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Occurrence of Multidrug Resistance among *E. coli* O157:H7 Isolated from Stool Samples Obtained from Hospitalized Children

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Abstract

A survey of the antimicrobial resistance pattern of *Escherichia coli* O157:H7 strains obtained from stool samples collected from children with diarrhea attending General hospital Warri, General Hospital Agbor, Eku General Hospital and University of Benin teaching Hospital was carried out. All isolates were obtained using standard microbiological and biochemical procedures. Serological analysis to detect *E. coli* O157 strains was carried out using the dry spot *E. coli* O157 test kit. Antimicrobial susceptibility testing was carried out using disc diffusion method. A total of 46 *Escherichia coli* isolates were obtained from the 60 stool samples. All *Escherichia coli* isolated were 100% resistant to cefixime. The lowest level of resistance was observed in nitrofuratoin (15%). Serotypes O157 exhibited 100% resistance to ceftazidime, cefuroxime and cefixime. The ability of *E. coli* O157 strains to transfer antimicrobial resistance traits by conjugation was detected using *Pseudomonas aeruginosa* as recipient. High level of resistance transferred was observed. The ease of transfer exhibited by *E. coli* O157 strains amongst children in this study is an issue of concern. As such, an early identification and understanding of the epidemiology of this resistance will enable the development of preventive strategies which can curtail this emerging resistance, thereby facilitating a timely and appropriate public health response.

Keywords: Diarrhoea; *E. coli* O157; Resistance

Introduction

The continued spread of multidrug resistant pathogens remains a huge public health problem worldwide. Chemotherapeutic agents employed in the treatment of serious infections have experienced a steady diminishing efficacy due to this scourge. This is even more highlighted in developing countries where misuse of drugs, poor regulation of over-the-counter sales of drugs, inadequately equipped diagnostic laboratories and relatively inadequate healthcare provision among other factors that serve to promote antibiotic resistance are widespread [1]. Diarrhoea is one of the leading causes of death in children in developing countries [2]. UNICEF/WHO [3] described diarrhoea as the passage of loose or watery stools at least three times per day or more frequently than normal for an individual. *E. coli* O157:H7 infections in children under the age of 5 have been associated with risk factors such as domestic use of contaminated water, premature weaning, bottle-feeding, and malnutrition [3-5]. In its report in 2008, the Word Health Organization stated that the highest number of diarrheagenic *E. coli* isolated in their study belonged to the O157:H7 serogroup [6]. Several studies have documented an observed decline in the mortality rates of diarrhoea infections in children worldwide and have suggested that this might be due to improved exclusive breastfeeding practices as well as the awareness generated towards the efficacy of oral rehydration treatments in reducing the mortality rates of these infections [7,8]. Despite this decline, however, the burden of diarrhoea and its mortality in children still exists in developing countries and it is reported that Africa and Asia account for 80% of children deaths due to diarrhoea with Nigeria ranking second with an estimated annual total of 151,700 child deaths due to diarrhoea [3]. Limited reports on the occurrence of *E. coli* O157:H7 infections in children in Nigeria, and more specifically, Delta State as well as the increase in documentations of multidrug resistant pathogens in this serotype have necessitated this study. This study was designed to detect the occurrence of multidrug resistant *E. coli* O157:H7 serotypes in children (0-5 yrs) having diarrhoea in Central Hospital Warri, Delta State, Central Hospital, Agbor, Delta State, Eku General Hospital, Delta State, and University of Benin Teaching Hospital, Edo State as well as to determine the transmissibility of these plasmid-borne resistance genes through conjugation experiments.

Materials and Methods

Sample collection

Stool samples were collected from children (0-5 yrs) having diarrhea using a sterile universal container and was labelled. A total of sixty samples were collected in sterile leak-proof universal containers within 4 months. These were transported immediately in ice packs to the Delta State University Microbiology laboratory for analysis.

Isolation and identification of *E. coli* O157:H7

The samples were inoculated into 5 ml of sterile MacConkey broth in sterile test tubes and incubated for 24 hours. A loopful was taken from the test tubes individually, inoculated onto freshly prepared Eosin methylene blue (EMB) agar plates, and incubated at 37°C for 24 hours. Observed colonies were subcultured onto freshly prepared nutrient agar plates and incubated at 37°C for 24 hours. Presumptive *E. coli* colonies were subjected to confirmatory gram staining and biochemical tests as described Cheesebrough [9]. Confirmation of *E. coli* O157 was done by testing for agglutination with *E. coli* O157 antisera (Oxoid).

Antimicrobial susceptibility test

The antimicrobial sensitivity of each of the isolates was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute [10]. The antibiotics used include Ceftazidime (Caz) 30 µg, Cefuroxime (Crx) 30 µg, Gentamicin (Gen) 10 µg, Cefixime (Cxm) 5 µg, Ofloxacin (Ofl) 5 µg, Augmentin (Aug)

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30 µg, Nitrofurantoin (Nit) 30 µg and Ciprofloxacin (Cpr) 5 µg. The inhibition zones obtained were interpreted in accordance with the zone diameter chart supplied by the CLSI [10].

Curing of isolates

Multi-drug resistant isolates were that were resistant to gentamicin and sensitive to nitrofuratoin were selected for plasmid curing [11]. Plasmid curing was carried out using Sodium Dodecyl Sulphate (SDS). An SDS solution was added to Lauria-Bertani (LB) broth. An LB normal strength was prepared and an inoculum of 100 µL of the isolate was then used to seed the SDS containing LB. The cultures were incubated overnight with shaking at 45°C and sub cultured for 6 days. A dilution from the culture was plated on EMB agar. To confirm loss of antimicrobial resistance, antimicrobial susceptibility was carried out as previously described.

Conjugation experiment

Conjugation was carried out using the method described by Thompson [11,12] and Kreuzer and Massey [13]. *Pseudomonas aeruginosa* strain that is Gentamicin sensitive and nitrofuratoin resistant obtained from the department of Microbiology, Delta State University Research laboratory was used as recipient. Donor and recipient strains were incubated separately on nutrient broth at 37°C for 24 hrs. Fifty microliter (50 µl) each of donor and recipient broth culture were transferred to the same spots on Mueller Hinton Agar plates supplemented with Nitrofurantoin (30 µg/ml) and Gentamicin (30 µg/ml) for selection of Trans conjugants. Incubation followed at 37°C for 24 hours. The Trans conjugants were then screened for antibiotic resistance as previously described.

Results and Discussion

Diarrhea remains a global public health problem significantly higher burden in developing countries evidenced by the higher incidences of childhood morbidity and mortality due to diarrhea in developing countries [8,14,15]. In several of the reports of diarrhea incidence in children, *Escherichia coli* have been implicated as being important etiologic agents [15-17]. In this study, a total of 46 *Escherichia coli* non-repetitive isolates were obtained from the 60 samples cultured, corroborating these aforementioned findings. A more alarming trend, however, is the increasingly high prevalence of multidrug-resistant diarrheagenic *E. coli* organisms, especially in developing countries. The rate of multidrug resistance in this study is in concordance with reports which have documented high resistance in *E. coli* causing diarrhea infections in children [18-20]. A total of 45 out of the 46 isolates obtained in this study were resistant to at least 3 of the antibiotics used, with highest resistance rates observed against the cephalosporins viz cefixime (100%), cefuroxime (98%), and ceftazidime (91%) (Tables 1 and 2). Much of the reasons for these high rates of resistance are related to the fact that reports have shown that antibiotics, despite not being required for the treatment of acute diarrhea, are widely prescribed for these forms of infections [21]. In children, this is made worse as cheap drugs are available over the counter and the wide majority of parents are unaware that antibiotics rarely alter the course of diarrhoeal infections and so administer one form of antibiotic or the other to their wards whenever a diarrheal infection is suspected. The continued use and abuse of these drugs thus allows for the selection of resistant strains which are easily disseminated. At this point, education of the masses on the management of diarrheal infections as well as the implementation of more stringent policies governing the availability of antibiotics is advised.

Twelve isolates (an incidence rate of 20%) were identified to belong to the serogroup O157 using the dry spot *E. coli* O157 test kit (oxoid) from the sixty stool samples collected. All 12 *E. coli* O157 harbored plasmids as indicated by the plasmid curing results.

S/N	Antimicrobial Agent	No of Resistant Isolates (%)
1	CXM – Cefixime	46 (100)
2	CRX – Cefuroxime	45 (98)
3	CAZ – Ceftazidime	42 (91)
4	AUG– Augmentin	32 (70)
5	GEN – Gentamicin	28 (61)
6	CPR – Ciprofloxacin	23 (50)
7	NIT – Nitrofurantoin	7 (15)
8	OFL – Ofloxacin	13 (28)

Table 1: Antimicrobial resistance profile of *E. coli* isolates.

S/N	Resistance Markers (No)	No of *E. coli* Isolates
1	CAZ, CXM (2)	1
2	CAZ, CRX, CXM (3)	2
3	CAZ, CRX CXM (3)	4
4	CAZ, CRX, GEN, CXM (4)	1
5	CAZ, CRX, CXM, AUG (4)	4
6	CRX, CXM, AUG, CPR (4)	4
7	CAZ, CRX, GEN, CXM, AUG (5)	6
8	CAZ, CRX, CXM, AUG, NIT (5)	3
9	CAZ, CRX, GEN, CXM, CPR (5)	2
10	CAZ, CRX, GEN, CXM, AUG, NIT (6)	2
11	CAZ, CRX, GEN, CXM, AUG, CPR (6)	4
12	CAZ, CRX, GEN, CXM, CPR, OFL (6)	4
13	CAZ, CRX, GEN, CXM, AUG, CPR, OFL (7)	7
14	CAZ, CRX, GEN, CXM, AUG, CPR, OFL, NIT (8)	2

Table 2: Multidrug resistance distribution of *E. coli* isolates.

S/N	Antimicrobial Agent	Symbol	No of Resistant Isolates (%)
1	CXM – Cefixime	CXM	12 (100)
2	CRX – Cefuroxime	CRX	12 (100)
3	CAZ – Ceftazidime	CAZ	12 (100)
4	AUG– Augmentin	AUG	8 (67)
5	CPR – Ciprofloxacin	CPR	8 (67)
6	GEN – Gentamicin	GEN	12(100)
7	OFL – Ofloxacin	OFL	5 (42)
8	NIT – Nitrofurantoin	NIT	2 (17)

Table 3: Antimicrobial resistance distribution of *E. coli* O157:H7 isolates.

Compared with other reports, our result is similar to other reports in India and Iraq [20-22] but suggests a rise in the prevalence of diarrhea due to *E. coli* O157:H7 in children in Nigeria [23-26]. In addition to contamination of water sources, animals consumed for food such as cattle and goats have been implicated as being asymptomatic carriers of *E. coli* O157:H7 strains [27,28]. Although the exact reasons for this increase in occurrence in comparison with other prevalence reports were not investigated in this study, Isibor and Ekundayo [26] suggested that the previous low reports of *E. coli* O157:H7 occurrence in Nigeria could be attributed to the inability of many medical laboratories in the country to detect its presence. Furthermore, in Nigeria, most clinicians do not readily request for the specific culture of these strains, much less in infected children. Multidrug resistance has also been reported in *E. coli* O157 strains. The antibiotic resistance patterns of *E. coli* O157 isolates is shown in Tables 3 and 4. All isolates of *Escherichia coli* O157 strains were 100% resistant to ceftazidime, cefuroxine and cefixime, 67% resistant to augmentin and ciprofloxacin, 58% resistant to gentamicin,

S/N	*E. coli* Isolate Number	Donor Resistance Profile	No of Markers	Resistance Markers Transferred	No of Markers
1	EC[17]	CAZ, CRX, GEN, CXM, AUG, **CPR**	6	CAZ, CRX, GEN, CXM, AUG	5
2	EC[18]	CAZ, CRX,GEN, CXM, OFL, AUG, **CPR**	7	CAZ, CRX, CXM, OFL, AUG	5
3	EC[19]	CAZ, CRX, GEN, CXM, AUG	5	CAZ, CRX, CXM, AUG	4
4	EC[21]	CAZ, CRX, CXM, GEN, **OFL, CPR**	6	CAZ, CRX, CXM, GEN	4
5	EC[22]	CAZ, CRX, GEN, CXM, OFL, AUG, CPR, **NIT**	8	CAZ, CRX, GEN, CXM, OFL, AUG, CPR	7
6	EC[24]	CAZ, CRX, GEN, CXM, **CPR**	5	CAZ, CRX, GEN, CXM	4
7	EC[28]	CAZ, CRX, GEN, CXM, AUG	5	CAZ, CRX, CXM, AUG	4
8	EC[30]	CAZ, CRX, GEN,CXM, OFL, AUG, CPR, **NIT**	8	CAZ, CRX, CXM, OFL, AUG, CPR	6
9	EC[31]	CAZ, CRX, GEN, CXM, AUG, **OFL, CPR**	7	CAZ, CRX, CXM, AUG	4
10	EC[37]	CAZ, CRX, GEN, CXM	4	CAZ, CRX, GEN, CXM	4
11	EC[40]	CAZ, CRX, GEN, CXM, AUG	5	CAZ, CRX, GEN, CXM, AUG	5
12	EC[41]	CAZ, CRX, GEN, CXM, **CPR**	5	CAZ, CRX, GEN, CXM	4

NB: Markers not transferred are in bold print.

Table 4: Transfer of Resistance Markers from *E. coli* Isolates to *Pseudomonas aeruginosa*.

42% resistant to ofloxacin and 17% resistant to Nitrofurantoin. In addition to the abuse of antibiotics in the control of diarrhea infections in children by their parents earlier stated, the indiscriminate use of antibiotics by livestock farmers could also have contributed to this high resistance rates. These high multidrug resistant rates of *E. coli* O157:H7 isolates, while alarming, are consistent with other findings in Nigeria and in other developing countries [28-30]. This represents a major problem not just to the chemotherapeutic control of diarrheal infections but also other infections caused by several genera within the family Enterobacteriaceae. This is largely due to the ease of transmissibility of these resistance genes from *E. coli* cells to other members of the family Enterobacteriaceae and Pseudomonads. This could greatly confound treatment of other non-diarrhea *E. coli* infections as well as infections by Enterobacteriaceae and Pseudomonads. To detect the transmissibility of these resistance genes, conjugation experiment was carried out. Table 4 also shows the antimicrobial resistance patterns of the transconjugant *Pseudomonas aeruginosa* strain used as recipient in the experiment. The conjugation rates in this experiment were 100% efficient for the transfer of ceftazidime, cefuroxine and cefixime, augmentin, and gentamicin resistance traits while 75% of the ofloxacin-resistant isolates transferred their resistance markers. Transfer of resistance traits by conjugation was less efficient for the transfer of ciprofloxacin resistance traits as only 2 out of 8 (25%) ciprofloxacin-resistant *E. coli* isolates transferred ciprofloxacin resistance markers. Nitrofurantoin resistance was not accounted for because the recipient strain harbourednitrofuratoin resistance There are several reports of high efficiency of transfer by conjugation of antimicrobial resistance traits by *E. coli* O157 strains [21,31]. The potentially grave implications of this are related to the fact that several factors within developing countries including urban migration, overcrowding and improper sewage disposal allow the easy exchange of antibiotic-resistant bacteria between individuals as well as the exchange of resistance genes among bacteria [1]. The inability of the *E. coli* O157 strains to transfer nitrofurantoin resistance could be due to the presence of the resistance markers on the chromosomes albeit it is essential to point out that transferrable plasmid-mediated resistance remain an important mechanism of nitrofurantoin resistance in *Escherichia coli* [32].

The emergence of multidrug resistance in the already notorious pathogen, *E. coli* O157 in Nigeria has grave public health consequences. More so, this study has highlighted the ease with which resistance markers to the commonly used antibiotics can be transferred even inter-generically. This therefore elicits urgent measures for the control of this scourge. Efficient management of the spread of this resistant serotype requires the involvement of many stakeholders. There is the need for effective government policies to help strictly control the availability of certain drugs to the general population. In Nigeria, even prescription drugs can be readily obtained over the counter without prescription from a clinician. The government is also saddled with the responsibility of providing better healthcare and diagnostic facilities to improve the detection of serotypes such as *E. coli* O157 which are not detectable using the existent diagnostic protocols in the country. Also, administration of antibiotics by healthcare providers needs to be strictly based on laboratory sensitivity tests results, whilst reducing empirical administration to the barest minimum. Finally, extensive education programs directed at members of the public to educate them on the hazards of self-medication, as well as the non-antibiotic control of acute diarrhea in children need to be instituted.

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