ORIGINAL PAPER

https://dx.doi.org/10.4314/njpr.v17i1.13

Nig. J. Pharm. Res. 2021, 17 (1) pp 119-129
ISSN 0189-8434 **e-ISSN 2635-3555** **Available online at http://www.nigjpharmres.com**

Molecular Characterization of Extended Spectrum Beta – Lactamase Producing *Escherichia Coli* Isolated from Pregnant Women with Urinary Tract Infections Attending Ante–Natal Clinics in Ilorin Metropolis

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article.

ABSTRACT

Background: The emergence of multidrug- resistance Enterobacteriaceae especially in *E. coli* bacteria associated with Urinary Tract Infections (UTIs) in pregnancy is a serious menace globally posing health challenges and confounding successful empirical treatment as well as increasing pregnancy – related complications.

Objectives: The aim of this study is to determine the phenotypic and genotypic characteristics of Extended Spectrum Beta – Lactamases (ESBLs) producing *E. coli* (ESBLs – EC) isolates in pregnant women attending ante – natal clinics within Ilorin - Kwara State, Nigeria.

Materials and methods: A total of 53 non - repeated *E. coli* isolates from urine samples of pregnant women were presumptively identified using standard bacteriological method and confirmed by commercially available Microgen® Identification Kits. Phenotypic detection of ESBLs was determined using antibiotics susceptibility test and double disc synergy Method for screening and confirmation respectively. Polymerase Chain Reaction (PCR) was further used for the genotypic detection of ESBLs genes.

Results: A total 88.67% (47/53) of *E. coli* exhibited resistance to the cephalosporins of which aztreonam was the highest (75.47%) and the least was cefpodoxime (35.84%) while 85.10% were confirmed positive for ESBL production. The genotypic detection showed the most occurring genotype was bla_{TEM} (50%) bla_{OXA} (27.7%), bla_{GES} (22.5%), bla_{SHV} (15%), bla_{CTXM} and bla_{VEB} (7.5%) while sixty – four (64%) of isolates co – harbored two or more gene. Bla_{TEM} and bla_{OXA} were dominant.

Conclusion: This study showed high resistance of *E. coli* to the third generation cephalosporins harboring different ESBL genes which increases UTIs complexity and limit therapeutic options in pregnancy. Therefore, continuous monitoring of resistance in *E. coli*, effective appraisal of antibiotic control policies and rational use of antibiotics is therefore encouraged.

Keywords: Extended Spectrum Beta - Lactamases, *E. coli*, pregnant women

INTRODUCTION

Antimicrobial resistance among *E. coli* associated with UTIs is currently a major cause of increasing antimicrobial resistance (Mukherjee *et al.*, 2013). *E. coli* accounts for about 80 – 90% of causal agent in community acquired Urinary Tract Infections (UTIs), 50% of nosocomial UTIs (Flores-mireles *et al.*, 2015) and up to 75% - 90% uncomplicated UTIs in pregnancy (Matuszkiewicz-Rowińska *et al.*, 2015). This is associated with irrational and excessive use of the first – line drugs such as the beta – lactam antibiotics use in the empirical treatment of UTIs (Bischooff *et al.*, 2018), which has led to an alternate shift to the use of broad and extended-spectrum cephalosphorins (Thakuria and Lahon, 2013). The consequent increased resistance among these antibiotics was ascribed to the production of beta-lactamases such as the Extended Spectrum Beta – Lactamases (ESBLs) among *E. coli* which has contributed remarkably to the high level prevalence of UTIs observed globally (WHO, 2015; Munita and Arias, 2016).

Extended Spectrum Beta - Lactamase enzymes are hydrolyzers of antibiotics such as penicillins, cephalosporins (first, second and third-generations) and aztreonam via destruction of their beta – lactam rings and as a result reducing susceptibility hence conferring resistance but their activities are inhibited *in - vitro* by inhibitors such as clavulanate, tazobactam and sulbactam (Bradford, 2001). These enzymes have been detected and reported in a wide range of Gram-negative Bacilli such as *Pseudomonas aeruginosa*, Enterobacteriaceae (*Klebsiella, Proteus, Salmonella* species) and *E. coli* (Rahman *et al.*, 2018).

Globally, the recent emergence of rapidly disseminating *E. coli* isolates which are resistant to extended-spectrum cephalosporins are of serious concern (Bradford, 2001) capable of acquiring and dissemination of resistance primarily via horizontal genes transfer (Munita and Arias, 2016). Others acquires resistance via mutation of existing genes either by deletion, addition, substitution of genes as well as via vertical gene transfer (Paterson and Bonomo, 2005; (Munita and Arias, 2016). Majorly encoded and commonly described in clinical and environmental settings include $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{OXA} and bla_{SHV} (Paterson and Bonomo, 2005). Other uncommon ESBL genes include bla_{VEB}, GES, BES -1, PER, SFO, BEL and TLA (Naas *et al.*, 2008) which are capable of increasing disease complexity, human and economic burden (Medina and Castillo-pino, 2019).

Several phenotypic detection of ESBLs among Enterobacteriaceae from different samples have been conducted by some researchers in Ilorin and different parts of the country with little or no attention on molecular studies (Igwe *et al.*, 2014; Yusuf *et al*., 2017; Faari *et al.*, 2015; Olowo-okere *et al*, 2018 Sa'adu *et al.*, 2019 and Amadu *et al*., 2019). The existing paucity of documented information in Ilorin, Kwara state necessitated this study, which seeks to determine the prevalence of ESBLs producing *E. coli* (ESBL – EC), establish their antibiotics resistance profile of and molecularly characterized the genotypes of these ESBL - EC associated with UTIs among pregnant women within Ilorin. This will provide appropriate information to clinicians that will enhance empirical treatment of UTIs thus improving general wellbeing.

METHODOLOGY

Study Area

This study was conducted within Ilorin Metropolis. Ilorin Metropolis consists of three Local Government Areas namely; Ilorin West, Ilorin East and Ilorin South as indicated (Figure 1). According to the National Population Commission (NPC) has a land size of about 150sq km and a population of 777,667 inhabitants (NPC, 2010). The metropolis according to the Kwara State Ministry of Health (KMOH) harbors three categories of hospitals namely; tertiary, secondary and primary (public or private) health centers (KMOH, 2019).

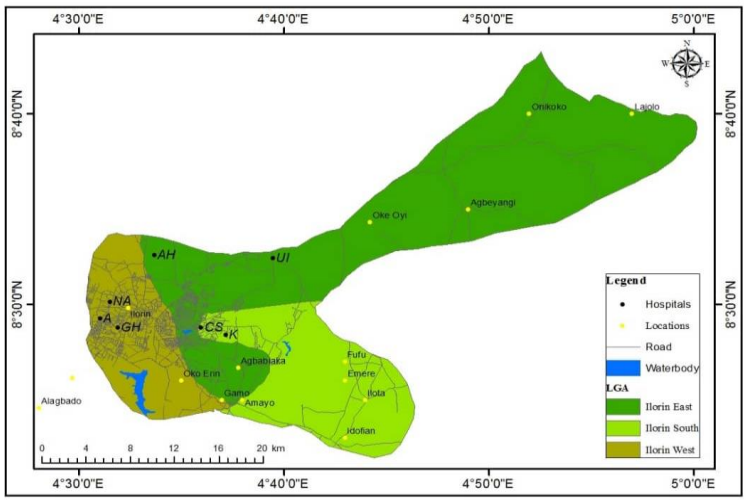

Figure 1. Map of Ilorin metropolis showing the different Local Government Areas and health centers (Map generated from Map Library, 2019 and coordinates obtained using Garmin ETrex 10).

Sampling techniques and study population

Purposive and proportionate convenient sampling techniques were used for the selection of health facilities with ante – natal clinics and pregnant women respectively. A cross-sectional study of pregnant women attending selected antenatal clinics in selected health centers within Ilorin metropolis, Kwara State was conducted. From the three categories of hospitals (Tertiary, Secondary and Primary), seven (7) centers with antenatal clinics were selected for the study. Only pregnant women who were not on antibiotics within the time of recruitment and those who were willing were conveniently recruited for the study.

Sample collection and Identification of bacterial Isolates

A total of 420 urine samples were collected from 7 (seven) selected ante – natal clinics between February, 2018 to April, 2019. Mid – stream urine samples were collected in wide – mouthed sterile universal containers (Tadesse *et al.,* 2014). Description on how to collect samples and the need for prompt delivery was emphasized to participants. On delivery, urine samples were carefully labeled and transported in cold chain to the Pharmaceutical Microbiology and Biotechnology Laboratory, University of Ilorin for immediate analysis. The urine samples were cultured using standard bacteriological methods (Cheesbrough, 2006) and identification was performed using Microgen® GN-ID A + B Kits (Microgen Bioproducts Ltd., Camberley, UK) (Bello *et al.,* 2020).

Bacterial Strains

A total of 53 (26.06%) of non – duplicated isolates of *E. coli* isolates were obtained from all urine samples. These include UI (n=10), NA (n =17), AH (n =10), A (n =2), CS (n =2), K (n=5) and GH (n = 7) were used in this study. All identified *E. coli* isolates were stored in cryovials containing 25% glycerol (Invitrogen, USA) and Luria-Bertani broth at −40°C until needed.

Phenotypic detection of Extended Spectrum Beta – Lactamases

Screening test was performed using the Disc diffusion antibiotics susceptibility testing method as described by Clinical and Laboratory Standard Institute (CLSI, 2017). A 24 hours freshly sub - cultured *E. coli* isolates were emulsified into 5mL saline water, turbidity were adjusted to 0.5 Mafarland turbidity and inoculated on MHA plates. Discs of ceftazidime (30 µg), cefpodoxime (10 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and aztreonam (30 µg) were carefully placed at equidistance on the inoculated MHA plates. Prepared plates were allowed to pre – diffused for 45 minutes and incubated at 37°C for up to 24 hours.

After 18 hours incubation, zones of inhibition were carefully measured in millimeter using a meter rule. Bacteria isolates were classified according to standard recommendations of Clinical Laboratory Standard Institute (CLSI, 2017). *Escherichia coli* showing zones of inhibitions of $\leq$ 17mm, 22mm, 27mm, 25mm and 27mm for cefpodoxime (10 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and aztreonam (30 µg) respectively were regarded as ESBL positive and subjected to confirmatory test.

Furthermore, confirmatory test was performed using the Double disk synergy test (DDST) (CLSI, 2017). Aseptically, prepared 0.5 MaFarland turbidity of *E. coli* isolates were prepared and inoculated on MHA

plates and allowed to stand for 5 minutes. Discs of amoxicillin-clavulanic acid (30µg) was placed centrally on the MHA plates and ceftazidime (30 µg) and cefotaxime (30 µg) were carefully placed at a distance of distance of 15 mm away from the central disc using a sterile forceps. Plates were prepared in duplicates and *E. coli* ATCC 25922 was used as a negative control. Test plates were allowed to pre – diffused for 45 minutes and incubated at 37ºC for up to 24 hours. After 18 hours incubation, the presence of key – hole effect was considered positive for ESBLs with any isolate that has increased zone of inhibition around the cephalosporin antibiotic discs expansion towards the center disc of amoxicillin-clavulanic acid.

Genotypic Detection of Extended Spectrum Beta – Lactamases

The genomic DNA of ESBL producing *E. coli* were extracted using bacteria genomic DNA extraction Kit (Norgen Biotek Corporation, Canada) for the presence of ESBLs genes using standard PCR (Bubpamala *et al.,* 2018). The detection ESBL genes was performed using the primer sequences as indicated in Table 1.

Table 1: Selected primers used for Polymerase Chain Reaction

Primer Name	Target Genes	Amplicon size (bp)	Primer Sequence (5'- 3')	References
TEM – F	bla_{TEM}	700	ATTCTTGAAGACGAAAGGGC	Zhao *et al.,* 2018
TEM – R			ACGCTCAGTGGAACGAAAAC	
OXA – F	bla_{OXA}	650	ACACAATACATATCAACTTCGC	Ferreira *et al*., 2011
OXA – R			AGTGTGTTTAGAATGGTGATC	
CTXM-F	bla_{CTXM}	870	TCTTCCAGAATAAGGAATCCC	Mshana *et al.,*2016
CTXM-R			CCGTTTCCGCTATTACAAAC	
SHV –F	bla_{SHV}	850	CACTCAAGGATGTATTGTG	Zhao *et al.,* 2018
SHV –R			TTAGCGTTGCCAGTGCTCG	
GES – F	bla_{GES}	900	ATG CGC TTC ATT CAC GCA C	Bubpamala *et al.,* 2018
GES – R			CTA TTT GTC CGT GCT CAG G	
VEB – F	bla_{VEB}	570	CGA CTT CCA TTT CCC GAT GC	Bubpamala *et al.,* 2018
VEB – R			GGA CTC TGC AAC AAA TAC GC	

A 25 µl volume reaction mixture containing 12.5 µl of the master mix (New England Biolabs ®), 0.5 µl of the forward and reverse primers each, 5 µl of the template DNA and 6.5 µl of the nuclease free water. For the six (6) genes, initial denaturation was achieved at 94◦C for 3 minute for 1 cycle while denaturation at 94◦C for 1 minutes for 30 cycles. For the *bla*TEM, *bla*CTX-M and *bla*OXA genes annealing was set at 53.70◦C, 53.20 and 53.20 respectively. While *bla*SHV genes at 57◦C, *bla*GES at 56.10◦C and *bla*VEB at 55.40◦C for 1 minute and 35 cycles. Furthermore, initial and final elongation were set at 68◦C for 1 and 5 minutes of 35 cycles respectively.

Finally, the PCR products were elucidated on a 1% agarose gel electrophoresis at 100mV for 45 minutes using PCRSizer (100 – 1000bp) DNA molecular ladder (Norgen Biotec®) and bands images were viewed using the UV- Gel documentation unit (Pavez *et al.,* 2019).

Statistical analysis

Data obtained were analyzed using descriptive statistics such as frequency, percentages, bar chart and tabular presentations.

RESULTS

Phenotypic detection of Extended Spectrum Beta – Lactamases

Fifty- three (53) non- repeated uropathogenic *E. coli* isolates isolated from pregnant women attending selected antenatal clinics within Ilorin metropolis exhibited varying degree of resistance to different cephalosporins used in accordance to the CSLI guideline (Figure 2). The screening test showed that 47 (88.67%) of *E. coli* isolates exhibited resistance to different cephalosporins with highest resistance observed with aztreonam 40 (75.47%), followed by cefotaxime 37(69.81%), ceftazidime 33(62.26%), ceftriaxone 30 (56.61%) and cefpodoxime 19 (35.84%). Five (5) strains (13KB, 78NA, 83UI, 83A and 27GH) were susceptible to all the antibiotics tested.

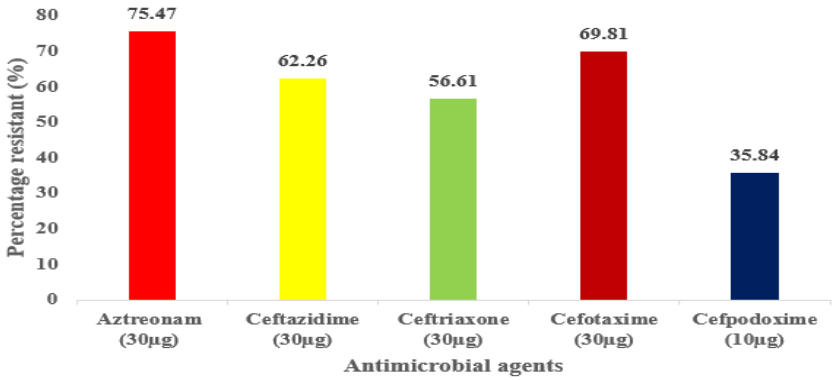

Figure 2. Resistance profile of Uropathogenic *E. coli* against selected antimicrobial agents.

Confirmatory Test

Plate 1 displayed positive (A) and negative (B) plates of ESBL – EC confirmed using DDST. Percentage distribution of ESBLs showed that 40 (85.10%) and 7 (14.89%) were confirmed positive and negative respectively (Figure 3).

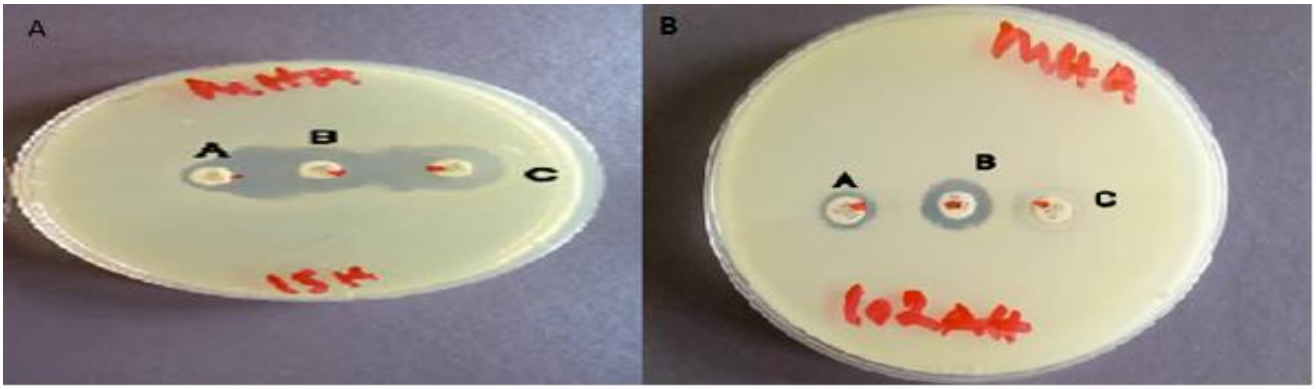

Plate 1. (A) Positive plate showing the key – hole inhibition **(B)** negative plates of ESBL – EC by DDST method**.**

Keys:

A - Cefotaxime (30µg)

B - Amoxcillin clavunic acid (20:10 µg)

C - Ceftazidime (30 µg).

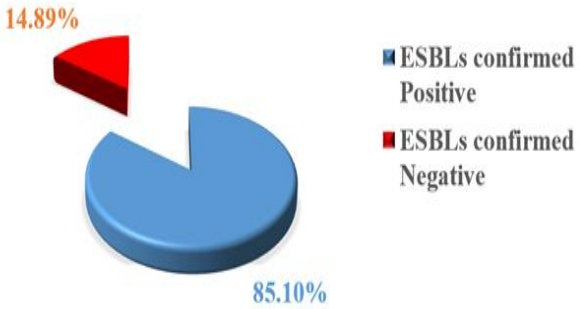

Figure 3: Percentage distribution of ESBL – EC confirmed by DDST.

Genotypic detection of Extended Spectrum Beta – Lactamases

Plate 2 shows a gel image of the ESBL genes, expressed at at varying base pair (bp). Of all the 40 (85.10%) phenotypic confirmed ESBLs – EC, 25 (62.5%) carried blaTEM, SHV, CTXM, OXA, GES and VEB respectively. The result showed that blaTEM was the most prevalent 50% (20/40), followed by blaOXA 27.5% (9/40), blaGES 22.5% (9/40), blaSHV 15% (6/40) and blaCTXM/VEB 7.5% (3/40) as indicated (Figure 4). Furthermore, 64% (16/25) of isolates co – harbored two or more genes, with bla_{TEM} and OXA accounting as the most predominant combined genotypes 20% (5/25), TEM and GES 8% (2/25), TEM, GES and OXA 8% (2/25) as well as 4% (1/25) each for other observed multiple genotypes (Table 2).

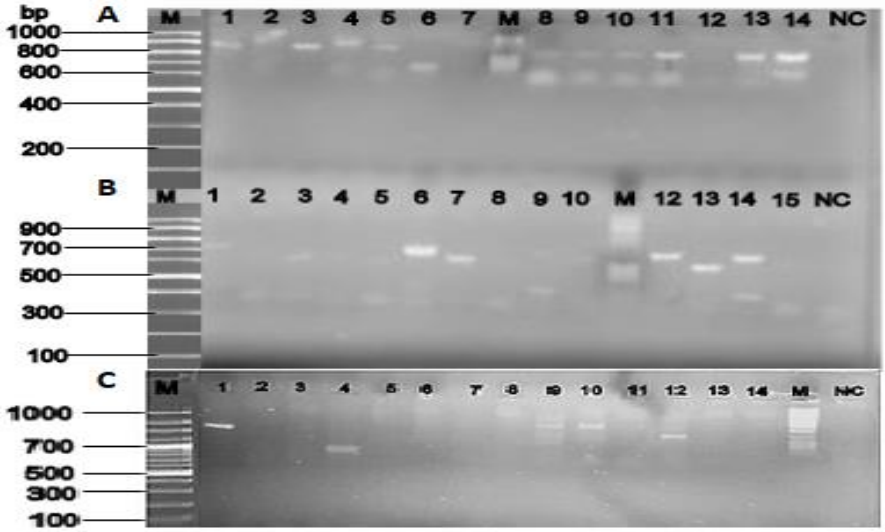

Plate 2. Image of agarose gel amplification of PCR products for selected ESBL genes

Keys:

A: Lane 2, 4 – GES; 1, 3, 5 – CTX-M; L 6 – VEB; L 8 -11, 13, 14 – TEM.

B: L6 –TEM; L7, 12, 14 – OXA; L13 – VEB.

C: L1, 9 -10 – SHV; L4 -VEB and L12 -TEM.

M: Molecular Ladder

NC: Negative control

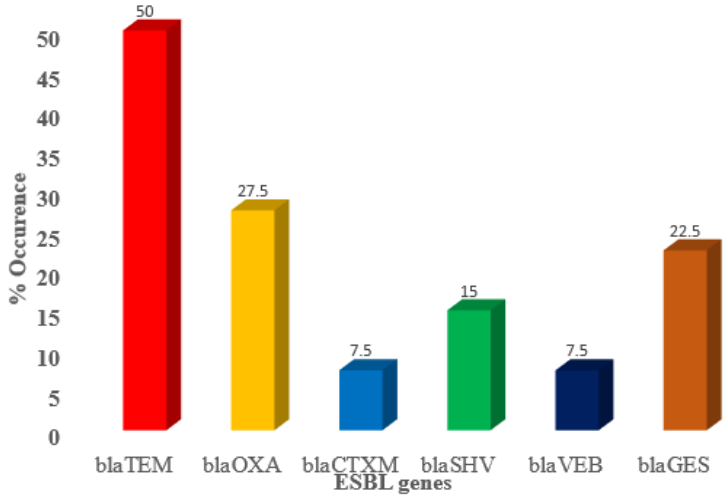

Figure 4: Occurrence of selected ESBL genes within Ilorin metropolis

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Table 2: Genotypic resistance pattern of uropathogenic ESBL - EC

Resistant Genotypes						F	%
TEM						7	28
SHV						1	4
OXA						1	4
TEM	GES					2	8
TEM	OXA					5	20
GES	OXA					1	4
GES	SHV					1	4
TEM	GES	OXA				2	8
TEM	CTXM	SHV				1	4
TEM	VEB	SHV				1	4
TEM	VEB	GES				1	4
GES	OXA	CTXM	SHV			1	4
TEM	VEB	GES	OXA	CTXM	SHV	1	4
TOTAL						**25**	**100**

DISCUSSION

In this study, high (88.67%) resistance to cephalosporins was observed among the Uropathogenic *E. coli*. This is in concordance with the 88.70% reports in Pakistan (Riaz and Bashir, 2015) and disagrees with the 59.7% reported by Hassuna *et al.,* (2020) among *E. coli* isolates associated UTI in Egypt. However, higher rate of 95. 71% of reported by Nwafia *et al.,* (2019) among *E. coli* isolated from clinical samples in Enugu – Nigeria. High resistance rates was observed among ESBLs - EC to aztreonam (75.47%), cefotaxime 37(69.81%) and the least was cefpodoxime (35.84%). This coincides with several with several reports (Al-Muhanna *et al.,* 2016; Alyamani *et al.,* 2017; Mahato *et al.,* 2019; Ghaddar *et al.,* 2020). This observed resistance to cephalosporins and monobactam is most likely due to selective pressure as a result of inappropriate use, misuse and hence the paradigm shift from first – line antibiotics to third generation cephalosporin antibiotics within Ilorin metropolis posing serious challenge to successful UTI empirical treatment. Furthermore, this shows the likelihood of ESBL – mediated resistance mechanism (Ghafourian, *et al.,* 2014) and underscores the importance of usage of a wide range cephalosporins in the phenotypic screening process as against the commonly used ceftazidime and cefotaxime.

In our study, 85.10% of uropathogenic *E. coli* were phenotypically confirmed ESBL –EC by double disc synergy test. This is in consistence with the 85% reported in Pakistan (Ishfaq *et al.,* 2017) and differs disagrees with the 23.60% was reported Maiduguri (Mohammed *et al.,* 2016) among uropathogenic *E. coli* isolated from pregnant women. However, increasing prevalence of ESBL – EC has been documented globally (Olowe *et al.,* 2015; Olowo-okere *et al.,* 2018; Tsaku *et al.,* 2019). This suggests that the varying ESBLs prevalence differ with different geographical locations, time, health facilities and vehicular sources of *E. coli*. Furthermore, the reliability, sensitivity, specificity, economic value in addition to the ease of use of this method premise its extensive use (Ishfaq *et al.,* 2017).

The high discriminative properties of molecular methods in eliminating false positive results was observed in our study. Of the 85.10% confirmed ESBL producing *E. coli*, 62.50% harbored the bla genes. This is similar to the of 62.34% reported in southeastern Nigeria (Ugwu *et al.,* 2020), but differs disagrees with lower rate 44.60% in Korea (Jeong *et al.,* 2004) and 40.3% Nepal (Pandit *et al.,* 2020) respectively among uropathogenic *E. coli*. This increasing emergence and development of ESBL genes is alarming and of global threats thereby preventing successful UTIs treatment and increasing human burden.

The high prevalence of bla_{TEM} (50%) ESBL genes observed in this study was in concordance to the findings of 47.8% among ESBL producing uropathogenic *E. coli* in India (Bajpai *et al.,* 2017). Higher occurrence of bla_{TEM} 75%, 83.8% and 93.47%

were documented among ESBL producing uropathogenic *E. coli* in Bhubaneswar, Nepal and Egypt respectively (Jena *et al.,* 2017; Pandit *et al.,* 2020; Hassuna *et al.,* 2020) and lower than 26.3% reported in Benue (Abba *et al.,* 2019). Bla_{TEM} are plasmid mediated resistant genes that has been reported among Enterobacteriaceae including *E. coli* (Rahman *et al.,* 2018) conferring resistance to the penicillin and cephalosporins (Bush and Bradford, 2016).

This study reported the detection of two (2) uncommon clinically described ESBL genes of bla_{VEB} (7.50%) and bla_{GES} (22.50%) and to the best of our knowledge reported for the first time in Ilorin -Nigeria. Although a low prevalence report of bla_{VEB} and GES have been reported from uropathogens in Anambra (Ugwu *et al.,* 2020) and bla_{VEB} in pigs and chickens in Nsukka – Southeastern Nigeria (Chah *et al.,* 2018). This supports the rare clinical descriptions of these genes belonging to the class A (Naas *et al.,* 2008). This suggest incursion of emerging ESBL genes in study area which may be attributed to mutational changes in genomic sequences of ESBL genes among ESBL – EC facilitated by continuous changes in antibiotics resistant trend, behavioral, demographic and environmental factors among pregnant women.

The detection of more than one ESBL genes among ESBL - EC was a common phenomenon in this study accounting for 64% of *E. coli* isolates co-harboring greater than two resistant genes. This vary from disagrees with of 56.51% and 88.37% co – habitation reported among ESBL – EC from pregnant women in Beirut and Bhubaneswa respectively (Jena *et al*., 2017 and Ghaddar *et al*., 2020). Furthermore, ten (10) different genotypes combinations were observed and the most dominant was $bla_{TEM/OXA}$ (20%). This high genotypes co-habitation contrast the findings of disagrees with Riaz and Bashir, 2015 and Ghaddar *et al*., 2020 which reported three and four different genotypes combinations. Although, the occurrence of bla_{TEM} and bla_{OXA} as the most dominant co-harbored genes is similar to the findings of Riaz and Bashir, (2015) however with a higher frequency of 44.2%. This differ to the findings of Nwafia, *et al.,* (2019) which states the highest genotype combination of $bla_{TEM/SHV/CTXM}$ (20%) among clinical *E. coli* isolated from a tertiary hospital in Nigeria as this scenario is not unlikely among the Enterobacteriaceae (Malik and Elhag, 2019). This genotypes complexity among these uropathogenic *E. coli* circulating within Ilorin metropolis could be the confounding factor militating against effective empirical treatment, management and control of *E. coli* associated UTIs in pregnant women.

CONCLUSION

High prevalence of ESBLs – EC isolates was observed within Ilorin metropolis characterized by isolates with multiple genotypes. Therefore, control policies such as providing antibiotic stewardship, regulating the use of cephalosporin and increased antibiotics surveillance should be encouraged.

ETHICAL CONSIDERATIONS

Ethical clearance was obtained from the Ethical Review Committee of the University of Ilorin Teaching Hospital and State Ministry of Health (UITH/CAS/ 189/19B /789 and MOH/ KS/EU/777/206). Prior to sample collection, permission was obtained from the Head, Department of Obstetrics and Gynecology housing the Ante – natal units. In addition, oral consent was obtained from the pregnant women that met the study criteria.

ACKNOWLEDGEMENTS

The authors wish to extend their sincere gratitude to the management of University of Ilorin Teaching Hospital, State Ministry of Health and private hospitals for helping out during sample collection. The authors wish to thank all the participating pregnant women for their support and corporation. The authors also like to appreciate all technical staff of the Department of Pharmaceutical Microbiology and Biotechnology for support and assistance throughout the study.

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Conflict of Interest: None declared
Received: February 04, 2021
Accepted: May 03, 2021