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Multidrug resistant *Escherichia coli* isolated from asymptomatic school going children in Kibera slum, Kenya

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Pathogenic *Escherichia coli* are of different types, currently grouped into six groups depending on the virulence gene(s) they possess. This study isolated pathogenic *E. coli* from 580 stool samples obtained in the month of August, 2016. The samples were obtained from asymptomatic school going children in one of the biggest urban slums in Kenya. Ten primary schools were randomly sampled and 40 to 80 stool samples collected from each school depending on the school population. Both gender and age were considered when sampling. Data obtained was analysed using single factor ANOVA to test association between school location and levels of infection with pathogenic bacteria. A total of 244 (17%) samples had *E. coli*. Out of these, 38 (6.5%) were shown to have one or a combination of the pathogenic genes, namely: *ipaH, virF, st2, daaE, eae, aafII, stx1, bfp, lt* and *stII* and were thus classified into seven groups. Of the pathogenic isolates 35 (21.2%) were multidrug resistant. There was an association between school location and the prevalence of pathogenic bacteria. In conclusion, asymptomatic school going children in the slum were found to be infected with multidrug resistant pathogenic *E. coli*.

Key words: Enteropathogenic, *E. coli,* multidrug resistance, school going children, asymptomatic.

INTRODUCTION

Escherichia coli form the normal flora of human and other mammals as the gut microbiota (Kaper et al., 2004; Fratamico et al., 2016). *E. coli* colonizes the gastrointestinal tract of infants within hours of birth, establishing mutual benefits with the host (Kaper et al., 2004). On colonization, *E. coli* will remain harmless to the host, only causing infection when the mutual environment is disturbed and in immune suppressed hosts. Pathogenic *E. coli* are of different types depending on the virulence gene(s) they possess (Kaper et al., 2004).

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Differentiating pathogenic strains has largely depended on serotyping methods (Fratamico et al., 2016).

Though serotypes are useful in pathogenic *E. coli* identification, they cannot be used to characterize the clinical syndromes of the strains. It is also a time consuming and expensive method, requiring well trained manpower. Identification of the specific virulence factors(s) in the pathogenic bacterial has been achieved through genotypic methods that detect specific genes encoding the pathogenic characteristics of the *E. coli* (Kaper et al., 2004). Based on the presence of virulence genes and ability to cause disease pathogenic *E. coli,* were classified as following pathotypes: Enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) shiga toxin– producing *E. coli* (STEC) and diffusely adherent *E. coli* (DAEC). Hybrid pathotypes have also been described including the enteroaggregative hemorrhagic *E. coli* (EAHEC) carrying both the STEC and EAEC associated genes (Fratamico et al., 2016), such hybrid was reported by Franz et al. (2011) as EAHEC serotype 0104:H4.

E. coli are one of the bacterial pathogens of international concern regarding antibacterial resistance according to WHO (2014). *E. coli* can develop resistance through different ways but mostly through mutation, resulting in fluoroquinolone resistance. The bacteria can also acquire mobile genetic elements including; plasmids, transposons, integrons and gene cassettes resulting in resistance to penicillins (ampicillin or amoxillin) as well as to third generation cephalosporins (WHO, 2014). Resistance to cephalosporins has been shown to be caused by extended spectrum beta-lactamases (ESBLs), enzymes which destroy beta–lactam antibiotics. *E. coli* has the ability to acquire ESBLs, causing them to be resistance to Beta-lactams (Downie et al., 2013). The presence of diarrheagenic *E. coli* in asymptomatic individuals has been reported in several studies and it is attributed to a number of observations including, development of protective antibodies early in life against diarrheagenic *E. coli,* as well as host susceptibility. This has been shown in children growing up in endemic areas (Quiroga et al., 2000). Though the actual process leading to diarrheal is not clearly understood (Donnenberg and Finlay, 2013), non-specific host barriers, such as internal microbiota, intact mucus layer and epithelial cell layers have been postulated to prevent diarrheal episodes (Levine and Robin, 2012). Bacterial factors inclining some pathogens to diarrhoea has also been shown in EPEC as a factor (Hu and Torres, 2015). Research clearance was from National Commission for Science, Technology and Innovation (NACOSTI) and Ethical clearance through the Kenyatta University Ethical Review Committees. The objective of this study was to determine the prevalence of pathogenic *E. coli* infection in asymptomatic school going children and establish their antibiotic resistance patterns using phenotypic and genotypic methods.

MATERIALS AND METHODS

Study area

The study was carried out in primary schools located in Kibera informal settlement, Nairobi County, Kenya. Kibera is located at an altitude of 1670 m above sea level, at longitude 1° 17° East and latitude 36°, 50° South and it is about 140 Km South of Equator. Due to poor infrastructure and drainage the slum is heavily polluted by human and animal excreta, organic and inorganic waste littering the open sewage lines. The area can be characterised by lack of sufficient sanitation and poor water supply which results in frequent waterborne disease outbreaks (Feikin et al., 2011).

Escherichia coli culture

Five grams of faecal sample was weighed and suspended in 45 ml of buffered peptone water to make a 1:9 (10^{-1}) dilution. Further, a 10-fold serial dilutions were made; 10^{-2}, 10^{-3}, 10^{-4} in sterile physiological saline solution (0.85%). Chromocult coliform agar (Merck Millipore Corporation, Germany) was prepared according to the manufacturer's instructions and kept at 50°C in water bath prior to use. One millilitre of the diluted and vortexed samples (10^{-3}, 10^{-4}) was transferred into well labelled sterile petri dishes and 15 ml of the cooled medium was added. The plates were thoroughly mixed avoiding spillage. The media was allowed to solidify at room temperature before being incubated at 44.5°C, 18 h. Characteristic colonies were examined, counted and recorded as total thermo-tolerant coliforms (appearing as salmon red) or total *E. coli* (appearing as dark-blue to violet colonies). *E. coli* were confirmed by overlaying the dark-blue to violet colonies with Kovacs reagent and observing for a cherry-red colour change. Four characteristic colonies of *E. coli* per stool sample were sub-cultured on to clean Chromocult agar to obtain distinct colonies. Colonies embedded in the media were sub-cultured by piercing the pour plate agar using a sterile straight wire. The pure cultures as well as a pool of all the four different isolates from a single sample were stored in skimmed milk at -80°C for further characterization.

DNA preparation from bacteria colonies and multiplex polymerase chain reaction (PCR)

Only pure bacterial cultures were used for DNA preparation. Three distinct colonies were picked from agar plates containing pure culture with a sterile wire loop and suspended in 0.5 ml sterile distilled water. The suspension was then placed in a water bath and heated to boil for 30 min. After cooling to room temperature the suspension was centrifuged at 2000 g and the supernatant was decanted and stored at -20°C as DNA templates.

Due to the large number of *E. coli* isolates, first the pools were sub-cultured, DNA isolated and tested for the presence of pathogenic *E. coli* using multiplex PCR. For pools that were positive, individual isolates making that specific pool were individually analysed. The PCR was carried out using a Veriti 96 wells thermocycler (Applied Biosystems, model 9902, Singapore) in 0.2 ml PCR tubes. A 25 µl reaction mix was prepared by mixing 12.5 µl of PCR master-mix (Taq polymerase 0.05 U/µl, 0.4 mM of each dNTP, 4mM $MgCl_2$) (Biolabs Inc., New England) with ten pairs of specific primers (10 pmol) at 0.5 µl each (Vidal et al., 2005) and 2.5 µl DNA template. Primers used for amplification of products encoding for pathogenic genes in *E. coli* are shown in Table 1.

E. coli were amplified with a program of initial heating at 94°C for

Table 1. Primers used for patho-typing *E. coli* isolates.

Primer sequence 5'-3'	PCR product size (pb)	Encoded gene	References
F-CTC GGC ACG TTT TAA TAG TCT GG R-GTG GAG AGC TGA AGT TTC TCT GC	933	*ipaH*	Vidal et al. 2005
F-AGC TCA GGC AAT GAA ACT TTG AC R-TGG GCT TGA TAT TCC GAT AAG TC	618	*virF*	Vidal et al. 2005
F-ATC CTA TTC CCG GGA GTT TAC G R-GCG TCA TCG TAT ACA CAG GAG C	584	*stx2*	Berlutti et al. 1998
F-GAA CGT TGG TTA ATG TGG GGT AA R-TAT TCA CCG GTC GGT TAT CAG T	542	*daaE*	Vidal et al. 2005
F-TCA ATG CAG TTC CGT TAT CAG TT R-GTA AAG TCC GTT ACC CCA ACC TG	482	*eae*	Stacy-Phipps et al. 1995
F-CAC AGG CAA CTG AAA TAA GTC TGG R-ATT CCC ATG ATG TCA AGC ACT TC	378	*aafII*	Vidal et al. 2005
F- CAG TTA ATG TGG TGG CGA AGG R- CAC GCA ACA ATG TAA CCG CTG	348	*stx1*	Berlutti et al. 1998
F-GGA AGT CAA ATT CAT GGG GGT AT R- GGA ATC AGA CGC AGA CTG GTA GT	300	*bfp*	Stacy-Phipps et al. 1995
R-TCC TTC ATC CTT TCA ATG GCT TT F-GCA CAC GGA GCT CCT CAG TC	218	*lt*	Stacy-Phipps et al. 1995
R-AAA GGA GAG CTT CGT CAC ATT TT F-AAT GTC CGT GCG TTA GGA C	129	*stII*	Stacy-Phipps et al. 1995

5 min followed by 94°C, 1.5 min minutes denaturation, 1.5 min at 60°C primer annealing and at 72°C for 1.5 min elongation for 35 cycles, with a final extension of 72°C for 7 min. The PCR products were kept at -20°C until gel electrophoresis was done. The PCR products were visualized following electrophoresis in a 1.5% agarose (Genetics analysis grade, Fisher Scientific, New Jersey) gel stained with 0.02% ethidium bromide and amplicons identified against molecular marker (50 bp DNA ladder, England Biolab) run long side the samples. A UV transilluminator digital camera (Gelmax 125 imager, Cambridge UK) with a UVP software interphase computer (Upland CA, USA) was used to visualize DNA bands relative to the molecular weight maker. For confirmation the positively identified PCR products were submitted for sequencing (ABI 3500XL Genetic Analyzer).

Antimicrobial sensitivity test (AST) for confirmed *E. coli isolates*.

Bacteria inoculum for the antimicrobial resistance test was prepared by touching the top of at least 2 to 3 well isolated colonies with a sterile wire loop and then transferred into sterile normal saline solution (0.85% NaCl). The inoculum was emulsified on the inside of the tube to avoid clumping of the cells and turbidity adjusted to a 0.5 McFarland. A standard bacterium, *E. coli* ATCC 25922 was included in every test as antibiotic sensitivity breakpoint control. Six antimicrobial discs were placed on a single Muller Hinton agar plate with a sterile multidisc dispenser. One hundred and sixty five (165) *E. coli* isolates were tested against twelve antimicrobial agents namely; Tetracycline (30 mg, TE); Ciprofloxacin (5 mg, CIP); 30 mg Naladixic acid (NA); 25 mg Trimethoprim-Sulfamethaxone (SXT); 30 mg Ceftriaxone (CRO); 10 mg Amoxycillin-clucianic acid (AMC); 30 mg Ceflazime (CAS); 10 mg Ampicillin (AMP); 30 mg Chloramphenicol (C); 10 mg Gentamycin (CN); 10 mg Streptomycin (S), CXM; 30 mg Cefuroxicin (CXM) according to the CLSI (2012) guidelines. The 12 antimicrobial agents are classified into 9 subclasses by CLSI (2012).

Genotypic characterization of bacteria isolates for antimicrobial resistance

Seventy five of the 165 *E.coli* isolates tested for antimicrobial sensitivity by the disc diffusion test were selected for genotypic characterization based on their phenotypic profiles. The isolates were tested for the presence of genes encoding for resistance to tetracyclines, quinolones and sulfonamides. Presence of four tetracycline resistance genes; *tet A, tet B, tet C* and *tet O* and two sulfonamides resistance genes namely *sul1* and *sul2* was tested. Multiplex PCR was carried out and primers used for amplification of products encoding for the resistant genes to the three antibiotic classes (Table 2).

RESULTS

A total of 221 stool samples had *E. coli* isolated, out of which 38 (6.5%) were pathogenic *E. coli*. These were distributed at different frequencies in the 11 schools studied with 4 (36%) schools having none isolated (Table 3). Using multiplex PCR, 83/555 (15%) pooled *E. coli* positive samples were shown to have one or a combination of the virulence genes and were thus classified into seven groups. Seventeen (3.1%) of the pools had the *eae* gene and were classified as atypical enteropathogenic. Thirty two (5.8%) pooled isolates were

Table 2. Primers used for identifying tetracyclines and sulphonamides encoding genes in selected bacteria isolates.

Primer sequence 5'-3'	PCR product (pb)	Encoded gene	References
F-GTGAAACCCAACATACCCC R-GAAGGCAAGCAGGATGTAG	577	*Tet A*	Randall et al., 2004
F-CCTCAGCTTCTCAACGCGTG R-GCACCTTGCTGAGACTCTT	635	*Tet B*	Randall et al., 2004
F-ACTTGGAGCCACTATCGAC R-CTACAATCCATGCCAACCC	880	*Tet C*	Van et al., 2008
R-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	515	*Tet O*	Abdi-Hachesoo et al., 2014
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCTCGGTCTC	822	*Sul1*	Van et al., 2008
F-TTCGGCATTCTGAATCTCAC R-ATGATCTAACCCTCGGTCTC	285	*Sul2*	Van et al., 2008

Table 3. Prevalence of pathogenic *E. coli* per school in the study area.

Schools	No sample per school	*E. coli* (Isolated) per school	Pathogenic *E. coli* n (%)
A	59	42	6 (14.2)
B	38	2	2 (0)
C	62	37	3 (5.4)
D	44	0	0 (0)
E	79	42	10 (23.8)
F	19	15	3 (20)
G	80	49	14 (28.6)
H	53	1	1 (0)
I	30	0	0 (0)
J	69	30	1 (0.33)
K	47	3	1 (33.3)
Totals	580	221	38
% Prevalence in the study area			6.5%

enterohemorrhagic forming the highest number followed by enterotoxigenic at (13/555) 2.3%, diffusely adherent at (12/555) 2.16%, enteroaggregative at (4/555) 0.72%, typical enteropathogenic at (3/555) 0.54% and lastly enteroinvasive at (2/555) 0.36%. A hundred and twenty three (20%) single *E. coli* isolates from 113 faecal samples were subjected to PCR and characterized into 6 pathotypes, with EHEC having 33 (27%) isolates, EPEC (typical and atypical) with 30 (24%) isolates, DAEC at 28 (23%) isolates, ETEC at 26 (21%) isolates, EAggEC with 5 isolates and lastly EIEC had only one isolate (Table 4). Multiple infections with more than one pathogenic *E. coli* were observed in 10 (8.8%) individuals. Three (2.0%) children had EHEC and EAggEC combined infection, another 4 (3.5%) children had EPEC and EIEC. A combined infection of EHEC, EAggEC and DAEC was observed in one (0.9%) child. Also observed in single individual child were EHEC together with EIEC and EPEC with EAgg EC (Table 4).

Multidrug resistant profiles of *E. coli* isolates

One (0.6%) *E. coli* isolate was resistant to the eight antibiotics tested, 3 (1.8%) were resistant to seven drugs, 6 (3.6%) were resistant to six drugs, 18 (10.9%) were resistant to 5 drugs while 33 (20%) were resistant to 4 drugs. Thirty five (21.2%) were shown to be resistant to 3 drugs (Figure 1).

Phenotypically the antimicrobial subclass with the highest number of resistant isolates was sulfonamides with 115 (70%) isolates, this was followed by aminopenicillin with 97 (59%) isolates, tetracyclines had 91 (55%), aminoglycoside with 74 (45%) isolates, fluoroquinolones at 37 (22%) and phenicols had 24 (15%) resistant isolates. The other three subclasses had less than 10 (6%) resistant isolates (Figure 2).

Genes encoding for resistance to three antimicrobial subclasses were tested in 74 out of 165 phenotypically resistant isolates. One (1.4%) isolate had *TetA* gene, 6

Table 4. Molecular characterization of pooled positive *E. coli* from stool (N=555).

PCR genotype	Prevalence (n)	Genes demonstrated
Enterohemorrhagic *E. coli* (EHEC)	5.76%, (32/555)	*stx1,stx2, eae*
Atypical Enteropathogenic *E. coli* (EPEC)	3.06%, (17/555)	*eae*
Typical Enteropathogenic *E.coli* (EPEC)	0.54%, (3/555)	*eae, bfp*
Enterotoxigenic *E.coli* (ETEC)	2.34%, (13/555)	*lt, stII*
Diffuse adherent *E.coli* (DAEC)	2.16%, (12/555)	*daaE*
Enteroaggregative *E.coli* (EAggEC)	0.72%, (4/555)	*aafII*
Enteroinvasive *E.coli* (EIEC)	0.36%, (2/555)	*IpaH, VirF*

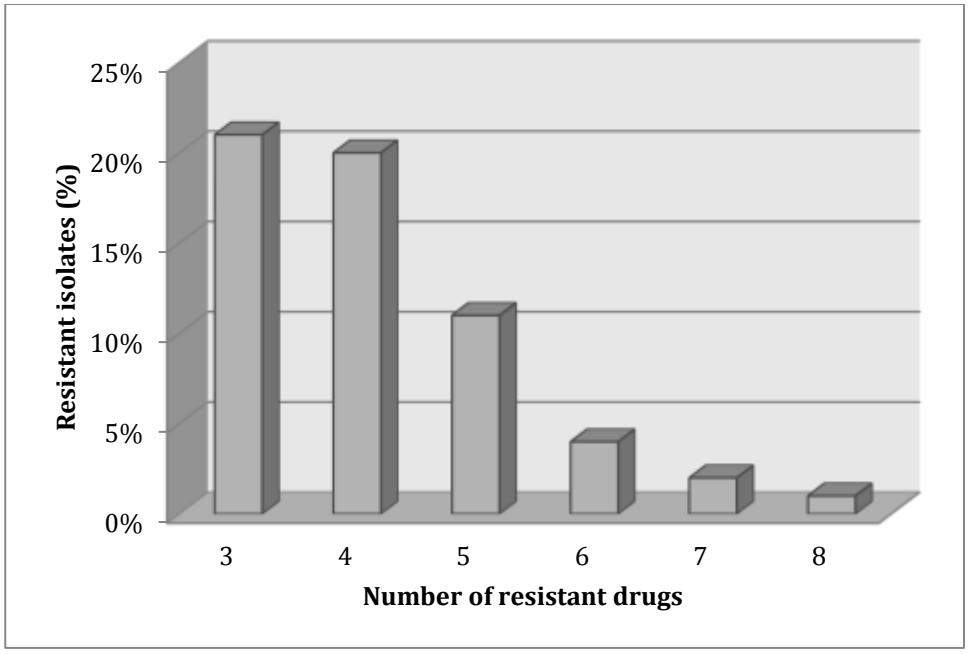

Figure 1. Percentage of multidrug resistant *E. coli*.

(8.1%) had *TetB* gene, 14 (19%) had *TetC* gene and none had *TetO* gene. Sulfonamides gene (*Sul1*) was only found in 10 (14%) isolates while none of the isolates had the *Sul2* gene.

Molecular sequencing of selected *E. coli* isolates

The *eae* gene encoding the outer membrane protein was demonstrated in 5 *E. coli* isolates submitted for gene sequencing. The *eae* gene in the 5 isolates had blastn alignment identity of 99%, 98 and 94% with standard *E. coli* O157:H7, EP 057 isolate and *E.coli* O157:H7, respectively. These similarities confirmed the identity of the PCR products obtained from *E. coli* isolated from the asymptomatic school going pupils. Heat labile enterotoxin (*Lt)* genes were demonstrated as eltB and eltA flanked by IS600 and IS1294. Two isolates processing the heat labile enterotoxin B encoding genes were submitted for sequencing. The sequences alignment with blastn gave a 99 and 84% identity for isolates 103a and 582a, respectively. Heat stable enterotoxin (*StII* gene) was shown in two isolates which were submitted for sequencing. The PCR products for the *StII* gene were sequenced and the sequences aligned using blastn giving 74 and 100% alignment respectively. One isolate with the *bfpA* gene encoding for major structure subunit of bundle forming pilus, had gene alignment identity at 99%.

DISCUSSION

Pathogenic *E. coli* were characterized using multiplex PCR to demonstrate the presence of characteristic virulence genes. A prevalence of 17% obtained in this study was much lower compared to Rono et al. (2014), who reported a prevalence of 34.2% in diarrheal

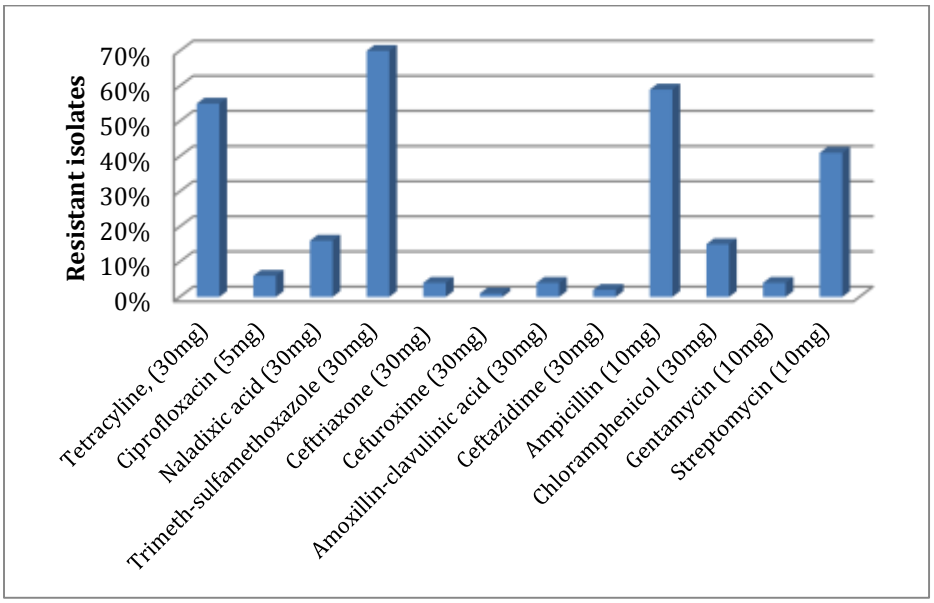

Figure 2. Drug resistant patterns of pathogenic *E. coli* isolates from stool samples, N=165.

samples. The difference in the observations being attributed to the fact that while this study sampled asymptomatic pupils, Rono et al. (2014) worked with diarrheal patients.

Molecular analysis of pathogenic *E.coli* isolates revealed that 13 (2.3%) samples had the enterotoxigenic (ETEC) genes, *LT* or the *ST 2*. Vidal et al. (2005) described ETEC having one or more enterotoxins; heat labile (LT) *LT1* and *LT2* or the heat stable (ST) as *STa* and STb. In the present study, three children had typical EPEC with both the *eae* and *bfp* genes. Another 17 pupils had atypical EPEC having only the *eae* gene. These findings compares well with those reported by Bugarel et al. (2011) and Vidal et al. (2005) who observed a ratio of 1:3 and 1:4, typical to atypical EPEC respectively. EPEC has proteins involved in attaching and effacement of the host cells microvilli. It has the EPEC adherence factor (EAF) plasmid and gene cluster encoding the bundle–forming pili (*bfp*) gene. Typical EPEC are those isolates with the EAF plasmid, those with the *bfp* gene but lack the EAF plasmid are classified as atypical EPEC. EIEC had the least number of isolates with only 2 (0.36%) children positive. This compares with other findings where none out of 509 stool samples yielded EIEC (Vidal et al., 2005). Rono et al. (2014) made comparable observations, with EIEC isolation being the second last frequently isolated, at 3 out of 100 samples. EIEC possess a gene located in a virulence plasmid (Plnv) 140 MDa encoding type III secretion system. Highest number of isolates were found to be EHEC, with 32 out of 555 children positive. The isolates had a combination of *eae* gene with either *stx1* or *stx2* genes. Several authors have reported different distributions in the prevalence of different pathogenic *E. coli*. Some have reported EAEC as the most frequent; others had EAggEC being the most frequent, while ETEC was shown by a different study as the most frequent. EPEC has also been identified by a different study as the most prevalent in Kenya (Sang et al., 2012a; Sang et al., 2012b; Makobe et al., 2012; Bii et al., 2005).

Unlike Vidal et al. (2005) who observed one patient out 509 with mixed enteropathogenic *E. coli*, this study had 10 individuals out of 555 with mixed enteropathogenic infections. While 9 of the 10 had combinations of two different enteropathogenic *E. coli*, one of the children had three pathogenic *E.coli* characterized (EHEC, EAggEC and DAEC). These findings are an indication that clinical symptoms alone, may not be conclusive in the diagnosis of entropathogenic *E. coli* infections. Resistance to sulfonamides was the highest at 70%, this was followed by ampicillin at 59%, tetracycline 55%, streptomycin 41%, nalidixic acid at 16% and chloramphenicol at 15%. The findings in this study agree with Christabel et al. (2012) who reported high resistance of environmental isolates from Kibera slums. Though the frequencies were not in agreement with this study, the general profile of resistance is comparable. In this study prevalence of resistance to cephalosporins (ceftriaxone, cefuroxime and ceftazidime) were very low at 4, 0.7 and 2%, respectively, resistance by *E. coli* to cephalosporins is mainly by the extended spectrum beta-lactamases (ESBLs) enzymes. These enzymes are able to destroy most of the beta-lactam antibiotics (WHO, 2014). ESBLs can be transferred between bacteria species. In a report on resistance to antibacterial drugs in selected bacteria of international concern WHO, (2014) the report gave a

range of resistance *E. coli* to third generation cephalosporin in thirteen African Countries at 2-70%. This was within the levels of this study which had 4% resistant to ceftriaxone.

Only 31 (18.7%) pathogenic isolates were sensitive to all the 12 antibiotics tested, 21 (12.7%) were resistant to only one of the 12 antibiotics, while the other 113 (68.5%) were resistant to more than 2 antibiotics. Isolates resistant to 3 or more antibiotics were 35 (21.2%) thus classified as multidrug resistant (MDR) isolates. Those resistant to any four of the 12 antibiotics were 33 (20%), those resistant to 5 antibiotics were 18 (10.9%), resistant to 6 antibiotics were 6 (3.6%) and those resistant to 7 antibiotics were 3 (1.8%) isolates. Multidrug resistant *E. coli* has been reported on environmental isolates at 40% from the same study area. These findings have slightly lower frequency compared to those reported in this study, which could be attribute to the differences between human isolates and environmental isolates. The findings however agrees with Kipkorir et al. (2016) who reported 42.2 % MDR *E. coli* isolates from faecal specimens obtained from patients ages 2 weeks to 82 years. The patients had gastroenteritis and the study was in Kitale, Kenya. Oundo et al. (2008) also reported 65.5% MDR *E. coli* isolates from asymptomatic food handlers in Kenya, which was very close to this study's 68.5%. They however reported resistance to cefuroxime at 6.9% which was much higher than 0.7% in the current study, which could be attributed to difference in the age of sampled study subjects.

Sequenced *TetA* gene from enteropathgenic tetracycline resistant *E. coli* showed 99% homology with related *TetA* sequences in GenBank. The fact that 55% of tested *E. coli* were resistant to tetracycline and the demonstration of *TetA* genes in some of the isolates would suggest that genes were freely transferable between bacteria *in-vivo* in cases of multi-bacterial infections observed in this study. The homology observed is a frequent finding with tetracycline resistance encoding genes in *E. coli*. *TetA* gene has been reported by Abdi-Hachesoo et al. (2014) to be located on bacterial mobile elements and thus horizontally transferable among bacteria strains. The 99 and 100% homology obtained after sequences alignment of the virulence genes confirmed identity and virulence of the pathogenic *E. coli* isolates. This proved that diarrheagenic *E. coli* were indeed isolated from asymptomatic children in the current study. Similar findings have been reported by Quiroga et al. (2000), who demonstrated the presence of EPEC, DAEC, ETEC and EAgEC in symptomatic infants. The authors also described 7.5 months of life as the earliest time of *E. coli* colonization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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