OPEN ACCESS Freely available online

Clinical Pediatrics: Open Access

Research Article

Pathogenic *Escherichia coli* Strains and their Antibiotic Susceptibility Profiles in Cases of Child Diarrhea at Addis Ababa University, College of Health Sciences, Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

Benyam Zenebe[1*], Tesfaye Sisay[2], Gurja Belay[3], Workeabeba Abebe[4]

[1]Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia; [2]Department of Biotechnology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia; [3]Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia; [4]Department of Pediatrics and Child Health, School of Medicine, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

ABSTRACT

Background: 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 specialized hospital, Addis Ababa, Ethiopia.

Methods: A purposive study which included 98 diarrheic children less than five years of age was conducted at Addis Ababa university college of health sciences, Tikur Anbessa specialized hospital, Addis Ababa, Ethiopia to detect pathogenic *E. coli* biotypes. Stool culture was used to identify presumptive *E. coli* isolates. Presumptive isolates were confirmed by biochemical tests and anti-microbial susceptibility tests were performed on confirmed *E. coli* isolates by disk diffusion method. DNA was extracted from confirmed isolates by heating method and subjected to PCR for the presence of virulence genes. Amplified PCR products were analyzed by agarose gel electrophoresis. Data were collected on child demographics and clinical conditions using administered questionnaires. 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; the distribution of pathogenic *E. coli* biotypes among different age groups and between the sexes were determined by using descriptive statistics.

Results: Out of 98 stool specimens collected from diarrheic children less than 5 years of age, 75 presumptive *E. coli* isolates were identified by culture; further confirmation by biochemical tests showed that only 56 of the isolates were *E. coli*; 29 of the isolates were found in male children and 27 of them in female children. Out of the 58 isolates of *E. coli*, 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.

Correspondence to: Benyam Zenebe, Department of Microbial, Cellular and Molecular Biology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, Ethiopia, Tel: +251911627679; E-mail: bennyboy6219@gmail.com

Received: 04-Jul-2023, Manuscript No. CPOA-23-25401; **Editor assigned:** 06-Jul-2023, PreQC No. CPOA-23-25401 (PQ); **Reviewed:** 20-Jul-2023, QC No. CPOA-23-25401; **Revised:** 11-Jul-2024, Manuscript No. CPOA-23-25401 (R); **Published:** 18-Jul-2024, DOI: 10.35248/2572-0775.24.9.271

Citation: Zenebe B, Sisay T, Belay G, Abebe W (2024) Pathogenic *Escherichia coli* Strains and their Antibiotic Susceptibility Profiles in Cases of Child Diarrhea at Addis Ababa University, College of Health Sciences, Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia. Clin Pediatr. 9:271.

Conclusion: This study found that the isolation rate of *E. coli* and the involvement of antibiotic resistant pathogenic *E. coli* in diarrheic children are prominent and hence focus should be given on the diagnosis and antimicrobial sensitivity testing of pathogenic *E. coli* at Addis Ababa university college of health sciences Tikur Anbessa specialized hospital. Among antibiotics tested, cefotitan could be a drug of choice to treat *E. coli*.

Keywords: *E. coli*; Pathogenic; Diarrhea; Children; Antibiotic susceptibility

Abbreviations: EAEC: Entero Aggregative *E. coli*; EHEC: Entero Hemorrhagic *Escherichia coli*, STEC: Shigatoxin producing *Escherichia coli*; EPEC: Entero Pathogenic *Escherichia coli*; HGT: Horizontal Gene Transfer; EMB: Eosin Methylene Blue; BFP: Bundle Forming Pilus; MMR: Methyl Directed Mismatch Repair; SD: Standard Deviation; WHO: World Health Organization; PCR: Polymerase Chain Reaction; MDR: Multi Drug Resistance; DNA: Deoxyribonucleic Acid

INTRODUCTION

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 double stranded 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.

The reference *E. coli* genome (k-12) 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 [1].

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 [2].

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 [3].

A study on mutation rates of *E. coli* has shown that mutation rates in isolated pathogenic *E. coli* are higher than the average bacterial population with defect in methyl directed mismatch repair being the predominant underlying cause. However, high mutation rates due tomethyl directed mismatch repair were demonstrated in both pathogenic and commensal *E. coli* strains in another independent survey of mutation rates of *E. coli* populations isolated from distinct environments. 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.

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 [4].

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*; Entero Pathogenic *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 [5].

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* contains 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*); entero aggregative *E. coli* produce pic and other toxins.

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 [6].

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 [7].

MATERIALS AND METHODS

Study area

This study was conducted at Tikur Anbessa 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 2500 m above sea level.

The average annual temperature and rainfall are 21°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 college of health sciences.

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 [8].

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 50 gm 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.

Isolation of *E. coli*

Broth specimens were be inoculated on MacConkey agar and incubated aerobically at overnight. Lactose fermenting colonies on MacConkey agar were then sub cultured in to eosin methylene blue and incubated aerobically at 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 [9].

Biochemical characterization of *E. coli* isolates

Presumptive *E. coli* isolates were further characterized for their biochemical activity using the biochemical tests Indole, Methyl red, Vogues Proskuer and Citrate utilization (IMViC). Bacterial isolates that exhibited IMViC pattern of (+ + - -) respectively were considered as *E. coli* isolates.

Indole test

A sterilized test tube containing 4 ml of tryptophan broth was inoculated aseptically by taking an inoculum from 18 hrs 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 [10].

Vogues-Proskuer (VP) test

The medium was inoculated with an inoculum taken from an 18 hour-24 hour pure culture and incubated aerobically at 37°Cfor 24 hours. 1 ml of the broth was transferred to a clean test tube following 24 hours of incubation. The remaining broth was re-incubated for an additional 24 hours. 0.6 ml of 5% alpha-naphthol was added to the 1 ml broth and next 0.2 ml of 40% KOH was added. By gently shaking to expose the medium to atmospheric oxygen, the tube was allowed to remain undisturbed for 10 minutes-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 [11].

Citrate utilization test

Simmons citrate agar was inoculated on the slant by touching the tip of a needle to a colony that is 18 hours to 24 hours old and incubated at 37°C for 24 hours. Development of blue color on the slant surface due to the alkaline carbonates and bicarbonates produced as byproducts 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 result.

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 (5 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), 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°C 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°C [12]. 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.

Virulence gene detection

DNA extraction: Biochemically confirmed *E. coli* isolates were grown in nutrient broth at 37°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°C for 10 minutes. The lysate was then centrifuged again as before and 50 µl of the supernatant used directly as template for PCR.

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 25 µl reaction volume in a PCR master mix (Himedia; India, 2017) containing 1 µ1 of each primer (EVS1, EVC2), 2.5 µl 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 µ1 of 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°C; 30 cycles each consisting, 40 s at 95°C, 40 s at 55°C, 30 s at 72°C; and a final extension of 1 cycle for 8 min. at 72°C [13].

To detect the *EAE* (intimin) gene of EPEC and EHEC strains, a reaction was set up in a 25 µl reaction tube in a PCR master mix (Himedia; India, 2017) containing 1 µ1 of each primer (EAE1 and EAE2), 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35 mm of each dNTP, 1 µ1 of 50 Mmol $MgCl_2$, 0.5 µl of Taq polymerase enzyme, 15 µ1 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°C; 35 cycles, each consisting of 40 s at 95°C, 60 s at 55°C and 60 s at 72°C; and a final extension of 1 cycle for 10 min. at 72°C.

To detect the *bfp* (bundle forming pillus) gene of typical EPEC strains, a reaction was set up in a 25 µl reaction tube in a PCR master mix (Himedia; India, 2017) containing 0.5 µ1 of each primer (BFPF, BFPR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35 mm 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°C; 30 cycles, each consisting of 40 s at 95°C, 40 s at 57°C, 30 s at 72°C; and a final extension of 1 cycle for 8 min at 72°C [14].

To detect *hylA* (hemolysin) gene of EHEC, reaction components were mixed in a 25 µl reaction tube in a PCR master mix (Himedia; India, 2012) containing 1 µ1 of each primer (EHECF, EHECR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35 mm 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°C; 30 cycles, each consisting of 40 s at 95°C, 1 min. at 45°C, 1 min at 72°C; and a final extension of 1 cycle for 10 min at 72°C [15].

To detect *aatA* (antiagrgation transporter gene) of EAEC strain reactions were set up in a 25 µl reaction tube in a PCR master mix containing 1 µ1 of each primer (EAECF, EAECR) containing 2.5 µl of PCR buffer with 17.5 mmol of $MgCl_2$, 1 µl of 0.35 mm 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°C; 30 cycles each consisting of, 40 s at 95°C, 1 min. at 45°C, 30 s at 72°C ; and a final extension of 1 cycle for 10 min. at 72°C [16].

All amplifications were carried out in a thermal cycler (applied BiosystemsStepOne™ real-time PCR system thermal cycling block).

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. 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 carried out in the PCR assays used were as indicated in Table 1 [17].

Table 1: Primer gene sequence and PCR conditions.

Primer	Nucleotide sequence	Target gene	Pathogenic *E. coil* strain	Denaturing	Annealing	Extension	Product size (Bp)	Cycles	Reference
EAE1	F:5'AAACAG GTGAAAC TGTTGCC3'	*eae eae*	EPEC/ EHEC	94°C,2 min.	55°C, 60 s	72°C, 60 s	490	35	Khan, et al.
EAE2	R: 5'-CTCTGC AGATTAAC CTCTGC-3'								
EVS1	F: 5'-ATCAGT CGTCACT CACTGGT-3'	*Stx2 Stx2*	STEC/ EHEC	94°C, 2 min.	55°C, 6°C	72°C, 60 s	110	30	Khan, et al.
EVC2	R: 5'-CTGCT GTCACAGT GACAAA-3'								
EHEC	F: 5'-ACGAT GTGGTTTA TTCTGGA-3'	*hlyA*	EHEC	95°C, 3 min.	45°C, 40 s	72°C, 30 s	165	30	Paton and Paton, et al.
EHEC	R:5'-CTTCA CGTCACCA TACATAT-3'	*hlyA*							
EAEC	F: 5'-CTGGC GAAAGACT GTATCTAT-3'	*aatA*	EAEC	95°C, 3 min.	45°C, 40 s	72°C, 30 s	630	30	Chattaway, et al.
	R: 5'-CAATGT ATAGAAAT CCGCTGTT-3'								
EAEC		*aatA*							
BFP	F: 5'-AATGG TGCTTGCG CTTGCTG C-3	*bfpA bfpA*	EPEC	95°C, 3 min.	57°C, 40 s	72°C, 30 s	324	30	Christian, et al.
	R: 5'-GCCGCT TTATCCAA CCTGGTA-3'								

Note: EPEC: Enteropathogenic *E. coli*; EHEC: Enterohemorrhagic *E. coli*; STEC: Shiga-like Toxin producing *E. coli*; EAEC: Enteroaggregative *E. coli*

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.

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.

RESULTS

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

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 and female children and among three age groups of children involved in the study [19].

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

Age in months	Sex		Total
	M	F	
0 month-6 months	4 (4.1%)	2 (2%)	6 (6.1%)
7 months-24 months	13 (13.26%)	10 (10.24%)	23 (23.5%)
25 months-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

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 and 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 [20].

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.

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%)

Resistance pattern of *E. coli* isolates

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, compound sulphonamide, gentamicin, ampicillin	4	7.1
6	Neomycin, compound sulphonaamide, gentamicin, ampicillin, streptomycin, tetracycline,	4	7.1
7	Neomycin compund sulphonaamide, gentamicin, ampicillin, streptomycin, tetracycline, trimethoprim	15	26.8
8	Neomycin, compound sulphonaamide, gentamicin, ampicillin, streptomycin tetracycline, trimethoprim, ciprofloxacin	14	25
11	Neomycin, compound sulphonaamide, gentamicin, ampicillin, streptomycin tetracycline, trimethoprim, ciprofloxacin, chloramphenicol, norfloxacin, cefotitan	1	1.8
Total		56	100

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 1-5.

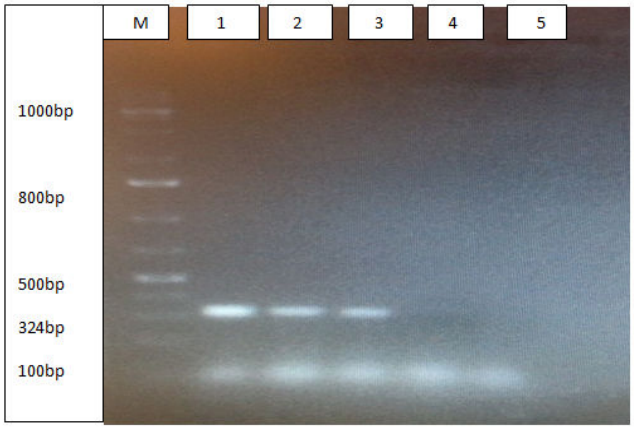

Figure 1: Amplified *bfp* gene, 324 bp amplicon, separated on 1.5 % agarose gel electrophoresis: M=Marker, 1=T8142, 2=T4163, 3=T008, 4=T1185, 5=negative control.

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

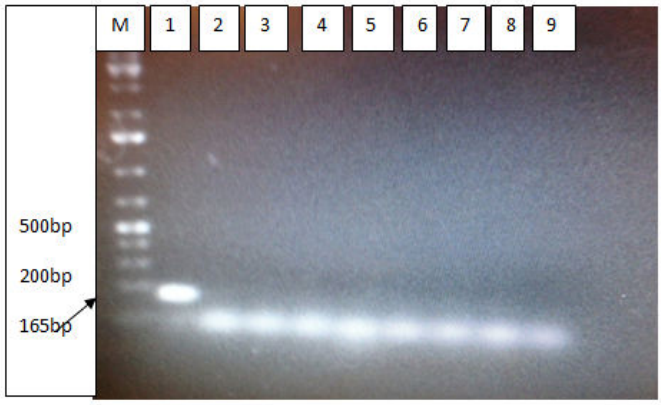

Figure 2: Amplified *hlyA* gene, 165 bp amplicon, separated on 1.5 % agarose gel electrophoresis.

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.

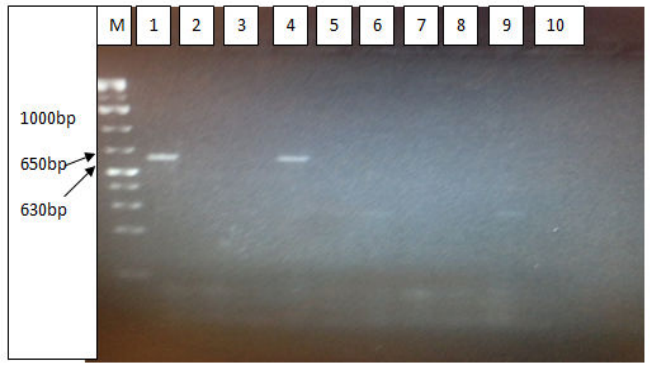

Figure 3: Amplified *aatA* gene, 630 bp amplicon, separated on 1.5 % agarose gel electrophoresis: M=Marker 1=T544, 2=T0438, 3=T1186, 4=T2934, 5=T7142, 6=T7147, 7=T7141, 8=T8142, 9=T2115, 10=negative control.

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.

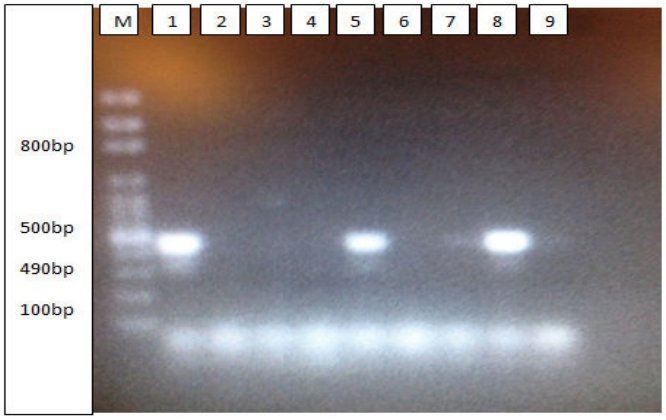

Figure 4: Amplified eae gene, 490 bp amplicon, separated on 1.5% agarose gel electrophoresis.

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.

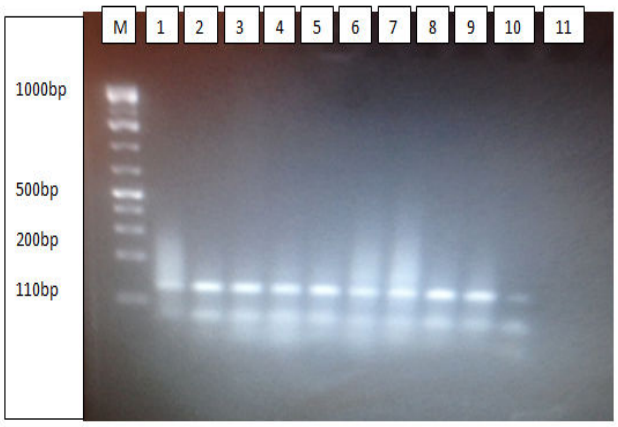

Figure 5: Amplified *stx2* gene, 110 bp amplicon, separated on 1.5% agarose gel electrophoresis: M=marker, 1=27D (positive control), 2=T845, 3=T1186, 4=T7142, 5=T8141, 6=T2934, 7=T8171, 8=T7147, 9=T543, 10=T1185, 11=negative control.

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. Entero Hemorrhagic *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 Entero Aggregative *E. coli* (EAEC). Finally isolates with more than one virulence gene were considered as mixed pathotypes. Five virulence genes were detected and 25 pathotypes (six categories) of *E. coli* were identified based on which virulence genes/s they contained; Entero Pathogenic *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						Total
	Atypical EPEC (*eae+*)	STEC (*stx2+*)	Typical EPEC (*eae+, bfp+*)	EAEC (*aatA+*)	Mixed pathotypes (*aatA+, stx1+*)	EHEC (*hlyA+, stx*)	
0 months-6 months	1	0	0	1	0	0	2
7 months-24 months	4	3	1	2	1	1	12
24 months-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

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 *aat*A, 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.

	Antibiotic resistance frequency of *E. coli* pathotypes			
Antibiotics	**Typical EPEC (*eae*+)**	**STEC (*stx1*+)**	**Atypiccal EPEC (*eae*+ *fp*+)**	**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 specialized hospital, 2017.

Number of drugs resisted	Resisted drugs	Number of isolates that showed MDR	Percent
4	Neomycin, compound sulphonamide, gentamicin, ampicilin	2	8

6	Neomycin, compound sulphonamide, gentamicin, ampicillin, streptomycin, tetracycline	1	4
7	Neomycin, compound sulphonamide, gentamicin, ampicillin, streptomycin, tetracycline, trimethoprim	6	24
8	Neomycin, compound sulphonamide, gentamicin ampicillin, streptomycin, tetracycline, trimethoprim, ciprofloxacin	9	36
11	Neomycin, compounds sulphonamide, gentamicin, ampicillin, streptomycin, tetracycline, trimethoprim, ciprofloxacin	1	4
Total	Chloramphenicol, norfloxacin, cefotitan	25	100

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* is rarely included in the range of target organisms in many studies in Africa. 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 Addis Ababa university, college of health sciences, Tikur Anbessa specialized hospital. Lower results were reported by other studies in Ethiopia (204/422, 48.3%), Sudan (211/437, 48%) and Italy (75/160, 46.9%). The occurrence of *E. coli* was similarly rated in all age groups examined and in both sexes of the children involved in the current study. This finding is in contrast with another study conducted in Ethiopia which reported that the isolation rate of *E. coli* was high in children aged 6 months-23 months. Antibiotic resistance rate of *E. coli* isolates in this study was generally high (>40%). Similar results were reported by studies made in Kenya and Eastern Romania which indicated that *E. coli* isolates showed high level of resistance to commonly use and locally available antimicrobial agents. But this finding is in contrast with reports of a study conducted in Nicaragua which show 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 result is in agreement with a previous study made in Ethiopia in which high level of resistance to ampicillin (86.8%), and tetracycline (76%) was documented. But the finding is in contrast with reports of a study made in Nepal in which 91.7% *E. coli* isolates were susceptible to gentamycin and 75% isolates were susceptible to tetracycline. It is also in contrast with a study made in Sudan which reported that 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. Resistance to ciprofloxacin and norfloxacin at low levels was documented in a similar study in Kenya.

On the other hand, 100% susceptibility of *E. coli* isolates to chloramphenicol was reported by studies from Nepal and Sudan in contrast to the present study. Multidrug resistance was observed in all isolates of *E. coli* in the present study. This is in contrast with a study conducted in Mexico 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 a study made in Nicaragua in 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 in the present study 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 a study conducted in Costarica in 2010 (77%). Lower figures were reported by studies from Tanzania (22.9%) and Burkina Faso (24%) in 2011 and 2013 respectively. A study conducted in Mexicoin 2005 reported 14% isolation rate of diarrheagenic *E. coli* from children and a similar study conducted in North Western Italy in 2011 reported 13.33% isolation rate. 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 in Costarica in 2010. But the finding in the present study is in contrast with findings from Burkina Faso and Sudan in 2013 and 2015 respectively which reported that EAEC were the most frequent pathotypes. A study in India also reported higher occurrence of typical EPEC strains than atypical ones in 2010 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). A 3.8% isolation rate of mixed pathotypes was reported in India in 2010. A study made in Brazil IN 2012 found that infection by mixed pathotypes was generally rare. 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 results of a study made in Vietnam in 2005 in which low sensitivity of *E. coli* isolates was documented towards ampicillin, chloramphenicol and ciprofloxacin. A study made in Iran in 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. 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 and virulence determinants that help bacterial cells to have adaptability and fitness in different ecologies can render bacteria both pathogenic and resistant. *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. Co-evolution of both virulence and resistance can be an explanation for the increased resistance pattern in the pathotypes with plasmid coded virulence genes *aat*A 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 reports of a study made in Costa Rica in 2010 in which pathogenic *E. coli* exhibited less prominent resistance. But the finding of the present study is in agreement with a finding from Peru in 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. 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 a study made in Iran in 2011 in isolated EPEC pathotypes. Similarly 86.4% multidrug resistance of *E. coli* pathotypes was reported in studies made in Iran in 2014. 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. 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.

CONCLUSION

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*.

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.

DECLARATION

I, Benyam Zenebe, declare that the information in this manuscript is true and correct. I believe it contains no material I would not want to include in my research publication. I acknowledge that this is a result my MSc thesis work which was conducted under the supervision of my advisers professor Tesfaye Sisay, Professor Gurja Belay, and Professor Workabeba Taye.

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.

AVAILABILITY OF DATA AND MATERIALS

The datasets used of this study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING

This study was funded by the thematic research project entitled "biotechnological approaches to harness microbe host environment interactions for sustainable development of health and agricultural productivity" at the institute of biotechnology, college of natural and computational sciences, Addis Ababa University; the study was also supported by the department of microbial, cellular and molecular biology, college of natural and computational sciences, Addis Ababa university.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Addis Ababa college of natural sciences microbial, cellular and molecular biology, Addis Ababa university college of natural sciences institute of biotechnology; Addis Ababa university college of health sciences Tikur Anbessa specialized hospital.

REFERENCES

1. Alekshun MN, Levy SB. Molecular mechanisms of antibacterial multidrug resistance. Cell. 2007;128(6):1037-1050.
2. Amaya E, Reyes D, Vilchez S, Paniagua M, Mollby R, Nord CE, et al. Antibiotic resistance patterns of intestinal *Escherichia coli* isolates from Nicaraguan children. J Med Microbiol. 2011;60(2):216-222.
3. Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F. Diarrheagenic *Escherichia coli* in acute gastroenteritis in infants in North-West Italy. New Microbiol. 2011;34(1):45-51.
4. Ansari S, Sherchand JB, Parajuli K, Mishra SK, Dahal RK, Shrestha S, et al. Bacterial etiology of acute diarrhea in children under five years of age. J Nepal Health Res Counc. 2013;10(22):218-223.
5. Adugna A, Kibret M, Abera B, Nibret E, Adal M. Antibiogram of *E. coli* serotypes isolated from children aged under five with acute diarrhea in Bahir Dar town. Afr Health Sci. 2015;15(2):656-664.
6. Blattner FR, Plunkett III G, Bloch CA, Perna NT, Burland V, Riley M, et al. The complete genome sequence of *Escherichia coli* K-12. Science. 1997;277(5331):1453-1462.
7. Bonkoungou IJ, Haukka K, Osterblad M, Hakanen AJ, Traore AS, Barro N, et al. Bacterial and viral etiology of childhood diarrhea in Ouagadougou, Burkina Faso. BMC Pediatrics. 2013;13(1):1-6.
8. Cambrea SC. Antibiotic susceptibility of *Escherichia coli* strains isolated in a pediatric population from South Eastern Romania. J Pediatr Infect Dis. 2014;9(3):157-162.
9. Chandra M, Cheng P, Rondeau G, Porwollik S, McClelland M. A single step multiplex PCR for identification of six diarrheagenic *E. coli* pathotypes and *Salmonella*. Int J Med Microbiol. 2013;303(4):210-216.
10. Chattaway MA, Dallman T, Okeke IN, Wain J. Enteroaggregative *E. coli* O104 from an outbreak of HUS in Germany 2011, could it happen again?. J Infect Dev Ctries. 2011;5(6):425-436.
11. Clermont O, Bonacorsi S, Bingen E. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Appl Environ Microbiol. 2000;66(10):4555-4558.
12. Perez C, Gomez-Duarte OG, Arias ML. Diarrheagenic *Escherichia coli* in children from Costa Rica. Am J Trop Med Hyg. 2010;83(2):292.
13. Croci L, Delibato E, Volpe G, de Medici D, Palleschi G. Comparison of PCR, electrochemical enzyme-linked immunosorbent assays, and the standard culture method for detecting *Salmonella* in meat products. Appl Environ Microbiol. 2004;70(3):1393-1396.
14. Croxen MA, Finlay BB. Molecular mechanisms of *Escherichia coli* pathogenicity. Nat Rev Microbiol. 2010;8(1):26-38.
15. Croxen MA, Law RJ, Scholz R, Keeney KM, Wlodarska M, Finlay BB. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin Microbiol Rev. 2013;26(4):822-880.
16. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417-433.
17. Edwards PR, Ewing WH. Identification of enterobacteriaceae. 3rd ed. Burgess Publ. Co., Minneapolis, Minn. 1972.
18. Estrada-Garcia T, Cerna JF, Paheco-Gil L, Velazquez RF, Ochoa TJ, Torres J, et al. Drug-resistant diarrheogenic *Escherichia coli*, Mexico. Emerg Infect Dis. 2005;11(8):1306.
19. Giedraitiene A, Vitkauskiene A, Naginiene R, Pavilonis A. Antibiotic resistance mechanisms of clinically important bacteria. Medicina. 2011;47(3):19.
20. Gordon DM, Clermont O, Tolley H, Denamur E. Assigning *Escherichia coli* strains to phylogenetic groups: multi – locus sequence typing versus the PCR triplex method. Environ Microbiol. 2008;10(10):2484-2496.