Journal of Advances in Medicine and Medical Research

22(10): 1-10, 2017; Article no.JAMMR.31023
Previously known as British Journal of Medicine and Medical Research
ISSN: 2231-0614, NLM ID: 101570965

Prevalence and Antibiotic Susceptibility Patterns of *Escherichia coli* O157:H7 in Children 0 – 24 Months in Calabar South L. G. A. of Cross River State, Nigeria

Kingsley Hovana Enyi-Idoh[1*], Okort Agbor Akwa[2], Ini Ubi Bassey[1], Veronica David Idim[3] and Stephen Ugoeze Egeonu[4]

[1]Department of Microbiology, Faculty of Biological Science, University of Calabar, Calabar, Nigeria.
[2]Department of Biological Sciences, Cross River University of Technology, Calabar, Cross River State, Nigeria.
[3]Department of Chemical Sciences, Cross River University of Technology, Calabar, Cross River State, Nigeria.
[4]Federal Medical Center, Jalingo, Taraba State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors KHEI and OAA designed and arranged the research from conception to end. Authors VDI and SUE were involved in collecting the samples under the supervision of author OAA. Authors IUB, OAA and VDI performed the laboratory analyses while authors KHEI, OAA and IUB prepared the manuscript and proofread by author KHEI. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMMR/2017/31023
Editor(s):
(1) Faris Q. B. Alenzi, Department of Medical Laboratories, College of Applied Medical Sciences Salman bin Abdulaziz University (Al-Kharj), Saudi Arabia.
Reviewers:
(1) Auwal Sani, Ahmadu Bello University, Nigeria.
(2) Shweta Sharma, Dr. Ram Manohar Lohia Hospital Hospital, India.
(3) Victor A. Amadi, St. George's University, Grenada.
Complete Peer review History: http://www.sciencedomain.org/review-history/20040

Original Research Article

Received 15th December 2016
Accepted 18th January 2017
Published 14th July 2017

ABSTRACT

Diarrhea continues to be the scourge of children across the world especially in developing countries like Nigeria. Despite efforts by World Health Organization and other agencies, much work still remain to be done in combating diarrheal diseases caused by *Escherichia coli* O157:H7 in the

**Corresponding author: E-mail: kingenyi@gmail.com;*

developing world including Nigeria. One hundred and fifty (150) children attending five nursery schools including Native nursery/primary school (Anantiga), Bank-dee nursery/primary school (Musaha), Golden nursery/primary school (Ekpo-Abasi), JOESAM nursery/primary school (Yellow Duke) and Ideal nursery/primary school (Palm street) in five major localities in Calabar South LGA between July 2014 and June 2015, were recruited in this study. Socio-demograhpic factors such as age, sex, weaning practices and parents' occupation/educational level were obtained using structured questionnaires. Stool samples were examined macroscopically and microscopically, and stool culture was done using MacConkey agar and Sobitol MacConkey agar. Antibiotics sensitivity and serology tests were carried out. *E. coli* O157:H7 was identified in 14(9.33%) out of the 150 stool samples examined. The highest number of isolates was recovered from the 6 – 12 months age group having 9 (64.29%) in total. No isolates were recovered from the age group 0 – 5 months or from exclusively breastfeed infants. However, 5 (7.35%) isolates were recovered from infants that received mixed feeding while 9 (27.27%) from infants who received no breastfeeding ($P > 0.05$). Of the isolates, 12 (85.71%) were sensitive to amoxicillin, 10 (71.43%) to pefloxacin, 8 (57.14%) to gentamicin, 17 (73.91%) to sporfloxacin, 16 (69.57%) to ciprofloxacin and 7 (50.00%) to ampiclox, 4 (28.57%) were sensitive to zinacef and rocephin while only 2 (14.29%) were sensitive to streptomycin and septrin. No isolates were sensitive to erythromycin. *E. coli* O157:H7 is an important bacterial agent in infantile diarrhea. Improvement in nutritional status, weaning practices, socioeconomic status and personal hygiene will lead to a reduction in the spread and incidence of diarrhea due to bacterial agents (pathogens), especially *E. coli* O157:H7.

Keywords: Diarrheal disease; exclusively breastfed infants; children under 24 months; Escherichia coli O157:H7; Calabar South LGA.

1. INTRODUCTION

Between 1960 and 1961, a study of the etiology in infantile diarrhea was conducted in Houston and the most commonly defined pathogen during this period was *Escherichia coli* 0157:H7 with a remarkable pattern of multiple antibiotic resistances [1]. From discovery to the present understanding of public health importance, *E. coli* was first suspected as being a cause of children's diarrhea in the 1940s, when nursery epidemics of severe diarrhea were found to be associated with particular serotypes of *E. coli* [2]. These specific serotypes, designated enteropathogenic *E. coli*, were epidemiologically incriminated as the cause of the outbreaks [3].

Diarrheal disease is a global public health problem causing considerable morbidity and mortality among infants and young children especially in developing countries [4,5]. It is reported that over five million deaths per year occur in children under the age of five years as a result of diarrhea in developing countries [6]. The significant reduction in children mortality observed in recent years has been attributed to the practices of exclusive breast feeding as well as recourse to oral rehydration therapy in the treatment of diarrhea [4].

Infections due to feacal water contamination are caused by a host of enteric infectious agents including bacteria, viruses, fungi and parasites. Among the bacterial pathogens *E. coli* O157:H7 is an important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries [7].

The history of enterotoxigenic *E. coli* began in 1956 in Calcutta [8]. The researchers injected live strains of *E. coli* isolated from children and adults with cholera–like illness.

From previous studies, it has shown that *E. coli* strains isolated from symptomatic children in different regions of the word have the following data for prevalence - % of subjects-, Bangladesh (18%), Mexico (33%), Peru (7.4%), Egypt (20%), Nigeria (21%), Argentina (18.3), India (12-24%) and Nicaragua (38%) [9].

Previous studies have highlighted the high incidence of community acquired *E. coli* O157:H7 infection in the first six months following childbirth [10,11]. The infection is relatively more severe in younger children than older adults with infants having a greater propensity for developing diarrhea during initial colonization with *E. coli* O157:H7 than subsequent exposures [11]. Parents' (especially mothers) educational standards, unhealthy and improper weaning and early bottle feeding are possible factors that could facilitate high prevalence rates of infection with *E. coli* O157:H7 in developing countries [12].

In Nigeria, cases of gastroenteritis due to *E. coli* O157:H7 have been reported. What is not well established however is the serotypes suspected to be associated with *E. coli* O157:H7 infection in children in Nigeria [10].

Escherichia coli, commonly found humans and warm-blooded animals gut, are harmless but strains like enterohaemorrhagic *E. coli* can cause very severe foodborne diseases. In 2016, WHO was notified of an outbreak of Enterohaemorrhagic Shiga toxin-producing Escherischia coli (STEC) O157 PT34 in England and Wales by the National IHR Focal Point for the United Kingdom.

Later in the year 158 cases had been identified, of which 105 were classified as confirmed cases and 53 as probable. Haemolytic uraemic syndrome (HUS) was reported in seven of the cases. Two cases with confirmed E. coli infection listed as a causative factor died. Cases are distributed throughout the UK, with the majority (91%) residing in England. Onset dates for cases range from 31 May 2016 to 5 July 2016 [13].

2. MATERIALS AND METHODS

2.1 Study Population

The study group consisted of infants and young children (with or without fever) who have not taken antimicrobial agents in the preceding weeks with ages between O to 24 months (from birth to 2 years) attending five nursery schools from five most important areas in Calabar South Local Government Area of Cross State, Nigeria. The areas include: Anantigha, Musaha, Ekpo-Abasi, Yellow Duke, and Palm Street. With the aid of the serial numbers of children in the class register, all even numbered children were selected for the study. Ethical approval was given by Cross River State Ministry of Health—Research and Ethics Committee (CRSH-REC). Permission was also obtained from Primary Education Board and informed written consent was obtained from parents or caregivers specifically mothers of infants and from school authorities. Parents of infants were briefed on the aims and objectives of the study in order to get their consent. Only those who returned consent forms were recruited for the study. A total of 150 stool samples were collected from the infants recruited in this study from August, 2015 to June, 2016.

2.2 Collection of Samples

The sample size was determined using the formula, $n = Z^2@/2/4E^2$ [14] where n is the required sample size, $Z^2_@/2$ is to be obtained from the table of normal curve areas and E is used in estimating the true proportion, resulting in sample size of 150.

Loop full of stool samples were collected from infants in Anantigha (Native nursery/primary school), Musaha (Bank-dee nursery/primary school), Ekpo-Abasi (Golden nursery/primary school), Yellow Duke (JOESAM nursery/primary School) and Palm Street (Ideal nursery/primary school) all located in Calabar South Local Government Area of Cross River State. The samples were put in leak-proof wide-mouth sterile capped containers (which were labeled using the serial numbers of infants in the class registers) containing Cary-Blair transport medium and transported immediately to Microbiology Laboratory of Cross River University of Technology (CRUTECH) Calabar, for analysis.

A pretested questionnaire was used to obtained information on the age, sex, anthropometry, breastfeeding status, occupational/educational status of the parents (especially mothers).

The social class was determined using classification of social class as proposed by [7]. This classification used the parental occupation and educational attainment to determine the social class. The nutritional status was determined using the modified Wellcome classification [15].

2.3 Processing of Samples

All stool samples were analyzed following the guideline provided by Cheesbrough and Cowan for stool examination [16,17].

2.4 Macroscopy

Cross appearance of all the stool samples were noted, especially consistency (formed, semi-formed and unformed or fluid), presence of mucus and blood.

2.5 Microscopy

A small portion of stool specimen was suspended in a drop of saline and placed on a

slide, then examined microscopically for pus cells and red blood cells [16].

2.6 Isolation and Maintenances of Pure Culture

Primary isolation of *E. coli* was performed using MacConkey agar medium and transferred onto Sorbitol MacConkey agar medium. A loop full of the stool sample was transferred from Cary Blair medium onto the surface of MacConkey medium using surface plating technique. All plates were incubated aerobically at 37℃ for 18 to 24 hours. The plates were observed for growth (morphology and pigmentation) of characteristic *E. coli* colonies. Representative pink/red colonies were then transferred to Sorbitol MacConkey agar plates and observed for translucent/colourless colonies which were subsequently sub cultured onto Nutrient agar plates by streaking. Purified discrete colonies were refrigerated in nutrient agar slants at 4℃ for further biochemical test [17].

2.7 Identification and Biochemical Characterization of Isolates

All lactose fermenting (pink/red) colonies on MacConkey agar plates and non-sorbitol fermenting (colourless/translucent) colonies on Sorbitol MacConkey agar plates on the Nutrient agar slant were Gram stained and subjected to urease, citrate utilization, indole biochemical tests and motility test.

2.8 Serotyping of Isolates

Isolates identified and confirmed as *E. coli* were suspended in saline and mixed on a reaction card with commercial *E.coli* typing serum and screened by a rapid latex agglutination slide test (OXOID, Hampshire, RG24 8PW, UK, *E. coli* O157:H7 latex test kit). *E. coli* gave 4+ agglutination immediately in type specific serum. The specific antiserum employed was *E. coli* O157:H7. *E. coli* serotyping was based on the presence or absence of three kinds of antigens: A, L and B, all of which mask the reaction and must be destroyed by heat before agglutination in O antiserum takes place. Ability of the bacteria to agglutinate antibodies specific for those antigens: O antigen (somatic), H antigen (flagella) and K antigen (capsular) surface profiles were considered positive reactions.

2.9 Antimicrobial Susceptibility Testing

The susceptibility of the isolates to standardized antibiotics was determined on Mueller–Hinton agar plates using modified Kirby-Bauer disc diffusion method, as recommended by Clinical Laboratory Standards Institute [18], and interpreted through zone size of inhibition. All isolates were tested for sensitivity to the following commercially obtained antibiotics: pefloxacin (10 µg), gentamicin (10 µg), ampiclox (30 µg), zinacef (20 µg), amoxicillin (30 µg), rocephin (25 µg), ciprofloxacin (10 µg), streptomycin (30 µg), septrin (30 µg) and erythromycin (10 µg).

Six to eight hours old broth culture of *E. coli* O157:H7 were carefully spread uniformly over Iso-sensitest agar (Oxoid Ltd., England, Code No. (M471). Each antibiotic disc was picked from its container using sterile forceps and placed at the center of each plate. The discs were carefully pressed to ensure direct contact with the test organism. All plates were incubated at 37℃ for 24 hour, after which observations were made and interpreted through zone size of inhibition [19,16].

2.10 Data Analysis

Data were analyzed using simple frequencies and percentages, Chi-squared test and a probability of p= 0.05 as the level of significance was used for comparing the significant difference between male and female, susceptibility of the antibiotics, ages, personal hygiene and educational background of mothers. A P- value of less than or equal to 0.05 ($P \leq 0.05$) was considered to be statistically significant.

3. RESULTS

A total of 150 stool samples of infants and young children, ages between birth to two years (0 – 24 months) were examined for the presences of *E. coli* O157:H7. Of the total number of samples examined 14 (9.33%) were positive for *E. coli* O157:H7. Infants 6–12 months showed the highest number of *E. coli* O157:H7 isolates, 9 (64.29%). This was followed by children 13-18 month old with 3 (21.43%) isolates, while the 19 – 24 months children showed only 2 (14.29%) isolates. However infants in the age range 0 – 5 months had no *E. coli* O157:H7 present in their stool samples. Females accounted for a higher percentage of infection 7 (11.86%) than males 7 (7.70%). The difference between these groups

and between males and females was not statistically significant (p >0.05) (Table 1, Fig. 1).

Table 2 shows the percentage distribution of stool forms in infants with *E. coli* O157:H7. Macroscopic stool analysis showed four forms of stool samples: Watery stool, watery and mucoid stool, loosed and mucoid stool and loosed stool. Watery stool samples yielded the highest percentage number of isolates, with a total of 6 (24.00%). Others showed that 6(19.36%) were watery/mucoid and 2(6.06%) were loose/ mucoid. However, loose and soft formed had no isolates. The results signify that the children were not properly cared for base on either poor drinking water source or contaminated food.

The correlation of feeding pattern of infants with number of *E. coli* O157:H7 isolates in Calabar showed that children who did not breast feed showed the highest number of *E. coli* O157:H7 isolates of 9 (27.27%) organisms and mixed-fed children only 5 (7.35%).

It was observed that exclusively breast fed infants did not have *E. coli* O157:H7 by the absence of any isolates from this group. This difference was statistically significant (p<0.05) (Table 3). This result implies that the method of feeding adopted by mothers could invariably be the reason for the low immunity of the infants to fight disease (importance of colostrums in breast milk).

Table 4 shows the correlation between maternal educational level and numbers of *E. coli* O157:H7 isolates. Of these, 8 (28.28%) *E. coli* O157:H7 isolates were recovered from infants with mothers who had received only primary education or had no education at all, 4 (8.16%) from infants with mothers who had up to secondary education, and 2 (2.82%) from infants with mothers who had secondary education and above. The results were not found to be statistically significant (p<0.05). Thus this result implies that nursing mothers from this study area with infants positive for *E. coli* O157:H7 have low level of education which could be a factor in the inadequate child care and predisposes the infants to *E. coli* O157:H7 infection.

The antibiotic susceptibility pattern of *E. coli* O157:H7 positive isolates showed that 12 (85.71%) were found to be sensitive to amoxicillin, 10 (71.43%) were sensitive to perfloxacin, 8 (57.43%) to ciprofloxacin and gentamicin, 7 (50.00%) to ampiclox, 4 (28.57%) to zinacef and rocephin, and 2 (14.29%) were sensitive to streptomycin and septrin, while complete resistance was observed for erythromycin.

Table 1. Percentage prevalence of *E. coli* O157:H7 infection in relation to age and sex of infants examined

Age group (months)	No. of samples examined	Females examined N (%)	No. of positive females N (%)	Males examined N (%)	No. of positive Males (+) N (%)	Total No (+) N (%)
0-5	17	7 (11.86)	0 (0.00)	10 (10.99)	0 (0.00)	0 (0.00)
6-12	64	25 (42.40)	4 (57.14)	39 (42.86)	5 (71.43)	9 (64.29)
13-18	38	15 (25.42)	2 (28.57)	23 (25.27)	1 (14.29)	3 (21.43)
19-24	31	12 (20.33)	1 (14.29)	19 (20.88)	1 (14.29)	2 (14.29)
Total N=	**150,**	**59 (39.33)**	**7 (11.86)**	**91 (60.67)**	**7 (7.70)**	**14 (9.33)**

N = Number, P > 0.05

Table 2. Stool analysis for number of *E. coli* O157:H7 isolated from stool samples examined

Stool form	No. of samples examined	No. of females examined N (%)	No. of females (+) N (%)	No. of males examined N (%)	No. of males (+) N (%)	Total no (+) N (%)
Watery	25	12 (20.69)	4 (33.33)	11 (11.99)	2 (8.00)	6 (24.00)
Watery/Mucoid	31	7 (12.07)	2 (28.57)	24 (23.91)	4 (16.67)	6 (19.36)
Loosed/Muciod	33	10 (17.24)	1 (10.00)	23 (22.83)	1 (4.35)	2 (6.06)
Loosed	45	16 (27.59)	0 (0.00)	27 (29.35)	0 (0.00)	0 (0.00)
Softformed	16	13 (46.43)	0 (0.00)	11 (11.96)	0 (0.00)	0(0.00)
Total,	**150,**	**58 (38.67)**	**7 (12.07)**	**92 (61.33)**	**7 (7.61)**	**14 (9.33)**

N = Number, P> 0.05

Table 3. Correlation of feeding pattern of infants with number of *E. coli* 0157:H7 isolated in Calabar South L.G.A.

Types of feeding	No. of samples examined N (%)	No. of samples (+) N (%)
Exclusive breast feeding	49 (32.69)	0 (0.00)
Mixed feeding	68 (45.33)	5 (7.35)
No Breast feeding	33 (32.00)	9 (27.27)
Total	**150**	**14 (9.33)**

P > 0.05

Table 4. Correlation of mother's level of education to numbers of *E. coli* O157:H7 infection in Calabar South L.G.A.

Level of education	No. of samples examined (%)	No. of samples (+) (%)
Above secondary education	71 (47.33)	2 (2.82)
Up to secondary school level	49 (32.67)	4 (8.16)
Primary school and below	30 (20.00)	8 (28.67)
Total	**150**	**14 (9.33)**

P<0.05

Table 5. Percentage in-vitro antibiotic susceptibility pattern of *E. coli* O157:H7 Isolated in Calabar South L.G.A.

Antibiotics	Abbreviation	Potency (µg or mcg)	Zone of inhibition (mm)	Results	No. of Isolates sensitive N (%)	No. of isolate Resistant N (%)
Amoxicillin	AM	30	20-31	S	12 (85.71)	2 (14.29)
Pefloxacin	PEF	10	15-28	S	10 (71.43)	4 (28.57)
Ciprofloxacin	CPX	10	13-15	M	8 (57.14)	6 (42.86)
Gentamicin	GN	10	13-15	M	8 (57.14)	6 (42.86)
Ampiclox	APX	30	>12	M	7 (50.00)	7 (50.00)
Zinacef	Z	20	<10	R	4 (28.57)	10 (71.43)
Rocephin	R	25	<10	R	4 (28.57)	10 (71.43)
Streptomycin	S	30	< 5	R	2 (14.29)	12 (85.71)
Septrin	SEX	30	< 3	R	2 (14.29)	12 (85.71)
Erythromycin	E	10	<1	R	0 (0.00)	14 (100.00)

Key: S – Strongly sensitive; M – Moderately sensitive; R – Resistance

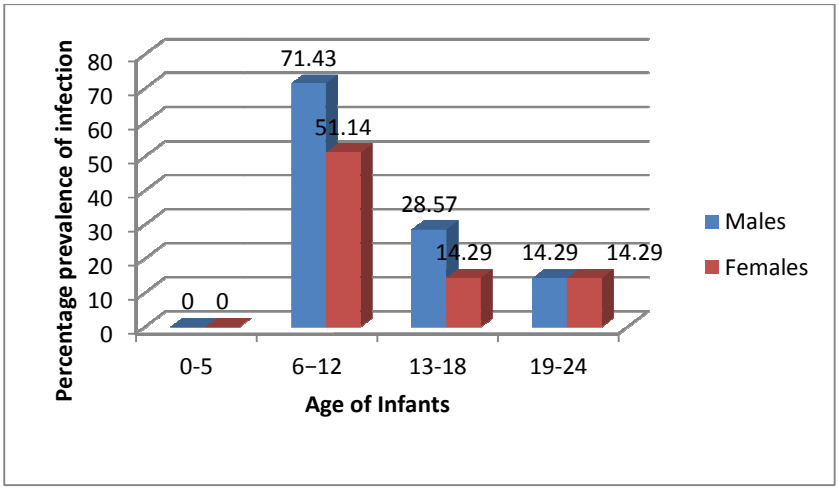

Fig. 1. Percentage prevalence of *E. coli* O157:H7 infection according to age and sex of infants

Fig. 1 depicts the percentage of the prevalence of *E. coli* O157:H7 observed according to the age and sex of the infants tested. Infants 6 to 12 months had the highest occurrence of the bacteria with males having a higher prevalence of 71.43% and females 51.14%.

Except for the agglutination reaction of *E. coli* O157:H7, other biochemical test results were the same for all *E. coli* isolated based on the biochemical and serological test conducted for the 14 bacterial isolates.

4. DISCUSSION

E. coli O157:H7 has been identified as an important cause of infants and young children diarrhea in developing countries, and diarrhea is the leading cause of morbidity and mortality worldwide, especially in tropical countries where it is responsible for more than half the deaths of children under 2 years of age [20,21]. A total 0f 150 stool samples were examined in this study. A frequency of 14 (9.33%) positive *E. coli* O157:H7 in infants have been established (Table 1). Previous study showed a lower prevalence rate of 26% [22] for children with diarrhea who attended Jos University Teaching Hospital in Nigeria. This difference may be attributed to the number of children who had exclusive breast feeding in both studies. Only 6.0% of mothers in the Jos Teaching Hospital Study exclusively breast fed compared to the Forty nine (32.69%) of mothers in the present study. It has also been reported that milk (colostrums) of mothers living in *E. coli* O157:H7 endemic areas contain high levels of Ig A antibody against *E. coli* O157:H7 [12,21].

The frequency of positive *E. coli* O157:H7 was found to be higher among subjects in the age group 6-12 months (64.29%). At this age, children have started being ambulatory and show geophagic tendencies and putting their hands and other contaminated items in their mouths. In contrast, there were no isolates from infants younger than 6 months of age, probably because of the fewer tendencies to put contaminated objects into their mouths. These findings are similar to the reports published by other studies that observed that the highest incidence of gastroenteritis in children was found within the 7 – 12 months age group, when weaning practices begin in many parts of the world [22,23,24,25].

The results obtained in this study has shown that children below 6 month of age had exclusive breast feeding, while children 6 – 12 months may have had breast and mixed feeding, or had stopped breast feeding completely. This study is therefore in agreement with previous studies that highlight the protective role of breast milk against *E. coli* O157:H7 [6,12].

It is also on record that poor hygiene during food preparation and faulty weaning practices may also contribute to increase *E. coli* O157:H7 amongst 6–12 months children. Similarly, low *E. coli* O157:H7 isolation rate in children of over 12 months may be attributed to the development of immunity [6,12].

The incidence of *E. coli* O157:H7 in females (11.86%) was found to be slightly higher than in males (7.70%). This finding is not in agreement with earlier studies of [26] 1994 in Lagos, 1991 in Ondo State [27] and 2012 in Abia State [23], where they observed higher prevalence in males than females. However, the difference was not statistically significant. The slight difference may be because most female infants are nursed by mothers who are not well educated and of low socioeconomic status and do not practice good weaning methods and equally are poor in personal hygiene or that females portend to be generally more active than males and more likely to put particles from the ground in their mouths [28].

Of the stool samples analyzed, a higher number of *E. coli* O157:H7 was Isolated from watery stools 6(24.00%) and 6(19.36%) from watery/muciod stools, than from other forms of stool sampled. However, no isolate was recovered from infants with loose stool. This study agrees with previous study which state that acute watery diarrhea is a common clinical feature in diarrhea due to *E. coli* O157:H7 [28]. However, no bloody stools were sampled. The difference however was not found to be statistically significant.

It has been alluded to that mothers' level of illiteracy may be a predisposing factor that contributes to the potentials of *E. coli* O157:H7 infection among infants and young children [6]. In this study, it is revealed that infants whose mothers had above secondary education had the lowest infection rate of 2 (2.82%), followed by those with secondary education, 4 (8.16%) and primary education, 8 (28.28%), though the differences was not found to be statistically significant. The insignificant difference may be

due to the unequal participation of infants whose mothers are uneducated.

The in-vitro antibiotic susceptibility profile of the isolates showed that most of the isolates were sensitive to amoxacillin, peprofloxacin and gentamycin. High sensitivity rates was also recorded for ciproflaxacin and ampliclox. These antibiotics are expensive and may have adverse side effects.

Diarrhea due to *E. coli* O157:H7 has been shown to be self –limiting and oral rehydration therapy seem to be a most effective treatment approach [29]. Criticism on the grounds of drug toxicity and the apparent increase of antimicrobial resistance has made the use of antibiotics of very little importance [11,30]. Equally, it has not yet been proven scientifically that in-vitro high rates of sensitivity can equally be the same in-vivo since environmental conditions are not the same [11]. High rates of resistance were recorded for erythromycin (100%), streptomycin and septrin 2 (14.29%) each, and zinacef and rocephin 4 (28.57%). Bacterial resistance to these antibacterials may be because they are cheap and easily obtained over the counter OTC resulting in drug abuse and plasmid-mediated antibiotic resistance commonly found among *E. coli* strains [31]. The antibiotic findings in this study is in agreement with previous studies by other researchers in and outside Nigeria [31,23, 28,29].

5. CONCLUSION

In conclusion, oral rehydration therapy and electrolytes replacement should be standard treatment approach in the prevention and correction of rapid dehydration following *E coli* O157:H7 diarrhea. Susceptibility of *E. coli* O157:H7 and side effects of the drugs should be considered before embarking on antibacterial therapy. Government control of over-the-counter purchase of antibiotics should be strengthened to prevent antibacterial abuse/misuse.

Nursing mothers have to be educated on the importance of good personal hygiene and the need to breast-feed infants for at least 12 months for the child to have the maximum protective function that breast milk will provide against bacterial pathogens in the first year of life. Center for disease control and prevention (CDC), the public health and regulatory officials and the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) recently investigated a multistate outbreak of products from Adams Farm Slaughterhouse. The products were recalled after eleven people were infected with the outbreak strain STEC O157:H7 in the five states. Seven people were hospitalized while one person developed hemolytic uremic syndrome. No deaths were reported. Forty-five percent of ill people were female.

There has been several outbreaks of Shiga toxin-producing *Escherichia coli* O157:H7 (STEC O157:H7) infections in five states of America, suspected to have come from beef, veal, and bison and flour in many24 States with 63 reported cases and 17 hospitalizations. There were no deaths.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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