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Prevalence and Antibiotic Resistant Patterns of *Escherichia coli* among Children from Selected Hospitals in Onitsha, Anambra State, Nigeria

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Abstract Infections by drug resistant *Escherichia coli* cause significant morbidity and mortality especially in developing countries. This study was conducted to determine the prevalence and antibiotic resistant pattern of E*scherichia coli* among children from selected hospitals in Onitsha, Anambra State, Nigeria. Two hundred and forty six stool samples were collected from diarrheal and non-diarrheal children aged ≤12 years attending outpatient clinic at St. Charles' Borromeo Specialist Hospital and General Hospital, Onitsha using sterile screw capped bottles. Structured questionnaire was used to obtain demographic data of the children. With an applicator swab, stool samples were inoculated directly onto MacConkey agar and Eosin Methylene Blue (EMB) agar using streak plate method and incubated at 37^{o}C for 24 h. Colonies were identified based on their morphology, Gram's stain and biochemical reactions and molecular characteristics. Antimicrobial susceptibility and resistance of the isolates was determined by standard disc diffusion method on Mueller Hinton agar. The gene encoding resistance for the highest resisted antibiotic was studied. The data obtained was statistically analyzed. Out of 246 samples collected from the hospitals, 206 (83.70 %) were positive for *Escherichia coli*. The molecular identification revealed the presence of *Escherichia coli* RPKN4. Prevalence of *E. coli* was higher in children with diarrhea (61.2%), males (54.9%), age group 6 – 9 (39.3%), those attending primary school (58.3%) and those whose mothers had tertiary education (74.8%). Prevalence of *E. coli* was statistically significant with gender (p = 0.001), age (p = 0.000) and child's educational level (p=0.040). The *E. coli* isolates were highly resistant (45.63 %) to ampicillin, and had least resistance to gentamicin (0.49%) and nitrofurantoin (0.49%). All the isolates resistant to ampicillin were positive for bla TEM genes. The study showed high prevalence of *Esch*erichia *coli* which are resistant to many of the antibiotics among children from the selected hospitals.

Keywords: *antibiotics, resistance, diarrhea, escherichia coli, prevalence, ampicillin*

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1. Introduction

Escherichia coli, is a commensal bacterium of the family Enterobacteriaceae. They inhabit the gut of vertebrate and are involved in numerous intestinal and extra-intestinal infections as opportunistic pathogen. It is a common cause of urinary tract, blood stream, food borne infections, cholecystitis, respiratory illnesses and meningitis in neonates and is linked with community associated as well as nosocomial infections [1,2]. It can also serve as a genetic reservoir for plasmid-mediated antibiotic resistance that can be transferred to other pathogens and thus, represent an important index pathogen for understanding the epidemiology of antibiotic resistance [3]. The pathogenic types (pathotypes) of *E. coli* have been linked to a wide array of human illness and significant food borne outbreaks [4].

Escherichia coli is one of the leading causes of diarrhea, together with other pathogens such as rotavirus, coronavirus, *Cryptosporidium parvum*, or in combination of these. Diarrheal diseases have contributed so much in child undernourishment and deterioration in growth and development. Besides other typical infections, diarrhea caused by *E coli* may even be more harmful than rotavirus infections. Children are more prone to repeated diarheagenic *E. coli* as a result of immature immune system [5].

There are six fundamentally recognized strains of *E. coli (*DEC*)* that are related to diarrhea based on their different clinical features, virulence factors, and serotypes grouping. These include enteroaggregrative *E. coli* (EAEC),

enteroinvasive *E. coli*, enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli (*EPEC*)*, diffusely adherent *E. coli* (DEAC), and enterotoxigenic *E. coli* (ETEC) [5].

Antibiotic resistance is the capability of bacteria to resist medication that could once inhibit them [6]. A growing number of infections such as pneumonia, tuberculosis, gonorrhea and salmonellosis are becoming more difficult to treat due to resistance to antibiotics by the causative agents [7].The emergence and spread of antibiotic resistance have been attributed to many factors including existing methods of antibiotic use, practices of healthcare providers, perception of users in the community, over-the-counter availability, socio-demographic factors and safe water and sanitation practices [6,8]. The alarming emergence of antibiotic resistance has become an international public health problem and children in low resource settings represent a high risk group [7]. Resistant strains are prevalent in both community and healthcare settings and although great strides have been made in reducing mortality due in part to implementation of oral rehydration therapy and appropriate feeding practices, enteric infections are still a significant cause of morbidity worldwide [9]. Few microorganisms have shown the ability to develop resistance to as many classes of antibiotics as those in family Enterobacteriaceae.

The past two decades have witnessed tremendous rise in the emergence and spread of *E. coli* resistance strains to major classes of antibiotics like beta lactams, quinolones, amino glycosides, sulfonamides and fosfomycin and has recently spread to last resort antibiotic classes such as the polymyxins and carbapenems [10]. Among the mechanisms of *E. coli* resistance to the antibiotics are limiting the absorption of antibodies, changing the location of the target of the antibodies and disrupting the action of the antibodies or flow pumps. These mechanisms may be present on the bacterial chromosome which can occur naturally in all strains or be acquired by plasmid [11].

In the year 2017, diarrhea accounted for about 1.6 million deaths worldwide, of which one third of the population were children under 5 years old. It was estimated that in 2021, 340,000 children under 5 years and 33,816 children between the ages of 5 – 14 years would die of diarrhea globally, with highest death rate in low income and developing countries [12]. Presence of diarrheagenic *E. coli* and their resistance to antibiotics is not frequently studied in many countries including Nigeria [13], hence, the need for this research. This study was, therefore, undertaken to investigate the prevalence and antibiotic resistant patterns of *Escherichia coli* among children from selected hospitals in Onitsha, Anambra State, Nigeria.

2. Methods

Study area

This study was conducted in Onitsha, a city situated in the eastern bank of the Niger River in Anambra state, Nigeria [14]. Onitsha is located between latitudes 6°08' and 6°10' North and between longitudes 6°46' and 6°49'East [15]. Onitsha metropolitan city, is known for its river port and also an economic hub for commerce, industry and education. The city has a population of about 3.553million people with an area of 52km^2. The city borders the local government areas of Idemili North and Oyi to the east, Anambra East to the north, Ogbaru to the south and Delta State to the west. Onitsha has two seasons, the wet season and the dry season with temperature range from 67°F to 88°F.

Study Design

The research was hospital-based descriptive cross-sectional study conducted among children ≤ 12 years of age who are outpatients at General Hospital and St Charles' Borromeo Specialist Hospital Onitsha from September to November, 2023.

Ethical clearance

Ethical clearance was obtained from the ethical committee of the concerned hospitals. Parents or guardians of the children were notified and oral consent obtained from them.

Study Population and Size

The study population comprised of 146 diarrheal and 100 non-diarrheal children aged ≤12 years who have not taken any antibiotics in the past 2 weeks prior to enrolment and who attended General Hospital and St. Charles' Borromeo Specialist Hospital, Onitsha.

The sample size was calculated using Yamane's [16] formula:

$n = N/1+Ne^2$ where:

n=sample size

N=population size

e= margin of error

Assuming a margin of error 0.09%, the sample size is two hundred and forty- six (246).

Sample collection

Stool samples of children were collected in sterile screw capped bottles and transported in an ice pack to the Department of Applied Microbiology and Brewing laboratory, Nnamdi Azikiwe University, Awka. Samples were processed within 2 h of collection.

Demographic data

Structured questionnaire was used to obtain information on the age, gender, and educational level of the children. Information on the source of their drinking water and educational level of their mother or guardian was also obtained.

Isolation of *Escherichia coli*

Using an applicator swab, stool samples were inoculated directly onto MacConkey agar and Eosin Methylene Blue (EMB) agar using streak plate method. The plates were thereafter incubated at 37°C for 24 h [17].

Identification of isolates:

Colonies of the isolates were identified based on their morphology, Gram's stain and biochemical reactions and molecular characteristics. The colonies were macroscopically observed and the biochemical tests which include catalase, indole, Voges-Proskauer, methyl red, oxidase, motility, carbohydrate fermentation, urease and citrate were performed according to the method of Cowan and Steel [18].

Molecular identification of isolates

Five of the isolates were randomly selected and subjected to molecular identification to ascertain their identity.

DNA extraction

A 200µl of test sample was added to a microcentrifuge tube. 200µl of Biofluid and Cell Buffer and 20µl of Proteinase K was added to it and mixed thoroughly for 10 – 15 sec. using a vortex mixer and the tube incubated at 55^{o}C for 10 min. on a heating block to digest the sample. Genomic Binding Buffer (i.e. 420µl) was added to the digested sample and mixed thoroughly with a vortex mixer for 10-15 sec. The mixture was transferred to a Zymo-Spin™IIC-XL column in a collection tube and centrifuged at 12,000 x g for 1min. The collection tube was discarded with the flow through. 400µl DNA Pre-Wash Buffer was added to the spin column in a new collection tube and centrifuged at 12,000 x g for 1 min. The collection tube was emptied and 700µl g DNA Wash Buffer was added to the spin column and centrifuged at greater than or equal to 12,000 x g for 1 min. The spin column was transferred to a clean microcentrifuge tube. Fifty microliter (50µl) of DNA Elution Buffer was added directly to the matrix and incubated for 5 min at room temperature, then centrifuged at maximum speed for 1 min to elute the DNA. The eluted DNA will be stored at <-20^{o}C for future use.

Polymerase chain reaction (PCR) Procedure

A 12.5µl of One Tag Quick- Load 2x Master Mix with standard buffer(New England Biolabs Inc), 0.5µl of each forward and reverse primers; 8.5µl of nuclease free water and 3µl of DNA template was used to prepare 25µl reaction volume of the PCR cocktail. The reaction was gently mixed and transferred to a thermocycler. Amplification conditions for the PCR was as follows: Initial denaturation for 30 sec at 94^{o}C followed by 35 cycles of denaturation at 94^{o}C for 20 sec, primer annealing at 56^{o}C for 45 sec and strand extension at 68^{o}C for 1 min. Final extension at 68^{o}C for 5 min on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA band visualized with Ethidium bromide.

Agarose Gel Electrophoresis

Two percent agarose gel was prepared by dissolving 1.2g of agarose in 60ml of IX TAE buffer. The mixture was heated to a clear solution using a microwave oven and allowed to cool to about 50^{0}C. Three microliter of ethidium bromide was added into the solution and mixed thoroughly. The agarose preparation was carefully poured into a gel tray, with the gel comb in place and allowed to solidify. The tray was loaded into the gel tank and IX TAE buffer was poured into the tank, making sure that the gel was properly submerged. The gel comb was carefully removed. Five microliter of amplicon was loaded into the wells. The tank was connected to the power pack and set to run at 100V for 20min after which it was viewed on a gel documentation system.

The sequences of the isolates were subjected to basic local alignment search tool (BLAST).

Antibiotic susceptibility testing

The susceptibility test was determined by standard disc diffusion method on Mueller Hinton agar plates as recommended by the Clinical Laboratory Standards Institute [19]. Antibiotic discs used contained ampicillin (10µg), amoxicillin/clavulanic acid (30µg), cefoxitin (30µg), ciprofloxacin (250µg), imipenem (30µg), ceftazidine (30µg), amikacin (30mcg), nitrofurantoin (50mcg), gentamicin (10µg) and levofloxacin (30µg) (AB Biodisk, Sweden). Broth culture matching 0.5 McFarland Standard was spread inoculated on the medium with sterile swab sticks and allowed to stand for 5 min. With the aid of sterile forceps, antibiotics discs were applied to the surface of the inoculated plates. Each disc was gently pressed down onto the agar to ensure complete contact with the agar surface. The plates were incubated at 37^{o}C for 18-24 h and the diameter zones of inhibition measured.

Detection of Ampicillin Resistant Genes

The isolates that resisted Ampicillin were used for the test

The presence of ampicillin resistance genes in *E. coli* DNA extracts was determined by conventional PCR. The conventional PCR was performed in thermocycler (ABI, USA) in 25 volume reaction containing 6µl of template DNA, 1µl of each of the primers, 12.5µl Phusion PCR master-mix (2x)(High Fidelity, USA),1µl DMSO and 2.5µl of nuclease free water. Amplification procedure consisted of initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec., extension at 72°C for 30 sec. and final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel electrophoresis.

Statistical Analysis

Data obtained were statistically described in terms of frequency and percentages. The Pearson Chi-Square test was applied to analyze association of *E. coli* with the obtained variables. The resistance pattern of *E .coli* strains was analyzed using independent samples T- test and One –Way analysis of variance (ANOVA). All p–values will be based on 2-tailed tests of significance where $p \leq 0.05$ is considered statistically significant.

3. Results

Table 1. Morphological, Gram stain and biochemical reactions of isolates

Morphol. Gram Stain	Biochemical reactions											
charact.	rxn.	Carbohydrates										
		MOT	CAT	IND	VP	MR	UR	CIT	OXD	GLU	SUC	LAC
Greenish col. –ve	+	+	+	-	+	-	-	-	+	v	+	
on EMB agar and												
pinkish red on												
MacConkey agar												

Key: Morphol. Charact = Morphological characteristics, rxn. = reaction, col. = colonies, EMB = Eosin Methylene Blue, -ve = Negative, + = Positive, v = Varied, MOT= Motility, CAT = Catalase, IND = Indole, VP = VogeProskauer, MR = Methyl Red, UR = Urease, CIT = Citrate, OXD = Oxidase, GLU = Glucose, SUC = Sucrose, LAC = Lactose.

Based on the morphological features, Gram stain reaction and biochemical tests (Table 1), stool samples for 206 (83.7%) children tested positive for *Escherichia coli*. Morphological features presented *E. coli* as greenish-black colonies on Eosin methylene blue agar and as pinkish red

colonies on MacConkey agar. Isolates were Gram negative and motile. Basic biochemical characteristics of the isolates for preliminary identification, showed positive reactions for catalase, indole, methyl red and negative for Voges Proskauer, urease, citrate and oxidase tests. While all the isolates fermented glucose and lactose, their fermentation of sucrose varied. Molecular characteristics identified isolates as *Escherichia coli* RPKN4 showing 93% to 99% identity, and having 1409 base pairs in query coverage of 100%.

Prevalence of *E. coli* based on demographic data of the children

The prevalence of *E. coli* based on the socio-demographic data of the children and other factors are represented in Table 2. The prevalence was higher among those with diarrhea (61.2%), male (54.9%), age group 6-9 years (39.3%), those attending primary schools (58.3%), children whose mothers had tertiary education (74.8%) and those whose source of drinking water is sachet water 170 (82.5%).

Antibiotic resistance pattern of *Escherichia coli* isolates

The antibiotic resistance pattern among *Escherichia coli* (n = 206) isolated from participants is as represented in Table 3. Isolates showed high resistance to ampicillin (45.63%), moderate resistance to cefoxitin (16.50%), amoxicillin/clavulanic acid (13.11%), ceftazidine (12.62%) and levofloxacin (9.71%) and low resistance to amikacin (0.97%), gentamicin (0.49%) and nitrofurantoin (0.49%).

Table 2. Prevalence of *E. coli* based on socio-demographic data and others factors

Characteristics	Frequency	Percentage	$\chi 2$	OR(95% CI)	p-value
variable		(%)			
Presence of diarrhea			8.700	0.802(2.199 – 1.397)	0.005*
Yes	126	61.2			
No	80	38.8			
Gender			10.907	1.526(1.970 - 0.444)	0.001*
Females	93	45.1			
Males	113	54.9			
Age			30.364	4.533(6.079 – 1.546)	0.000*
<1	2	1			
1 – 5	60	29.1			
6-9	81	39.3			
10 – 12	63	30			
Child's educational level			13.791	0.519(0.983 – 0.464)	0.040*
Play group	13	6.3			
Nursery	38	18.4			
Primary	120	58.3			
Secondary	35	17			
Mother's educational level			0.13	0.415(0.509 – 0.094)	0.908
Primary	7	3.4			
Secondary	45	21.8			
Tertiary	154	74.8			
Source of drinking water			5.040	1.811(2.290 – 0.479)	0.031*
Tap/borehole	31	15			
Sachet	170	82.5			
Bottled	5	2.4			

Keys: χ^2= chi square, CI = confidence interval, OR = odds ratio, p value = level of significance, * = statistically significant

Table 3. Antibiotic resistance pattern of *Escherichia coli*

Antibiotics			Number of *E. coli* isolates (206)							
			Sensitive	Intermediate	Resistant	%Resistant				
AMP			112	-	94	45.63				
CEF			172	-	34	16.50				
AMC			179	-	27	13.11				
CEZ			162	18	26	12.62				
LEV			174	12	20	9.71				
AMI			200	4	2	0.97				
GEN			197	8	1	0.49				
NIF			205	-	1	0.49				

Keys: AMP = ampicillin, CEF = cefoxitin, AMC = amoxicillin/clavulanic acid, CEZ = ceftazidine, LEV = levofloxacin, AMI = amikacin, GEN = gentamicin, NIF= nitrofurantoin **% -** percentage.

Detection of Ampicillin Resistant Genes

All the isolates that were resistant to ampicillin were positive for bla TEM gene.

4. Discussion

Escherichia coli are very important members of the intestinal microbiota as well as pathogens of public health importance affecting both children and adults worldwide [20]. A total of 246 faecal samples were processed, to determine the prevalence of *Escherichia coli* and their resistant pattern to antibiotics in the study area. Two hundred and six (83.7%) samples out of 246 faecal samples examined tested positive for *Escherichia coli* with 126 (61.2%) samples from children with diarrhea and 80 (38.8%) samples from non-diarrhea children. This result agrees with the prevalence rates of 88.3% and 89.22% recorded in studies among children in Eastern Cape, South Africa and China by Omolajaiye *et al.* [5] and Patil *et al.* [21] respectively. Contrary to our finding Olaniran *et al.* [22], reported a low prevalence rate of 22% of *E. coli* in diarrheic children in Ile-Ife. However, a higher prevalence of 95% was reported among children in Ethiopia [23]. As suggested by Wolde *et al.* [24], the disparity in the results from different researchers may be attributed to factors like the geographic locations, ages of children involved, sample size, season and methods of sampling. Male participants in this study recorded a higher prevalence rate (54.9%) than females (45.1%), which is statistically significant (p value = 0.001). This corroborates the finding of Tatar *et al.* [25] in Iran, in which they observed a prevalence rate of 55.7% in males and 44.2% in females. The high prevalence rate observed in male children may be due to the fact that they are generally more active and care- free when compared to their female counterparts. Although Mwape *et al.* [26] recorded a prevalence rate (46.9%) of EPEC among males in Lusaka, Omolajaiye, *et al.* [5], reported a high prevalence rate (52.63%) of *E. coli* among female children in Eastern Cape, South Africa.

A lower rate (1%) of recovery of *E. coli* was observed in children less than a year old while the prevalence (39.9%) was highest in age group 6-9 years. The prevalence among the different age groups was statistically significant (p-value = 0.000). The higher rate of isolation of *E. coli* among children in age group 6-9 years may be attributed to their exposure to contaminated adult foods or lack of proper hand washing before meal [24]. Most children in this age group usually lack personal and nutritional hygiene and are generally oblivious of the health implications of such actions.

The lowest prevalence was recorded among children attending play group facilities (6.3%). The low prevalence for playgroup might be due to the fact that parents are generally more attentive to children in this category and usually try to provide basic health care for them because of their tender age and dependence. Their caregivers are also quick to inform the appropriate persons in cases of ill-health. Furthermore, government approved healthcare centers which conduct immunization exercises and also render primary treatment to ailments like diarrhea and uncomplicated fever/ colds in children under 5 years of age are within the reach of most residents of Onitsha. As observed in this study, *E. coli* prevalence was highest in children attending primary schools (58.3%), p=0.040. The children in primary school are mostly in age group 6-9 years, who are very active and unconcerned of hygienic practices. Maternal education has previously been regarded as a proxy indicator for socioeconomic status (SES) of a family [27]. Results obtained showed no significant association between maternal educational level and prevalence of *E. coli*, even though the highest prevalence was observed among children whose mothers had tertiary education (74.8%). This may be attributed to the large sample size (154) obtained from this group. In contrast, Olaniran *et al* [22] reported a high prevalence (50%) of *E. coli* among children whose mothers had no formal education.

Considering the source of drinking water of the children, the prevalence of *E. coli* was found to be significantly higher (p value – 0.031) in participants drinking sachet water (82.5%) than in other participants. This shows that some sachet water produced in Onitsha, Nigeria are of poor standard water quality. The result of the study supports the findings of Afiukwa *et al.* [28], who reported that 26.6% of sachet water sampled in Enugu and Onitsha in South Eastern Nigeria were contaminated with coliforms. This result contradicts the findings of Olaniran *et al.* [28] and Getahum and Adane [29], in their studies on diarrheal children in Southwest Nigeria and Northeastern Ethiopia respectively. They reported high prevalence of *E. coli* among those that use tap water.

Antibiotic resistance is an international health issue, entailing the transfer of bacteria and genes between humans, animals and the environment [30]. Unprofessional use of antibiotics by humans and farms, poor hygiene and sanitation, and inefficient prevention and control of infections in health-care settings are considered important basis in the emergence of antibiotic-resistant bacteria [20,31]. Also implicated in antibiotic resistance is non-adherence to standard treatment procedures especially in sub-Saharan Africa, and the use of fake or substandard drugs, and the unauthorized sale of antibiotics without appropriate prescription [32,33].

In this study, the highest drug resistance (45.63%) in *E. coli* was recorded in ampicillin. The result is in line with the work of Buonsenso *et al.* [34], who reported 47.3% resistance of *E. coli.* Higher resistance (81% - 100%) of *E. coli* to ampicillin had been reported by many researchers [5,9,21,28,35,36]. It has been suggested that the high resistance to ampicillin may have been caused by the presence of beta-lactamase enzyme in the bacteria or as a result of the reduction of affinity of existing penicillin binding proteins [37]. Another possible adduced reason for the high resistance to ampicillin is the frequent use or misuse due to its easy availability and affordability [8,37]. All the isolates that were resistant to ampicillin were found to be positive for the bla TEM gene and this observation supports the work of Mustapha *et al.* [38] on the detection of ampicillin resistance in India.

Relatively low resistance levels were observed with amikacin (0.97%), nitrofurantoin (0.49%) and gentamicin (0.49%). This result is in line with the studies of Olaniran *et al.* [22] and Bartoloni *et al.* [39] who also recorded low resistance rates against these antibiotics. The non - resistance of *E. coli* to imipenem and ciprofloxacin

observed in this study agrees with the reports of David *et al*.[10], Singh *et al.* [36], Salleh *et al.* [40], Nji *et al.* [41], who also did not record any resistance to the antibiotics. The non-resistance of *E. coli* to imipenem and ciprofloxacin which are fluoroquinolones, corroborates with the findings of Hiedary *et al*. [42], who recommended fluoroquinolones as first- line drugs in the treatment of diarrhea caused by *Escherichia coli*.

5 .Conclusion

From the study conducted high prevalence rate of *Escherichia coli* was observed among children from selected hospitals. Children mostly affected were children with diarrhea, males, primary school children, 6-9 years age bracket and whose source of drinking water is sachet water. The *E. coli* isolated were observed to be resistant to most antibiotics especially Ampicillin. There is therefore, need for proper diagnosis and antibiotic sensitivity testing before drug administration.

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Contributions of Authors

ECC conceived, designed the study, and reviewed the manuscript, NGP collected, processed samples and did laboratory analysis, INS & AVN, carried out the literature search, ECM statistically analyzed the data and ACG wrote the first draft of the manuscript. All authors critically reviewed and approved the manuscript.

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Conflict of Interest

Authors declare no conflict of interest.

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