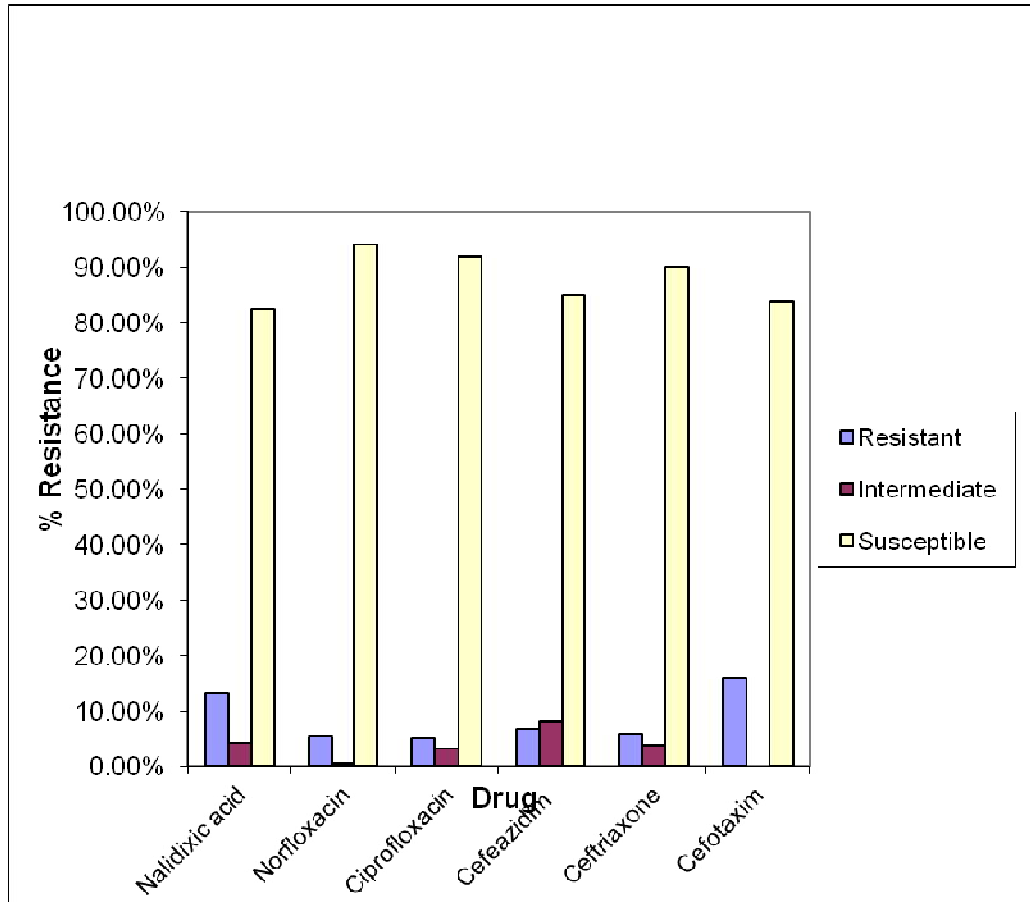


Figure 4.9 Fluoroquinolone resistance in bacterial isolates from Machakos

4.7.3 Minimum inhibitory concentrations for bacteria isolates

The minimum inhibition concentrations for ETEC isolates indicated raised MICs especially for cephalosporins (0.16µg/ml) and cephalosporins (>32µg/ml) compared to the normal range (0.002- 4) and (0.16-32) respectively implying a possibility for

limited antimicrobial therapy tables 4.6. This was observed in all 45 tested ETEC isolates. Other *E. coli* pathotypes (EIEC, EPEC and EAEC) also demonstrated raised MICs to different antibiotics ranging from cotrimoxazole to cephalosporins. However, there was reduced MICs to gentamicin implying that it can be prescribed by clinician for treatment of diarrhoea. This was observed in all 58 isolates of other *E. coli* pathotypes. Shigellae isolates showed the highest raised minimum inhibitory concentrations compared to ETEC and other *E. coli* pathotypes especially on cephalosporins, an indication of limited options in the treatment to reduce the shading of resistant strains from people infected with different Shigellae strains (Table 4.7).

Table 4.7 MIC ranges for ETEC, Other *E. coli* and Shigellae isolates.

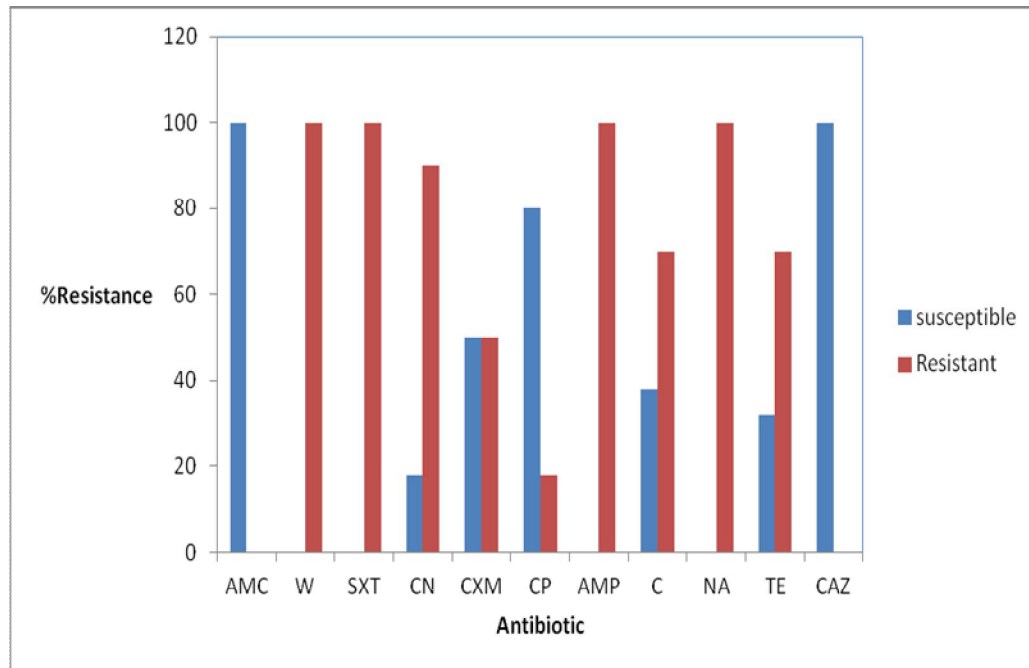
Antibiotic	ETEC		Other <i>E.coli</i>		Shigellae		Range
	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	MIC ₅₀	MIC ₉₀	
Contrimoxazole	>2/38	>4/38	>2/38	>4/38	>2/38	>4/38	0.002 - 32
Amikacin	>16	>16	>16	>16	>16	>16	0.16 - 256
Gentamicin	4	6	4	6	4	8	0.16 - 256
Chloramphenicol	>16	64	>16	64	>16	64	0.16 - 256
Ciprofloxacin	0.08	0.16	0.08	0.4	0.16	0.4	0.002 - 4
Cefuroxime	>8	>16	>16	>16	>16	>16	0.16 - 256
Cefotaxime	>32	>32	16	>32	> 8	32	0.16 - 32
Ceftazidime	>32	>32	>32	>32	8	32	0.16 - 256
Tetracycline	4	8	4	8	32	64	1.5 - 256

The antibiotics with raised MIC₅₀ and MIC₉₀ were fluoroquinolones for ETEC, *E. coli* pathotypes and Shigellae. Gentamicin was reported with low MIC pointing out that it can now be used for treatment of infection due to diarrhoea. Gentamicin had been out of shelves for the last ten years as the bacteria were resistant to it.

All the isolates showed increased minimum inhibition concentrations at both MIC₅₀ and MIC₉₀ indicating that 50% or 90% of the microorganisms could be inhibited respectively. This indicates a possibility of limited choice in antimicrobial agents for management of bacterial diarrhoeal diseases especially in the curtailing of shading of enteric pathogens. Shigella isolates presented with the highest increase in minimum inhibition concentrations.

4.7.6 Drug susceptibility testing after conjugation

Drug susceptibility testing after conjugation on 38 isolates showed 33/38 (86.8%) transferred resistance to the recipient *E. coli* K12. These were distributed as Shigellae (13/38; 34.2%), ETEC (15/38; 39.5%) and EPEC(5/38;13.2%) . The recipient was 100% resistant to ampicillin, cotrimoxazole and nalidixic acid for 33/38 MDR strains indicating that resistance was transmissible (Figure 4.10). The plasmids ranged from 1.8kb to 35.5 kb.



Key: AMC: Co-amoxiclav, W: Trimethoprim SXT: Sulfamethoxazole, CN: Canamycin, CXM: Cefuroxime, CIP: Ciprofloxacin, AMP: Ampicilin, C: Chloramphenicol, NA: Nalidixic acid, TE: Tetracyclin, CAZ: Cefetazidine.

Figure 4.10 Drug susceptibility of the transconjugant bacteria

4.8 Extended spectrum beta lactamases

Out of the 300 samples from the Machakos District Hospital tested, 18 (6%) were positive for ESBL production by double disk diffusion DDD. The 18 DDD and E test-positive strains (3 *Klebsiella* SPP and 15 *E. coli*) were retested with Vitek (GNS-532 card), and 17 of these strains (94.4%) were subsequently found to be ESBL positive. One strain (5.6%) tested ESBL negative by Vitek. The cefotaxime ESBL strip detected the presence of ESBL activity in 18 (100 %) of the isolates tested. All the 18 (100 %) isolates demonstrated phantom phenomena that indicated them to be ESBL positive by DDD (Figure 4.11).



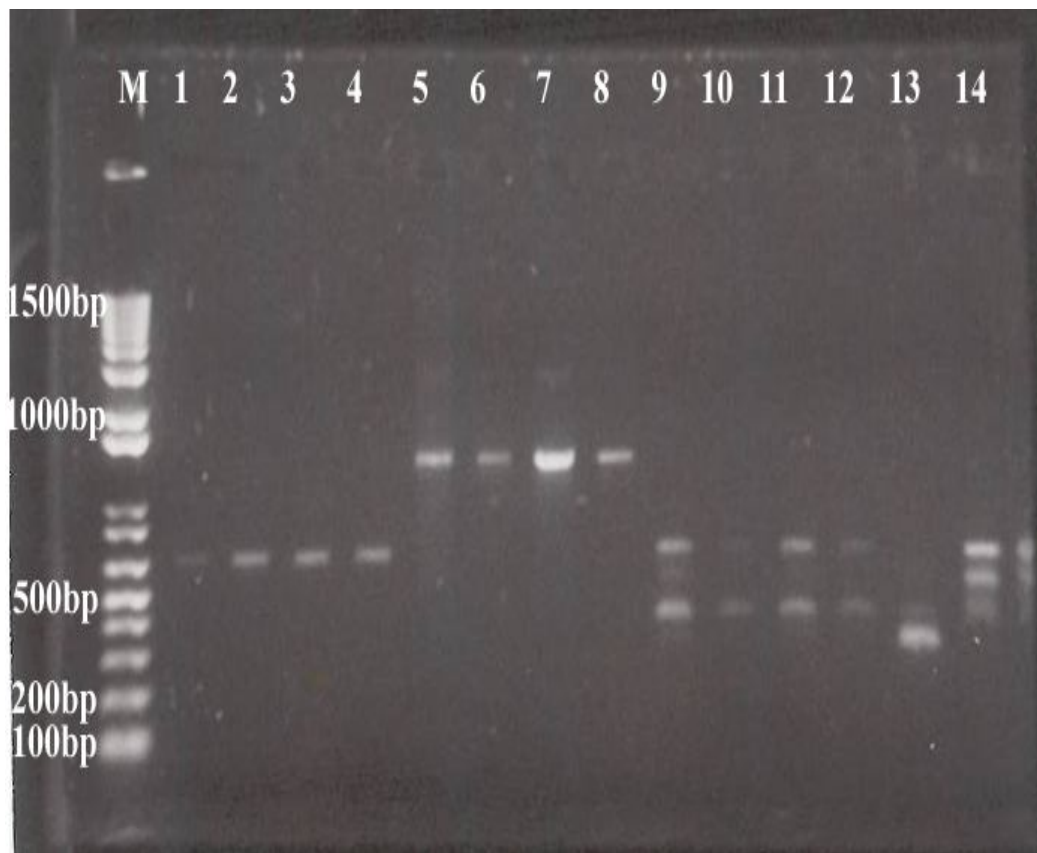
Figure 4.11 ESBL activity for phantom phenomena by Double Disc Diffusion method

4.9 Detection of gyrase A, B and Topoisomerase iv genes by PCR and sequencing

4.9.1 Gyrase A, B and Topoisomerase detection

The mutations that were responsible for fluoroquinolone resistance in the *gyrA*, *gyrB*, *parC*, and *parE* genes of *E. coli*, *Shigella* spp. and *Salmonella* spp were investigated by mPCR using primers that were highly specific (Plate 4.7). From the isolates tested, 30/300(10%) were positive for the above genes. The genes were distributed as follows: *gyrA* (17/30, 36%) *gyrB* (7/30, 23.3%) topoisomerase (*parC* 3/30, 10%) *parE* 3/30, 10%). Out of the 17/30 *gyrA* genes 9 were extracted from ETEC, 6 from *Shigellae*, 2 from Atypical *E.coli* and none from *Salmonellae*. *GyrB* resistant genes were distributed as follows: two from ETEC, THREE FROM *Shigellae*, two from

atypical EPEC and none from Salmonellae. Topoismerase genes (*parC* and *parE*) were only recovered from Shigellae. The genes were isolated from children below age of five years and those above 40 years.



Lane M represents 100bp molecular maker, Lanes 1-4 gyraseA, 5-8 gyrase B, 9-13 *par C*, and 14 *par E*.

Plate 4. 7 PCR products of the *GyrA*, *B*, *par C* and *par E* quinolone resistance genes in *E. coli* and *Shigella* spp

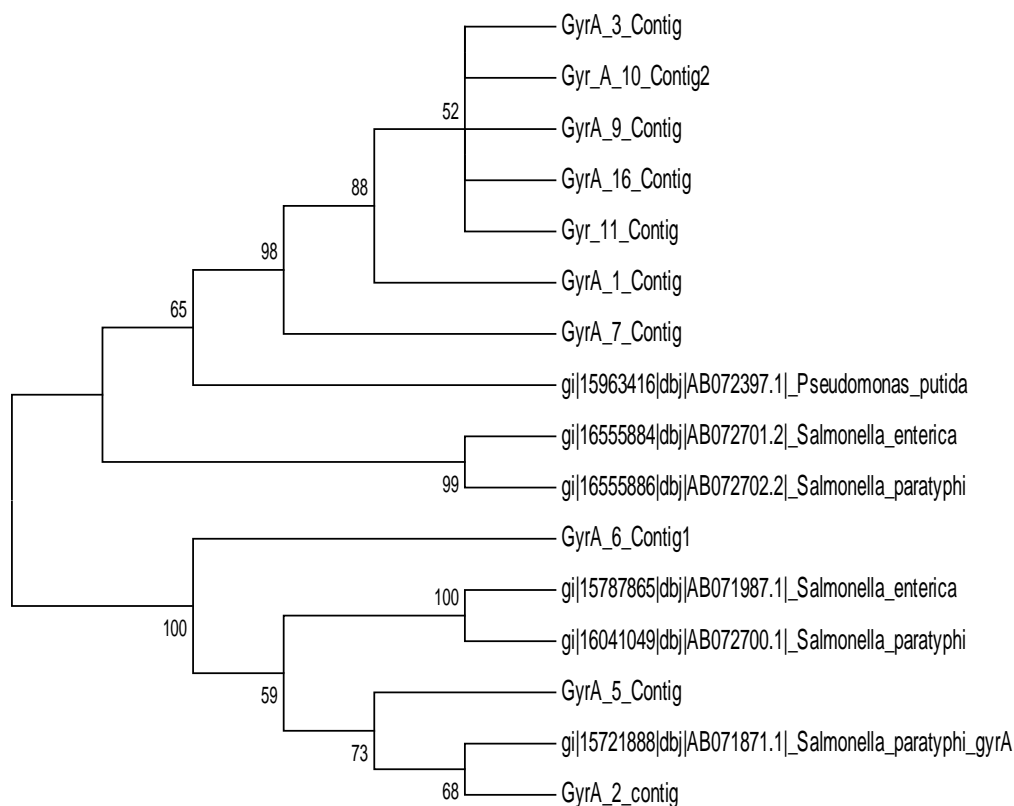
4.9.2 Sequences for quinolone resistance determining regions

Sequencing was carried out on all the 30 isolates that were resistant to quinolones. All sequences of the quinolone resistance-determining region of the *gyrA* gene (17/30, 56.7%) in the isolates which showed decreased susceptibilities or complete

resistance had a single or double mutation at either the Ser-83 or the Asp-87 codon. There was no mutations observed in the *gyrB* (7/30, 23.3%), *par C* 3/30, 10%) or *par E* (3/30, 10%) genes (Plate 4.7 , Table 4.8).

4.9.3 Sequence alignment for quinolone resistance regions

The sequences were aligned as analysed using MRbayes software and phylogenetic tree drawn using the fig tree for determination of relatedness of the isolates in terms of *GyrA* gene mutations (17/30, 56.7% Figure 4.16). The tree yielded five clusters. Isolates from this study clustered successfully with those from the gene bank bearing the *GyrA* resistance genes. The conversions and comparisons in base alignment was analysed by ESS using tracer software and black colour outcome indicated a success in the conversions from the 17/30 (36%) isolates and a dendrogram drawn by neighbour joining (Figure 4.12). Gyr7 was the only different isolate (1/30, 3.3%). It was routed out of the other sequences implying that it was different. It was clustered as an out group and this will require further work to be done. The other *gyrA* resistant isolates (16/17, 94.1%) clustered 100% with sequences of quinolone resistance genes obtained from the gene bank. This implied that *GyrA* resistance genes were responsible for observed resistance in ciprofloxacin, nalidixic acid and norfloxacin.



The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 21.7019)). The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 76 positions in the final dataset.

Figure 4.12 Consensus tree of Gyrase A genes and its nearest neighbours.

Both single (50%) and double(50%) amino acid switch were thought to be the cause for resistance to the quinolones (Table 4.8). Mutations were detected at amino acid 83 and 87.

Table 4.8 Fluoroquinolone resistance and amino acids substitution in bacteria isolates from Machakos District Hospital.

Strain	MIC (μ/ml)			Amino acid switch	
	CIP	NOR	NA	<u>GyrA</u>	
MKS <i>S. dysenteriae</i>	0.5	0.03	1	ser ₈₃	Ser ₈₇
MKS <i>EPEC</i>	0.09	0.19	>256	Ser ₈₃ -Leu	Asp ₈₇
MKS <i>ETEC</i>	6	12	>256	Ser ₈₃ -Leu	Asp ₈₇ - Gly
MKS <i>ETEC</i>	200	250	>256	Ser ₈₃ -Leu	Asp ₈₇ - Gly
MKS <i>S. flexneri</i>	0.012	0.023	1.25	Ser ₈₃	Ser ₈₇
MKS <i>S. boydii</i>	64	250	>256	Ser ₈₃ -Leu	Asp ₈₇ - Asn
MKS <i>S. sonnei</i>	0.16	0.014	>256	Ser ₈₃ -Leu	Ser ₈₇

Key: MKS denotes Machakos

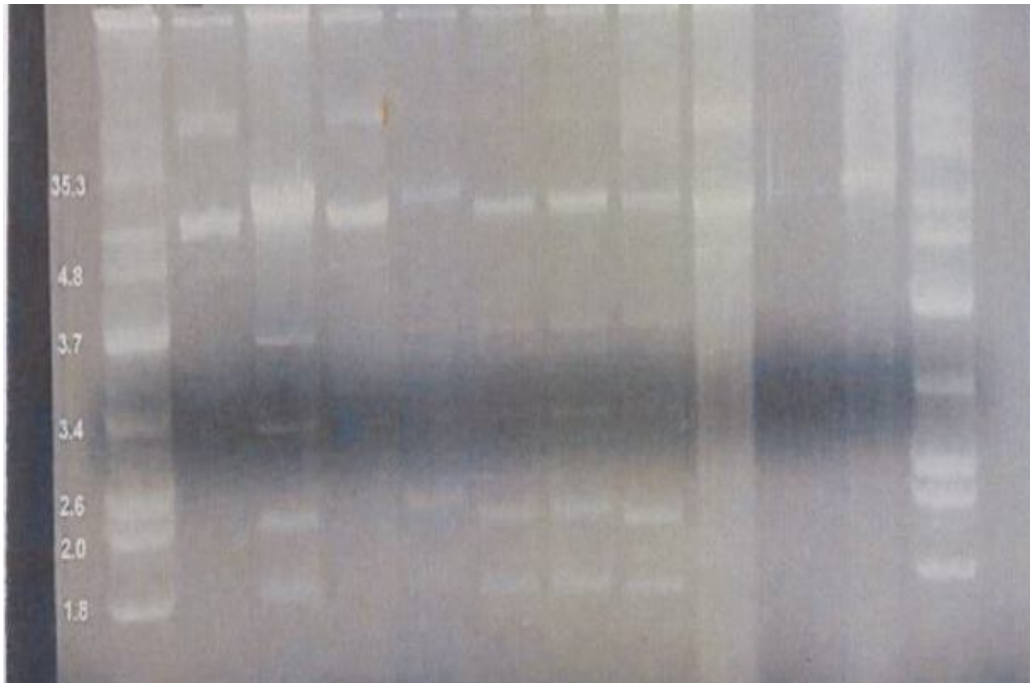
4.10 Test for invasiveness

All 67 *Shigella* strains harbored the invasive plasmid and had the ability to amplify for *ipaH* gene.

4.10.1 Plasmid profile analysis

Analysis of plasmid DNA revealed that out of the 33 multidrug resistant strains tested (33/33, 100%) contained multiple plasmids whose sizes ranged from 1.8 to 35 kb, forming a number of unique banding patterns as shown in Plate 4.8. All strains carried more than two plasmids. They all transferred their resistance phenotypes to four antibiotics (Ampicillin, Nalidixic acid, cotrimoxazole and chloramphenicol) to the recipient *E. coli* K12 100% sensitive for the above antibiotics. After conjugation, the transconjugant *E. coli* K12 was 100% resistant to Ampicillin, Nalidixic acid, cotrimoxazole and chloramphenicol. This confirmed the fact that resistance observed to a range of antibiotics was plasmid transmitted.

M 1 2 3 4 5 6 7 8 9 10 M



Lanes M indicates molecular marker *E. coli* 39R and V517MDa respectively; lanes 1 to 8 MDR strains, lanes 9 antibiotic sensitive *E. coli* strain and lane 10 *Shigella* strain.

Plate 4.8 Plasmid profiles of multidrug resistance *Shigella* strains

CHAPTER FIVE

5.0 DISCUSSION

5.1 Prevalence of ETEC (LT, ST, LTST, CFA, CS)

Enterotoxigenic *E. coli* is one of the common causes of diarrhoea in the developing countries. In the current study, ETEC prevalence was 11%. This was higher compared with the studies done in Kenya where the prevalence was reported at 3% (Waiyaki *et al.*, 1985; Jiang *et al.*, 2002). A study by Ngugi did not yield any ETEC (Ngugi *et al.*, 1985). In Nigeria (Okeke *et al.*, 2003), ETEC was detected at a lower rate compared with those detected in previous Kenyan studies (Sang *et al.*, 1997; Waiyaki *et al.*, 1985). In Peru, a detection rate of 5.3% was realized (Rivera *et al.*, 2010). Due to the differences in the occurrences of ETEC, the implementation of immunoprophylactic measures for the control of ETEC diarrhoea, should be first be assessed and variations in the phenotypic, genotypic, and pathogenic properties of the bacterium at hand should be identified. It is worth noting that this is the first time that the presence of CS19 in ETEC in Kenya has been documented. Studies done elsewhere also show that CS6 is increasing throughout the world (Claudia *et al.*, 2011; Rodas *et al.*, 2010; Rivera *et al.*, 2010; Shaheen *et al.*, 2004; Paniagua *et al.*, 1997). In this study, it was also noted that a significant low recovery of ETEC strains expressing CS6 and CS19 compared with the frequency of isolation of ETEC strains expressing other CF types from children below five years was obtained. Similarly, the current study noted that the detection of CFA/I-expressing ETEC isolates appeared to be more associated with children below 5 years of age and this finding agrees with those obtained from studies conducted by Claudia *et al.* (2011) in Brazil

and Ireland, Shaheen *et al.* (2004) in Egypt, Richard *et al.* (2007) in Zambia, Eric *et al.* (2001) in England and Peruski *et al.* (1999) in Bangladesh.

The rates of recovery of ETEC and the identities of the CFs reported in this study are similar to those from longitudinal community-based epidemiological studies of ETEC in northern Egypt and hospital-based studies in other disparate locations, such as Indonesia (Subekti *et al.*, 2003; von Sonnenburg *et al.*, 1981) but differ with findings in Peru (Rivera *et al.*, 2010; Paniagua *et al.*, 1997) where the recovery rate was 5.3%. The rates of identification of ETEC isolates expressing CS6 and CS19 were similar in the entire study implying that these two CFs are persistent and are likely to withstand adverse conditions. This makes them ideal vaccine candidates for prevention of diarrhoea due to ETEC. CS6 and CS19 have been tested for animal model in University of Maryland and shown to be safe and successful. CS1 and CS3 were detected at a rate of 5.6%. These findings indicate that ETEC isolates expressing CS6 are more likely to be recovered from children below 5 years of age. They were, however, recovered significantly less than ETEC isolates expressing CFA/I in children less than 5 years of age. In this study other ETEC CF types, such as CS3 alone were infrequently recovered at 2.8%. A similar observation was reported in Argentina and Brazil (Viboud *et al.*, 1999; Rodas *et al.*, 2010). On the other hand, this finding differs from the results obtained in Mexico (Arne *et al.*, 2010), Bangladesh (Blackburn *et al.*, 2009; Karina *et al.*, 2009) and Egypt (Qadri *et al.*, 2000) where this CF component was identified at a higher percentage. It should be noted that the prevalence of infection due to ETEC isolates expressing CS6 or CS19 has been increasing in the world (Li *et al.*, 2004; 2009; Sherlock *et al.*, 2005;

Steinsland *et al.*, 2004; Jiang *et al.*, 2000; Qadri *et al.*, 2000). The highest rate of recovery of ETEC isolates expressing a detectable CF was during the warm months. This finding is in agreement with observations made in Egypt (Qadri *et al.*, 2007; Rao *et al.*, 2003). It should be also noted that the recovery of ETEC isolates expressing CS6 or CS19 did not appear to vary by season as other CFs. From Studies carried out in the Judea area of the West-Bank of Israel (Wolk 1997) and by Sizemore in Malawi (Sizemore *et al.*, 2004) similar observations were made, implying that CS6 and CS19 are stable and are viable candidates for the elaboration of a vaccine for prevention of diarrhoea due to ETEC.

In the present study, there were ETEC isolates (36/98, 36.7%) without detectable CF. This observation is in agreement with findings from work done in Egypt (Shaheen *et al.*, 2004; Steinsland *et al.*, 2003; 2004) and the Asian region (Serichantalergs *et al.*, 1997). In the latter studies, approximately 50% of the ETEC isolates identified lacked detectable CF expression based on either dot blot analysis or DNA hybridization assays (Rao *et al.*, 2003; Shaheen *et al.*, 2004; Steinsland *et al.*, 2004). It was therefore anticipated, based on the findings from Rao *et al.*, (2003) that a large proportion of the ETEC isolates recovered in the current study, would also lack a detectable CF antigen but this was not the case. This could be due to the use of specific oligonucleotide primers in the Multiplex Polymerase Chain Reaction in the current study being more sensitive than the dot blot used in Rao's study (Rao *et al.*, 2003). The percentage of ETEC isolates where a CF was not detectable expressed LT and these results were in agreement with those obtained from studies that used genotypic detection methods (Rivera *et al.*, 2010; Mandomando *et al.*, 2007; Steffen

et al., 2004; Nishimura *et al.*, 2002; Merz *et al.*, 2000). The lack of an identifiable CF in ETECs may have been due to the loss of the plasmid harbouring the CF genetic element, down regulation of the CF genes, a mutation within the genetic locus, or expression of a CF not covered by the mPCR panel (Rodas *et al.*, 2011). To account for differences due to age or diarrhoea markers associated with ETEC isolates expressing one of the five major CFs found, the corresponding patients' demographic and clinical data were related according to the ETEC CF identified. Although ETEC infection can cause profuse watery diarrhoea with little or no fever or vomiting, previous reports of ETEC isolates recovered from hospitalized infants elsewhere found the infection to be commonly associated with fever and vomiting (Nweze, 2010; Rodas *et al.*, 2009; Shaheen *et al.*, 2004; WHO 1990).

In the current study, 5.3% of the children below three years of age with ETEC infection suffered from dehydration. In addition, vomiting and fever were reported in children shedding ETEC strains. The observation of severe diarrhoea markers for example, dehydration and the number of stools per day in some of the children may be attributed to the presence of strains that are more virulent due to the presence of additional virulence factors (Long *et al.*, 2010; Huang *et al.*, 2006; Alam *et al.*, 2006). It could also be that the presence of severe diarrhoeal symptoms may be the result of an immunologically naïve child's first encounter with an ETEC pathogen.

E. coli pathotypes are diverse microorganisms composed of several clones, the genetic background of each encoding for distinct combinations of virulence determinants. Accordingly, use of the conventional classification of *E. coli* by O- and

H-antigen serotyping may restrict appreciation of the genetic relationship of different diarrhoeagenic *E. coli* pathotypes (Chakraborty *et al.*, 2001; Pacheco *et al.*, 2001). Phenotypic and genotypic heterogeneity has been demonstrated among ETEC isolates of similar serogroups, such as O153 and O20 (Sullivan 2005; Maiden 1998). ETEC isolates expressing CS5 plus CS6 appear to be genetically distinct from ETEC isolates expressing CFA/I (Li *et al.*, 2004). In the present study, the isolates fitted within two of the five indistinguishable strain patterns found within the CFA/I-expressing ETEC isolates and may have resulted in diarrhoea with diverse severities according to patients' presentations. The adaptive ability of *E. coli* pathogens (Qadri *et al.*, 2000) within the host gastrointestinal tract or in the external environment is not clear.

An age-dependent isolation rate has been observed for ETEC isolates expressing CFA1 and CSs, suggesting a naturally acquired immunity in older children (Rao *et al.*, 2005) and this was reflected in the current study. In the current study, the decline in the rate of infection with ETEC isolates expressing CFA/I that was seen from the age of 10 years and above supports this idea that older children shed off ETEC or build up immunity due to frequent exposure and this underlies the importance of developing an ETEC vaccine since the risk factors for acquiring ETEC infection are present among children up to one year of age (Arne *et al.*, 2010; Qadri *et al.*, 2006). *In vitro* experiments have demonstrated that environmental components have an impact on the expression of ETEC virulence factors (Fleckenstein *et al.*, 2006; Glenn *et al.*, 2005). Little is known about the *in vivo* influence of environmental stress and the adaptability of ETEC as well as other *E. coli* pathotypes within their groups. The

successful recovery of ETEC CFAS and CS might have been attributed to the modern techniques which are both specific and sensitive. Many researchers have tried this but failed before.

5.2 Prevalence of other *E. coli* pathotypes

From the current study, different *E. coli* pathotypes were detected among them Enteropathogenic *E. coli* (EPEC), Enteroinvasive *E. coli* (EIEC) Enteroaggregative *E. coli* (EAEC) and colonizing necrotising factors (CNF). These were isolated from (42.6%) patients. This was higher compared to studies done by Sang *et al.* (1996; 1997) where the recovery rate was less 22%. However, Sang in His study in the Masai community in Kenya detected *E. coli* 0157:H7 which was not detected in the current study. Diarrhoeal disease remains a public health problem worldwide and the role of EPEC as a cause of diarrhoea in children, particularly in developing countries, is now well established (Vilchez *et al.*, 2009; Trabulsi *et al.*, 2002). *E. coli* is known to be a normal flora of the gut, however, the acquisition of virulence genes from other pathogenic enteric bacteria make it virulent. The proven virulence determinants of EPEC include genes within the LEE, notably intimin (the outer membrane protein product of the *eae* gene), and Bfp, which is encoded by *EAF* (Al-Galas *et al.*, 2007). The key role of *EAF* gene in promoting the virulence of EPEC was established by Levin *et al.*, (1985), who showed that an *EAF*-negative derivative strain of EPEC, E2348/69, is markedly less virulent for adult volunteers than the wild-type strain. The same study showed that an atypical EPEC strain, E128012, which intrinsically lacks *EAF*, is also virulent in volunteers. This observation established that certain

EPEC strains do not require *EAF* to cause disease. In the current study most EPEC only carried the *eae* gene and is in agreement with Levin *et al.*, (2001). These intrinsically *EAF*-negative strains were originally called Class II EPEC (Feng *et al.*, 1998) but are now more generally referred to as atypical EPEC. They are characterized by the presence of *LEE* and the absence of factors encoded by *EAF*, in particular, *Bfp*. In this way, atypical EPEC resemble EHEC, which are able to cause diarrhoea despite their lack of *Bfp*. Indeed, persuasive evidence indicates that the most prevalent EHEC strain, serotype O157:H7, evolved from an atypical EPEC strain of serotype O55:H7 (Bryce *et al.*, 2005; Feng *et al.*, 1998).

Atypical EPEC, EAEC and EIEC accounted for 54.6% % (Table 4.4) of all the cases in the study; 21% of cases being attributable to a mixture of other bacterial causes. However, the prevalence of EAEC in patients below 10 years of age and that obtained from participants above 11 years was the same. In contrast, atypical EPEC was isolated significantly more often from patients below 5 years than those above 6 years, regardless of when the sample was collected.

In the current study (5.6%) of *eae*-bearing strains identified in patients with gastroenteritis were atypical EPEC and these were thought to have caused the disease. In other regions, the evidence from volunteer studies and reports of outbreaks of diarrhoea with atypical EPEC (Bischoff *et al.*, 2005; Yatsuyanagi *et al.*, 2003; Viljanen 1990) indicates the role of atypical EPEC in disease as controversial. Originally, atypical EPEC were grouped with EPEC but were then segregated because they lacked *EAF*. Justification for this division stemmed from the observation that *EAF*-bearing EPEC far outnumber atypical EPEC as the cause of

infantile diarrhoea in less-developed countries and of diarrhoea outbreaks in general (Vilchez *et al.*, 2009). In diarrhoeal reports from countries as diverse as Iran, Poland, Brazil, Japan, South Africa, and the United Kingdom, atypical EPEC strains have outnumbered typical strains as a cause of gastroenteritis (Cooke., 2010; Spano *et al.*, 2008; Nishikawa *et al.*, 2002; Paciorek, 2002; Bouzari *et al.*, 2000; 2001). Atypical EPEC were also more frequent than typical strains in aboriginal children hospitalized for diarrhoea in the Northern Territory of Australia (Kukuruzovic *et al.*, 2002), a finding that was reflected in the current study. In the present study 17 of *eae*-bearing strains identified in patients with gastroenteritis were atypical EPEC.

As for EPEC in general, atypical EPEC were originally incriminated as intestinal pathogens by virtue of their epidemiologic association with cases of diarrhoea (Trabulsi *et al.*, 2002). Although atypical EPEC generally are serotypes which differ from *EAF*-positive EPEC (and other pathotypes of diarrhoeagenic *E. coli*), the 12 O-serogroups recognized by the World Health Organization as EPEC include both typical and atypical varieties (Robins-brown, 2004; Trabulsi 2002). Some of the atypical EPEC strains within these serogroups carry accessory virulence-associated determinants such as the EHEC hemolysin (commonly found in serotypes O26:H11 and O111ac:H8). Some strains also carry *astA*, the gene for enteroaggregative heat-stable enterotoxin, *EAST1*, which is frequently found in serotypes O55:H7, O119:H2, and O128:H2 (Bryce *et al.*, 2005; Viera *et al.*, 2001; Trabulsi 2002). Atypical EPEC strains of non-EPEC serogroups generally do not express these factors. In the present study, none of the 17 atypical strains was positive for *ehxA* required for the production of EHEC hemolysin.

The virulence of atypical EPEC despite their lack of *Bfp*, which typical EPEC require to cause severe disease, suggests that these bacteria carry an adhesin analogous to Bfp that augments their ability to colonize the intestine. Previous studies, however, have shown only a low frequency of known *E. coli* adhesins, including aggregative adherence fimbriae, P fimbriae, S fimbriae, PAP pili, and afimbrial adhesins in atypical EPEC strains (Vernacchio *et al.*, 2006). Although atypical EPEC may carry an adhesin equivalent to Bfp, which remains to be discovered, these bacteria may use any known *E. coli* adhesins to bind to the intestine (Adachi *et al.*, 2002). This suggestion is supported by the marked heterogeneity of atypical EPEC in terms of adhesion pattern and the observation that adhesins other than Bfp can restore cell-binding capacity to EAF-cured strains of typical EPEC (Francis *et al.*, 1991).

5.3 Prevalence of Enteroaggregative *E. coli*

In the current study, *E. coli* isolates from the stools of patients with diarrhoea in Machakos County were investigated for the presence of EAEC. EAEC was the most prevalent (35%) among other *E. coli* pathotypes and this observation agrees with results from a study done by Oundo *et al.*, (2008). Reports from other developing countries with similar conditions as Kenya have shown that EAEC are significantly associated with diarrhoea (Martin *et al.*, 2003; Scaletsky *et al.*, 2002) and this was in agreement with the findings in the current study; EAEC were the most prevalent pathotype isolated. EAEC as a causal agent for diarrhoea has not been given the attention it deserves in Kenya. In the current study, EAEC was found in all diarrhoeal stools. The recovery rates of EAEC in stool showed significant association

with diarrhoea ($X^2 = 3.85$, $P = 0.021$) and this finding is in agreement with that reported by Oundo *et al.* (2008). Human infections due to EAEC are primarily associated with the consumption of faecally contaminated foodstuffs.

According to statistical data from the Center for Health Statistics in Mongolia and Nicaragua, the major bacterial enteric pathogen was *Shigella* (83%), followed by *Salmonella* (8.2%) and *E. coli* (4%) as the least (Vilchez *et al.*, 2009). This was contrarily to findings in the current study in which *E. coli* was the most prevalent (53%) followed *Shigella* (21%) and *Salmonella* was the least prevalent (0.3%). However, the significance of *E. coli* as an enteric pathogen has been underestimated, because the virulence factors of DEC have not been fully evaluated due to shortage of resources. The finding that EAEC is the most prevalent (13.7%) and most common pathotype in patient stools is important for future studies of the epidemiology of EAEC as causal agent for diarrhoea in Kenya. EAEC was recovered at 35% compared to 15.1% of cases in Mongolia and this appeared to be the most prevalent pathogen among DEC categories. Studies carried out in Brazil, India, southwest Nigeria, Congo, and Iran have noted that EAEC strains are important emerging agents of pediatric diarrhoea (Scaletsky *et al.*, 2002, Okeke *et al.*, 2000; Bouzari *et al.*, 2001). The pathogenic mechanisms of EAEC infection are only partially understood and are most consistent with mucosal colonization followed by secretion of enterotoxins and cytotoxins (Spano *et al.*, 2008). There appears to be significant heterogeneity of virulence among EAEC isolates, in part due to lack of specificity in the HEp-2 adherence assay. A few reports have evaluated the prevalence of EAEC virulence genes in strains isolated from case-control studies

(Spano *et al.*, 2008; Piva *et al.*, 2003; Elias *et al.*, 2002; Okeke *et al.*, 2000; Vila *et al.*, 2000).

5.4 Prevalence of other enteric pathogenic bacteria

Other than the targeted Entero-toxicogenic *E. coli*, the current study also detected other enteric bacteria pathogen such as *Shigella* spp and *Klebsiella* spp. The prevalence for *Shigella* spp was higher than that reported from other studies done in Kenya (Ngugi *et al.*, 1985) as well as other regions in Africa (Cooke *et al.*, 2010; Aibinu *et al.*, 2007). The recovery of *Salmonella* spp was the least compared with those reported in other studies done in Kenya (Kariuki *et al.*, 2004) and West Africa (Aibinu *et al.*, 2007) thus implying that Salmonellae are not common cause of diarrhoea in Machakos County.

In the current study, a total of 13 *S. flexneri* isolates were identified, primarily by using the standard biochemical and serological methods. However, only 66% of the isolates could be definitively serotyped using commercially available antisera (Denka Seiken Tokyo, Japan). The close relatedness between *E. coli* and *Shigella* spp makes serological identification a crucial step in the diagnosis of *Shigella* infection (Coimbra, 2001a). *S. flexneri* has eight serotypes, of which serotypes 1 to 5 are further classified into 12 sub-serotypes. *S. dysenteriae* has 12, *S. boydii* 18 and *S. sonnei* does not have any serotype. This classification scheme for *S. flexneri* is not comprehensive, because atypical strains or newer sub-serotypes are being isolated from different parts of the world including Egypt and Bangladesh (Qadri *et al.*, 2004;

El-Gendy, 1999; Carlin 1984 and 1989). These serologically atypical strains displayed conflicting agglutination patterns, reacting strongly with serotype 4-specific antisera.

On the basis of biochemical tests, 10 of the 13 *S. flexneri* isolates (76.9%) were mannitol negative but utilized sodium acetate. The remaining 3 (23.1%) were mannitol positive but did not utilize sodium acetate. The *S. flexneri* subgroup is characteristically mannitol positive, but variants in each serotype that do not utilize mannitol have been reported (Cook *et al.*, 2010). *S. flexneri* serotypes 4 and 6 appear to be the most common among the mannitol-negative varieties of *S. flexneri* but apparently these do not occur as frequently as their mannitol-positive counterparts. Utilization of sodium acetate by the isolates was in accordance with the standard results for *S. flexneri* serotype 4. Mannitol-negative serobiotypes of *S. flexneri* 4a are able to utilize sodium acetate, whereas their mannitol-positive counterparts rarely utilize sodium acetate (Amieva, 2005). On the other hand, *S. flexneri* 4b does not utilize sodium acetate. Vargas indicated that 43% of the mannitol-negative and approximately 8% of the mannitol-positive 4a strains were weakly positive in reaction with sodium acetate following incubation for 2 to 7 days (Vargas *et al.*, 1999). Similar results were obtained in the current study where the majority of the non-mannitol-fermenting strains showed strong positive reactions on extended incubation to 48 hours.

All of the mannitol-negative isolates in the present study were able to utilize xylose, mannose, and maltose, while the mannitol-positive isolates were not able to utilize these sugars. Another important distinction between these two groups was that those

that were mannitol positive were able to produce indole within 24 hours whereas mannitol negative failed to produce it. Arabinose was utilized by all of the strains, but a slight variation was observed between the two groups in terms of incubation time. The mannitol-negative isolates showed a positive reaction after overnight incubation, but the mannitol-positive isolates had to be incubated for more than 3 days for utilization of arabinose. Detailed biochemical studies, particularly of the utilization of mannitol, sodium acetate, and xylose and production of indole, confirmed that all of the isolates belonged to serotype 4 of *S. flexneri*, but grouping at the subserotype level based on biochemical tests was not possible due to variable reactions. According to Walker *et al.*, (2010), 82% of the mannitol-positive and 3% of the mannitol-negative strains of *S. flexneri* 4a are able to ferment raffinose, but none of the strains in the present study showed a positive reaction in raffinose fermentation. The overall criteria for this group did not agree completely with those for any sub-serotypes of *S. flexneri* type 4. Among the mannitol-negative strains, variations were observed in some biochemical reactions too. However, the common characteristics of these groups did not correlate with those of any of the sub-serotypes of *S. flexneri* type 4 meaning that they harboured features other those observed in *S. flexneri* type 4 and could be new serotypes.

As stated above, serotyping and biochemical techniques could not identify all species of *Shigella* and since antibiotic resistance is a major phenotypic trait, particularly for clinical isolates, it can potentially be informative in exploring the characteristics of an untypeable *Shigella* strain (Leibovitz *et al.*, 2000). Resistance in the current study was thought to be due to acquisition of resistance plasmids and possession of

quinilone resistance genes. The isolates were shown to be multidrug resistant (Chloramphenicol (28%), Cotrimoxazole (78%), Co-amoxiclav (70%) Erythromycin (98%) Ciprofloxacin (5%) and Tetracycline (56%). The finding from this study differs with that from Hossain working in Bangladesh (Hossain, 1998) where ciprofloxacin was 100% sensitive. Although in Bangladesh ciprofloxacin-resistant strains of *S. flexneri* have not yet been detected, nalidixic acid- and mecillinam-resistant strains of *S. flexneri* are frequently isolated there (Hossain, 1998). Interestingly, 11% of the *Shigella* strains were found to be sensitive to all of the antibiotics commonly used for the treatment of shigellosis in Bangladesh. This was contrarily to the findings of the current study in Machakos, Kenya where the strains were multi drug resistant. The overall susceptibility patterns of the test strains focus on the fact that the strains were not frequently exposed to expanded- or broad-spectrum antibiotics. Multiple antimicrobial resistance among *Shigella* isolates is an important problem in developing countries, including Kenya.

Although there is little information available on the association of plasmid profiles of *S. flexneri* strains and their serotypes, published reports have revealed a heterogeneous plasmid population in strains of *S. flexneri*, with most plasmids being smaller than 6 MDa (Nordmann, 2005). The presence of additional plasmids in patterns related to particular serotypes suggests that plasmid profiles may be useful in distinguishing between serotypes of *S. flexneri* (Wise *et al.*, 2007). In this study, plasmid profiling could not distinguish the *Shigella* isolates according to their major groups. Strains that were mannitol-positive (23.3%) showed an identical plasmid pattern which could not be distinguished from that of the mannitol-negative strains.

The current study showed that, 47.1% of the 67 strains were resistant to multiple antibiotics, of which 38% strains harbored the middle-range plasmid. The association observed between plasmid profiles and drug resistance patterns suggests that plasmids has epidemiological significance and should be evaluated carefully. To confirm this, conjugation experiments were carried out. Conjugal transfer of these plasmids to an *E. coli* K-12 strain of the plasmid demonstrated that resistance against ampicillin, tetracycline, chloramphenicol and trimethoprim-sulfomethoxazole was conferred by the plasmid as shown by respective drug resistance patterns after conjugation.

Invasiveness is an important property of pathogenic *Shigella* spp. The present study reviewed the invasive characteristics of all of the strains by PCR, since these were isolated from clinical cases. All isolates were invasive. Although the cardinal feature in the pathogenesis of *S. flexneri* infection involves the invasion of epithelial cells, it nevertheless has been reported that *S. flexneri* also produces an enterotoxin of mainly two types, SHET-1 and SHET-2. In the current study, it was found that the *sen* gene (which encodes ShET-2) was absent but the *set1* gene (which encodes ShET-1) was present. These findings were essentially similar to the ones reported by Noriega *et al* (1995), in which the *set1* gene has been shown to be exclusively present in *S. flexneri*.

PFGE has been employed to successfully discriminate strains of a variety of bacteria, including *S. dysenteriae* type 1 (Talukder *et al.*, 2001). Studies have shown that *NotI* gave the best discrimination among the strains, since it has a long-range DNA cutting site and cuts the DNA infrequently (Rodas *et al.*, 2010). Hence, this endonuclease

was used for typing of all isolates in the current study. Of the 13 atypical strains of *S. flexneri* type 4, a PFGE pattern was obtained (Figure 4.9B) and the PFGE pattern showed an identical banding pattern with one to three band differences

5.5 Susceptibility testing

5.5.1 Antibiotic susceptibility profiles of ETEC, other *E. coli* pathotypes and other pathogenic bacterial isolates

From the current study, multiple antimicrobial resistance patterns of the *Shigella* spp, *E. coli*, *Klebsiella* spp and *Salmonella* spp particularly to the drugs most frequently used in Kenya, that is amoxicillin / ampicillin, trimethoprim/sulfamethoxazole and chloramphenicol. The results are similar to other studies done in Kenya (Kariuki *et al.*, 2002:2004; Sang *et al.*, (1996:1997). However, the current study differs in gentamycin susceptibility where it indicates it as sensitive while the other four studies indicates it as resistant. The data also indicate that tetracycline, ampicillin, augmentin, trimethoprim/sulfamethoxazole and chloramphenicol are least likely to be effective in the treatment of infections due to diarrhoea in Machakos County. Similar observations have been reported in different regions in other parts of this country (Kariuki *et al.*, 2002; 2004; Sang *et al.*, 1997). Similarly, high proportions of multidrug-resistant *E. coli*, *Shigella*, *Salmonella* and *Klebsiella* isolates in children and adults have also been reported from Lagos and Ogun states in Nigeria (Aibinu *et al.*, 2007; Opintan *et al.*, 2007), Accra region of Ghana (Mills-Robertson *et al.*, 2002), and Lima in Peru (Rivera *et al.*, 2010). In contrast to the studies in Ghana,

Kenyan isolates not only resisted the drugs mentioned but also quinolones and third generation cephalosporins.

Susceptibility testing of *E. coli* isolates revealed high resistance to the locally used antibiotics comparable to those shown by the closely related Shigella. This underlines the usefulness of *E. coli* as a surveillance tool, atleast for infections due to gram negative bacteria. A good percentage of *E. coli* (34.9%) isolates from patients belonged to diarrhoeagenic strains and possessed virulent genes. More than half of the *E. coli* isolates in this study presented as normal stool *E. coli* since bacteria isolated from both children and adults as non pathogenic *E. coli* were also resistant to the same antibiotics.

Both diarrhoeagenic and commensal resistant *E. coli* may constitute a potential reservoir for resistance genes that can be transmitted horizontally to other bacteria. This was observed in the transconjugant when pathogenic and non pathogenic *E. coli* were used as donors. Out of 30 pathogenic *E. coli* tested 28 successfully transferred plasmids to the respective recipient. From the 8 nonpathogenic *E. coli* tested 5 successfully transferred plasmids to *E. coli* K12. The transconjugant was observed to be resistant to ampicillin and nalidixic acid implying that non pathogenic *E. coli* had also acquired resistant plasmids. The high proportions of resistant bacteria (and particularly those resistant to tetracycline, which is generally not used in children) in samples from children less than 5 years indicate the acquisition of resistant bacteria by the children rather than resistance induced through antimicrobial treatment. Another major finding was that the transconjugant did not acquire resistance plasmid

for cephalosporins contrarily to the finding from Lagos, Nigeria from both human and animal samples where the cephalosporins resistance plasmids were transmissible (Aibinu *et al.*, 2007). The findings from the current study agree with those from a population-based study, which indicate that children acquire resistant *E. coli* isolates from household contacts (Lietzau *et al.*, 2007; CDC, 2004).

Resistance to β -lactam antibiotics or chloramphenicol was observed more frequently among isolates obtained from infants below 5 years when compared with older children that were between 6-10, and 11-16 years. The association was statistically significant ($X^2 = 3.38$; $P < 0.05$) and this group may be representative for the overall harboring of resistant enteric bacteria by children in the County. These data suggest that infants may acquire the commensal enteric flora from their parents who have likely been more exposed to antibiotics than their older children. Notably, a high prevalence of resistant *E. coli* has been shown for adults from other parts of Africa (Diniz-Santos *et al.*, 2005; Bopp *et al.*, 2003; Newman *et al.*, 2002). With increasing age, the children may lose some of the resistant strains acquired originally. This view was supported by findings from older children between the ages of 11-16 years of age for whom *E. coli*, *Shigella* spp and *Klebsiella* spp showed reduced resistance. The data also indicate that the commonly used can no longer be considered as first-line treatment options for infections due to *E. coli*, *Shigella*, *Salmonella* and *Klebsiella* in people with diarrhoeal illness in Machakos County. In addition, 18/300 (6%) isolates produced extended-spectrum β -lactamases. This has also been reported in Cameroon though their figures were 2% higher than those reported in the current study (Gangoue *et al.*, 2007).

To determine if resistance was transferrable, *in-vitro* Conjugation was performed on the 38 of the MDR strains comprising of pathogenic and none pathogenic *E. coli*, *Shigella*, *Salmonella*, and *Klebsiella* using *E. coli* K12 as the recipient. The transferability of resistance was observed in 20/38 transcojugant.

The conjugative transfer of resistance was investigated in the 38 isolates exhibiting the most prevalent resistance phenotype (resistance to ampicillin, chloramphenicol, tetracycline, trimethoprim, and sulfonamides, amikacin and nalidixic acid and third generation cephalosporins). Conjugative transfer of resistance traits was observed in 20 of the 38 isolates tested. In most cases, the co-transfer of several resistance traits was observed, thus leading to the suggestion that there is linkage of the resistance genes in the same transferable plasmid. Though the recommended way to contain diarrhoea is oral rehydration, it is prudent to administer antibiotics in order to control the shedding of resistance strains from the infected patients to the environment and water systems.

5.5.2 Extended - Spectrum Beta Lactamases (ESBLs)

From the current study, 18/300 (6%) of the cephalosporins resistant bacteria exhibited beta lactamases enzyme that is responsible for the resistance. The available data on ESBL in Kenya was performed on blood and urine samples whose prevalence was higher than what was reported in the current study (Kariuki *et al.*, 2002). There is currently a great need for reliable and efficient tests to detect ESBLs in clinical isolates of Enterobacteriaceae as their contribution to the spread of antibiotic resistance is real. Conventional susceptibility testing methods on their own

may fail to offer reliable susceptibility results for β -lactam antibiotics when testing those species that harbour ESBLs. For more reliable results, the use of freshly prepared media is recommended (1 day old) and 18-24 hours old isolates should be used when carrying out DDD.

Currently, most clinical Laboratories in Kenya do not use standard methods for the detection of ESBLs, or do not test for it at all just like other resource restrained countries (Vera *et al.*, 2004). In addition, the majority of clinical laboratories do not routinely identify Enterobacteriaceae to the genus and/or species levels due to unavailability of resources, skills and techniques. The automated Vitek system used in the detection of ESBLs will only validate a result once the organism has been identified to species level. If the system detects the presence of an ESBL resistance mechanism in strains of *Klebsiella* spp. and / or *E. coli*, it then flags it and utilizes its expert software and applies it to the final susceptibility results. If Beta lactams are found to be resistant to ESBL activity, the strain is then flagged as resistant, regardless of whether the *in vitro* test indicates susceptibility. Since the interpretation is done with software and an internal system, it totally reduces to the minimum or even zero the issue of bias in the interpretation of results.

For the past 40 years, *Klebsiella* spp and pathogenic *E. coli* that are resistant to aminoglycosides have been known to cause outbreaks of hospital-acquired infection (David *et al.*, 2004; Casewell and Phillips., 1981). The discovery of plasmid-mediated ESBL production by *Klebsiella* spp and *E. coli*, together with plasmid-mediated aminoglycoside resistance, in the early 1980s (Vinue *et al.*, 2008; Casellas *et al.*, 1989) signaled a major new problem with antibiotic resistance. In the current

study, 6% of isolates (*Klebsiella* spp and *E. coli*) were found to produce ESBL. Many clinicians and other health care providers may be unaware of the problem of ESBL production by gram-negative bacilli.

In developed countries, information on resistance to vancomycin especially by *E. faecium* as indicator organism is readily available to clinicians routine laboratory reports. However, there is no universally accepted applicable marker of the presence of ESBLs. Although resistance of *K. pneumoniae* to ceftazidime is a useful marker for presence of ESBLs, fewer than 50% of *Klebsiella* isolates reported in the United States undergo testing for susceptibility to ceftazidime, a practice that is not done in Kenya (Kariuki *et al.*, 2004). Moreover, some types of ESBL-producing organisms appear susceptible to ceftazidime according to standard methods, and ceftazidime resistance may be due to mechanisms other than ESBL production (Monnet *et al.*, 1997). Unfortunately, many laboratories in Kenya as well as elsewhere in the world do not test for ESBL production (CLSI, 1998).

Awareness of ESBL production by *K. pneumoniae* and *E. coli* is clinically important. The implications in clinical settings are that in the absence of infection control measures, ESBL-producing organisms can readily pass horizontally from patient to patient. Reliable laboratory methods are now available (DDD, E-strip and VITEK) by which ESBL production can be detected by clinical microbiology laboratories. These methods, which have also been promoted by the CLSI (1998), rely on initial screening tests and follow-up confirmatory tests. It is believed therefore that clinicians should not have to specifically request these tests; rather, all *K. pneumoniae* and *E. coli* isolates should undergo routine screening by clinical

microbiology laboratories. Isolates suspected of producing ESBLs should not be reported as susceptible to third-generation cephalosporins or cefepime until follow-up confirmatory tests are performed.

From this study, it has been shown that the Vitek system is easy to perform and without any subjective interpretation of results, reported false-positive detection of ESBL activity with one strain of *E. coli*. This strain of *E. coli* had been reported as positive by the DDD and E-strip methods. The best combination by use of E-strip was cefotaxime and ceftazidime. Although the percentage of false-positive *E. coli* ESBL strains was only 5.6% it is still a concern that the strain was misreported as ESBL producer by E-strip and DDD antibiotic susceptibility to β -lactam antibiotics. There appears to be no obvious reason for these results. It is, however, unlikely to arise from a technical error, as the Vitek is a highly standardized system.

The DDD test requires careful spacing of discs as indicated earlier for accurate results and careful interpretation of zone sizes. It is therefore technically demanding. In reported studies, the DDD test was able to detect 82% and 88 % of ESBL-positive strains, respectively (Thomson and Sanders, 1992) contrarily to the findings from this study (100% and 94.4%). The limitations of this test have been described elsewhere (Vinué *et al.*, 2008; Bush, 1996; Thomson and Sanders, 1992). It has been reported that cepodoxime achieves a 100 % sensitivity rate in detecting ESBLs in tested isolates, cefotaxime 92 %, and ceftazidime 82 % (Appleton and Hall, 2001). In contrast, the results on isolates from the current study showed that cefotaxime was the most efficient combination for the detection of ESBLs, with a sensitivity rate of 100%; the values for cepodoxime and ceftazidime were 50 % and 90%, respectively.

The commercially available ESBL E-test strip is a quantitative technique, and is widely regarded as the 'gold standard' for detection in clinical laboratories of ESBL production (Crowley, 2001; M'Zali *et al.*, 2000). In this study, it detected 100 % of the test isolates and this was only possible when both cefotaxime and ceftazidime strips were used in conjunction and not with cefodoxime. As noted above, the ceftazidime E-test strip was less sensitive when solely used; this could be due to the possibility that there were other ceftazidime-hydrolysing β -lactamases in some strains that were not sensitive to clavulanic acid, which could have reduced the sensitivity of the test. Thus, E- tests with both cephalosporins are recommended; however, this makes the technique expensive, and most clinical laboratories in Kenya would only use it for confirmation rather than routine testing.

It should be noted that in this study that one *E. coli* was falsely reported as ESBL positive by the two methods. For all ESBL positive *K. pneumoniae* in this study, the Vitek test was accurate, but it should be noted that this study only included Vitek ESBL - positive strains by the other two methods, and was therefore not an evaluation of the Vitek ESBL test itself per se. To the best of the author's knowledge, this is the first report on ESBL in Kenya reported from isolates in stool samples. The other two documentations available on ESBL were from blood and urine (Kariuki *et al.*, 2001; Kariuki *et al.*, 2004).

5.5.3 Detection of Gyrase A, B and topoisomerase IV genes by PCR and Sequencing

The isolates from the current study exhibited resistance to the three generations of fluoroquinolones ranging from 4 to 12%. Studies done in Kenya have indicated resistance to fluoroquinolones (Oundo *et al.*, 2008; Kariuki *et al.*, 2004; Sang *et al.*, 1997). Resistance reported in this study was higher (upto 12%) than what has been reported before. Fluoroquinolones have become the first-line drugs for the treatment of bacillary dysentery and typhoid fever (Pazhani *et al.*, 2011; Felmingham *et al.*, 2007). Some *Shigella dysenteriae*, *S. flexineri*, *S.boydii*, *S. sonnei*, *E. coli* and *Salmonella enterica* serovar typhi strains that exhibit decreased susceptibilities or complete resistance to fluoroquinolones have been reported (Mendez *et al.*, 2009; Menendez *et al.*, 2004; Hirose *et al.*, 2001).

Several clinical treatment failures after the administration of ciprofloxacin and other fluoroquinolones to patients with dysentery and typhoid fever due to strains with decreased susceptibilities and /or resistance to fluoroquinolones have also been reported elsewhere (Martinez-Martinez *et al.*, 2003; Olsen *et al.*, 2001). The emergence and spread of these organisms have been reported in developing countries including Kenya and Nigeria (Kariuki *et al.*, 2004; Aibinu *et al.*, 2007). There is evidence that the incidence of strains that are resistant to nalidixic acid and that exhibit decreased susceptibilities to the most recent fluoroquinolones used for the treatment of shigellosis and typhoid fever is increasing (Strahilevitz *et al.*, 2009; Tran *et al.*, 2002).

In most strains in the current study, the acquired fluoroquinolone resistance was attributed to mutations in the genes encoding DNA gyrase (GyrA) while studies in

other regions indicate mutations in GyrA, B and DNA topoisomerase IV (ParC, ParE) (Kato *et al.*, 1990). The single *Salmonella* spp isolated in the study was sensitive to quinolones. The MICs of several fluoroquinolones, including nalidixic acid, norfloxacin and ciprofloxacin were determined by the E-test. The criterion for ciprofloxacin resistance was an MIC of ≥ 4 $\mu\text{g/ml}$, according to the NLSI breakpoint criteria for members of the family *Enterobacteriaceae* (NLSI, 2008). The criterion for decreased susceptibility or resistance to ciprofloxacin used in the present study was an MIC between ≥ 0.25 and < 4 $\mu\text{g/ml}$, and that for ciprofloxacin susceptibility was an MIC < 0.25 $\mu\text{g/ml}$ (Raghunath *et al.*, 2008; Koutsolioutsou *et al.*, 2001).

From the current study, 43 isolates were resistant to ciprofloxacin. This was in contrast to the study findings by Sinha *et al.*, (2004) where typical resistant strains for ciprofloxacin were never found among the strains tested in his work. In the current study, the resistant *Shigella dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei* and *E. coli* isolates to fluoroquinolone had single mutation in the *gyrA* gene, at either position 83 or 87. This was also contrary to the results of Kariuki *et al.*, 2004 findings where mutations were detected in GyrA, B and topoisomerase.

In quinolone resistance, there are three groups distinguished among the strains in which resistance is observed *in vitro* on the basis of the ciprofloxacin MICs and *gyrA* mutations. The first group consisted of strains which were susceptible to fluoroquinolones and which had no mutations in the QRDR of the *gyrA* gene and this was in agreement with the work done by (Piddock, 1998 and 2002; Konstantinos *et al.*, 2008). The second group consisted of strains which exhibited slightly reduced susceptibilities to fluoroquinolones by MICs (or intermediate by disc diffusion and

which had only a single mutation in the QRDR of the *gyrA* gene. The third group consisted of strains which were resistant to fluoroquinolones with a single mutation in the QRDR of the *gyrA* gene. These findings indicate that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of *Shigella* spp, *E. coli*, *Klebsiella* spp or infections due to *Salmonella*.

Alterations at position 83 or 87 of the GyrA amino acid sequence have been described for *Salmonella* strains (Pazhani *et al.*, 2011; Kariuki *et al.*, 2004; Piddock *et al.*, 2002; D'Ignazio *et al.*, 2005). Double mutations at positions 83 and 87 of the GyrA amino acid sequence were also reported in clinical isolates of serovar Schwarzengrund, which caused nosocomial infections in the United States and which exhibited ciprofloxacin resistance (Rodas *et al.*, 2011). Although strains with high-level fluoroquinolone resistance due to double mutations at codons 83 and 87 in the GyrA amino acid sequence were not found in clinical isolates of *Shigella* spp, *Klebsiella* spp and *E. coli* in the current study, several cases of the failure of treatment for shigellosis due to strains with decreased susceptibilities or total resistance to fluoroquinolones have been reported elsewhere (Ouyang-Latimer *et al.*, 2011; Smith *et al.*, 2010; Felmingham *et al.*, 2007; Tran *et al.*, 2002; Olsen *et al.*, 2001).

5.5.4 Plasmid analysis

All the multidrug resistant *E. coli*, *Shigella*, and *Klebsiella* isolates contained multiple plasmids ranging from 1.8 kb to 35.5 kb. No plasmid was isolated from fully susceptible isolates. Based on conjugation results, it was shown that 33/38 MDR isolates transferred their fully resistance phenotype to five antibiotics

(ampicillin, chloramphenicol, tetracycline, nalidixic acid, and cotrimoxazole) to *E. coli* K12. There is an indication based on these findings that the multidrug resistant strains have been spreading in the County as well as the country (Kariuki *et al.*, 2004) and will slowly replace the fully sensitive strains due their survival advantage over the sensitive ones.

On the basis of the multiple plasmids carried by single isolates, two models are proposed to explain the findings of high prevalence of antibiotic-resistant bacteria in Machakos County where antibiotic exposure is considered minimal and there are no obvious sources of a sustained contamination from the exterior: A primitive selection of resistance in the setting, due to peculiar environmental conditions for example a consistent exposure to natural products with antibiotic activity in food or water. The introduction of resistant strains via occasional travelers to the district from Nairobi and/or animals [chicken] from antibiotic-exposed settings, followed by the local dissemination and maintenance of resistance in the absence of antibiotic pressure. In the first model, one would expect a limited number of resistance traits (restricted to natural compounds) and, possibly, the evolution of some original resistance genes that differ from those selected in antibiotic-exposed settings. In the second model, one would rather expect the dissemination of a limited number of resistant clones (or mobile genetic elements) carrying resistance genes typical of antibiotic-exposed settings as suggested by Khachatryan *et al.* (2006).

The molecular characterization of resistant isolates from samples, carried out in this study, provided some insights into this phenomenon. Resistance plasmids carried a

remarkable variety of acquired resistance genes (even resistance to synthetic agents, such as sulfonamides and trimethoprim) which were entirely like those encountered in isolates from antibiotic-exposed settings (Bartoloni *et al.*, 2006; Nordmann., 2005). There was 100% resistance to cotrimoxazole and this finding concurs with that of Bartoloni. Specialized elements carrying the resistance genes, such as the backbones of conjugative plasmids found, were similar to those described by Carattoli (Carattoli *et al.*, 2005). Overall, this scenario appears to be consistent with the model ascribing antibiotic resistance, observed in areas not commonly exposed to antibiotic use, to the dissemination of resistant bacteria and resistance genes from antibiotic-exposed settings rather than to an independent *in situ* selection. This view is further supported by the finding that similar resistance patterns and resistance genes are highly prevalent in commensal *E. coli* isolates from urban areas (Bartoloni *et al.*, 2006).

Despite the evidence for the expansion of some resistant clones, a remarkable heterogeneity was observed in the population structure of resistant bacteria (Figure 4.9). Similar findings do not support a simple model in which a few resistant strains are occasionally introduced in the remote setting and expand to replace the existing susceptible population but, rather, suggest either a sustained flow of diverse resistant strains from the exterior or the occurrence of substantial horizontal gene transfer and recombination phenomena involving resistance genes following the occasional introduction of resistance strains from the exterior into the remote settings (Enne *et al.*, 2005).

Whichever the mechanism is responsible for this genetic diversity, the reasons for maintaining the high prevalence of resistance in the absence of antibiotic pressure remain unexplained. Substantial exposure to natural antibiotics seems likely, since it is implying the presence of natural products that select for the same patterns of resistance traits that are common in commensal *E. coli* isolates from antibiotic-exposed areas (Bartoloni *et al.*, 2006). A possible explanation could be the selective advantage conferred by genetic determinants linked to resistance genes on the same plasmids (genes for colicins, iron uptake systems, and/or intestinal colonization factors) which could increase the fitness of the organisms for the intestinal ecosystem, or determinants for heavy metal resistance and/or additional metabolic pathways, which could facilitate survival in the environment of the remote setting.

The maintenance of different types of acquired resistance genes in such an “antibiotic-free” setting is consistent with research reports suggesting that expression of acquired antibiotic resistance in bacteria might not always involve a fitness cost otherwise, resistance genes would rapidly be lost (Enne *et al.*, 2005; Khachatryan 2006).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study sought to establish molecular epidemiology of Enterotoxigenic *E. coli* and other pathogenic bacteria associated with diarrhoea. Arising from the results the following conclusions are drawn:

- *E. coli* is a major cause of diarrhoea in patients reporting to Machakos District Hospital.
- In respect with prevalence, EAEC (13.7%), ETEC (11%), EIEC (8.3%), EPEC (4.3%), and CNF1 (2.7%), and EAEC was the most prevalent.
- ETEC CFAs and CS were more prevalent in children below five years.
- CS19 was detected and reported for the first time in Kenya.
- The prevalence CS6 has increased compared to other studies.
- CS19 and CS6 would be ideal candidates for incorporation in potential Kenyan-based vaccine.
- ETEC, other *E. coli* pathotypes and *Shigella* spp were heterogenous as shown by PFGE.
- Infections caused by atypical EPEC were more prevalent than those caused by typical EPEC.
- A significant proportion of the isolates were multidrug resistant; and this highlights the emerging threat posed in treatment and management of diarrhoeal diseases in Machakos County.

- Diarrhoea causing bacteria are sensitive to gentamicin, therefore, it should be adopted for treatment of diarrhea.
- Ciprofloxacin, Cephalosporins and gentamycin are recommended for treatment of diarrhoea in Machakos Hospital.
- There is emergence of ESBL that will limit the effectiveness of cephalosporins in treatment of diarrhoea.
- Double disc diffusion method is ideal in detection of resistance to cephalosporins.
- The emergence of ESBL will limit the effectiveness of cephalosporins in treatment of diarrhoea.
- Resistance to fluoroquinolones was due to gyrase A resistance gene.
- Resistance in gyrase A was caused by both single and double mutations at region 83 and 87.
- Single and double mutation led to similar rates of resistance to fluoroquinolones.
- Lack of clean water contributes to dissemination of diarrhoeal causing agents in Machakos District Hospital.

6.2 Recommendations

From the results emanating from this study, the following recommendations are made:

- There is need to carry out intensive case-controlled studies in the entire County in order to elucidate the role of *E. coli* pathotypes in the aetiology of diarrhoea and delineate the reservoir for the MDR pathotypes in all .
- Based on the diversity of the CFAs and CS detected, there is need for further studies which could lead to their incorporation in development of potential vaccines.
- The use of Genetic methods, such as multilocus sequence typing and multilocus variable-nucleotide tandem-repeat, restriction length polymorphism and microarrays analysis that will permit the greater resolution of ETEC strains expressing CFA I.
- With a detection of 6% and 8% observed for ESBL and resistance to quinolones, respectively in Machakos, surveillance and monitoring of the effectiveness of cephalosporins and quinolones in health-care settings in in the entire county should be studied.
- Active efflux may have contributed to ETEC resistance and studies must be mounted on the same.
- There is need for regular antimicrobial surveillance to monitor the effectiveness of Cephalosporins and fluoroquinolones.
- There is need for public health education on simple hand hygiene for management of diarrhoea
- The practice of sale of antibiotics over the counter should be investigated and prohibited.
- The adaptive ability of *E. coli* pathogens within the host gastrointestinal tract and/or the external environment need to be stried.

- Intiate studies with a view to establishing that CFA isolates induce age dependant immunity in old children need to be carried out as this will collaborate concept of immunoprophylaxis.
- There is need for further studies to obtain a more indepth understanding of the naturally acquired ETEC antifimbrial immune responses.
- The *in vivo* influence of environmental stress and the adaptability of ETEC as well as other *E. coli* pathotypes within their groups should be studies.
- Studies on the role of EAEC as a causal agent for diarrhoea should be studied.
- There is need for further characterization of atypical EPEC to delineate their role in cause of diarrhoea. Diarrhoea due to EPEC had been attributed to typical EPEC harbouring intimin and *Bfp* genes.

REFERENCES

- Abu-Elyazeed, R., Wierzba T. F., Mourad A. S., Peruski L. F., Kay B. A., Rao M., Churilla A. M., Bourgeois A. L., Mortagy A. K., Kamal S. M., Savarino S. J., Campbell J. R., Murphy J. R., Naficy A., Clemens J. D. 1999. Epidemiology of enterotoxigenic *Escherichia coli* diarrhoea in a pediatric cohort in a periurban area of Lower Egypt. *J. Infect. Dis.* 179:382-389.
- Adachi, J. A., Ericsson, C. D., Jiang, Z. D., DuPont, M. W., Pallegar, S. R., DuPont, H. L. 2002. Natural history of enteroaggregative and enterotoxigenic *Escherichia coli* infection among US travelers to Guadalajara, Mexico. *J Infect Dis* 185: 1681–1683.
- Adachi, J. A., Mathewson J. J., Jiang Z. D., Ericsson C. D., DuPont H. L. 2002. Enteric pathogens in Mexican sauces of popular restaurants in Guadalajara, Mexico, and Houston, Texas. *Ann. Intern. Med.* 136:884-887.
- Adachi, J. A., Jiang, Z. D., Mathewson, J. J., Verenkar, M. P., Thompson, S., Martinez-Sandoval, F., Steffen, R., Ericsson, C. D., DuPont, H. L. 2001. Enteroaggregative *Escherichia coli* as a major etiologic agent in traveler's diarrhoea in 3 regions of the world. *Clin Infect Dis* 32: 1706–1709.
- Afset, J. E., Bergh, K., Bevanger, L. 2003. High prevalence of atypical enteropathogenic *Escherichia coli* (EPEC) in Norwegian children with diarrhoea. *J Med Microbiol* 52: 1015–1019.
- Afset, J. E., Bruant G., Brousseau R., Harel J., Anderssen E., Bevanger L., Bergh K. 2006. Identification of virulence genes linked with diarrhoea due to atypical enteropathogenic *Escherichia coli* by DNA microarray analysis and PCR. *J. Clin. Microbiol.* 44:3703-3711.
- Afset, J. E., Bevanger L., Romundstam P., Bergh K 2004. Association of atypical enteropathogenic *Escherichia coli* (EPEC) with prolonged diarrhoea. *J. Med. Microbiol.* 53:1137-1144.
- Ahmed, H. M., Coulter J B., Nakagomi O., Hart C.A. 2006. Molecular characterization of Rota virus Gastroenteritis strains, Iraqi Kurdistan. *Emer. Infect Dis.* 12: 824-826.
- Ahmed, T., Haque, R., Mansur, A., Ahmed, S., Petri, W.A., Cravioto, A. 2009. Use of metagenomics to understand the genetic basis of malnutrition. *Nutr. Rev.*, 67: 201-206.
- Ahmed, T., Ali M., Ullah M. M., Choudhury I. A, Haque M. E., Salam M. A., Rabbani G. H., Suskind R. M., Fuchs G. J. 1999. Mortality in severely malnourished children with diarrhoea and use of a standardised management protocol. *Lancet* 353:1919-1922.

- Ahren, C. M., Gothefors L., Stoll B. J., Salek M. A., Svennerholm A. M. 1986. Comparison of methods for detection of colonization factor antigens on enterotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 23:586-591.
- Ahren, C., Jertborn M., Svennerholm A. M. 1998. Intestinal immune responses to an inactivated oral enterotoxigenic *Escherichia coli* vaccine and associated immunoglobulin A responses in blood. *Infect. Immun.* 66: 3311-3316.
- Ahren, C., Jertborn M., Svennerholm A. M. 2006. An inactivated triple oral enterotoxigenic *Escherichia coli* vaccine. *Infect. Immun.* 23:1890-1895.
- Aibinu, I., Peters A., Amisu K.O., Adesida S.A, Ojo M.O., Tolu O. 2007. Multidrug resistance in *E. coli* 0157 Strains and the Public Health Implication. *J Amer Sci.* 3: 3-7.
- Al- Gallas N., Olfa B., Aida B. 2007. Etiology and acute diarrhoea in children and adults in Tunisia, with emphasis on diarrhoeagenic *Escherichia coli*: Prevalence and molecular epidemiology. *Am J Trop Med Hyg*; 77: 571-582.
- Alam, M., Nur A. H., Ahsan S., Pazhani G. P., Tamura K, Ramamurthy T., Gomes D. J., Rahman S. R., Islam A., Akhtar F., Shinoda S., Watanabe H., Faruque S. M., Nair G. B. 2006. Phenotypic and molecular characteristics of *Escherichia coli* isolated from aquatic environment of Bangladesh. *Microbiol. Immunol.* 50:359-370.
- Alam, N. H., Ashraf H. 2003. Treatment of infectious diarrhoea in children. *Paediatr Drugs* 5:151-165.
- Alaullah G., Charles P., Khan S.K., Azharul I., Rahma, A., Qadri, F. 2011. Zinc treatment to under-five children: applications to improve child survival and reduce burden of disease. *J Popul Nutr.* 76:123-128.
- Alavandi SV., Manoranjita V., Vijayan K.K., Kalaimani N., Santiago TC. 2006. Phenotypic and molecular typing of *Vibrio harveyi* isolates and their pathogenicity to tiger shrimp larvae. *Lett Appl Microbiol.* 43:566-570
- Al-Gallas N., Ben Aissa R., Annabi T. K. 2002. Isolation and characterization of Shiga toxin-producing *Escherichia coli* from meat and dairy products. *Food Microbiol.* 34: 389-398.
- Al-Gallas N., Bahri O., Bouratbeen A., Ben Haasen A., Ben Aissa R. 2007. Etiology of acute diarrhoea in children and adults in Tunis, Tunisia, with emphasis on diarrhoeagenic *Escherichia coli*: prevalence, phenotyping, and molecular epidemiology. *Am J Trop Med Hyg.* 77:571-582.
- Altboum, Z., Barry E. M., Losonsky G., Galen J. E., Levine M. M. 2001. Attenuated *Shigella flexneri* 2a Delta *guaBA* strain CVD 1204 expressing enterotoxigenic *Escherichia coli* (ETEC) CS2 and CS3 fimbriae as a live mucosal vaccine against *Shigella* and ETEC infection. *Infect Immun.* 69:3150-3158.

- Amieva MR. 2005. Important bacterial gastrointestinal pathogens in children: a pathogenesis perspective. *Pediatr Clin North Am.* 52:749–77.
- Amisano G., Stefania F., Giuseppe M., Stefano C., Valentina T., Francesco S. 2011. Diarrhoeagenic *Escherichia coli* in acute gastroenteritis in infants in North -West Italy. *New Microbiol.* 34: 45-51.
- Anantha RP., McVeigh AL., Lee LH., Agnew MK., Cassels FJ., Scott DA. 2004. Evolutionary and functional relationships of colonization factor antigen i and other class 5 adhesive fimbriae of enterotoxigenic *Escherichia coli*. *Infect Immun.*72:7190–201.
- Andersson M., Björnham O., Svantesson M., Badahdah A., Uhlin BE., Bullitt E. 2011. A structural basis for sustained bacterial adhesion: Biomechanical properties of CFA/I pili. *J Mol Biol.* 3: 415-5.
- Anjum, M. F., Mafura M., Slickers P., Ballmer K., Kuhnert P., Woodward M. J., Ehricht R. 2007. Pathotyping *Escherichia coli* by using miniaturized DNA microarrays. *Appl Environ Microbiol.* 73:5692-5697.
- Appleton, A., Hall, S. 2000. Comparison of three combination discs for the detection of extended spectrum β -lactamases. Poster presented at the Third European Congress of Chemotherapy, Madrid, Spain, May 7–10.
- Applied Biosystems, 2002. BigDye® Terminator. One cycle Sequencing Kit. *ABI.* 1: 4337036.
- Aranda K., Fabbriotti S., Fagundes-Neto U., Scaletsky I. 2007. Single multiplex assay to identify simultaneously enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing *Escherichia coli* strains in Brazilian children. *FEMS Microbiol Lett.*267:145–150.
- Araujo J. M., Tabarelli G. F., Aranda K. R. 2007. “Typical enteroaggregative and atypical enteropathogenic types of *Escherichia coli* are the most prevalent diarrhoea-associated pathotypes among Brazilian children,” *J Clinical Microbiol.* 45: 3396–3399.
- Arne T., Rein A., Halvor S., James N., Pål P. 2010. Heat-Stable Enterotoxin of Enterotoxigenic *Escherichia coli* as a vaccine target. *Infect Immun.* 78: 1824–1831.
- Ashley, D. V., Walters CDockery-Brown., C., McNab A., Ashley D. E. 2004. Interventions to prevent and control foodborne diseases associated with a reduction in traveler's diarrhoea in tourists to Jamaica. *J. Travel Med.* 11:364-367.
- Aslani M. M., Alikhani M. Y. 2009. “Serotypes of enteropathogenic *Escherichia coli* isolated from children under 5 years of age”. *Iranian J Pub Health.* 38: 70–77.

- Ballmer, K., Korczak B. M., Kuhnert P., Slickers P., Ehricht R., Hächler H. 2007. Fast DNA serotyping of *Escherichia coli* by use of an oligonucleotide microarray. *J. Clin. Microbiol.* 45:370-379.
- Banatvala, N. 2001. The United States National Prospective Hemolytic Uremic Syndrome Study: microbiologic, serologic, clinical, and epidemiologic findings. *J. Infect. Dis.* 183:1063–1070.
- Bangar R., Mamatha B. 2008. Identification of enteroaggregative *Escherichia coli* in infants with acute diarrhoea based on biofilm production in Manipal, *South India*. *Indian J Med Sci.* 62:8-12.
- Banyai, k., Bogdan A., Kiszfalvi P., Molnar P. 2007. Emerging of Serotype G12 Rota viruses in Hungary. *Emerg Infect Dis.* 13: 916-919.
- Bartoloni, A., Pallecchi L., Benedetti M., Fernandez C., Vallejos Y., Guzman E., Villagran A., Mantella A., Lucchetti C., Bartalesi F., Strohmeyer M., Bechini A., Gamboa H, Rodriguez H., Falkenberg T., Kronvall G., Gotuzzo E., Paradisi F., Rossolini G. M. 2006. Multidrug-resistant commensal *Escherichia coli* in children, Peru and Bolivia. *Emerg. Infect. Dis.* 12:907-913.
- Bauchard P., Bauchard P., Germon A., Brée E., Oswald J., Hacker U. 2010. Dobrindt Pathogenomic comparison of human extraintestinal and avian pathogenic *Escherichia coli* – search for factors involved in host specificity or zoonotic potential. *Microb Pathog.* 49:105–115.
- Begum YA., Talukder KA., Nair GB., Khan SI., Svennerholm AM., Sack RB., Qadri F. 2007. Comparison of enterotoxigenic *Escherichia coli* isolated from surface water and diarrhoeal stool samples in Bangladesh. *Can J Microbiol.* 53:19-26.
- Berlanda Scorza, F., Doro F., Rodriguez-Ortega M. J., Stella M., Liberatori S., Taddei A. R., Serino L., Gomes Moriel D., Nesta B., Fontana M. R., Spagnuolo A., Pizza M., Norais N., Grandi G. 2008. Proteomics characterization of outer membrane vesicles from the extraintestinal pathogenic *Escherichia coli* DeltatolR IHE3034 mutant. *Mol. Cell Proteo.* 7:473-485.
- Bertin, Y., Boukhors K., Livrelli V., Martin C. 2004. Localization of the insertion site and pathotype determination of the locus of enterocyte effacement of Shiga toxin-producing *Escherichia coli* strains. *Appl Environ Microbiol.* 70:61-68.
- Bettger WJ., O'Dell BL. 1981. A critical physiological role of zinc in the structure and function of biomembranes. *Life Sci.* 28:1425–1438.
- Beutin L. 2006. Emerging enterohaemorrhagic *Escherichia coli*, causes and effects of the rise of a human pathogen. *J. Vet Med.* 53: 299-305.
- Beutin, L., Marchés, O., Bettelheim, K. A., Gleier, K., Zimmermann, S., Schmidt, H., Oswald, E. 2003. Hep-2 cell adherence, actin aggregation, and intimin types of

attaching and effacing *Escherichia coli* strains isolated from healthy infants in Germany and Australia. *Infect Immun* 71: 3995–4002.

Bhandari, N., Bahl R., Taneja S., Strand T., Molbak K., Ulvik R. J., Sommerfelt H., Bhan M. K. 2002. Substantial reduction in severe diarrhoeal morbidity by daily zinc supplementation in young north Indian children. *Pediatrics*. 109: 86-90.

Bhardwaj R, Majumdar S., Ganguly NK., Taneja N., Dutta S., Ramamurthy T., Chakraborti A. 2006. Characterization of adhesin variants in Indian isolates of enteroaggregative *Escherichia coli*. *FEMS Microbiol Lett*. 258: 274-83.

Biais N., Higashi DL., Brujic J., So M., Sheetz MP. 2010. Force-dependent polymorphism in type IV pili reveals hidden epitopes. *Proc Natl Acad Sci*. 107:11358–11363.

Bielaszewska M., Middendorf B., Kock R., Friedrich AW., Fruth A., Karch H., Schmidt MA., Mellmann A . 2008: Shiga toxin-negative attaching and effacing *Escherichia coli*: distinct clinical associations with bacterial phylogeny and virulence traits and inferred in-host pathogen evolution. *Clin Infect Dis*. 47:208-217.

Bielaszewska, M., Köck R., Friedrich A. W., von Eiff C., Zimmerhackl L. B., Karch H., Mellmann A. 2007. Shiga toxin-mediated hemolytic uremic syndrome: time to change the diagnostic paradigm? *PLoS*. 2:1019-1024.

Bingen-Bidois, M., Bingen-Bidois O., Clermont S., Bonacorsi M., Terki N., Brahimi C., Loukil D., Barraud E., Bingen. 2002. Phylogenetic analysis and prevalence of urosepsis strains of *Escherichia coli* bearing pathogenicity island-like domains. *Infect Immun.*, 70:3216–3226.

Binsztein N., Jouve M., Viboud G. 1991. Colonization factors of enterotoxigenic Enterotoxigenic *Escherichia coli* isolated from children with diarrhoea in Argentina. *J. Clin. Microbiol*. 29: 1893-8.

Birnboim H., Doly J. 1979. A rapid extraction procedure for screening recombinant Plasmid DNA. *Nucleic Acids Research*. 7: 1513-1525

Bischoff K.M., Bischoff D.G., White P.F., McDermott S., Zhao S., Gaines J.J., Maurer D.J. 2002. Characterization of chloramphenicol resistance in beta-hemolytic *Escherichia coli* associated with diarrhoea in neonatal swine. *J Clin Microbiol*, 40 : 389–394

Bischoff C., Luthy J., Altwegg M., Baggi F. 2005: Rapid detection of diarrhoeagenic *E. coli* by real-time PCR. *J. Microbiol. Methods*. 61:335-341.

Bjorn –Arne L., Lena A. 2007. Stusy of polymorphic variable number of tandem repeats loci in the ECOR collection and in a set of pathogenic *Escherichia coli* and *Shigella* isolates for us ein genotyping assay. *J Microbiol Meth*. 69: 197-205.

- Black, R. E. 1990. Epidemiology of travelers' diarrhoea and relative importance of various pathogens. *Rev. Infect. Dis.* 12(Suppl. 1):S73-S79.
- Black, R. E. 1990. Stunting growth developing countries. *J. Gen. Intern. Med.* 5:132-135.
- Black, R. E. 1993. Persistent diarrhoea in children of developing countries. *J. Pediatr. Infect. Dis.* 12:751-764.
- Black, R. E. 2003. Zinc deficiency, infectious disease and mortality in the developing world. *J. Nutr.* 133:1485S-9S.
- Black, R. E., G. Lopez de Romana, K. H. Brown, N. Bravo, O. G. Bazalar, H. C. Kanashiro. 1989. Incidence and etiology of infantile diarrhoea and major routes of transmission in Huascar, Peru. *Am. J. Epidemiol.* 129:785-799.
- Black, R. E., Brown K. H., S. Becker. 1984. Effects of diarrhoea associated with specific enteropathogens on the growth of children in rural Bangladesh. *Pediatr.* 73:799-805.
- Black, R. E., Merson M. H., Rowe B., Taylor P. R., Abdul Alim A. R., Gross R. J., Sack D. A. 1981. Enterotoxigenic *Escherichia coli* diarrhoea: acquired immunity and transmission in an endemic area. *Bull.* 59:263-268.
- Black, R. E., Merson M. H., Huq I., Alim A. R., Yunus M.. 1981. Incidence and severity of rotavirus and *Escherichia coli* diarrhoea in rural Bangladesh. Implications for vaccine development. *Lancet.* 102:141-143.
- Blackburn, D., Husband A., Saldana Z., Nada R. A., Klena J., Qadri F., Giron J. A. 2009. Distribution of the *Escherichia coli* common pilus among diverse strains of human enterotoxigenic *E. coli*. *J. Clin. Microbiol.* 47:1781-1784.
- Blanco M., Jesús B., Ghizlane D., Azucena M., María P., Gustavo V., María P., Felipe S., Enrique A., Jorge B. 2006. Typing of intimin (*eae*) genes from enteropathogenic *Escherichia coli* (EPEC) isolated from children with diarrhoea in Montevideo, Uruguay: Identification of two novel intimin variants (μ B and ζ R/ β 2B). *J Med Microbiol.* 55: 9 1165-1174.
- Blanco, M., Blanco, J. E., Blanco, J., Carvalho, V. M., Lopes Onuma, D. Pestana de Castro, A. F. (2004c). Typing of intimin (*eae*) genes in attaching and effacing *Escherichia coli* strains from monkeys. *J Clin Microbiol.* 42: 1382–1383.
- Blanco, M., Blanco, J. E., Mora, A. 2003. Serotypes, virulence genes, and intimin types of Shiga toxin (verotoxin)-producing *Escherichia coli* isolates from healthy sheep in Spain. *J Clin Microbiol.* 41: 1351–1365.
- Blanco M., Schumacher S., Tasara T., Zweifel C., Blanco J. E., Dahbi G., Blanco J. Stephan R. (2005). Serotypes, intimin variants and other virulence factors of *eae*

positive *Escherichia coli* strains isolated from healthy cattle in Switzerland. Identification of a new intimin variant gene (eae-ε2). *BMC Microbiol* 5: 23-7.

Boerlin White, 2006. Antimicrobial Therapy in Veterinary Medicine, *Blackwell Pub, Stafford*. 27–49.

Boerlin S., Boerlin P., Travis R., Gyles C. 2005. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied Environ Microbiol*. 6753–6761

Bolin I., Wiklund G., Qadri F., Torres O., Bourgeois A. L., Savarino S., Svennerholm A. M. 2006. Enterotoxigenic *Escherichia coli* with STh and STp genotypes is associated with diarrhoea both in children in areas of endemicity and in travelers. *J. Clin. Microbiol*. 44:3872-3877.

Bono J. 2009. Genotyping *Escherichia coli* 0157:H7 for its ability to cause disease in humans. *Miscellaneous* 14: 5-10.

Bopp CA., Brenner FW., Fields PI., Wells JG., Strockbine NA. 2003. *Escherichia, Shigella, and Salmonella*. *Clin Microbiol*. 56: 654-671.

Boschi-Pinto C., Velebit L., Shibuya K. 2008. Estimating child mortality due to diarrhoea in developing countries. *Bull WHO*.86:710–717.

Botelho B. A., Bando S. Y., Trabulsi L. R., Moreira-Filho C. A. 2003. “Identification of EPEC and non-EPEC serotypes in the EPEC O serogroups by PCR-RFLP analysis of the *fliC* gene”. *J Microbiol*. 54: 87–93.

Botelho B. A., Bando S. Y., Trabulsi L. R., Moreira-Filho C. A. 2003. “Identification of EPEC and non-EPEC serotypes in the EPEC O serogroups by PCR-RFLP analysis of the *fliC* gene,” *J Microbiol Methods*. 54: 87–93.

Bouckenooghe AR., DuPont HL., Jiang ZD., Adachi J., Mathewson JJ., Verenkar MP., Rodrigues S., Steffen R. 2000. Markers of enteric inflammation in enteroaggregative *Escherichia coli* diarrhoea in travelers. *Am J Trop Med Hyg*. 62:711-713.

Bouguenec Le C., Servin AL. 2006. Diffusely adherent *Escherichia coli* strains expressing Afa/Dr adhesins (Afa/Dr DAEC): hitherto unrecognized pathogens. *FEMS Microbiol Lett*. 256 :1574-6968.

Bouzari S., Jafari MN., Shokouhi F., Parsi M., Jafari A. 2000. Virulence-related DNA sequences and adherence patterns in strains of enteropathogenic *Escherichia coli*. *FEMS Microbiol Lett*. 185:89–93.

Bouzari, S., Jafari A., Azizi A., Oloomi M., Nataro J. P. 2001. Short report: characterization of enteroaggregative *Escherichia coli* isolates from Iranian children. *Am J Trop Med Hyg*. 65:13-14.

- Boxrud D., Pederson-Gulrud. 2007. Comparison of Multilocus variable number tandem repeat analysis Pulsed Field gel electrophoresis, and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. *J Clin Microbiol.* 43:536-543.
- Bradley Sack R. 2011. The discovery of cholera - like enterotoxins produced by *Escherichia coli* causing secretory diarrhoea in humans. *Indian J Med Res.* 133:171-80.
- Brooks J. 2005. Non-O157 Shiga toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *J. Infect. Dis.* 192:1422–1429.
- Brown K.. 2003. Diarrhoea and malnutrition. *J. Nutr.* 133:328-332.
- Bryce J., Boschi-Pinto C., Shibuya K., Black RE. WHO Child Health Epidemiology Reference Group. 2005. WHO estimates of the causes of death in children. *Lancet.* 365: 1147-1152.
- Bucardo F., Nordgren J., Carlsson B., Paniagua M., Lindgren, P.-E., Espinoza F. Svensson L. 2008. Pediatric norovirus diarrhoea in Nicaragua. *J Clin Microbiol* 46: 2573–2580.
- Bueris V., Sircili M.P., Taddei C.R., dos Santos M.F., Franzolin M.R., Martinez M.B., Ferrer S.R., Barreto M.L., Trabulsi L.R. 2007. Detection of diarrhoeagenic *Escherichia coli* from children with and without diarrhoea in Salvador, Bahia, Brazil. *Mem Inst Oswaldo Cruz.* 102: 839-844.
- Bush K.. 1996. Is it important to identify extended-spectrum β -lactamase producing isolates? *Eur J Clin Microb Infect Dis.* 15: 361–364.
- Caeiro J P., Mathewson ., Smith ., Jiang ., Kaplan ., DuPont H L. 2003. Etiology of outpatient pediatric nondysenteric diarrhoea: a multicenter study in the United States. *J Pediatr Infect Dis.* 18:94-97.
- Caeiro J P., Estrada-Garcia M T, Jiang Z D, Mathewson J J, Adachi J A, Steffen R, DuPont HL. 1999. Improved detection of enterotoxigenic *Escherichia coli* among patients with travelers' diarrhoea, by use of the polymerase chain reaction technique. *J Infect Dis.* 180:2053-2055
- Carattoli A., Bertini AVilla., L., Falbo V, Hpkins K. L., Threlfall E. J. 2005. Introduction of plasmid by PCR typing. *J. Microbiol. Methods.* 63:219-228.
- Carlin N. I. A., Lindberg A. A., Bock K., Bundle D. R. 1984. The *Shigella flexneri* O-antigenic polysaccharide chain. Nature of the natural repeating unit. *Eur J. Biochem.* 139:189-194.
- Carlin N. I. A., Rahman M., Sack D. A, Zaman A., Kay B. A., Lindberg A. A.. 1989. Use of monoclonal antibodies to type *Shigella flexneri* in Bangladesh. *J. Clin. Microbiol.* 27:1163-1166.

- Casellas J M., Goldberg M. 1989. Incidence of strains producing extended spectrum β -lactamases in Argentina.. *Infection*. 17:434-6.
- Casewell M W., Phillips I. 1981. Aspects of the plasmid-mediated antibiotic resistance and epidemiology of *Klebsiella* species. *Am J Med.*; 70:459-62.
- Centers for Disease Control and Prevention 2004. Defeating Cholera: Clinical presentation and Management for Haiti Cholera outbreak. *MMWR*. 329: 78-82.
- Centers for Disease Control and Prevention 2004. National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS): Human Isolates Final Report. Atlanta, Georgia: US Department of Health and Human Services, CDC. Available at <http://www.cdc.gov/narms/annual/2002/2002ANNUALREPORTFINAL.pdf>.
- Centers for Disease Control and Prevention 2011. Health Information for Travelers to developing world. 620: 126-131.
- Centers for Disease Control and Prevention 2012. Enteropathogenic *Escherichia coli* (EPEC). 800: 232-4636.
- Centers for Disease Control. 2001. Diagnosis and Management of Foodborne Illnesses: A Primer for Physicians. *MMWR*. 50(RR02):1-69.
- Centers for Disease Control. 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach—United States, September 2006. *MMWR*. 55:1045–1046.
- Chakraborty, S., J. S. Deokule, P. Garg, S. K. Bhattacharya, R. K. Nandy, G. B. Nair, S. Yamasaki, Y. Takeda, and T. Ramamurthy. 2001. Concomitant infection of enterotoxigenic *Escherichia coli* in an outbreak of cholera caused by *Vibrio cholerae* O1 and O139 in Ahmedabad, India. *J. Clin. Microbiol.* 39:3241-3246.
- Chapman P A., Siddons C A. 1997. A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from cases of bloody diarrhoea, non-bloody diarrhoea and asymptomatic contacts. *J. Med. Microbiol.* 44:267-271.
- Chattopadhyay S., Tchesnokova V., McVeigh A., Kisiela DI., Dori K., Navarro A., Sokurenko EV., Savarino SJ. 2012. Adaptive evolution of class 5 fimbrial genes in enterotoxigenic *Escherichia coli* and its functional consequences. *J Biol Chem*. 287:6150-8.
- Chattopadhyay S., Sinha S S., Bhattacharya SK., Nair GB., Ramamurthy T. 2004. An unusually high level of quinolone resistance associated with type II topoisomerase mutations in quinolone resistance-determining regions of *Aeromonas caviae* isolated from diarrhoeal patients. *Res Microbiol.* 55: 827-9.
- Cheng AC., McDonald JR., Thielman NM. 2005. Infectious diarrhoea in developed and developing countries. *J Clin Gastroenterol.* 39:757-773.

- Cheng HD., Frankel G. 2005. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. *FEMS Microbiol Rev* 29:83–98.
- Chowdhury F., Yasmin A., Begum M. 2010. Concomitant ETEC infection induces increased immune responses to *Vibrio cholera* O1 Antigens in patients with cholera in Bangladesh. *J Inf Imm.* 78: 2117-2122.
- Christoph H., Sabine M., Birgit H., Klaus P., Jens W., Colin R. 2010. A set of novel multiplex Taqman real-time PCRs for the detection of diarrhoeagenic *Escherichia coli* and its use in determining the prevalence of EPEC and EAEC in a university hospital. *Annals of Clin Microbiol and Antimicrobiol*, 9: 1476-1482.
- Claudia R., John D., Matilda N., Volga I., Åsa S. 2011. Clonal relatedness of enterotoxigenic *Escherichia coli* (ETEC) strains expressing LT and CS17 isolated from children with diarrhoea in La Paz, Bolivia. *Plos one*. 2011: 9-14
- Claudia R., Rosalía I, Jorge B., Jesus E., Gudrun W., Ann-Mari S., Åsa S., Volga I. 2011. Enterotoxins, colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhoea in Bolivia. *Braz J Infect Dis*. 15: 2-8.
- Clemens, J. D., Sack D. A., Chakraborty J., Rao M. R., Ahmed F., Harris J. R., van Loon F., Khan M. R., Yunis M., Huda S. 1990. Field trial of oral cholera vaccines in Bangladesh: evaluation of anti-bacterial and anti-toxic breast-milk immunity in response to ingestion of the vaccines. *Vaccine* 8:469-472.
- Clemens J., Savarino S., Abu-Elyazeed R., Safwat M., Rao M., Wierzba T., Svennerholm A. M., Holmgren J., Frenck R., Park E., Naficy A.. 2004. Development of pathogenicity-driven definitions of outcomes for a field trial of a killed oral vaccine against enterotoxigenic *Escherichia coli* in Egypt: application of an evidence-based method. *J. Infect. Dis*. 189:2299-2307.
- Clermont O., Maiwenn O., Claire H., Laure D., Sylvain B., Monique K, Jérémy G, Bertrand P, Eric O, Erick D. 2011. Animal and human pathogenic *Escherichia coli* strains share common genetic backgrounds. *Infect Genet Evol*. 11: 654–662.
- Clinical and Laboratory Standards Institute, CLSI. 1998. Performance standards for antimicrobial susceptibility testing; 3rd informational supplement. 5: 12-18.
- Clinical and Laboratory Standards Institute (CLSI). 2008. Performance standards for antimicrobial susceptibility testing; 18th informational supplement. 28: 63-98.
- Cocolin L., Manzano M, Cantoni C, Comi G. 2003. A multiplex-PCR method to detect enterohemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* in artificially contaminated foods. *Int. J. Hyg. Environ. Health*: 89:159-164.
- Cohen ML. 2000. Changing patterns of infectious disease. *Nature* 406:762–7.

- Cohen M., B., Nataro J. P., Bernstein D. I., Hawkins J., Roberts N., Staat M. A. 2005. Prevalence of diarrhoeagenic *Escherichia coli* in acute childhood enteritis: a prospective controlled study. *J. Pediatr.* 146:54-61.
- Cohen M. B., Nataro J. P., Bernstein D. I., Hawkins J., Roberts N. 2010. TrbB from conjugative plasmid F is a structurally distinct disulfide isomerase that requires DsbD for redox state maintenance. *J Bacteriol.* 193: 4588–4597.
- Coimbra R., Lenormand P., Grimont F., Bouvet P., Matsushita S., Grimont P. A. D. 2001a. Molecular and phenotypic characterization of potentially new *Shigella dysenteriae* serotype. *J. Clin. Microbiol.* 39:618-621.
- Connor B. 2010. Persistent Travelers' Diarrhoea. In: Travelers' Health: Yellow book; Chapter 4: The Post-travel Consultation. Available from: <http://wwwnc.cdc.gov/travel/yellowbook/2010/chapter-4/persistent-travelers-diarrhoea.aspx>.
- Cooke M. 2010. Causes and management of diarrhoea in children in a clinical setting. *S Afr J Clin Nutr.* 23:42-46.
- Cravioto A R., Reyes E., Ortega R. 1988. Prospective study of diarrhoeal disease in a cohort of rural Mexican children: incidence and isolated pathogens during the first two years of life. *Epidemiol. Rev.* 101:123-129.
- Cravioto A., Eslava C., Lopez-Vidal Y., Cabrera R. 1998. Strategies for control of common infectious diseases prevalent in developing countries. *Academic Press*, London. 8: 577-586.
- Cravioto, A., Reyes R. E., Trujillo F., Uribe F., Navarro A., De La Roca J. M., Hernandez J. M., Perez G., Vazquez V. 1990. Risk of diarrhoea during the first year of life associated with initial and subsequent colonization by specific enteropathogens. *Am J Epidemiol.* 131:886-904.
- Crowley D. B. 2001. Extended-spectrum β -lactamases in blood culture isolates of *Klebsiella pneumoniae*: seek and you may find! *J Antimicrob Chemother* 47: 728–729.
- Crump J. 2002. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. *N. Engl. J. Med.* 347:555–560.
- Cunliffe N., Ngwira B., Dove W. 2012. Epidemiology of rotavirus infections in children in Blantyre, Malawi, 1997-2007. *J Infect Dis.* 88: 56-64
- Daniels N. A., Neimann J., Karpati A., Parashar U. D., Greene K. D., Wells J. G., Srivastava A., Tauxe R. V., Mintz E. D., Quick R. 2000. Travelers' diarrhoea at sea: three outbreaks of waterborne enterotoxigenic *Escherichia coli* on cruise ships. *J. Infect. Dis.* 181:1491-1495.

- Danielsson M. L., Mollby R., Brag H., Hansson N., Jonsson P., Olsson E., Wadstrom T. 1979. Enterotoxigenic enteric bacteria in foods and outbreaks of food-borne diseases in Sweden. *J. Hyg.* 83:33-40.
- Darfeuille A., Aubel D., Chauviere G., Rich C., Bourges M., Servin A., Joly B. 1990. Adhesion of enterotoxigenic *Escherichia coli* to the human colon carcinoma cell line Caco-2 in culture. *Infect. Immun.* 58:893-902.
- David L. P., Wen C. K., Anne V.G., Sunita M., Jose M. C. , Herman G., Lutfiye M., Gordon T. 2004. International Prospective Study of *Klebsiella pneumoniae* Bacteremia: Implications of Extended-Spectrum β -Lactamase Production in Nosocomial Infections. *Ann Intern Med.* 140:26-32.
- Diemert J. 2006. Prevention and Self-Treatment of Traveler's Diarrhoea. *Clin. Microbiol.* 19: 583-594.
- Dietrich, R., Moravek M., Burk C., Granum P. E., Märtlbauer E. 2005. Production and characterization of antibodies against each of the three subunits of the *Bacillus cereus* nonhemolytic enterotoxin complex. *Appl Environ. Microbiol.* 71:8214-8220.
- D'Ignazio J., Camere MA., Lewis DE. 2005. Novel, single-dose microsphere formulation of azithromycin versus 7-day levofloxacin therapy for treatment of mild to moderate community-acquired pneumonia in adults. *Antimicrob Agents Chemother.* 49:4035-41.
- Diniz-Santos D., Santana J., Barretto J., Andrade M., Silva L. 2005. Epidemiological and microbiological aspects of acute bacterial diarrhoea in children from Salvador, Bahia, Brazil. *Braz J Infect Dis* 9: 77-83.
- Donta, S., Smith D. 1974. Stimulation of steroidogenesis in tissue culture by enterotoxigenic *Escherichia coli* and its neutralization by specific antiserum. *Infect. Immun.* 9:500-505.
- Dupont C., Zigha A., Rosenfeld E., Schmitt P. 2006. Control of enterotoxin gene expression in *Bacillus cereus* F4430/73 involves the redox-sensitive Res DE signal transduction system. *J. Bacteriol.* 188:6640-6651.
- DuPont H. L., Evans D. G., Evans D. J., Satterwhite T. K.. 2003. Antitoxic immunity in cholera and enterotoxigenic *Escherichia coli* (ETEC) diarrhoea. *Pharmacol. Ther.* 13:249-255.
- DuPont H. L., Formal S. B., Hornick R. B., Snyder M. J., Libonati J. P., Sheahan D. G., LaBrec E. H., Kalas J. P. 1971. Pathogenesis of diarrhoea. *N. Engl. J. Med.* 285:1-9.
- DuPont, H. L., Jiang Z. D., Ericsson C. D., Adachi J. A., Mathewson J. J., DuPont M. W., Palazzini E., Riopel L. M., Ashley D., Martinez-Sandoval F. 2001. Rifaximin

versus ciprofloxacin for the treatment of travelers' diarrhoea: a randomized, double-blind clinical trial. *Clin. Infect. Dis.* 33:1807-1815.

Echeverria P., Pitarangsi C., Eampokalap B., Vibulbandhitkit S., Boonthai P., Rowe B. 1983. A longitudinal study of the prevalence of bacterial enteric pathogens among adults with diarrhoea in Bangkok, Thailand. *Diagn. Microbiol. Infect. Dis.* 1:193-204.

Echeverria P., Taylor D. N., Leksomboon U., Bhaibulaya M., Blacklow N. R., Tamura K., Sakazaki R. 1989. Case-control study of endemic diarrhoeal disease in Thai children. *J. Infect. Dis.* 159:543-548.

Echeverria P., Seriwatana J., Chityothin O., Chaicumpa W., Tirapat C. 1982. Detection of enterotoxigenic *Escherichia coli* in water by filter hybridization with three enterotoxin gene probes. *J. Clin. Microbiol.* 16:1086-1090.

Echeverria P., Seriwatana J., Leksomboon U., Tirapat C., Chaicumpa W., Rowe B. 1984. Identification by DNA hybridisation of enterotoxigenic *Escherichia coli* in homes of children with diarrhoea. *Lancet* 1:63-66.

Echeverria P., Verhaert L., Ulyangco C. V., Komalarini S., Ho M. T., Orskov F., Orskov I. 1978. Antimicrobial resistance and enterotoxin production among isolates of *Escherichia coli* in the Far East. *Lancet.* 11: 589-592.

Eitan E. Z., Joshua A. L., Michael S. ., Mor N., Kobi B., Ilan R., Jerrold R., Turner N., Efrat Z., B. 2011. Bundle-forming pilus retraction enhances enteropathogenic *Escherichia coli* infectivity. *Mol. Biol. Cell* 22: 2436-2447

El-Gendy A., El-Ghorab N., Lane E. M., Elyazeed R. A., Carlin N. I. A., Mitry M. M., Kay B. A., Savarino S. J., Peruski L. F. 1999. Identification of *Shigella flexneri* subserotype 1c in rural Egypt. *J. Clin. Microbiol.* 37:873-874.

Elias W. P., Barros S. F., Moreira C. G., Trabulsi L. R. Gomes T. A. 2002. Enteroaggregative *Escherichia coli* strains among classical enteropathogenic *Escherichia coli* O serogroups. *J. Clin. Microbiol.* 40: 3540-3541.

Elias W. P., Czczulin J. R., Henderson I. R., Trabulsi L. R., Nataro J. P. 1999. Organization of biogenesis genes for aggregative adherence fimbria II defines a virulence gene cluster in enteroaggregative *Escherichia coli*. *J. Bacteriol.* 181:1779-1785.

Elliot E. J. 2007. Acute gastroenteritis in Children. *BMJ* 334:35-40

Enne V. I., Delson A. A., Davis G. R., Haywars S. L., Roe J. M., Benett P. M. 2005. Assessment of the fitness impacts on *Escherichia coli* of acquisition of antibiotic resistance genes encoded by different types of genetic element. *J. Antimicrob. Chemother.* 56:544-551.

Eric R. H., Thomas F., Wierzba C., A°hre N., Malla R., Samir A., Wagdy F., Fouad Y., Mohamed S., Young J., Ann-mari S., John D., Stephen J. 2001. Induction of systemic antifimbria and antitoxin antibody responses in Egyptian children and adults by an oral, killed enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine. *Infect and Imm.* 5:2853–2857.

Ericsson C. D. 2003. Travellers' diarrhoea. *Int. J. Antimicrob. Agents* 21:116-124.

Estrada-Garcia T., Lopez-Saucedo C., Thompson-Bonilla R. 2009. “Association of diarrhoeagenic *Escherichia coli* pathotypes with infection and diarrhoea among Mexican children and association of atypical enteropathogenic *E. coli* with acute diarrhoea,” *J Clin Microbiol.* 47: 93–98.

Evans D., Evans G. 1973. Three characteristics associated with enterotoxigenic *Escherichia coli* isolated from man. *Infect. Immun.* 8:322-328.

Evans D., Evans J., Tjoa W. S., DuPont H. L. 1978. Detection and characterization of colonization factor of enterotoxigenic *Escherichia coli* isolated from adults with diarrhoea. *Infect. Immun.* 19:727-736.

FairBrother J., Nadeau E., Gyles C. 2005. *Escherichia coli* in postweaning diarrhoea in pigs: an update on bacterial types pathogenesis and prevention strategies. *An He Res reviews* 6: 17-39.

Faruque A. S., Salam M. A., Faruque S. M., Fuchs G. J.. 1998. Aetiological, clinical and epidemiological characteristics of a seasonal peak of diarrhoea in Dhaka, Bangladesh. *Scand J. Infect. Dis.* 30:393-396.

Faruque A., Malek A., Khan S., Huq M., Salam Sack D. 2004. Diarrhoea in elderly people: aetiology, and clinical characteristics. *Scand J. Infect. Dis.* 36:204-208.

Felmingham D., Canton R., Jenkins SG. 2007. Regional trends in beta-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001-2004. *J Infect.* 55:111-8.

Feng P., Lampel KA., Karch H., Whittam TS. 1998. Genotypic and phenotypic changes in the emergence of *Escherichia coli* O157:H7. *J Infect Dis.* 177:1750–3.

Feng P.C., Feng, S.R., Monday D.W., Lacher E. 2007. Genetic diversity among clonal lineages within *Escherichia coli* O157:H7 stepwise evolutionary model. *Emerg Infect. Dis.* 13 :1701–1706

Fleckenstein J. M., Roy K., Fischer J. F., Burkitt M.. 2006. Identification of a two-partner secretion locus of enterotoxigenic *Escherichia coli*. *Infect. Immun.* 74:2245-2258.

Forsberg C., Petzold G. 2007. Diarrhoea case management in low and middle income countries- an unfinished agenda. *Bull WHO*; 85(1).

- Francis CL., Jerse AE., Kaper JB., Falkow S. 1991. Characterization on interactions of enteropathogenic *Escherichia coli* O127:H6 with mammalian cells in vitro. *J Infect Dis.* 164:693–703.
- Frankel G., Giron J A., Valmassol J., Schoolnik G K. 1989. Multi-gene amplification: simultaneous detection of three virulence genes in diarrhoeal stool. *Molecular Microbiol.* 3:1729-34.
- Freedman D. J., Tacket C. O., Delehanty A., Maneval D. R., Nataro J., Crabb J. H. 1998. Milk immunoglobulin with specific activity against purified colonization factor antigens can protect against oral challenge with enterotoxigenic *Escherichia coli*. *J. Infect. Dis.* 177:662-667.
- Fukui Y., Sawabe T (2007) Improved one-step colony PCR detection of *Vibrio harveyi*. *Microbes Environ* 22:1-10
- Gaastra W., Svennerholm A. M. 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol.* 4:444-452.
- Gaastra W., de Graaf F. K. 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. *Microbiol. Rev.* 46:129-161.
- Gannon V P J., Rashed M., King R K., Golsteyn -Thomas E. J. 1993. Detection and characterization of the eae gene of Shiga-like toxin-producing *Escherichia coli* using polymerase chain reaction. *J. Clin. Microbiol.* 31:1268-1274.
- Garrido P., Blanco M., Moreno-Paz M., Briones C., Dahbi G., Blanco J. E., Blanco J. Parro V. 2006. STEC-EPEC oligonucleotide microarray: a new tool for typing genetic variants of the LEE pathogenicity island of human and animal Shiga toxin-producing *Escherichia coli* (STEC) and enteropathogenic *E. coli* (EPEC). *Clin Chem* 52: 192–201.
- Geornaras I., John H., Alexander H. 2001. Genotypic analysis of *Escherichia coli* strains from Poultry Carcasses and their Susceptibilities to antimicrobial agents. *App Environ Microbiol.* 98: 1940-1944.
- Geyer A., Crewe-Brown H. H., Greeff A. S., Fripp P. J., Steele A. D., Van Schalkwyk T. V., Clay C. G. 1993. The microbial aetiology of summer paediatric gastroenteritis at Ga-Rankuwa Hospital in South Africa. *East Afr. Med. J.* 70:78-81.
- Gibbons R. A., Sellwood R., Burrows M. 1977. Inheritance of resistance to neonatal *Escherichia coli* diarrhoea in the pig: examination of the genetic system. *Theor. Appl. Genet* 51:65-70.
- Gill D. M., Richardson S. H. 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin. *J. Infect. Dis.* 141:64-70.

Gillespie S. H., Hawkey P. M. 2006. Principles and Practice of Clinical Bacteriology. *Chichester* 2:1360-72.

Girardeau J.P., Girardeau L., Lalioui A.M., Said C., De Champs C., Le Bouguenec. 2003. Extended virulence genotype of pathogenic *Escherichia coli* isolates carrying the *afa-8* operon: evidence of similarities between isolates from humans and animals with extraintestinal infections. *J. Clin. Microbiol.*, 41:218–226

Giraud E., Brisabois A., Martel J. L., Chaslus-Dancla E. 1999. Comparative studies of mutations in animal isolates and experimental in vitro- and in vivo-selected mutants of *Salmonella spp.* suggest a counterselection of highly fluoroquinolone-resistant strains in the field. *Antimicrob. Agents Chemother.* 43:2131-2137.

Giraud E., Cloeckart A., Kerboeuf D., Chaslus-Dancla E. 2000. Evidence for active efflux as the primary mechanism of resistance to ciprofloxacin in *Salmonella enterica* serovar Typhimurium. *Antimicrob. Agents Chemother.* 44:1223-1228.

Glass RI., Svennerholm A-M., Stoll BJ. 1983. Protection against cholera in breast-fed children by antibodies in breast-milk. *N Engl J Med.* 308:1389–1392.

Glenn G. M., Francis D. H., Danielsen E. M. 2009. Toxin-mediated effects on the innate mucosal defenses: implications for enteric vaccines. *Infect. Immun.* 77:5206-5215.

Glenn G. M., Frech S. A., Kenney R. T., Ellingsworth L. R. 2005. Transcutaneous immunization: antigen and adjuvant delivery to the skin. *Drugs Pharm. Sci.* 155:769-788.

Golden BE., Golden MHN. 1985. Zinc, sodium and potassium losses in the diarrhoeas of malnutrition and zinc deficiency. In: Mills CF, Bremner I, Chesters JK, editors. Trace elements in man and animals TEMA 5. Aberdeen: Rowett Research Institute; pp. 228–232.

Gómez-Duarte O. G., Octavio A., Delfina U., Jing B., Julio G., Oscar M., Marta P., Ketty M., Gregorio Y., Castro N. 2010. Detection of *Escherichia coli* Enteropathogens by Multiplex Polymerase Chain Reaction from Children's Diarrhoeal Stools in Two Caribbean–Colombian Cities. *Foodborne Pathog Dis.* 7: 199–206.

Gould L. H. 2009. Hemolytic uremic syndrome and death in persons with *Escherichia coli* O157:H7 infection, foodborne diseases active surveillance network sites, 2000-2006. *Clin. Infect. Dis.* 49:1480–1485.

Gould L. H. 2009. Recommendations for diagnosis of Shiga toxinproducing *Escherichia coli* infections by clinical laboratories. *MMWR Recommend. Rep.* 58:1–14.

Greenwood Z., Black J., Weld L. 2008. Gastrointestinal infection among international travelers globally. *J Travel Med.*15:221–228.

Guarino A., Albano F., Ashkenazi S. 2008. European Society for Paediatric Gastroenterology, hepatology and Nutrition/European Society for paediatric Infectious Diseases Evidence-based Guidelines for the Management of Acute Gastroenteritis in Children in Europe. *JPGN*: 46:S81–S122.

Guerena-Burgueno F., Hall E. R., Taylor D. N., Cassels F. J., Scott D. A., Wolf M. K., Roberts Z. J., Nesterova G. V., Alving C. R., Glenn G. M. 2002. Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. *Infect. Immun.* 70:1874-1880.

Gutiérrez-Cázares Z., Qadri F., Albert M. J., Girón J. A. 2000. Identification of enterotoxigenic *Escherichia coli* harboring Longus type IV pilus gene by DNA amplification. *J. Clin. Microbiol.* 38:1767-1771.

Hall E. R., Wierzba T. F., Ahren C., Rao M. R., Bassily S., Francis W., Girgis F. Y., Safwat M., Lee Y. J., Svennerholm A. M., Clemens J. D., Savarino S. J. 2000. Induction of systemic antifimbria and antitoxin antibody responses in Egyptian children and adults by an oral, killed enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine. *Infect. Immun.* 69:2853-2857.

Hammerum A., Ole E. 2009. Human health hazards from antimicrobial resistant *Escherichia coli* of Animal origin. *Food safety* 48: 916-21.

Hamond A., Mangia R., Beatriz C., Joao R., Kinue I., Ana B., Pacheco L., Ferreira V., Lu M. 2004. Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* (ETEC) strains isolated in Rio de Janeiro city, Brazil. *Immun and Med. Microbiol.*40:2011-2015.

Harper W.M. 1980. Statistics 3rd edition. Macdonald and Evans Ltd, Estover, Plymouth, *United Kingdom*.

Harris A.M., Chowdhury F., Begum Y.A., Khan A.I., Faruque A.S., Svennerholm A.M., Harris J.B., Ryan E.T., Cravioto A., Calderwood S.B., Qadri,F. 2008. Shifting prevalence of major diarrhoeal pathogens in patients seeking hospital care during floods in 1998, 2004, and 2007 in Dhaka, Bangladesh. *Am J Trop Med Hyg.* 79: 708-714.

Harro C., David S., Louis B., Walker R., Barbara N., Andrea F., Subhra C., Charlotte B., Michael J., 2011. A Combination Vaccine Consisting of Three Live Attenuated Enterotoxigenic *Escherichia coli* Strains Expressing a Range of Colonization Factors and Heat-Labile Toxin Subunit B Is Well Tolerated and Immunogenic in a Placebo-Controlled Double-Blind Phase I Trial in Healthy Adults. *Clin Vac Immunol.* 354:340-238.

Hedican E. B. 2009. Characteristics of O157 versus non-O157 Shiga toxin-producing *Escherichia coli* infections in Minnesota, 2000-2006. *Clin. Infect. Dis.* 49:358–364. 15.

Heuvelink A E., van de Kar N C A J, Meis J F G M, Monnens L A H, Melchers W J G. 1995. Characterization of verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uremic syndrome in Western Europe. *Epidemiol. Infect.* 115:1-14.

Heyman D L. 2004. Control of Communicable Diseases Manual, 18th edn. Washington, DC: *Amer Pub Health Assoc.* pp. 234-239.

Hien B.T., Scheutz F., Cam P.D., Serichantalergs O., Huong T.T., Thu T.M., Dalsgaard A. 2008. Diarrhoeagenic *Escherichia coli* and *Shigella* strains isolated from children in a hospital case-control study in Hanoi, Vietnam. *J Clin Microbiol.* 46: 996-1004.

Hill DR., Ford L., Lalloo DG. Oral cholera vaccines: 2006. Use in clinical practice. *Lancet.* 6:361-73.

Hirose K., K. Tamura H., Sagara H. Watanabe K. 2001. Antibiotic susceptibilities of *Salmonella enterica* serovar Typhi and *S. enterica* serovar Paratyphi A isolated from patients in Japan. *Antimicrob. Agents Chemother.* 45:956-958.

Holmgren J., Kaper J.. 2009. Oral cholera vaccines. In: Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R. 2009. *New gen vaccines.* 49:506–17.

Honda T., Khan M., Miwatani T. 1983. Grouping of ETEC by Hydrophobicity and its relation to haemagglutination and enterotoxin production. *FEMS Microbiol. Lett.* 17: 273-276.

Hong N., Kiyofumi O., Jiro M., Takayuki E. 2007. Rapid and specific identification of 5 human pathogenic Enteroinvasive *Escherichia coli* species by multiplex polymerase chain reaction targeted to *dnaJ* gene. *J. Diag Microbiol.* 5: 16-20.

Hossain M. A., Rahman M., Ahmed Q. S., Malek M. A., Sack R. B., Albert M. J. 1998. Increasing frequency of mecillinum-resistant *Shigella* isolates in urban Dhaka and rural Matlab, Bangladesh: a 6 year observation. *J. Antimicrob. Chemother.* 42:99-102.

Huang D. B., H. L. Dupont, 2004. “Enteraggregative *Escherichia coli*: an emerging pathogen in children,” *Seminars in Pediatric Infectious Diseases*, 15: 266–271.

Huang D. B., Brown E. L., DuPont H. L. 2008. “Seroprevalence of the enteraggregative *Escherichia coli* virulence factor dispersin among USA travellers to Cuernavaca, Mexico: a pilot study,” *J Med Microbiol.* 57: 476–479.

- Huang DB., Mohanty A., DuPont HL., Okhuysen PC., Chiang T. 2006. A review of an emerging enteric pathogen: enteroaggregative *Escherichia coli*. *J Med Microbiol*: 55: 1303–1311.
- Huang D., Alakananda M., Herbert L., DuPont P., Tom C. 2006. A review of an emerging enteric pathogen: enteroaggregative *Escherichia coli*. *J Med Microbiol*. 10: 1303-1311.
- Hunter S., Paul V. 2005. Establishment of universal sized standard Pulsed-Gel Electrophoresis protocol: Converting the national data base to the new size atandard. *J Clin Microbiol* 43: 1045-1050.
- Iman K. B., Emad A. A., Entsar A. ., Rania S. L. 2011. Enteropathogenic *Escherichia coli* Associated with Diarrhoea in Children in Cairo, Egypt. *J Med Microbiol*: 57: 47-49.
- Insenbarger D W. 2001. Prospective study of the incidence of diarrhoea and prevalence of bacterial pathogens in a cohort of Vietnamese children along the Red River. *Epid and Infect*.127: 229-236.
- Isidean SD., Riddle MS., Savarino SJ., Porter CK. 2011. A systematic review of ETEC epidemiology focusing on colonization factor and toxin expression. *Vaccine*. 29 :6167-78.
- Iwanaga M., Song T., Higa N., Kakinohana S., Toma C., Nakasone N. 2002. “Enteraggregative *Escherichia coli*: incidence in Japan and usefulness of the clump-formation test,” *J Infect Chemother*. 8: 345–348.
- Jann K., B Jann. 1992. Capsules of *Escherichia coli*, expression and biological significance. *Can. J. Microbiol*. 38:705-10.
- Javier de la Cabada B., Herbert L. D. 2011. New Developments in Travelers’ diarrhoea. *Gastroenterol Hepatol*. 7: 88–95.
- Jay M. T. 2004. A multistate outbreak of *Escherichia coli* O157:H7 infection linked to consumption of beef tacos at a fast-food restaurant chain. *Clin. Infect. Dis*. 39:1–7.
- Jerse A E., Martin W C., Galen J E, Kaper J B. 1990. Oligonucleotide probe for detection of the enteropathogenic *Escherichia coli* (EPEC) adherence factor of localized adherent EPEC. *J. Clin. Microbiol*. 28:2842-2844.
- Jeuland M., Cook J., Poulos C., Clemens J., Whittington D. 2009. Cost-effectiveness of new-generation oral cholera vaccines: A multisite analysis. *Value Health*. 12:899-908.
- Jiang Z., Lowe B., Verenkar MP., Ashley D., Steffen R., Tornieporth N., von Sonnenburg .F, Waiyaki P., DuPont HL. 2002. Prevalence of enteric pathogens among international travelers with diarrhoea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay). *J Infect Dis*. 32: 1234-1239.

- Jiang ZD., Lowe B., Verenkar M.P, Waiyaki P. 2002. Prevalence of enteric pathogens among international travelers with diarrhoea acquired in Kenya (Mombasa), India (Goa), or Jamaica (Montego Bay) *J Infect Dis.*185:497–502.
- Jiang, Z. D., Mathewson J. J., Ericsson C. D., Svennerholm A. M., Pulido C., DuPont H. L. 2000. Characterization of enterotoxigenic *Escherichia coli* strains in patients with travelers' diarrhoea acquired in Guadalajara, Mexico, 1992-1997. *J. Infect. Dis.* 181:779-782.
- Jiang Z.-D., Okhuysen P. C., Guo D.-C., He R., King T. M, DuPont H. L., Milewicz D. M. 2003. Genetic susceptibility to enteroaggregative *Escherichia coli* diarrhoea: polymorphism in the interleukin-8 promoter region. *J. Infect. Dis.* 188:506-511.
- Jo M.Y. 2004: Prevalence and characteristics of *Escherichia coli* O157 from major food animals in Korea. *Int. J. Food Microbiol.*, 95: 41-49.
- John B., Rogger L., Lata K., Joy G., Wells P. 2003. Epidemiology of sporadic bloody diarrhoea in Rural Western Kenya. *Amer Socf Trop Med Hyg.* 86: 671-677.
- Johnson K. E., Thorpe C. M., Sears C. L. 2006. The emerging clinical importance of non-O157 Shiga toxin-producing *Escherichia coli*. *Clin. Infect. Dis.* 43:1587–1595.
- Jolley K. A., Chan M.S., Maiden M. C. J. 2004 distributed multi-locus sequence typing (MLST) databases. *BMC Bioinformatics.* 5:86-93.
- Kam K., Cindy Y. 2008. Evaluation and validation of PulseNet standardised Pulsed-Field gel Electrophoresis protocol for subtyping *Vibrio parahaemolyticus*: An international multicentre collaborative study. *J Clin Microbiol* 46: 2766-2770.
- Kandakai-Olukemi Y. T., Mawak J. D., Onojo M. M. 2009. “Isolation of enteropathogenic *Escherichia coli* from children with diarrhoea attending the national hospital in Abuja, Nigeria,” *Shiraz E-Med J.* 10: 99–106.
- Kaper, J. B., Nataro J. P., Mobley H. L. 2004. Pathogenic *Escherichia coli*. *Nat. Rev. Microbiol.* 2:123-140.
- Karina M., Ariadna C., Maria A. L., Juan C. 2009. Longus, a type IV pilus of enterotoxigenic *Escherichia coli*, is involved in adherence to intestinal epithelial cells. *J. Bacteriol.* 192: 2791-2800.
- Kariuki S., Oundo JO., Muyodi J., Lowe B., Threshfall E J., Hart CA. 2000. Genotypic of multidrug – resistance *Salmonella enterica* serotype typhimurium from two regions of Kenya. *FEMS Immunol Med Microbiol.* 29: 9-13.
- Kariuki S., Oundo JO., Muyodi J., Lowe B., Threshfall E J., Hart CA. 2001. Genotypic of multidrug – resistance *Salmonella enterica* serotype typhimurium from Kenya. *FEMS Immunol Med Microbiol.* 29: 56-61

- Kariuki S., Revathi G., Gaguya F., Yamo V., Muyodi J., Hart CA. 2002. Lack of clonal relationship between non typhi Salmonella strain types from human and those those isolated from animals living in close contact. *FEMS Immunol Med Microbiol.* 33: 165-71.
- Kariuki S., Revathi G., Kariuki N., Muyodi J., Mwituria J., Munyalo A., Kagendo D., Murungi L., Antony CA. 2004. Increasing prevalence of multi-drug resistance non- typhoidal Salmonellae, Kenya. *Int. J Antimicrobiol Agents.* 25: 38 – 43.
- Kariuki S., Revathi G., Kariuki N., Muyodi J., Mwituria J., Munyalo A., Kagendo D., Murungi L., Antony CA. 2004. Characterization of multi-drug resistance typhoid out breaks in Kenya. *J Clin Microbiol.* 43:1477-1482.
- Karmali M A., Steele B T., Petric M., Lim C. 1983. Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stool. *Lancet.* 59: 619-620.
- Karmali M A., Petric M., Bielaszewska M. 1999. Evaluation of microplate latex agglutination method (Verotex-F assay) for detecting and characterizing verotoxins (Shiga toxins) in *Escherichia coli*. *J. Clin. Microbiol.* 37:396-399.
- Kato J., Nishimura Y., Imamura R., Niki H., Hiraga S., Suzuki H. 1990. New topoisomerase essential for chromosome segregation in *E. coli*. *Cell.* 63:393-404.
- Kaur P., Chakraborti A., Asea A. 2010. Enteroaggregative *Escherichia coli*: An emerging enteric foodborne pathogen. *Interdisc Perspect on Infect Dis.* 10.1155-1165.
- Kehl S. C. 2002. Role of the laboratory in the diagnosis of enterohemorrhagic *Escherichia coli* infections. *J. Clin. Microbiol.* 40:2711–2715
- Keskimaki M., Marjut E., Heidi P., Tarja H. 2001. EPEC, EAEC and STEC in stool specimens: Prevalence and molecular epidemiology of isolates. *Diag Microbiol Infect Dis.* 40: 151-156.
- Keusch G.T. 2003. The history of nutrition: Malnutrition, infection and immunity. *J. Nutr.* 133, 336-340.
- Khachatryan A. R., Hancock D. D., Besser T. E., Call D. R. 2006. Antimicrobial drug resistance genes do not convey a secondary fitness advantage to calf-adapted *Escherichia coli*. *Appl Environ. Microbiol.* 72:443-448.
- Khaing-Khaing –Gyi. 2009. Use of rehydration therapy for management of children under five years with Diarrhoea in Thailand. *J Biomed Biotechnol.* 45:123-129.
- Kheyami AM., Cunliffe NA., Hart CA. 2006. Rotavirus infection in Saudi Arabia. *Ann Saudi Med* 16: 861-867.

- Kilic I. H., Ozaslan M., Karsligil T., Karagöz I. D., Zer Y. 2007. "Investigation of diarrhoea agents in children less than 5 years of age in summer in Gaziantep, Turkey." *Pakistan J Biological Scie* 17: 2915–2919.
- Kimata K., Shima T., Shimizu M., Tanaka D., Isobe J., Gyobu Y., Watahiki M., Nagai Y. 2005. Rapid categorization of pathogenic *Escherichia coli* by multiplex PCR. *Microbiol Immunol.* 49:485-492.
- Kingombe CI., Cerqueira-Campos ML., Farber JM. 2005. Molecular strategies for the detection, identification, and differentiation between enteroinvasive *Escherichia coli* and *Shigella spp.* *J Food Prod:* 68:239-45.
- King-Ting L., Rohani Y., Chew-Chieng Y., Savithri P., Kwai-Lin T. 2009. Characterization of multidrug resistant ESBL-producing *Escherichia coli* isolates from Hospitals in Malaysia. *J Biomed Biotechnol.* 2009: 165- 637.
- Kiranmayi B., Krishnaiah N. 2010. Detection of *Escherichia coli* O157:H7 prevalence in foods of animal origin by cultural methods and PCR technique. *Vet World.* 3: 13-16.
- Klena JO., McCracken Jr GH., Feigin RD. 2005. Primer design for Multiplex PCR for detection of enterotoxigenic *Escherichia coli*. *Pediatrics.* 195:759–82.
- Konstantinos Z., Vardakas., Ilias I., Siempos., Alexandros G., Zoe A., Ioanna P., Matthew E. F. 2008. Respiratory fluoroquinolones for the treatment of community-acquired pneumonia: a meta-analysis of randomized controlled trials. *CMAJ.* 179: 1503-1510.
- Koutsolioutsou A., Martins EA., White DG., Levy SB., Demple B. 2001. A soxRS-constitutive mutation contributing to antibiotic resistance in a clinical isolate of *Salmonella enterica* (serovar Typhimurium). *Antimicrob Agents Chemother.* 45:38-43.
- Kukuruzovic R., Robins-Browne RM., Anstey NM., Brewster DR. 2002. Enteric pathogens, intestinal permeability and nitric oxide production in acute gastroenteritis. *J. Pediatr Infect Dis.* 21:730–9.
- Kwena, A.M., Terlouw, D.J., de Vlas, S.J., Phillips-Howard, P.A., Hawley, W.A., Friedman, J.F., Vulule, J.M., Nahlen, B.L., Sauerwein, R.W., Terkuile, F.O. 2003. Prevalence and severity of malnutrition in pre-school children in a rural area of western Kenya. *Am. J. Trop. Med. Hyg.* 68: 94-99.
- Landry P. 2006. Diarrhoea and vaccines: Current developments. *Rev Med Suisse.* 2:1240-4.
- Latife I., Teoman Z., Altan A., Figen K., Jülide S., Diba N. 2011. The prevalence of enterotoxigenic *E. coli* isolated from the stools of children aged 0-10 years with diarrhoea in mid-anatolia region, Turkey. *Braz. J. Microbiol.* 42: 117-123.

Leibovitz E., Janco J., Piglansky L. 2000. Oral ciprofloxacin vs. intramuscular ceftriaxone as empiric treatment of acute invasive diarrhoea in children. *Pediatr Infect Dis J.* 19:1060-67.

Leonor R, Elsa C., Rocío V. 2011. Malnutrition and gastrointestinal and respiratory infections in children: A public health problem. *Int. J. Environ. Res. Public Health.* 8:1174-1205.

Levine M.M., Svennerholm A.M. 2001. Enteric vaccines: present and future. (2nd ed.). *Decker.* pp 239-245.

Levine MM., Nataro JP., Karch H., Baldini MM., Kaper JB., Black RE. 1985. The diarrhoeal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. *J Infect Dis.*152:550–9.

Levine M. 1987. *Escherichia coli* that cause diarrhoea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. *J. Infect. Dis.* 155:377-389.

Li, X., Mariano N., Rahal J. J., Urban C. M., Drlica K.. 2004. Quinolone-resistant *Haemophilus influenzae* in a long-term care facility: nucleotide sequence characterization of alterations in the genes encoding DNA gyrase and DNA topoisomerase. *Antimicrob Agents Chemother.* 48:3570-3572.

Li, Y. F., Poole S., Nishio K., Jang K., Rasuloova F., McVeigh A., J S., Savarino, Xia D., Bullitt E. 2009. Structure of CFA/I fimbriae from enterotoxigenic *Escherichia coli*. *Proc. Natl. Acad. Sci.* 106:10793-10798.

Lietzau S., Raum E., von Baum H. 2007. Household contacts were key factor for children's colonization with resistant *Escherichia coli* in community setting. *J Clin Epidemiol* 60:1149–55.

Liu M., Ruan X., Zhang C., Lawson SR., Knudsen DE., Nataro JP., Robertson DC., Zhang W. 2011. Heat-labile- and heat-stable-toxoid fusions (LTR₁₉₂G-STaP₁₃F) of human enterotoxigenic *Escherichia coli* elicit neutralizing antitoxin antibodies. *Infect Immun.* 79:4002-9.

Liu M., Xiaosai R., Thomas A., Casey I., Weiping Z. 2011. A Tripartite fusion, FaeG-FedF-LT₁₉₂A2:B, of enterotoxigenic *Escherichia coli* (ETEC) Elicits Antibodies that neutralize cholera toxin, inhibit adherence of K88 (F4) and F18 fimbriae, and protect pigs against K88ac/heat-labile toxin infection. *Clin Vaccine Immunol* 18 : 1593-1599.

Long, Jorge L., Jose´ I., Meredith H., Abdullah A., Herbert D., Nanda N., Teresa E. 2010. Associations between mucosal innate and adaptive immune responses and resolution of diarrhoeal pathogen infections. *Infect and immune.* 122: 1221–1228.

- Lopez-Campistrous A., Semchuk P., Burke L., Palmer-Stone T., Brox SJ., Broderick G., Bottorff D., Bolch S., Weiner JH., Ellison MJ. 2005. Localization, annotation, and comparison of the *Escherichia coli* K-12 proteome under two states of growth. *Mol Cell Proteomics*. 4:1205-9.
- Lundkvist J., Steffen R., Jönsson B. 2009. Cost-benefit of WC/rBS oral cholera vaccine for vaccination against ETEC-caused travelers' diarrhoea. *J Travel Med*. 16:28-34.
- Lutz G., Susann S., Birgit M., Franz J., Elke M., and Ralf E. 2010. Rapid Microarray-based genotyping of enterohemorrhagic *Escherichia coli* serotype O156:H25/H-/Hnt isolates from cattle and clonal relationship analysis. *App Env Microbiol*. 201: 5510-5519.
- Maiden M., Bygraves C., Feil G., Morelli J. E., Russell R., Urwin Q., Zhang J., Zhou, K., Zurth, D. A., Caugant, I. M., Feavers, M., Achtman, B., Spratt G. 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci*. 95: 3140-3145.
- Maity T. K., Rakesh K., Misra A. K. 2010. Prevalence of enteropathogenic *Escherichia coli* isolated from *Chhana* based Indian sweets in relation to public health. *Indian J Microbiol*. 50: 463-467.
- Mandomando I. M., Eusébio V., Macete J., Sergi S., Fatima A., Xavier A., Jahit Sa. 2007. Etiology of diarrhoea in children younger than 5 years of age admitted in a rural hospital of southern Mozambique. *Am J Trop Med Hyg*. 76:522-527.
- Manning, S. D. 2007. Surveillance for Shiga toxin-producing *Escherichia coli*, Michigan, 2001-2005. *Emerg. Infect. Dis*. 13:318-321
- Mariana A, Honda T., Wetprasit N., Miwatani T. 2000. Pilus colonization factors of enterotoxigenic *Escherichia coli* isolated from infantile diarrhoeal patients in Papua New Guinea. *American J Trop Med Hyg*. 41: 220-223.
- Martin A. J., Qadri F., Wahed M. A., Ahmed T., Rahman A. S., Ahmed F., Bhuiyan N. A., Zaman K., Baqui A. H., Clemens J. D., Black R. E. 2003. Supplementation with zinc, but not vitamin A, improves seroconversion to vibriocidal antibody in children given an oral cholera vaccine. *J. Infect. Dis*. 187:909-913.
- Martin, A. J., Faruque A. S., Faruque S. M., Sack R. B., Mahalanabis D. 1999. Case-control study of enteropathogens associated with childhood diarrhoea in Dhaka. Bangladesh. *J. Clin. Microbiol*. 37:3458-3464
- Martin, A. J., Faruque S. M., Faruque A. S., Neogi P. K., Ansaruzzaman M., Bhuiyan N. A., Alam K., Akbar M. S. 1995. Controlled study of *Escherichia coli* diarrhoeal infections in Bangladeshi children. *J. Clin. Microbiol*. 33:973-977.

- Martinez-Martinez L., Pascual A., Garcia I., Tran J., Jacoby G. 2003. Interaction of plasmid and host quinolone resistance. *J Antimicrob Chemother.*51:1037–9.
- McConnell, M. M., Hibberd M. L., Penny M. E., Scotland S. M., Cheasty T., Rowe. B.1991. Surveys of human enterotoxigenic *Escherichia coli* from three different geographical areas for possible colonization factors. *Epidemiol. Infect.* 106:477-484.
- McDaniel, T K, Jarvis K G, Donnerberg M S, Kaper J B. 1995. A genetic locus of the enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc. Natl. Acad. Sci.*92:1664-1668.
- Meléndez, G. 2011. Abstract. Fundación Mexicana para la Salud-FUNSAUD. Available online: <http://www.informador.com.mx/mexico/2010/198618/6/afecta-desnutricion-a-18-millones-de-mexicanos-menores-de-cinco-anos.htm>.
- Mendez A E, Pitart C., Ruiz J., Marco F., Gascón J., Vila. 9. J. 2009. Evolution of antimicrobial resistance in enteroaggregative *Escherichia coli* and enterotoxigenic *Escherichia coli* causing traveller's diarrhoea. *J Antimicrob Chemother.* 64 : 343-7.
- Menendez R., Torres A., Zalacain R. 2004. Neumofail Group. Risk factors of treatment failure in community acquired pneumonia: implications for disease outcome. *Thorax.* 59:960-5.
- Meraz IM., Jiang ZD., Ericsson CD. 2008. Enterotoxigenic *Escherichia coli* and diffusely adherent E. coli as likely causes of a proportion of pathogen-negative travelers' diarrhoea a PCR-based study. *J Travel Med.*15:412–418.
- Merz, A. J., So M., Sheetz M. P. 2000. Pilus retraction powers bacterial twitching motility. *Nature* 407:98-102.
- Mickelsen P. A. 1997. The use of molecular strain typing has become a standard practise. *Clin.Microbiol. Newsletter.* 19:137-144.
- Mills-Robertson F., Addy ME., Mensah P. 2002. Molecular characterization of antibiotic resistance in clinical *Salmonella typhi* isolated in Ghana. *FEMS Microbiol Lett.* 215:249–53.
- Mondal D., Minak J., Alam M., Liu Y., Dai J., Korpe P., Liu L., Haque R. 2012. Contribution of enteric infection, altered intestinal barrier function, and maternal malnutrition to infant malnutrition in Bangladesh. *Clin Infect Dis.* 54:185-92.
- Monnet DL., Biddle JW., Edwards JR., Culver DH., Tolson JS., Martone WJ. 1997. Evidence of interhospital transmission of extended-spectrum β -lactam-resistant *Klebsiella pneumoniae* in the United States, 1986 to 1993. The National Nosocomial Infections Surveillance System. *Infect Control Hosp Epidemiol:* 18:492-8.

- Nataro J. P., a Kaper J. B. 1998. Diarrhoeagenic *Escherichia coli*. *J Clin. Microbiol.* 11:142-150.
- Nataro J. P., Serowatana J. A., Fasano A., Maneval D. R., Guers L. D., Noriega F., Dubovsky, F. Levine M. M., Morris J. G. 1995. Identification and cloning of a novel plasmid-encoded enterotoxin of enteroinvasive *Escherichia coli* and *Shigella* strains. *Infect Immun.* 63:4721-4728.9
- Nayak N., Steven L. F., Aaron M., Lynne S. 2004. Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infect Genetics Evolution.* 9:430-440.
- Newman MJ., Seidu A. 2002. Carriage of antimicrobial resistant *Escherichia coli* in adult intestinal flora. *West Afr J Med* 21:48–50.
- Ngugi JM., Sang FC., Gatheri UZ., Waiyaki PG., Ichoroh CG. 1985. *In vitro* antimicrobial susceptibility of *Shigella* serotypes isolated from patients with diarrhoea in Kwale District. *KEMRI/KETRI Ann Scie Proc.*
- Nicholas A., Daniels J. 2005. Enterotoxigenic *Escherichia coli*: Travelers' diarrhoea comes home. *Oxford J Med Infect Dis* 42: 335-336.
- Nishikawa Y., Zhou Z., Hase A., Ogasawara J., Kitase T., Abe N., Nakamura H., Wada T., Ishii E. Haruki K. 2002. Diarrhoeagenic *Escherichia coli* isolated from stools of sporadic cases of diarrhoeal illness in Osaka City, Japan between 1997 and 2000: prevalence of enteroaggregative *E. coli* heat-stable enterotoxin 1 gene-possessing *E. coli*. *Jpn J Infect Dis* 55: 183–190.
- Nishimura L. S., Giron J. A., Nunes S. L., Guth B. E. 2002. Prevalence of enterotoxigenic *Escherichia coli* strains harboring the longus pilus gene in Brazil. *J. Clin. Microbiol.* 40:2606-2608.
- Nordmann P., Laurent P. 2005. Emergence of plasmid-mediated resistance to quinolones in Enterobacteriaceae. *J. Antiml Chemother* 56: 463–469.
- Noriega F. R., Liao F. M., Formal S. B., Fasano A., Levine M. M. 1995. Prevalence of *Shigella* enterotoxin 1 among *Shigella* clinical isolates of diverse serotypes. *J. Infect. Dis.* 172:1408-1410.
- Nunes E. B., Saridakis H. O., Irino K., Pelayo J. S. 2003. Genotypic and phenotypic characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from children with and without diarrhoea in Londrina, Brazil. *J Med Microbiol.* 52: 499–504.

- Nunes E. B., Saridakis H. O., Irino K., Pelayo J. S. 2011. Characterization of attaching and effacing *Escherichia coli* (AEEC) from children with diarrhoea in Londrina, Brazil. *J Med Microbiol* 52: 499–504.
- Nweze E. 2010. Aetiology of diarrhoea and virulence properties of diarrhoeagenic *Escherichia coli* among patients and healthy subjects in southeast Nigeria. *J Health Popul Nutr.* 3: 245-252.
- Ochoa T. J., Barletta F., Contreras C., Mercado E. 2008. New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. *Trans. R. Soc. Trop. Med. Hyg.* 102:852-856.
- Okeke IN., Ojo O., Lamikanra A., Kapper JB. 2003. Etiology of acute diarrhoea in adults in southwestern Nigeria. *J Clin Microbiol* 41: 4525–4530.
- Okeke I. N., Lamikanra A., Czeczulin J., Dubovsky F., Kaper J. B., Nataro J. P. 2000. Heterogeneous virulence of enteroaggregative *Escherichia coli* strains isolated from children in southwest Nigeria. *J. Infect. Dis.* 181:252-260.
- Oksuz O., Arici M., Kurultay S., Gumus, T. 2004: Incidence of *Escherichia coli* O157:H7 in raw milk and white pickled cheese manufactured from raw milk in Turkey. *Food Control.* 15: 453-456.
- Olesen B., Neimann J., Bottiger B., Ethelberg S., Schiellerup P., Jensen C., Helms M., Scheutz F., Olsen KE., Kroghfelt K.. 2005: Etiology of diarrhoea in young children in Denmark: a case-control study. *J Clin Microbiol*, 43:3636-3641.
- Olsen S. J., DeBess E. E, McGivern T. E., Marano N., Eby T., Mauvais S., Balan, V. K. Zirnstein G., Cieslak P. R., Angulo F. J. 2001. A nosocomial outbreak of fluoroquinolone-resistant *Salmonella* infection. *N. Engl. J. Med.* 344:1572-1579.
- Olsvik Ø., Strockbine N A. 1993. PCR detection of heat-stable, heat-labile, and Shiga-like toxin genes in *Escherichia coli*. *ASM, Washington DC.* 641- 647.
- Onyango M D., Beniam G., Eliud N. 2009. Phenotypic and genotypic analysis of clinical isolates of *Salmonella* serovar Typhimurium in western Kenya. *J Infect Dev Ctries* 9: 685-694.
- Opintan J., Newman MJ. 2007. Distribution of serogroups and serotypes of multiple drug resistant *Shigella* isolates. *Ghana Med J* 41:8–29.
- Osek J. 2001. Multiplex Polymerase Chain Reaction assay for identification of enterotoxigenic *Escherichia coli* strains. *J Vet Diagn Invest.* 13: 308-311.
- Oundo J., Samuel K., Hamadi B., Faith M., Yoshio I. 2008. High incidence of enteroaggregative *Escherichia coli* among food handlers in three areas of Kenya: A possible transmission route of travelers' diarrhoea. *J Travel Med.* 15: 31–38.

Ouyang-Latimer J., Syed J., Audrey V., Zhi-Dong J., Kaur G., Savio R., Ranjan K., Thandavaryan R., Santanu C., Robin M K., Robert S., Herbert L. 2011. *In Vitro* antimicrobial susceptibility of bacterial enteropathogens isolated from international travelers' to Mexico, Guatemala, and India from 2006 to 2008. *Antimicrobiol Agents Chemother.* 55: 874-878.

Pacheco A. B., Ferreira L. C., Pichel M. G., Almeida D. F., Binsztein N., Viboud. G. I. 2001. Beyond serotypes and virulence-associated factors: detection of genetic diversity among O153:H45 CFA/I heat-stable enterotoxigenic *Escherichia coli* strains. *J. Clin. Microbiol.* 39: 4500-4505.

Pacheco G., Alessandro B., Lucia Pal., Marta B., Connie F., Yolanda V., Elisa G. 2006. Multidrug-resistant Commensal *Escherichia coli* in children, Peru and Bolivia. *Emerg Infect Dis*: 12: 6-10.

Paciorek J. 2002. Virulence properties of *Escherichia coli* faecal strains isolated in Poland from healthy children and strains belonging to serogroups O18, O26, O44, O86, O126 and O127 isolated from children with diarrhoea. *J Med Microbiol.* 51:548-56.

Paniagua M., Espinoza F., Ringman M., Reizenstein E., Svennerholm A. M., Hallander H. 1997. Analysis of incidence of infection with enterotoxigenic *Escherichia coli* in a prospective cohort study of infant diarrhoea in Nicaragua. *J. Clin. Microbiol.* 35:1404-1410.

Paredes-Paredes M., Pablo C., Jose F., Jamal A., Ranjit S., Alexei G., Clinton A., Lily G., Parvathy N., Herbert D. 2011. Seasonality of Diarrhoeagenic *Escherichia coli* Pathotypes in the US Students Acquiring Diarrhoea in Mexico. *J Travel Med.* 18:121-5.

Parvath A., Dafini C A. 2011. Distribution of putative virulence genes and antimicrobial drug resistance in *Vibrio harveyi*. *Indian J. Microbiol.* 51: 332-337.

Paton A W., Manning P A., Woodrow M C., Paton J C. 1998. Translocated intimin receptors (Tir) of Shiga-toxigenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from patients with Hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. *Infect. Immun.* 66:5580-5586.

Pazhani G.P., Chakraborty S., Fujihara K., Yamasaki S., Ghosh A., Nair G.B., Ramamurthy T. 2011. Molecular characterization and antimicrobial susceptibility patterns in enteric pathogens. *J. Clin. Microbiol.* 56: 3052-0357.

Pelletier D., Frongillo E.A. 2003. Changes in child survival are strongly associated with changes in malnutrition in developing countries. *J. Nutr.*, 133; 107-119.

Peruski L. F., Kay B. A., El-Yazeed R. A., El-Etr S. H., Cravioto A., Wierzba T. F., Rao M., El-Ghorab N., Shaheen H., Khalil S. B., Kamal K., Wasfy M. O., Svennerholm A. M., Clemens J. D., Savarino S. J. 1999. Phenotypic diversity of enterotoxigenic *Escherichia coli* strains from a community-based study of pediatric diarrhoea in periurban Egypt. *J. Clin. Microbiol.* 37: 2974-2978.

Piddock LJV. 2002. Fluoroquinolone resistance in *Salmonella* serovars isolated from humans and food animals. *FEMS Microb Rev.* 26:3-16.

Piddock L. J., Ricci V., McLaren I., Griggs D. J.. 1998. Role of mutation in the *gyrA* and *parC* genes of nalidixic-acid-resistant salmonella serotypes isolated from animals in the United Kingdom. *J. Antimicrob. Chemother.* 41:635-641.

Piva I. C., Pereira A. L., Ferraz L. R., Silva R. S., Vieira A. C., Blanco J. E., Blanco M., Blanco J., Giugliano L. G. 2003. Virulence markers of enteroaggregative *Escherichia coli* isolated from children and adults with diarrhoea in Brasilia, Brazil. *J. Clin. Microbiol.* 41:1827-1832.

Porter CK., Riddle MS., Tribble DR. 2010. The epidemiology of travelers' diarrhoea in Incirlik, Turkey: a region with a predominance of heat-stabile toxin producing enterotoxigenic *Escherichia coli*. *Diagn Microbiol Infect Dis.* 66:241-247.

Prere MF., Bacrie SC., Baron O., Fayet O. 2006: Bacterial aetiology of diarrhoea in young children: high prevalence of enteropathogenic *Escherichia coli* (EPEC) not belonging to the classical EPEC serogroups. *Pathol Biol.* 54:600-602.

Puiprom O. C., Gangnonngiw S. 2009. Identification of colonization factors of enterotoxigenic *Escherichia coli* with PCR-based technique. *Epidemiol Infect.* 138: 519-24.

Qadri F., Svennerholm A. M., Faruque A. S., Sack R. B. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin. Microbiol.* 18:465-483.

Qadri F., Saha A., Ahmed T., Al Tarique A., Begum Y. A., Svennerholm A. M. 2007. Disease burden due to enterotoxigenic *Escherichia coli* in the first 2 years of life in an urban community in Bangladesh. *Infect. Immun.* 75: 3961-3968.

Qadri F., Ahmed F., Ahmed T., Svennerholm A. M. 2006. Homologous and cross-reactive immune responses to enterotoxigenic *Escherichia coli* colonization factors in Bangladeshi children. *Infect. Immun.* 74: 4512-4518.

Qadri F., Das S. K., Faruque A. S., Fuchs G. J., Albert M. J., Sack R. B., Svennerholm A. M. 2004. Enterotoxigenic *Escherichia coli* and *Vibrio cholerae* diarrhoea in Bangladesh. *Emerg Infect Dis.* 11: 1266-1270.

- Qadri F., Das S. K., Faruque A. S., Fuchs G. J., Albert M. J., Sack R. B., Svennerholm A. M. 2000. Prevalence of toxin types and colonization factors in enterotoxigenic *Escherichia coli* isolated during a 2-year period from diarrhoeal patients in Bangladesh. *J. Clin. Microbiol.* 38:27-31.
- Queenan AM., Bush K. 2007. Carbapenemases: the versatile beta-lactamases. *Clin Microbiol.* 20:440-58.
- Raffaelli R. M. 2007. Child care-associated outbreak of *Escherichia coli* O157:H7 and hemolytic uremic syndrome. *Pediatr. Infect. Dis. J.* 26:951–953.
- Raghunath D. 2008. Emerging antibiotic resistance in bacteria with special reference to India. *J. Biosci.* 33: 593–603.
- Rao M. R., Abu-Elyazeed R., Savarino S. J., Naficy A. B., Wierzba T. F., Abdel-Messih I., Shaheen H., Frenck R. W., Svennerholm A. M., Clemens J. D. 2003. High disease burden of diarrhoea due to enterotoxigenic *Escherichia coli* among rural Egyptian infants and young children. *J. Clin. Microbiol.* 41: 4862-4864.
- Rao M. R., Wierzba T. F., Savarino S. J., Abu-Elyazeed R., El-Ghoreb N., Hall E. R., Naficy A., Abdel-Messih I., Frenck R. W., Svennerholm A. M., Clemens J. D. 2005. Serologic correlates of protection against enterotoxigenic *Escherichia coli* diarrhoea. *J. Infect. Dis.* 19: 1562-1570.
- Rappelli P., Folgosa E., Solinas M., Dacosta J., Pisanu C., Sidat M., Melo J., Cappuccinelli P., Colombo MM. 2005. Pathogenic enteric *Escherichia coli* in children with and without diarrhoea in Maputo, Mozambique. *FEMS Immunol Med Microbiol* 43: 67–72.
- Richard I., Walker' ., Teresa A. 2007. Ad Hoc ETEC Technical Expert Committee.. Analysis of strategies to successfully vaccinate infants in developing countries against enterotoxigenic *E. coli* (ETEC) disease. *Vaccine.* 14: 2545–2566.
- Rivera F. P., Ochoa T. J., Maves R. C., Bernal M., Edina A. M., Meza F. R., Mercado E., Gil A. I., Hall E. R., Huicho L., Lanata C. F. 2010. Genotypic and phenotypic characterization of enterotoxigenic *Escherichia coli* strains isolated from Peruvian children. *J Clin Microbiol*; 50:1-8.
- Robins-Browne R. M., Bordun A. M., Tauschek M., Bennett-Wood V. R., Russell J., Oppedisano F., Lister N. A., Bettelheim K. A., Fairley C. K Sinclair., M. I., Hellard M. E. 2004. *Escherichia coli* and community acquired gastroenteritis, Melbourne, Australia. *Emerg Infect Dis.* 10:1797-1805.
- Rodas C., Klena JD., Nicklasson M., Iniguez V., Sjöling A. 2009. Clonal relatedness of enterotoxigenic *Escherichia coli* (ETEC) strains expressing LT and CS17 isolated from children with diarrhoea in La Paz, Bolivia. *PLoS One.* 6: 11-19.

- Rodas C., Mamani R., Blanco J., Blanco JE., Wiklund G. 2010. Enterotoxins, colonization factors, serotype and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhoea in Bolivia. *Braz J Infect Dis* 15: 132–7.
- Rodas C., Mmani R., Blanco J. 2011. Enterotoxins, Colonization factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* 9ETEC0 strains isolated from hospitalized children with diarrhoea in Bolivia. *Braz J Infect Dis*. 15: 132-7.
- Rodas C., Volga I., Qadri F., Gudrun W., Svennerholm A., Åsa S. 2009. Development of Multiplex PCR assays for detection of enterotoxigenic *Escherichia coli* colonization factors and toxins. *J Clin Microbiol*. 47: 1218–1220.
- Rodas C., Hermos Marcie J., Linda L., Alexander J. 2011. Shiga Toxin-Producing *Escherichia coli* in Children: Diagnosis and clinical manifestations of O157:H7 and Non-O157:H7 infection. *J Clin microbial*.54: 955–959
- Rodrigo T., Waldir P., Monica A., Gomes A. 2009. An overview of atypical enteropathogenic *Escherichia coli*. *FEMS Microbiol Lett*. 297:137–149. 10: 1574-6968.
- Roy K., Scott B., Firadausi Q. 2010. Enterotoxigenic *Escherichia coli* elicits immune responses to multiple surface proteins. *Infect. Immun*. 78: 3027-3035.
- Ruiting L., Chehani M.A., Kathy D., Marina B., Martinez P. R. 2004. Molecular evolutionary relationships of enteroinvasive *Escherichia coli* and *Shigella* spp. *Infect. Immun*. 72:5080-5088.
- Sabrina M., Samwel Y., Mecky I., Nina L., Haima M. 2007. Identification of diarrhoeagenic *Escherichia coli* isolated from infants and children in Dar es Salaam, Tanzania. *J Microbiol*. 354: 123-130.
- Sack R. B., Svennerholm A. 2000. Safety and immunogenicity of an oral, inactivated enterotoxigenic *Escherichia coli* plus cholera toxin B subunit vaccine in Bangladeshi adults and children. *Vaccine* 18:2704–2712.
- Saidi H., Abdulsudi A I. 2010. Persistent child malnutrition in Tanzania: Risks associated with traditional complementary foods (A review). *Afri Jf Food Sci* 4: 679 – 692.
- Sánchez J., Holmgren J. 2005. Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhoea. *Curr Opin Immunol*. 17:388-98.
- Sang WK., Oundo JO., Mwituria JK., Waiyaki PG., Yoh M., Iida T., Honda T. 1997. Multidrug-resistant enteroaggregative *Escherichia coli* associated with persistent diarrhoea in Kenyan children. *Emerg Infect Dis*. 3: 373-4.

- Sang WK., Saidi SM., Yamamoto H., Ezaki T., Iida T., Yoh M., Honda T. 1996. Hemorrhagic colitis due to *Escherichia coli* 0157:H7 in Kenya. *J Trop Paed* 42:238-243
- Scaletsky I. C., Fabbicotti S. H., Silva S. O., Morais M. B., Fagundes-Neto U. 2002. HEp-2-adherent *Escherichia coli* strains associated with acute infantile diarrhoea, Sao Paulo, Brazil. *Emerg. Infect. Dis.* 8:855-858.
- Scavia G., Staffolani M., Fisichella S.. 2008. Enteroaggregative *Escherichia coli* associated with a foodborne outbreak of gastroenteritis. *J Med Microbiol*, 57: 1141–1146.
- Schaible U.E, Kaufmann S.H.E. 2007. Malnutrition and infection: Complex mechanisms and global impacts. *PLoS Med.* 4: 115-120.
- Schmidt H., Knop C., Franke S., Aleksic S., Heeseman J., Karch H. 1995. Development of PCR for screening of enteroaggregative *Escherichia coli*. *J. Clin. Microbiol.* 33:701-705.
- Serichantalergs O., Nirdnoy W., Cravioto A., LeBron C., Wolf M., Svennerholm A. M., Shlim D., Hoge C. W., Echeverria P. 1997. Coli surface antigens associated with enterotoxigenic *Escherichia coli* strains isolated from persons with traveler's diarrhoea in Asia. *J. Clin. Microbiol.* 35:1639-1641.
- Shabir A. M., Nigel A. C., Duncan S., Desirée W., Mari K., Cheryl L., Bagrey., John C., Paul H. G., Brigitte B. C., Htay H., Kathleen M. N. 2010. Effect of human Rotavirus vaccine on severe diarrhoea in African infants. *Engl J Med*; 362:289-298.
- Shah N., DuPont HL., Ramsey DJ. 2009. Global etiology of travelers' diarrhoea: systematic review from 1973 to the present. *Am J Trop Med Hyg.* 80:609–614.
- Shaheen H. I., Abdel Messih I A, Klena J.D, Mansour A. .2009. Phenotypic and Genotypic analysis of enterotoxigenic *Escherichia coli* in samples obtained from Egyptian children presenting to referral Hospitals. *J Clin Microbiol.* 52: 189-197.
- Shaheen H. I., Khalil S. B., Rao M. R., Abu Elyazeed R., Wierzba T. F., Peruski L. F., Putnam S., Navarro A., Morsy B. Z., Cravioto A., Clemens J. D., Svennerholm A. M., Savarino S. J. 2004. Phenotypic profiles of enterotoxigenic *Escherichia coli* associated with early childhood diarrhoea in rural Egypt. *J. Clin. Microbiol.* 42:5588-5595.
- Sheikh A., Shamsuzzaman S., Ahmad SM., Nasrin D., Nahar S., Alam MM., Al Tarique A., Begum YA., Qadri SS., Chowdhury MI., Saha A., Larson CP., Qadri F. 2010. Zinc influences innate immune responses in children with enterotoxigenic *Escherichia coli*-induced diarrhoea. *J Nutr.* 140:1049-56.

- Sherlock O., Vejborg R. M., Klemm P. 2005. The TibA adhesin/invasin from enterotoxigenic *Escherichia coli* is self recognizing and induces bacterial aggregation and biofilm formation. *Infect. Immun.* 73:1954-1963.
- Sizemore D. R., Roland K. L., Ryan U. S. 2004. Enterotoxigenic *Escherichia coli* virulence factors and vaccine approaches. *Vaccines* 3:585-595.
- Sjöling Å., Wiklund G., Savarino S., Cohen D., Svennerholm AM. 2007. Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* (ETEC) toxins and colonisation factors. *J Clin Microbiol.* 45:3295–3301.
- Smith M G., Jordan D., Chapman T A, Chin J J C. 2010. Antimicrobial resistance and virulent gene profiles in multi drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post weaning diarrhoea. *Veterinary Microbiol.*20:1313-1317
- Spano LC., Sadovsky AD., Segui PN., Saick KW., Kitagawa SM., Pereira FE., Fagundes-Neto U., Scaletsky IC. 2008. Age-specific prevalence of diffusely adherent *Escherichia coli* in Brazilian children with acute diarrhoea. *J Med Microbiol.* 57:359–363.
- Stacy-Phipps S J., Mecca J., Weiss J B. 1995. Multiplex PCR assay and simple preparation method for stool specimens detect enterotoxigenic *Escherichia coli* DNA during the course of infection. *J. Clin. Microbiol.* 33:1054-1059.
- Steffen R., Nadia T., Sue-Ann C., Santanu C., Ana-Maria C., Françoise C., Norbert D., Herbert L., Frank V. 2004. Epidemiology of travelers': Details of a global Survey. *J Travel Med.* 11: 231-238.
- Steffen R., Sack DA., Riopel L., Jiang ZD., Stürchler M., Ericsson CD., Lowe B., Waiyaki P., White M., DuPont HL. 2003. Therapy of travelers' diarrhoea with rifaximin on various continents. *Am J Gastroenterol.* 98:1073-8
- Steffen R., David A. , Lise R., Zhi-Dong J., Matius S., Charles D. , Brett L., Peter W., Mike W., Herbert L D: 2003. Therapy of travelers' diarrhoea with rifaximin on various continents. *Jf Gastroenterol.* 67: 342-347.
- Steffen R. 2005. Epidemiology of Travelers' diarrhoea. *Clinical infect Dis.* 112: 23-28.
- Steinsland H., Lacher DW., Sommerfelt H., Whittam TS. 2010. Ancestral lineages of human enterotoxigenic. *Escherichia coli.* *J Clin Microbiol* 48: 2916–2924.
- Steinsland H., Valentiner-Branth P., Gjessing H.K., Aaby P., Molbak K H.2003. Sommerfelt Protection from natural infections with enterotoxigenic *Escherichia coli*: longitudinal study. *Lancet.* 362: 286–291.

- Steinsland H., Valentiner-Branth P., Aaby P., Molbak K., Sommerfelt H. 2004. Development and evaluation of genotypic assays for the detection and characterization of enterotoxigenic *Escherichia coli*. *Diag Microbio Infect Dis*. 45: 97-105.
- Strahilevitz J., Jacoby GA., Hooper DC., Robicsek A. 2009. Plasmid-10. mediated quinolone resistance: a multifaceted threat. *Clin Microbiol Rev*. 22: 664-89.
- Subekti, D. S., Lesmana M., Tjaniadi P., Machpud N., Sukarma T, Daniel J. C., Alexander W., Campbell J. R., Corwin A. L., Beecham III H. J., Simanjuntak C., Oyoyo B. A. 2003. Prevalence of enterotoxigenic *Escherichia coli* (ETEC) in hospitalized acute diarrhoea patients in Denpasar, Bali, Indonesia. *Diagn. Microbiol. Infect. Dis*. 47:399-405.
- Sullivan C. B., Diggle M. A., Clarke S. C. 2005. Multilocus sequence typing: data analysis in clinical microbiology and public health. *Mol. Biotechnol*. 29: 245-254.
- Surjawidjaja H. A., Lesmana M. 2004. Growth inhibition of enteric pathogens by zinc sulphate: An in vitro study. *Med Princ Pract*.13:286–289.
- Svennerholm A. 2011. From cholera to enterotoxigenic *Escherichia coli* (ETEC) vaccine development. *Indian J Med Res* 133: 1890-1898.
- Svennerholm AM., Tobias J. 2008. Vaccines against enterotoxigenic *Escherichia coli*. *Expert Rev Vaccin*.7: 795–804.
- Svennerholm A. M., Steele D. 2004. Progress in enteric vaccine development. Best Practice. *Res. Clin. Gastroentrol*. 18:421- 445.
- Talukder K. A., Dutta D K, Safa A., Ansaruzzaman M., Hassan F., Alam K., Islam K. M., Carlin N. I. A., Nair G. B., Sack D. A. 2001. Altering trends in the dominance of *Shigella flexneri* serotypes and emergence of serologically atypical *S. flexneri* strains in Dhaka, Bangladesh. *J. Clin. Microbiol*. 39:3757-3759.
- Tamatsukuri S., Yamato K., Sibata S., Leano F., Honda T. 1992. Detection of a heat labile enterotoxin gene in ETEC by densitometric evaluation using specific probes. *Eur. J. Clin. Microbiol. Infect. Dis*. 10: 1048-1053.
- Tarantola A., Vaucel J., Laviolle C., Quilici M., Thiolet J., Fournier J. A. 2008. cluster of *Vibrio cholerae* O1 infections in French travelers to Rajasthan (India). *J Travel Med*. 15:273-7.
- Tarkka E., Åhman H., Siitonen A. 1994. Ribotyping as an epidemiologic tool for *Escherichia coli*. *Epidemiol. Infect*. 112:263-274.
- Tenover F C., Arbeit R D, Goering R V. 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for

healthcare epidemiologists. Molecular Typing Working Group of the Society for Healthcare Epidemiology of America. *Infect. Control Hosp. Epidemiol.* 18:426-39.

Thomazini CM., Samegima DA., Rodrigues MA., Victoria CR., Rodrigues J. 2011. High prevalence of aggregative adherent *Escherichia coli* strains in the mucosa-associated microbiota of patients with inflammatory bowel diseases. *Int J Med Microbiol.* 301:475-9.

Thomson K. S., Sanders C. C. 1992. Detection of extended-spectrum β -lactamases in members of the family Enterobacteriaceae – comparison of the double-disk and 3-dimensional tests. *Antimicrob Agents Chemother.* 36: 1877–1882.

Thornton C., Selvin P. 2004. Inhibition of PCR amplification by phytic acid, and treatment of bovine fecal specimens with phytase to reduce inhibition. *J. Microbiol Methods.* 59: 43-52.

Tobias J., Holmgren J., Hellman M., Nygren E., Lebens M., Svennerholm AM. 2010. Over-expression of major colonization factors of enterotoxigenic *Escherichia coli*, alone or together, on non-toxigenic *E. coli* bacteria. *Vaccine.* 28:6977–84.

Torrell JM., Aumatell CM., Ramos SM., Mestre LG., Salas CM. 2009. Reduction of travellers' diarrhoea by WC/rBS oral cholera vaccine in young, high-risk travellers. *Vaccine.* 27: 4074-7.

Torres A. G., Zhou X., Kaper J. B. 2005. Adherence of diarrhoeagenic *Escherichia coli* strains to epithelial cells. *Infect Immun.* 73: 18–29.

Torres, M. E. M. C., Pérez, F., Schelotto, F. 2001. Etiology of children's diarrhoea in Montevideo, Uruguay: associated pathogens and unusual isolates. *J Clin Microbiol* 39: 2134–2139.

Trabulsi L. R., Keller R., Tardelli Gomes T. A. 2002. Typical and atypical enteropathogenic *Escherichia coli*. *Emerg Infect Dis.* 8:508-513.

Tran JH., Jacoby GA. 2002. Mechanism of plasmid-mediated quinolone resistance. *Proc Natl Acad Sci.* 99:5638-42.

Turner AK., Stephens JC., Beavis JC., Greenwood J., Gewert C., Randall R., Freeman D., Darsley MJ. 2011. Generation and characterization of a live attenuated enterotoxigenic *Escherichia coli* combination vaccine expressing six colonization factors and heat-labile toxin subunit B. *Clin Vaccine Immunol.* 2011. 18 : 2128-35.

Turner KM., Feil EJ. 2007. The secret life of the multilocus sequence type. *Int J Antimicrob Agents;* 29: 129–135.

Turner A. K., Terry T. D., Sack D. A., Londono-Arcila P., Darsley M. J. 2001. Construction and characterization of genetically defined aro omp mutants of enterotoxigenic *Escherichia coli* and preliminary studies of safety and immunogenicity in humans. *Infect. Immun.* 69:4969-4979.

- Usein C.R., Tatu-Chitoiu D., Ciontea S., Condei M., Damian M. 2009. *Escherichia coli* pathotypes associated with diarrhoea in Romanian children younger than 5 years of age. *Jpn J Infect Dis.* 62: 289-293.
- Valentiner B. 2003. Cohort study of Guinean Children: Incidence, pathogenicity, conferred protection, and attributable risk for enteropathogens during the first two years of life. *J Clin Microbiol.* 41: 4238-4245.
- Vargas M., Gascon J., Jimenez MT., Vila J. 1999. Prevalence of *Shigella* enterotoxins 1 and 2 among *Shigella* strains isolated from patients with traveler's diarrhoea. *J Med Micro.* 320: 23-31.
- Vaux S., Carbonne A., Thiolet J M., Jarlier V., Coignard B. 2011. Emergence of carbapenemase-producing Enterobacteriaceae in France, 2004 to 2011. *Eurosur.* 16: 22-28.
- Vera E., Rodríguez- María R., Carlos H. S. 2004. Phenotypic confirmation of extended-spectrum B-lactamases (ESBL) in clinical isolates of *Eschreichia coli* and *Klebsiella pneumoniae* at the San Juan Veterans Affairs Medical Center. *Puerto Rico Health Sci J.* 23: 207-215.
- Vernacchio L., Vezina R. M., Mitchell A. A., Lesko S. M., Plaut A. G. Acheson D. W. 2006. Diarrhoea in American infants and young children in the community setting: incidence, clinical presentation and microbiology. *J Pediatr Infect Dis.* 25: 2-7.
- Vesikari T., Karvonen A., Prymula R. 2007. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study. *Lancet.* 370:1757-1763
- Viboud G. I., Jouve M. J., Binsztein N., Vergara M.M., Rivas M., Quiroga S., Svennerholm A. M. 1999. Prospective cohort study of enterotoxigenic *Escherichia coli* infections in Argentinean children. *J. Clin. Microbiol.* 37 : 2829-2833.
- Vidal RM., Valenzuela P., Bakerk. 2009. Characterization of most prevalent colonization factor antigens present in Chilean clinical enterotoxigenic *Escherichia coli* strains using new multiplex chain reaction. *Dign. Microbiol Infect Dis.* 65: 217-23.
- Vieira M. A. M., Andrade J. R. C., Trabulsi L. R., Rosa A. C. P., Dias A. M. G., Ramos S. R. T. S., Frankel G, Gomes T. A. T. 2001. Phenotypic and genotypic characteristics of *Escherichia coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry *eae* and lack the EPEC adherence factor and Shiga toxin DNA probe sequences. *J Infect Dis* 183, 762-772.
- Vila J., Vargas M., Henderson I. R., Gascon J., Nataro J. P. 2000. Enteroaggregative *Escherichia coli* virulence factors in traveler's diarrhoea strains. *J. Infect. Dis.* 182:1780-1783.

- Vilchez S., Daniel R., Margarita P., Filemon B., Roland M., Andrej W. 2009. Prevalence of diarrhoeagenic *Escherichia coli* in children from León, Nicaragua. *J Med Microbiol*. 58: 630-637
- Viljanen MK., Peltola T., Junnila SY., Olkkonen L., Jarvinen H., Kuistila M., *et al.*, 1990. Outbreak of diarrhoea due to *Escherichia coli* O111:B4 in schoolchildren and adults: association of Vi antigen-like reactivity. *Lancet*. 336:831-4.
- Vinué L., Martínez S., del Pozo J.P., Sáenz Y., Torres C., Zarazaga-Logroño M., ES. 2008. High prevalence and diversity of extended-spectrum beta-lactamases in faecal *Escherichia coli* isolates of healthy humans. *ECCMID*: 22-24.
- von Sonnenburg F., Tornieporth N., Waiyaki P. 1981. Risk and aetiology of diarrhoea at various tourist destinations. *Lancet*; 356:133-134.
- von Wright A., Bridges B. 1981. Effect of gyrB-mediated changes in chromosome structure on killing of *Escherichia coli* by ultraviolet light: experiments with strains differing in deoxyribonucleic acid repair capacity. *J. Bacteriol*. 146:18-23.
- Vu Nguyen T., Le v., Le Huy. 2006. Etiology and epidemiology of diarrhoea in children in Hanoi, Vietnam. *Int J Infect Dis* 10: 298-308.
- Vu Nguyen, T., Van P., Huy, C. L., Gia K., Weintraub N A. 2005. Detection and characterization of diarrhoeagenic *Escherichia coli* from young children in Hanoi, Vietnam. *J Clin Microbiol* 43, 755-760.
- Waiyaki P., Ngugi J., Sang W., Ichoroh G. 1985. Isolation of enteropathogenic *Escherichia coli* from community water sources in Nyanza Province, Kenya. *KEMRI/ KETRI Ann Sci Proc*. pp 56-61.
- Wajima T., Sabui S., Fukumoto M., Kano S., Ramamurthy T., Chatterjee NS., Hamabata T. 2011. Enterotoxigenic *Escherichia coli* CS6 gene products and their roles in CS6 structural protein assembly and cellular adherence. *Microb Pathog*. 51:243-9.
- Walker N., Fischer Walker CL., Bryce J. 2010. Standards for CHERG reviews of intervention effects on child survival. *Int J Epidemiol*; 39:21-31.
- Weinke T., Liebold I., Burchard GD., Frühwein N., Grobusch MP., Hatz C., 2008. Prophylactic immunisation against traveller's diarrhoea caused by enterotoxin-forming strains of *Escherichia coli* and against cholera: Does it make sense and for whom? *Travel Med Infect Dis*. 6:362-7.
- Weintraub A. 2007. Enteroaggregative *Escherichia coli*: epidemiology, virulence and detection. *J. Med. Microbiol*. 54: 4-8.

Weiping Z., Emil M., Berberov J., Jessica F. 2006. Significance of heat stable heat labile enterotoxins in porcine colibacillosis in an additive model for pathogenicity studies. *Infect Immun.* 54: 3107-3114.

Wenneras C., V. Erling. 2004. Prevalence of enterotoxigenic *Escherichia coli*-associated diarrhoea and carrier state in the developing world. *J. Health Popul. Nutr.* 22:370-382.

WHO, 2011. EHEC Outbreak in Germany May 2011. WHO. Vol. C/123-6

WHO, 2012. Enterohaemorrhagic *Escherichia coli* (EHEC). WHO media centre 2012.

WHO, UNICEF, World Bank. State of the world's vaccines and immunization, 3rd ed. Geneva: World Health Organization; 2009. p. 1-208. Available from: http://whqlibdoc.who.int/publications/2009/9789241563864_eng.pdf

WHO. 1990. Programme for the Control of Diarrhoeal Diseases. A manual for the treatment of diarrhoea—for use by physicians and other senior health workers. WHO, Geneva, Switzerland.

WHO. 1993. Global Health Situation III. *WER* 6:33.

WHO. 1999. Generic protocol to estimate the burden of Shigella diarrhoea and dysenteric mortality. *B*: 99:26.

WHO. Cholera vaccines. *Wkly Epidemiol Rec* 2001; 76:117-25.

WHO.2006. Future directions for research on enterotoxigenic *Escherichia coli* vaccines for developing countries. *Weekly Epidemiological Record.* 81: 97-104.

Wilson G., Easow J M., Mukhopadhyay C., Shivananda P G. 2006. Isolation and antimicrobial susceptibility of *Shigella* from patients with acute gastroenteritis in Western Nepal; *Indian J. Med. Res.* 123 145–150.

Wise T., Subarna R., Debduitta B., Thanasekaran K., Ghosh R., Sathya P., Bharadwaj A., Singhanian A., Sugunan P., Vijayachari C. 2007 Emergence of fluoroquinolone resistance in *Shigella* isolated from Andaman and Nicobar Islands, India. *Indian J Med Research.* 2007:12-17.

Wolk M., Ohad E., Shafran R., Schmid I., Jarjoui E. 1997. Enterotoxigenic *Escherichia coli* (ETEC) in hospitalised Arab infants from Judea area—West Bank, Israel. *Public Health* 11:111-117.

Yates J. 2005. Travelers' diarrhoea. *Am Fam Physician:* 71: 5-11

Yatsuyanagi J., Saito S., Miyajima Y., Amano K., Enomoto K. 2003. Characterization of atypical enteropathogenic *Escherichia coli* strains harboring the

astA gene that were associated with a waterborne outbreak of diarrhoea in Japan. *J Clin Microbiol.* 41:2033–9.

Yavzori M., Porath N., Ochana O., Dagan R, Orni-Wasserlauf R, Cohen D. 1998. Detection of enterotoxigenic *Escherichia coli* in stool specimens by polymerase chain reaction. *Diagn. Microbiol. Infect. Dis.* 31:503-509.

Yoder J., Shawn C., Victor P., Xinfang M., Kate K., Mark S. 2006. Outbreak of enterotoxigenic *Escherichia coli* infection with an unusually long duration of illness. *Oxford J Clin Infect Dis.* 42: 1513-1517.

Appendix 1: Informed consent agreement

TITLE OF STUDY: MOLECULAR EPIDEMIOLOGY OF ENTEROTOXIGENIC *Escherichia coli* AND OTHER ENTERIC PATHOGENIC BACTERIA IN MACHAKOS DISTRICT HOSPITAL IN MACHAKOS COUNTY, KENYA.

INSTITUTIONS: Kenya Medical Research Institute, Nairobi, Kenya; United States Army Medical Research Unit – Kenya, Nairobi, Kenya.

INSTITUTIONS: Kenya Medical Research Institute, Nairobi, Kenya; United States Army Medical Research Unit – Kenya, Nairobi, Kenya.

PRINCIPAL INVESTIGATORS: Bonventure Juma, MSc; Dr. Samuel Kariuki PhD, Dr. Wallace Bulimo, PhD, Dr. Peter Waiyaki, PhD, and Prof. Marion Mutugi, PhD.

INCLUSION CRITERIA: All outpatient from three months old and above complaining of diarrhoeal illness and have given consent to participate in the study.

EXCLUSION CRITERIA: Patients unwilling to give stool or consent for participation in the study and those who are less than three months old.

INFORMED CONSENT

You/Your child is being asked to participate in a research study conducted by the investigators listed above. You/Your child's participation is entirely voluntary. If you do not want to participate, there will be no penalty. You will not lose any benefits to which you/your child is entitled. You may stop your child's participation at any time. If you choose for you/your child to leave the study, you should return to the study doctor and inform him or her.

You should read the information below and ask questions about anything you do not understand before deciding whether or not to participate. You will be given a copy of the consent form to keep.

PURPOSE OF THE STUDY

We are interested in finding out more about the germs that cause diarrhoea in Machakos County, Kenya. We will carry out laboratory tests to determine the effective drugs to treat diarrhoea, the toxins present and the genetic relatedness.

PROCEDURES

If you volunteer to participate in this study, we would ask you to do the following things:

STUDY TESTS

During this study, you/your child will be asked to provide stool in a cup.

QUESTIONNAIRE

If you allow you/your child to participate, we will ask you some questions. These include questions about your child's age, your job, where you live, how many times your child had diarrhoea before coming to the hospital, recent medications your child has received, source of water, other illness your child had with the diarrhoea such as headache or vomiting. You do not have to answer any question that makes you uncomfortable.

STATEMENT OF CONSENT FOR SAMPLE STORAGE AND FUTURE TESTING

Once testing is complete, you/your child's stool isolates will be stored indefinitely at the Kenya Medical Research Institute. We will only use a code to identify the stored isolates, not your child's name. We would like to use your child's isolate in future research studies on germs that cause diarrhoea. You or your child will not receive payment for any future value the isolate may be found to have. You or your child will not receive any notice of future uses of the isolates. Your child does not have to donate his/her sample for future research. Any future research involving your child's isolate will be reviewed by an ethical review committee to ensure the use is confidential, ethical and meets all government guidelines. At times, we will need to ship isolates obtained from your child's isolate to America or Egypt for further analysis.

I agree to have my/child's isolates stored for future use.

_____ I agree

_____ I do not agree

Subject Name _____

Subject Signature/Fingerprint _____ Date _____

Witness Name _____

Witness Signature _____

POTENTIAL RISKS AND DISCOMFORTS

There is no risk from providing stool. Participation in this study will not delay medical care your child would normally receive.

BENEFITS

The benefit of taking part in the study is that you/your child will have tests done, free of charge, to determine the cause of your child's diarrhoea and to determine the best way to treat it. In addition, a better understanding of diarrhoeal illnesses may eventually lead to better treatments and preventive measures.

COMPENSATION

There is no direct compensation to volunteers for their participation.

CONFIDENTIALITY

All information collected about you/your child will remain confidential. You/Your child's name will not appear on any publication or report from this or other studies. You/Your child's name will not be kept in the computerized research database. All samples will be labeled only with a unique study number, not you/your child's name. Representatives of the Walter Reed Army Institute of Research are eligible to review research records as part of their responsibility to protect human subjects in research. Questionnaires and consent forms will be kept in a locked file at the US Army Medical Research Unit-Kenya storage facility for 10 years following completion of the study. Laboratory data sheets will be made available only to Principal Investigators, clinical personnel who require this information to treat your child, or to members of the Ministry of Health who require this information for legal reasons or to study an outbreak.

PARTICIPATION

You/Your child's participation in this study is entirely voluntary. You are free to withdraw from this study at any time.

MEDICAL CARE FOR RESEARCH RELATED INJURY

Should you/your child be injured as a direct result of participating in this research project, you will be provided medical care, at no cost for that injury. You or your child will not receive any injury compensation, only medical care. You should also understand that this is not a waiver or release of your child's legal rights. You should discuss this issue thoroughly with one of the principal investigators or his or her designees before you enroll in this study.

YOUR RIGHTS AS A RESEARCH VOLUNTEER

If you have any questions about the study, your rights as a research volunteer, or a research-related injury, you should contact Mr. Bonventure Juma at Box 606 Village Market, Nairobi, Kenya, tel 020-2713689, or the doctor on duty at the clinic. The Chairman of the Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 20-272251.

WRITTEN CONSENT

I, _____(Name) having attained my ____ birthday and full capacity to consent for my child named: _____(Subject's name), do hereby consent to his/her participation in the research study:

“Molecular Epidemiology of Enterotoxigenic Escherichia coli and other enteric pathogenic Bacteria in Machakos District, Machakos County, Kenya” under the direction of Mr. Bonventure Juma. The methods and means by which the study will be conducted and the risks which may be reasonably expected have been explained to me by _____. I have been given the opportunity to ask questions concerning this research study, and any such questions have been answered to my full and complete satisfaction.

I understand that I may at any time during the course of this study cancel this consent agreement and withdraw myself/ child from the study without prejudice.

SIGNATURE OF RESEARCH SUBJECT

Name of Subject: _____

Subject's/Fingerprint: _____ Date: _____

Guardian's Signature: _____ Date: _____

Permanent Address: _____

SIGNATURE OF WITNESS

My signature as witness certifies that the subject signed this consent form in my presence as his or her voluntary act.

Witness's Name: _____

Witness's Signature: _____ Date: _____

Study Number: _____

UCHUNGUZI HUU NI JUU YA: Elimu kuhusu magonjwa ya kuhara katika Hospitali ya Machakos.

TAASISI: Kenya Medical Research Institute, Nairobi, Kenya, United States Army Medical Research Unit Kenya, Nairobi, Kenya.

ENEO: Machakos District Hospital.

WACHUNGUZI WAKUU: Mr. Bonventure Juma, MSc, Dr. Samuel Kariuki, PhD, Dr. Peter Waiyaki, PhD, Dr. Wallace Bulimo and Prof. Marion Mutugi, PhD.

KANUNI KWA WANAOSHIRIKI: Wagonjwa wote ambao wametimiza miezi mitatu na zaidi wanaouguu ugonjwa wa kuhara na wamesharuhusiwa na wazazi au walezi wao kushiriki kwenye uchunguzi.

KANUNI KWA WASIOSHIRIKI: Wagonjwa wanaoona ugumu wa kutoa kinyesi au choo chao au kutoa ruhusa kwa wanaoshiriki kwenye uchunguzi au wale ambao hawajatimiza umri wa miezi mitatu.

MAELEZO KUHUSU RIDHAA AU RUHUSA: Wewe/Mtoto wako anaombwa kushiriki katika utafiti huu wa uchunguzi unaoongozwa na wachunguzi ambao majina yao yameorodheswa hapo juu.

Kushiki kwako/ mtoto wako kunatokana na kujitolea kwake. Iwapo hutaki kushiriki, hutakuwa na adhabu yeyote, hutapoteza faida yoyote ambayo wewe/mtoto wako anapaswa kupewa. Unaweza kumwachisha mtoto wako wakati wowote iwapo hataki

aendelee kushiriki. Ukiamua kumwachisha, unaombwa kurudi kwa daktari wa uchunguzi ili uweze kumwaarifu.

Unapaswa kusoma taarifa ifwatayo na uulize maswali yoyote kuhusu jambo lolote ambalo hujalifahamu kabisa kabla ya kuamua kushiriki au kutoshiriki. Utapewa fomu ili uweze kuweka.

KUZUDI LA UCHUNGUZI:

Tuna hamu sana ya kufanya uchunguzi kuhusu vijidudu vinavyosababisha ugojwa wa kuhara katika Machakos.

Pia tutafanya uchunguzi katika maabara kudhihiriha dawa zinazofaa zaidi kwa kutibu ugonjwa wa kuhara.

TARATIBU:

Iwapo umejitolea kushiriki katika uchunguzi huu, tungependa kuwaomba mfanye yafwatayo:

UCHUNGUZI:

Wakati wa uchunguzi, mtoto wako ataombwa kuleta kinyesi au choo. Mtoto wako atahitaji kutia choo kwenye kikombe atakachopewa.

MASWALI:

Ukiruhusu mtoto wako kushiriki, tutakuuliza maswali yafwatay. Umri wa mtoto wako, kazi yako, mahali unapoishi, mtoto wako ameshahara mara ngapi kabla ya kumpeleka hospitalini na madawa amashapokea, chemichemi ya maji mnayotumia au mahali mnapotoa maji, magojwa mengine ambayo yanambatana na ugojwa wa kuhara kama kwa mfano kuumwa na kichwa au kutapika. Unaweza kuliacha swali lolote ambalo hupendelei kujibu.

MAELEZO KUHUSU RIDHIA KWA UHIFADHI WA SAMPULI KWA UCHUNGUZI WA SIKU ZA USONI:

Mara uchunguzi ukisha fanywa, sampuli ya choo cha mtoto wako itawekwa pahali pasipo thahiri au wazi katika Kenya Medical Research Institute (KEMRI). Tutaitumia tarkimu au nambari kuitambulisha sampuli ambayo imehifadhiwa. Si jina la mtoto wako tukependa kuitumia ya mtoto wako kwa uchunguzi kwa siku zijazo dhidi ya vijidudu vinavyo sababisha ugonjwa wa kuhara.

Wewe au mtoto wako hatapoea malipo yoyote kutokana na dhamana yoyote itakavyopatikana kwa choo cha mtoto wako kwa siku za baadaye. Wewe ahata mtoto wako hataokea notisi yoyote kuhusu matumizi ya sampuli ya mtoto wakokwa siku za baadaye.

Mtoto wako hatahitajika kutoa sampuli ya utafiti siku za baadaye. Utafiti wowote siku za baadaye ambao utashirikisha sampuli ya mtoto wako, utachunguzwa na kamati ya uchunguzi wa maadili kuhakkikisha kuwa matumizi ya kisiri, maadili yanayofaa mwongozo wa serikali.

Nimekubali kumruhusu mtoto wangu kutoa sampuli itakayihifadhiwa kwa matumizi ya siku za usoni.

_____nakubali

_____sikubali

Jina _____ la
mshiriki _____ Tarehe _____

Mshiriki/alama ya kidole _____

Jina la shahidi _____

Muhuri (sahihi ya shahidi _____)

HATARI ZISIZIDHIHIRIKA NA PIA HALI ISIYO NA RAHA.

Hakuna hatari yoyote inayoweza kutokana na utoaji wa kinyesi au choo. Kushiriki kwenye uchunguzi huu hakutayachelewesha matumizi ya madawa kwa mtoto wako. Mtoto wako atatumia madawa jinsi inavyotakikana.

FAIDA.

Faida inayotokan na kushiki kwenye uchunguzi huu ni kwamba: Mtoto wako atachunguzwa bila malipo yoyote kudhihirisha chanzo cha ugonjwa wake. Na pia kutafuta njia bora saidi ya kuutibu ugonjwa huu. Saidi ya hayo, kuelewa kinaganaga kwa ugonjwa unaweza kutuongoza kwa utafiti unaofaa wa kuzuia ugonjwa huu.

FIDIA

Hakuna fidia yoyote wajitoleaji wataipokea baada ya kushiriki.

SIRI.

Habari zote ambazo zitakusanywa kuhusu mtoto wako zitakuwa za kiziri daima. Jina la mtoto halitaonyeswa kwenye tangazo au ripoti yoyote kutokana na uchunguzi huu au mwingine. Jina la mtoto wako halitawekwa kwenye kompyuta ya utafiti au data base. Sampuli zote zitabandikwa tu tarakimu au nambari siyo ya kawaida. Si jina la mtoto wako. Wasimamisi wa Walter Reed Army Institute of Research wanastahili kuchunguza rekodi za uchunguzi kwa ajili, hii ndiyo moja wako ya majukumu yao kutunza majina ya watu wanaoshiriki kwenye uchunguzi. Maswali na fomu za ruhusa zitawekwa katika faili ambazo zitafungiwa katika US. Army Medical Research Unit – Kenya. Fomu hizi zitahifadhiwa kwa miaka kumihadi wakati uchunguzi utakapokamilika.

Karatasi ya mambo yasiokubaliwa kuwa hakika ya maabara (lab data sheet) zitapatikana tu kwa wachunguzi wakuu., madaktari au wauuguzi wa kliniki ambao wahitaji habari hii kwa matibabu ya mtoto wako, au mwanachana wa wizara ya afya anahitaji habari hii kwa sababu halali au kuchunguza mlipuko.

KUSHIRIKI

Kushiriki kwa uchuzi huu unatokana na kujitolea kwako. Lazima mtu ajitolee kwa hiyari (dhati) . Uko huru kujiondoa kwenye uchunguzi huu wakati wowote.

UTUNZAJI WA TABIBU DHITI YA MADHARA YOYOTE KUTOKANA NA UTAFITI.

Mtoto wako akijeruhiwa wakati huu atapewa matibabu ya bure. Wewe au mtoto wako hatapokea fidia yoyote au malipo yoyote. Atapokea tu matibabu. Wewe unastahili kufahamu kuwa, utafiti huu si haki halali ya mtoto wako. Unapaswa kujadili jambo hili kwelikweli na mmoja wa wachunguzi au mshauri wake kabla ya kujiandikisha kwenye uchunguzi huu.

HAKI YAKO KAMA MJITOLEAJI WA UTAFITI.

Iwapo una swali lolote kuhusu uchunguzi huu, haki yako kama mjitoleaji wa utafiti au kuhusu madhara yoyote kutokana na utafiti, unapaswa kuwasiliana na Bw. Juma katika sanduku la post 606, Village Market, Nairobi, Kenya; nambari ya simu 020 2722541 au Daktari wa zamu katika kliniki. Mwenye kiti wa Kenya National Ethical Review Committee, c/o Kenya Medical Research Institute Sanduku la post; 54840, Kenya, nambari ya simu, 020 2722541.

MAANDIKO YA RIDHIA. (UTOAJI WA RUHUSA).

Mimi _____ (Jina) nimetiza umri wa miaka _____ nimetoa ruhusa kwa mtoto wangu _____ nimekubali kushiriki kwake katika utafiti wa uchunguzi Elimu kuhusu magonjwa ya kuhara katika nchi ya Kenya chini ya usimamizi wa Bw. Willie Sang, Daktari Shery Bedno, na Daktari Jane Mbui. Mbinu na njia ambazo uchunguzi huu utafanywa na hatari yoyote inayoweza kutokana, tayari nimehaeleswa na _____.

Nimeshapewa idhini ya kuuliza maswali kuhusu utafiti huu na tayari hayo maswali nimeniridhika na majibu yote. Nafahamu kwamba ninaweza kuondoka wakati wowote au kuondoa mtoto wangu bila chuki yoyote.

MUHURI (SAHIHI) YA MSHIRIKI WA UCHUNGUZI:

Jina _____ la _____ mshiriki _____
Tarehe: _____

Alama _____ ya _____ kidole _____ ya _____
mshiriki _____ Tarehe _____

Mhuri (sahihi) ya mlezi _____

Anwani _____ ya _____
kudumu _____

MUHURI YA SHAHIDI

Muhuri yangu kama shahidi inathibitisha ya kwamba mtu huyu alikubali kutia fomu hii sahihi mbele yangu kama kitendo cha kujitolea.

Jina la shahidi_____

Muhuri ya shahidi_____

Tarehe_____

Nambari ya uchunguzi_____

Appendix 2: Questionnaire

MOLECULAR EPIDEMIOLOGY OF ENTEROTOXIGENIC <i>Escherichia coli</i> IN MACHAKOS DISTRICT HOSPITAL IN EASTERN PROVINCE, KENYA			
PLEASE USE THIS FORM FOR ALL INTERVIEWS AND REQUESTS FOR DIARRHOEA INVESTIGATION. The following information is required to aid the diagnosis and epidemiology of Diarrhoea. Please complete ALL sections: SURNAME _____ FORE NAME (S) _____ SEX: <input type="checkbox"/> M <input type="checkbox"/> F. AGE _____ (yrs) DOB __ / __ / __ P.O.R _____ IF NOT HUMAN, PLEASE CHECK HERE: <input type="checkbox"/> FOOD <input type="checkbox"/> WATER.			
Clinical details <input type="checkbox"/> Headache <input type="checkbox"/> Myalgia <input type="checkbox"/> Lethargy <input type="checkbox"/> Malaise <input type="checkbox"/> Vomiting <input type="checkbox"/> Arthralgia <input type="checkbox"/> Diarrhoea <input type="checkbox"/> Stomach cramps. <input type="checkbox"/> No symptoms <input type="checkbox"/> Died <input type="checkbox"/> Medical screen <input type="checkbox"/> Other (specify) _____	Occupation <input type="checkbox"/> Farmer - arable <input type="checkbox"/> - livestock <input type="checkbox"/> Farm worker - arable <input type="checkbox"/> - livestock worker <input type="checkbox"/> Outdoor - manual <input type="checkbox"/> - with animals <input type="checkbox"/> Fish - farmer <input type="checkbox"/> - worker <input type="checkbox"/> - filleter <input type="checkbox"/> Abattoir - worker <input type="checkbox"/> - butcher <input type="checkbox"/> Indoor - manual <input type="checkbox"/> - office <input type="checkbox"/> - domestic <input type="checkbox"/> Water worker - sewage <input type="checkbox"/> - plumber <input type="checkbox"/> Veterinarian <input type="checkbox"/> Medical <input type="checkbox"/> Military <input type="checkbox"/> Teacher <input type="checkbox"/> Student <input type="checkbox"/> Housewife <input type="checkbox"/> Retired <input type="checkbox"/> Unemployed <input type="checkbox"/> Other (specify) _____	Water contact <input type="checkbox"/> Water sport <input type="checkbox"/> - swimming <input type="checkbox"/> - canoeing <input type="checkbox"/> Fishing <input type="checkbox"/> River <input type="checkbox"/> Lake <input type="checkbox"/> Pond <input type="checkbox"/> Ditch <input type="checkbox"/> Sewage <input type="checkbox"/> No known contact <input type="checkbox"/> Other (specify) _____ *How do you treat your drinking water? <input type="checkbox"/> Boiling <input type="checkbox"/> Chemicals <input type="checkbox"/> Filter <input type="checkbox"/> Other (specify) _____ * What is your water source? <input type="checkbox"/> River <input type="checkbox"/> Borehole <input type="checkbox"/> Tap water *What type of toilet do you use? <input type="checkbox"/> VIP <input type="checkbox"/> Pit latrine <input type="checkbox"/> Bush <input type="checkbox"/> River <input type="checkbox"/> Flash toilet	Animal contact <input type="checkbox"/> Farm livestock <input type="checkbox"/> - cattle <input type="checkbox"/> - sheep <input type="checkbox"/> Dogs <input type="checkbox"/> Rats <input type="checkbox"/> Mice <input type="checkbox"/> Other animals (specify in Additional info. box) _____ <input type="checkbox"/> No known contact Type of contact <input type="checkbox"/> Occupational <input type="checkbox"/> Recreational (specify in Additional info. box) _____ <input type="checkbox"/> Wound / abrasion <input type="checkbox"/> Immersion <input type="checkbox"/> Other (specify) _____
Recent travel to Diarrhoea endemic regions. <input type="checkbox"/> Yes <input type="checkbox"/> No If YES, please give details (when / where) in additional info. Box		Macroscopic examination of stool: <input type="checkbox"/> Watery <input type="checkbox"/> Mucoid <input type="checkbox"/> Bloody <input type="checkbox"/> Mucoid/bloody	
Date of onset of symptoms: _____ Date of antibiotic treatment: _____ Antibiotic treatment: _____ Specimen type: Date collected: Reference No. _____		Additional information:	
Requesting laboratories test: <input type="checkbox"/> PCR <input type="checkbox"/> Serology <input type="checkbox"/> wet mount <input type="checkbox"/> PFGE <input type="checkbox"/> Sequencing <input type="checkbox"/> Other (specify): _____ Results: _____ SITE: <input type="checkbox"/> Machakos Hospital Address: Contact Doctor / Clinical Officer: _____ Date: __ / __ / __		DRL use only Serology <input type="checkbox"/> POSITIVE <input type="checkbox"/> NEGATIVE Infecting serogroup _____ Completed <input type="checkbox"/> Yes <input type="checkbox"/> No Lab no: _____	

Appendix 3: Ethical Review committee approval



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: kemri-hq@nairobi.mimcom.net; director@kemri.org Website: www.kemri.org


KEMRI/RES/7/3/1

23rd November 2005

Mr. B. Juma,
CMR,
NAIROBI

Thro'

Director,
CMR,
NAIROBI

Forwarded. 
25/11/05

Dear Sir,

RE: SSC Protocol No. 989 (Revised) – Molecular epidemiology of diarrhoeagenic *Escherichia coli* in Machakos District Hospital in Eastern Province of Kenya, by B Juma et al (CMR)

Thank you for your letter dated 23rd November 2005.

The issues raised in the 127th Meeting of the KEMRI/National Ethical Review Committee have been addressed adequately and the protocol is therefore granted approval.

You may commence your study.

R. C. M. Kithinji
R. C. M. Kithinji,
For: SECRETARY,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE