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Research Article

Detection of Escherichia coli Strains, Determinants and Antibiogram from Diarrheic Children in Southwest Ethiopia

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Abstract

Pathogenic *E. coli* strains could cause diarrheal infection both in animal and human hosts due to their virulence factors. Molecular detection of the virulent *E. coli* strains with antibiogram and determinants for the occurrence of diarrhea in children was conducted in Jimma. Cross-sectional study with purposive sampling was undertaken to isolate bacteria from stool samples of 68 diarrheic children, identified conventionally, virulence factors were identified and antimicrobial sensitivity patterns were tested. Socio-demographic data was obtained using a structured questionnaire. *E. coli* was recovered from 47.1% (32/68) (95% CI: 35.0-59.0) of the children. There was a statistical difference by age, presence of caretaker for the child, manure contact, hand washing before meal and exclusive breast milk feeding ($P < 0.05$). The conspectus isolation rate of pathogenic strains was 43.7% (95% CI: 26.0-62.0). Five (15.6%) of *Enteropathogenic E. coli (EPEC)* strains, 9 (27.1%) of Shiga toxin like producing *E. coli* (*STEC*) strains were detected. Neomycin, 92.9% (13/14), followed by sulphonamides, 64.3% (9/14), were the highest resisted by STEC and *EPEC* strains. The study demonstrated involvement of *EPEC* and *STEC* in children suffering from diarrhea. The existence of multiple antimicrobial resistant isolates calls for prudent use of antimicrobials. Household hygiene practices and antimicrobial judiciousness could aid to limit the occurrence of pathogenic *E. coli* infection.

Keywords: *Children; Escherichia coli; Ethiopia; Antimicrobial Resistance; Risk Factors; Virulence Genes*

Abbreviations

BP: Base Pair; CI: Confidence Interval; CLSI: Clinical Laboratory and Standard Institute; DACA: Drug Administration and Control Authority; dNTP: Deoxyribo Nucleotide Triphosphate; *EaeA*: Effacing and Attaching; EVT: EnteroVero Toxin; IMViC: Indole, Methyl Red, Voges Proskauer and Citrate Utilization; ISO: International Organizations for Standardization; OPEDJZ: Office of Planning and Economic Development for Jimma Zone; P: Probability; PCR: Polymerase Chain Reaction; SPSS: Software Package for Social Science; Stx: Shiga Toxin; χ^2: Chi-Square

Introduction

Diarrheal illnesses are the leading cause of death in children under the age of five worldwide [1]. Despite the fact that co-contamination of multiple enteric bacteria pathogens greatly worsens the diarrheal illness, single contamination is occasionally recorded [2,3]. Among the bacterial infections, *E. coli* diarrhea in children is a prevalent and deadly disease that affects children under the age of five months [4,5].

Diarrheagenic strains of *E. coli* strains that are as sources of gastrointestinal problems are classified based on epidemiological and clinical characteristics, virulence determinants and serotype association [6,7]. Because of new strains of *E. coli* emerge, it is critical to keep researches on the levels of diarrheagenic *E. coli*. To this reason, PCR has been widely employed to detect pathogenic *E. coli* strains in a quick and accurate manner [8,9].

Data on the illness burden caused by *E. coli* in Ethiopian children is very limited. According to a serological report from Bahir Dar town, the overall *E. coli* isolation rate from children under the age of five was 48.3% [10]. However, the possibility of pathogenic *E. coli* strains causing diarrhea in children are still not defined. This makes it difficult to adopt effective control and prevention measures. Furthermore, most pathogenic bacteria that cause infection in this country have high level of resistance to antimicrobials [11]. Uncontrolled antimicrobial use from lack of specific pathogenic strain identification could exist. This leads to an increase in antibiotic resistance and has a substantial impact. Therefore, this study was conducted to identify pathogenic *E. coli* strains, their antibiogram and associated determinants in diarrheic children admitted to public health institutions in Jimma, Southwest Ethiopia.

Materials and Methods

Study subjects, design and sample size

Children with diarrhea under five years of age admitted to public health institutions in Jimma were included. Cross sectional study with purposive sampling were chosen based on the availability of clinical diarrheic cases and willingness parents to participate. The health status of each child was determined by the pediatricians. Diarrheic children were defined as those who had an abnormal stool consistency and/or showed indicators of dehydration, including sunken eyes, diarrhea, and weakness [12]. Additionally, during the period of sample collection, pre-tested and structured questionnaire were issued for parents to assess relevant information. Sample size of the study comprised 68 diarrheic children.

Sample collection procedure

Medical laboratory personnel collected stools samples from non-treated diarrheic children. A sufficient amount (25 - 50g) of stool samples were collected and transferred to 50ml sterile screw caped universal bottles. Then, it was transported in an ice box under cold circumstances to Microbiology Laboratories in the Jimma and Addis Ababa Universities.

Isolation and identification

The isolation and identification of *E. coli* were done following standard procedures [13,14]. The samples were homogenized using a vortex mixer for approximately 30s upon arrival at the laboratory, either immediately or after overnight storage in a refrigerator at 4°C and thawing at ambient temperature. When there is a small amount of sample present, 25g of stool sample was swirled into 225 ml of sterile buffered peptone water (Himedia, India) at a 1:9 ratio. The pre-enriched samples were homogenized in the flask for two minutes before being incubated aerobically for 24 hours at 37°C.

A loop full (0.1 ml) dilution of pre-enriched broth was inoculated onto sterile MacConkey agar (Himedia, India) and incubated. Lactose fermenting colonies were extracted and inoculated into EMB agar medium (Himedia, India) for metallic sheen appearance of *E. coli*. The cell morphology and purity of all of the isolates were examined using gram staining having a distinct appearance. Biochemical assays were used to further process *E. coli* isolates with the IMViC pattern (+ + - -) [13,15].

Detection of pathogenic strains

Glycerol stocks of *E. coli* isolates were thawed at room temperature and inoculated into nutrient broth and incubated at 37°C for 24 hours. In an autoclaved eppendorf tube, 1.5 ml of the culture was pipetted and centrifuged at 13000 rpm for 10 minutes. The bacterial

pellet was lysed by boiling for five minutes in 50 liters of nucleus-free water in a water bath at 95°C. The lysate was centrifuged once more, and an aliquot of the supernatant was transferred to another autoclaved eppendorf tube; the extracted DNA was then utilized directly as PCR template [16]. The purity, intactness and quantity of extracted DNA were measured using UV spectroscopy to control the possible contaminates.

Three types of PCR assays were performed based on the component optimization of the compatible enzyme utilized. The first PCR assays was performed independently to detect the presence of *eaeA* (450bp) gene with 5′-AAACAGGTGAAACTGTTGCC-3′ forward and 5′-CTCTGCAGATTAACCTCTGC-3′ reverse EAE primers. The second assay of *stx1* (110bp) gene was with 5′-ATCAGTCGTCACTCACTGGT-3′ EVS forward and 5′-CTGCTGTCACAGTGACAAA-3′ EVC reverse primers. Both assays had a 25 µl reaction volume contained 12 µl of nucleus free water, 1 µl of 0.5 µmol of each primers, 2.5 µl of PCR buffer with 2 µl of 1.5 mmol $MgCl_2$, 2 µl of solution S, 1 µl of 0.35 mmol of dNTPs, 0.5 µl of 1U Taq polymerase enzyme (Solis Biodine), and 3 µl of template DNA. The third PCR assay was to detect *stx2* gene (350bp) with 5′-CAACACTGGATGATCTCAG-3′ forward and 5′-CCCCCTCAACTGCTAATA-3' reverse EVT primers. Its 25 µl reaction volume contained 14 µl of nucleus free water, 1 µl of 0.5 µmol of each primer, 2.5 µl of PCR buffer with 2 µl of 1.5 mmol $MgCl_2$, 1 µl of 0.35 mmol of each dNTPs, 0.5 µl of 1U Taq polymerase enzyme (Himedia, India), and 3 µl of template DNA [17,18].

All reaction mixtures were amplified using 35 cycles, each consisting of 3 minutes initial denaturation at 95°C, 60s denaturation at 95°C, 60s annealing at 55°C and 60s elongation at 72°C in thermal cycler (TC-412; Version 34.1, USA) [19]. An additional extension step of 10 minutes at 72°C was conducted. Along with the samples, a negative control (PCR grade water in place of the template) and a known pooled positive *E. coli* gene as a positive control for each primer involved were included. The minimum criteria to classify *E. coli* as EPEC or STEC were the *eae* gene for EPEC and stx encoding *stx*1 and *stx*2 genes for STEC [17,18]. The amplified PCR products were then evaluated by gel electrophoresis at 120 volt for 45 minutes in 2% agarose (Conda, cat.8010.11) containing ethidium bromide (0.5g ml^{-1}) and a 100bp DNA ladder [16].

Testing for antimicrobial resistance

Pathogenic *E. coli* strains were tested for antimicrobial susceptibility with a panel of routinely used drugs using the Kirby-Bauer disk diffusion test, as per the CLSI (M100-S25). The findings were analyzed using CLSI guidelines, and the isolates were divided into three groups: susceptible, intermediate, and resistant [20].

Questionnaire survey

A structured questionnaire that had been pre-tested for its validity was given out to the participants. It was given to parents or caretakers of the children during admission to public health institutions to assess the general family life standard, ways of handling the children and hygienic practices of the family.

Analytical statistics

Data collected from surveys and laboratory tests was filtered and recorded into a Microsoft Excel spread sheet 2007 and computed using SPSS version 20.0 software (SPSS INC. Chicago, IL). The study subjects were described using descriptive analysis in relation to risk variables. Using the person's X^2 test, the relationships between *E. coli* isolates and risk factors, as well as the presence of different virulent genes and diarrheal infection due to positive *E. coli*, were investigated. Effects were considered statistically significant when the p-value was <0.05.

Results

Occurrence of *Escherichia coli* in child diarrhea

Of the 68 diarrheic stool samples, 32 (47.1%) were *E. coli* positive presumptively. The occurrence of bacteria differed significantly by age, presence of caretaker for the child, manure contact, hand washing before meal and exclusive breast milk feeding ($P < 0.05$). A higher

isolates of *E. coli* was detected in children of age group 6 - 12 months (19.1%), who did not have caretaker (36.8%), exposed to manure (29.4%), did not wash their hand before meal (36.8%) and those who did not get only breast feed until six month of age (36.8%) (Table 1).

Variable	Category	Number Examined	Number Positive (%)	Category Proportion (%)	Samples Proportion (%)	χ^2	P-Value
Age (months)	<5	10	8	80.0	11.8	10.91	0.031
	6-12	24	13	54.2	19.1		
	13-36	20	9	45.0	13.2		
	37-60	14	2	14.3	3.0		
Sex	Female	38	18	47.4	26.5	0.01	0.954
	Male	30	14	46.7	20.6		
Caretaker	No	44	25	56.8	36.8	4.77	0.032
	Yes	24	7	29.2	10.3		
MC	Yes	24	20	83.3	29.4	19.59	0.001
	No	44	12	27.3	17.7		
HWBM	No	30	25	83.3	36.8	28.35	0.001
	Yes	38	7	18.4	10.3		
EBF6M	No	33	25	75.75	36.8	21.20	0.001
	Yes	35	7	20.0	10.3		
Each Total		68			47.1		

Table 1: *Association of E. coli occurrence with determinants in diarrheic children.*

MC: Manure Contact; HWBM: Hand Washing Before Meal; EBMF6M: Exclusive Breast Feeding for Six Months; χ^2: Chi Square.

Description of socio-demographic pattern

Of the 68 diarrheic children included in the study 48 (70.6%), each 12 (17.6%), were from Jimma Hospital, Shinen Gibe Hospital, Mendera Qochi Health Center and Olana Lema Health Center. The remained 20 (29.4%), each 10 (14.7%), of diarrheic children were obtained from Bocho Bore and Jimma Health Centers. From all parents of the sampled children, 59 (86.8%) used toilet for defecation and the remained were not in which children were susceptible to diarrheal infection because they did not pay attention to hygiene. Nursing homes and day cares are also places where adults and kids can get the infection. Thirty (44.1%) of the parents reared domestic animals in home and 23 (33.8) used animals products as supplement feed source.

Detection of virulence genes

The overall PCR assays detection rate of the three genes investigated from positive isolates in children was 14 (43.7%) out of 32 presumptive isolates. Five (15.6%) of the isolates carried only *eaeA*, characteristic of EPEC strains; 3 (9.4%) of the isolates carried only *stx1* and 6 (18.7%) of the isolates carried only *stx2* genes, both characteristics of shiga toxin like producing *E. coli* (STEC) strains, which are the highly prevalent strains detected in this study (Table 2). There were no isolates possessing combination of *stx1*, *stx2* and *eaeA* genes, thus EHEC strains were not observed and the revealed *EPEC* type is atypical in this study. All of the virulence genes were found to be substantially linked to *E. coli* diarrhea ($P < 0.05$). Typical results from the PCR experiments are indicated in figure 1 below.

Sample source	Genes	*E. coli* strain	Isolates examined	Positive isolates (%)	Diarrheic samples (%)	χ^2	P (95% CI)
Children	*EaeA*	EPEC	32	5 (15.6)	5 (7.4)	7.62	0.006 (0.000-0.092)
	Stx1	STEC	32	3 (9.4)	3 (4.4)	4.26	0.039 (0.076-0.362)
	Sx2	STEC	32	6 (18.7)	6 (8.8)	9.49	0.002 (0.000-0.089)
	Stx1+EaeA	EHEC	32	0	-	-	-
	Stx2+EaeA	EHEC	32	0	-	-	-
	Stx1+Stx2+EaeA	EHEC	32	0	-	-	-
	Total			14 (43.7)	14 (20.6)		

Table 2: *PCR based detection rate of virulence genes in E. coli isolates in diarrheic children.*

EPEC: Enteropathogenic E. coli; EHEC: Enterohemorrhagic E. coli; STEC: Shiga-Like Toxin Producing E. coli; χ^2: Chi Square; CI: Confidence Interval.

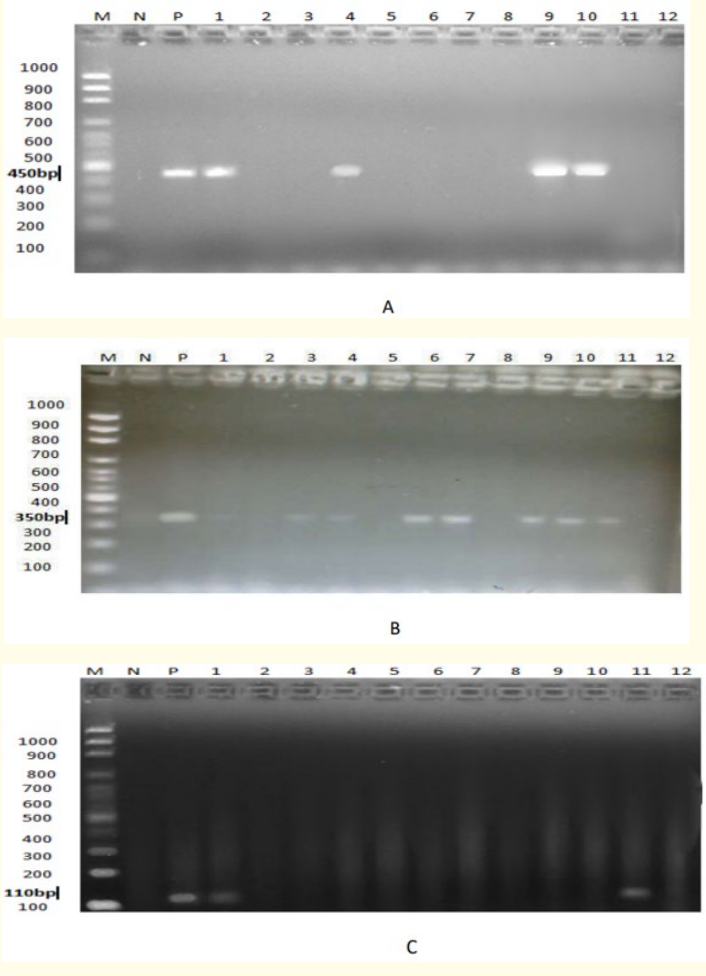

Figure 1A-1C: *Amplification of virulence genes in E. coli isolates from children.*

Key: M=Marker; N=Negative Control; P= Positive Control; Numbers =1-12 Representative Sample Numbers; Figure 1A=eaeA gene with 450bp; Figure 1B=Stx2 gene with 350bp; and 1C=Stx1 gene with 110bp.

Antimicrobial resistance profiles

The antimicrobial resistance pattern of the pathogenic *E. coli* isolates (14) is shown in table 3 below. In both EPEC and STEC strains, neomycin 13 (92.9%) resistance was found to be the most resistant pathogen, followed by sulphonamides 9 (64.3%) resistance. Resistance to ciprofloxacin, gentamycin and norfloxacin was recorded in only *stx2* STEC *E. coli* strains. *EaeA* 4 (80%), *stx1* gene 3 (100%) and *stx2* 6 (100%) positive *E. coli* isolates showed highest resistance for neomycin; whereas *eaeA,* 1 (20%), *stx1,* 1 (33.3%), and *stx2,* 1 (16.6%), positive *E. coli* isolates showed the least resistance for streptomycin. Amoxicillin, chloramphenicol and cefoxitin resistance were not observed.

Antimicrobials	AM Resistant Pathogenic *E. coli* strains (%)			
	***EPEC (EaeA +)* (n = 5)**	***STEC (Stx1+)* (n = 3)**	***STEC (Stx2+)* (n = 6)**	**All (n = 14)**
Amoxicillin	-	-	-	-
Chloramphenicol	-	-	-	-
Ciprofloxacin	-	-	1 (16.6)	1 (7.1)
Cefoxitin	-	-	-	-
Gentamycin	-	-	1 (16.6)	1 (7.1)
Neomycin	4 (80)	3 (100)	6 (100)	13 (92.9)
Norfloxacin	-	-	1 (16.6)	1 (7.1)
Oxytetracycline	3 (60)	2 (66.7)	1 (16.6)	6 (42.9)
Streptomycin	1 (20)	1 (33.3)	1 (16.6)	3 (21.4)
Sulfonamides	3 (60)	2 (66.7)	4 (66.7)	9 (64.3)
Trimethoprim	3 (60)	2 (66.7)	2 (33.3)	7 (50)

Table 3: *Antimicrobial resistance profiles of pathogenic E. coli strains isolated from diarrheic children*

AM: Antimicrobial; n: Number.

Discussion and Conclusion

The current occurrence of *E. coli* isolates from diarrheic children (47.1%) was parallel with the finding in Bahir Dar (48.3%) [10]. However, the rate was higher than the finding in Mozambique (22.6%) [21], and in Australia (31%) [22]; but, lower than that of in Kenya (86.5%) [23]. Differences in socio-demographics, genetic resistance among human races, agro-climatic and sample size variance could all contribute to the disparity [24].

Different determinants associated with *E. coli* attributed for the occurrence of diarrhea in children. Diarrhea and presence of *E. coli* was observed in all age groups although children under 5 months of age were more likely to be diarrheic than the other age groups which coincided with previous studies elsewhere [23]. This could be owing to their immature immunity and the development of receptors for pathogenic strain ligands, such as EPEC intimin, in younger children [25]. Children who did not have caretaker were also more likely to be diarrheic with *E. coli* infection than the cared ones, in accordance with the report in Bolivia [26]. Children who had contact with cattle manure were again more likely to be diarrheic than those with no contact as supported by a report in Kenya [27], which could be attention to hygiene is marginal [28]. Hand washing habit before feeding the child had a significant association with the presence of *E. coli* infection in diarrheic children. This is in consistent with the finding in Bahir Dar [10] and Bolivia [26]. Diarrheic children who did not get exclusive breast milk feeding in six months of age were also more likely to be susceptible for diarrhea. This finding is in agreement a finding in Kenya [29] in which the number of diarrheic children was the least when complimentary feeding was initiated after six months of age.

Fourteen (43.7%) of 32 presumptive isolates were tested positive for all virulent genes involved in EPEC and STEC strains. The detection rates of *eaeA* (15.6%), *stx1* (9.4%) and *stx2* (18.7%) genes were in parallel with the findings in Iran [30], Argentina [31] and 18.2% for Scotland [32] respectively from diarrheic children. The present finding was higher than the report in Brazil [33] and Tunisia [34]. However, the result was lower than that of Iran [35], Scotland [32] and Chili [35] diarrheic children. This variation reported in different areas could be due to geographical [36], season of sample taken [37], sample size, age of sampled children and the detection methods used. In the present study, higher frequency of *stx2* gene over *stx1* and *eaeA* genes of *E. coli* strains was observed. The combination of the *eaeA* and *stx2* genes is more prevalent than the combination of the *eaeA* and *stx2* genes. The relatively greater prevalence of *stx2* gene in this study compared to the *stx1* gene of *EHEC* suggests that *E. coli* groups are contained within mobile genetic components and can be passed across strains to form emerging strains [38]. *E. coli* producing *stx2* are more pathogenic [39] and higher *stx2* gene finding of the present study might in agreement.

The presence of resistance among the EPEC and EHEC genes were investigated. Neomycin followed by sulphonamides was recorded as highest resistant. *Stx2* positive EHEC predominated over the resistance profiles *stx1* of EHEC and *eaeA* of EPEC, which is consistent with prior findings [40]. In this work, neomycin resistance of *E. coli* virulence genes neared 100% in Indian strains [41]. In contrast, 39% neomycin resistant Stx encoded *E. coli* strains in Germany are higher [42]. The differences could be attributable to the difference in sensitivity test method employed, frequency of medications, and resistance gene transfer across isolates from different locations [43]. Resistance genes could be passed across related bacteria, habitats, and food products, which could disseminate several antimicrobial resistance genes in *E. coli* isolates, was also linked to the development of resistance [44,45].

In conclusion, high rate of *E. coli* isolation from diarrhoeic children with a considerable proportion of pathogenic *E. coli* strains detected. Presence of a caretaker, breastfeeding, age and hygiene were all determinants of *E. coli* infection in diarrheic children. EHEC *stx2* gene was shown to have a higher rate of *stx2* positive pathotypes than its *stx1* and EPEC *eaeA* genes. Drug resistant *E. coli* strains were found considerably. The high percentage of *E. coli* isolation suggests that the illness is widespread in the area. Because of time and resource constraints, not all virulence genes and serotypes were characterized in this work in addition to the sample size limitation. It is necessary to promote parents' perceptions of the value of exclusive breast milk feeding, personal hygiene and safe socialization of their children. Pathotypes, *EPEC* for *eaeA* and *EHEC* for shiga toxin are common in diarrheic children and might be addressed when designing control and prevention measures. In order to advise effective antimicrobial treatment, proper antibiotic prescription in human and veterinary practices, as well as constant monitoring of resistance trends in bacterial pathogens in general, and *E. coli* in particular, is required.

Authors Contributions

All authors contributed for this work.

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Transparency Declarations

All the authors have no conflicts of interest to this manuscript.

Ethics Statement

The Institutional Review Board (HIRPG/248/07) of Jimma University Institute of Health Sciences gave its ethical approval. This clearance was documented in each peasant association health centers from where the samples were taken. The setting of verbal consent is approved by the IRB to be sufficient based on the procedure outlined; emphasizing the stool sample type in which the protocol clearly defined the modality of which the verbal consent must be obtained. Therefore, verbal informed consent was obtained from the parents and household leaders, where samples from the children were taken, through explaining the aim of the research that could not harm the subjects in any circumstances. This study was also conducted in accordance with the Declaration of Helsinki.

Supplementary Materials

Supplementary material associated with this article will be found from the corresponding author on reasonable request.

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