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Original Research Article

Multiple-Resistant Commensal *Escherichia Coli* from Nigerian Children: Potential Opportunistic Pathogens

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Abstract

Purpose: *The antimicrobial susceptibility and virulence traits of 150 strains of Escherichia coli characterized as commensals recovered from faecal samples from pre-school age children in Ile-Ife, Nigeria were evaluated in order to determine their potentials for pathogenicity and their contribution to antibiotic resistance in the community.*
Methods: *The isolates were identified using conventional biochemical methods. The presence or absence of virulence traits was determined using phenotypic and genotypic (polymerase chain reaction) methods. Their susceptibility to antibiotics was determined using the disk diffusion method.*
Results: *Possession of virulence properties including encapsulation (89.3 %), haemolysin production (24.8 %) and colicinogenicity (11.3 %) was detected among the strains and susceptibility of the strains to multiple antibiotics showed that the strains were highly resistant to cefalothin (100 %), streptomycin (94.0 %), tetracycline (92.0 %), and trimethoprim (89.3 %) while resistance to the quinolones was low (3.3 - 14.0 %).*
Conclusion: *The possession of virulence properties by antibiotic resistant strains of commensal E. coli may enhance their potential as extraintestinal pathogens.*

Keywords: *Escherichia coli, Virulence traits, Haemolysin, Colicin, Capsule, Antibiotic resistance, Drug resistance.*

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INTRODUCTION

Pathogenic strains of *E. coli* are able to cause gastrointestinal infections because they possess virulence factors which are responsible for their pathogenicity. They include enteropathogenic *E. coli*, enteroaggregative *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enterohaemorrhagic *E. coli*, and diffusely adherent *E. coli* [1]. Other proposed classes are cell detaching *E. coli* and cytolethal distending toxin producing *E. coli*. Non-pathogenic strains of *E. coli* referred to as commensal strains are facultative residents of the gastrointestinal tract where they remain harmless. This commensal bacterium can, however, cause extraintestinal diseases in their hosts.

The pathogenic potential of the *E. coli* strains is determined by the host's endogenous factors, as well as the genetic structure and ecological distribution of the strains in the particular host. Human infections by *E. coli* strains commonly occur in immuno-compromised individuals or when the normal gastrointestinal barrier is broken [2], enabling opportunistic pathogens to evade host surveillance mechanism. In this way the commensals can have access to other parts of the body such as the urinary tract, blood or wounds. Some of these commensals have been shown by phenotypic tests to possess some

virulence traits which can contribute to their ability to act as pathogens outside the gastrointestinal tract. Furthermore, high prevalence of antibiotic resistance have often been reported in surveillance studies involving commensal *E. coli* [3,4]. Similar to pathogenic strains, commensal *E. coli* are exposed to the selective pressure of antimicrobial agents, and they have often been used as indicator for the dissemination of acquired resistance genes [4]. The aim of this study is to determine the virulence traits and status of antimicrobial drug resistance in commensal *E. coli* in a cohort of pre-school age children.

EXPERIMENTAL

Study population

Pre-school children under the age of 2 years (mean, 8.2 months) residing in Ile-Ife, South Western Nigeria were recruited for the study. The subjects were healthy male (68) and female (82) children attending day care centres situated within the town. The children involved in the study were those who had not taken antimicrobial agents within 2 weeks preceding sample collection. The study was conducted according to international guidelines [5] and ethical approval was obtained from the Health Research Ethics Committee, Institute of Public Health, Obafemi Awolowo University, Ile-Ife (IPHOAU/12/21). All specimens were collected with the informed consent of the parents or guardians of the children.

Sample collection

Stool specimens were collected by means of sterile cotton applicators applied to the stools of the children. The samples were transferred directly onto the surface of MacConkey agar plates and streaked for isolated colonies. Organisms producing colonies with typical *E. coli* morphology after 24 or 48 h incubation at 37 ^{o}C were transferred to fresh MacConkey and Eosine Methylene Blue agar plates and onto nutrient agar (Oxoid, England). Each isolate was subjected to standard routine microbiological and biochemical tests for identification and stocks of each were maintained in the laboratory in cryovials (Nalgene, Rochester, NY, USA) by cryopreservation and also in nutrient agar stabs stored at –20 ^{o}C in a freezer and 4 ^{o}C in a refrigerator, respectively.

Polymerase chain reaction analysis

All strains were evaluated by multiplex PCR, using the method of Aranda *et al.* [6] for differentiation of diarrhoeagenic strains and commensals. The genes investigated and primers used are described in Table 1. PCR primers were designed by MWG Biotech Inc, USA. Recombinant Taq DNA polymerase (BioLabs, England) was employed for all PCR reactions.

Screening for verocytotoxin production

Since verocytotoxin and colicin D production are frequently coded by a single plasmid. Isolates were screened for colicin production.

Test for colicin production

Colicin production was determined by the agar overlay method [7]. Organisms producing colicin produced clear zones of inhibition in the mat of indicator *E. coli* K-12 strain after incubation at 37 ^{o}C for 18 h.

Haemolysin production

All *E. coli* isolates were tested for the production of haemolysin on nutrient agar plates containing 4 % citrated whole human blood. The plates were observed for blood free zones surrounding the colonies indicating lyses.

Exopolysaccharide production

To distinguish between encapsulated and non-encapsulated strains, the combined positive and negative staining method of Okeke and Lamikanra [8] was employed. When observed with the microscope, the isolates appeared red against a blue background, while the presence of capsule was observed as a clear zone around each cell.

Antibiotic susceptibility tests

The standard disc agar diffusion method approved by the Clinical Laboratory Standard Institute (CLIS) [9] was used for susceptibility testing. The agents tested were ampicillin (10 µg), chloramphenicol (30 µg), streptomycin (30 µg), gentamicin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (30 µg), sulphonamide (1000 µg) all supplied by Remel, Kansas, USA and cotrimoxazole (25 µg) (Abtek Biologicals, UK). All plates were incubated at 37 ^{o}C for 18 h.

The diameters of zones of inhibition were measured in mm, and interpreted in accordance with the manufacturer's recommendations. *E. coli* NCTC 10418 was used as control.

Table 1: PCR primers used in the study

Primer	Primer Sequences (5' to 3')	Target gene or probe	Amplicon size (bp)
eae1	CTGAACGGCGATTACGCGAA	*eae*	917
eae2	CCAGACGATACGATCCAG		
BFP1	AATGGTGCTTGCGCTTGCTGC	bfpA	326
BFP2	GCCGCTTTATCCAACCTGGTA		
EAEC1	CTGGCGAAAGACTGTATCAT	CVD432	630
EAEC2	CAATGTATAGAAATCCGCTGTT		
LTf	GGCGACAGATTATACCGTGC	LT gene	450
LTr	CGGTCTCTATATTCCCTGTT		
STf	ATTTTTMTTTCTGTATTRTCTT	ST gene	190
STr	CACCCGGTACARGCAGGATT		
IpaH1	GTTCCTTGACCGCCTTTCCGATACCGTC	ipaH	600
IpaH2	GCCGGTCAGCCACCCTCTGAGAGTAC		
Stx1f	ATAAATCGCCATTCGTTGACTAC	stx1	180
Stx1r	AGAACGCCCACTGAGATCATC		
Stx2f	GGCACTGTCTGAAACTGCTCC	stx2	255
Stx2r	TCGCCAGTTATCTGACATTCTG		

Statistical analysis

Data derived from the study was analyzed using Microsoft Excel 2007. Frequencies and percentages were calculated for the study variables and Chi-square test was used to determine the significance of differences between values. $P \leq 0.05$ was considered significant.

RESULTS

A total of 150 *E. coli* strains categorised as commensals were recovered from the subjects involved in the study. There was no significant difference between the rate of isolation of commensal *E. coli* from female and male subjects nor was there any significant relationship between faecal carriage and the age of the children ($p > 0.05$).

Some of these isolates possessed some virulence markers as shown in Table 2. These markers include colicin production, haemolysin production, encapsulation and possession of bundle forming pilli. The strains recovered were statistically associated with encapsulation ($p < 0.05$), 24.7 % produced haemolysin, 11.3 % produced colicin, while 8.0% possessed bundle forming pilli (BfpA). Some possessed up to two different virulence markers concurrently while 2.0 - 2.7 % possessed up to three virulence markers concurrently (Table 3).

The result of the susceptibility test is shown in Table 4. Resistance to ampicillin was observed among all the strains recovered in this study. High resistance rates were observed for streptomycin (94.0 %), tetracycline (92.0 %), trimethoprim (89.3 %) and gentamicin (88.0 %). Significantly lower resistance rates ($p<0.05$) were observed for the quinolones; nalidixic acid (14.0 %), ciprofloxacin (7.3 %) and ofloxacin (3.3 %).

Table 2: Occurrence of virulence markers among commensal *E. coli*

Virulence marker	Possession of at least one marker (n=150)	
	No. (%) positive strains	***No. (%) negative strains***
Colicin	17 (11.3)	133
Haemolysin	37 (24.7)	113
Capsule	134 (89.3)*	16
BfpA	12 (8.0)	138

*$p < 0.05$

Table 3: Occurrence of multiple virulence markers among commensal *E. coli*

Virulence marker	N (%) positive strains n=150
Possession of at least two markers	
Colicin + Capsule	20 (11.6)
Colicin + Haemolysin	4 (2.7)
Colicin + BfpA	0 (0)
Haemolysin + Capsule	41 (27.3)
Haemolysin + BfpA	3 (2.0)
Capsule + BfpA	12 (8.0)
Possession of at least three markers	
Colicin + Haemolysin + Capsule	4 (2.7)
Colicin + Haemolysin + BfpA	0 (0)
Haemolysin + Capsule + BfpA	3 (2.0)

The isolates displayed multiple antibiotic resistance patterns (MAR index = 0.564), most of them being found to be simultaneously resistant to between 3 and 10 antibiotics. Figure 1 shows

the incidences of multiple resistances among the commensal *E. coli* recovered from the children.

Table 4: Antibiotic resistance profile of commensal *E.coli* from subjects

Antibiotic	Resistance (%)
Ampicillin	100[a]
Cefalothin	100[a]
Streptomycin	94[a]
Gentamicin	88[a]
Tetracycline	92[a]
Trimethoprim	89.3[a]
Chloramphenicol	52[b]
Sulphonamide	70[c]
Cotrimoxazole	70[c]
Nalidixic acid	14[d]
Ciprofloxacin	7.3[e]
Ofloxacin	3.3[f]

a – f denotes significantly different prevalence of resistance values. There is no significant difference in resistance prevalence values obtained for antibiotics within the same group.

DISCUSSION:

The molecular tests for virulence genes present in diarrhoeagenic *E. coli* carried out on the strains involved in this study using polymerase chain reaction revealed that none of the strains possessed the sets of genetic determinants of virulence associated with the diarrhoeagenic classes of *E. coli.* Phenotypic tests on the other hand showed that some of these strains had some virulence markers which include the possession of capsules, haemolysin production and colicinogenicity.

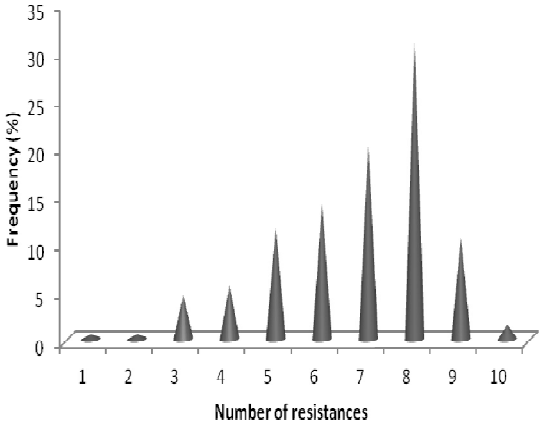

Figure 1: Profile of multiple antibiotic resistances among commensal *E. coli* from subjects.

E. coli strains are normally non-capsulate and encapsulated variants are more common in isolates from invasive infections than from faecal strains [10]. Majority of the recovered faecal strains from the study population however possessed capsules. Capsular polysaccharides are known to render bacterial surfaces hydrophilic and negatively charged, making the bacterium resistant to entrapment in mucus. In addition, capsules contribute to virulence by protecting bacteria from phagocytosis and possibly from serum killing, in part by blocking activation of the alternative complement pathway [11]. This property is attributed to the sialic acid content which reduces the ability of bacteria surface to activate complement by alternative pathway [11].The implication of this is that these encapsulated strains may be difficult to eliminate by phagocytosis if for instance they cause systemic infection in their host. With 89.3% of the recovered strains being encapsulated, it appears that encapsulated strains of *E. coli* are predominant in the gut flora of pre-school age children in the study environment. Further work on strains from individuals of different ages and from different localities may reveal more information on the prevalence of encapsulated strains of *E. coli* in human faecal samples.

Apart from the ability of these strains to escape phagocytosis within the host, some of them produce α-haemolysin, which is the most common cytolytic protein secreted by haemolytic *E. coli* strains. Haemolysin activity may contribute to persistence of these *E. coli* strains by attacking enterocytes and releasing membrane lipids as the main nutrient source for the bacteria [11]. Specifically, alpha-hemolysin causes the lysis of erythrocytes, leading to the release of intracellular iron [12] which may also enhance the survival and persistence of these strains.

Some of the recovered strains also possess the ability to competitively exclude or displace other strains by the production of bacteriocins known as colicins. This property has been reported to be associated with uropathogenic strains and half of the characterized colicin plasmids promote a significant defense against bacteriophage predation [13] and is correlated with increased strain virulence. The possession of colicin by these strains implies that they have the ability to colonise the host GIT and other sites to a greater extent than other strains and thus become persistent. Colicin production also enhances the invasion and establishment success of the producing strains [14]. Numerous studies have shown that colicinogenic *E. coli* rapidly out-compete their colicin sensitive counterparts, due to the lethality of colicin production [14]. These characters may make treatment of opportunistic infections caused by

these strains to be problematic especially when the strains are not susceptible to available antimicrobial agents.

The *E. coli* strains recovered in this study were found to be resistant to a number of older antimicrobial agents (ampicillin, tetracycline, trimethoprim, trimethoprim-sulfamethoxazole, chloramphenicol, and streptomycin). This is in agreement with previous reports of high resistance rates in commensals of study participants from developing countries [5-6].Comparative analysis with previous data available for studies carried out about 12 years earlier [15] indicated a significant increase in the resistance rates to most of the antibiotics and the appearance of resistance to ciprofloxacin. The observed phenomenon probably reflects an increased use of antimicrobial drugs in these children. However, this cannot explain the appearance of resistance to ciprofloxacin, a drug which was not prescribed in this age group at the time samples were collected. A likely explanation for this could be the multiple antibiotic resistance property of the isolates which suggests that ciprofloxacin resistance already exists among the block of resistances carried by the isolates.

Another property observed among the isolates in this study is multiple antibiotic resistances which have also been observed among strains of *E. coli* studied by various authors in developing and developed countries [17-18]. This property could be transferred by conjugation, to other organisms, pathogenic or not, especially where the multiply resistant strain have the ability to persist in the guts by virtue of the ability of such strains to produce any of colicin, capsule, and haemolysin or all concurrently as observed among some of the recovered strains.

CONCLUSION

The study shows the pathogenic potential of commensal *E. coli* in children and their potential as reservoirs of multiple resistance genes that could be transferred to pathogens. This study underscores the urgent need for constant antibiotic resistance surveillance and measures to prevent opportunistic infections in hospital and the community settings.

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