Original Research Article

Molecular Detection of Extended Spectrum and AmpCβ-Lactamase Resistance in *Escherichia coli* Isolated from Diarrheic Children in Lafia, Nasarawa State, Nigeria

UNDER PEER REVIEW

ABSTRACT

Aims: This study evaluated the presence of extended spectrum β-lactamase (ESBL) and AmpC β-lactamase resistance genes in *E. coli* from stool of diarrheic children in some hospitals in Lafia metropolis, Nigeria.

Methodology: A total of 70 stools sample of children were obtained from Dalhatu Araf Specialist Hospital, Lafia, M & D Hospital, Olivet Medical Centre and Sandaji Medical Centre. *Escherichia coli* were isolated and identified using standard microbiological methods. Antimicrobial susceptibility of the isolates was tested using Clinical and Laboratory Standards Institute (CLSI) method. The phenotypic detection of ESBL and AmpC β-lactamase production in some antibiotic resistant isolates were carried out using disc method. The molecular detections of ESBL and AmpC resistance genes were carried out using Polymerase Chain reaction (PCR) method.

Results: Of the 70 samples, the occurrence of *E. coli* was 100%. The isolates were highly resistant to ampicillin (97.14%), ciprofloxacin (90.00%), sulfamethoxazole/trimethoprim (84.29%), streptomycin (78.57%), amoxicillin/clavulanic acid (70.00%); moderate to gentamicin (38.57%), ceftazidime (37.14%) and cefotaxime (30.00%); and less resistant to cefoxitin (15.71%) and imipenem (8.57%). Twenty-one (30.00%) isolates were jointly resistant to both cefotaxime and ceftazidine. Of this number, 66.67% (14/21) were phenotypically confirmed ESBL producers; and the occurrences of ESBL resistance genes were: 7.14% (*SHV*), 42.86% (*CTX-M*) and 50.00% (*TEM*). Out of 11isolates resistant to cefoxitin, 4(36.36%) were phenotypically confirmed as AmpC β-lactamase producers; and the occurrence of AmpC genes were: 50.00% (*CIT*), 25.00% (*FOX*) and 25.00% (*MOX*).

Conclusion: The isolates were least resistant to imipenem and cefoxitin and highly resistant to ampicillin, ciprofloxacin and sulfamethoxazole/trimethoprim. *TEM and CTX-M* ESBL genes were more frequent than *SHV*. *CIT* AmpC gene was more frequent than *FOX* and *MOX*.

Keywords: Escherichia coli, Extended spectrum, AmpC, β-lactamase, stool, diarrhea, children

1. INTRODUCTION

Diarrhea, defined by the World Health Organization (WHO), as "the passage of three or more loose or liquid stools per day, or more frequently than is normal for the individual" [1], has been reported to be a leading cause of morbidity and mortality in children [2]. *Escherichia coli* (*E. coli*) is a major cause of diarrheal disease particularly in developing countries [2], [3].

Treatment of bacterial diarrhea has employed antibiotics [4], [5]. However, the use of antibiotics has been reported to be one of the factors contributing to the emergence of bacterial resistance, a global health challenge [1], [5], [6].The continuous emergence, development and spread of pathogenic organisms that are resistant to antibiotics are a cause of increasing concern to health care practice [1]. Beta-lactam antibiotics are antibiotics that have a beta-lactam ring nucleus as part of their molecular structure, and typically include penicillinderivatives(penams), cephalosporins (cephems), monobactams, and carbapenems [7], they are the most widely used class of drugs for the treatment of bacterial infections and have been prescribed for over 70 years [8], [9], [10], [11], [12].
Resistance mechanisms in bacteria against β-lactam antibiotics include: β-lactamase production and alteration of the penicillin-binding protein (PBP) target site [13], [14]. The production of β-lactamases, which hydrolyzes the β-lactam ring, is among the most frequently encountered mechanisms in *E. coli* [15].
Extended-spectrum β-lactamases (ESBLs) are a rapidly evolving group of β-lactamases which share the ability to hydrolyze third-generation cephalosporins and aztreonam. The ESBLs have contributed to the dramatic increase in resistance to new generation beta-lactam agents throughout the world [11]. These enzymes are usually plasmid-encoded and have the capacity to hydrolyze many antibiotics including penicillins, cephalosporins, and aztreonam and are inhibited by clavulanic acid [16]. Extended-spectrum β-lactamases are frequently encoded by genes located on different transferable genetic elements, a variety of epidemiological situations have been documented, ranging from sporadic cases to large outbreaks [17]. Moreover, ESBL-producing strains are often resistant to antibiotics of other classes (sulfonamides, aminoglycosides, quinolones) which complicates the treatment strategies in many hospitalized patients [5], [18].

Amp-C beta-lactamases are bacterial enzymes that hydrolyze third-generation extended spectrum cephalosporins and cephamycins engendering resistance to these categories of antibiotics [19]. Thus, the onslaught of AmpC resistance represents a major challenge for clinicians as it renders third-generation cephalosporins increasingly ineffective [20].

Several studies worldwide have reported on ESBL resistance in *E. coli* from stool of diarrheic children [5], [14], [21], [22], [23], [24], [25], [26], [27], [28] [29]. There are few reports in Nigeria on ESBL production in *E. coli* isolated from stool of diarrheic patients [30], [31], [32], [33], [34]. However, no report is known to us on ESBL and AmpC β-lactamase resistance in *E. coli* from diarrheic patients of any age group in the study area. This study thus investigated the antibiotic resistance profile and molecular basis ESBL and AmpC β-lactamase resistance in *E. coli* from diarrheic children accessing selected hospitals in Lafia, Nigeria.

2. MATERIAL AND METHODS

2.1 Antibiotic Discs

The antibiotic discs (potency) used in this study were from Oxoid Ltd (U.K.) and include: Amoxicillin (AMX: 10 µg), Amoxicillin-Clavulanic acid (AMC: 30 µg), Cefotaxime (CTX: 30 µg), Ceftazidime (CAZ: 30 µg), Imipenem (IPM: 30 µg), Ciprofloxacin (CIP: 5 µg), Co-trimoxazole (SXT: 25 µg), Gentamicin (CN: 10 µg), Streptomycin (S: 10 µg) and Cefoxitin (FOX: 30µg).

2.2 Equipment

The equipment used for this study include: Autoclave (Certoclav, Model SM280E, Surgifriend Medicals, England), Gel electrophoresis machine (Max Fill Scie-plas Model HU10 serial no 5237), Laminar air flow cabinet (PCR-8 re-circulating laminar flow pre station, Labcaire product 220/240v), Microscope (Model CME 1349522X, Leica, USA),

UNDER PEER REVIEW

Spectrophotometer (Eppendorf Biophotometer 8.5mm, Lichtstrahihohe), UV illuminator (Vilberb Lourmat TFX-35-M serial no NoV02 8104), Centrifuge (Model 5417R: Lab-line Instrument Inc USA), Microwave oven (HINARI Life Style 800watts model MX310TCSL), Oven (Hotbox Size One, Galengkamp, U.K.), Incubator (Model 12-140E, Quincy Lab Inc), Refrigerator/Freezer (Model PRN 1313 HCA, BEKO, Germany), Thermocycler (Model TC-312, Techne, England), Electronic weighing balance (Model QT 600: Lab-line Instrument Inc USA),Vortex machine (Touch plate Super Mixer, CAT No 1291, Lab-line Instrument Inc USA), and GelDoc system (Biorad, U.K.).

2.3 Chemicals and Reagents

The reagents and chemicals used were: acridine orange, carbol fuschin, crystal violet, ethanol, xylene, creatinine, pottasium hydroxide and Kovac's reagents, obtained from BDH chemical Ltd, England; ethydium bromide, iodine solution, EDTA and Glycerol, obtained from Sigma chemical Ltd, England; and agarose gel from Schwarz/ Mann Biotech., Germany.

2.4 Primers

The primers used in this study are as given in the Table 1.

Table1: Primers and expected amplicon size for each gene

S/N	Target genes	Sequence	Amplicon size	References
1	*bla*$_{TEM}$	5´-TCGGGGAAATGTGCGCG-3´ 5´-TGCTTAATCAGTGAGGCACC-3´	972	[15]
2	*bla*$_{SHV}$	5´-GGGTTATTCTTATTTGTCGC-3´ 5´-TTAGCGTTGCCAGTGCTC-3	615	[15]
3	*bla*$_{CTX-M}$	5´-ACGCTGTTGTTAGGAAGTG-3´ 5´-TTGAGGCTGGGTGAAGT-3´	857	[15]
4	*MOX-M (F)* *MOX-M (R)*	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520	[35]
5	*CIT-M (F)* *CIT-M (R)*	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462	[35]
6	*FOX-M (F)* *FOX-M (R)*	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190	[35]

F = Forward; R = Reverse

2.5 Study Location

This study was carried in some selected hospitals namely: Dalhatu Araf Specialist Hospital, Olivet Medical Center, Sandaji Medical Center, M&D Hospital and Peace Laboratory & Diagnostic Centers within Lafia Metropolis, Nigeria.

2.6 Ethical Clearance

UNDER PEER REVIEW

The ethical approval for this study was obtained from the ethical committees of the selected hospitals.

2.7 Sample Collection

A total of seventy (70) stool samples of diarrheic patients attending the selected hospitals were collected using sterile container and transported using ice pack to Microbiology Laboratory of Dalhatu Araf Specialist Hospital, Lafia for analysis.

2.8 Isolation of *Escherichia coli*

Escherichia coli was isolated from the stool of the diarrheic children as earlier described [2]: A loopful of stool was streaked on MacConkey agar (Oxoid Ltd, U.K) and incubated at 37ºC for 24 h. A pinkish colony from MacConkey agar plates were further streaked on Eosine methylene blue agar (Oxoid Ltd, U.K) and incubated at 37ºC for 24 h. Greenish metallic sheen colonies that grew on the Eosine methylene blue agar were presumptively selected as *E. coli*.

2. 8.1 Identification of *Escherichia coli*

The presumptive *E. coli* was Gram-stained, and biochemically tested using Indole, Methyl red, Voges-Proskauer and Citrate tests (“IMViC”) to confirm its identity as *E. coli* as earlier described [36].

2.9 Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testing of the bacterial isolates was carried out as earlier described [37]. Briefly, three (3) pure colonies of the isolates were inoculated in to 5 ml sterile 0.85% (w/v) NaCl (normal saline) and the turbidity of the bacteria suspension will be adjusted to the turbidity equivalent to 0.5 McFarland’s standard. The McFarland’s standard was prepared as follows: 0.5 ml of 1.172% (w/v) $BaCl_2.2H_2O$ was added into 99.5 ml of 1% (w/v) H_2SO_4.

A sterile swab stick was soaked in standardized bacteria suspension and streaked on Mueller-Hinton agar (Oxoid Ltd, U.K) plates and the antibiotic discs were aseptically placed at the center of the plates and allowed to stand for 1 h for pre-diffusion. The plates were incubated at 37°C for 24 h. The diameter zone of inhibition in millimeter was measured and the result was interpreted in accordance with the susceptibility break point earlier described [37].

2.10 Determination of Multiple Antibiotic Resistance (MAR) Index of the isolates

The MAR index of the isolates was determined using the formula [6]:

$$\text{MAR Index} = \frac{\text{No. antibiotics isolate is resistant to}}{\text{No. of antibiotics tested}}$$

2.11 Extended Spectrum β-Lactamase Production Test

The phenotypic confirmatory test for ESBL production by isolates resistant to cefotaxime and ceftazidime was carried out using Double-Disc Synergy Test (DDST) method earlier described [38]. Briefly, 10^5 cfu/ml bacterial suspension was streaked on sterile Mueller-Hinton agar plates and amoxicillin-clavulanic acid (30 µg) disc was placed at the centre of the plate. Cefotaxime (30 µg) and ceftazidime (30 µg) discs were then placed 15 mm (edge-to-edge) from the centre disc. Enhancement of zone of inhibition in the area between the amoxicillin-clavulanic acid disc and any one of the β-lactam discs compared with the zone of inhibition on the far side of the drug disc was interpreted as indicative of the presence of an ESBL in the tested strain.

UNDER PEER REVIEW

2.12 Confirmatory Test for AmpC β-Lactamase Production

The confirmatory Test for AmpC β-Lactamase against *E. coli* isolates whose diameter zone of inhibition of cefoxitin were <18mm was carried out as follow; the swab stick was soaked in a standardized *E. coli* isolates resistance to cefoxitin suspension (10^5 cfu/ml) and streaked on MHA plates and 30 µg cefoxitin disks were placed at the centre of the plates and 30 µg cefotaxime disk was placed 20 mm away from cefoxitime disks and allowed to stand for 1 h for pre-diffusion at room temperature before they were incubated at 37°C for 24 h. A clover leaf zone of inhibition against the isolates after 24 h incubation was confirmed AmpC β-lactamase producers.

2.13 Molecular Detection of Extended Spectrum β-Lactamase and AmpC Genes

The confirmed ESBL isolates were subjected to a singleplex polymerase chain reaction to detect three (3) ESBL genes: bla_{SHV}, bla_{TEM}, $bla_{CTX\text{-}M}$ and the confirmed *AmpC* isolates were also subjected to multiplex PCR to detect three (3) genes: *MOX*, *CIT* and *FOX* genes.

2.13.1 DNA Extraction

DNA extraction was performed by boiling method as described [2]. Following purification on MacConkey agar, bacterial DNA was isolated from a 24-h culture in Luria-Bertani broth (LB broth) prepared according to the manufacturers' protocol.

The bacterial cells were harvested by centrifugation at 3200 rpm in a micro centrifuge for 2 min at room temperature and the supernatant was discarded. The pelleted cells were re-suspended in 1ml of sterile normal saline and the micro-centrifuge tubes were placed in the vortex for five seconds. Centrifugation was carried out at 3200 rpm for 1 min and the supernatant was discarded. 0.5 ml of sterile normal saline was added to the pellets and the tubes were vortexed for 5 sec after which they were heated in the block heater at 90°C for 10 min. immediately after heating, rapid cooling was done by transferring the tubes into the freezer for 10 minutes. Cell debris was removed after centrifugation was done at 3200 rpm for 1 min and 300 µl of the supernatant was transferred into a sterile 2 ml Eppendof tube as DNA and stored at -10°C until use.

Estimation of the concentration, purity and yield of the DNA sample was accessed using absorbance method (measurement of absorbance) with the spectrophotometer (Nanodrop 1000). For DNA concentration, absorbance readings were performed at 260nm (A_{260}) and the readings were observed to be within the instrument's linear range (0.1 – 1.0). DNA purity was estimated by calculating the A_{260}/A_{280} ratio and this was done by the spectrophotometer's computer software (where A_{260}/A_{280} ratio ranges from 1.7 – 1.9).

2.13.2 DNA Amplification of Extended Spectrum β-Lactamase Genes

Simplex Polymerase Chain Reaction (PCR) was performed in order to amplify the ESBL genes present in the isolates. The presence of $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} genes were tested for using previously published primer sets and conditions. The primer sequences and expected amplicon size for each gene are listed in Table 1.

The reactions were carried out in 20 µl reaction volume which was made up of 10 µl of Mastermix (Qiagen), 0.32 µl of primers (0.16 µl each of forward and reverse primers), 3 µl of DNA and 6.68 µl of nuclease free water. The primer concentration stood at 0.2 M. The reaction tubes were placed in the holes of the thermal cycler and the door of the machine was closed.

Conditions during the reactions were set as: 3 minutes of initial denaturation at 95°C, followed by 35 amplification cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 40 sec, initial extension at 72°C for 50 sec, final extension at 72°C for 3 min and a hold at 4°C infinitely.

UNDER PEER REVIEW

2.13.3 DNA Amplification of AmpC Genes

Multiplex Polymerase chain reaction (PCR) was performed in order to amplify the AmpC genes present in the isolates. The presence of *MOX*, *CIT* and *FOX* genes were tested for using previously published primer sets and conditions. The primer sequences and expected amplicon size for each gene are listed in Table 1.

The reactions were carried out in 25 µl reaction volume which was made up of 5 µl of Mastermix (Qiagen), 2.4 µl of primers (0.4 µl each of forward and reverse primers), 0.5 µl of $MgCl_2$, 1.5 µl of DNA and 15.6 µl of nuclease free water. The primer concentration stood at 0.4 M. The reaction tubes were placed in the holes of the thermal cycler and the door of the machine was closed.

Conditions during the reactions were set as: 3 min for initial denaturation at 94°C, followed by 35 amplification cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, initial extension at 72°C for 30 sec, final extension at 72°C for 7 min and a held at 4°C infinitely.

2.13.4 Agarose Gel Electrophoresis

Seven microliters of the amplified DNA were transferred into the wells of a 1.5% Agarose gel by stabbing the wells using a micropipette and this was done carefully to ensure that each well had only one sample. Each gel had one well which contained a DNA ladder (100 bp, Thermo Scientific) in order to estimate the size of the DNA amplicons. Electrophoresis was run at 125 volts for 20 min, after which the gels were viewed using ultra-violet trans-illuminator.

3. RESULTS AND DISCUSSION

3.1 Occurrence of *Escherichia coli*

The cultural, morphological and biochemical characteristics for identification of *Escherichia coli* is as shown in Table 2. The combined occurrence of *E. coli* in the study centers was 100%.

3.2 Antibiotic Resistance Profile

The antibiotic resistance profile of the isolates is as given in Table 3. The isolates were more resistance to ampicillin (97.1%), ciprofloxacin (90.0%) and sulphamethoxazole/trimethoprim (84.3%) but less resistance to cefotaxime (30.0%), cefoxitin (15.7%) and imipenem (8.6%).

3.2.1 Antibiotics Resistance Phenotypes

The various antibiotic resistance phenotypes of the isolates are as given in Table 4. The most common resistance phenotype was SXT-AMC-AMP-S-CIP with an occurrence of 10(14.3%).

3.2.2 Multiple Antibiotic Resistance (MAR) Index

The MAR index of *E. coli* isolates from stool of diarrheic children of underage attending selected hospitals in Lafia metropolis is as given in Table 5. All the *E. coli* isolates were MAR isolates MAR index of ≥0.2 and the most common MAR index were 0.4 and 0.5 and their percentage occurrence were 17.1%and 34.3% respectively as shown in Table 5.

3.2.3 Extended Spectrum β-lactamase Production

The phenotypic and genotypic confirmatory tests of ESBL production in the isolates jointly resistant to both cefotaxime and ceftazidine is as shown in Table 7. Out of 21 *E. coli* isolates jointly resistance to both ceftazidime and cefotaxime, 14 (66.7%) of the *E. coli* isolates were phenotypically confirmed ESBL producers as given in Table 7. The order of occurrence of

UNDER PEER REVIEW

ESBL resistance genes was: bla_{SHV} (76.5%)> bla_{TEM}(50.0%)>bla_{CTX-M} (42.9%) as given in Table 7. The DNA bands for *SHV, CTX-M* and *TEM* genes are as given in Plate 1, 2 and 3.

3.2.4 AmpC β-lactamase Production

The result of phenotypic and genotypic confirmatory test of AmpC β-lactamase Production in *E. coli* isolates resistant to cefoxitin from stool of diarrheic children attending some selected hospitals in Lafia metropolis, Nigeria is as shown in Table 8. Whereas the DNA bands for AmpC genes is as shown in plate 1, 2 and 3 respectively. Out of 11 *E. coli* isolates resistant to cefoxitin, 4(36.4%) were phenotypically confirmed AmpC β-lactamase producers as given in Table 8. The OF percentage occurrence of the AmpC genes were; *CIT* (50%)>*MOX* (25%) and *FOX* (25%) respectively as shown in Table 8.

UNDER PEER REVIEW

Table 2: Cultural, morphological and biochemical characteristics of *Escherichia coli* isolated from stool of diarrheic children attending selected hospitals in Lafia, Nigeria

Clinical characteristics	Morphological characteristics		Biochemical characteristics								Inference
	Gram stain	Morphology	Ind	Mr	Vp	Ct	Lac	Glu	Gal	Suc	
Pinkish colonies on MCA and greenish metallic sheen colonies on EMB agar	-	Rod	+	+	-	-	+	+	+	+	*E. coli*

MCA= Mac Conkey Agar; EMB= Eosin Methylene blue; - = Negative; + = positive; Ind = Indole; Mr = Methyl Red; Vp = Voges-Proskauer; Ct= Citrate; Lac= Lactose; Glu= Glucose; Gal =Galactose; Suc= Sucrose

UNDER PEER REVIEW

Table 3: Antibiotic Resistance Profile of *Escherichia coli* from stool of diarrheic children attending selected hospitals in Lafia, Nigeria

Antibiotics	Disc Content (µg)	No. (%) Resistance(n=70)
Ampicillin (AMP)	30	68 (97.1)
Amoxycillin/clavunate (AMC)	30	49 (70.0)
Ciprofloxacin (CIP)	5	63 (90.0)
Ceftazidime (CAZ)	30	26 (37.1)
Cefotaxime (CTX)	30	21 (30.0)
Cefoxitin (FOX)	30	11 (15.7)
Gentamicin (CN)	10	27 (38.6)
Impenem (IPM)	30	6 (8.6)
Streptomycin(S)	30	55 (78.6)
Sulphamethoxazole/trimethoprim (SXT)	25	59 (84.3)

IEW

Table 4: Antibiotic Resistance Phenotypes of *Escherichia coli* from diarrheic stool of children attending selected hospitals in Lafia, Nigeria

Antibiotic Resistance Phenotypes	No. (%) *E. coli* isolates (n=70)
AMP,CIP	1(1.43)
SXT,AMP	1(1.43)
SXT,AMP,S	1(1.43)
AMC,AMP,S	1(1.43)
SXT,AMP,CIP	2(2.86)
SXT,CN,AMP,CIP	3(4.29)
SXT,AMP,S,CIP	5(7.1)
SXT,CN,AMP,CIP	1(1.43)
SXT,AMC,AMP,S	1(1.43)
SXT,AMC,AMP,CIP	2(2.86)
SXT,CN,AMP,S,CIP	5(7.14)
FOX,AMC,AMP,S,CIP	1(1.43)
SXT,FOX,AMC,AMP,CIP	1(1.43)
SXT,CAZ,AMP,S,CIP	1(1.43)
SXT,CAZ,AMC,AMP,CIP	1(1.43)
CN,AMC,AMP,S,CIP	1(1.43)
CAZ,CTX,AMC,AMP,CIP	1(1.43)
SXT,AMC,AMP,S,CIP	10(14.29)
SXT,CAZ,AMC,AMP,S,CIP	1(1.43)
SXT,AMC,AMP,S,CIP,IPM	1(1.43)
SXT,CAZ,CN,AMP,S,CIP	1(1.43)
SXT,CN,AMC,AMP,CIP	2(2.86)
SXT,CAZ,CTX,AMC,S,CIP	1(1.43)
SXT,CN,AMC,AMP,S,CIP	3(4.29)
SXT,FOX,AMC,AMP,S,CIP	1(1.43)
CAZ,AMC,AMP,S,CIP	1(1.43)
CAZ,CTX,AMC,AMP,S,CIP	1(1.43)
SXT,CAZ,CN,AMC,S,CIP	1(1.43)
FOX,CNAMC,AMP,S,CIP,IPM	2(2.46)
SXT,CAZ,CTX,AMC,AMP,S,CIP	4(5.71)
SXT,FOX,CAZ,CTX,AMC,AMP,S	2(2.86)
SXT,CAZ,CN,AMC,AMP,S,CIP	1(1.43)
SXT,CTX,CN,AMC,AMP,S,CIP,IPM	1(1.43)
SXT,CAZ,CTX,AMC,AMP,S,CIP,IPM	1(1.43)
SXT,CAZ,CTX,CN,AMC,AMP,S,CIP	3(4.29)
SXT,FOX,CAZ,CTX,AMC,AMP,S,CIP	1(1.43)
SXT,FOX,CAZ,CTX,CN,AMC,AMP,S	1(1.43)
SXT,FOX,CAZ,CTX,AMC,AQMP,S,CIP,IPM	1(1.43)
SXT,FOX,CAZ,CTX,CN,AMC,AMP,S,CIP	1(1.43)

Table 5: Multiple Antibiotics Resistance (MAR) Indices of *Escherichia coli* isolated from stool of diarrheic children attending selected hospitals in Lafia, Nigeria

No of Antibiotics Resistance (a)	No. of Antibiotics tested (b)	MAR index (a/b)	Frequency (%)
9	10	0.9	2 (2.86)
8	10	0.8	7 (10.00)
7	10	0.7	9 (12.86)
6	10	0.6	10 (14.29)
5	10	0.5	24 (34.29)
4	10	0.4	12 (17.14)
3	10	0.3	4 (5.71)
2	10	0.2	2 (2.86)

Table 6: Extended Spectrum β-lactamase production and genes in *Escherichia coli* jointly resistant to cefotaxime and ceftazidime from stool of diarrheic children attending selected hospitals in Lafia, Nigeria

Isolates	No. (%) ESBL producers	No. (%) ESBL genes (n= 14)		
		CTX-M	*SHV*	*TEM*
E. coli (n=21)	14(66.67)	6(42.86)	1(7.14)	7(50.00)

Table 7: Phenotypic and Genotypic confirmation of AmpC β-lactamase production in cefoxitin resistance *Escherichia coli* from stool of diarrheic children attending selected hospitals in Lafia, Nigeria

Isolates	No. (%) AmpC β-lactamase producers	No. (%) AmpC genes (n = 4)		
		MOXCITFOX		
E. coli (11)	4 (36.36)	1(25.00)	2(50.00)	1(25.00)

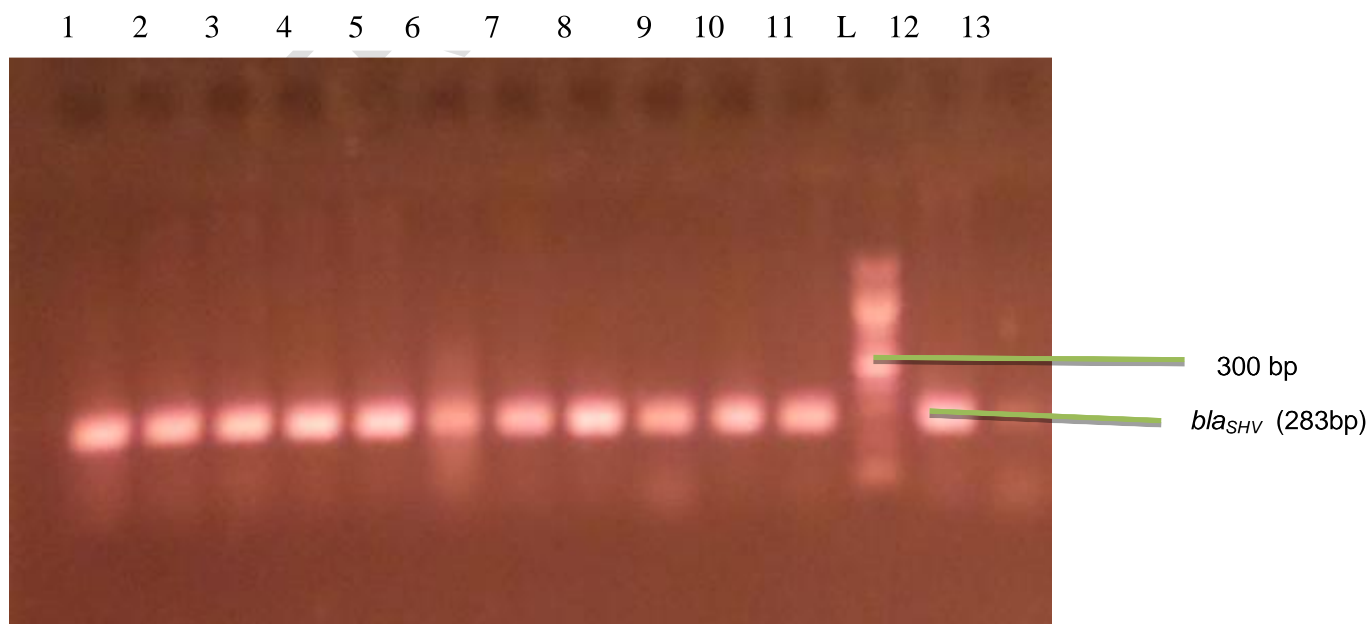

Plate 1: Agarose gel electrophoresis showing the bla_{SHV} bands, lane 1-13 represent bla_{SHV} gene bands while L represents the 1000bp molecular ladder.

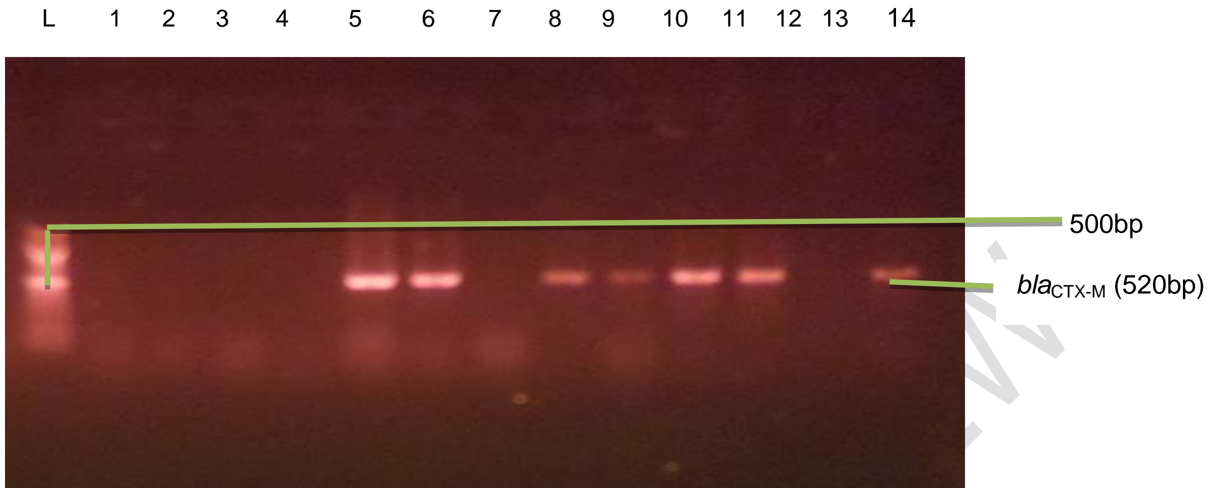

Plate 2: Agarose gel electrophoresis showing the *bla*$_{CTX-M}$ bands, lanes 5, 6, 8, 9, 10, 11, 12 and 14 represent *bla*$_{CTX-M}$ gene bands while L represents the 1000bp molecular ladder.

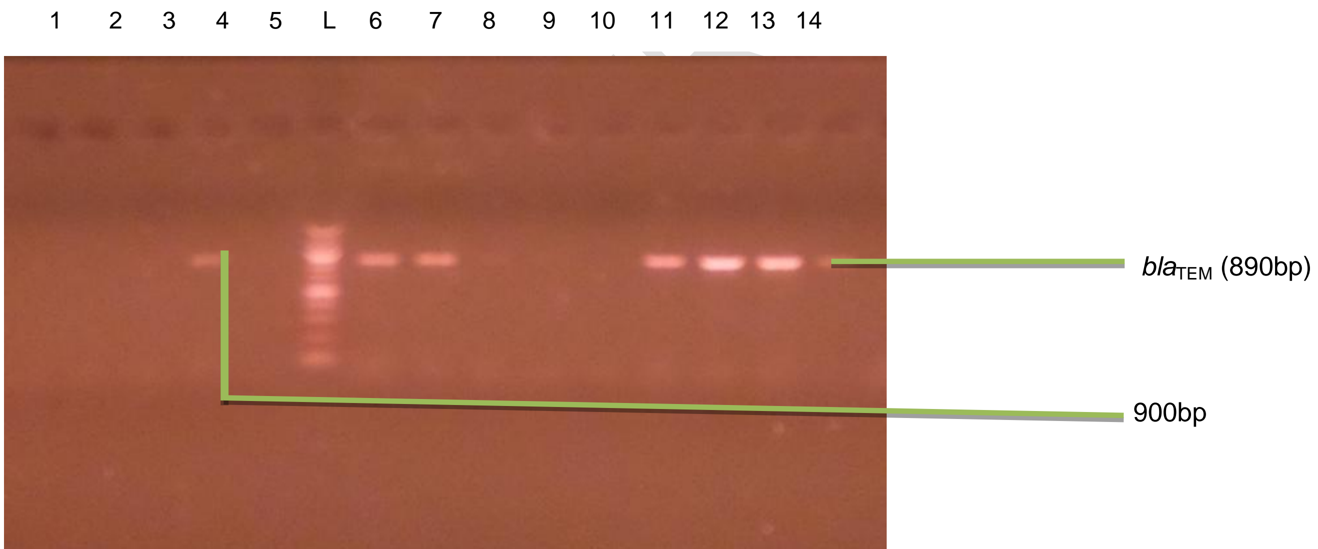

Plate 3: Agarose gel electrophoresis showing the *bla*$_{TEM}$ bands, lanes 4, 6, 7, 11, 12, 13 and 14 represent *bla*$_{TEM}$gene bands while L represents the 1000bp molecular ladder.

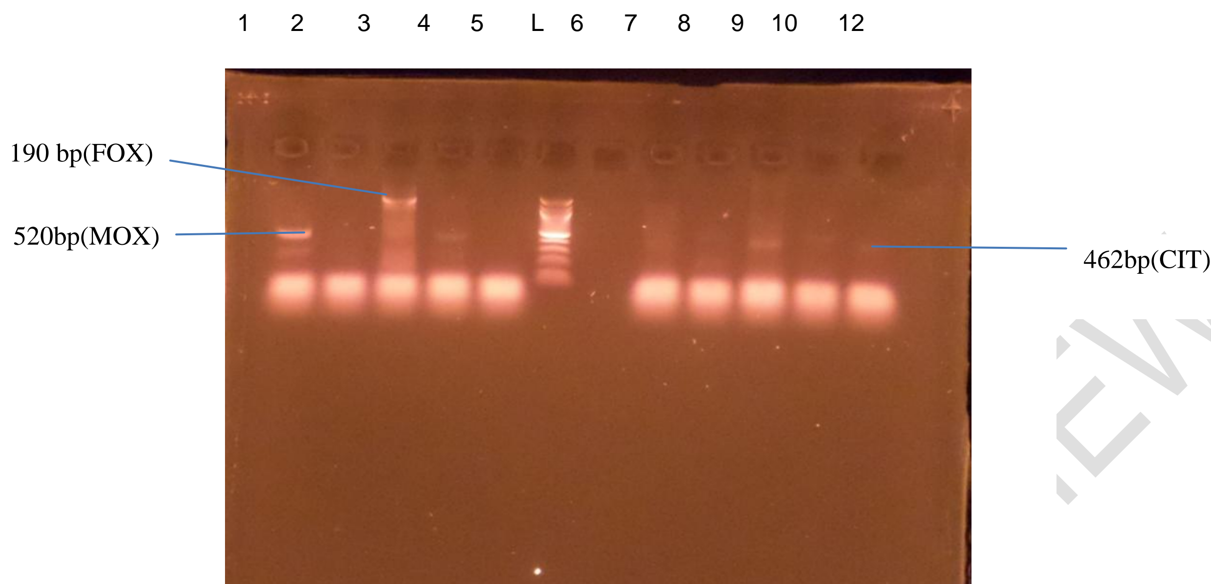

Plate 4: Agarose gel electrophoresis showing the *MOX*, *FOX* and *CIT* bands, while L represents the 1000bp molecular ladder.

The study investigated the antibiotic susceptibility and presence of ESBL and AmpC genes in *E. coli* from stool of diarrheic children attending selected hospitals in Lafia, Nigeria. From this study we observed that the detection rate of *E. coli* in stool of children with diarrhea was 100% and this however is in agreement with available data elsewhere [2] that *E. coli* is one of the major causes of diarrhea in diseases in children. From this study we also observed that the *E. coli* isolates from stool of children with cases of diarrhea were more susceptible to imipenem, cefoxitin, cefotaxime, ceftazidine and gentamicin. The high susceptibility of *E. coli* isolates to antibiotics mention was expected and this may be due to the fact that imipenem, cefoxitin, cefotaxime, ceftazidine, are very costly and not commonly prescribed and they are not likely to be abused and this however is in agreement with the early study reported [6] that antibiotics that are very costly are not likely to be abused. In another relation high susceptibility of *E. coli* isolates to gentamicin observed in this study may be due to the fact that drug is in injectable form and because of the pains and the discomfort of the injection they are not likely to be abused [39]. This finding is also in agreement with the study earlier reported [5]. The high susceptibility of *E. coli* isolates to ceftazidine and cefotaxime observed in this study is not in agreement with the study earlier reported [4], [5] reported high level of resistance of *E. coli* to cefotaxime (92.6%), ceftazidine (97.2%). Also, 89.7% and 79.5% *E. coli* isolates resistance to cefotaxime and ceftazidine was reported [4]. The high susceptibility of *E. coli* isolates observed in this study to cefotaxime and ceftazidine is in agreement with the study earlier described [40]. The low susceptibility of *E coli* isolates from stool of children with cases of diarrhea to ampicillin, amoxicillin/clavulanic acid, ciprofloxacin, streptomycin, and sulphamethoxazole/trimethoprim and this however may be due to misused, abused and used of substances and antibiotics. The low susceptibility of *E. coli* isolates to antibiotics mention is in agreement with study earlier described [5]. The occurrence of ESBL and AmpC β-lactamase production in *E. coli* isolates from stool of children with diarrhea cases observed in this study was not surprising and this finding is also in agreement with other reports [4], [40]. The occurrence of ESBL producers in *E. coli* isolates jointly resistant to ceftazidine and cefotaxime observed in this study is higher than 26.3% reported [5] and 48.7% [4]. The occurrence of *SHV* genes was higher *TEM and CTX-M* and this finding seems to disagree with the study reported [10], [14] reported that *CTX-M* gene is more prevalent in Enterobacteriaceae than *SHV* and *TEM* genes. The occurrence of *CTX-M, SHV* and *TEM* genes observed in this study is higher than that reported [38], [39]. Although from our study we observed that not all the *E. coli* isolates jointly resistance to both cefotaxime and ceftazidime were ESBL producers and this finding is also in agreement with the study earlier reported [40].

The detection of AmpC β-lactamase production in *E. coli* isolates resistance to cefoxitin observed in this study was expected and this however is in agreement with the study earlier reported [4]. The percentage

occurrence of AmpC β-lactamase producers in *E. coli* isolates in this study was higher than 7.2% reported [4], [40].

4. CONCLUSION

The *E. coli* isolates were more susceptible to gentamicin, imipenem, ceftazizidine, cefotaxime, and cefoxitin. Most of the isolates were confirmed ESBL and AmpC β-lactamase producers; ESBL genes (*SHV, CTX–M* and *TEM*) were detected; and *CIT* AmpC gene was more frequently detected than *MOX* and *FOX.* This suggest the need for further studies on molecular typing of ESBL and AmpC genes in *E. coli* isolates in the study area using a larger sample size.

CONSENT

All authors declare that written informed consent was obtained from the patients.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

UNDER PEER REVIEW

REFERENCES

1. WHO 2012; (www.who.int/topics/diarrhoea/en; Accessed: October 31st, 2016).
2. Abimiku RH, Ngwai YB, Nkene IH, Tatfeng YM. Molecular detection of diarrheagenicpathotypes of *Escherichiacoli* from diarrheic patients in Keffi, Nigeria. *Microbioz Journals, Journal of Microbiology and Biomedical Research.*2016;*2*(3): 1-6.
3. Walker CL, Aryee MJ, Boschi-Pinto C, Black RE. Estimating diarrhea mortality among young children in low- and middle-income countries. *PLOS ONE*. 2012;7:e29151.
4. Abdulkareem SS, Al-yassini HO, Muttaleb A, Nktel FNA, Nesseralla A,Noor SK, Al-Khafaji RKM, Noor MN. Occurrence of AmpC MBL, CRE and ESBL among diarrhegenic *Escherichia coli* recovered from Infantile diarrhea, Iraq. *International Journal of Microbiology, Genetics and Molecular Biology Research.* 2016; 2(2): 21-29.
5. Fody AM, Boubou L,Moussa A,Bawa HI,Konate A, Yaou C, Zongo C, Salaou C, Daouda A, Sidikou R, Traoure AB, Barro N. Phenotypic Detection of ESBL in multidrug resistant *E. coli* from clinical isolates in Niamey, Niger. African Journal of Microbiology Research. 2017; 11(18): 713-717.
6. Ngwai YB, Gyar SD, Pennap GRI, Makut MD, Ishaleku D, Corosi SM, Nkene IH, Uzoamaka N. Antibiogram of non-sorbitol fermenting *Escherichia coli* from sources and stool in Keffi, Nigeria. *NSUK Journal of Science and Technology*. 2014; vol. 4 (1 and 2).
7. Holten KB, Onusko EM. "Appropriate prescribing of oral beta-lactam antibiotics". *American Family Physician*. 2000; 62 (3): 611–20.
8. Crowder MW, Spencer J, Vila AJ. Metallo-beta-lactamases: novel weaponry for antibiotic resistance in bacteria. Acc Chem Res. 2006; 39(10):721-728.

9. Page MI, Badarau A. The mechanisms of catalysis by metallo beta-lactamases. *Bioinorganic Chemistry and Applied*: 2008; 576297.
10. Bush, K. Alarming β-lactamase mediated resistance in multiple resistance enterobactericea. Lurr. Opin. Microbiology. 2010; 13: 558-564.
11. Mollahaliloglu S, Alkan A, Donertas B, Ozgulcu S, Akici A. Assessment of antibiotic prescribing at different hospitals and primary health care facilities Saudi Pharm J. 2013; 21(3): 281–291.
12. Daumann LJ, Schenk G, Gahan LR. Metallo-β-lactamases and Their Biomimetic Complexes. *European Journal of Inorganic Chemistry*, 2014; 18:2856-2982.
13. HarrisPN, Ferguson JK. Antibiotic therapy for inducible AmpC beta-lactamase-producing Gram-negative bacilli: what are the alternatives to carbapenems, quinolones and aminoglycosides? *International Journal of Antimicrobial Agents.* 2012; 40: 297-305.
14. Day MJ, Rodriguez I, Van EZA, Dierikx C, Kadlee K, Schink A. Diversity of ST, Plasmids and ESBL genes among *E. coli* from humans, animals and food in germany, the Netherlands and the UK . Journal of Antimicrobial Chemotherapy. 2016; 10:485.
15. Ferjani S, Saidani M, Amine FS, Boubaker IBB. Prevalence and characterization of plasmid-mediated quinolone resistance genes in extended-spectrum*β*-lactamase-producing *Enterobacteriaceae* in a Tunisian hospital. *Microbial Drug Resistance*. 2015; 21(2):158–166.
16. Dashti AA, West P, Paton R, Amyes SG. Characterization of extended spectrum beta-lactamase (ESBL)-producing Kuwait and UK strains identified by the Vitek system, and subsequent comparison of the Vitek system with other commercial ESBL-testing systems using these strains. *Journal of Medical Microbiology*. 2006; 55: 417-421.
17. Fang H, Ataker F, Hedin G, Dornbusch K (2008) Molecular epidemiology of extended-spectrum beta-lactamases among *Escherichia coli* isolates collected in a Swedish hospital and its associated health care facilities from 2001 to 2006. *Journal of Clinical Microbiology*, 46: 707–712.
18. Sedighi, I., Mohammad R. A., Ali, R., Zahra, K. and Mohammad, Y. A. (2015). Dissemination of Extended-Spectrum β-Lactamases and Quinolone Resistance Genes among Clinical Isolates of Uropathogenic *Escherichia coli* in Children. *Jundishapur Journal of Microbiology*, 8(7): 1-6.
19. Jacoby GA. AmpC beta-lactamases. *Clinical MicrobiologyReview.* 2009; 22: 161-82.
20. Jameel N, Ejaz H, Zafar A, Amin H. Multidrug resistant AmpC β-lactamase producing *Escherichia coli* isolated from a paediatric hospital. Pak. J. Med. Sci. 2014; 30(1): 181–184.
21. El-Sharif A, Ali R. Molecular detection of TEM Type β lactamase producing Escherichia coli from diarrheic Egyptian children. *Arch. Clin. Microbiol.* 2012; 3(5):3
22. Ali MMM, Ahmed S, Klena JD, Mohamed ZK, Moussa TAA, Ghenghesh KS. Enteroaggregative Escherichia coli in diarrheic children in Egypt: Molecular characterization and antimicrobial susceptibility. The Journal of Infection in Developing Countries. 2014; 8(5):589-596.
23. Alizade H, Fallah F, Ghanbarpour R, Aflatoonian MR, Goudarzi H, Sharif H. Phylotyping of blaCTX-M-15 gene in extended spectrum beta lactamase producing Escherichia coli isolates from clinical samples in Iran. HVM Bioflux. 2014; 6(4): 169-173.
24. Ogefere HO, Ibadin EE, Omoregie R, Ilerhunwa I. Prevalence of Extended Spectrum β-Lactamase among Diarrheagenic Strains of Escherichia Coli among Children in Yenagoa, Nigeria. Sokoto Journal of Medical Laboratory Science 2016; 1(1): 7-12
25. Bai L, Wang L, Yang X, Wang J,Gan X, Wang W, Xu J, Chen Q, Lan R, Fanning S, Li F. Prevalence and Molecular Characteristics of Extended-Spectrum β-Lactamase Genes in Escherichia coli Isolated from Diarrheic Patients in China. Front. Microbiology. 2017; 8: 1-8.
26. Mandal A, Sengupta A, Kumar A, Singh UK, Jaiswal AK, Das P, Das S. Molecular Epidemiology of Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Pathotypes in Diarrheal Children from Low Socioeconomic Status Communities in Bihar, India: Emergence of the CTX-M Type. *Infectious Diseases*, (Auckl). 2017; 10: 1-11
27. Rabia AR, Wamba PN, Kimera SI, Mdegela RH, Mzulla A, Kahmis FA. Phenotypic Characterisation of *Escherichia coli* Isolates from Fish, Diarrheic and Healthy Children inZanzibar, Tanzania.*International Journal of Tropical Disease and Health*. 2017; 24(3):1-11.
28. Ingle DJ, Levine MM, Kotloff KL, Holt KE, Robins-Browne RMR. Dynamics of antimicrobial resistance in intestinal Escherichia coli from children in community settings in South Asia and sub-Saharan Africa. Nature Microbiol. 2018; 3: 1063-1073.
29. Xu Y, Sun H, Bai X, Fu S, Fan R, Xiong Y. Occurrence of multidrug‐resistant and ESBL‐producing atypical enteropathogenic Escherichia coli in China. Gut Pathogens. 2018; 10:8
30. Okeke IN, Fayinka ST, Lamikanra A. Antibiotic resistance in Escherichia coli from Nigerian students, 1986-1998. Emerg. Infec. Dis. 2000;6:393–396.
31. Aibinu IE, Peters RF, Amisu KO, Adesida SA, Ojo MO, Odugbemi T. Multidrug resistance in *E. coli* O157 strains and the public health implication. J. Am. Sci. 2007;3:22–33.

UNDER PEER REVIEW

32. Ifeanyi CIC, Ikeneche NF, Bassey BE, Morabito S, Graziani C, Caprioli A (2017). Molecular and phenotypic typing of enteropathogenic Escherichia coli isolated in childhood acute diarrhea in Abuja, Nigeria. J. Infect. Dev. Ctries. 2017; 11(7):527-535.
33. Ugwu MC, Edeani GI, Ejikeugwu CP, Okezie U, Ejiofor SO. Antibiotic Susceptibility Profile of Escherichia coli and Salmonella Causing Childhood Diarrhoea in Awka Municipality, South-eastern Nigeria. Clin. Microbiol. 2017; 6:277.
34. Ifeanyi CIC, Ikeneche NF, Bassey BE, Nazek A-G, Akpa AC, Isu RN. Characterization of Toxins and Colonization Factors of Enterotoxigenic Escherichia coli Isolates from Children with Acute Diarrhea in Abuja, Nigeria. Jundishapur Journal of Microbiology. 2018; 11 (1): e64269.
35. Ding H, Yang Y, Lu Q, Wang Y, Chen Y, Deng L. The prevalence of plasmid-mediated AmpC beta-lactamases among clinical isolates of *Escherichiacoli* and *Klebsiellapneumoniae* from five children's hospitals in China. *European Journal of Clinical Microbiology and Infectious Diseases.*2008; 27: 915-21.
36. Cheesebrough M. Medical Laboratory Manual for Tropical Countries. Cambridge: Cambridge University Press. 2006; Pp. 49-97.
37. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; 27nd Informational Supplement M100-S22*. Wayne, Pa, USA.2017.
38. Jarlier V, Nicolas MH, Fournier G. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. Review of Infectious Diseases. 1998; 10: 867-868.
39. Nkene IH, Ngwai YB, Omede MU, Samuel J, Envuladu EY, Abimiku RH. Extended spectrum beta-lactamase producing *Escherichia coli* from urine of symptomatic and asymptomatic subjects in Keffi, Nigeria. *International Journal of Research Studies in Biosciences.* 2015; 3(12): 1-5.
40. Amaya E, Reyes D, Vilchez S, Paniagua M, Miuby R, Nord CE, Weintraub A. Antibiotic Resistance Patterns of Intestinal *E. coli* isolates from Nicaraguan Children. *Journal of Medical Microbiology*. 2010; 216-222.

UNDER PEER REVIEW