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Pathogenic *Escherichia coli* strains and their antibiotic susceptibility profiles in cases of child diarrhea at Tikur Anbessa General Specialized Hospital, AAU College of Health Sciences, Addis Ababa, Ethiopia.

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES OF ADDISABABA UNIVERSITY IN PARTIAL FULFILMENT FOR THE REQUIREMENT OF MASTER OF SCIENCE IN APPLIED GENETICS.

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ABSTRACT

The prevalence and antibiogram of pathogenic *E. coli* strains which cause diarrhea vary from region to region, and even within countries in the same geographical area. In Ethiopia, diagnostic approaches to *E. coli* induced diarrhea in children less than five years of age are not standardized. The aim of this study was to determine the involvement of pathogenic *E. coli* strains in child diarrhea and determine the antibiograms of the isolates in children less than 5 years of age with diarrhea at Addis Ababa University College of Health Sciences Tikur Anbessa General Specialized Hospital, Addis Ababa, Ethiopia. Children (n=98) (47 males & 51 females), aged 01 month to 60 months suffering from diarrhea were included in this study and stool specimens were collected and processed from January 2017 to March 2018. Fecal specimens were inoculated on MacConkey and Eosin Methylene Blue agar to grow *E. coli*. *E. coli* isolates were biochemically confirmed using biochemical tests indole, methylred, Vogues proskuer and citrate. Antimicrobial susceptibility profiles were determined using Kirby Bauer disk diffusion method. Heating method was used to extract DNA from *E. coli* isolates. 58 *E. coli* isolates were detected out of which 25 pathotypes belonging to different classes of pathogenic strains: STEC, EPEC, EHEC, EAEC were detected by using PCR technique. Pathogenic *E. coli* exhibited high rates of antibiotic resistance to many of the antibiotics tested. Moreover, they exhibited multiple drug resistance. The involvement of antibiotic resistant pathogenic *E. coli* in diarrheic children was prominent and hence focus should be given on the diagnosis and antimicrobial sensitivity testing of pathogenic *E. coli* at AAU CHS Tikur Anbessa General Specialized Hospital. Among antibiotics tested, Cefotitan could be a drug of choice to treat *E. coli.*

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V. ACRONYMS

EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
STEC	Shigatoxin producing *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
HGT	Horizontal gene transfer
EMB	Eosin Methylene Blue
bfp	Bundle forming pilus
MMR	Methyl Directed Mismatch Repair
CHS	College of Health Sciences
CNS	College of Natural Sciences
SD	Standard deviation
AAU	Addis Ababa University
PCR	Polymerase Chain Reaction

1. INTRODUCTION

1.1. Background Information

E. coli is a gram negative, rod shaped, facultative anaerobe bacterium that belongs to the family Enterobacteriaceae. It is normally found in the intestines of humans and is involved in the maintenance of the balance of the intestinal microbiota. The *E. coli* genome has a single circular chromosome with doublestranded DNA. Some strains may also contain extra chromosomal genetic elements such as plasmids. *E. coli* can be easily genetically manipulated and readily grows in the laboratory. Therefore, *E. coli* has always been a common and suitable model organism for different genetic studies (Nataro and Kaper, 1998; Rasko *et al.,* 2008).

The reference *E. coli* genome (k – 12 strain) has 4288 protein coding genes. Genome plasticity through horizontal gene transfer is high in *E. coli* as indicated by the presence of insertion sequences, phage remnants and additional sequences of unusual composition in the genome (Blattner *et al*., 1997).

Although most *E. coli* are commensals, some strains can be pathogenic due to additional virulence genes found in chromosomes and/or plasmids. Virulence genes can be generated by mutation; they can also be acquired from mobile genetic elements through horizontal gene transfer. Clusters of virulence genes can be found on plasmids or integrated into the chromosome in pathogenic strains. These clusters of genes also called pathogenicity islands are usually flanked by mobile genetic elements such as bacteriophages, insertion sequences, etc and often insert near transfer RNA genes (Wantanabe, 1963; Croxen and Finlay, 2010; Chandra *et al*., 2013).

E. coli bacteria evolve from distinctive clonal groups and most strains of *E. coli* (85-90%) can be assigned by simple PCR technique into different phylogenetic groups based on the presence/absence of the genes designated as *chuA* and *yjaA* and a DNA fragment designated as *TSPE4.C2*. Other more complex methods of phylogenetic grouping are enzyme electrophoresis and ribotyping. There are four known phylogenetic groups (A, B1, B2, D) of *E. coli* among which groups A and B1 have been found to be associated with intestinal human and mammalian pathogenicity (Ochman and Selander, 1984; Clermont *et al*., 2000; Gordon *et al*., 2008).

According to LeClerc *et al*. (1996), mutation rates in isolated pathogenic *E. coli* were found to be higher than the average bacterial population with defect in methyldirected mismatch repair being the predominant underlying cause. However, high mutation rates due to methyldirected mismatch repair were demonstrated in both pathogenic and commensal *E. coli* strains in an independent survey of mutation rates of *E. coli* populations isolated from distinct environments (Matic *et al*., 1997). Genome size of *E. coli* markedly varies between commensal and pathogenic strains and the extra genetic material in pathogenic strains can contain either virulence or fitness genes (Croxen *et al*., 2013).

The overall target genes for detection of pathogenic *E. coli* are varied between different studies. Pathogenic *E. coli* strains can be detected by polymerase chain reaction (PCR) which is a powerful molecular biology technique that can detect target DNA of many kinds of pathogens in various clinical specimens. PCR is preferred over other methods of detection because it gives rapid, reliable results with greater specificity and sensitivity (Mohammed, 2012; Tobias and Vutukuru, 2012).

Based on unique sets of virulence and colonization factors encoded in the chromosomal or episomal structures and target genes, pathogenic *E. coli* bacteria can be grouped in to different strains. Enteroinvasive *E. coli* strains (EIEC) possess invasion genes virF and ipaH; Enteropathogenic *E. coli* strains (EPEC) possess a pathogenicity island in their genome called locus of enterocyte effacement (LEE) which encodes for the target gene *intimin*, designated as *eae.* These strains are also called Attaching/Effacing *E. coli* because they attach intimately and efface cytoplasm and microvilli from the intestinal epithelial cells of their hosts. Typical enteropathogenic *E. coli* contain EPEC adherence factor (EAF) plasmid which codes for the target gene *bundle forming pillus* (*bfp*) in addition to LEE. Enterotoxigenic *E. coli* are detected by their heat labile and heat stable enterotoxin genes (*LT, ST*); shiga toxin producing *E. coli* also called verotoxin producing *E. coli* possess shiga toxin genes (*stx1* and *stx2*); enteroaggregative *E. coli* produce *pic* and other toxins (Moon *et al*., 1983; Rúgeles *et al*., 2010; Chandra *et al*., 2013).

Diarrhea is a clinical condition characterized by frequent bowel movement with loose stools and accompanying signs and symptoms like fever and vomiting. Acute diarrhea is a term used to describe the presence of three or more loose watery stools within 24 hours; dysentery indicates presence of blood and mucus in diarrheal stools; and persistent diarrhea is diarrhea lasting for more than 14 days. Children in developing countries get exposed to many bacterial enteric pathogens at very early age and suffer many episodes of diarrhea as a result; pathogenic *E. coli* is among these enteric pathogens. Pathogenic *E. coli* has been associated with diarrheal disease in different parts of Africa particularly among young children, HIV positive and visitors from abroad (Nataro and Kaper,1998; Okeke, 2009;Vilchez *et al*., 2009).

The rational management of infectious diarrhea requires highly selective use of laboratory tests for these varied etiologic agents, depending on the clinical and epidemiologic setting; information generated from the study of pathogenic *E. coli* permits a practical approach to the diagnosis and management of diarrhea (Guerrant *et al*., 2001).

1.2. Statement of the Problem

Studies of *E. coli* are of great importance because this organism occupies broad host range including humans and animals (Levy , 1997). Pathogenic *E. coli* bacteria are the most common pathogens responsible for diarrhea in children. When antimicrobial therapy is indicated, selection of specific antimicrobial agents should be based on information on local susceptibility patterns of the organism and there should be continuous monitoring of local susceptibility patterns. Few studies have evaluated antimicrobial resistance of *E. coli* in sub Saharan Africa and have shown that the prevalence of resistant strains is increasing (O'Ryan *et al.*, 2005). Administration of antibiotics without accurate diagnosis of etiologic agent exposes children to unwanted drug side effects and contributes to the emergence of resistant strains (WHO, 2009).

The burden of child diarrhea due to pathogenic *E. coli* strains being evident from the above literature, implementation of rapid and simple molecular techniques in developing countries may provide an important epidemiological tool for the surveillance of pathogenic *E. coli* from clinical specimens and for the effective management of child diarrhea due to pathogenic *E. coli*. Implementation of simple molecular techniques which use plasmid DNA as controls can be considered (Rúgeles *et al*., 2010).

In Ethiopia, diagnostic approaches to *E. coli* induced diarrhea in children less than five years of age are not standardized.

Pathogenic *E. coli* is mot usually considered as a possible cause of child diarrhea but diagnostic tests requested are mostly for *salmonella* and *shigella*. i.e, there is no appropriate laboratory diagnosis of pathogenic *E. coli.* Any bacterial growth other than *Salmonella* or *Shigella* on diarrheal stools is generally reported as '*NO SALMONELLA OR SHIGELLA'* and hence there are no detection procedures of possible pathogenic *E .coli* strains.

It is clear that current laboratory reports on bacterial isolates of stool specimens may not produce sound clinical judgment with regards to diarrhea due to pathogenic *E. coli* and hence there is a need to determine the real burden and antibiotic profiles of pathogenic *E. coli* strains in local hospitals in order to introduce effective diagnosis and management of children with *E. coli* induced diarrhea. Therefore, this study was conducted to determine the involvement of pathogenic *E. coli* strains in child diarrhea and determine the antibiogram of the isolates in children less than 5 years of age with diarrhea at Addis Ababa University College of Health Sciences Tikur Anbessa General Specialized Hospital, Addis Ababa, Ethiopia.

2. OBJECTIVES.

2.1. General Objective

- To investigate pathogenic *E. coli* strains and their antibiogram profile from stool specimens in diarrheic children less than five years of age at Addis Ababa University College of Health Sciences Tikur Anbessa General Specialized Hospital.

2.2. Specific Objectives

- To identify pathogenic *E. coli* strains from children less than five years of age with diarrhea by bacteriological and molecular techniques.
- To determine the antimicrobial susceptibility profiles of isolated pathogenic *E. coli* strains.
- To explore the occurrence of pathogenic *E. coli* among demographic and clinical features of diarrheic children

3. LITERATURE REVIEW

3.1. *Escherichia coli* genome dynamics & the emergence of pathogenic strains

The *E. coli* genome is among those bacterial species which show high genome variability with high degree of rearrangements. One reason for the pronounced evolutionary dynamics of *E. coli* species may be a result of high recombination frequencies at the open replication forks of their genomes that lead to symmetric translocation and inversion events. Insertion sequence (IS) elements may be important mediators of such gene rearrangements by offering multiple sequences among which recombination can be initiated. Insertion – deletion rearrangements are other factors that account for increased genome flexibility. Generally, *E. coli* and other related bacterial genomes show high structural and functional variability, and the degree of genome flexibility is dependent on the content of repeated and mobile sequences such as IS elements, plasmids and phages. Although most analyses have focused on pathogenic organisms and have found high variation in the genome content of these strains (Boucher *et al.*, 2001; Mira *et al*., 2002).

The *E. coli* genome consists of two parts: a core genome (a mosaic – like structure) containing genes which encode essential cellular functions; and flexible genomic parts which are interspersed variable regions. The core genome is phylogenetically relatively stable. It contains essential genes which are shared by all strains of the species – those genes whose functions are always needed. Genes in this region are not subject to transfer among strains except under rare conditions. Genes that belong to the flexible genomic regions code for factors involved in fitness and adaptation of the organism to different environments (Hacker and Carniel, 2001).

Many strains of *E. coli* carry mobile and accessory genetic elements such as plasmids, bacteriophages, genomic islands and others, which code for adaptive traits of the bacteria. Genome dynamics processes like gene transfer, genome reduction, rearrangements and point mutations contribute to the adaptation of the bacteria into different environments. Genetic elements which are found in the flexible region are involved in such genome dynamics processes (Hacker and Carniel, 2001).The different components of the core genome and the flexible gene pool of *E. coli* are depicted in figure 1.

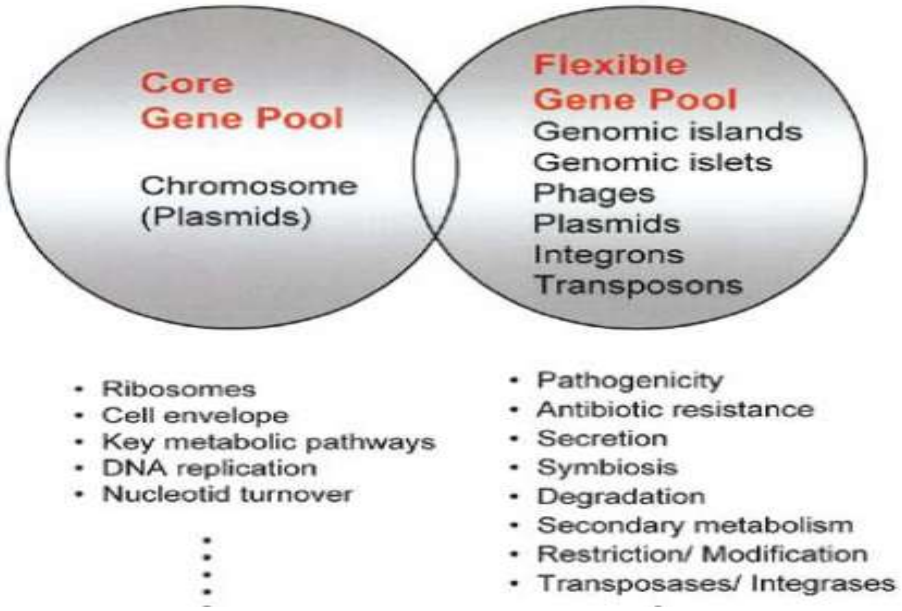

Figure 1: Model of the DNA pools in the genomes of prokaryotes (Hacker and Carniel, 2001)

There is high degree of flexibility in the *E. coli* genome as is seen in the fact that the genomes of many strains do share only a certain percent of their coding capacity, i.e, only a certain portion of the coding region of the *E. coli* genome is shared among different strains. The genome segment surrounding the replication terminus displays the most variation in chromosome size and organization of the *E .coli* genome most probably due to increased levels of recombination. (Boucher *et al.*, 2001; Dobrindt *et al*., 2010).

Horizontal gene transfer adds to genetic variation in genomic content among different strains of *E. coli*; the contribution of horizontal gene transfer is, in fact, greater than that of mutation. Horizontal gene transfer can take place between different organisms through transformation (uptake of DNA from the environment), transduction (packaging and transport of bacterial DNA by viruses), and conjugation (bacterial mating). Much of the horizontally transferred DNA is part of the flexible gene pool. Virulence genes of pathogenic strains are found on mobile genetic elements such as genomic islands (dynamic, ancient integrative elements in bacterial evolution which are important sources of genomic diversification and adaptation), bacteriophages, plasmids or transposons.

Virulence genes can thus be transferred from one *E. coli* to another or can be acquired from other species of bacteria and contribute to the development of adaptive characters of different pathogenic *E. coli* strains (Lawrence and Ochman, 1998; Hacker and Carniel, 2001; Boyd *et al*., 2009; Dobrindt *et al*., 2010). Genetic Processes involved in bacterial genome dynamics and evolution and the evolution of pathogenic strains are presented in figure 2.

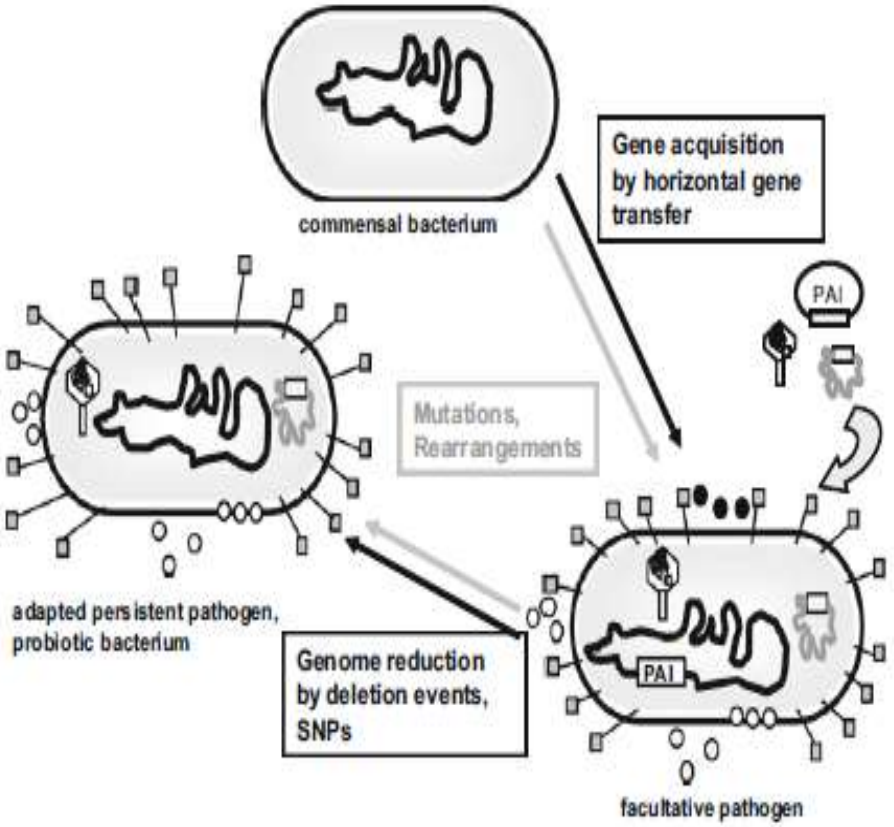

Figure 2: Processes involved in bacterial genome dynamics and evolution (Dobrindt *et al*., 2010).

Horizontal gene transfer may produce mosaic chromosomes comprised of genes which differ in ancestries and durations in the genome. Horizontally transferred genes in *E. coli* chromosomes mostly get inserted next to tRNA loci. This is a site where lysogenic phages get inserted; bacteriophages thus serve as vehicles for the introduction of horizontally transferred genes in these sites (Boyd *et al.,* 2009).

Horizontal gene transfer has contributed to the emergence of pathogenic strains of *E. coli*. The rate of horizontal gene transfer depends on: availability of environmental DNA; rate of integration of foreign DNA into recipient genome by conjugation, transformation, and transduction; degree of successful integration of foreign DNA into recipient genome; probability of fixation of the new allele; degree of the benefit resulted from the expression of the integrated gene. On an evolutionary timescale, increases in genome size due to the acquisition of horizontally transferred sequences are compensated by equivalent losses of DNA through deletion. These processes of gene gain and gene loss result in an extremely dynamic genome in which substantial amounts of DNA are introduced into and/or deleted from chromosomes which bring about change in adaptation characters of the species including pathogenicity (Boyd and Hartl, 1998; Lawrence and Ochman, 1998; Lawrence, 1999*).*

Mutation is another biological force that derives the emergence of pathogenic variants. Assessment of mutation rates of a species is important to understand the ecological and evolutionary processes of the species. Mutations may occur due to errors in DNA replication. There is generally low rate of spontaneous mutation in the genome of wild type *E coli* due to an enhanced accuracy of DNA replication and many enzymatic activities which control and repair DNA among which is methyl directed mismatch repair (MMR) which controls newly replicated DNA. In this enzymatic activity, mismatched bases, if any, are detected and enzymes are recruited to destroy the new DNA strand with mismatched bases; repolymerization is then undertaken using the old DNA strand as a template. Spontaneous mutation rate in wild type *E. coli* is thus lower than other microbes which do not undergo MMR (Elena and Lenski, 2003).

Although MMR is found in all domains of life, some bacterial strains isolated from natural environments often do not undergo this process in their genomes. In this case, MMR deficient strains are likely to be dominant after long term evolution. Loss of MMR increases mutation rate in *E. coli* by favoring base pair substitution mutations. Certain domestic *E. coli* strains may have lost their MMR capacity over evolutionary periods and thus exhibit higher genome mutability. Base pair substitution mutations usually occur in coding regions of the strains than in non coding regions.

This shows that MMR pathway mainly prevents errors of replication in the coding regions of the wild type *E. coli* genome rendering coding regions less susceptible to mutation than non coding regions; mutations occurring in non coding regions may be usually neutral and contribute less to the adaptability of the species (Elena and Lenski, 2003)

There are many factors that contribute to continuous adaptation as far as mutation is concerned: the amount of time required for a beneficial mutation to be fixed in a population, selective advantage of the beneficial mutation, the population size, and random genetic drift. Mutation and recombination of vertically inherited genes can give novel characters to offspring generation resulting in species diversification which may be much pronounced over long periods of time but horizontally acquired genes can better add to species diversity by integrating entirely new genes in to a species genome there by creating more novel characters with in short periods of time (Elena and Lenski, 2003; Lee *et al*., 2012).

In *E. coli*, horizontal gene transfer has greater potential of effecting species genetic diversification and hence emergence of pathogens than mutation. Mutations can promote adaptability of the species by modifying existing functions to some degree there by contributing to a gradual ecological expansion of the species while horizontal gene transfer renders the species effectively adaptive to a new habitat by integrating new DNA into the species genome there by giving the species genome completely new adaptive character – the introduction of foreign DNA is more effective in creating better adapted traits including pathogenicity than genomic rearrangements (Lawrence, 1999; Gogarten and Townsend, 2005; Lee *et al*., 2012). Figure 3 shows how horizontal gene transfer results in genetic diversification of a species.

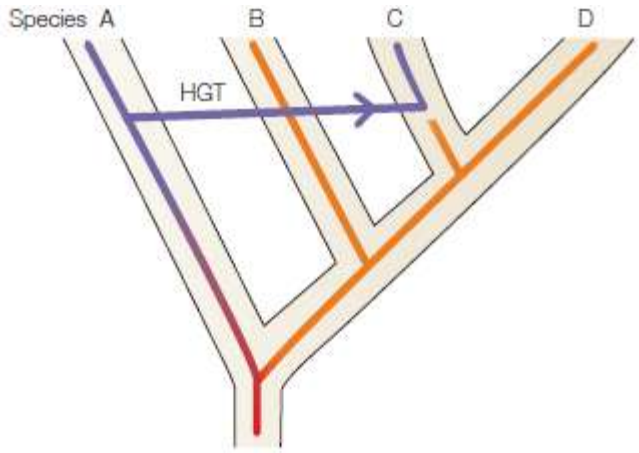

Figure 3: Explanations for unexpected phylogenetic distribution: the presence of a gene with characteristics that are typical for an unrelated group can be due to horizontal gene transfer (Gogarten and Townsend, 2005; Lee *et al*., 2012).

Generally, the gene content variation and degree of genomic alteration in *E. coli* is very high. This may be indicative of frequent acquisition of foreign DNA by horizontal gene transfer and processes of gene gain and deletion during the evolution of the genome of the bacteria (Blattner *et al*., 1997; Dobrindt *et al*., 2003; Archer *et al*., 2011).

3.2. Virulence mechanisms of pathogenic *E. coli* Strains

E. coli is a remarkably diverse organism. Acquisition of combination of various genetic elements enables the bacteria to become highly adapted pathogens capable of causing many diseases of worldwide distribution. Virulence factors found in pathogenic *E. coli* are mostly attributed to the presence of mobile genetic elements; both gain and loss of these mobile genetic elements are known to promote the adaptability and pathogenicity of different *E. coli* strains. Virulence strategies of pathogenic strains include: production of toxins to host organisms, involvement of host specific invasion plasmids, involvement of colonization factors, and adhesive characteristics (Croxen and Finlay, 2010).

The host attack strategy of pathogenic *E. coli* involves colonization of mucosal linings, evasion of host defenses, multiplication, and host damage. Diarrheagenic *E. coli* strains can colonize the intestinal mucosal linings without being prevented by peristalsis and the presence of other competitive intestinal micro flora.

Host specific fimbrial antigens of these diarrheagenic strains of *E. coli* allow their adherence to the mucosal linings of the small intestine (Nataro and Kaper, 1998). Diarrheagenic *E. coli* exhibit different adherence patterns as indicated in figure 4.

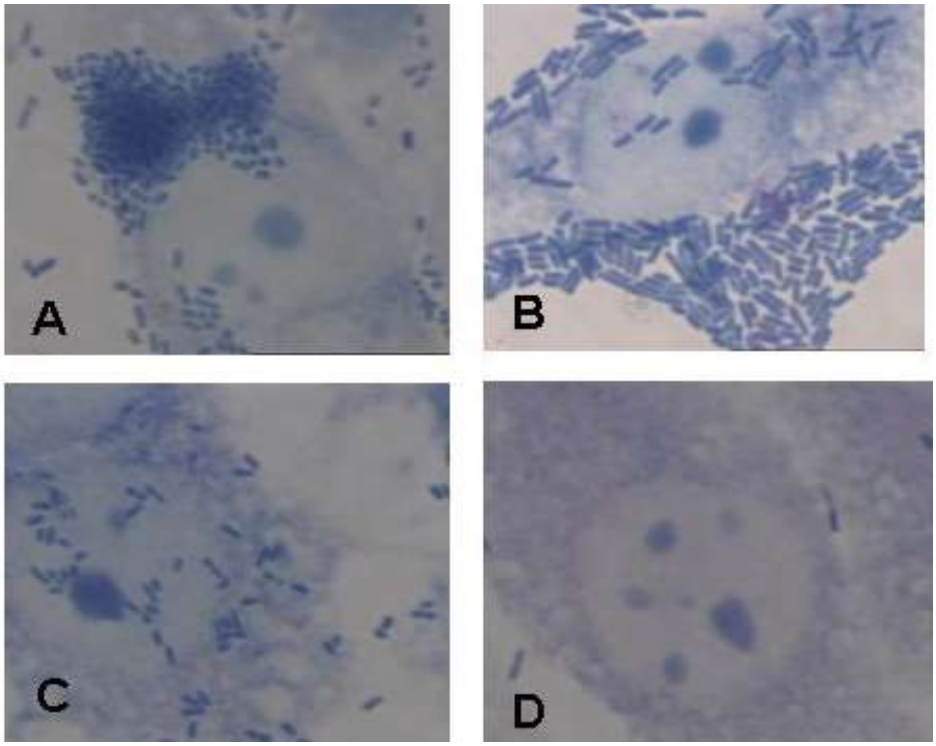

Figure 4: Adherence patterns of diarrheagenic *E. coli* to cultured epithelial cell A. Localized Adherence by enteropathogenic *E. coli*, B. Aggregative Adherence, C. Diffuse Adherence, and D. Non – adherent control strain (Okeke, 2009).

E. coli virulence genes are usually encoded on a DNA segment which is absent in commensal strains; transcriptional regulators encoded on pathogenicity islands or plasmids are known to regulate the expression virulence genes (Kaper *et al*., 2004). These virulence mechanisms that characterize pathogenic strains of *E. coli* are encoded by chromosomal, plasmid, and bacteriophages DNAs and are represented by several virulent genes including: *eae* (attaching and effacing lesions), *bfpA* (localized adherence), the gene encoding Enteroaggregative adherence, *ipaH* (Enteroinvasive mechanism), the genes encoding heat-labile toxin (LT) and heat-stable toxin (ST), and *stx1* and *stx2* (Shiga toxins) (O'Ryan *et al.*, 2005; Croxen and Finlay, 2010; Chandra *et al*., 2013).

Diarrheagenic *E. coli* are the most common bacteria detected in developing countries; they include the following clinically relevant pathotypes. Enteroaggregative *E. coli* (EAEC) exhibit aggregative adherence pattern on cultured epithelial cells and production of fimbrial colonization factors also called aggregative adherence factors (AAFs). They possess the following virulence factors which are plasmid encoded: aggregative adherence factor II (*aafII*), a heat stable toxin designated *astt*, a transcriptional activator designated *aggR* and a novel antiaggregation protein (dispersin) encoded by the *aap* gene. EAEC are known to cause acute and persistent diarrhea in infants. Enterotoxigenic *E. coli* (ETEC) are characterized by production of heat labile (LT) and/or heat-stable (ST) enterotoxins. Enteropathogenic *E. coli* (EPEC) bacteria possess a pathogenicity island known as the locus of enterocyte effacement (LEE) which encodes factors responsible for the attaching and effacing (A/E) phenomenon on host enterocytes as indicated in Figure 5.

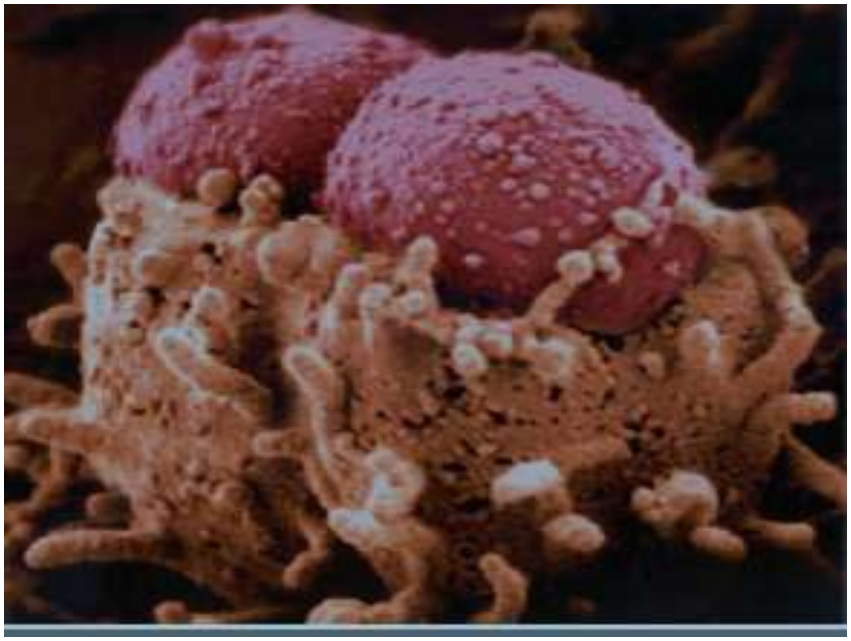

Figure 5: Attaching and effacing histopathology caused by EPEC and EHEC**.** The attaching and effacing histopathology results in pedestal-like structures, which rise up from the epithelial cell on which the bacteria perch (Kaper *et al*., 2004)

LEE is a 35 kb pathogenicity island (PAI) which contains genes encoding intimin (eae), a type III secretion system, a number of secreted (Esp) proteins, and an intimin receptor named Tir.

Some EPEC strains possess a 70–100 kb extra chromosomal plasmid which is called the EPEC adherence plasmid (EAF). This plasmid contains a collection of genes encoding a type IV pilus called the bundle forming pilus (BFP). BFP is known to mediate interbacterial adherence and adherence to host epithelial cells. Strains containing EAF are called 'typical EPEC' while those without EAF are called 'atypical EPEC'. EPEC are common causatives of acute endemic and epidemic diarrhea in infants, occasionally accompanied by persistent diarrhea. They attack the intestinal epithelium and produce a typical attaching and effacing (A/E) lesion.

Enterohemorrhagic *E. coli* (EHEC) which belong to Shiga toxin producing *E. coli* are characterized by invasion of the colonic epithelial lining of their host and production of Shiga toxins 1 and 2 (stx1 and stx2). EHEC also contain the pathogenicity island LEE; more than 200 serotypes of *E. coli* can produce Stx but most of these serotypes do not contain LEE. Enterotoxigenic *E. coli* (ETEC) are known to cause infantile diarrhea in developing countries and travelers diarrhea in all ages; they have been reported as causes of bloody diarrhea in few developing countries. They are also common causes of bloody diarrhea and hemolytic uremic syndrome in children <5 years of age; hemorrhagic colitis and diarrhea accompanied by thrombotic purpura in adults. Genes that produce different enterotoxins known as heat stable enterotoxins (ST) and heat labile enterotoxins (LT) and other colonization factors are mostly found on plasmids of these strains. Enteroinvasive *E. coli* (EIEC) constitutes a class of pathogenic *E. coli* with significantly lower prevalence which is phylogenetically related to Shigella species. These strains possess a virulence plasmid (pInv) that encodes different virulence genes for invasion including *virf , ipah, ipal*. They cause diseases similar to those caused by Shigella including diarrhea associated with fever and blood. EAEC, EPEC and ETEC are generally causatives of endemic watery diarrhea and have been commonly reported in children younger than 2 years of age (O'Ryan *et al.,* 2005; Croxen and Finlay, 2010; Rajendran *et al*., 2010; Tobias and Vutukuru, 2012; Chandra *et al*., 2013).

3.3. Laboratory diagnosis of Pathogenic *E. coli* strains in Diarrheic specimens

Selective use of clinical and laboratory diagnostic methods is of tremendous value to develop an effective approach to the evaluation and management of infectious diarrhea. There is a need for clear and applicable diagnostic guidelines to identify enteric pathogens that require specific therapy the lack of which can hinder appropriate management and treatment of many infections.

Although the patient's history and clinical findings may provide important clues as to the causes of diarrhea, an organism – specific diagnosis is required for some pathogens to prevent inappropriate treatment (Guerrant *et al*., 2001).

Many microorganisms including viruses, bacteria and parasites can cause infectious diarrhea and patients may even have mixed infections. But the contribution of these agents to the disease is not clearly known from different studies. The focus of many researches in the clinical area has been on agents that belong to certain specific areas such as: those which cause life – threatening illness, those which spread rapidly, or those which are vaccine controllable. Diarrheagenic *E. coli* are difficult to differentiate from commensals by conventional laboratory methods and hence they are less likely to be sought as causatives of infectious diarrhea; they have been featured as predominant causes of diarrhea only in studies that have attempted to encompass a broad range of pathogens (Okeke, 2009).

The identification of diarrheagenic *E. coli* pathotypes requires differentiation from non – pathogenic members of the normal intestinal flora. Besides, diarrheagenic *E. coli* infections are sometimes indistinguishable from viral gastroenteritis and hence identification of these strains could be helpful to deliver appropriate treatment for pathogen – specific illness. Many approaches have been employed to identify these pathogenic classes of *E. coli.* One approach is the use of serotypic markers which correlate with specific categories of diarrheagenic *E. coli* although these markers are insufficient to identify a strain as diarrheagenic.

Detection of diarrheagenic *E. coli* has mainly focused on the identification of characteristics of the bacteria which determine the virulence of these organisms such as *in vitro* phenotypic assays which correlate with the presence of specific virulence traits or detection of genes encoding these traits (Nataro and Kaper, 1998). One such useful phenotypic assay is the HEp – 2 adherence assay. The HEp – 2 adherence assay has mostly been used in the identification of Enteroaggregative *E. coli* pathotypes. The HEp – 2 adherence assays can be used to differentiate the three patterns of adherence: localized adherence, aggregative adherence, and diffuse adherence. Non – molecular methods (phenotypic assays, biochemical assays, and serotyping) can be used for the identification of diarrheagenic *E. coli* but they may not be solely sufficient to identify the main categories.

Molecular techniques including the use of nucleic acid – based probes as well as PCR methods are the most popular and most reliable techniques to differentiate diarrheagenic strains from members of stool normal flora and to discriminate between different pathogenic categories. DNA probes were first applied for detection of heat – labile (LT) and heat – stable (ST) enterotoxins in enterotoxigenic *E. coli* (ETEC) (Nguyen *et al*., 2005; Vilchez *et al*., 2009).

Today, gene probes are available for all diarrheagenic classes. Nucleic acid – based probes can be of oligonucleotide or polynucleotide types. Polynucleotide probes are derived from genes that encode a particular phenotype while oligonucleotide probes are derived from the DNA sequence of a target gene. Minor strain based differences among the virulence genes may generate false – negative results with oligonucleotideprobes, but oligonucleotide probes generate faster results than polynucleotide probes especially when screening for small genes is required. Recent probe techniques do not involve radio nucleotides to detect probe hybridization, i.e; they are nonisotopic (Estrada – García *et al*., 2005).

PCR is a molecular technique which is used to diagnose pathogenic microorganisms including *E. coli*. It is a powerful molecular biology technique for the detection of target DNA in various clinical specimens and for the detection of many kinds of pathogens. It is not only highly sensitive and specific, but it also provides rapid and reliable results. For stool samples, it can help to distinguish diarrheagenic *E. coli* isolates from those of the normal flora. PCR primers have been developed successfully for the different diarrheagenic *E. coli* pathotypes. Diarrheagenic *E. coli* strains are classified into different categories according to the presence of different virulence genes. In order to detect these categories of *E. coli* strains, it is necessary to perform several PCRs with different primers specific for these genes. (Nataro and Kaper, 1998; Nguyen *et al*., 2005; Vilchez *et al*., 2009).

3.4. Prevalence of pathogenic *E. coli* strains in diarrheic children

Diarrhea is a change in the normal bowel movement. This includes an increase in the water content, volume, or frequency of stools. Diarrhea may also be defined as a decrease in consistency of stool and an increase in frequency of bowel movements (>3 stools/ day). Infectious diarrhea is often accompanied by nausea, vomiting, or abdominal cramps (Guerrant *et al*., 2001).

A number of pathogens including bacteria, viruses and protozoa can cause infectious diarrhea. Pathogenic *E. coli* is among common bacterial pathogens which cause diarrhea in children less than 5 years of age (WHO, 2009).

Diarrhea associated global mortality rate of children less than five years of age is estimated to be around 1.87 million; the disease being one of the leading causes of morbidity and mortality in this age group especially in developing countries (Rajendran *et al*., 2010). Although the incidence of diarrhea due to pathogenic *E. coli* is more pronounced in developing countries, pathogenic *E. coli* strains have been found in studies of causative organisms of child diarrhea from developed countries also. In a study made in Italy in 160 samples collected from infants with gastroenteritis, *E. coli* were isolated in 75 of the samples (46.88%). Out of these, 10 samples (13.33%) were positive for pathogenic *E. coli*. 6 EPEC strains (two typical and four atypical) and four ETEC (one with *lt* and *st* genes, one with only *st* gene, two with *lt* genes) were detected. EAEC has been implicated as an emerging cause of sporadic diarrhea in children in the United States and as a cause of acute and persistent diarrhea globally (Okhuysen and DuPont., 2010; Amisano *et al.*, 2011).In a study made in pediatric children in Washington, 10 samples were found to have STEC out of 254 cases, STEC was one of 30 bacterial pathogens found in 29 (11.4%) of the cases involved in this study (Denno *et al*., 2012).

Risk factors for gastrointestinal illnesses like poor hygiene, inadequate access to safe drinking water, etc tend to show different distribution pattern between developed and developing countries; the occurrence of a causative pathogen differs accordingly. Until 2013, there was no precise information on the estimates of the distribution of enteric pathogens affecting children with diarrheal illnesses in developed and developing countries (Fletcher *et al*., 2013).

Generally, children living in developing countries have more diarrheal episodes with associated dehydration and a higher death rate than children living in developed countries. Nearly 1.4 billion Diarrheal disease episodes occurred among children less than 5 years of age every year in developing countries during the 1990s. This frequency remained constant until the early 2000s but with a decrease in deaths related to diarrheal disease episodes (O'Ryan *et al*., 2005).

The rate of child death due to diarrhea is estimated to be 12,600 per day in children less than 5 years of age living in Asia, Africa, and Latin America. Among other bacteria, *E. coli* plays a significant role in the occurrence of these diarrhea related deaths. The implication of *E. coli* as a causative of diarrheal diseases generally dates back to the 1920s. *E. coli* are among the early colonizers of the intestinal micro biota in infancy and live as commensals in mutual benefit with their human hosts. They usually do not cause disease except in immunocompromised hosts or when the gastrointestinal mucosal linings are perforated or broken due to different reasons. Commensal *E. coli* generally effectively compete with other normal flora to co1lonize the mucous layer of the mammalian colon although the genetic mechanisms behind this colonization are not well understood. Although most *E. coli* are harmless or rather beneficial to their hosts, few highly adapted clones of the species display specific virulence characteristics causing a broad spectrum of diseases (Nataro and Kaper, 1998).

Clinicians and epidemiologists have classified diarrheal episodes into three categories: acute diarrhea indicates the presence of three or more loose & watery stools within a 24 – hour period; dysentery or bloody diarrhea is the presence of visible blood and mucous in diarrheal stools, and persistent diarrhea is diarrhea which usually lasts for more than 14 days.

Children in developing countries may suffer repeated episodes of diarrhea as result of their exposure to diarrheagenic *E. coli* and other bacterial enteric pathogens at an early age. The predominance of a specific bacterial pathogen in a diarrheal condition generally shows age, time and geographical variation (Podewils *et al*., 2004; Nguyen *et al*., 2005).

Pathogenic *E. coli* strains cause high rates of persistent diarrhea with accompanying malnutrition and death in children in developing countries. In a five years survey of the etiology of child diarrhea conducted in China in 2015, diarrheagenic *E. coli* were the most frequently isolated bacteria (Yu *et al*., 2015). In a similar study made in Mexico, ETEC was found to be the most prevalent strain (18/62) among pathogenic *E. coli* followed by EAEC (26/62) (Estrada – García *et al*., 2005).

In another study made in India in 2010, EPEC was the most common pathogenic strain in children (9.9%). 6.1% of children positive for the EPEC pathotypes were infected with typical EPEC and 3.8% with atypical EPEC. ETEC was found in 4.1% cases while EHEC, EIEC, and DAEC were detected in 2%, 1%, and 0.5% cases respectively. Co infections with more than one pathogenic strain were seen in 3.8% cases. Analysis of association of pathogenic strain with child age and clinical features showed a significant association between lower age in months and the detection of typical EPEC; isolation of EAEC strains was found to be significantly associated with vomiting in this study (P = 0.03) (Rajendran *et al*., 2010).

Diarrheagenic *E. coli* are the most common cause of diarrhea in children less than five years of age next to rotavirus. In a prevalence study which was conducted to evaluate the number of *E. coli* pathotypes among children with diarrhea in Costa Rica from August 2005 to August 2007, a total of 40 (77%) strains were identified as pathogenic *E. coli*; 22% EIEC (9/40), 15% (6/40) EAEC and EPEC, 12% (5/40) EHEC, 10% (4/40) DAEC, and 5% (2/40) ETEC were detected. Among the EPEC isolates, *eae* A positive strains were negative for *bfp* A gene; only two strains were found to harbor *bfp* A gene indicating that atypical EPEC strains positive for *eae* A and negative for *bfp* A are prevalent in Costa Rica (Christian *et al.*, 2010).

A study made in Khartoum, Sudan in 437 children indicted that diarrhea in children was mainly caused by bacterial infection and that 48 % of these bacterial infections were caused by *E. coli*. The most frequent type of *E. coli* in this study was EAEC (43%), followed by EPEC (29 %), ETEC (18 %) and EIEC (9 %). The age and gender distribution in this study had the following pattern: 5% were aged 0–6 months, 6% were 7–24 months, 5% were 25–36 months, 18% were 37–48 months and 66% were 49–60 months; 240 (55 %) were boys and 197 (45 %) girls, the ratio of male to female being 1 : 0.82. The rate of diarrhoea was significantly higher (P, 0.0001) in the oldest group (49–60 months) and in male children. 98% of children in this study had watery diarrhoea while 2% had bloody diarrhoea; 53% of children presented with fever, 55% of the children with vomiting and 60% with dehydration (Saeed *et al*., 2015).

A total of 280 children (172 boys and 108 girls) aged 0 – 60 months with diarrhoea were involved in a study conducted in children aged less than five years of age with diarrhoea in Dare Salaam, Tanzania. Out of these, 235 (83.9%) children presented with acute watery diarrhoea, 27 (9.6%) with persistent diarrhoea; 79 children (28.2%) were found to be malnourished in this study. The most common pathogen, isolated among 64 children (22.9%), was diarrheagenic *E. coli* which included 41(64.1%) EAEC, 13 (20.3%) EPEC, and 10(15.6%) ETEC strains harboring stable enterotoxins (Moyo *et al*., 2011).

In a similar study conducted to assess the bacterial and viral etiology of childhood diarrhea in Ouagadougou, Burkina Faso, 283 children with diarrhea; 49% aged 0 –12 months, 34% aged 13–24 months and 17% aged 25 –59 months old were involved. Virulence genes of *E. coli* were found in 24% of the patients; the most frequent strain was EAEC (12%), followed by EPEC (8%) and ETEC (4%). STEC and EIEC were found only in one patient each (Bonkoungou *et al.*, 2013).

A cross-sectional study conducted in Bahirdar in 422 children less than five years of age with diarrhea from December 2011 to February 2012 came up with an overall *E. coli* isolation rate of 48.3% and isolates belonging to enteropathogenic group were the most commonly isolated pathotypes (Ayrikim Adugna *et al.*, 2015).

3.5. Antibiotic susceptibility profiles of pathogenic *E. coli* strains in diarrheic children

Diarrhea, generally, is self – limiting and antimicrobial agents are not usually recommended for treatment. But there are cases where antimicrobial treatment may be required. These include: patients who are travelers to endemic area, to shorten the course of the disease to prevent malnutrition and other consequences, to prevent transmission in community. In this case, information on the antibiotic susceptibility profiles of pathogens can be used for selecting appropriate antimicrobial therapy (Amaya *et al*., 2011).

Besides shortening illness and reducing morbidity, appropriate antimicrobial treatment can be life saving in devastating infections. Emergence of strains that are resistant to commonly used antimicrobial agents may lead to treatment failures and hence determinations of antimicrobial susceptibility may be of importance. There has been a decrease in the effectiveness of antibiotics in treating common infections in recent years, and many untreatable strains of Enterobacteriaceae are emerging.

This is majorly attributed to high rates of antibiotic use in high – income countries which leads to selection pressure that sustain resistant strains. Antibiotic use is also increasing in middle – income countries where there is high rate of hospitalization with rising incomes. Resistance to antibiotics is generally a result of microbial mutations and selection pressures from antibiotic use which give mutated strains a competitive advantage. The global burden of antibiotic resistance is evident in that nearly 25, 000 people die each year in Europe due to infections by antibiotic – resistant bacteria and 23 000 deaths occur every year in the USA due to a similar condition.

Empirical therapy with broad – spectrum antibiotics may facilitate the emergence of drug resistance among pathogens that spread easily from person to person. Hence, knowledge of the local patterns of susceptibility of pathogenic strains can be helpful in the choice of antibiotics to prevent resistance (Guerrant *et al*., 2001; Laxminarayan *et al*., 2013).

Different studies have documented patters of antibiotic sensitivity of pathogenic *E. coli* from different places. In a study conducted in Mexico in 2005 diarrheagenic *E. coli* were identified in 62 (14%) of 430 patients. Out of the 170 strains identified, 105 (62%) were multidrug resistant, 145 (85%) were resistant to tetracycline, 124 (73%) to ampicillin, 127 (75%) to trimethoprim – sulfamethoxazole, 29 (17%) to chloramphenicol, 4 (2%) to gentamycin, and none to ciprofloxacin and cefotaxime. Atypical EPEC was significantly less resistant ($p<0.05$) to ampicillin and trimethoprim – sulfamethoxazole than ETEC and EAEC in this study (Estrada – García *et al*., 2005).

In another study undertaken to determine the antimicrobial resistance pattern of *E. coli* isolates from stool specimens of children in Leo´ n, Nicaragua between March 2005 and September 2006, it was shown that resistance to ampicillin was found in 67.7% (225/332) of the diarrheagenic *E. coli* isolates, resistance to trimethoprim – sulfamethoxazole was found in 71.6% (238/332) of the isolates, resistance to chloramphenicol was found in 9.3% (31/332) of the isolates and no resistance to imipenem was observed; the level of antibiotic resistance was low in all *E. coli* isolates for other antimicrobial agents (Amaya *et al*., 2011).

Another study in Kathmandu, Nepal demonstrated that 100% of isolated *E. coli* were susceptible to Chloramphenicol, 91.7% isolates were susceptible to Gentamicin, Fluoroquinolones, and 3rd generation Cephalosporin, 75.0% isolates were susceptible to Tetracycline, and 41.7% isolates were resistant to Ampicillin (Ansari, *et al*., 2013). A cohort study of 1,034 infants was conducted in Lima, Peru to determine antimicrobial susceptibility pattern of diarrheagenic *E. coli.* High levels of resistance to ampicillin (85%), cotrimoxazole (79%), tetracycline (65%), and nalidixic acid (28%) were found; DAEC and EAEC showed higher degree of resistance to these antibiotics than EPEC and ETEC in this study (Ochoa *et al*., 2009).

Sometimes pathogens may not be susceptible to locally available antimicrobial agents. Antibiotic susceptibility profile of *E. coli* strains isolated from stool of pediatric children at Constanta Clinical Infectious Diseases Hospital, Romania was determined in 2012. Nalidixic acid, amoxicillin – clavulanic acid and trimethoprim – sulfamethoxazole were the most commonly used antimicrobials to treat children in this area, but *E. coli* isolates showed a decreased susceptibility to these antibiotics. *E. coli* isolates also showed lower sensitivity to ampicillin and tetracycline in this study (Cambrea, 2014).

E. coli isolates showed a high level of resistance to commonly used antimicrobials such as tetracycline, ampicillin and trimethoprim – sulphamethoxazole in a study done in four provinces of Kenya to assess the prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea. 63/73 (86%) of the isolates were resistant to ampicillin, whereas 64/73 (87.6%) were resistant to trimethoprim – sulphamethoxazole and 50/73 (68%) were resistant to tetracycline from the four study regions. This study also demonstrated emergence of resistance to ciprofloxacin, nalidixic acid, norfloxacin and gentamycin at low levels (Sang *et al*., 2012).

Faecal specimen from 50 children less than 5 years of age were collected and assessed for microbiological profile of enteric pathogens in a study conducted at University of Benin Teaching Hospital, Nigeria. The isolates were found to be most resistant to doxycycline followed by ciprofloxacin while they were most susceptible to amoxicillin (Akinnibosun and Nwafor, 2015).

Isolated *E. coli* strains exhibited high levels of antimicrobial resistance to ampicillin (86.8%), tetracycline (76%) and cotrimoxazole (76%) in a study conducted in Bahirdar in 422 children less than five years of age with diarrhea from December 2011 to February 2012 (Ayrikim Adugna *et al.*, 2015).

4. MATERIALS AND METHODS

4.1. Study Area

This study was conducted at Tikur Anbessa General Specialized Hospital, Addis Ababa University College of Health Sciences, Addis Ababa. Addis Ababa is the capital and largest city of Ethiopia, located on a well-watered plateau surrounded by hills and mountains at an altitude of about 2500m above sea level. The average annual temperature and rainfall are 21^{o}C and 1800 mm, respectively. Tikur Anbessa Hospital is the largest hospital in the country and functions under the authority of the Addis Ababa University Health Sciences College.

4. 2. Study Population

Patients included in this study were children under 5 years old with 3 or more loose stools in 24 hours or with an episode of bloody diarrhea. Children that received previous antimicrobial drug treatment were excluded from this study (Estrada-García *et al*., 2005)

4.3. Study Design and Sampling Methodology

The study was purposive type, i.e., samples were collected from all children less than five years of age with diarrhea. Sample collection was done from January 2017 to March 2018. Approximately 50gm of feces was collected from children with diarrhea in accordance with standard laboratory specimen collection procedures. Specimens from diarrheic children were collected in sample cups on to which buffered peptone water was added for enrichment. Specimens were labeled with unique sample identification numbers, transported in ice box to biomedical laboratory of Microbial, Cellular and Molecular Biology at College of Natural Sciences, Addis Ababa University and inoculated in to primary culture media within the same day of collection.

4.4. Isolation of *E. coli*

Broth specimens were be inoculated on MacConkey Agar and incubated aerobically at $37^{0}c$ overnight. Lactose fermenting colonies on MacConkey Agar were then sub cultured in to eosin Methylene blue and incubated aerobically at $37^{0}c$ overnight. Green metallic sheen colonies on Eosin Methylene Blue were considered as presumptive *E. coli* isolates.

Presumptive isolates were stored in nutrient broth for further identification by biochemical tests. All the isolates were also stained by Gram stain to determine cell morphology and purity of the isolates.

4.5. Biochemical Characterization of *E. coli* isolates

Presumptive *E. coli* isolates were further characterized for their biochemical activity using the biochemical tests indole, methylred, Vogues proskuer and citrate utilization (IMViC). Bacterial isolates that exhibited IMViC pattern of **(+ + - -)** respectively were considered as *E. coli* isolates (Edwards and Ewing, 1972)

Indole test

A sterilized test tube containing 4 ml of tryptophan broth was inoculated aseptically by taking an inoculum from 18 to 24 hrs culture on EMB. The broth was incubated at 37°C for 24 – 28 hours. 0.5 ml of Kovac's reagent was added to the broth culture and the presence or absence of ring was observed.

Formation of a pink color in the reagent layer on top of the medium within seconds of adding the reagent was considered as positive result. Absence of ring formation considered as a negative result.

Vogues – Proskuer (VP) test

The medium was inoculated with an inoculum taken from an 18-24 hour pure culture and incubated aerobically at 37^{o}c for 24 hours. 1ml of the broth was transferred to a clean test tube following 24 hours of incubation. The remaining broth was reincubated for an additional 24 hours. 0.6ml of 5% alpha-naphthol was added to the 1ml broth and next 0.2ml of 40% KOH was added. By gently shaking to expose the medium to atmospheric oxygen, the tube was allowed to remain undisturbed for 10-15 minutes. Observation of a pink-red color development was considered as a positive VP test. A negative VP test was demonstrated by the appearance of a yellow color on the surface of the medium. Development of a copper-like color was also interpreted as negative.

Methyl red test

Following 48 hours of incubation, 2.5 ml of the broth was transferred to a clean test tube. Five drops of methyl red indicator were added. Development of a stable red color on the surface of the medium after the addition of methyl red indicator was interpreted as positive test.

A negative methyl red test was demonstrated by the development of a yellow color on the surface of the medium

Citrate utilization test

Simmons Citrate Agar was inoculated on the slant by touching the tip of a needle to a colony that is 18 to 24 hours old and incubated at 37^{o}C for 24 hours. Development of blue color on the slant surface due to the alkaline carbonates and bicarbonates produced as by – products of citrate catabolism increasing the pH was considered as positive result. The absence of color change (the medium remains deep green) was considered as negative re

4.6. Antimicrobial Sensitivity Testing

The antimicrobial susceptibility/resistance profiles of the bacterial isolates were determined using Kirby – Bauer – disk diffusion method. Disks impregnated with the following antibiotics were used: Trimethoprim (5µg), chloramphenicol (30 µg), ciprofloxacin (5µg), ampicillin (10 µg), neomycin (10 µg), gentamycin (10 µg), tetracycline (30 µg), Compound sulfonamides (300 µg), chloramphenicol (30 µg), Cefotetan (30 µg), Norfloxacin (10 µg) and streptomycin (25 µg). Pure bacterial colonies were inoculated into 7 ml of Tryptophan soya broth and incubated at 37 ºC for 18 hours until turbidity is seen and were compared to the 0.5 McFarland standards.

Mueller – Hinton Agar was used as plating medium. Fifteen minutes after inoculation of the plates using sterile swabs, the antibiotic impregnated disks were applied on the surface of inoculated plates with sterile forceps. All the disks were gently pressed down onto the agar with forceps. The plates were inverted and then incubated aerobically for 18 hours at 37 ºC. The diameters of the zones of inhibition were measured to the nearest whole millimeter using the transparent ruler and were interpreted as susceptible, intermediate and resistant based on the recommendations of Clinical Laboratory Standards Institute (Wayne, 2016).

4.7. Virulence gene detection

4.7.1. DNA extraction

Biochemically confirmed *E. coli* isolates were grown in nutrient broth at 37^{0}C overnight. Exactly 1.5 ml of the culture was spun by centrifugation at 5000 g for 10 min.

The bacterial pellet was lysed by adding 50 µl of double distilled water and boiling in a water bath at 95^0C for 10 minutes. The lysate was then centrifuged again as before and 50 µl of the supernatant used directly as template for PCR (Croci *et al*., 2004)

4.7.2. Detection of virulence gene sequences by PCR

After extraction, the bacterial DNA was subjected to PCR for the presence of virulence genes. According to the annealing temperatures of the different primers used, five PCR assays were performed. The PCR experiments were carried out using the following protocols.

To detect the presence of *stx2* (shigatoxin) genes of STEC and EHEC, a reaction was set up in a 25µl reaction volume in a PCR master mix (Himedia; India, 2017) containing 1µ1 of each primer (EVS1, EVC2), 2.5µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35 mm of each dNTP, 0.5 µ1 of Taq polymerase enzyme, 14 µ1of double distilled water and 3 µl of template DNA. The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c; 30 cycles each consisting, 40 s at 95^0c, 40 s at 55^0c, 30 s at 72^0c; and a final extension of 1cycle for 8 min. at 72^0c.

To detect the *eae* (intimin) gene of EPEC and EHEC strains, a reaction was set up in a 25µl reaction tube in a PCR master mix (Himedia; India, 2017) containing 1µ1 of each primer (EAE1 and EAE2), 2.5µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35mm of each dNTP, 1µ1 of 50Mmol $MgCl_2$, 0.5µl of Taq polymerase enzyme, 15 µ1of sterile distilled water and 3 µl of template DNA. The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c ; 35 cycles, each consisting of 40 s at 95^0c, 60 s at 55^0c and 60 s at 72^0c; and a final extension of 1cycle for 10 min. at 72^0c.

To detect the *bfp* (bundle forming pillus) gene of typical EPEC strains, a reaction was set up in a 25µl reaction tube in a PCR master mix (Himedia; India, 2017) containing 0.5µ1 of each primer (BFPF, BFPR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35mm of each dNTP, 0.3 µl of Taq polymerase enzyme, 16.2 µl of sterile distilled water and 3 µl of template DNA. The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c; 30 cycles, each consisting of 40 s at 95^0c, 40 s at 57^0c, 30 s at 72^0c; and a final extension of 1cycle for 8 min at 72^0c.

To detect *hylA* (hemolysin) gene of EHEC, reaction components were mixed in a 25µl reaction tube in a PCR master mix (Himedia; India, 2012) containing 1µ1 of each primer (EHECF, EHECR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35mm of each dNTP, 0.3 µl of Taq polymerase enzyme, 16.2 µl of sterile distilled water and 3 µl of template DNA. Then, the reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c ;30 cycles, each consisting of 40 s at 95^0c, 1min. at 45^0c, 1min at 72^0c; and a final extension of 1cycle for 10 min at 72^0c.

To detect *aatA* (antiagrgation transporter gene) of EAEC strain reactions were set up in a 25µl reaction tube in a PCR master mix (Himedia; India, 2012) containing 1µ1 of each primer (EAECF, EAECR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35mm of each dNTP, 0.3 µl of Taq polymerase enzyme, 16.2 µl of sterile distilled water and 3 µl of template DNA. The reaction mixture was amplified with an initial denaturation of 1 cycle for 3 min. at 95^0c ;30 cycles each consisting of,40 s at 95^0c, 1min. at 45^0c, 30s at72^0c ; and a final extension of 1cycle for 10 min. at 72^0c.

All amplifications were carried out in a thermal cycler (Applied Biosystems StepOne™ Real-Time PCR System Thermal Cycling Block).

4.7.3. Agarose Gel Electrophoresis

Amplified PCR products were analyzed by agarose gel electrophoresis at 120 volt for 30 minutes in 1.5% agarose containing ethidium bromide (0.5 µg ml^{-1}) using a marker DNA ladder of 100 bp (Himedia; India, 2017). The products were visualized with ultraviolet illumination and imaged with gel documentation system (Biored Gel Doc XR, USA). Details of primer gene sequences and the different reaction temperatures to be carried out in the PCR assays used were as indicated in table 1.

Table 1**:** Primer gene sequence and PCR conditions

Primer	Nuclotide sequence	Target gene	Pathogenic *E. coli* strain	Denaturing	Annealing	Extension	Product size (Bp)	Cycles	Reference
EAE1	F:5'AAACAGGTGAAACTGTTGCC3'	*eae*	EPEC/EHEC	94^0c,2 min.	55^0c,60s	72^0c,60s	490	35	Khan *et al.*(2002)
EAE2	R:5'-CTCTGCAGATTAACCTCTGC-3'	*eae*							
EVS1	F:5'-ATCAGTCGTCACTCACTGGT-3'	*Stx2*	STEC/EHEC	94^0c,2 min.	55^0c,60s	72^0c,60s	110	30	Khan *et al.*(2002)
EVC2	R:5'-CTGCTGTCACAGTGACAAA-3'	*Stx2*							
EHEC	F: 5'-ACGATGTGGTTTATTCTGGA-3'	*hlyA*	EHEC	95^0c,3 min.	45^0c,40s	72^0c,30s	165	30	Paton and Paton.(1998
EHEC	R:5'-CTTCACGTCACCATACATAT-3'	*hlyA*							
EAEC	F:5'CTGGCGAAAGACTGTATCTAT-3'	*aatA*	EAEC	95^0c,3 min.	45^0c,40s	72^0c,30s	630	30	Chattaway *et al.*(2011)
EAEC	R:5'CAATGTATAGAAATCCGCTGTT-3'	*aatA*							
BFP	F:5'AATGGTGCTTGCGCTTGCTGC-3	*bfpA*	EPEC	95^0c,3 min.	57^0c,40s	72^0c,30s	324	30	Christian *et al.*(2010
	R:5'GCCGCTTTATCCAACCTGGTA-3'	*bfpA*							

EPEC = Enteropathogenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; STEC= Shiga – like toxin producing *E. coli*; EAEC= Enteroaggregative *E. coli*

4.8. Questionnaire Survey

Questionnaire for data collection was prepared and administered to the attendants of children from whom specimens were collected. Data were collected on child demographics and clinical condition such as child age, sex, residence, onset of diarrhea, clinical diagnosis, history of previous illness, RVI status, BMI, household members and history of illness, etc.

4.9. Ethical approval

Before the start of the work, the proposal was submitted to the Ethics committee of Addis Ababa University College of Natural Sciences to get Ethical approval to conduct the study (CNSDO/237/09/2017). During sample collection the objectives of the work was explained to the parents of children visiting hospitals in order to get consent of the parents or attendants of children. In addition, all samples were collected by health professionals.

4.10. Data Management and Analysis

Data describing the diarrheagenic conditions suggestive of *E. coli* infection observed on children along with age were classified filtered and coded using Microsoft Excel® 2007. The data were then exported to SPSS windows version 20.0 (IBMSPSS INC.Chicago, IL) for statistical analysis. The prevalence of *E. coli* strains from the total diarrheic children and the prevalence of pathogenic strains from total *E. coli* isolates along with their susceptibility profiles were determined by using descriptive statistics.

4.11. Limitation of the study

The current study encountered the following limitations. Additional primers like those of ETEC and DAEC were not optimized and strains identified due to lack of positive controls.

Sample size of the study is small which makes it difficult to make statistical generalizations. The study concentrated in only one hospital in Addis Ababa. Other hospitals were not included because the occurrence of child diarrhea in other hospitals was low. The study had to be completed within a specified period of time because it was conducted for academic fulfillment. More children would have been enrolled in the study had it not been for shortage of time.

5. RESULTS

Children (n=98) (47 males & 51 females), aged 01 month to 60 months with a mean age of 31.9 months suffering from diarrhea were included in this study.

5.1. Occurrence of *E. coli* in diarrheic children

Out of 98 stool specimens collected from diarrheic children less than 5 years of age, 75 were found to be positive for *E. coli* based on colony characteristic on EMB; further confirmation by IMViC tests showed that only 56 of the isolates were *E. coli*. The frequency of *E. coli* isolates in male children constituted 29.6% of diarrheic children included in the study while 27.5% of the isolates were found in female children. Table 2 shows the occurrence of *E. coli* isolates between male & female children and among three age groups of children involved in the study.

Table 2: Age and sex distribution of diarrheic children with *E. coli* at AAU CHS Tikur Anbessa General Specialized Hospital, Addis Ababa, 2017.

	Sex		Total
Age in months	M	F	
0 – 6 months	4 (4.1%)	2 (2%)	6 (6.1%)
7 – 24 months	13 (13.26%)	10 (10.24%)	23 (23.5%)
25 - 60 months	12 (12.2%)	15 (15.3%)	27 (27.5%)
Total	29/98 (29.6%)	27/98(27.5%)	56/98 (57.1%)

In addition, data on the clinical characters of the children were collected using questionnaires and analyzed. Table 3 shows the occurrence of *E. coli* among potential risk factors that could expose children to *E. coli* infection.

Table 3: Clinical characters of diarrheic children and occurrence of *E. coli* at AAU CHS Tikur Anbessa General Specialized Hospital, Addis Ababa, 2017.

Risk factors			*E. coli* occurrence
Animal Contact	YES	28	17
	NO	70	39
Habit of eating undercooked food	YES	33	20
	NO	65	36
Habit of boiling water	YES	16	9
	NO	82	47
Family history of illness	YES	27	12
	NO	71	44
Previous history of illness	YES	32	20
	NO	66	36

5.2. Antimicrobial susceptibility profiles of *E. coli* isolated from diarrheic children

E. coli isolates were tested against 11 antibiotics to determine their susceptibility patterns. The isolates were resistant to most of the antibiotics used. High percentage of resistance was observed for neomycin (94.6%), ampicillin (87.5%), and compound sulfonamides (83.9%).

The isolates showed low resistance towards Ciprofloxacin & Norfloxacin (33.9%, 26.8%), and to Chloramphenicol (32.1%). Least resistance was exhibited by the isolates towards Cefotetan among the antibiotics tested as indicated in table 4.

Table 4: Antibiotics susceptibility profiles of *E. coli* isolated from diarrheic children less than 5 years of age at AAU CHS Tikur Anbessa General Specialized Hospital, 2017.

	Resistance pattern of *E. coli* isolates		
Antibiotics	Susceptible	Resistant	Intermediate
Trimethoprim	11/56 (19.6%)	43/56 (76.8%)	2 /56(3.6%)
Ciprofloxacin	35/56 (62.5%)	19/56 (33.9%)	2/56 (3.6%)
Ampicillin	4/56 (7.1%)	49/56 (87.5%)	3/56 (5.4%)
Neomycin	1/56 (1.8%)	53/56 (94.6%)	2/56 (3.6%)
Gentamicin	3/56 (5.4%)	40/56 (71.4%)	13/56 (23.2%)
Compound sulphonamide	1/56 (1.8%)	47/56 (83.9%)	8/56 (14.3%)
Tetracycline	8/56 (14.3%)	48/56 (85.7%)	_
Chloramphenicol	38/56 (67.9%)	18/56 (32.1%)	_
Cefotetan	46/56 (82.1%)	10/56 (17.9%)	_
Norfloxacin	40/56 (71.4%)	15/56 (26.8%)	1/56 (1.8%)
Streptomycin	6/56 (10.71%)	43/56 (76.8%)	7/56 (12.5%)

Multidrug resistance was observed in all isolates of *E. coli.* All isolates were found to be resistant to at least 4 antibiotics. The highest prevalence of multi drug resistance was resistance to 7 antibiotics tested: trimethoprim, ampicillin, neomycin, gentamycin, compound sulfonamide, streptomycin, and tetracycline among the antibiotics tested. One of the isolates exhibited resistance to all 11 antibiotics tested and 4 of the isolates exhibited resistance to 10 out 11 antibiotics tested.

Data on the multidrug resistance pattern of the isolates are summarized in table 5.

Table 5: Multi drug resistance of *E. coli* isolated from diarrheic children less than 5 years of age at AAUCHS Tikur Anbessa General Specialized Hospital, 2017.

Number of drugs resisted	Resisted drugs	Number of isolates that showed MDR	Percent
4	Neomycin Compund sulphonamide Gentamicin Ampicilin	4	7.1
6	Neomycin Compund sulphonaamide Gentamicin Ampicilin Streptomycin Tetracycline	4	7.1
7	Neomycin Compund sulphonaamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim	15	26.8
8	Neomycin Compund sulphonaamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim Ciprofloxacin	14	25.0
11	Neomycin Compund sulphonaamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim Ciprofloxacin Chloramphenicol Norfloxacin Cefotitan	1	1.8
Total		56	100.0

5.3. Occurrence of *E. coli* pathotypes (pathogenic strains) in diarrheic children

Five pairs of primers (reverse and forward) were optimized according to their annealing temperatures and different virulence genes of *E. coli* were detected by PCR. Specimens were pooled to obtain control strains with the desired genes; in addition, positive controls from previous experiments at biotechnology laboratory, college of natural sciences were used in parallel with the current samples. Many PCR experiments were run to detect virulence genes and to identify pathotypes of *E. coli.* It was difficult to incorporate all the images generated from the PCR experiments in the paper due to space limitation. Selected agarose gel images which represent each virulence gene generated from the different PCR runs according to specific base pairs are presented in figures 6 to 10.

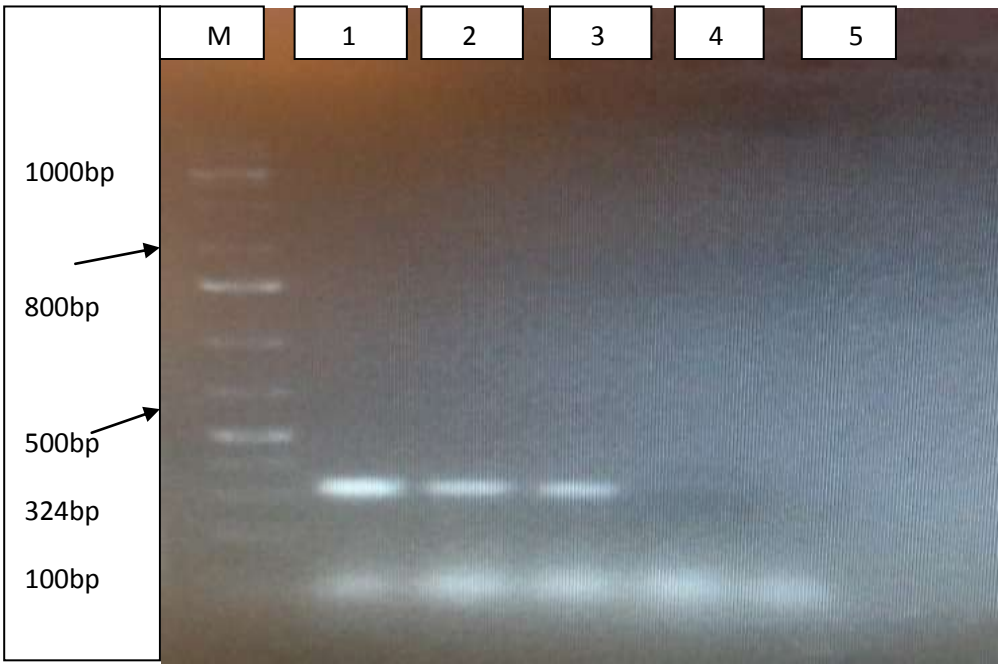

Figure 6: Amplified *bfp* gene, 324 bp amplicon, separated on 1.5 % agarose gel electrophoresis: M=Marker, 1=T8142, 2 =T4163, 3=T008, 4=T1185, 5=Negative Control.

T8142 was obtained from pooled specimens during optimization process and was treated as a positive control in this run. T4163 & T008 are positive samples, T1185 is a negative sample.

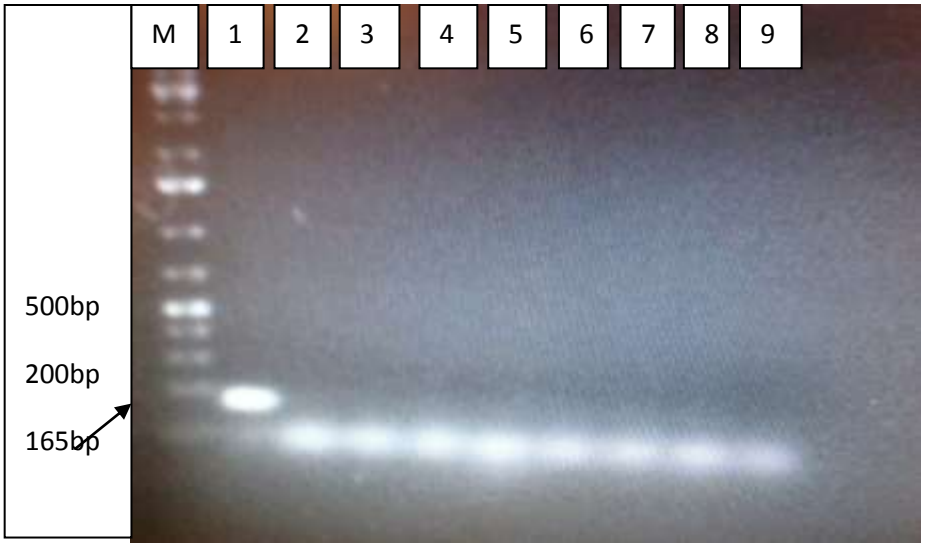

Figure 7: Amplified *hlyA* gene, 165 bp amplicon, separated on 1.5 % agarose gel electrophoresis:

M=Marker, 1=T004, 2=T4163, 3=T008, 4=T001, 5=T1185, 6=T002, 7=T5677, 8=T8142, 9= Negative Control. Only one isolate of *E. coli*, T004 was positive for *hlyA* gene out of 56 isolates.

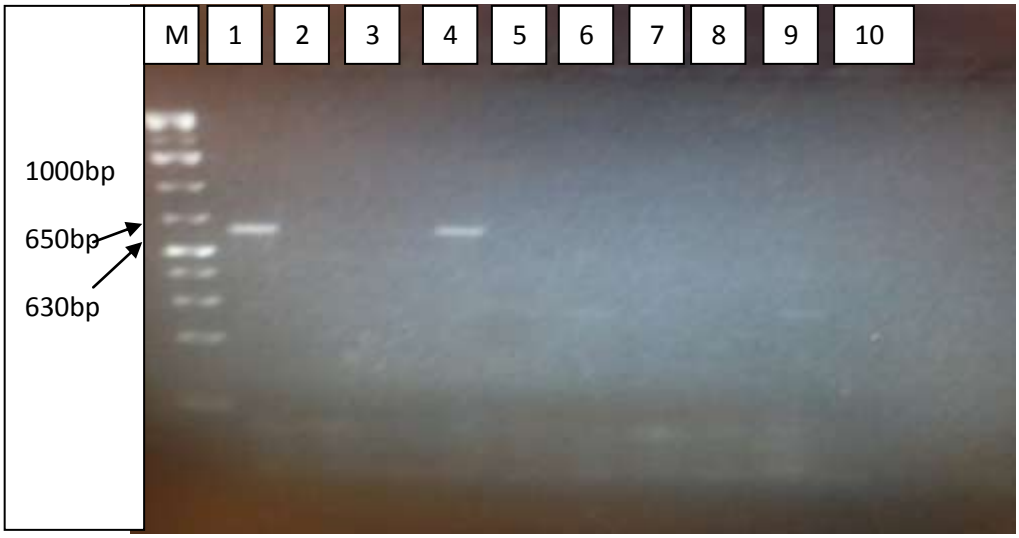

Figure 8: Amplified *aatA* gene, 630 bp amplicon, separated on 1.5 % agarose gel electrophoresis: M=Marker 1=T544,2=T0438, 3=T1186, 4=T2934, 5= T7142, 6= T7147, 7= T7141, 8= T8142, 9= T2115, 10= Negative Control.

T544 was obtained from pooled specimens during optimization process and was treated as a positive control in this run. T2934 is also positive for *aatA* gene.

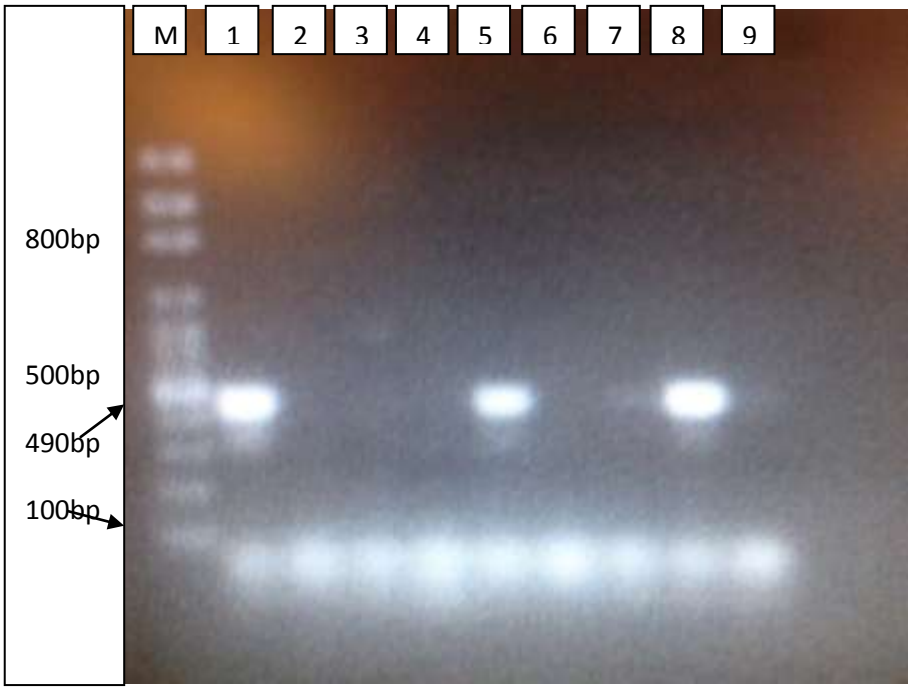

Figure 9: Amplified *eae* gene, 490 bp amplicon, separated on 1.5 % agarose gel electrophoresis:

M=Marker, 1= T001, 2= T6028, 3= T7898, 4= T8316, 5= T0342, 6= T7849, 7= T0438, 8= T8311, 9= Negative Control.T001 was obtained from pooled specimens during optimization process and was treated as a positive control in this run. T0342 and T8311 are also positive for *eae* gene.

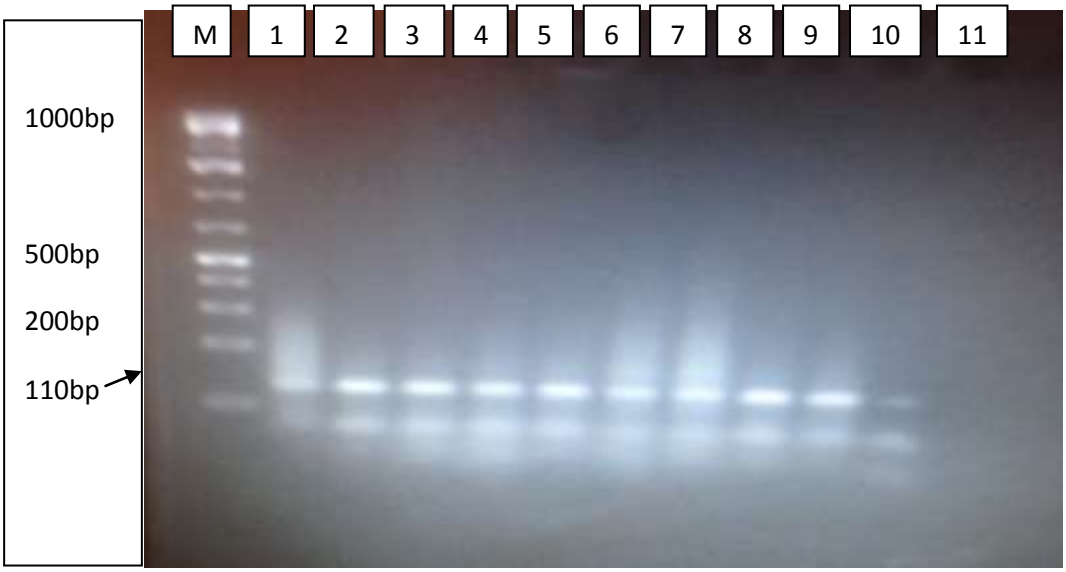

Figure 10: Amplified *stx2* gene, 110 bp amplicon, separated on 1.5 % agarose gel electrophoresis:

M= Marker, 1= 27D (Positive Control), 2= T845, 3= T1186, 4= T7142, 5= T8141, 6= T2934, 7= T8171, 8= T7147, 9= T543, 10= T1185, 11= Negative Control.

Based on the different virulence genes detected, *E. coli* pathotypes/ strains were identified as follows. Enteropathogenic *E. coli* (EPEC) strains were identified as those positive for *eae (intimin)* gene; shigatoxin producing *E. coli* (STEC) strains were identified as those positive for *stx2*; enterohemorrhagic *E. coli* strains were identified as those isolates positive for eae and *stx2*. Enteropathogenic *E. coli* are further classified into typical (positive for additional bundle forming pillus, *bfp*, gene) and atypical (negative for *bfp*) strains. Enterohemorrhagic *E. coli* (EHEC) strains were further supported by their plasmid gene hemolysin (*hlyA)* gene. Isolates of *E. coli* with the plasmid gene *aatA* were considered as enteroaggregative *E. coli* (EAEC). Finally isolates with more than one virulence gene were considered as mixed pathotypes (Paton and Paton., 1998; O'Ryan *et al.*, 2005; Christian *et al*., 2010; Croxen and Finlay, 2010; Rajendran *et al*., 2010; Chattaway *et al*., 2011; Tobias and Vutukuru., 2012; Chandra *et al*., 2013)

Five virulence genes were detected and 25 pathotypes (six categories) of *E. coli* were identified based on which virulence genes/s they contained; Enteropathogenic *E. coli* (EPEC) was the most prevalent category with atypical EPEC being more common than typical EPEC.

Mixed pathotypes with more than one virulence gene constituted for only 1% in the present study. Summary of the findings is presented in table 6.

Table 6: Frequency of different *E. coli* pathotypes detected from diarrheic children less than 5 years of age at AAU CHS Tikur Anbessa General Specialized Hospital, 2017.

Virulence gene detected	Frequency among *E. coli* isolates; N (%)	Frequency among diarrheic children; N (%)	Pathotypes / strains designation
eae	9 (16%)	9/98 (9.1%)	Atypical EPEC
Eae + bfp	3 (5.4%)	3/98 (3.1%)	Typical EPEC
Stx2	8 (14.3%)	8/98 (8.1%)	STEC
aatA	3 (5.4%)	3/98 (3.1%)	EAEC
Stx1+eae	1 (1.8%)	1/98 (1%)	EHEC
hlyA	1 (1.8%)	1/98 (1%)	EHEC
Stx1 + aatA	1 (1.8%)	1/98 (1%)	Mixed pathotypes
Total pathotypes	25/56 (44.6%)	25/98 (25.5%)	

The distribution of *E. coli* pathotypes among the age groups of children is shown in table 7.

Table 7: Occurrence of *E. coli* pathotypes among different age categories of diarrheic children less than 5 years of age at AAU CHS Tikur Anbessa General Specialized Hospital, 2017.

Age group	Pathotypes detected						
	Atypical EPEC (eae +)	STEC (stx2+)	Typical EPEC (eae +, bfp+)	EAEC (aatA+)	Mixed pathotypes (aatA+, stx1+)	EHEC (hlyA+,stx)	Total
0 – 6 months	1	0	0	1	0	0	2
7 – 24 months	4	3	1	2	1	1	12
24 – 60 months	4	5	2	0	0	0	11
Total	9 (36%)	8 (32%)	3 (12%)	3 (12%)	1 (4%)	1 (4%)	25 (100%)

Detection of the virulence gene *eae* was also analyzed among the clinical characters of the children and the results are presented in table 8.

Table 8: Clinical characters of diarrheic children less than 5 years of age and occurrence of *E. coli eae* gene at AAU CHS Tikur Anbessa General Specialized Hospital, 2017

Risk factor			eae occurrence
Animal Contact	YES	17	4/56
	NO	39	8/56
Habit of eating undercooked food	YES	20	5/56
	NO	36	7/56
Habit of boiling water	YES	9	2/56
	NO	47	10/56
Family history of illness	YES	12	3/56
	NO	44	9/56
Previous history of illness	YES	20	5/56
	NO	36	7/56

5.4. Antibiotic susceptibility profiles of *E. coli* pathotypes identified from diarrheic children

Drug susceptibility profiles of the different pathotypes detected showed that the pathotypes exhibited higher resistance to most of the antibiotics tested. Lower resistance was exhibited by the pathotypes towards Cefotetan.

One interesting observation is to see that plasmid encoded virulence genes, bfp and aatA, are associated with increase in resistance, actually 100% resistance which may be a potential indicator for co-carriage of both virulence and resistance gene on the plasmid. The results are summarized in the table 9.

Table 9: Distribution of resistance profiles among different pathotypes of *E. coli* detected from children less than 5 years of age at AAU CHS Tikur Anbessa General Specialized Hospital, 2017.

Antibiotics	Antibiotic resistance frequency of *E. coli* pathotypes			
	Typical EPEC (*eae*+)	STEC (*stx1*+)	Atypiccal EPEC (*eae* + *bfp*+)	EAEC *(aatA+)*
Trimethoprim	9 (75%)	7 (77.8%)	3 (100%)	2 (50%)
Ciprofloxacin	6 (50%)	5 (55.6%)	1 (33.3%)	4 (100%)
Ampicillin	11(91.7%)	6 (66.7%)	3 (100%)	4 (100%)
Neomycin	12 (100%)	8 (88.9)	3 (100%)	2 (50%)
Gentamycin	10 (83.3%)	5 (55.6%)	3 (100%)	4 (100%)
Compound sulphonamide	10 (83.3%)	8 (88.9)	3 (100%)	4 (100%)
Tetracycline	11(91.7%)	8 (88.9)	3 (100%)	4 (100%)
Chloramphenicol	7 (58.3%)	3 (33.3%)	1 (33.3%)	3 (75%)
Cefotitan	2 (16.7%)	2 (22.2%)	1 (33.3%)	1 (25%)
Norfloxacin	6 (50%)	2 (22.2%)	1 (33.3%)	4 (100%)
Streptomycin	10 (83.3%)	2 (22.2%)	3 (100%)	2 (50%)

Multi drug resistance was observed in all categories of *E. coli* pathotypes. All of the pathotypes were found to be resistant to at least 4 antibiotics. The highest prevalence of multi drug resistance was resistance to 8 antibiotics tested: trimethoprim, ampicillin, neomycin, gentamycin, compound sulfonamide, streptomycin, chloramphenicol and tetracycline among the antibiotics tested. One of the isolates exhibited resistance to all 11 antibiotics tested. The results are summarized in table 10.

Table 10: Multi drug resistance of *E. coli* pathotypes isolated from children less than 5 years of age at AAUCNS Tikur Anbessa General Specialized Hospital, 2017.

Number of drugs resisted	Resisted drugs	Number of isolates that showed MDR	Percent
4	Neomycin Compund sulphonamide Gentamicin Ampicilin	2	8
6	Neomycin Compund sulphonamide Gentamicin Ampicilin Streptomycin Tetracycline	1	4
7	Neomycin Compund sulphonamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim	6	24
8	Neomycin Compund sulphonamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim Ciprofloxacin	9	36
11	Neomycin Compund sulphonamide Gentamicin Ampicilin Streptomycin Tetracycline Trimethoprim Ciprofloxacin Chloramphenicol Norfloxacin Cefotitan	1	4
Total		25	100.0

6. DISCUSSION

Despite the significant contribution that pathogenic *E. coli* strains have to the burden of diarrhea, their distribution in Africa is not well studied. Studies undertaken in few countries like Kenya, Nigeria and South Africa have been concentrated on certain localities and specific risk populations. This has led to the lack of capacity to detect diarrheagenic *E. coli* in patients with diarrhea. Diarrheagenic *E. coli* are rarely included in the range of target organisms in many studies in Africa (Okeke, 2009).

Pathogenic *E. coli* is not usually considered as a possible cause of child diarrhea but diagnostic tests requested at Tikur Anbessa Hospital are mostly for *salmonella* and *shigella*. i.e, there is no appropriate laboratory diagnosis for pathogenic *E. coli.* In the present study, *E. coli* was found in 56/98 (57.1%) pediatric children less than 5 years of age at AAU CHS Tikur Anbessa General Specialized Hospital. This figure is higher than results which were reported by Ayrikim Adugna *et al*. (2015) (204/422, 48.3%), Amisano *et al*. (2011) (75/160, 46.9%) and Saeed *et al*. (2015) (211/437, 48%).

The occurrence of *E. coli* was similarly rated in all age – groups examined and in both sexes of the children involved in the study. This finding is in contrast with Ayrikim Adugna *et al*. (2015) which reported that the isolation rate of *E. coli* was high in children aged 6 – 23 months. Antibiotic resistance rate of *E. coli* isolates in this study was generally high (>40%). Similar results were reported by Sang *et al*. (2012) which indicated that *E. coli* isolates showed high level of resistance to commonly used antimicrobial agents and Cambrea, (2014) which showed that *E. coli* isolates exhibited decreased susceptibility to locally available antimicrobial agents. But this finding is in contrast with Amaya *et al*.(2011), which asserted that *E. coli* isolates exhibited low resistance to most antimicrobial drugs and that *E. coli* have not reached high level of resistance to commonly used antibiotics.

In the present study, *E. coli* isolates showed high resistance rates towards many antimicrobial drugs including ampicillin, trimethoprim, gentamycin and tetracycline. This is in agreement with Ayrikim Adugna *et al*. (2015), in which high level of resistance to ampicillin (86.8%), and tetracycline (76%) was documented.

But the finding is in contrast with Ansari *et al*. (2012), in which 91.7% *E. coli* isolates were susceptible to gentamycin and 75 % isolates were susceptible to tetracycline. It is also in contrast with Saeed *et al*. (2015) in which 94% *E. coli* isolates were susceptible to Gentamicin.

Low resistance was exhibited by *E. coli* isolates in the present study towards Chloramphenicol, Ciprofloxacin, and Norfloxacin; Cefotetan being the antibiotic to which the isolates showed the highest susceptibility. This is in agreement with Sang *et al*. (2012) in which resistance to Ciprofloxacin and Norfloxacin at low levels was documented. On the other hand, Ansari *et al*. (2012) and Saeed *et al*. (2015) reported 100% susceptibility of *E. coli* isolates to Chloramphenicol which is in contrast with the present study.

Multidrug resistance was observed in all isolates of *E. coli* in the present study. This is in contrast with Estrada – García *et al*. (2005) in which multidrug resistance was reported in 58% of isolates. The highest prevalence of multi drug resistance of *E. coli* isolates in the present study was resistance to 7 antibiotics tested: trimethoprim, ampicillin, neomycin, gentamycin, compound sulfonamide, streptomycin, and tetracycline.

This is in contrast with Amaya *et al*. (2011) in which the most prevalent multidrug resistance in *E. coli* isolates was resistance to 2 antibiotics. Factors like mutation which give selective advantage to resistant strains may be involved in the high antibiotic resistance observed in the present study. The high antibiotic resistance rate observed may indicate that the number of potential commensal reservoirs of resistance genes which can be transferred to pathogens is on increase and that an increase in the emergence of drug resistant pathogenic *E. coli* strains is a possibility. The finding in the present study may be a useful indicator of bacterial antibiotic resistance in the local community.

25/56 (44.6%) of the *E. coli* isolates in this study were found to be pathogenic classes carrying different virulence genes. This study investigated the identity of Pathogenic *E. coli* strains that occurred in child diarrhea using gene specific primers. Thus, the pathogenic strains named STEC, EPEC, EHEC, and EAEC were identified from processed samples. Higher frequency of pathogenic strains than the present study was reported in Christian *et al*. (2010) (77%). Lower figures were reported by Moyo *et al*. (2011) (22.9%) and Bonkoungou *et al*. (2013) (24%).

Estrada – García *et al*. (2005), reported 14 % isolation rate of diarrheagenic *E. coli* and Amisano *et al*. (2011) reported 13.33%.

Atypical EPEC were the most prevalent pathotypes in the present study being found in 16 % of *E. coli* isolates followed by STEC which constituted for 14.3 % of *E. coli* isolates. Similar finding was reported by Christian *et al*. (2010). But the finding in the present study is in contrast with Saeed *et al*.(2015) and Bonkoungou *et al*.(2013) which reported that EAEC were the most frequent pathotypes. Similarly, Rajendran *et al*. (2010) reported higher occurrence of typical EPEC strains than atypical ones which is in contrast with the present study.

The high isolation rate of pathogenic strains in the present study may be attributed to increased evolution of pathogens through horizontal gene transfer of mobile genetic elements harboring virulence genes and may be an indication that there is tremendous environmental ecological niche which is a reservoir of virulence genes. Extensive hospital and environment based virulence studies are required to come up with more conclusive data.

The isolation of mixed pathotypes (isolates possessing more than one virulence gene) was 1.8% in the present study (1/56). Rajendran *et al*. (2010) reported a 3.8% isolation rate of mixed pathotypes. A study made in Brazil found that infection by mixed pathotypes was generally rare (Nunes *et al*., 2012). Taking more than one colony of bacteria from the original culture for extraction of bacterial DNA may increase the likelihood of identifying mixed pathotypes from a single patient.

Diarrheagenic *E. coli* in the present study exhibited high resistance rates towards many of the antimicrobials tested; low resistance rates were observed towards Cefotitan. This is in agreement with Nguyen *et al*., 2005 in which low sensitivity of *E. coli* isolates was documented towards Ampicillin, Chloramphenicol and Ciprofloxacin.

Mitra *et al*. (2011) reported 100% resistance of EPEC strains to ampicillin which is in agreement with the present study in which resistance to ampicillin was 100% for atypical EEC strains.

Pathotypes with plasmid coded virulence genes showed increased resistance rates in the present study as indicated in table 11 in the result section. This may be an indication of co – carriage of virulence and resistance genes on the plasmids of the pathotypes.

Antibiotic resistance may be usually associated with environmentally acquired extra chromosomal mobile genetic elements including plasmids, transposons, and integrons apart from chromosomal mutations which are usually considered to be rare (Alekshun and Levy, 2007).

E. coli are known to show high genetic flexibility in that resistance and virulence genes can be transferred together from one bacteria to another through horizontal gene transfer. Resistance occurs more effectively due to transfer of resistance genes than chromosomal mutation. Plasmids encoding genes that confer resistance to different classes of antimicrobial agents including cephalosporins, fluoroquinolones, aminoglycosides & virulence determinants that help bacterial cells to have adaptability and fitness in different ecologies can render bacteria both pathogenic and resistant (Giedraitienė *et al.*, 2011). *E. coli* plasmids can thus carry both virulence and resistance genes. Accessory genetic materials in bacteria can acquire resistance genes there by promoting their transmission between bacteria. Among other mobile genetic elements, plasmid – mediated transmission is the most common mechanism of such horizontal transfer of resistance genes (Davies and Davies, 2010).

Co – evolution of both virulence and resistance can be an explanation for the increased resistance pattern in the pathotypes with plasmid coded virulence genes *aatA* and *bfp* in the present study. This can be an area of extended research on association between virulence and resistance.

Pathotypes generally showed high resistance patterns in the present study with atypical EPEC and EAEC showing the highest resistance rates. This is in contrast with Christian *et al*., 2010 in which pathogenic *E. coli* exhibited less prominent resistance. But the finding of the present study is in agreement with Ochoa *et al*., 2009 in which differential resistance patterns were exhibited by individual *E. coli* pathotypes with EAEC strains exhibiting higher resistance level than EPEC. The high antibiotic resistance rates in the present study may be attributed to the presence of commensal resistant bacteria which act as reservoirs of resistance genes that are transferred to pathogenic strains (Okeke, 2009).

100 % multi drug resistance was observed in all pathotypes of *E. coli* in the present study. The most prevalent multidrug resistance among isolated pathogenic strains was resistance to 8 antibiotics tested: trimethoprim, ampicillin, neomycin, gentamycin, compound sulfonamide, streptomycin, chloramphenicol and tetracycline among the antibiotics tested. This result is higher than results reported by other studies.

70.6 % multi drug resistance was reported in Mitra *et al*. (2011) in isolated EPEC pathotypes. 86.4% multidrug resistance of EAEC pathotypes was reported in a study made in Iran (Khoshvaght *et al*., 2014). Haghi *et al.* (2014) reported 86.4% multi drug resistance of *E. coli* pathotypes. Factors like duration of hospital stay, self medication, Poor Patient's compliance; environmental conditions may be involved in the emergence of multi drug resistant strains (Tula *et al.*, 2015).

Pathotypes which harbor chromosomal virulence genes were identified in higher numbers in the present study while pathotypes which harbor plasmid virulence genes like EAEC were comparably few. This may be due to loss of integrity of plasmids as a result of delay in bacterial DNA extraction after isolation of the bacteria.

7. CONCLUSION AND RECOMMENDATION

The isolation rate of *E. coli* in this study was high. *E. coli* exhibited high rates of antibiotic resistance to many of the antibiotics tested including ampicillin, gentamycin, chloramphenicol and tetracycline. Moreover, all *E. coli* exhibited multiple drug resistance. This is an indication that there is a need for extensive study on the occurrence, risk factors and genetic back ground of antimicrobial resistance of *E. coli* in the study area. The involvement of antibiotic resistant pathogenic *E. coli* in diarrheic children is prominent and hence diagnosis and antimicrobial sensitivity testing procedures need to be incorporated in to routine laboratory practices at AAU CHS Tikur Anbessa General Specialized Hospital. Determination of antibiogram before antibiotic Prescription for effective treatment is recommended. Among antibiotics tested, Cefotitan was found to be the most effective drug against isolates of *E. coli*.

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ANNEX I. English version of Information Sheet and informed consent

Title of Research Study: PATHOGENIC E.COLI STRAINS AND THEIR ANTIBIOTIC SUSCEPTIBILITY PROFILES IN CASES OF CHILD DIARHEA AT AAUCHS TIKUR ANBESSA GENERAL SPECIALIZED HOSPITAL

Dear Client,

My name is Benyam Zenebe G/yesus. I am a graduate student of Applied Genetics at Addis Ababa University College of Natural Sciences. I am requesting you to allow involving your child in my MSc thesis - a study to investigate Pathogenic *E. coli* trains and their Antibiotic Susceptibility Profiles in cases of child diarrhoea. Before you decide whether to take part in the study, I would like to introduce you what the research is about. It is up to you to decide whether or not to take part. You will also be asked to sign a consent form. You can change your mind at any time and withdraw from the study without giving a reason. You are encouraged to ask questions or raise concerns at any time about the nature of the study and you are welcome to call me if you would like to get any further information. My phone number is 0941610878.

Purpose of the study: This is an MSc thesis and the purpose of the study is to investigate pathogenic strains of *E. coli* in under five children with diarrhoea.

Procedure and selection criteria: I would like to take a stool specimen from your child and check for the stated etiologies in a laboratory. All children less than five years of age with diarrhea can be included in this study and there are no any other criteria.

Benefits and risks: Additional laboratory diagnosis will be offered to your child free of charge because of your participation in this study and this will help in choosing an appropriate therapy for your child. No risks will happen to your child because of participation in this study.

Result treatment and confidentiality: Information generated from this study will be used to write an MSc thesis project besides the treatment given to your child. The results of the study and your response of the questionnaire interview will be treated with full confidentiality and anyone who takes part in the research will be identified by code numbers only.

At the end of the research I will write a report and the results may be published in different journals and presented in different conferences. Your participation will not be identifiable from any publications. This study has been reviewed and approved by the Research Ethics Committee at Addis Ababa University College of Natural Sciences.

By signing below I acknowledge that I have read and understand the above information. I am aware that I can discontinue my participation in the study at any time.

Signature_____ Date_____

ANNEX II. Amharic version of Information Sheet and informed consent

የጥናቱ ርእስ፡- በአዲስ አበባ ዩኒቨርሲቲ ጤና ሳይንስ ኮሌጅ ጥቁር አንበሳ ሆስፒታል የህፃናት የተቅማጥ በሽታን የሚያመጡ የኢሸርሽያ ኮላይ ባክቴሪያ ዝርያዎች ስርጭትና ለተለያዩ መድኃኒቶች ያላቸው የተላምዶ ሁኔታን ማጥናት፡፡

ውድ መላሾች

ስሜ ቢንያም ዘነበ ገ/የሱስ ይባላል፡፡ በአዲስ አበባ ዩኒቨርሲቲ የተፈጥሮ ሳይንስ ኮሌጅ የአፕላይድ ጀነቲክስ የድህረ ምረቃ ተማሪ ነኝ፡፡ አሁን የምጠይቅዎ ልጅዎን በመመረቂያ ማሚያ ጥናቴ ላይ ለማሳተፍ እንዲፈቅዱልኝ ነው፡፡ በዚህ ጥናት ላይ ከመሳተፍዎ በፊት ስለጥናቱ ሁኔታ ልገልፅልዎ እወዳለሁ፡፡ ጥናቱ ላይ ለመሳተፍ ወይም ላለመሳተፍ መብትዎ ሙሉ በሙሉ የተጠበቀ ነው፡፡ ጥናቱ ከተጀመረ በኋላም በማንኛውም ሰዓት ተሳትፎዎን የማቋረጥ ማለትም የልጅዎ ውጤት ጥናቱ ላይ እንዳይካተት የመጠየቅ ሙሉ መብት አለዎት፡፡ በማንኛውም ሰዓትና ሁኔታ ማብራሪያ ከፈለጉ በ 0941610878 ይደውሉልኝ፡፡

የጥናቱ ዓላማ፡- በህፃናት ላይ የተቅማጥ በሽታን የሚያመጡ የኢሸርሽያ ኮላይ ባክቴሪያ ዝርያዎችና ለተለያዩ መድኃኒቶች ያላቸውን ተላምዶ ማጥናት፡፡

ጥናቱ ላይ የመሳተፍ ሁኔታ፡- ከልጅዎ የዓይነ ምድር ናሙና የሚወሰድ ሲሆን የተጠቀሱት ታህዋስያን መኖር አለመኖራቸውን በላቦራቶሪ ውስጥ ይታያል፡፡ ዕድሜያቸው ከአምስት ዓመት በታች የሆነና የተቅማጥ በሽታ ያለባቸው ህፃናት በሙሉ በዚህ ጥናት ውስጥ መካተት ይችላሉ፡፡ ጥናቱ ሌላ ተጨማሪ መስፈርት የለውም፡፡

የጥናቱ ጥቅምና ጉዳት፡- በዚህ ጥናት ላይ ልጅዎን በማሳተፍዎ ለልጅዎ የላቦራቶሪ ምርምራ በ ነፃ የሚሰጥ ሲሆን በ ምርመራው መሰረትም አስፈላጊው ህክምና የሚሰጥ ይሆናል፡፡ በዚህ ጥናት ላይ ልጅዎን በማሳተፍዎ ምክንያት በልጅዎ ላይ የሚደርስ ምንም ዓይነት ጉዳት የለም፡፡

የጥናቱ ውጤት አጠቃቀምና ሚስጢራዊነት፡- ከዚህ ጥናት ላይ የሚገኙ ውጤቶች ለ ልጅዎ ከሚሰጠው ህክምና በተጨማሪ ለ ሁለተኛ ዲግሪ መመረቂያ ፅሑፍ ማሚያ የሚውሉ ሲሆን ሚስጥራዊነታቸው የተጠበቀ ነው፡፡ በጥናቱ ላይ የሚሳተፉ ህፃናት ናሙና በመለያ ቁጥር እንጂ በስም አይመዘገብም፡፡ ስለ ትብብርዎ አመሰግናለሁ፡፡

ልጄ በዚህ ጥናት ላይ እንዲሳተፍ እፈቅዳለሁሁ፡

ፊርማ _____ **ቀን** _____

ANNEX III. English version of Questionnaire for stool specimen collection from diarrheic children.

1. Age_____
   _____
2. Sex_____
3. Identification nuber_____
4. Family address_____
5. Father's educational status (please specify)_____
6. Mother's educational status (please specify)_____
7. Father's occupation_____
8. Mother's occupation_____
9. 6. Clinical diagnosis of the child_____
10. Duration of diarrhea_____
11. Family member with similar illness

Yes ☐ No ☐

12. Blood in stool

Yes ☐ No ☐

13. RVI status

Positive ☐ Negative ☐

14. Child previous history of similar illness

15. Yes ☐ No ☐

15. If your response is yes, how was the illness managed?

16. If there was drug medication, which drug was given?

17. Child Body Mass Index

Underweight ☐ Normal ☐ Overweight ☐

18. Frequency of diarrhea

3 – 5times /day ☐ >5 times/day ☐

19. Is there a habit of eating undercooked food in your family? Yes ☐ No ☐

20. Contact with animals. Yes ☐ No ☐

21. Practice of boiling to sterilize drinking water. Yes ☐ No☐

ANNEX IV. Amharic version of Questionnaire for stool specimen collection from diarrheic children.

1. ዕድሜ _____
2. ዖታ _____
3. መለያ ቁጥር _____
4. የቤተሰብ አድራሻ_____
5. የአባት የትምህርት ደረጃ (ይጥቀሱ)_____
6. የእናት የትምህርት ደረጃ (ይጥቀሱ)_____
7. የአባት ስራ _____
8. የእናት ስራ_____
9. ለህፃኑ ህመም በሐኪም የተሰጠው ስም _____
10. ተቅማጡ የቆየበት ጊዜ _____
11. በቤተሰብዎ በተመሳሳይ ህመም የተጠቃ ሰው አለ?

    አዎ ☐ አይደለም ☐
12. የህፃን የዓይነ ምድር ሁኔታ

    ደም የተቀላቀለበት ☐ ደም ያልተቀላቀለበት ☐
13. የህፃን የኤች አይ ቪ ምርመራ ውጤት

    ፖዝቲቭ ☐ ነጌቲቭ☐
14. ህፃንዎ ከዚህ በፊት በተመሳሳይ ህመም ተጠቅቶ ነበር

    አዎ ☐ አይደለም ☐

15. አዎ ካሉ ምን ዓይነት የ ሕክምና አገልግሎት ተሰጠው

16. መድሐኒት ተሰጥቶ ከነበር የመድሐኒቱን ዓይነት ይጥቀሱ

17. የህፃን ቁመትና ክብደት ተመጣጣኝነት

    ከልክ በታች ☐ ትክክለኛ ☐ ከልክ በላይ ☐

18. የተቅማጥ ድግግሞሽ በቀን

    3-5 ጊዜ ☐ ከ5 ጊዜ በላይ ☐

19. በቤተሰብ ውስጥ በደንብ ያልበሰለ ምግብ የመብላት ልምድ

አለ ☐ የለም ☐

20. ህፃን ከ ለማዳ እንስሳት ጋር

ንክኪ አለው/አላት ☐ ንክኪ የለውም/የላትም ☐

21. በቤተሰብ ውስጥ ለ መጠጥ የሚሆን ውሃን የማፈላት ልምድ

አለ ☐ የለም ☐

Declaration

I, the undersigned, declare that this master thesis is my original work and has not been presented for a degree in any other university and that all sources of materials that were used for this thesis have been correctly acknowledged.

Name: Benyam Zenebe

Date----------

Signature----------

This thesis has been submitted for examination with our approval as advisor:

Dr. Tesfaye Sisay

Date----------

Signature----------

Dr. Gurja Belay

Date----------

Signature----------

Date----------

Signature----------