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PATHOGENIC *E.COLI* AND OTHER PATHOGENIC GRAM NEGATIVE ENTERIC STRAINS FROM FOECAL SAMPLES OF CHILDREN WITHOUT DIARRHOEA LIVING IN MUKURU SLUMS, NAIROBI
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PATHOGENIC *E.COLI* AND OTHER PATHOGENIC GRAM NEGATIVE ENTERIC STRAINS FROM FOECAL SAMPLES OF CHILDREN WITHOUT DIARRHOEA LIVING IN MUKURU SLUMS, NAIROBI

P. M. IMPWI, P. WAMBUGU, A. N. KIMANG'A and S. KARIUKI

ABSTRACT

Background: **Diarrhoea remains a major public health problem among children and adults in developing nations such as Kenya. The risk of infection is higher in children due to their developing immunity, relatively poor hygiene and habits especially those living in informal settlements where water supply and sanitation are inadequate.**
Objectives: **To determine the prevalence of selected enteric pathogens from children without diarrhoea attending two clinics in Mukuru as well as the anti-microbial resistance patterns, and pathogenicity of *E. coli* isolated.**
Design: **A cross sectional study.**
Setting: **Mukuru slum, Nairobi County.**
Subjects: **Three hundred and twenty two children of ages five and below.**
Results: **Mukuru Kwa Njenga; *E. coli* 34.6%, *Salmonella spp.* 1.3%, *Shigella* 0.7%, *Citrobacter spp.* 2.3%, *Klebsiella spp.* 5.3%, *Proteus spp.* 7.0%, No growth 2.3%. Mukuru Kwa Reuben; *E. coli* 63.4%, *Salmonella* 0.6%, *Shigella* 0.6%, *Citrobacter spp.* 1.2%, *Klebsiella spp.* 14.3%, *Proteus spp.* 16.1%, No growth 3.7%. No significant difference among the organisms isolated in both clinics (p = 0.982). Ampicillin, Amoxycillin/ Clavulanic, cefoxitin had high resistance, while gentamicin was 100% susceptible. 46.6% *E. coli* isolates were positive for at least one of the eight virulence genes tested.**
***Conclusion: Salmonella, Shigella* and *pathogenic E.coli* associated with diarrhoea and presence of resistance genes were identified in foecal samples of children without diarrhoea living in Mukuru informal settlements in Nairobi. The major concern from the findings of this study was the emerging high resistance of *E.coli* that was observed to cephamycin (Cefoxitin).**

ITRODUCTION

An asymptomatic carrier (healthy carrier or just carrier) is a person or other organism that has contracted an infectious disease, but who displays no symptoms. Although unaffected by the disease themselves, carriers can transmit it to others (1). A carrier may be one with a latent infection and which appears healthy. Other types of carriers are the incubatory carrier, when the animal is not yet showing clinical signs or a convalescent carrier when it has passed the clinical stage. A study done in Bristol University of UK showed that the children can be carriers of antibiotic-resistant bacteria (2).

Gastroenteritis or infectious diarrhoea is a medical condition characterised by inflammation (*"-itis"*) of the gastrointestinal tract that involves both the stomach (*"gastro"*-) and the small intestine (*"entero"*-), resulting in some combination of diarrhoea, vomiting, and abdominal pain and cramping (3). Diarrhoea is the passage of three or more liquid stools within 24 hours or any number (more than three times) of loose stools accompanied with mucous within 24 hours as per WHO definition (4). Diarrhoeal disease is a public health problem that mostly affects children in developing countries, where approximately 1.9 million children die each year (5), or roughly accounting for all 15% of all child deaths (6). This disease is also responsible for chronic malnutrition due to malabsorption, and it

has impacts on cognitive development, in particular semant fluency. In developing areas, due to the poor hygiene and sanitation, enteric bacteria and parasites are highly prevalent (7). Infective diarrhoea is one of the leading causes of morbidity and mortality among children under five years in the developing world and can be caused by a wide range of viruses, bacteria, or parasites (8). Gram-negative bacteria are the major cause of diarrhoea and they belong in a class of bacteria that do not retain the crystal violet stain used in the Gram staining method of bacterial differentiation, making positive identification possible. They have a thin peptidoglycan layer of cell wall sandwiched between an inner cell membrane and a bacterial outer membrane. Some of them cause diarrhoea infection to human beings and they include; *E. coli*, *salmonella*, *Shigella*, among others.

World Health Organization, Global Burden of Disease estimates, 2004 update showed that nearly three quarters of child deaths are due to diarrhoea where Uganda was ranked number 9 with a total of 29,300 child deaths annually, Kenya number 10 with 27,400 total child deaths annually and Tanzania number 13 with 23,900 total child deaths annually (4)

In Kenya <5 mortality rate was 128 per 1000 live births while the number of under-five deaths was 189,000 as per the WHO 2008 annual report (4). An investigation was initiated as a consequence of several cases of diarrhea in a nursery ward for preterm babies in Nairobi, Kenya (9). Ten lactose-positive colonies were isolated from the stools of each of 30 neonates, regardless of whether they had diarrhea; 229 strains were identified as *Escherichia coli* and 65 strains were identified as *Klebsiella pneumonia* (9). A study on bacterial diarrhoea diseases involving children below five years of age in Kenya, the KEMRI/JICA Research and Control of Infectious Diarrhoea Project (between 1990 and 1995) reported the first confirmed case of hoemorrhagic colitis due to *E. coli* serotype O157:H7 in Kenya (10).

From studies done previously in Kenya on different settings, among the major causes of diarrhoea in children under five are pathogenic *E.coli, Shigella* and *Salmonella spp* (11,13).

MATERIALS AND METHODS

Study area and sampling: This was a laboratory based cross sectional study of children aged five years and below who presented at the selected outpatient clinics (Reuben Centre and Medical Missionaries of Mary) for routine checkup/immunisation or treatment of other diseases other than diarrhoea. The two clinics are public dispensaries located in Mukuru informal settlement, which is about 15 km east of the Nairobi city centre. Basic demographic information including age and sex was collected on enrolment. A total sample size of 322 study participants (161) from each clinic, was calculated and enrolled in the study using systematic random sampling. Stool samples were collected from the children without diarrhoea, rectal swab was obtained when could not produce the fecal sample. The foecal specimen was placed in a Carry Blair transport media using a sterile swab then in a cool box and transported to Kenya Medical Research Institute, Centre for Microbiology Research laboratories. The study was approved by Kenya Medical Research Institute (KEMRI) ethical review committee. Consent was obtained before inclusion from parents or guardians of the participants after complete explanation of the study content and purpose.

Isolation of the bacterial: For isolation, part of the samples were directly inoculated on MacConkey agar while another part was enriched in Selenite-F broth and then sub-cultured onto the following plating media; Xylose Lysine Deoxychocolate (XLD) agar(Oxoid, Basingstoke, UK) for identification of *Salmonela* and *Shigella,* MacConkey agar (Oxoid, Basingstoke, UK) was used for all enterics while Sorbitol MacConkey agar(Oxoid, Basingstoke, UK) was used as a medium of choice for *E. coli 0157:H7* and it contains sorbitol instead of lactose as in the ordinary MacConkey medium. Nearly all isolates of *E. coli 0157:H7* ferment D-sorbitol slowly and appear colorless on SMAC while non E. coli colonies appear pinkish. After inoculation all media were incubated at 36C (+/- 1°C) in a non-CO2 incubator for 18-24 hours (overnight) then observed for characteristic growth of organisms. The suspected colonies were then picked and biochemical test performed for confirmation (14,15). Isolates from the biochemical testing which were strongly indicative of a particular organism such as *Salmonella spp,* and *Shigella spp* were serotyped using commercial antisera (Remel Europe, Dartford Kent, DA2 6PT, UK) by visible agglutination method following the manufacturer's instructions.

Anti-microbial susceptibility testing: Anti-biotic susceptibility testing was performed on the *E. coli*isolates using the Kirby – Bauer disc diffusion technique (16). The anti-biotics used included; Cefpodoxime (CPD 10μg), ceftazidime (CAZ 30μg), cefotaxime (CTX 30μg), Cefoxitin (FOX 30μg), Cefepime (FEP 30μg), ampicillin (AMP 10μg), Amoxycillin/Clavulanic 2:1 (AMC 30μg), ciprofloxacin (CIP 5μg), sulphamethoxazole/trimethoprim (SXT 25μg), chloramphenicol (C 30μg), gentamicin (CN 10μg), streptomycin (S 25 μg), nalidixic acid (NA 30μg) and tetracycline (TE 30μg). All anti-biotic discs were from Oxoid, Basingstoke, UK. These anti-biotics were chosen on the basis of their use in the management of enteric bacterial infections. The concentrations of the antimicrobial disks were selected based on the internationally recognised

standards and guidelines on anti-microbial routine testing and reporting on *enterobacteriaceae* provided by the Clinical and Laboratory Standard Institute (2012). The innocula for susceptibility testing was compared against the McFarland 0.5 turbidity standard with the *E. coli* ATCC 25922 strain being used as the test standard. The interpretation of results was according to Clinical Laboratory Standard Institute (CLSI) guidelines (17).

E. coli DNA extraction and Amplification: DNA extraction was done by boiling method. 1 ml of PBS was transferred to a 1.5-ml Eppendorf tube. Using inoculation loop (wire), a loop full of bacteria was picked from a plate (pure colonies) and transferred to the Eppendorf tube. The suspension was Shaken or vortexed just before use to make a homogeneous cell suspension. Centrifuge at 14,000 rpm for 5 min. Supernatant was discarded and the pellet re-suspended in 100 µl TE, and then boiled (or heated at 95°C) for 5-10 minutes and transfer it directly to ice. The lysed DNA was diluted 10 fold in TE before amplification. A ventilation hole was made in the lid of the Eppendorf tube using a needle (15).

Multiplex PCR for categorisation of diarrhoeagenic *E. coli* (DEC) into EAEC, ETEC, EPEC, STEC and EIEC was done using primers (0.2 μmol, HPSF purification) for identification of the genes. The specificity of each primer set was confirmed by monoplex PCR and then multiplex PCR was carried out using reference strains(18). Briefly, the optimised PCR protocol was carried out with a 25μl mixture containing 20μl PCR buffer, PuReTaqready to go PCR beads (GE Health care) were used for, 0.5 μl of each primer set and 1μl of DNA template. Two master mixes were used depending on the primers, master mix 1 comprised of eae, aggR, Stx and bfpprimers, while master mix 2 contained *LT, ST* and *ipaH* primers. A monoplex PCR was done for *INV* gene. Primer sequences used for the detection of virulence genes were described by Gomez-Duarte *et al*.(19). The β-lactamase genes were screened by PCR using primers bla_{TEM}, bla_{SHV} and bla_{CTX-M}genes were detected using a PCR procedure previously described by Kiiru *et al.* (20). The PCR was carried out in a thermal cycler using a programme of initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 92°C for 30sec, annealing at 59°C for 30sec, and extension at 72°C for 30sec; and final extension at 72°C for 5 min. The PCR products was then separated by electrophoresis on a 2% agarose gel (AmpliSize; Bio-Rad Laboratories) stained with ethidium bromide and visualised by UV transillumination(21,22)B1, D and A Diarrhoeagenic *E. coli* reference strains EHEC (*stx1*,*stx2*), EPEC (*eae*, *bfp*A) and ETEC (*st* and *lt*) were used as positive controls. Negative control used was the master mix plus water instead of DNA.

Statistical Analysis: Data were entered in SPSS version 17.0 software and analysed using chi-square. Chi-square was used to describe associations and P-value equal or less than 0.05 was considered as statistically significant.

RESULTS

The study targeted children without diarrhoea living in Mukuru slums, Nairobi. From the study, 322 samples were collected from both sites whereby each site had 161 samples. From Mukuru kwa Reuben 68(42%) were females while 93(57.8%) were males, while from Mukuru Kwa Njenga 73(45%) were females and 88(54.7%) were males. Out of 161 samples collected from Mukuru Kwa Njenga *E. coli* had the highest prevalence of 34.6%,*Salmonella* spp. 1.3% and *Shigella* 0.7%.There were 2 *Salmonella enteritidis* and 2 *Salmonella typhi*, 1 *Shigella sonnei* and 1 *Shigella flexneri*. Other bacteria isolated were*Citrobacter spp*. 2.3%,*Klebsiella spp*. 5.3% and *Proteus spp*. 7.0%. Seven samples collected in the same site had no growth. At Mukuru Kwa Reuben *E. coli* also had the highest prevalence of 63.4% while *Salmonella typhi* and *Shigella sonnei* had the lowest prevalence of 0.6% each. Other isolated bacteria were; *Citrobacter spp.* 1.2%, *Klebsiella spp*14.3% and *Proteus spp* 16.1%. Out of 161 samples collected here six samples did not show any growth in both primary agars XLD and MacConkey). There was no association between the bacterial isolated and the clinics (χ^2n-1 = 0.714; d.f = 5; p = 0.982).

Table 1
Bacterial Isolated at Mukuru kwa Njenga and Mukuru kwa Reuben

	Mukuru kwa Njenga		Mukuru kwa Reuben	
Organisms isolated	Frequency	Percentage (%)	Frequency	Percentage (%)
Citrobacter spp	7	2.3	2	1.2
E. coli spp	104	34.6	102	63.4
Klebsiella spp	16	5.3	23	14.3
No Growth	7	2.3	6	3.7
Proteus spp	21	7.0	26	16.1
Salmonella spp	4	1.3	1	.6
Shigella spp	2	.7	1	.6
TOTAL	161	100.0	161	100.0

Prevalence of anti-microbial resistance in both regions was highest for ampicillin, Amoxycillin/Clavulanic, cefoxitin, sulphamethoxazole/trimethoprim and Tetracycline. The anti-microbials with the lowest resistance were; cefpodoxime, ceftazidime, chloramphenicol and cefepime. Gentamicin had no resistant thus it was 100% susceptible.Cefoxitin which is a cephamycin recorded 54% resistance at Mukuru kwa Njenga and 64.3% resistance at Mukuru Kwa Reuben. 60% (105/174) of the *E. coli* isolated exhibited resistance to more than three of the 12 anti-biotics tested while 10% (18/174) isolates were susceptible to all the anti-biotics used. There was no significant difference in the prevalence of antimicrobial resistance at Mukuru kwa Njenga, (χ^2n-1 = 3.284; d.f = 10; p = 0.974 and Mukuru kwa Reuben, χ^2n-1 = 0.857, df =12, p =1.000). Resistance genes were identified from some (20 isolates) of the multiple drug resistance (MDR) *E.coli* in which only three types of resistance genes identified;CMY 2(25%), TEM 1 5(62.5%) and SHV 1(1.1%). One isolate carried multiple resistance genes which were CMY and TEM 1.

Table 2
Anti-biotic Susceptibility Profiles of the E.coli Isolates (n=87)

Anti-biotic	Mukuru Kwa Njenga Frequency of resistant isolates	%	Mukuru Kwa Reuben Frequency of resistant isolates	%
CPD	2	2.3	14	16.1
CAZ	3	3.4	1	1.1
CTX	5	5.7	3	3.5
FOX	47	54	56	64.4
FEP	3	3.3	2	2.3
AMP	52	59.8	52	59.7
AMC	52	59.8	54	62.1
CIP	4	4.6	3	3.4
SXT	36	41.4	45	51.7
C	3	3.4	4	4.5
CN	0	0	0	0
S	17	19.5	23	26.4
NA	7	8	9	10.3
TE	22	25.3	28	32.2

Out of 103(50%) *E.coli* isolates from foecal samples of apparently healthy children 48(46.6%) carried at least one of nine virulence genes tested and specific for five known diarrhoeagenic *E. coli*. EAEC *(aggR),* EPEC *(eae&bfpA)*, ETEC *(ST&LT)*, EHEC *(stx1& stx2)* and EIEC *(ipaH&inv) bfpA* had the highest percentage followed by *aggR* while *inv* and *ipaH* had the lowest percentages. There was no significant difference in the prevalence of virulence genes between the two Clinics (χ2n-1 = 0.000, d.f =4, p =1.000).

Table 3
Classification of pathogenic E. coli

Pathogenic *E. coli*	Frequency of isolates	Percentage (%)
EAEC	13	27.1
EPEC	18	37.4
ETEC	13	27.1
EHEC	2	4.2
EIEC	2	4.2

DISCUSSION

Detection of enteric bacterial pathogens: The study was to characterise pathogenic *E.coli* and other pathogenic gram negative enteric strains from foecal samples of children without diarrhoea living in Mukuru slums, Nairobi. The emphasize was put on three enteric bacteria which are *Salmonella, Shigella* and *E.coli.* At Mukuru Kwa Reuben *E. coli* was the most isolated with 63.4% while *Salmonella typhi* and *Shigella sonnei* were the least isolated of 0.6%. At Mukuru Kwa Njenga the isolation rate was *E. coli* 34.6%. *Salmonella spp.* 1.3% and *Shigella spp* 0.7%, while the frequency rate for *salmonella spp* was 2 *S. enteritidis* and 2 *S. typhi.* For *Shigella spp* there was 1*S.frexneri*and 1 *S. sonnei.* The findings are almost similar to those of a study done in Finland on Prevalence of Diarrhoeagenic *Escherichia coli* in Finns with or without Diarrhoea during a Round-the-World Trip, which showed entero-pathogenic bacteria were present in 33% of the 127 non-diarrhoeal samples; diarrheagenic *E. coli* strains were found in 26% of these. As a single pathogen, *E. coli* was found in 24% of samples (23). The apparently healthy children (non-diarrhoeal samples) tend to carry the pathogenic enterics same as diarrhoea samples, whereby *E.coli* was the most common followed by *Salmonella* and *Shigella* was the least of all. A study done in Kenya (Mbagathi District Hospital) found that *Escherichia coli* (93.83%) was the most commonly isolated pathogen followed by *Salmonella* 3.7% and *Shigella* 2.4%) (12)median of 26.0 months and age range between 2-60 months. The bacterial isolation rates were ETEC 9.1%, EPEC 6.8% and EAEC 12.3%, *Salmonella paratyphoid* (10.4%) Other organisms isolated were *Citrobacter spp., Klebsiella spp.* and *Proteus spp.*

Anti-microbial susceptibility of E. coli isolated from foecal samples and Resistance genes: Anti-microbial resistance (AMR) threatens the effectiveness of successful treatments for infections and is a public health issue with local, national and global dimensions. In low-income countries, AMR frequently occurs in micro-organisms that are likely to be transmitted in the community such as those that cause pneumonia, diarrhoea diseases, typhoid fever, tuberculosis, sexually transmitted diseases and malaria. Resistance to anti-microbial agents renders drugs for these illnesses ineffective, resulting in the need for wide-scale use of broad-spectrum agents, in the process creating a major global threat.

In this study Ampicillin, Amoxycillin/ Clavulanic, cefoxitin and sulphamethoxazole/ trimethoprim had the highest resistance. This finding is consistent with related studies done in Eastleigh City Council Health Centre, Kenya and another one done in Four Province of Kenya which found a high level of isolates to be resistant to the anti-biotics prescribed most commonly in Kenya, such as amoxicillin, ampicillin but disagree on tetracycline which is not highly resistance (11,10). Cefoxitin which is a cephamycin at Mukuru kwa Njenga it recorded 54% resistance while in Mukuru Kwa Reuben it had 64.3% resistance. This is in contrast with a study that was carried out in Kenya which showed that cefoxitin was effective against majority (60%) of the total isolates (20). Cefpodoxime, ceftazidime, chloramphenicol and cefepime recorded the lowest resistance while gentamicin recorded 100% susceptible. This is similar to a study that was done in Eastleigh City Council Health Centre, Kenya where gentamicin was sensitive to all isolates (11). Many developing countries rely heavily on β-lactam anti-biotics which has posed a big threat due to the enormous resistance. Currently most of the studies and researches are geared towards the diversity of β-lactamase genes (bla) is the base for understanding the resistance and can help in development of more effective antibiotics.This study only selected 20 isolates of *E.coli* with resistance to cefoxitin, Ampicillin and Amoxycillin/Clavulanic and 8(40%) tested positive for virulence genes. 2(25%) tested positive for bla_{CMY}, 5(62.5%) for $bla_{TEM\ 1}$ and 1(1.1%) for bla_{SHV}. One isolate carried multiple resistance genes and tested positive for $bla_{CMY}$and bla_{TEM1}. This study agrees with a study done in Kenya in which the carriage and diversity of blaTEM 1 was high while SHV-type ESBL genes was also low (20). In a similar study done in Czech Republic during 2007 to 2009 bla_{TEM} and bla_{SHV} genes were detected in isolates with ampicillin resistance (24).

Prevalence of E. coli isolates harboring virulence marker: Out of 103(50%) *E.coli* isolates from foecal samples of apparently healthy children 48(46.6%) carried virulence genes tested. EPEC was the most common pathogenic *E. coli* isolated (37.4%) followed by EAEC and ETEC both with 27.1%, the least common were EHEC and EIEC both with 4.2%. A study in Egypt that involved both diarrhoearing and non-diarrhoearing (as controls) reported that the non-diarrhoearing had no EPEC while in this study EPEC had the highest percentage (25). In another study carried out on Children with and without Diarrhoea in Switzerland EPEC was commonly isolated which agrees with this study but disagrees on EAEC which were rarely isolated, ETEC and EIEC were not isolated from children with no diarrhoea (26). This study disagrees with a study done in Meru, Kenya whereby EAEC was commonly isolated followed by ETEC and EPEC the least (12).

In conclusion, the major concern from the findings of this study is the emerging high resistance of *E.coli* that was observed to cephamycin (Cefoxitin), thus, we suggest that further studies to be done in order to identify all the genes that confer resistance to Cefoxitin. Due to the identification of pathogenic bacterial associated with diarrhoea in foecal samples of children without diarrhoea living in Mukuru informal settlements in Nairobi Community health

education on personal hygiene, treatment of human waste and access to treated water should be enhanced so as to reduce the carriage of anti-microbial resistance and pathogenic gram negative enteric strains among pre- school children.

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