

# Antimicrobial Resistance Profile and Extended Spectrum Beta-Lactamase Resistance Genes in *Escherichia coli* from Patients in General Hospital, Karshi, Abuja, Nigeria

## ABSTRACT

**Aims:** This study investigated the antimicrobial resistance profile and extended spectrum beta-lactamase resistance genes in *Escherichia coli* of from urine of patients sourced from General Hospital, Karshi, Abuja, Nigeria

**Study design:** Cross-sectional study

**Place and Duration of Study:** Department of Microbiology, Nasarawa State University, Keffi, between August 2022 and February 2023.

**Methodology:** A total of 120 samples were collected from patients. *Escherichia coli* was isolated from the samples using standard microbiological methods. Antibiotic susceptibility testing was evaluated as described by the Clinical and Laboratory Standards Institute (CLSI). The detection of ESBL production in *E. coli* isolates was carried out using double disc synergy test. In addition, molecular detection of ESBL genes was carried out using Polymerase Chain Reaction (PCR) method.

**Results:** The prevalence of *E. coli* was 17.5% (21/120), out of which two (2) of the positive isolates (9.5%) were male, and 19 (90.5%) female. Antibiotic resistances in the isolates in decreasing order were as follows: sulphamethoxazole / trimethoprim (SXT: 81.0%), amoxicillin/clavulanic acid (AMC: 61.9%), ofloxacin (OFX: 66.7%), cefotaxime (CTX: 53.4%), gentamicin (CN: 42.9%), ceftriaxone (CRO: 33.3%), imipenem (IPM: 33.3%), meropenem (MOR: 42.4%), nitrofurantoin (NET: 20.3%) and ciprofloxacin (CIP: 23.8%). The commonest antibiotic resistant resistance phenotype was CIP-OFX-SXT (23.8%). Multiple antibiotic resistance (MAR) was observed in 90.5% (19/21) of the isolates, with the common MAR index being 0.3 (23.8%). Six of the twenty one beta-lactam resistant isolates (28.5%) were confirmed ESBL producers. The 6 ESBL positive isolates carried *bla* genes as follows: *bla*<sub>TEM</sub> (1/6, 16.7%) and *bla*<sub>CTX-M</sub> (1/6, 16.7%). *bla*<sub>SHV</sub> only was not found in any of the isolates.

**Conclusion:** The *E. coli* isolates from urine of patients in General Hospital, Karshi, Abuja, Nigeria was less resistant to ciprofloxacin, nitrofurantoin, meropenem and imipenem. This implies that the antibiotics are useful in the treatment of infection caused by *E. coli*. Also, ESBL-positive *E. coli* isolates harbored ESBL genes, with *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub> as the most common.

**Keywords:** *Escherichia coli*; Urine; antibiotic; resistance; ESBL; genes

## 1. INTRODUCTION

Urinary tract infections (UTIs) are one of the popularly known diseases that are encountered in medical and clinical practice and are caused mainly by organisms such as *Escherichia coli* [1, 2, 3, 4]. Antimicrobial resistance during treatment in patients with UTIs is an increasing global concern [5]. The recent emergence and global dissemination of *E. coli* resistant to antibiotics due to the production of extended-spectrum b-lactamases (ESBLs) is a public health concern [5, 6, 7]. There is currently lack of epidemiological surveillance studies in Abuja that have investigated the molecular characterization of *E. coli* strains circulating in the healthcare settings, particularly the General hospitals.

The objectives of this study were to detect *E. coli* in urine from General Hospital in Karshi, Abuja. The results of the present study might be helpful and aid the understanding of the situation in UTIs caused by *E. coli*.

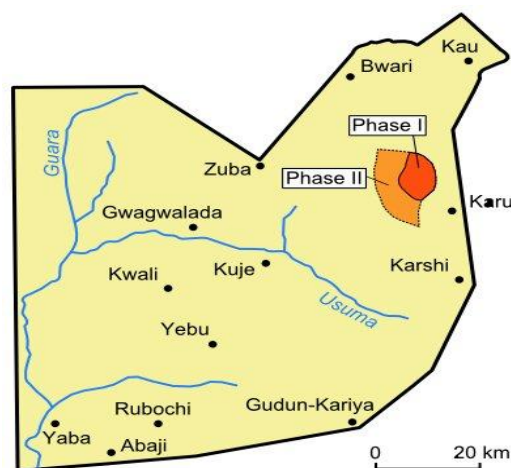
## 2. MATERIAL AND METHODS

### 2.1 Sample collection

A total of 120 urinary samples were randomly collected using appropriate sterile sample containers and transported to the Microbiology Laboratory at the Nasarawa State University, Keffi, for analysis. The samples were collected from Karshi General Hospital, a secondary health facility located in Karshi town, a suburb in the Federal Capital Territory, Abuja, Nigeria. Samples were collected between August 2022 and February 2023.

### 2.2 Study area

The study was carried out at Karshi General Hospital, a secondary health facility located in Karshi town, a suburb in the Federal Capital Territory, Abuja, Nigeria. Karshi town is made up of a large population of farmers and traders.



Map 1: Map showing Karshi, Federal Capital Territory (Ajobiewe, 2021) [8]

#### 2.2.1 Isolation of *Escherichia coli*

*Escherichia coli* was isolated by inoculating 1ml of urine sample into 9 ml of nutrient broth (NB: Oxoid Ltd., UK) and incubated (Quincy Lab Inc. Model12-140E, USA) at 37°C for 24 h. Following incubation, a loopful of the 24- h broth was streaked on MacConkey agar (MCA: Oxoid Ltd., UK) plate and incubated at 37°C for 24 h. Pinkish colonies from the 24-hour MCA plates were further streaked on Eosine Methylene Blue agar (EMB: Oxoid Ltd., UK) plates and incubated at 37°C for 24 h. Colonies with a greenish-metallic sheen appearance were selected as presumptive *E. coli* [9].

#### 2.2.2 Identification of *Escherichia coli*

Presumptive colonies were phenotypically characterized based on morphological, Gram staining, catalase and oxidase tests. Gram-negative, catalase positive and oxidase negative isolates were subjected to biochemical tests including Indole, Methyl Red-Voges-Proskauer, Citrate, Nitrate Reduction Test, Urease Test, H<sub>2</sub>S production motility test, etc. as described in the Bacteriological Analytical Manual, 2019 [10] and Cheesbrough [9]. The API20E system (Analytical Profile Index) (BioMerieux™, USA), was used to confirm the suspected isolates as described in the manufacturer's manual. Colonies with a characteristic pink color on MCA, which grew with a greenish-metallic sheen on EMB agar, Gram-negative, rods, indole positive, citrate negative, methyl-red positive, Voges-Proskauer negative, urease

negative, nitrate reduction positive test indicated *E. coli*. The bacterium was stored in the refrigerator on nutrient agar (Oxoid Ltd, UK) slants and reactivated by sub-culturing on MCA for use in further research.

### 2.3 Antimicrobial Susceptibility Testing

The antibiotic susceptibility test for *E. coli* isolates from urine was carried out using the Kirby-Bauer disc diffusion method as modified by the Clinical and Laboratory Standards Institute (CLSI) [11]. Briefly, 5 colonies of *E. coli* isolates were inoculated into 5 ml of Mueller-Hinton broth (MHB: Oxoid Ltd, UK) and incubated at 37°C for 24 h after which the 24-h MHB was standardized to the turbidity equivalent to 0.5 McFarland Standard. The 0.5 McFarland Standard was prepared as follows: 99.5 ml of 1% ( $\frac{v}{v}$ ) H<sub>2</sub>SO<sub>4</sub> + 0.5 ml of 1.172% ( $\frac{w}{v}$ ) BaCl<sub>2</sub>.2H<sub>2</sub>O. A sterile cotton swab stick was dipped into the standardized *E. coli* suspension and streaked on Mueller-Hinton Agar (MHA: Oxoid Ltd, UK) plates. Antibiotics discs (Oxoid Ltd, UK) were gently placed 15mm apart on the MHA surface using a pair of sterile forceps and the plates were allowed to incubate at room temperature for 1 h before re-incubating at 37°C for 17 h. The discs used included: Amoxicillin/Clavulanic acid (AMC): (10/20 µg), Sulphamethoxazole/ Trimethoprim (SXT :) (25 µg), Ceftriaxone (CRO): (30 µg), Cefotaxime (CTX :) (30 µg), Nitrofurantoin (NET :) (30 µg), Ofloxacin (OFX :) (5 µg), Gentamicin (CN :) (10 µg), Meropenem (MOR :) (30 µg), Ciprofloxacin (CIP :) (5 µg) and Imipenem (IPM :) (30 µg). Following incubation, the diameters of the zones of inhibition were measured to the nearest millimeter (mm) using a ruler. The result of the susceptibility test was interpreted using susceptibility breakpoint earlier described by CLSI [11].

### 2.4 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 by Giriya et al. [12] with modification. Briefly, 10<sup>5</sup>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 ceftriaxone (30 µg) discs were then placed 15 mm (edge-to-edge) from the disc at the centre. 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.

#### 2.4.1 Determination of Multiple Antibiotic Resistance (MAR) Index

The multiple antibiotic resistance index (MARI) of the *E. coli* isolates were determined as described by [13]. MARI is defined as resistance to at least two (2) antibiotics, hence obtaining a MAR value higher than 0.2 indicated a significant and high risk source of acquiring the multidrug resistant *E. coli* from the tested samples

$$\text{MAR Index} = \frac{a \text{ (Number of antibiotics isolate is resistant to)}}{b \text{ (Number of antibiotics tested)}}$$

(Where a= number of antibiotics to which an isolate is resistant to, while b= number of antibiotics against which isolate was tested).

#### 2.4.2 Molecular detection of ESBL resistance genes

##### 2.4.2.1 DNA extraction

The bacterial DNA was extracted by boiling method as described by Abimikuet *et al*, 2016 [13] with minor modification. Ten (10) milliliters of the broth culture of the bacterial isolate was dispensed in 1 ml Luria-Bertani broth (LB) and was spun at 14000 rpm for 3 mins. The supernatant was discarded, and the pellet was resuspended in 1 ml sterile distilled water and centrifuged at 14000 rpm for 10 mins. After discarding the supernatant, 100 µl of sterile distilled water was added to the tube and vortexed. The tube was centrifuged again at 14000 g for 10 min, and the supernatant was discarded carefully. The cells were re-suspended in 500 µl of normal saline and heated at 95°C for 20 mins. The heated bacterial suspension was cooled on ice for 10 mins and spun at 14000 rpm for 3 mins. The supernatant containing the DNA was transferred to a 1.5-ml microcentrifuge tube and stored at -20°C for other downstream reactions.

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 260 nm ( $A_{260}$ ) 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).

#### **2.4.2.2 DNA amplification of target genes by polymerase chain reaction**

To detect the presence of *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes, a simplex Polymerase Chain Reaction (PCR) was performed as described by Tama *et al* 2019 [14]. The primer sequences and expected amplicon sizes for each gene are listed in Table 1. The reactions were carried out in 20 µl reaction volume made up of 10 µl of Mastermix (Inqaba Biotech, South Africa), 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. [14]. The thermocycler (Model TC-312, Techne, England) conditions were set as follows: 3 mins 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.

#### **2.4.2.3 Agarose gel electrophoresis**

Exactly 7 µl of the amplified DNA was transferred into the wells of a 1.5% Agarose gel containing ethidium bromide (0.3 µg/lm) (Sigma Aldrich, USA). A DNA ladder (1500 bp, Inqaba Biotech, South Africa) was used 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 (Vilber Lourmat TFX-35-M serial no NoV02 8104, France).

### **3. RESULTS AND DISCUSSION**

#### **3.1 Prevalence of *Escherichia coli***

A total of 120 samples were collected, out of which 21 (17.5%) were *E. coli* positive. Two (2) of the positive isolates (9.5%) were male, and 19 (90.5%) female. In relation to age, the age group 21-30 years had more patients for this study (13, 72.2%). The prevalence of *E. coli* in the samples and in relation to gender and age is as shown in Table 2 and Table 3.

#### **3.2 Antibiotic Resistance Profile of the *Escherichia coli* isolates**

The highest resistance observed was to Sulphamethoxazole/Trimethoprim (81.0%), and the least resistance was to ciprofloxacin (23.8%). Low resistance was also observed to imipenem (33.3%), meropenem (33.3%) and ceftriaxone (33.3%). The antibiotic resistance profile of the *E. coli* isolates is shown in Table 4 and Table 5.

### 3.3 Antibiotic Resistant Phenotypes of the *Escherichia coli* isolates

The common phenotype among the isolates observed was CIP-OFX-SXT (23.8%). The antibiotic resistant phenotypes of the *E. coli* isolates is shown in Table 4.

### 3.4 Multiple Antibiotic Resistance (MAR) Index

Nineteen (19) of the isolates were MAR isolates, showing resistance to at least two antibiotics tested. The commonest MAR index was 0.3 (23.8%). The MAR indices of the isolates is as shown in Table 6.

### 3.5 Phenotypic confirmation of Extended-Spectrum Beta-Lactamase production

All of the 21 beta-lactam resistant isolates tested showed enhanced zones of clearing towards the amoxicillin-clavulanic acid disc when examined by DDST method.

### 3.6 Molecular Detection of Extended-Spectrum Beta-Lactamase Genes

The distribution of the ESBL resistance genes screened for in the ESBL-positive *E. coli* isolates is as follows: of the 21 ESBL-positive isolates screened, 6 (28.5%) carried the *bla* genes. 1 (16.7%) harbored *bla*<sub>CTX-M</sub> and 1 (16.7%) carried *bla*<sub>TEM</sub>. None harbored *bla*<sub>SHV</sub> only. Some isolates carried two *bla* genes or more (either of the combinations: *bla*<sub>CTX-M</sub>, and *bla*<sub>TEM</sub>; *bla*<sub>TEM</sub> and *bla*<sub>SHV</sub>; and *bla*<sub>TEM</sub>*bla*<sub>CTX-M</sub> and *bla*<sub>SHV</sub>).

**Table 1. Primers and their sequences**

S/N	Target genes	Sequence	Amplicon size (bp)	References
1	<i>bla</i> <sub>TEM</sub>	5'-TCGGGGAAATGTGCGCG-3' 5'-TGCTTAATCAGTGAGGCACC-3'	972	[15]
2	<i>bla</i> <sub>SHV</sub>	5'-GGGTTATTCTTATTTGTGCGC-3' 5'-TTAGCGTTGCCAGTGCTC-3'	615	[16]
3	<i>bla</i> <sub>CTX-M</sub>	5'-ACGCTGTTGTTAGGAAGTG-3' 5'-TTGAGGCTGGGTGAAGT-3'	857	[16]

**Table 2. Occurrence of *Escherichia coli* in the urine of patients in relation to Gender**

Gender	No. (%) <i>E. coli</i> (n=21)
Male	2(9.5)
Female	19 (90.4)
Total	21 (100.0)

**Table 3. Occurrence of *Escherichia coli* from urine of patients Karshi General Hospital in Abuja Municipal Area, Nigeria in relation to age**

Age (yrs)	n=120
≤ 10	0(0.0)
11-20	0(0.0)
21-30	13(72.2)
31-40	7(11.7)
41-50	0(0.0)
>50	1(8.3)
<b>Total</b>	<b>21(17.5)</b>

**Table 4. Antimicrobial resistance phenotypes of *Escherichia coli* isolated from Urine from General Hospital, Karshi, Nigeria**

Antimicrobial Resistance Phenotypes	Frequency (n=21)
CRO	1(4.8)
AMC	1(4.8)
AMC, SXT	1(4.8)
CTX, CN	1(4.8)
CIP, OFX, SXT	5(23.8)
CTX, AMC, CRO, SXT, OFX	3(14.3)
AMC, CN, OFX, CIP	1(4.8)
CTX, OFX, CRO, CN, SXT, NET	1(4.8)
CTX, AMC, CN, SXT, CIP, IMP, MOR	1(4.8)
CTX, AMC, CRO, CN, SXT, IMP, MOR	2(9.5)
AMC, OFX, CN, SXT, CIP, NET, IMP, MOR	1(4.8)
CTX, AMC, OFX, CRO, CN, SXT, CIP, NET, IMP, MOR	3(14.3)

AMC=Amoxicillin/Clavulanic acid; CTX=Cefotaxime; CRO=Ceftriaxone; CIP=Ciprofloxacin; CN=Gentamicin; IMP=Imipenem; OFX=Ofloxacin; MOR=Meropenem; NET=Nitrofurantoin; SXT=Sulfamethoxazole/Trimethoprim

**Table 5. Antimicrobial Resistance of *Escherichia coli* isolated from urine of patients attending Karshi General Hospital in Abuja Municipal Area, Nigeria**

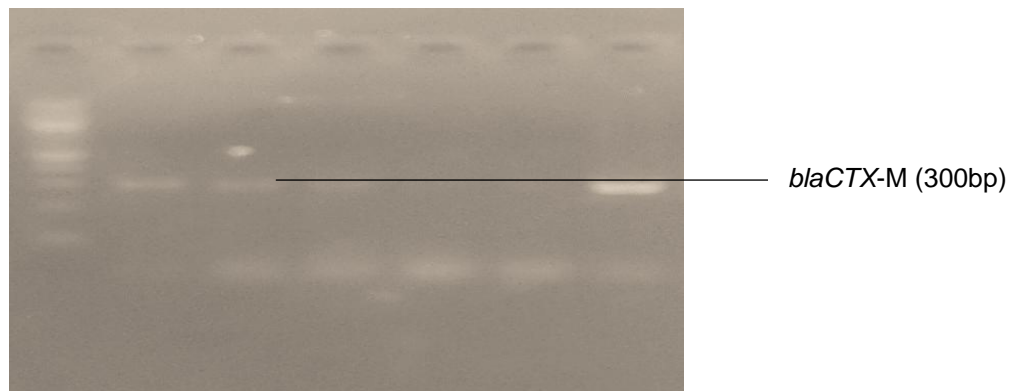
Antimicrobials	Disc content (µg)	(n=21)
AMC	30	13(61.9)
CTX	30	11(53.4)

CRO	30	7(33.3)
CIP	5	5(23.8)
CN	10	9(42.9)
IMP	30	7(33.3)
OFX	5	14(66.7)
MOR	30	7(33.3)
NET	30	7(33.3)
SXT	25	17(81.0)

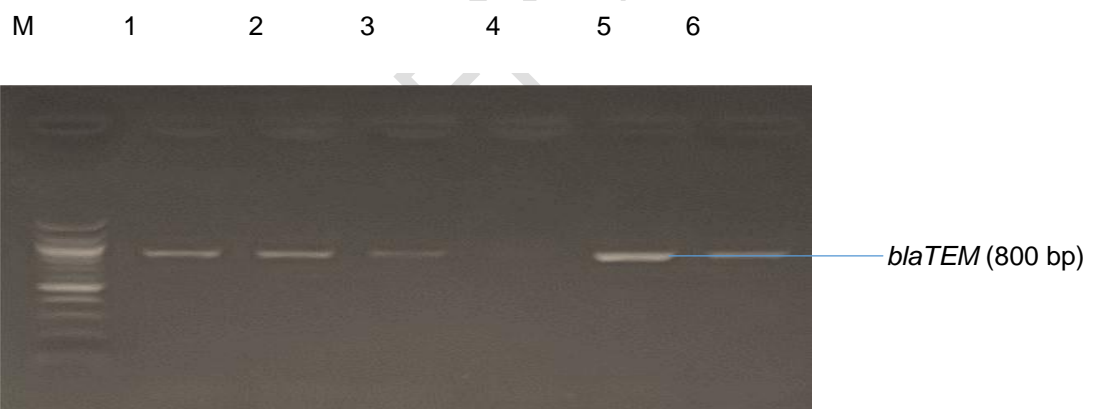
**Table 6. Multiple Antibiotic Resistance (MAR) Index of *Escherichia coli* isolated from Urine from General Hospital, Karshi, Nigeria**

No of antibiotics isolate resistant to (a)	No. of antibiotics tested (b)	MAR Index ( $\frac{a}{b}$ )	No. (%) MAR isolates (n=21)
10	10	1.0	3(14.3)
9	10	0.9	0(0.0)
8	10	0.8	3(13.3)
7	10	0.7	1(4.8)
6	10	0.6	1(4.8)
5	10	0.5	0(0.0)
4	10	0.4	4(19.0)
3	10	0.3	5(23.8)
2	10	0.2	2(9.5)
1	10	0.1	2(9.5)

\*MAR isolates are those with resistance to at least two antibiotics [24]



**Figure 1:** Agarose gel electrophoresis of the amplified *bla*CTX-M genes from the *E. coli* isolates. Lanes 1-3 and 6 represent the *bla*CTX-M bands while Lanes 4 and 5 were negative for *bla*CTX-M. Lane M represents the 1500bp molecular ladder.



**Figure.2:** Agarose gel electrophoresis of the amplified *bla*TEM genes from the *E. coli* isolates. Lanes 1-3, 5 and 6 represent the *bla*TEM bands while Lanes 4 was negative for *bla*TEM. Lane M represents the 1500bp

There has been an increase in the incidence of UTIs in recent years globally, which is caused by a variety of microorganisms, particularly Gram-negative bacteria [Gajdacset al][17]. This study revealed that *E. coli* was likely the uropathogen responsible for infection, with an occurrence rate of 17.5%, which is similar to studies in Keffi carried out by Nkene et al [17] and Bassey et al in Abuja [18], but less than 31, 19%, 59, 85% and 55, 0% in the studies conducted by Ekenget al. [18], Farooq et al. [20] and Amadu et al. [21] respectively. The prevalence of the infected women (19/21; 90.4%) was higher than in men (2/21; 9.5%). The high percentage occurrence of the urinary isolates in female than the male patients in our study is not in agreement with the findings of Shitu et al. [22] which reported high prevalence

in males (37.5%) than the females (36.5%) but agreed with the study conducted by Bassey *et al.* [18], Amadu *et al.* [21] and Nkeneet *et al.* [17]. There are many factors that can affect the interpretation of UTIs in women, particularly *E. coli*. Among these factors are the anatomical structure of the urogenital system, and women have a shorter distance between the urethral and anal opening [23].

The occurrence of the isolates in relation to age of patients in our study was highest in age group 21-30 yrs. The high occurrence of the isolates in age group 21-30 yrs may be because individuals within this age group may be sexually active, with high risk of UTIs. The high occurrence observed contradicts the findings in a study by Amadu *et al.* [21] and Nkeneet *et al.* [17] who reported high prevalence of urinary *E. coli* in age group 61-70 yrs (29.5%) and  $\geq 50$  yrs (31.8%) but was consistent with the high prevalence in age group 21-30 yrs (20.5%) observed in a study conducted in Abuja by Bassey *et al.* [18].

In this study, the antimicrobial susceptibility testing of *E. coli* showed that 17 and 14 (81.0% and 66.7%) of the isolates were highly resistant to sulfamethoxazole/trimethoprim and ofloxacin respectively. This finding is similar to studies conducted by Shitu *et al.* [22] and Meduguet *et al.* [24] which reported 82.9% and 88.4% respectively. High resistance of isolates may primarily be attributed to the diversity of genes in various bacteria that regulate the efflux of antibiotics from the cell [24]. Furthermore, the high resistance of the isolates to amoxicillin/clavulanic acid (13, 61.9%), sulfamethoxazole/trimethoprim (17, 81.0%) and ofloxacin (16, 66.7%) may be due to misuse, ineffective therapy, lack of compliance to dosage schedules and prolonged use of single agent for treatment of bacterial diseases which is common in the developing countries like Nigeria [18]. Although it has been suggested that certain Penicillins, when taken with  $\beta$ -lactamase inhibitors (clavulanic acid), are useful in treating some infections caused by bacteria that produce ESBLs [25], in this study, 66.7% of *E. coli* were resistant to Amoxicillin-clavulanic acid. This finding is in agreement with a similar laboratory-based recent study conducted in Turkey [26], while less of it was found in a study conducted in Poland [27].

In the current study, approximately 33.3% of *E. coli* were resistant to Imipenem. This finding was slightly lower when compared with other results from studies in Iraq (95.2%) [28,29], and in Baghdad (100%) [30]. It is disturbing that from recent data, *E. coli* have been resistant to Imipenem in UTIs such as observed in Duhok city [31] and Erbil city [32], as well as in Saudi Arabia [33]. In other words, *E. coli* resistance to Imipenem is steadily on the rise, and this could be due to the use of over-the-counter antibiotics and the ease of buying antibiotics without a prescription. According to Adamu *et al.*, the public health sector in Nigeria has not verified the guidelines for how to control the selling of antibiotics [34]. In addition, ciprofloxacin (23.8) and nitrofurantoin (33.3%) had the least resistance against these isolates in this study. A similar result was found for Nitrofurantoin in a study conducted in Ethiopia [35].

It has been noted that *E. coli* which produce extended-spectrum  $\beta$ -lactamase (ESBL) are becoming more common in the community globally [36]. In this study, all isolates of *E. coli* were subjected to phenotypic and molecular detection of ESBL production/genes and 6 (28.5%) were positive for ESBL production. The prevalence rate ESBL-producing *E. coli* was similar when compared with studies in Qatar [37] and Iran [38], (30.0% and 30.5% respectively, but lower than other studies 41% in Turkey [39], and higher than reports in Sweden 16.8% [40] and Spain (2%)[41]. The isolates of *E. coli* were subjected to PCR using TEM, CTX-M, and SHV-specific primers for the detection of ESBL genes. Out of the 21 isolates, six (6, 28.5%) carried ESBL genes, TEM and CTX-M, respectively. The detection of both  $bla_{TEM}$  and  $bla_{CTX-M}$  resistance genes in the ESBL-producing *E. coli* suggests that these genes may be responsible for the observed resistance to beta-lactam antibiotics.

## CONCLUSION

This study shows that *E. coli* is one of the most predominant bacteria among urinary patients in the hospital, and especially among women. It also shows significant number of *E. coli* with ESBL production. The ESBL-producing *E. coli* was less resistant to ciprofloxacin, nitrofurantoin, meropenem and imipenem, hence these are considered to be the effective antibiotic choices for the treatment of UTI infections. In addition, ESBL infection are increased in the urinary tract because of two  $\beta$ -lactamase genes, *bla*<sub>CTX-M</sub> and *bla*<sub>TEM</sub>. Effective monitoring of antibiotic prescription and use will be helpful to reduce and manage the cases of antibiotic resistance.

## ETHICAL APPROVAL

Ethical approval was sought and gotten from Health Research Ethics Committee of the Federal Capital Territory Administration before the commencement of the study.

## COMPETING INTERESTS

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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