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Distribution of TEM, SHV, OXA AND CTX-M Genes among ESBL- producing *E. coli* Isolates from Patients with Urinary Tract Infections.Olley Mitsan¹, Maureen U. Okwu², Osakue Eguagie Osareniro³, Alao James⁴

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Abstract

The emergence of extended-spectrum beta-lactamases (ESBLs) are as a result of transmission among Gram negative bacteria, usually by horizontal gene transfer. The epidemiology of ESBL genes is changing rapidly and shows marked geographic differences in distribution of genotypes. In this study, isolation of extended- spectrum beta- lactamase producing *E. coli* in patients with bacteriuria in parts of Edo state, Nigeria was carried out using the urine of patients with UTI in selected teaching hospitals namely : University of Benin Teaching Hospital (UBTH), Irrua Specialist Teaching Hospital, Irrua (ISTH) and Igbinedion University Teaching Hospital, Okada (IUTH). A total of 3,378 urine samples were cultured into Blood agar, Cysteine lactose electrolyte deficient (CLED) agar and MacConkey agar and incubated aerobically at 37°C. Isolated *E. coli* had resistance of 38.5%, 43.9%, 36.4%, 38.5% and 52% to cefuroxime, ceftazidime, cefixime, cefttriaxone and cefotaxime respectively. Phenotypic detection of ESBL producing *E. coli* was done using the double disk synergism. ESBL- producing *E. coli* had prevalence of 1.5%, genomic DNA extraction was carried out on the *E. coli* that were ESBL positive and hybridization effected using specific primers for the assayed genes. The BlaTEM, BlaCTX, BlaOXA and BlaSHV genes had prevalence of 33.3%, 53.3%, 6.7% and 6.7% respectively. This study has established the presence of these resistant genes among the *E. coli* isolates from patients with urinary tract infections.

Keywords: *Extended spectrum beta-lactamase, E. coli***Introduction**

Extended-spectrum beta-lactamases (ESBLs) are a large and rapidly evolving group of enzymes able to hydrolyse oxyimino-cephalosporins and monobactams, which can be inhibited by clavulanate, sulbactam or tazobactam (Paterson and Bonomo, 2005). ESBLs are responsible for mediating resistance to beta lactams and their accurate detection should be a solution to hampering resistance in infections. These enzymes vary in their substrate affinities and catalytic efficiencies, whilst beta lactams differ in their penetration rates into bacterial cells (Bradford, 2001; Kaye *et al.*, 2004). The beta lactamase genes are located on the large plasmids that confer resistance to other classes of antimicrobial agents and are readily transmissible from strain to strain and between different species of enteric gram-negative bacilli (Paterson and Bonomo, 2005).

The epidemiology of ESBL genes is changing rapidly and shows marked geographic differences in distribution of genotypes of blaCTX-M β-lactamases (Hawkey and Jones, 2009). The most prevalent ESBL types have evolved through point mutation of key amino acid substitution in the parent TEM and SHV enzymes (Gniadkowski, 2001) and others are mobilized from environmental bacteria. The selection pressure that drives the emergence of ESBL has actually been attributed to the intense use of oxyimino beta lactams mainly the third generation cephalosporins (Madeiros and Crellin, 1997). The tourist traffic as a way of life experienced in the state is a predisposing factor to abuse of life style and ultimately abuse of antibiotics.

Aim of The Study

The aim of this study is to determine the antibiotic susceptibility pattern of ESBL-producing *Escherichia coli* and the genes responsible for resistance.

Study Areas

This study was conducted in three selected hospitals located in the three senatorial districts of Edo state namely: University of Benin Teaching Hospital, Benin City, Irrua Specialist Teaching Hospital Irrua and Igbinedion University Teaching Hospital, Okada. However, the State has a high presence of residents from across the country and the world because of its cosmopolitan tendencies. Benin City the capital has a history of being one of the foremost destinations of Europeans during their exploration of the African continent many centuries ago (Ogbeifun, 2006), this is a reflection of their occupation which is mainly tourism and hospitality.

Study Design

The study was based on case report involving patients with urinary tract infection, no control group is involved. Participants are In and Out - patients attending the hospital within the month of March 2018 to September 2019.

Sample Size Determination

The sample size of this study was determined using the statistical formula adopted by Fischer (2004).

$$n = \frac{z^2(p)(1-p)D}{c^2}$$

Where:

n = Estimated sample size

z = value of 95% confidence level = 1.96 at 5% level of confidence

p = Prevalence rate of ESBL taken as 56% = 0.56

c = confidence interval

D = Design effect which is usually 1

For present study z value at 95% confidence level is 1.96

Existing prevalence from other study (Egbebi and Famurewa, 2011) = 56% (0.56)

Confidence interval = 5% (0.05)

$$n = \frac{1.96^2(0.56)(0.44)}{(0.05)^2}$$

$$n = 0.946 \div 0.0025 = 378$$

10% attrition of the estimated sample size is calculated and added to the sample size. This is to take care of wrong sample collection

: 10% of 378

$$= 37.8 \text{ approx. } 38$$

$$31 + 305$$

Total estimated sample size = 416

A total of three thousand three hundred and seventy-eight (3,378) urine samples were collected from the three hospitals. All samples were transported to Medical Microbiology Laboratory, Igbinedion University Teaching Hospital, and Okada in ice pack bags and were analyzed immediately.

Sample Collection

Mid-stream urine samples were collected into sterile urine bottles from each of the patients with the history of urinary tract infection at the designated foci. Samples were collected into sterile, labeled universal containers after informed consent. All patients on antibiotics prior to the commencement of the research were included likewise patients with impaired renal or on catheter were included.

Screening of Urine for Bacteria

Culture method described by Chessbrough (2010) was used for the isolation of bacteria. Briefly, mid-stream urine was cultured on blood agar (BA), MacConkey agar (MCA) and Cysteine lactose electrolyte deficient (CLED) plates. All cultures were incubated aerobically at 37°C for 24 hours.

Identification of Bacterial Isolates

All bacterial isolates were subjected to taxonomic analysis by the determination of their morphological, physiological and biochemical characteristics as adopted by previous authors (Chessbrough, 2010; Cowan, 1999; Fawole and Oso, 1988; Holt *et al.*, 1994).

Susceptibility Testing

In vitro susceptibility of the pure bacterial

species to twelve different antibiotics was determined using Kirby-Bauer disk-diffusion technique (Bauer *et al.*, 1996; CLSI, 2012).

Phenotypic detection of ESBL-producing *E. coli*:

In accordance with CLSI, (2012), antibiotics disk of ceftazidime, cefoxime, ceftriaxone and amoxicillin/clavulanic acid were used for the screening of ESBL production by phenotypic method. The test was performed by the DDDT and DDST as previously described (Chikwendu *et al.*, 2011; Jarlier *et al.*, 1988; Kumar *et al.*, 2014). Phenotypic detection of ESBLs was defined by an increase ≥ 5 mm in the inhibition zone around clavulanic acid. *E. coli* ATCC 35218 was used as positive ESBL producer.

Singleplex PCR for TEM, CTX, SHV and OXA10

Nineteen (19) positive ESBL- producing *E. coli* isolates obtained were tested for the presence of TEM, CTX, SHV and OXA 10 genes. Using the PCR techniques and methods described previously (Hasman *et al.*, 2005; Mesa *et al.*, 2006; Olesen *et al.*, 2004), the Deoxyribonucleic Acid (DNA) was extracted from phenotypic ESBLs – positive *E. coli* strains by boiling the organism in a broth and centrifuging at 10,000rpm for 5minutes and dried for 10 minutes. Purified DNA is then amplified in an Eppendorf master cycle; samples were separated using 1.5% agarose gel electrophoresis and viewed under UV light using a Clinx photo documentation system at appropriate ladder as molecular weight marker.

Statistical Analysis

The distribution of isolates and resistant genes computed, chi square and significant values were

evaluated using SPSS version 23 software. Level of significance was $p < 0.05$.

Results

A total of 3,378 urine samples were cultured from three locations, that is University of Benin Teaching Hospital, Irrua Specialist Teaching Hospital, Irrua and Igbinedion University Teaching Hospital, Okada, Edo state. Results obtained had *E. coli* having a prevalence 34% (Table1). Other pathogens isolated are *Klebsiella* species, *Citrobacter* species, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis* and *Candida* species, with varying degree of prevalence. The chi square computed for the various locations had p value < 0.0001 indicating an association of *E. coli* with urinary tract infections.

The susceptibility profile for the antimicrobial agents as shown in Table 2 had Cefotaxim, cefuroxime, ceftazidime and cefixime having resistance of 52%, 38.5%, 43.9% and 36.4% respectively.

The prevalence of ESBL producing *E. coli* is depicted in Table 3 with a prevalence of 1.5%. ESBL- producing *E. coli* did not show an association with regard to location and is not statistically significant.

Table 4 shows the distribution of resistant genes possesses by the ESBL – producing *E. coli*. The genes: BlaTEM, blaSHV, blaOXA10 and BlaCTX had prevalence of 33.3%, 6.7%, 6.7% and 53.3% respectively. Plates 1, 2, 3, 4 and 5 are gel plates for the resistance genes assayed (see appendix).

Table 1: Distribution of Microbial Isolates

Isolates	N= 3378		Prevalence	X ²	p- value
	Positive n (%)	Negative n (%)			
<i>Klebsiella</i> sp.	146 (4.3)	3,232 (95.7)	7.4		
<i>Citrobacter</i> sp	47 (1.4)	3331 (98.6)	2.4		
<i>S. aureus</i>	311 (9.2)	3067 (90.8)	15.9		
<i>S. epidermidis</i>	57 (1.7)	3321 (98.3)	2.9		
<i>E.coli</i>	1180 (34.9)	2,198 (65.1)	60.2	1273.7	< 0.00001
<i>S. faecalis</i>	6 (0.2)	3,372 (99.8)	0.3		
<i>C. albicans</i>	213 (6.3)	3,165 (93.7)	10.9		

Table 2: Susceptibility profile of *Escherichia coli* showing resistance to antimicrobial agents

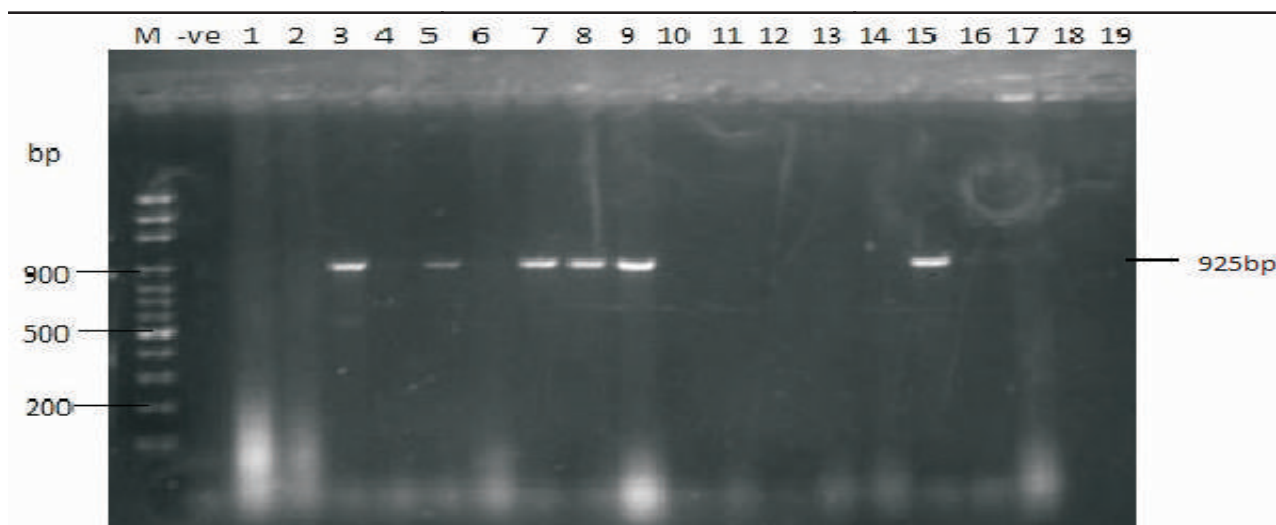
Antimicrobial	N	(%)
Cefuroxime	505	42.8
Gentamycin	416	35.3
Ofloxacin	257	21.8
Ceftazidime	566	50
Cefixime	457	38.7
Augmentin	811	68.7
Ciprofloxacin	260	22
Cloxacillin	1093	92.6
Ampicillin	1120	94.9
Nitrofurantoin	88	7.5
Ceftriaxone	464	39.3
Cefotaxime	673	57

Table 3: Distribution of ESBL- producing *Escherichia coli*

No of isolates	ESBL Positive N (%)	ESBL Negative N (%)	Prevalence
1120	17 (1.5)	1103 (98.5)	1.5

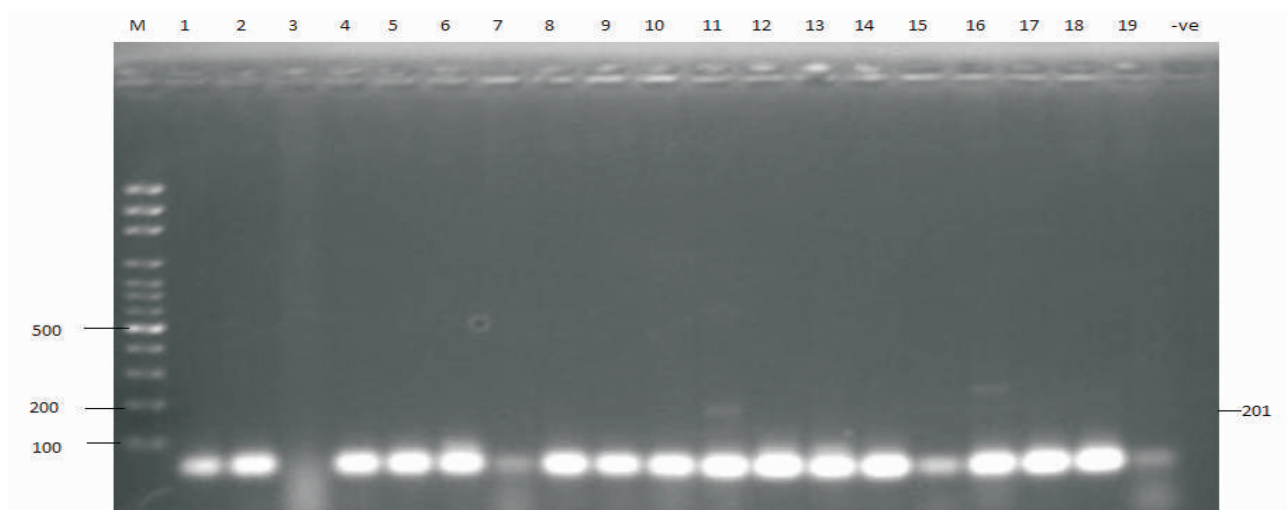
Table 4: Prevalence and distribution of genes in extended-spectrum beta-lactamase producing *E. coli*

Resistance genes	N = 15	Prevalence
blaTEM	5	33.3
blaSHV	1	6.7
blaOXA 10	1	6.7
BlaCTX	8	53.3



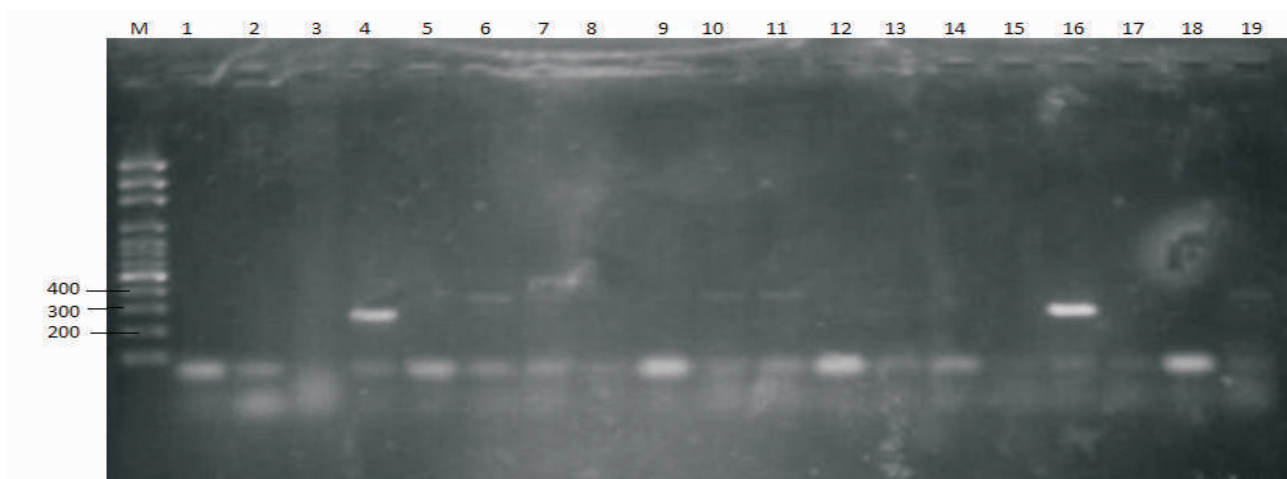
Only samples 3, 5, 7, 8, 9, and 15 showed the presence of the blaTEM gene

Plate 4.2: Identification of BlaTEM gene



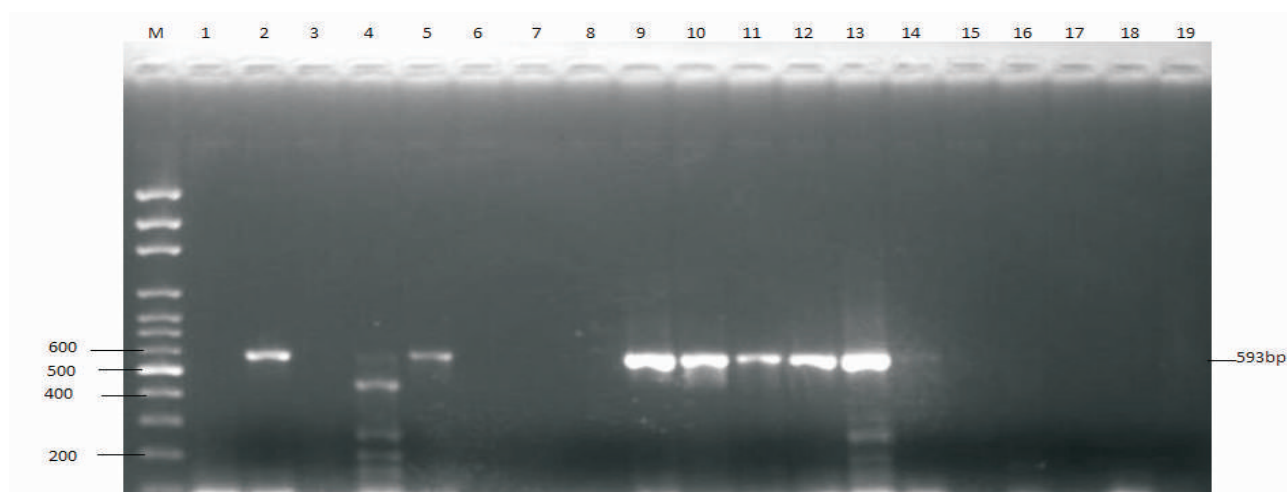
Only sample 11 showed an amplicon size of 201bp corresponding to the presence of *blaSHV* gene. Sample 16 also showed amplification but with an amplicon size of about 250bp.

Plate 4.3: Identification of *BlaSHV* genes



Samples 4 and 16 showed an amplicon size of 276bp corresponding to the presence of *blaOXA 10* (Oxa group 1). While samples 6, 10, 11, and 19 shows an amplicon size of about 400bp.

Plate 4.4: Identification of *BlaOXA* gene



Samples 2, 4, 5, 9, 10, 11, 12, 13, and 14 were all positive for *blaCTX*.

Plate 4.5: Identification of *BlaCTX* genes



Discussion

Antibiotic resistance has become a menace in Nigeria, as it has rendered the use of certain antibiotic in the treatment of certain infections ineffective. In the past decade, ESBL producing Gram negative bacilli, especially *E. coli*, have emerged as serious pathogen in suspected Urinary tract infections (Azekhueme *et al.*, 2005; Behroozi *et al.*, 2010; Bhatt *et al.*, 2012; Parajuli *et al.*, 2017).

A total of 3,378 non repetitive MSU samples were collected from three different locations within Edo state were analyzed in this study. *Escherichia coli* accounted to 60.2% as the most prevalent pathogen implicated in UTI (Table 1). This accords with other studies producers (Ebie *et al.*, 2001; Hooper, 2005; Omonigho *et al.*, 2001; Pullukon *et al.*, 2007). However, two earlier studies in Benin city (Urban settlement) Nigeria indicate *Staphylococcus aureus* as the predominant isolate (Omoregie and Eghafona, 2009; Omoregie *et al.*, 2008). These studies however were on asymptomatic patients as compared to this current study which was on patients presenting with symptoms of UTI.

Similarly, Obiogbolu *et al.* (2009) in his study in Akwa metropolis, South Eastern Nigeria also reported *E. coli* having a high incidence of 54% among patients with UTI. Likewise, in a study conducted by Jumbo *et al.* (2011), *E. coli* also accounted for 52.5% incidence rate at the Imo State University Teaching Hospital, Orlu.

Other pathogens isolated in a fairly high prevalence include *S. aureus* and *Klebsiella* species with varying frequency of occurrence, the similarities and differences in the type and distribution of uropathogens in the current study and other studies conducted thus far in different countries may be due to source of the specimens, patients involved and the various environmental conditions and host factors. Standard hygiene practices, education, population and the socioeconomic standards of the different sources of specimen collected may be responsible for the differences observed in uropathogens from the different studies (Ani and Mgbechi, 2008).

Antimicrobial resistance is now accepted as a major problem in public health and patient care (Shankar *et al.*, 2002). It is more troublesome to developing countries (Shankar *et al.*, 2002). In

this study, *E. coli* was highly resistant to ampicillin (94.9%) and least resistant to nitrofurantoin (7.5%) (Table 2). This is similar to report of different studies (Bhandari *et al.*, 2016; Neupane *et al.* 2016). Nitrofurantoin is a synthetic nitrofurantoin antimicrobial agent that has been in use for more than 50 years and continues to be effective for the treatment of uncomplicated UTIs in the ambulatory setting (Hooper, 2005). A low resistance to nitrofurantoin (7.5%) are in tandem with previous studies carried out in Kwara and Kaduna State (Aziz *et al.*, 2006 and Haruna *et al.*, 2014) and also consistent with other studies in South east Nigeria (El-Astal, 2005; Jumbo *et al.*, 2011; Uwaezuoke and Ogbulie, 2006), and other part of Africa; Cameroon (Mokube *et al.*, 2013), Ethiopia (Tessema *et al.*, 2007; Yismaw *et al.*, 2012), all having a low of resistance rate of about 10% to Nitrofurantoin. The low resistance to nitrofurantoin may be attributed to its ability to achieve very high urine concentrations (Rajesh *et al.*, 2011). Likewise surveys in the USA and Canada on *E. coli* urinary isolates found 1.1% resistance (Zhanel *et al.*, 2006) which is similar to the resistance rate in France which was 1.8% among *E. coli* urinary isolates (Honderlick *et al.*, 2005), studies done have also found the antibiotic effective, *in vitro* against *E. coli* strains, that are ESBL- producers (Hooper, 2005; Pullukon *et al.*, 2007). These results suggest that nitrofurantoin is a suitable, effective, and cheap alternative drug in the treatment of ESBL-producing *E. coli* related UTI (Tasbakan *et al.*, 2012). Likewise, susceptibility results of ciprofloxacin and ofloxacin seen in this study had resistance of 22% and 21.8% respectively. The quinolones are gradually becoming resistant due to their excessive pressure prevalent in an environment in which antibiotics are freely available without restriction (Babalola and Lamikanra, 2002; Manjunath *et al.*, 2011). Moreover, resistant to third generation cephalosporins as exhibited by ESBLs often co-exists with resistance to other antibiotics (Hanberger *et al.*, 2013).

Identifying ESBL producing organism is a major challenge in clinical settings and due to the selective pressure caused by heavy use of expanded spectrum cephalosporins, lapses in effective infection control measures and affinity of these enzymes for different substrates, outbreaks are increasing (Shankar *et al.*, 2002).

The prevalence of ESBL producing *E. coli* varies among countries likewise in locations. In this study, the prevalence of ESBL-producing *E. coli* was 1.5% as shown in Table 3. This low prevalence is in accordance with previous studies (Akujobi and Ewuru, 2010; Iroha *et al.*, 2008; Khuruna *et al.*, 2002) done in India, Nigeria (Calabar and Nnewi) and likewise in Europe recorded by Arpin *et al.* (2009) and Rodriguez *et al.* (2009) had prevalence of 1.1% and 1.4% for studies done in France and Spain respectively and however in contrast to previous reports (Mathur *et al.*, 2002, Azekhueme *et al.*, 2005, Tankhiwale *et al.*, 2004 and Ogefere *et al.*, 2015) for studies carried out in Nagpur, Benin, and New Delhi (India) and Calabar respectively, with prevalence higher rates of 47%, 44.3%, 52% and 58% respectively among other Gram negative bacilli. This low prevalence recorded in this study may be due to non-usage of beta lactam group of antimicrobial but rather a wide spread administration of empirical drugs such as the quinolones whose mode of action is not on cell wall synthesis but rather inhibition of DNA gyrase, study design and variance in geographical distribution of clinical isolates.

A variety of enzymes were detected among the ESBL producing *E. coli* isolates, this is similar to results previously reported by Rodriguez *et al.* (2009) and Spanu *et al.* (2002). The most frequently encountered mechanism of resistance to beta lactam found in this study was the production of BlaTEM and BlaCTX genes. The prevalence of resistant genes harbored by the ESBL producing *E. coli* isolates constituting 33.3% and 53.3% of the genes from the location respectively. This result was in accordance with reports (Mojtaba and Behnaz, 2011; Olatunji *et al.*, 2016) in which BlaTEM and BlaCTX were the predominant genes isolated from uropathogenic *E. coli*. However low prevalence was observed in BlaSHV and BlaOXA 10 genes as shown in Table 3.

The blaCTX-M group is unique among ESBL genes. CTX-M was initially identified in 1989 and its pandemic (CTX-M) has changed the prevalence of ESBLs, and it has rapidly become the dominant ESBL worldwide (Canton and Coque, 2006). Previous reports suggest that the CTX-Ms had several different environmental origins, in contrast to TEM and SHV, with single ancestors (Barlow *et al.*, 2008). CTX-M not only is associated with nosocomial outbreaks but is

also mostly found in community acquired infections. The high degree of blaCTX genes in this present study could have two potential origins: the cosmopolitan nature of the environment or selective pressure on a particular generation of cephalosporin class of antibiotic.

Conclusion

Extended - spectrum beta-lactamase-producing *E. coli* plays a major role in drug resistance, which sometimes results to therapeutic failures, economic waste and mortality. The presence of BlaTEM, BlaCTX, BlaSHV and BlaOXA genes in the isolated *E. coli* strains indicates a potential to possible spread to other bacteria. According to the results of this study, *E. coli* contributes a significant rate of infection as compared to other organisms; the use of appropriate antibiotic will help in its treatment as well as preventing the spread of antibiotic resistant strains.

Likewise, it was observed that *E. coli* possess resistant genes thereby conferring resistance to the cephalosporin class of antibiotic hence emphasis should be placed on other classes of antibiotic such as flouoroquinones and the nitrofurantoin in the treatment of UTI since they exhibit minimal resistance rate.

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Conflict of interest

There is no conflict of interest.

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