ENTEROPATHOGENIC ESCHERICHIA COLI STRAINSFROM DIARRHOEIC STOOLSAMPLES OF CHILDREN BELOW 5 YEARS OF AGE IN DAMATURU, YOBE STATE, NIGERIA

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ABSTRACT

Enteropathogenic *Escherichia coli* (EPEC) is an important cause of deaths mostly in infants and young children with diarrhoea worldwide. This study investigated Enteropathogenic *Escherichia coli* strains in diarrhoeic stool samples of children below 5 years of age in Damaturu, Yobe State, Nigeria. *Microscopy, culture and* antibiotic susceptibility tests were carried out on *s*tool specimens obtained from children with diarrhoea. All isolated *Escherichia coli* were investigated for virulence *eae* and *eaf* genes of EPEC strains using Polymerase Chain Reaction method. Information on risk factors of diarrhoea was obtained using the questionnaire. Out of 307 children, 154 (50.2%) were male and 153 (49.8%) female, majority 107 (34.9%) were 3 years old. A total of 175 (57.0%) *Escherichia coli* were isolated, 19 (10.9%) were identified to be enteropathogenic *Escherichia coli* of these, 17 (89.5%) were atypical (carries *eae* genes) while only 2 (10.5%) were typical (harbours *eaf* genes). Multidrug resistance was observed in some of the isolates, the EPEC were resistant to Reflacin (47.4%), Ciprofloxacin (36.8%), Augmentin (36.8%), Septrin (36.8%). The major factor that predispose children to diarrhoea are poor hygiene practices. *Escherichia coli* was the most prevalent bacterial causing diarrhoea and atypical EPEC is the predominant strain circulating among these children.

KEYWORDS: **Diarrhoea, Enteropathogenic *Escherichia coli*, *eae* and *eaf* genes, children**

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INTRODUCTION

Diarrhoea has been well documented as the commonest symptom of gastrointestinal infections and the leading cause of death among children less than 5 years of age in developing countries (UNICEF/WHO, 2009; You *et al*, 2010; Black *et al*., 2010; Onanuga *et al*., 2014). Diarrhoea is caused by non-infectious agents and infectious pathogens which include bacteria, viruses and protozoa. *Escherichia coli* is the most frequently isolated bacterial pathogen from cases of diarrhea all over the world (UNICEF/WHO, 2009; Okeke *et al*., 2012; Al-Gallas *et al*., 2007; Nweze, 2010).

Enteropathogenic *Escherichia coli* (EPEC) have been identified as the predominant pathotype among the several diarrheagenic *E. coli* infecting children worldwide (Nguyen *et al*., 2005; Onanuga *et al.,* 2014; Ifeanyi *et al.,* 2015). The diarrheagenic *E. coli* pathotypes are described based on epidemiologic, clinical features and specific virulence determinants genes associated with the diarrhoeal disease caused by them (Nataro and Kaper, 1988; Kaper and Nataro, 2004; Yu *et al.,* 2018). Virulence determinants genes, the eae (intimin) and bfpA (bundle forming pilus) are used for identification and division of EPEC into typical and atypical strains (Nataro and Kaper, 1998; Afset *et al*., 2004; Nguyen *et al*., 2006). EPEC is a major diarrheagenic *E. coli* linked with infant diarrhea in the developing world (Kaper and Nataro, 2004; Ochoa *et al.,* 2008). The prevalence of EPEC infection varies with study populations, age, geographic region, socioeconomic characteristic and methods of detection and diagnosis (Ochoa *et al.,* 2008; Ifeanyi *et al.,* 2015; Odetoyin *et al*., 2016).

Infections with *E. coli* pathotypes are indistinguishable based on clinical findings of diarrhoea which are often accompanied by fever, vomiting, and dehydration in children (Vilchez *et al*., 2009). Poor laboratory facilities in most hospitals settings for serological and molecular tests limited the identification and reporting of aetiology of diarrhoea in *E. coli* and other enteric bacteria thus under-reporting the different pathotypes of diarrhoeagenic *E. coli*. The few available reports of diarrheagenic *E. coli* in Nigeria are from researches in Federal Capital Territory (Onanuga *et al.,* 2014; Ifeanyi *et al.,* 2015), South-West, (Okeke *et al*., 2012; Odetoyin *et al*., 2016) and South-East (Nweze, 2010). This study investigated the prevalence of Enteropathogenic *Escherichia coli* strains in diarrhoeic stool of children below 5 years of age in Damaturu, Yobe State, Nigeria.

MATERIALS AND METHODS

Study area

The study was carried out in Damaturu, the capital of Yobe State, North-Eastern Nigeria. The state has 17 Local Government Councils, the major ethnic group includes Manga, Fulani, Bolewa, Hausa and Kanuri. The State has 3 Tertiary Health Facilities, 13 Secondary Health Facilities and 517 Public Health Centres, that is, all the Local Government Areas Headquarters have Government runs Hospitals, and Private Clinics that provide services to the population (Historical Documentation of Yobe State, Directorate of Information, 2016).

This study was carried out in General Sani Abacha Specialist Hospital Damaturu Km 3, Gujba Road, Yobe State, Nigeria. The hospital has 300-bed spaces and 11 different wards. The hospital was selected because it has an emergency paediatric ward, children ward, paediatric department, as well as a high population of paediatric patients. The hospital attends to about 1720 children per month out of which at least 282 are diarrhoea cases (Information obtained from the Monthly Summary Form Medical Record Unit of the hospital, 2017).

Ethical approval

Ethical approval was obtained from the Medical Research Ethics Committee, Ministry of Health, Damaturu, Yobe State, Nigeria (Ref. No. MOH/GEN/474/VOL.1). Permission to use the patients for the study was also obtained from Hospital authority and Clinicians involved. The concept of the study was explained to the Parents/Guardians of the children and informed consent was obtained for the participation of their children in the study.

Study population

Children less than 5 years with diarrhoea as confirmed by the clinicians, and whose mother or guardians consented and signed the informed consent form to participate in the study were included. Children over 5 years of age and those whose parents or guardians did not consent were excluded.

Collection and processing of stool samples

Demographic information such as name, sex and age of children, hygiene, socio-economic status and general information on clinical history was obtained from the patients on pre-validated questionnaires. Sterile plastic universal bottles with scoop were given to the parent to collect fresh stool samples which were transported to the Laboratory and were processed within 4 hours of collection.

The stool samples were examined macroscopically for the presence of blood and mucous, physical characteristics such as appearance, odour, colour and consistency were also noted. The stool samples were inoculated on MacConkey agar (Oxoid, UK), and subcultured Eosin Methylene Blue agar (Oxoid, UK), all incubations were performed at 37°C for 18 - 24 hours. Isolates were Gram stained, the Gram-Negative isolates were identified using Microbact Identification System (12E) Oxoid as described by the Manufacturer. Antibiotics susceptibility tests were performed on all confirmed *Escherichia coli* using the disc diffusion method (Clinical Laboratory Standards International, 2013).

Identification of enteropathogenic *Escherichia coli* strains by PCR method

DNA Template was prepared from an overnight culture isolate of *E. coli* on Trypticase Soy agar by suspending three colonies in 200 uL deionised water in Eppendorf tube, vortex briefly. The supernatant was removed, the pellet was re-suspended in 200 uL deionised water, boiled at 100°C for 10 minutes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was transferred into a sterile Eppendorf tube as the DNA template and

stored at -20°C. The DNA was amplified using the primers used previously by Vidal *et al.* (2004), these were designated bfp1 & bfp2 and eae-F &eae-R. The bfp primers amplify the 397 bp of the gene that encodes the bundle forming pills also called EPEC adherence factor (EAF), while the eae primers amplify the 492 bp plasmid localized within the locus of enterocyte effacement (LEE) Pathogenicity Island.

PCR

All the PCR reactions were performed in 20 µl final volume containing 2 µl of the template DNA, 4 µl of PCR mix (containing Hot Start Taq polymerase, 1.0U, x 1 PCR buffer (50mM KCl, 10mM Tris –HCl, pH 8.3), 0.2 mM of each dNTP and 1.5 mM of $MgCl_2$), 0.2 uL each of the forward and reverse primers for 10 picomoles each) and an additional 2uL of 5mM $MgCl_2$ to give a final concentration of 2mM plus 11.2 uL of deionised water.

The thermocycling conditions for all the PCRs were as follows: 95 °C for 2 minutes, 95 °C for 15s, 52 °C for 8 s, and 10 s at 72 °C for 30 cycles, with a final 2 minutes extension, and all the PCRs were performed in the Applied Biosystem Thermal Cycler (Applied Biosystem, USA). Amplified samples were evaluated by 1.5% agarose gel electrophoresis in Tris–borate–EDTA buffer containing 10 mg/mL of ethidium bromide, visualized with a UV transilluminator.

Data analysis

Data were analyzed using statistical package for social scientist software, IBM (SPSS) version 5.0 (2016). Regression analysis tests with the level of significance set at p-value <0.05. P-value for each of the carriage risk factors was identified to determine the most significant factors.

Results

Out of 307 children, 154 (50.2%) were male and 153 (49.8%) female, majority 107 (34.9%) were 25 -36 months old (Table 1). The macroscopic characteristic of stool samples from children with diarrhoea is presented in Table 2. 219 (71.34%) of the samples were watery and 257 (83.71%) have observable mucus. The bacteria isolated from the stool samples includes *Escherichia coli* 175 (57.0%), *Enterobacter gergoviae* 35 (11.40%), *Serratia liquefaciens* 26 (8.47%), *Acinetobacter iwofii* 26 (8.47%), *Salmonella arizonae* 18 (5.86%), *Citrobacter diversus* 18 (5.86%) and *Citrobacter freundii* 9 (2.93%). Out of 175 *Escherichia coli* isolated only 19 (10.9%) were identified as enteropathogenic *Escherichia coli* using the molecular method, of these 17 (89.5%) carries *eae* genes with the size of 492 bp while only 2 (10.5%) harbours *eaf* genes with size 397 bp (Figure 1).Enteropathogenic *Escherichia coli* were classified as typical when they harbour both *eae* and *eaf* genes while they are classified as atypical when they habours either *eae or eaf genes (*Alikhani, *et al*., 2006).

The antibiotic resistance of the bacteria isolated from the stool samples is presented in Table 3. *Escherichia coli 152* (86.7%), *Acinetobacter iwofii 22* (84.6%), *Citrobacter freundii* 7 (77.8%), *Enterobacter gergoviae 23* (65.7%) and *Citrobacter diversus* 7 (50.0%) were highly resistant to Augmentin, also multiple drug resistance was observed in some of the isolates. Besides, the antibiotics susceptibility of the Enteropathogenic, *Escherichia coli* showed that they were resistant to Reflacin 9 (47.4%), Ciprofloxacin 7 (36.8%), Augmentin 7 (36.8%), Septrin 7 (36.8%) Table 3. The major pre-disposing factor for diarrhoea among the children is poor hygiene practices (Table 4).

Table 1: Age-sex distribution of children with diarrhoea

Age (Month)	Sex		Total
	Male n (%)	Female n (%)	N (%)
0 - 12	26 (16.9)	9 (5.8)	35 (11.4)
13 – 24	23 (14.9)	17 (11.1)	40 (13.3)
25 - 36	62 (40.3)	45 (29.4)	107 (34.9)
37 – 48	21 (13.6)	76 (49.7)	97 (31.6)
49 - 60	22 (14.3)	6 (3.9)	28 (9.1)
Total	154 (50.2)	153 (49.8)	100 (100%)

Table 2: Macroscopic characteristics of stool samples from children with diarrhoea

Characteristic	Number (percentage)
Texture	
Watery	219 (71.34)
Semi-Solid	38 (12.37)
Solid	50 (16.29)
Colour	
Brown	184 (59.94)
Green	35 (11.40)
Black	88 (28.66)
Other features	
Mucoid	257 (83.71)
Bloody	23 (7.49)

Table 3: Antibiotic resistance of *E. coli* and EPEC isolated from children with diarrhoea

Antibiotics	*A. iwofii*	*S. liquefaciens*	*S. arizonæ*	*E. gergoviæ*	*C. freundii*	*C. diversus*	*E. coli*	EPEC
OFX (10µ)	8 (30.8)	7 (26.9)	6 (33.3)	14 (40.0)	6 (66.7)	6 (33.3)	47 (26.7)	5 (26.3)
PEF (10µ)	14 (53.9)	13 (50.0)	4 (22.2)	25 (71.4)	4 (44.5	4 (22.2)	69 (40.0)	9 (47.4)
CPX (10 µ)	0 (0.0)	5 (27.8)	0 (0.0)	17 (48.6)	1 (11.1)	7 (38.9)	4 (2.23)	7 (36.8)
AU (30µ)	22 (84.6)	11 (42.4)	5 (27.8)	23 (65.7)	7 (77.8)	9 (50.0)	152 (86.9)	7 (36.8)
CN (10µ)	9 (34.6)	8 (30.7)	7 (38.9)	12 (34.3)	3 (33.3)	3 (33.3)	56 (32.0)	2 (10.5)
S (30µ)	0 (0.0)	2 (7.7)	1 (5.6)	4 (11.4)	0 (0.0)	0 (0.0)	2 (1.2)	0 (0.0)
CEP (10µ)	4 (15.4)	5 (19.2)	0 (0.0)	1 (2.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
NA (30µ)	8 (30.8)	9 (34.6)	6 (33.3)	7 (20.0)	3 (33.3)	0 (0.0)	43 (24.6)	6 (31.6)
SXT(30µ)	6 (23.1)	10 (38.5)	2 (11.1)	11 (31.4)	5 (55.6)	3 (16.7)	67 (38.3)	7 (36.8)
PN (30µ)	11 (42.3)	12 (46.2)	7 (38.9)	15 (42.9)	3 (33.3)	6 (33.4)	48 (27.4)	5 (26.3)

Key:
OFX=Tarivid, PEF=Reflacin, CPX=Ciprofloxacin, AU=Augmentin, CN=Gentamycin, S=Streptomycin, CEP=Ceporex, NA=Nalidixic Acid, SXT=Septrin, PN=Ampicilin

TABLE 4: Predisposing factors for diarrhoea in children under five years

S/n	Risk factors	Í ÞÖNÑØ	ĜBŃÕŒD	Ő-value
1.	Hands washing after remover of diapers			
	Yes	118	4	
	No	189	171	0.042
2.	Hands washing with soaps after toilet			
	Yes	63	8	
	No	244	167	0.036
3.	Hands washing with soaps before serving food			
	Yes	4	0	
	No	303	175	0.047
4.	Hands washing with soaps and water after defecation.			
	Yes	29	3	
	No	278	172	0.011
5.	Do you use antibiotics whenever your child is indisposed?			
	Yes	264	167	
	No	43	8	0.035
6.	Is the child stooling?			
	Yes	307	175	0.046
	No	0	0	
7.	Does the child experience abdominal pains?			
	Yes	179	125	
	No	128	50	0.028
8.	Does the child have fever?			
	Yes	193	128	
	No	128	47	0.034
9.	Do you maintain long nails?			
	Yes	235	163	
	No	72	12	0.028
10.	Is the child on admission for more than 2 days?			
	Yes	273	156	
	No	34	19	0.061
11.	Does your child experience watery stool?			
	Yes	219	155	
	No	88	20	0.032
12.	Does the child wash hands before eating?			
	Yes	92	24	
	No	215	151	0.031

Fig. 2.0: Geologic Map of Eastern Dahomey Basin (Modified after Billman, 1976).

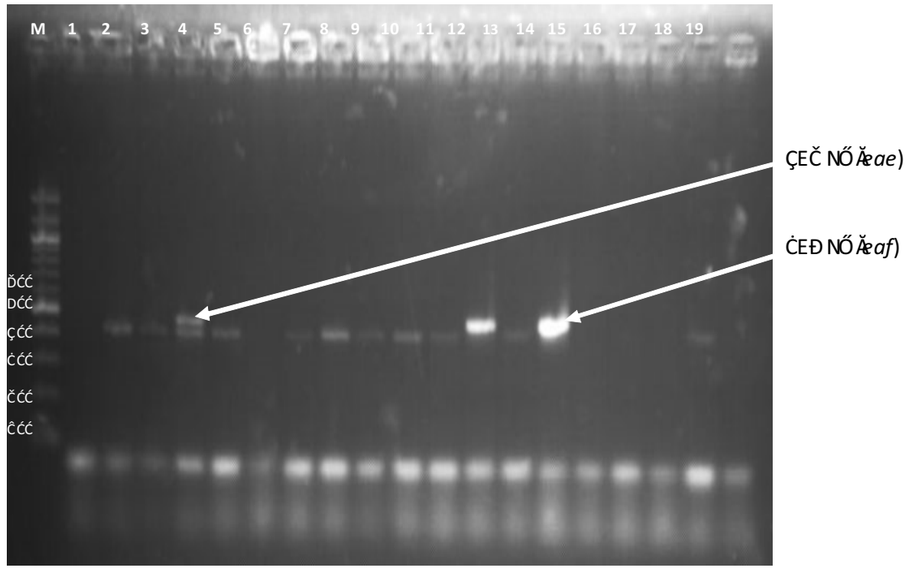

Figure 1: Detection of *eae* and *eaf* genes of enteropathogenic *E. coil* (EPEC) among isolated *E. coli*

Legend: M = Maker, 1-19 = Enteropathogenic *E coli,* 492 bp eae genes = 17 (atypical *Escherichia coli)* isolates, 397bp carrying eaf genes = 2 (Typical *Escherichia coli)* isolates

DISCUSSION

Diarrhoea due to bacterial infection is an important cause of morbidity mortality in infants and young children under the age of 5 years in most developing countries including Nigeria (Adegunloye, 2005). Most of the children diagnosed with diarrhoea in this study were under the age of 3 years. This finding is line with the report of Karambu *et al*., (2013).

Majority of the stool samples from the children were mucoid and watery, only few were bloody. *Acinetobacter iwofii*, *Serratia liquefaciens*, *Salmonella arizonae*, *Enterobacter gergoviae*, *Citrobacter freundii, Citrobacter diversus* and *Escherichia coli* were isolated from the stool samples. Okeke *et al*., (2012) isolated similar bacteria from stool samples of children. Although most of these isolates are normal flora of human, report have shown that they cause opportunistic bacterial infections in patient with low immunity (*Ku et al*., *2012)*. The roles of *A. iwoffii, C. freundii Salmonella arizonae* in gastroenteritis have been reported (Regalado *et al*., 2013; Mahajan *et al*., 2003). *Escherichia coli* (57.0%) is the most prevalence isolate in this study. This high prevalence confirms that *Escherichia coli* remains the major cause of diarrhoea in children (Yu and Kaper 1992, Onanuga *et al*., 2014).The result of the antibiotic susceptibility showed that *Escherichia coli* (86.7%), *Acinetobacter iwofii* (84.6%), *Citrobacter freundii* (77.8%), *Enterobacter gergoviae (*65.7%) and *Citrobacter diversus* (50.0%) were highly resistant to Augmentin. This is worrisome because Augmentin is known to possess high level spectrum of antibacterial activity compared to other broad-spectrum antibiotics and may be attributed to inappropriate use, overuse, high prevalence of sub-standard and adulterated drugs in our patent medicine and pharmacy stores.

This research work was able to identify the main pre-disposing factor to acquisition of diarrhoea in Damaturu as hand washing and poor hygiene. These were basically observed on the part of the parents and the children. Other factors include non-washing of hands before serving meals, as well as non-washing of hands after changing of diapers. These factors were also observed by some authors (Nguyen *et al., 2005;* Scaletsky *et al.*, 2010; *Lanata*

et al., 2011; Theresa and Carmen, 2012).

The molecular characterization identified (10.9%) *Escherichia coli* to be Entropathogenic *Escherichia coli*. The identification of *eae* genes (89.5) indicated that atypical entropathogenic *Escherichia coli* are the most prevalent *Escherichia coli* causing diarrhoea in children in Damaturu, Yobe state. This was also observed by Trabulsi *et al*, (2002), Afset *et al*. (2003); Scaletsky *et al*, (2010), Tauschek *et al*, (2013) in similar studies. This is contrary to findings of Martinez-Medina and Garcia-Gil, (2014) that reported higher prevalence of typical *Escherichia coli* isolates than atypical species. Studies have significantly associated atypical EPEC with endemic (Scaletsky *et al*., 1999; Vieira *et al*., 2001) and outbreaks of diarrhoea (Jenkins *et al*., 2003). Furthermore, atypical EPEC are more prevalent than typical EPEC in both developing and developed countries also, the duration of diarrhea with atypical EPEC is significantly longer than that caused by other pathogens (Afset *et al*., 2003; Ochoa *et al*., 2008).

Conclusion

Escherichia coli isolates is the most predominant bacteria causing diarrhoea in Damaturu, Yobe State and the most common of the Enteropathogenic strain identified were the atypical type. The bacteria isolates showed very high antibiotic resistance to Augumentin. There is a link between diarrhoea and poor personal hygiene in children under 5 years of age at Damaturu, Yobe state, Nigeria

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