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Characterization of Selected *Escherichia coli* Pathovars and Their Antimicrobial Resistance Patterns among Diarrheal Children under the Age of Five Years from Machakos County, Kenya

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Abstract **Background:** Diarrheal diseases constitute an important cause of death among children under the age of five years globally. These diseases are caused by diarrheagenic *Escherichia coli* including enteropathogenic *Escherichia coli* and enterotoxigenic *Escherichia coli* among other agents. Treatment and management of diarrheal diseases including EPEC and ETEC is complicated by rapidly developing problem of antimicrobial resistance. **Methods:** Stool samples were collected from children under the age of five years attending Machakos Level 5 hospital. *Escherichia coli* was isolated and identified by culture-based techniques followed by multiplex polymerase chain reaction using primers specific for virulence genes associated with EPEC and ETEC pathovars. Confirmed EPEC and ETEC pathovars were subjected to a panel of eight antimicrobial agents. **Results:** Both EPEC and ETEC were detected in 29/118 (24.6%) samples collected during the study period. Prevalence of EPEC was higher 18 (15.3%) compared to ETEC that was detected in 11 (9.3%) samples analyzed. ETEC appeared to be more resistant to ampicillin (90.9%, 66.7%), trimethoprim (81.8%, 77.8%), gentamicin (45.5%, 22.2%), chloramphenicol (27.3%, 16.3%), cefuroxime (18.2%, 5.6%) and ciprofloxacin (9.1%, 5.6%) compared to EPEC respectively. On the other hand, EPEC displayed higher resistance against nalidixic acid (38.9%, 36.4%) and tetracycline (33.3%, 18.2%) compared to ETEC isolates. **Conclusion:** The role of EPEC and ETEC as a cause of infantile diarrhea cannot be underestimated in Machakos County, Kenya since they are both pathogenic and resistant to commonly used antimicrobial agents.

Keywords: *Escherichia coli, children, diarrhea, antimicrobial resistance*

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1. Introduction

Diarrheal diseases in Kenya are among the leading causes of morbidity and mortality in children below five years with bacteria accounting for up to 30% of all cases of infantile diarrhea [1] Enteropathogenic *Escherichia coli* (EPEC) is a major etiological agent of infantile diarrhea and is also responsible for morbidity in industrialized countries. Diarrhea is incriminated to cause over 2 million deaths of infants worldwide [2,3]. Several studies in Kenya have demonstrated that EPEC is the most predominant diarreagenic *E. coli* pathovar [1,4]. Different DEC pathovars include: enteropathogenic *E.coli* (EPEC), Enterohaemorrhagic *E.coli* (EHEC), Enterotoxigenic *E.coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [5]. EPEC is an inhabitant of the small intestines and is associated with the attachment and physical alteration of the integrity of the intestine [6]. EPEC causes either watery or bloody diarrhea. The pathogenesis of EPEC depends on the expression of locus of enterocyte effacement (LEE); a chromosomal pathogenicity island. The LEE contains a number of different genes, including *eae*, which has an essential role in inducing a characteristic lesion formation in the intestinal epithelium, termed as attaching and effacing (A/E) lesion [7]. The *eae* gene encodes intimin, a 94 kDa outer-membrane protein that is responsible for the intimate adherence between bacterial and enterocyte membranes [8,9]. Enterotoxigenic *E. coli* (ETEC) is recognized as a diarrheagenic pathovar of *E. coli* causing diarrhea in children from low and middle income countries and adults travelling from developed to

developing nations. Enterotoxigenic *E. coli* is defined by its ability to produce either or both heat-stable (ST) or heat-labile (LT) enterotoxins. The organism colonize the surface of the small bowel mucosa and express their enterotoxins, leading to secretion of electrolytes and water. In Kenya, several studies on the causes of diarrhea have been carried out and proved the existence of EPEC and ETEC as important aetiological agents [1] but little has been conducted to infer their distribution among different age groups and regions including Machakos County.

Antimicrobial resistance (AMR) is a serious threat to treatment and management of bacterial infection including infantile diarrhea globally [10]. Emergence and spread of antibiotic resistant bacteria including DECs poses the risk of development of infections the have limited treatment options thus resulting in high morbidity and mortality [11,12]. Infection by antibiotic resistant pathogens is also responsible for prolonged hospitalization [13,14,15] that translates to economic pressure. Antibiotic resistance among enterobacteriaceae is not uncommon [12,16]. Although several studies have been conducted on prevalence of AMR among gut *E. coli* isolates, information on resistant pathovars is still limited. This study aimed at characterizing *E. coli* pathovars associated with diarrhea in children below five years and antimicrobial resistance patterns of these isolates.

2. Materials and Methods

2.1. Specimen Collection

A total of 130 stool samples were collected from study participants attending Machakos Level 5 hospital that met the study inclusion criteria that consisted of children below five years of age presenting with diarrhea after obtaining formal consent from their parents or guardians. Scientific and ethical guidelines were adhered to during stool samples collection based on the requirements that were obtained from Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) (P0046/3380) during the proposal review. All stool samples collected were transferred into Cary-Blair transport media (Oxoid, Basingstoke, UK), labeled with a unique identification number, and placed in an insulated cooler box with ice packs before transporting them to Centre for Microbiology Research laboratories at KEMRI within three days for processing.

2.2. Isolation of Bacteria

Bacteria isolation was performed by plating all collected stool samples on MacConkey agar plates (Oxoid, Basingstoke, UK) that were incubated at 37°C for 18-24 hours. Colonies that appeared pink indicating lactose fermentation were sub-cultured onto MacConkey agar (Oxoid, Basingstoke, UK) plates to obtain pure isolates that were further identified based on their motility, indole production from decomposition of tryptophan, lysine decarboxylation, citrate utilization, urease production and reaction of triple sugar iron agar (Oxoid, Basingstoke, UK). Isolates that were motile and decarboxylated lysine, produced indole from decomposition of tryptophan, acid slant/acid butt and gas without H_2S on triple sugar iron agar but did neither produced urease nor utilized citrate were identified as *E. coli.* Pure *E. coli* isolates were suspended in tryptone soy broth supplemented with 50% glycerol and stored at -80°C as they awaited subsequent analysis.

2.3. DNA Isolation

Several colonies of pure *E.coli* isolates obtained from an overnight growth on tryptone soy agar (Oxoid, Basingstoke, UK) were suspended in 500 µl of nuclease free water and mixed thoroughly using a vortex. This was followed by boiling the bacteria suspension at 95^{o}C for 10 minutes then chilling them on ice. The supernatant rich in DNA was harvested after centrifugation at 12,000 ×g for 5 minutes and stored at -20°C [17].

2.4. Multiplex Polymerase Chain Reaction

Characterization of the two selected *E. coli* pathovars; EPEC and ETEC was conducted using a multiplex PCR that targeted 3 virulence genes; *eae*A, SLT1 and SLTII (Table 1). Multiplex PCR reaction mix consisted of 12.5 µl of DreamTaq mastermix (ThermoScientific), 0.625 µl of each of the three primers (Bioserve Biotechnologies, Laurel, MD.USA), 2.0 µl of DNA template and nuclease free water to the final volume of 25 µl. Multiplex PCR amplifications was performed on PTC-200 thermocycler (MJ Research Inc. USA) using the following conditions; initial denaturation at 95°C for 3 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 63^{o}C for 30 seconds and extension at 72°C for 30 seconds with a final extension at 72°C for 5 minutes [5]. DNA isolated from ATCC 43887, ATCC 933J and ATCC 933W strains were used as controls. The amplified PCR products were analyzed by electrophoresis using 2% agarose gels in Tris-boric acid EDTA (TBE) buffer containing EZ-vision dye (VWR Life Science). Product sizes was determined against 100 bp gene ruler (100-BP DNA ladder: MBI Fermentas, UK).

Table 1. Primer sequences used in this study

Primer	Gene sequence 5' to 3'	Product size (bp)	Reference
*eae*A	TGAGCGGCTGGCATGAGTCATAC TCGATCCCCATCGTCACCAGAGG	241	[4]
SLTI	TTTCCCCTCTTTTAGTCACTCAACTG GGCAGGATTACAACAAAGTTCACAG	160	[4]
SLTII	CCCCCTCTCTTTTGCACTTCTTTCC TGCTCCAGCAGTACCATCTAACCC	423	[4]

2.5. Antimicrobial Susceptibility testing for Selected *E. coli* Pathovars

Characterized *E. coli* pathovars were screened for AMR using Kirby-Bauer disk diffusion method [18]. A suspension of characterized *E. coli* pathovar isolates equivalent to 0.5 McFarland standard was uniformly spread on Mueller-Hinton agar (Oxoid, Basingstoke, UK) plates before carefully placing antimicrobial disks on their surface. Antimicrobial disks used in this study included: ciprofloxacin (5 µg), nalidixic acid (30 µg), cefuroxime (30 µg), tetracycline (10 µg), ampicillin (10 µg), chloramphenicol (30 µg), trimethoprim/Sulphomethoxazole (25 µg) and Gentamicin (10 µg). Zones of inhibition were measured after incubation at 37°C for 18 hours and interpreted according to Clinical Laboratories Standards Institute (CLSI) guidelines [19]. *E. coli* ATCC 25922 was used for quality control during antimicrobial susceptibility testing.

3. Results

3.1. Prevalence of Selected *E. coli* Pathovars among Children below the Age of Five Years

A total of 118 *E. coli* isolates were recovered from 130 stool samples collected from children under investigation. Two pathovars that included EPEC and ETEC were detected in 29/118 (24.6%) based on presence or absence either of the three virulence genes; *eae*, SLTI and SLTII. The most prevalent pathovar was EPEC that was detected in 18 (15.3%) samples compared to ETEC that showed a prevalence of 9.3% (11) (Figure 1).

3.2. Antimicrobial Susceptibility Patterns of Selected *E. coli* Pathovars

The two isolated *E. coli* pathovars were subjected to eight (8) different antimicrobial agents that included ciprofloxacin (5 µg), nalidixic acid (30 µg), cefuroxime (30 µg), tetracycline (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), trimethoprim/Sulphomethoxazole (25 µg) and Gentamicin (10 µg). Measurements of zones of inhibition produced by each pathovar was used to classify them as sensitive or resistant based CLSI guidelines. ETEC appeared to be more resistant to ampicillin (90.9%, 66.7%), trimethoprim/Sulphomethoxazole (81.8%, 77.8%), gentamicin (45.5%, 22.2%), chloramphenicol (27.3%, 16.3%), cefuroxime (18.2%, 5.6%) and ciprofloxacin (9.1%, 5.6%) compared to EPEC (Figure 2). On the other hand, EPEC displayed higher resistance against nalidixic acid (38.9%, 36.4%) and tetracycline (33.3%, 18.2%) compared to ETEC isolates (Figure 2).

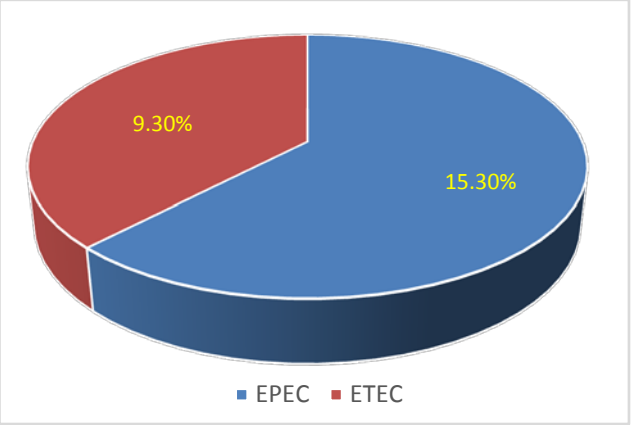

Figure 1. Prevalence of selected *E. coli* pathovars

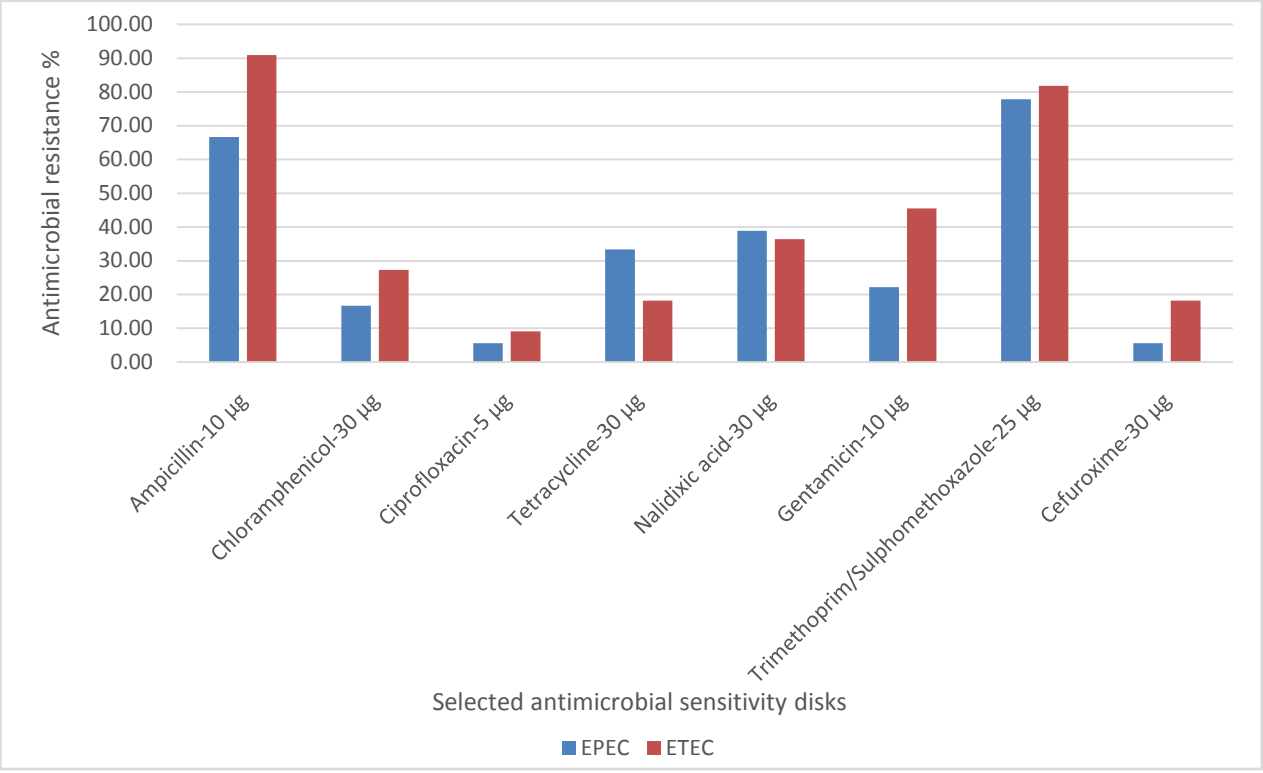

Figure 2. Antimicrobial susceptibility patterns of selected *E. coli* patrhovars

4. Discussion

Infantile diarrhea is a major public health problem responsible for high cases of morbidity and mortality in developing countries [20]. Despite the fact that *E. coli* are known to be essential part of normal gut flora, some strains are highly pathogenic with ability to cause infections including infantile diarrhea. This study focused on detection of two pathovars; EPEC and ETEC among children under the age of five presenting with diarrhea. Treatment and management of microbial infections including EPEC and ETEC is complicated by rapidly developing antimicrobial resistance [21]. The two pathovars; EPEC and ETEC detected in 24.6% (29) of all samples analyzed have been previously reported in other studies among children from different regions [2,4,22]. The observed higher prevalence of EPEC pathovar (15.3%) compared to ETEC (9.3%) in this study depicts a similar pattern that was previously reported [23].

EPEC remains one of the major causes of infantile diarrhea [24,25]. A study conducted in Asia that utilized both serological and PCR techniques reported higher prevalence of EPEC (30%) [26] reinforcing the fact that this pathovar remains a public health concern in low and middle income countries. This is also emphasized by high prevalence of EPEC (52.6%) that was observed in a similar study in Brazil [27]. Several studies in different regions of Kenya have also demonstrated high prevalence of EPEC [4,22,28]. On the other hand, lower prevalence of EPEC has been reported in Kenya [29]. and this could be attributed to reduced factors of transmission propagated by environmental sanitation.

ETEC is associated with childhood diarrhea globally and has been demonstrated in stool samples in different regions in developing countries [30]. The observed lower prevalence of ETEC among Peruvian children aged below 24 months (5.3%) [31] compared to 9.3% reported in this study could be due to the range of age-group inclusion. A similar study conducted in Columbia reported 2.4% prevalence of ETEC [32]. Higher prevalence (10.5%) has been reported in similar age group of children in Nairobi [33].

Rapid development and spread of AMR has become a public health problem across the globe [34]. This has resulted in escalating morbidity and mortality due to limited treatment options to manage them [35]. Unlike in previous studies where antimicrobial susceptibility profiles has been reported collectively for all diarrhegenic *E. coli*, this study conducted antibiotic sensitivity for each of the two identified pathovars; EPEC and ETEC. The two pathovars; EPEC and ETEC were resistant to all antimicrobial agents used in this study. However, ETEC were more resistant to all antimicrobial agents used in this study except nalidixic acid and tetracycline. DECs isolated from hospitalized paediatric patients in India have been reported to be resistant against ampicillin (97.3%) and trimethoprim (96.0%) [36] which is consistent to the findings of a study conducted in a selected Nairobi City hospital in Kenya [23]. These findings are consistent with resistance patterns of ETEC isolates in this study. On the other hand Webale *et al* [37] reported lower AMR against trimethoprim (61.4%) and ampicillin (55.2%) but demonstrated reduced susceptibility to tetracycline, chloramphenicol and gentamycin.

Both EPEC and ETEC are causes of infantile diarrhea that cannot be underestimated in Machakos Level 5 hospital, Kenya owing to the fact besides being diarrheagenic, they are also resistant to commonly used antimicrobial agents.

Conflict of Interest

The authors do not have any competing interests.

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