Detection of Enteropathogenic *Escherichia coli* among Children under Five Years with Diarrhoea in Kano, Nigeria

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Abstract: Enteropathogenic *Escherichia coli* (EPEC), is among the most important pathogens infecting children worldwide and one of the main causes of diarrhoea. EPEC infection is often under diagnosed during routine microbiological analysis, especially in resource constrained settings and therefore the use of serological and molecular test could help to determine the distribution of EPEC and its clinical significance. The study was carried out to investigate the occurrence of EPEC as a cause of diarrhoea in children younger than 5years of age and to detect their virulence genes. During the study period, a total of 280 faecal specimen from children with diarrhoea and 20 from healthy children were collected and screened for *E.coli* using biochemical tests. The confirmed *E. coli* isolates were serologically tested with EPEC polyvalent and monovalent antisera to detect EPEC serotypes. The EPEC serotypes were screened for the presence of the attaching and effacing (*eae*A) and bundle- forming pilus (b*fp*A), gene by PCR assay. Furthermore, antimicrobial susceptibility pattern of the EPEC serotypes were determined by Kirby Bauer disc diffusion method. The isolates were also screened for Extended Spectrum βeta-lactamase (ESBL) production by double disc synergy. The study revealed that EPEC was detected in 19 (6.7%) of the test samples but negative in the control group. The EPEC serotype O55: K59 (B5), had the highest frequency of occurrence. The b*fp*A and *eae*A genes were detected in 31.6% and 15.7% of the EPEC isolates respectively. Typical EPEC (*eae*A+, b*fp*A+) was detected in one isolate, while atypical EPEC was detected in seven isolates. The antimicrobial susceptibility revealed that EPEC isolates were highly resistant to ampicillin (100%), tetracycline (84.2%), and trimethoprim (89.4%) but were sensitive to ciprofloxacin (95%), ceftriaxone (84.2%), ceftazidime (79.0%) and amoxicillin-clavulanate (79.0%). Three (3) isolates were found to produce ESBL. The investigation including the use of serotyping and molecular technique, are necessary to allow precise identification and epidemiological study of these pathogens. Multidrug resistant EPEC can be associated with infantile diarrhoea.

Keywords- Enteropathogenic *E.coli*, Typical EPEC, Atypical EPEC, Bundle- forming pilus, Intimin, Diarrhoea

INTRODUCTION

Globally, diarrhoeal diseases are a public health problem causing considerable morbidity and mortality among children under five years of age especially in the developing countries (Okeke *et al*., 2000). During the period from 1950 to 1970s, it was estimated that 4.6 million children died annually from diarrhea in developing world. Mortality due to diarrheoa declined to approximately 3.3 million annually in the 1980s (Moyo *et al*., 2007). In 2017, it has been estimated that diarrhea accounts for approximately 8% of all deaths among children under 5 years of age worldwide. This translates to over 1,300 young children dying each day, or about 480,000 children a year, despite the availability of a simple treatment solution (UNICEF, 2020). According to the Kano Multiple Indicator Cluster Study (KMICS,2018), one in five Kano children die before the age of five years.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of diarrhoea among infants in developing countries (Al-Hilali and Almohana, 2011; O'Ryan *et al*., 2005; Clarke, 2001). The EPEC pathogenesis is based on an intimate adherence of bacteria to the intestinal epithelium cells, leading to the development of lesions called "attaching and effacing" (A/E) lesions (Mohammadzadeh *et al*., 2013; Chen and Frankel, 2005).The locus of enterocyte effacement (LEE) pathogenicity island harbors virulence genes, responsible for A/E lesions (Mohammadzadeh *et al*.,2013;Schmidt and Hensel,2004). The *eae*A gene is located in the LEE and mediates intimate adherence of EPEC to the translocated intimin receptor (Tir) (Yamamoto *et al*., 2009).

EPEC are classified into typical and atypical strains based on the presence of a plasmid called the *E.coli* adherence factor (EAF). *E. coli* strains that are *eae*+*bfp*A+ are classified as typical EPEC (tEPEC). Most of these strains belong to the classic O:H serotypes , and produce the localized adherence (LA) phenotype associated with the production of bundle-forming pili (BFP). Conversely, *E. coli* strains that are *eae*+*bfp*A− are classified as atypical EPEC (aEPEC) (Afset*et al*., 2004)

E.coli is often detected among diarrhoeic children in Kano, Nigeria, but its serological and molecular parameters are not routinely evaluated and hence the rate of diarrhoea due to EPEC remains undefined. This study investigated the prevalence of EPEC as a cause of infectious diarrhea in children under 5years in Kano, along with their antibiotic susceptibility pattern to provide baseline data for epidemiological control.

METERIALS AND METHODS

Study Design

This cross sectional study was carried out among children under five years of age with diarrhoea attending Hasiya Bayero Paediatric Hospital, Kano to establish the prevalence of typical and atypical Enteropathogenic *E. coli* strains and their antimicrobial susceptibility profile. Diarrhoea case was defined as three or more liquid or semi- liquid stools defecation per day. Healthy children who had no diarrhoea complain during the previous month were selected as the control group.

Ethical Approval and Informed Consent

This study was approved by Kano State Ministry of Health (Ref: MOH/Off/797/T.I/1194) prior to commencement. The ethics sub- committee of the ministry; operational research advisory committee approved the use of both verbal informed consent for children with illiterate parent and written informed consent for children with literate parent.

Sample Size Determination

Sample size was determined using the formula by Daniel (1999) shown below to determine an adequate sample size and estimate the population prevalence with a good precision.

$$n = \frac{Z^{2\times} P (1-P)}{d^2}$$

Where n= required sample size

Z= confidence interval for 95% CI is 1.96

P= 15% prevalence obtained from a study in Gwagwalada, Abuja (Adebola *et al*., 2014)

d= margin of error at 5% i.e. 0.05

$$n = \frac{(1.96)^2 \times 0.15 \times (1- 0.15)}{(0.05)^2}$$

n= 200

Questionnaire

Information on Socio-demographic factors, date of symptoms onset, diarrhoea type and symptoms were obtained using a structured questionnaire.

Sample Collection

Fecal samples were obtained as rectal swabs or stool from each of the children in the study. Stool samples were collected from the patients in clear, transparent, sterilized wide mouthed bottle. In the case of rectal swab, a cotton swab was inserted in the rectum, rotated gently and removed. The fecal specimen was transported to the laboratory in sterilized Cary Blair transport medium for the culture and isolation of *E.coli* (Lautenbach *et al*., 2005).

Laboratory Analysis

Isolation and Identification of Bacterial Isolate

All stool specimens were cultured on MacConkey agar and the plates were incubated at 37^{o}C for 24hrs. Pink colonies MacConkey agar were then sub-cultured on Eosine Methylene Blue agar at 37^{o}C for 24hrs to observe the Green metallic sheen. The *E. coli* suspected colonies were further subjected to biochemical tests.

Identification of EPEC by Serology

The isolates biochemically identified as *E. coli* were serotyped by slide agglutination test kit (Guangdong Huankai Microbial Sci. & Tech. Co., ltd., China) according to manufacturer's protocols to detect the EPEC serotypes.

Colonies were first tested with polyvalent O antisera for EPEC separately and positive isolates were subcultured and retested using the following EPEC monovalent O;K antisera: (O26:K60(B6), O55: K59(B5), O111: K58(B4), O119: K69(B14), O126: K71(B16), O86: K61(B7), 0114: K90(B), O125: K70(15), O127: K63(B8), O128: K67(B12), O44: K74(L), 0112: K66(B11), 0124: K72(B17), O142: K86(B), O18c: K77(B21).

Molecular Identification of EPEC

The Enteropathogenic *E. coli* strains were further subjected to Polymerase Chain Reaction (PCR) to detect the presence of virulence genes (*eaeA* and *bfpA*) known to cause infantile diarrhea using a specific primer against each gene (Table 1).

DNA Isolation (Extraction)

DNA from each confirmed EPEC isolate was extracted according to the protocol of Norgen Biotek Kit.

Polymerase Chain Reaction Assay

After *E. coli* DNA extraction, the templates were subjected to polymerase chain reaction for detection of virulence genes using two sets of (Forward and Reverse) primers (Inqaba Biotec West Africa Ltd) as listed in Table 1

Table 1 - Primers, Genes and Cycling Procedures

Category	Target	Sequence(5'-3')	Annealing temp(^{o}C)	No of cycles	Amplicon size(bp)	Reference
EPEC	*eaeA*	F,CATTATGGAACGGCAGAGGT R,ATCTTCTGCGTACTGCGTTCA	55(1 min)	35	790	Beaudry *et al.* (1996)
EPEC	*bfpA*	F,AATGGTGCTTGCGCTTGCTCG R,GCCGCTTTATCCAACCTGGTA	60 (1 min)	30	326	Gunzburg *et al.* (1995)

The primers for the virulence genes shown in Table 1 were diluted based on manufacturer's instruction. PCR was performed using reaction mixture of 50µL containing 25µL 2X master mix, 21µL water, 2µl primer and 2µL DNA template. Amplification reactions were performed in a thermal cycler and for all amplification reactions; the mixture was heated at 94^{o}C for 5 min prior to thermocycling. The mixture was held at 57^{o}C for 7 min after the final cycle before cooling at 4^{o}C.

Agarose Gel Electrophoresis

The PCR products were evaluated on a 1.5% (w/v) Agarose gel at 100 mV for 60 minutes and a molecular weight marker was run concurrently. The DNA bands were visualized and photographed under UV light (Hedge *et al*., 2012; Vidal, 2004)

Antimicrobial Susceptibility Testing

Susceptibility of isolated EPEC serotypes to different antibiotics was determined by Kirby- Bauer disc diffusion technique as specified by the Clinical and Laboratory Standard Institute (CLSI, 2017). The antibiotic discs (All Oxoid U*K*) used in this study were amoxicillin- clavulanate (20/10µg), ceftriazone (30µg), ceftazidime (30µg), gentamycin (10µg), Tetracycline (30µg), ciprofloxacin (5µg), ampicillin (10µg), trimethoprim (5µg).

A sterile wire loop was used to transfer 3 colonies of EPEC isolates to a srew- capped tube containing 4mL of normal saline and turbidity was adjusted to 0.5 McFarland turbidity standards. Within 15minutes after standardization of the inocula, a sterile cotton swab was immersed into bacterial suspension. The swab was then streaked evenly on the surface of the plate in three different planes by rotating the plate to get a uniform distribution of the inoculum. The inoculum was allowed to dry for 15minutes at room temperature with lid closed.The antibiotics were then placed on the inoculated plate using a sterile forcep 10-15mm away from the edge of the petridish and 24mm gap between the discs.

The plates were incubated at 37^{o}C for 24hours.After incubation, each plate was examined, and the diameter of complete inhibition zone was measured with a ruler. Measurement of diameter in millimeter was made in two directions at right angle to each other through the centre of each disc and the average of the two readings was taken. The zone of inhibition in growth produced by each antimicrobial agent on the test organisms were categorized into Susceptible (S comprising intermediate), and Resistant (R) (CLSI, 2017)

Phenotypic Screening of Extended Spectrum β-Lactamases

EPEC isolates were further tested for Extended-Spectrum βeta lactamase (ESBL) production by the double disk diffusion (DDD) test using ceftriaxone (30µg) and ceftazidime discs (30µg) with amoxicillin-clavulanate (*Oxoid, UK*). A positive test for ESBL was indicated by formation of zone of inhibition towards clavulanic acid disc (CLSI, 2012).

Statistical Analysis

The possible association between socio-demographic factors and clinical symptoms of infected subjects was assessed using chi-square tests and implemented using SPSS version 20. A P- value of < 0.05 was considered to be statistically significant.

RESULTS

Isolation and Identification of *Escherichia coli*

In total, 88 (31.4%) isolates were recovered as pure isolates of *E. coli* out of the 280 diarrhoea samples screened (Fig. 1).

Identification of Enteropathogenic *Escherichia coli* (EPEC) by Serotyping

Of the 88 *E.coli* isolated, 19 EPEC serotypes belonging to classic EPEC serogroups were identified. The O55: K59 (31.5%) serotype had the highest frequency of occurrence, followed by O44: K74 with a prevalence of 21%, 0114: K90 having 15.8% and O128: K67 with 10.5%. Other serogroups obtained were one each (Table 2).

Identification of EPEC Virulence Gene by Polymerase Chain Reaction (PCR)

The 19 isolates that were positive to the EPEC antisera were genotyped to detect the presence of the EPEC virulence genes. Three 3 (15.7%) isolateswere positive for the *eae*A gene, 6 (31.6%) were positive for the b*fp*A gene respectively (Table 2).

Antimicrobial Susceptibility Testing

A total of 19 EPEC strains were tested for antimicrobial susceptibility pattern as shown in table 3. The highest sensitivity was observed against Ciprofloxacin 18 (95%), followed by ceftriaxone 16 (84%) and ceftazidime 15 (79%). Isolated EPEC strains were highly resistant to ampicillin 19 (100%), trimethoprim 17 (89%) and tetracycline 16 (84.2%). Multidrug resistance was observed in 5 (26.3%) isolates.

Detection of Extended Spectrum β-Lactamase Producing EPEC

Figure 2shows the distribution of ESBL producers. Three (3) of the EPEC isolates were found to display resistance pattern of ESBL producers.

Statistical Analysis

Table 4 below shows the level of association between socio- demographic factors and clinical symptoms of infected subjects indicated by Chi- square (χ^2) and level of significance (P-value). The present result reveals that diarrhoea caused by EPEC is statistically associated with age with the majority of cases occurring in children between 1 – 12 months of age group (χ^2= 20.236df= 4 (P< 0.05) P= 0.0004). The distribution of EPEC was higher in male (8.0%, P= 0.33) than in female (5.0%). There was no statistical significant association between EPEC infection and duration of diarrhoea, symptoms and diarrhoea type as a result of P- value greater than 0.05.

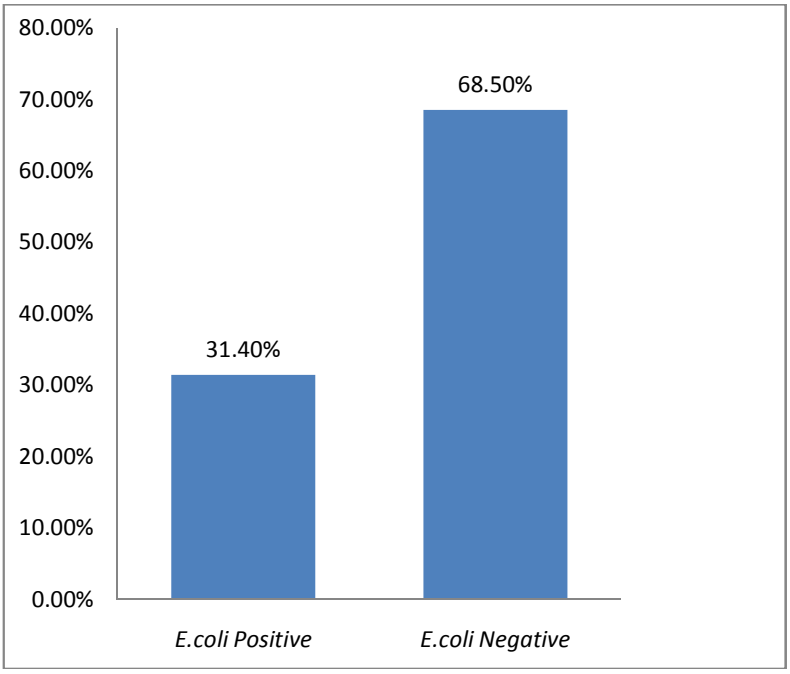

Figure 1 – Distribution of *Escherichia coli* among the study participants.

Table 2: Serological and Molecular Characterization of Typical and Atypical EPEC Serotypes

Isolate no	EPEC serotypes		PCR results		Virulence
	Polyvalent	Monovalent	*eae*A	b*fp*A	category
E1	2	O55:K59(B5)	+	+	Typical EPEC
E2	3	O44: K74(L)	-	+	Atypical EPEC
E3	2	O125: K70(B15)	-	-	Negative
E4	2	O114: K90(B)	-	-	Negative
E5	2	O55:K59(B5)	-	+	Atypical EPEC
E6	2	O114: K90(B)	-	+	Atypical EPEC
E7	3	O44: K74(L)	-	-	Negative
E8	1	O86: K61(B7)	-	-	Negative
E9	2	O55:K59(B5)	-	+	Atypical EPEC
E10	1	O128: K67(B12)	-	+	Atypical EPEC
E11	2	O55:K59(B5)	-	-	Negative
E12	3	O44: K74(L)	-	-	Negative
E13	1	O128: K67(B12)	+	-	Atypical EPEC
E14	3	O44: K74(L)	-	-	Negative
E15	3	O126: K71(B16)	-	-	Negative
E16	2	O111: K58 (B4)	-	-	Negative
E17	2	O114: K90(B)	+	-	Atypical EPEC
E18	2	O55:K59(B5)	-	-	Negative
E19	2	O55:K59(B5)	-	-	Negative

Key- *eae*A- attaching and effacing gene; b*fp*A- bundle forming pilus gene

Table 3- Summary of Antibiotic Susceptibility Profile of EPEC Strains

Antimicrobial agent	Susceptibility	
	No (%) S	No (%) R
Ampicillin	0 (0.0)	19 (100)
Amoxicillin- clavulanate	14 (73.6)	5 (26.3)
Ceftriaxone	16 (84.2)	3 (16.0)
Gentamycin	13 (68.4)	6 (32.0)
Tetracycline	3 (16.0)	16 (84.2)
Ciprofloxacin	18 (95.0)	1 (5.3)
Ceftazidime	15 (79.0)	4 (21.0)
Trimethoprim	2 (11)	17 (89.4)

Key- S- sensitive R- resistant

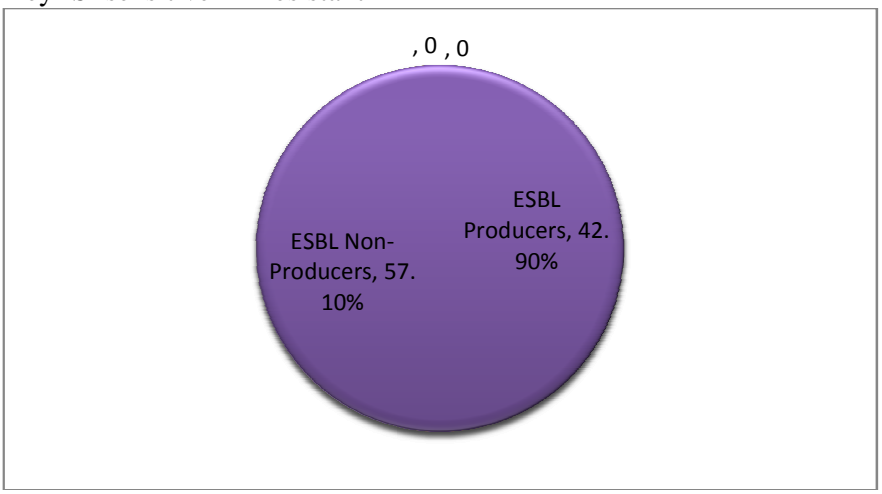

Figure 2 - Distribution of EPEC strains with Extended Spectrum β – Lactamase (ESBL) Production.

Table 4- Occurrence of EPEC Based on Socio-Demographic Factors and Clinical Symptoms of Infected Subjects.

Features	No. tested	No. (%) positive cases	χ^2	P- value
Age (months)				
1-12	130	14 (10.8)	20.326	0.0004
13- 24	102	4 (3.9)		
25- 36	25	1 (4.0)		
37- 48	17	0 (0.0)		
49- 60	6	0 (0.0)		
Sex				
Male	162	13 (8.0)	0.936	0.33
Female	118	6 (5.0)		
Symptoms				
Fever	127	3 (2.4)	1.498	0.47
Vomiting	186	7 (3.8)		
Abdominal pain	29	2 (6.9)		
Diarrhoea type				
Watery	206	15 (7.3)	2.149	0.54
Mucoid	48	4 (8.3)		
Bloody	8	0 (0.0)		
Loose	18	0 (0.0)		
Duration of diarrhea (days)				
1- 2	192	11 (5.7)	2.002	0.37
3- 4	56	4 (7.1)		
>5	32	4 (12.5)		

DISCUSSION

Enteropathogenic *E.coli* has been identified as an important cause of infantile diarrhea in developing countries, but the incidence has varied greatly in different studies. In this study, the overall prevalence of EPEC was 6.7% which is lower compared to other studies in Nigeria and outside Nigeria. Bukar *et al*. (2014) reported 15% in Maiduguri, Adebola *et al.* (2014) reported 15% in Abuja, Ome and Nonye reported 19.5% in Aba respectively. These differences may be due to changes in the distribution of this pathotype from region to region and within countries in the same region. The study is in agreement with a previous study conducted in Egypt (5.2%) (Behiry *et al*., 2011).

The WHO has considered isolates in the 12 O serogroups to be EPEC strains; 026, O55, 086, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Trabulsi *et al*., 2002). However, the serological investigation revealed 8 serogroups of the 12 *E.coli* serogroups described by WHO as EPEC strains. It was found that 055: K59 (B5) was the most common EPEC isolates in this study. This is in agreement with the previous study by Alikhani *et al*. (2006) and Al-Hilali and Al- Mohana (2011) in Iraq where O55 serogroup was the most prevalent.

The b*fp*A gene was detected in 31.6% of the EPEC isolates screened. The occurrence of b*fp*A in the present study was analogous to that recorded by other studies such as Alikhani *et al*. (2006) who found that 31.5% strains in Iran gave positive results for the b*fp*A gene. In South Africa, Obi *et al*. (2004) showed that the b*fp*A gene coding for EPEC was most frequently detected (22.7%) among EPEC isolates.

In this study, atypical EPEC is the most frequently isolated serotype which is in consonance with the recent findings in developing and developed countries (Nguyen *et al*., 2005) Remarkably, these findings differ from previous reports by Nataro *et al*. (1998) and Ratchtrachenchia *et al*. (2004) which suggested that the prevalence of atypical EPEC is limited to industrialized countries. Even though the etiologic role of this sub- pathotype of EPEC in diarrhoea has not been definitively established, this finding underscores the emergence of atypical EPEC worldwide.

The use of antibiotics to treat EPEC is not routinely recommended; however understanding the antibiotic susceptibility of these pathogens is important as intestinal *E .coli* strains may serve as a reservoir of antibiotic resistance genes (Imdad *et al*., 2018). In addition, antimicrobial therapy may be indicated in children with diarrhoea due to EPEC once identified, and in children with persistent diarrhea. The susceptibility test results showed that 95% of the isolates were resistant to at least one antibiotic while 39% of the isolates exhibited resistance to at least three antibiotics with first panel antibiotics showing higher resistance than reserve antibiotics. Highest resistance was detected against Ampicillin (100%), followed by trimethoprim (89.4%) and tetracycline (84.2%). The findings could be attributed to the fact that these antibiotics are relatively cheap and can be illegally acquired over the counter without prescription (Emacalar *et al*., 2011). High resistance against these antibiotics is in line with a study carried out in Yola by Abdullahi *et al*. (2018). A study carried out by Sang *et al*. (2012) reported high level resistance to ampicillin (95.0%), trimethoprim (95.0%) and tetracycline (81.0%).

ESBLs producing isolates have hydrolytic and inactivating effect on Oxyimino-aminothiazolylcephalosporins with exception of cefoxitin leading to their resistance (Odonkor and Addo, 2011; Kiiru*et al*., 2012). These plasmid mediated enzymes are caused by mutations of TEM-1 & TEM-2 (Temoniera enzyme) and SHV-1 (sulfhdryl variable enzyme) (Odonkor and Addo, 2012) and are commonly found in Enterobacteriaceae family. In this study, 3 (15.7%) of the EPEC strains were found to produce ESBL.

The prevalence of EPEC strains was dominant in children 1 – 12months of age in this study which conforms to the report that EPEC infection is primarily a disease of infants younger than two years of age (Vilchez *et al*., 2009). It is also in agreement with the previous study of Farfan- Garcia *et al*. (2017), Tian *et al*. (2016) and Shamki *et al*. (2014) with higher number of subjects from age group 0- 12months. The reason for the high incidence of isolates in this age group could be due to the fact that children within this age group on their own cannot differentiate between what to eat and what not to eat, they have not learnt the rudiment of adherence to aseptic or hygienic practice. The study revealed that 13 (8.0%) of EPEC infected children were males and 6 (5.0%) were females. These findings were similar to the study of Tian *et al*. (2016) (58.1% male and 41.9% female), Haghi *et al*. (2014) (60.7% male and 37% female) and Natarajan *et al*. (2018) (63% male and 37% female) who had more male subjects than female. However this observation is at variance with the study of Farfan- Garcia *et al*. (2017), Ifeanyi *et al*. (2015) and Shamki *et al.* (2014) with 55.6%, 55.0% and 51.3% female dominance in their respective reports. The reason for higher number of male subjects cannot be established but Kano Multiple Indicator Cluster Survey (KMICS, 2018) reported that diarrhoea is more common in male than in female children in Kano. Most of the subjects had mucoid type diarrhea which is in agreement with a previous study in Abuja, Nigeria by Ifeanyi *et al*. (2015) which may be due to the quality of water used at home. In addition to the clinical state of diarrhoea among the subjects, other clinical symptom that was predominant among the subjects was vomiting which accounts for 7 (3.8%). This findings is in contrast with KMICS (2018) who reported high rate of fever among the diarrhoeic children.

CONCLUSION

The current observations showed that EPEC is a causative agent of childhood diarrhoea among children under 5 years in the study area with a prevalence rate of 6.7%. Age group 1- 12months (10.8%) were found to be the most frequently affected. The 055: K59 (B5) serotype was the most predominant EPEC serotype with 31.5% prevalence rate. Atypical EPEC was more prevalent (36.8%) compared to typical EPEC (5.2%).The EPEC isolates were highly resistant to ampicillin (100%) and trimethoprim(89.4%). The susceptibility result also suggests that ciprofloxacin (95.0%) is the most effective drug against the isolates.

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