PREVALENCE OF ENTEROHAEMORRHAGIC *ESCHERICHIA COLI* O157:H7, SHIGA TOXIN GENES AND ANTIBIOTIC RESISTANCE IN CHILDREN AGED 0-5 YEARS WITH DIARRHOEA IN IBADAN, OYO STATE

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ABSTRACT

Diarrhoea is a predominant cause of childhood illness and death, Enterohaemorrhagic *Escherichia coli* (EHEC) have increasingly been recognised as an important cause of diarrhea all over the world. This study investigates the prevalence, presence of shiga toxin (Stx) and antibiotic resistance of Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 in children with diarrhoea.
Stool samples collected from children less than five year with diarrhoea were cultured, bacteria isolated were identified and antibiotics susceptibility testing was performed using standard methods. Entrohaemorrhagic *E. coli* O157:H7 and Stx were screened using serology and Polymerase Chain Reaction respectively. Structured questionnaires were administered to determine factors that predispose the children to diarrhoea. A total of 301/370 (81.4%) stool samples yielded bacterial growth, 261/301 (86.7%) were gram negative bacilli, 188/261 (72.0%) *Escherichia coli,* 163/188 (86.7%) were EHEC O157: H7. Out of the 5 EHEC O157:H7 only 2 possessed *Stx* genes, 1 have *Stx* 2 while the other have both *Stx* 1 and *Stx* 2 gene. *Escherichia coli* were resistant to Tetracycline 98.4%, Ampicillin 83.0%, Cefuroxime 76.5%, Augmentin 62.9% and Gentamycin 51.4%, all the EHEC O157:H7 were resistant to Tetracycline and Ampicillin. Diarrhoea in the children were significantly associated with hand wash after toilet, eating pastries, sources of drinking water and the educational level of parent/caretaker (p-values = 0.04, 0.00, 0.00 and 0.03 respectively).
The presence of EHEC O157:H7 carrying Stx 1 and Stx 2 gene as well as resistant to all antibiotics tested is a pointer for more stringent and better screening for diarrhoea infections in children.

Key words: Entrohaemorrhagic *Escherichia coli,* EHEC O157:H7, Shiga toxin (Stx 1 and Stx 2)

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INTRODUCTION

Infectious diarrhoea is one of the leading public health problems worldwide and the major cause of morbidity and mortality in all ages especially children less than five years in developing countries (UNICEF/WHO, 2009; Cooke, 2010; Bonkoungou *et al*., 2013; WHO, 2013). Diarrhoea is defined as the passage of loose or watery stools at least three times per day, or more frequently than usual for an individual. Most cases of childhood diarrhoea are mild, and acute cases can lead to significant fiuid loss and dehydration or death (Cheng, 2005; UNICEF/WHO, 2009; WHO, 2013). The aetiological agents for acute diarrhea include a wide range of viruses, bacteria and parasites (Bonkoungou *et al.,* 2013; WHO, 2013). The most important cause of acute watery bacteria diarrhoea in young children is *Escherichia coli* (Ecuador *et al.,* 2012; Kadhim and Abbas, 2013). *Escherichia coli* is a member of the family Enterobacteriaceae, different strains have been identified in diarrhoea, and the most common is *Escherichia coli* O157:H7 (Okeke *et al*., 2003). EHEC has increasingly been recognised as an essential public health problem worldwide since they were first implicated in diarrhoea in 1983 (Burland *et al*., 1998). *Escherichia coli* O157:H7 causes hemorrhagic colitis, hemolytic uremic syndrome and death. Several outbreaks of *Escherichia coli* O157:H7 have been reported in different parts of the world (Raji *et al*., 2006) and were linked with the consumption of raw or

undercooked foods (Hiko *et al*., 2008; Oloyede *et al*., 2016).
Few studies have attempted to look at the prevalence of EHEC O157:H7 in diarrhogenic children in Nigeria (Olorunshola *et al*., 2000; Okeke *et al*., 2000; Okeke *et al*., 2003; Yilgwan and Okolo, 2012; AbdulAziz, 2016). The prevalence of *Escherichia coli* O157:H7 in Nigeria is undoubtedly underestimated because the bacteria is not sought for in routine laboratory diagnosis of diarrhoea infection as such most of the available information are report of presumptive identification based on the inability of the strain to ferment sorbitol rather than serology.
Furthermore, the unavailability or high cost of polyvalent or monovalent antisera for serology might also be responsible for the underestimation of enterohaemorrhagic *Escherichia coli* O157:H7 in Nigeria. This study determines the prevalence, presence of shiga toxin genes and antibiotic resistance of Enterohaemorrhagic *Escherichia coli* O157:H7 in children aged 0-5 years with diarrhoea in Ibadan, Oyo State

MATERIALS AND METHODS

Study Area

This study was carried out in three selected government hospitals Oni and Sons Memorial Children Hospital, Ade-Oyo General Hospital and Otunba Tunwase Children Emergency Ward (OTCHEW) University College Hospital Ibadan, Oyo State Nigeria.

Selection Criteria

Children aged 0-5 years old, with acute diarrhea (passage of three or more loose stools in 24 hours) with or without clinical symptoms of an enteric ailment (nausea, cramps, faecal urgency, abdominal pain, vomiting, dehydration, or dysentery) and whose mother or caretaker consented to participate in the study were included. Children more than 5 years old and whose mother or caretaker did not give their consent were excluded

Sample Collection

Oral and written informed consent were obtained from parents or caretakers and general information which includes sex, age, clinical history, socio-economic status and living conditions of the patients were obtained on questionnaires. Fresh stool samples were collected in sterile wide mouth plastic universal containers and sterile swab stick was used to collect rectal sample from those that could not product stool sample. Sample and questionnaire were labeled with 3 digits numbers and were transported in ice packs to the laboratory for immediate processing.

Processing of samples

Stool samples collected were cultured on MacConkey agar and incubated aerobically at 37°C for 24 hours. The isolates were Gram stained, all Gram negative bacteria were identified biochemically with MICROBACT 24E Identification System following the manufacturer's manual. Identified *Escherichia coli* were inoculated onto Sorbitol MacConkey agar plate and were incubated aerobically at 37°C for 24 hours, non-sorbitol fermenting colonies were collectively presumed to be Enterohaemorrhagic O157:H7

Escherichia coli O157: H Serotyping

Non-Sorbitol fermenting colonies were tested for O157:H7 antigen using Latex test kit (Oxoid). A colony was emulsified in 40µL of saline on two circles on a Reaction Card with an applicator stick. A drop of O157 Test Latex was added to one circle, and a drop of O157 Control Latex was added to the other circle. The Reaction Card was rocked carefully for 30 seconds and observed for agglutination, which indicates Positive. O157 positive isolates were similarly tested for H7 antigen using a drop of H7 Test Latex and a drop of H7 Control Latex and observed for agglutination, which indicates Positive.

Antibiotics Susceptibility Test

Escherichia coli was inoculated into sterile peptone water, incubated for 2 hours and the turbidity of culture was adjusted to that of 0.5 MacFarland standard (equivalent to $1.5x10^8$cfu/ml of innoculum). A sterile cotton swab was dipped into the inoculum, the excess was drained by pressing the swab on the side of the tube and swab was used to streak the surface of sterile Mueller-Hinton agar plate. Antibiotic disks were asceptically mounted on the inoculated plate, the zones of inhibition produced after incubated at 37°C for 24 hours was measured to the nearest millimeter (mm). A control test was carried out simultaneously using *Escherichia coli ATCC*

35218. The susceptibility results were interpreted using Clinical Laboratory Standards International (2013).

Extraction of DNA

The DNA of *E. coli* was extracted using Norgens Biotek Corp Kit (Canada) following the manufacturer's instructions. An 18-24 broth culture of the isolates was centrifuged 2000 rpm for 10 minutes, the supernatant discarded; 100µL of phosphate buffered saline (PBS) was added to the cell pellet and was mixed gently by vortexing. Protenaise K (20µL), lysis solution (300µL) was added mixed by vortexing and was incubated at 56°C for 20 minutes. The lysate mixture (750µL) was introduced into assembled spin column; the column was capped and centrifuged for 2 minutes at 8,000 rpm. The flow through was discarded, the spin column was attached to collecting tube, 500µL of Wash Solution I was added and was centrifuged for 1 minutes at 8000 rpm. The flow through was discarded and the spin column was attached to another collection tube, 500µL of Wash Solution II was added and was centrifuged at 14000 rpm for 1 minutes. The column was spun further for 2 minutes to dry 14000 rpm and the collection tube was discarded. The DNA was obtained by dispencing 200µL of Elution Buffer into the column attached to the elution tube provided, was allowed to incubated at room temperature for 1 minute and was centrifuged at 8000 rpm for 1 minute. The Purified DNA stored at -20°C for use.

Polymerase Chain Reaction (PCR)

The extracted DNA of EHEC O157/H7 isolates were screened for possession of *Stx1* and *Stx2* pathogenic genes using the primer sequences published by Sarimehmetogiu *et al*., (2009) obtained from a Jena Biosciences, Germany. The multiplex PCR was carried out in a 50 µL reaction mixture containing 5µL of the DNA of sample and PCR master mix which contains a premix of sequenced primers for EHEC O157/H7

(F-TGTAACTGGAAAGGTGGAGTATACA,
R- GCTATTCTGAGTCAACGAAAAATAAC stx_1 - 210 bp,
F- GTTTTTCTTCGGTATCCTATTCC,
R- GATGCATCTCTGGTCATTGTATTAC stx_2- 484 bp),

PCR buffer, Magnesium chloride, dNTPs, and Taq Polymerase enzyme in optimized concentrations. The PCR reaction mixture was put in the Thermal cycler (Master Cycler Gradient Eppendorf, Hamburg, Germany) programmed at 95°C for 5 minutes to activate the Taq polymerase enzyme followed by 34 cycles of denaturation of the double-stranded DNA at 95°C for 30 seconds, primer annealing at 55°C for 60 seconds, elongation at 72°C for 60 seconds and final extension at 72°C for 5 minutes. The amplified products were loaded on 1.5% Agarose gel, stained with Ethidium Bromide ran for 15 minutes in electrophoresis tank containing and visualised under the ultraviolet light using the Trans-illuminator (Bio-Rad, Italy).

Ethical permission

Ethical Approval was obtained from Oyo State Ethical Review Committee, Ministry of Health, Ibadan, in Oyo State, Nigeria and permission to use the hospitals were obtained from the Head of each hospital.

Data Analysis

The data generated in the study and questionnaires were reported as percentages, statistical analysis of data was performed using SPSS version 20.0 and $p \leq 0.05$ was taken as significant.

RESULTS

Out of the 370 stool samples cultured 301 (81.4%) yielded bacterial growth, 261 (86.7%) were Gram negative bacilli. *Escherichia coli* accounted for 188 (72.0%) most predominant Gram negative bacilli (Table 1). Out of the 188 *Escherichia coli* sub cultured on Sorbitol MacConkey agar, 163 (86.7%) were non-sorbitol fermenters and only 5 (3.1%) agglutinates the EHEC O157:H7 antisera. Shiga toxin genes were detected in two of EHEC O157:H7 isolates, one of which carried both Stx1 and Stx2 genes while the other one carried only Stx2 gene.

Antibiotic Susceptibility test of *Escherichia coli* isolated from stool samples of children with diarrhoea

The susceptibility results showed that the *Escherichia coli* were resistant to Tetracycline 180 (98.4%), Ampicillin 152 (83.0%), Cefuroxime 140

(76.5%), Augmentin 116 (62.9%) and Gentmycin 94 (51.4%) (Table 2). The resistant pattern of EHEC O157: H7 is presented in (Table 3), all showed multiple resistant to more than three antibiotics, importantly all were resistant to Tetracycline and Ampicillin. At the same time, one of the isolates was resistant to all the antibiotics tested.

Factors Associated with the duration of diarrhea in children aged 0-5 years

The factors associated with diarrhea duration are presented in Table 3. Hand washing after toilet, eating pastries, sources of drinking water and the parent/caretakers educational level was found to be significantly associated with duration of diarhoea in children p-values = 0.04, 0.00, 0.00 and 0.03 respectively. Age, Sex and Educational status of the children were not significantly associated with the duration of diarrhea in the children p-values= 0.25, 0.63, and 0.09, respectively.

Table 1: Percentage Frequency Distribution of Gram-negative bacteria isolated from children with diarrhoea

Organisms	Frequency
Escherichia coli	188 (72.0)
Hafniaalvei	7 (2.7)
Acinetobacterbaumannii	8 (3.1)
Serratiamarcescens	6 (2.3)
Salmonella subsp1	1 (0.4)
Citrobacterdiversus	5 (1.9)
Enterobactergergoviae	5 (1.9)
Klebsiella pneumonia	1(0.4)
Klebsiellaoxytoca	6 (2.3)
Pseudomonas aeruginosa	17 (6.5)
Morganellamorganni	5 (1.9)
Enterobacter Cloacae	7 (2.7)
Proteus mirabilis	5 (1.9)
Total	**261 (100)**

Table 2: Antibiotic resistant of *Escherichia coli* isolated from stool samples

Antibiotics	Resistant Frequency (%)
Ciprofloxacin (10µg)	26 (14.2)
Ampicillin (33µg)	152 (83.0)
Augmentin (45µg)	116 (62.9)
Cefuroxime (30µg)	140 (76.5)
Tetracycline (80µg)	180 (98.4)
Ceftriaxone (60µg),	31 (16.9%)
Gentamycin (40µg)	94 (51.4)

Table 3: Antibiotic resistant pattern of EHEC O157: H7 from children with diarrhoea

Sample number	Antibiotics resistant pattern
024	(CXM, GEN, **TET, AMP**)
153	(CXM, AUG, CIP, CRO, GEN, **TET, AMP**)
154	(CXM, AUG, CRO, **TET, AMP**)
172	(AUG, GEN, **TET, AMP**)
236	(AUG, CRO, GEN, **TET, AMP**)

Key: CXM = Cefuroxime, AUG = Augmentin, CIP =Ciprofloxacin, CRO = Ceftriaxone GEN = Gentamycin, TET = Tetracycline, AMP = Ampicilin

Table 4: Factors associated with diarrhea duration in children

Variables		Duration of Diarrhea n (%) Low (<3days)	High (>3days)	Total	???	p-value
Sex	Male	110 (29.7)	98 (26.5)	208 (56.2)	1.29	0.25
	Female	76 (20.5)	86 (23.2)	162 (43.8)		
	Total	**186 (50.3)**	**184 (49.7)**	**370(100)**		
Age (months)	0 – 12	22(5.9)	27(7.3)	49(13.2)	7.09	0.63
	13 – 24	128(34.6)	125(33.8)	253(68.4)		
	>24 =5	49(13. 2)	24(6.5)	68(19.7)		
	Total	**199(53.7)**	**176(47.6)**	**370(100)**		
Hand washing after toilet	Yes	152 (41.1)	137 (37.0)	289 (78.1)	4.37	0.04
	No	32 (8.6)	49 (13.2)	81 (21.9)		
	Total	**184 (49.7)**	**186 (50.3)**	**370 (100)**		
Educational status of child	Creche	33 (8.9)	35 (9.5)	68 (18.4)	8.13	0.09
	Pre-Nursery	31 (8.4)	38 (10.3)	68 (18.6)		
	Nursery	29 (7.8)	19 (5.1)	48 (13.0)		
	Primary	22 (5.9)	10 (2.7)	32 (8.6)		
	Non	71 (19.2)	82 (22.2)	153 (41.4)		
	Total	**186(50.3)**	**184 (49.7)**	**370 (100)**		
Educational status of parent	Educated	138 (37.3)	110 (29.7)	248 (67.0)	8.69	0.03
	Not Educated	48 (13.0)	74 (20.0)	122 (33.0)		
	Total	**186 (50.3)**	**184 (49.7)**	**370 (100)**		
Eating pastries	Yes	80 (21.6)	121 (32.7)	201 (54.3)	17.35	0.00
	No	104 (28.1)	65 (17.6)	169 (45.7)		
	Total	**184 (49.7)**	**186 (50.3)**	**370(100)**		
Source of drinking water	Good	74 (20.0)	48 (13.0)	122 (33.0)	8.69	0.00
	Poor	110 (29.7)	138 (37.3)	248 (67.0)		
	Total	**184 (49.7)**	**186 (50.3)**	**370 (100**		

Significance is at $p < 0.05$

DISCUSSION

The World Health Organisation defined diarrhoea as the passage of three or more loose or liquid stools per day (or more frequent passage than is healthy for the individual) (WHO, 2013). The children recruited for this study were those clinically diagnosed as having diarrhoea by the Physicians in the centres. Diarrhoea infection is caused by variety of microbial agents (WHO, 2013). The isolation of bacteria from 81.4% of the stool samples in this study confirmed the bacterial aetiology of the diarrhoea in this study. Thirteen (13) bacteria species which includes *Escherichia coli* were isolated from the stool samples. Ifeanyi *et al*. (2010) isolated eight (8) different bacteria; this disparity may be attributed to the biochemical identification system used in this study which allowed for better speciation of the isolates. The identification of *Escherichia coli* 72.0% as the most predominant bacteria is in agreement with other reports (Vargas *et al*., 2004; Galadima and Kolo, 2014).

The result of this study showed that age, gender and educational level of the children does not predisposed to diarrhea. This finding corroborate the report of Isibor *et al*., (2013) that age and gender were not significant risk factors for diarrhoea infection in children. The prevalence of diarrhoea in children aged 0 -5 years in Ibadan, Nigeria was 81.4%, this is higher than 65.8% reported by Ifeanyi *et al*. (2010) but is in concordance with 83.1% reported by Ogbu *et al*. (2008) in a similar study in Abakaliki, South Eastern Nigeria.

The prevalence of Enterohaemorrhagic *Escherichia coli* O157:H7 in this present study is 3.1%, other studies have reported prevalence as low as 0.6% (Okeke *et al*., 2000),1.39% (AbdulAziz *et al*., 2016), 2.7% (Isibor *et al*., 2013) and as high as 6% (Olorunshola *et al*. 2000), 8.4% (Akinyemi *et al*., 1998). These variations could be related to differences in sample size, age, study population or area, and specificity of methods used for identification. The pathogenicity of Enterohemorrhagic *Escherichia coli* is associated with possession of Shiga toxin (stx1,stx2) genes (Gerrish *et al*., 2007), presence of shiga toxin in 2 of the EHEC O157:H7 indicates their virulence. Hospital laboratories should be upgraded with facilities to isolate and identify shiga toxin and other virulence gene in other to reduce the morbidity associated with strains.

Antibiotic resistance remains a significant threat worldwide to the control of bacterial infections. Very high antibiotic resistance rates were observed in all the *Escherichia coli* including the Enterohaemorrhagic *Escherichia coli*. EHEC O157:H7 isolated showed multi-drug resistance to at least 4 of the antibiotics tested. It is worthy to note that all the EHEC O157:H7 are resistant to Tetracycline and Ampicillin, 1 out of the 2 that possess shiga toxin gene was resistant to all the antibiotics tested in addition, this particular one also carried both stx1 and stx2 genes. Strains resistant to ampicillin, tetracycline and other antibiotics have been reported (Meng *et al*., 1998; Schroeder *et al*., 2002; Reyes *et al*., 2004; Hiko *et al*., 2008). The use of these antibiotics for treatment may compromise antibiotic therapy in patients with diarrhoea. The presence of EHEC:O157:H7 that has shiga toxins genes, and also exhibited multiple drug resistance has implications on treatment outcomes.

The duration of diarrhoea in the children is significantly associated with hand wash after toilet, eating of pastries, sources of drinking water as well as the educational level of parent/caretaker. The washing of hands with soap and water is the most effective way of reducing diarrhoea (Wilunda and Panza, 2006). The low prevalence of EHEC O157/H7 in this research work could be associated with the washing of hands with soap and water by most mothers or caretakers. In Nigeria, Enterohaemorrhagic *Escherichia coli* O157/H7 have been isolated from different sources which include vegetables, water, meat, cattle, sheep, goat and abattoirs (Olatoye, 2010; Chigor, 2010; Oloyede, 2016). Therefore, the transmission of Enterohaemorrhagic *Escherichia coli* O157/H7 and associated diseases involve multiple reservoirs which are potential health hazards to human.

CONCLUSION

E. coli is the most predominant bacteria agent of diarrhea among children (0-5years) in this study. The presence of EHEC O157: H7 carrying Stx1 and Stx2 gene as well as resistant to all antibiotics tested is a pointer for more stringent and better screening for diarrhoea infections in children.

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