Original Research Article

bla TEM, _bla_ SHV and _bla_ CTX-M-15 Extended spectrum beta-lactamase produced by _Acinetobacter baumanii, Enterobacter clocae_ and _Proteus mirabilis_ from pregnant women in three secondary health care facilities in south-south, Nigeria.

Abstract

Background\purpose

Treatments options are limited due to bacterial resistance to antibiotics hence increase in morbidity and mortality, and raise the risk of treatment failure. From Fact there is high urinary tract infection and increasing treatment failure among pregnant women and this has led to increased mortality and morbidity among pregnant women, and increased stay in the hospital.

This study was conducted to evaluate the prevalence of antimicrobial resistance and distribution of *bla*TEM, *bla*CTX-M-15 and *bla*SHV genes among *A. baumannii* , *P. mirabilis* and *E. clocae* strains isolated from urine samples from pregnant women attending antenatal at three secondary health care facilities south-south Nigeria.

Methods:

A. baumannii, P. mirabilis and *E. clocae* strains were isolated and identified using Microbact 24E. The disc diffusion and combined discs methods were used for testing antimicrobial susceptibility .The presence of ESBL was detected using Double Disk Synergy Test (DDST) and CHROMagar respectively. Plasmid extraction was carried out following the protocol of ZR Plasmid Miniprep-Classic extraction kit. Finally, the frequency of resistant genes including *bla*TEM, *bla*CTX-M-15 and *bla*SHV in selected 50 ESBL producing isolates was studied by PCR and using designed primers**.**

Result

A total of 252 clinical isolates was collected from three secondary health care facilities in south-south, Nigeria. ESBLs were found in 231 (92%) isolates. *bla*CTX-M-15 was the commonest genotype (58.3%), followed by *bla*SHV (43.3%) and *bla*TEM (43.3%).

Conclusion

ESBL positive strains of *Enterobacter clocae, Acinetobacter baumannii* and *Proteus mirabilis* are increasingly found in isolates from pregnant women. The widespread use of antibiotics has caused shifts in bacterial development to overcome the existing mechanisms of combating

bacterial infections. These strains become resistant to frequently used antibiotics and they can pass the gene to other bacterial strains, the quick detection of these strains in clinical laboratories an essential step. The frequency of genes encoded ESBL isolates of *Enterobacter clocae*, *Acinetobacter baumannii* and *Proteus mirabilis* may be due to abuse and misuse of antibiotics.

Key Word: ESBL *bla*gene , PCR, UTI

INTRODUCTION

Currently, the challenge of gradually increasing resistance to antibiotics has affected the entire world. The hydrolysis and inactivation of beta-lactam antibiotics, through the production of beta-lactamase has been one of the most main resistance mechanisms of many bacterial species, especially in the family Enterobacteriace (Akcam *et al.,* 2004). Gram-negative pathogens is increasingly associated with ESBLs, hence resulting in the resistance to beta-lactam antibiotics (Kimura *et al.,* 2007).

ESBL positive enterobacterial species are widely disseminating throughout the world (Timko, 2004).The main reason for development of resistance is mainly the selection and preferential growth of resistant bacteria, together with inhibition of susceptible strains from prolonged use of antibiotic. Extended-spectrum -lactamases (ESBLs) were first described in the 1980s and they have been detected in Gram-negative bacilli (Kiratin *et al*., 2008; Cheng *et al*., 2008; Morris *et al*., 2003).
A typical mechanism of AMR is the production of extended-spectrum beta-lactamase. (ESBL) enzymes, which confer resistance to penicillins, cephalosporins, and monobactams, but not to cephamycins and
carbapenems (Paterson and Bonomo, 2005; Pitout *et al*., 2005).
Presently there is an increase in the emergence of ESBL producing bacteria. The increasing resistant to beta-lactam antibiotics used in treating urinary tract infections (UTIs) has made the treatment very challenging and frequently resistant to many of the antimicrobial agents recommended for the treatment of such infections (Ben-Ami R, Rodriguez-Bano *et al.,* 2009). Most ESBLs belong to the CTX-M, SHV (Sulfhydryl variable) and TEM (Temoniera) families. Due to the production of multiple enzymes such as the inhibitor-resistant ESBL variants and plasmid-borne AmpC, ESBL phenotypes have become more complex (Mohanty *et al*., 2010). Commercial available chromogenic media such as CHROMagar(Paris, France) have been used to detect ESBL production. Chromogenic culture media is a rapid culture based methods used for detection of ESBL and presumptive organism identification. The media has a chromogenic enzyme substrate as a detection system. Chromogenic substrates consist of chromophor which is linked to an enzyme-recognizing part such as carbohydrate, amino acids or phosphate. Specific enzymes produced by the target micro-organism will cleave to the chromogenic substrate liberating the chromophor which highlight the micro organism by coloration of the grown colony (Gazin *et al.,* 2012). The aim of this study was to isolate and identify the types of extended spectrum beta-lactamases genes (ESBL) produced by *A.baumannii* and *Enterobacter clocae and Proteus mirabilis.*

Materials and Method

Sample Collection

The study was cried out within a period of six months. A total of 660 urine samples were collected from pregnant women attending antenatal at the three secondary health care facilities between July to December, 2018. All pregnant women who were not on any antibiotics and willing to participate were included in the studies, while those on any antibiotic therapy were excluded from the studies.

Mid stream clean- catch urine samples were collected and inoculated on MacConkey and CHROMagar ESBL and incubated at 37^{o}C for 24 hours. They were examined for growth and colony counts yielding bacterial growth of 10^5 /ml of urine were taken to be significant. Samples were Gram stained and also subjected to Microbact 24E identification.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility was determined by the disk diffusion method on Mueller-Hinton agar (Oxoid, UK) according to Clinical and Laboratory Standard Institute(CLSI) guidelines. ESBL-producing isolates were screened using double- disk synergy test in accordance with CLSI guidelines (CLSI, 2012). According to CLSIs guidelines isolates showing inhibition zone size of $\leq$ 22mm with Ceftazidime (30µg), $\leq$ 25mm with Cefotaxime (30 µg), $\leq$27mm with Azetronam (30 µg) and $\leq$22mm with Cefodoxime (10 µg) was identified as potential ESBL producers and shortlisted for confirmation of ESBL production(CLSI, 2010).*E. coli* ATCC 25922 and *S. aureus* 6571 were used as quality control strains.

Double Disk Synergy Test

Double disk synergy test as described by Jarlier *et al.,* [32] was used to confirm ESBL production. Test isolate was swabbed on the surface of Mueller Hinton agar, then placement of a ceftazidime disk close (20 or 30 mm) to an amoxicillin-clavulanate disk on a plate inoculated with the test organism. A clear extension the zone of inhibition around the disk towards the amoxicillin-clavulanate disk that is centrally placed indicates the production of ESBL. This extension occurred due to the fact that the clavulanic acid present in the augmentin disc inactivated the ESBL produced by the organism.

Innoculation was also done on CHROMagar ESBL, a completely new and innovative chromogenic medium designed specifically for the Screening of Extended Spectrum ß-Lactamase-producing Enterobacteria (ESBL) [33].Incubation was done for 18-24hrs. *Escherichia coli* produced pink to burgundy colouration of ß-glucuronidase-producing colonies *Klebsiella, Enterobacter, Serratia, Citrobacter* (KESC): green/blue to browny-green colouration of ß-glucosidase-producing colonies . *Proteeae (Proteus, Providencia, Moraganella)* produced dark to light brown colouration.

Ethical consideration

Ethics committee of Akwa Ibom State Ministry of health, provided ethical clearance for the study.Participants' privacy and confidentiality have been assured (no names have been used, only serial numbers were used) and all data and results have been handled and treated confidently. Ref:MH/PRS/99/VOL.IV/200

Statistical Analysis

The SPSS statistical package version (20.0) was used for statistical analysis. A p-value <0.05 was considered as statistically significant.

Plasmid DNA Analysis

Plasmid extraction was carried out using ZR Plasmid Miniprep-Classic extraction kit according to the manufacturers instruction

Table 1: Primer sequences used to amplify and β–lactamase genes by the PCR technique

Gene	Target	Primer	Product size (bp)	Reference
bla $_{TEM}$	β-lactam	F: ATAAAATTCTTGAAGACGAAA R: GACAGTTACCAATGCTTAATC	1080	Sharma *et al.*, 2010
bla $_{SHV}$	β-lactam	F:CACTCAAGGATGTATTGTG R: TTAGCGTTGCCAGTGCTCG	928	Sharma *et al.*,2010
bla $_{CTX-M-15}$	β-lactam	F:CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	550	Sharma *et al.*,2010

Detection of ESBL Genes types by PCR

ESBL producing isolates were amplified using bla TEM/SHVCTX-15, specific primers listed in Table 1. The reaction was performed in Gene Amp PCR system 9700 thermocycler (Thermo Electron Corporation, USA) under the following conditions: Initial denaturation at 94^{o}C for 5 minutes followed by 35 cycles of 30 seconds denaturation at 94^{o}C, 30 seconds annealing at 58^{o}C, 60 seconds extension at 72^{o}C and a final extension at 72^{o}C for 7 minutes. Polymerase chain reaction (PCR) products was separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide .A molecular marker (DNA laddah size range: 10 kb) was used to assess PCR product size.

RESULT AND DISCUSSION

During a six-month period, a total of 252 uropathogens from pregnant women attending antenatal at Government general hospital, Eket, Ikot Ekpene and Oron were identified. 231 isolates were confirmed as potentially ESBL producers using DDST and CHROMagar ESBL. Occurrence of ESBL isolates was as follows: *Enterobacter clocae* (57%), *A. baumanii* (13.5%), *Proteus mirabilis* (32%), (Table 2).

50 ESBL producing isolates were selected for plasmid DNA extraction and *bla*gene amplification: *P. mirabilis* n=12, *A. baumanii* n=20, *Enterobacter clocae* n=18. Plasmid DNA of size 10kb was extracted from the 50 isolates (Fig. 4). CTX-M-15 type ESBL gene was found in 17% of *P.mirabilis*, 25% of *A. baumanii* and 17% of *E. clocae,(*Fig. 3) *bla* TEM ESBL resistant gene was found in 8.3% of *P. mirabilis*, 35% of *A. baumanii* , none was found in *E. clocae (*Fig. 1) *bla* SHV resistant gene was found in 35% of *A. baumanii* , none was found in *E. clocae (Fig* 3). In this study, antimicrobial susceptibility testing of *A. baumannii, E. clocae*, and *P. mirabilis* isolates originally showed highly significant resistance to different types of antibiotics. This resistance can be due to the presence of specific genes of ESBL such as *bla*TEM, *bla*CTX-M-15 and *bla*SHV. Knowing the types and frequency of these genes helps us to make a good decision for the treatment process of patients effectively.

High level of multi-drug resistance including Cefotaxime (CTX), Ceftazidime,(CAZ), Amoxicillin/clavulanic acid (AMC) , Ofolxacin (OFX) and Amikacin (Ak) was observed among the isolates under the study. Various factors, such as the abuse of antibiotics, the spread of clonal resistant microorganisms, can cause the release of highly resistant pathogens. Previous studies showed that the prevalence of *A. baumannii* MDR isolates ranged from 32.7% to 100% [4, 5, 6, 7, 8]. Previous researches has reported that the prevalence of ESBL producing *E. clocae* isolates ranged from 18% to 75% [21, 22] while our studies reports a prevalence of 57% . In the present study 32% of *P. mirabilis* was ESBL producer was consistent with previous studies by Habibu and Orhue [23,24]

Among the mechanisms that create resistance to drugs, ESBLs play an important role in resistance to commonly used antibiotics such as penicillin and cephalosporins. ESBL genes, due to the widespread diffusion of pathogens in the community through plasmids and integrons, can further lead to an increase in resistance to drugs including MDR isolates [9].

Safari *et al*. reported that *SHV* (58%) and *TEM* (20%) were the highest numbers of ESBL genes in their study (Safari *et al.,*2015). Azhar *et al*. based on a study that conducted in Iraq, reported that *SHV* (25%) was the most frequently detected ESBL gene [28]. Reza *et al*., reported that *TEM* (52.1) was the most frequently detected ESBL gene [34]. Khalilzadegan and colleagues identified that *CTX*-M and *TEM* have most ESBL genes [26] While in our studies *bla*CTX-M-15 (58.3) was the most frequently detected gene.

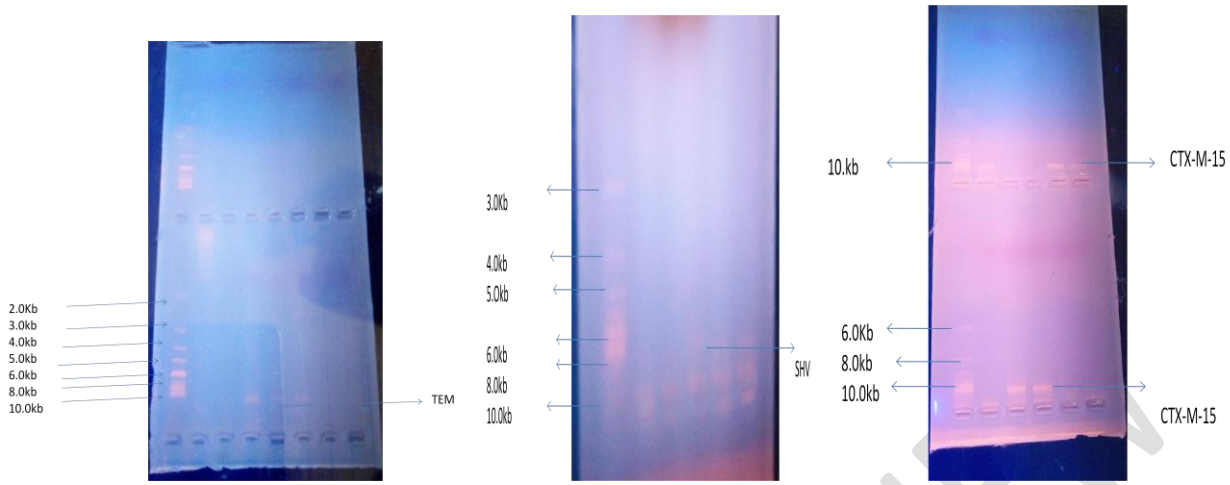
The reason to the observed differences in resistance patterns and the prevalence of *A. baumannii* , *E. clocae* and *P.mirabilis* in various investigations include the following; abuse and misuse of antibiotics, differences in the type of antibiotics used, long-term hospitalization, type of samples taken, differences in diagnostic methods used to identify genes, geographical conditions, gender and etc [29,30]

Table 2: Frequency of ESBL producing isolates across the three study area

Bacterial Isolates	Total	%
Acinetobacter baumanii	31	13.5
Acinetobacter haemolyticus	7	3
A. iwoffi	3	1.3
E. coli	10	4.3
Citrobacter youngae	1	0.4
Citrobacter freundii	1	0.4
Citrobacter diversus	1	0.4
Hafnia alvei	17	7.4
Staphlococcus aureus	17	7.4
Enterobacter clocae	57	24.8
S. maltophilia	12	5.2
Proteus mirabilis	32	13.9
Salmonella subspecies	7	3
P. stuarti	1	0.4
Klebsiella pneumoniae	2	0.7
Enterobacter hormaechei	4	1.7
Enterobacter gresoviae	1	0.4
Serratia marcescens	7	3
Seratia luquefaciens	1	0.4
Morganella morganii	6	2.6
Citrobacter sakazaki	2	0.4
Total	**231**	**100**

Table 3: Detection of *bla*$_{ESBL}$ genes of SHV, TEM and CTX-M-15 in ESBL producing *Proteus mirabilis*, *Acinetobacter baumanii* and *Enterobacter clocae*

Strain identification	No. of isolates tested	No. (%) positive isolates		
		bla$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M-15}$
Proteus mirabilis	12	1(8.3)	1(8.3)	2(16.7)
Acinetobacter baumanii	20	7(35)	7(35)	5(25)
Enterobacter clocae	18	0	0	3(16.6)
Total	**50**	**8(16)**	**8(16)**	**10(20)**



1 2 3

Figure 1: PCR product of *bla* $_{TEM}$ (Line 1 10kb DNA ladder,5, 7 lower gel) Figure 2: PCR product of bla$_{SHV}$ (Line 1:10kb DNA ladder, 4, 5, 6 lower gel) Figure 3:PCR product of *bla*$_{CTX\text{-}M\text{-}15}$ (Line 1:10kb DNA ladder, 2,5, and 6 upper gel, Line 1:10kb DNA ladder, 3,4 lower gel).

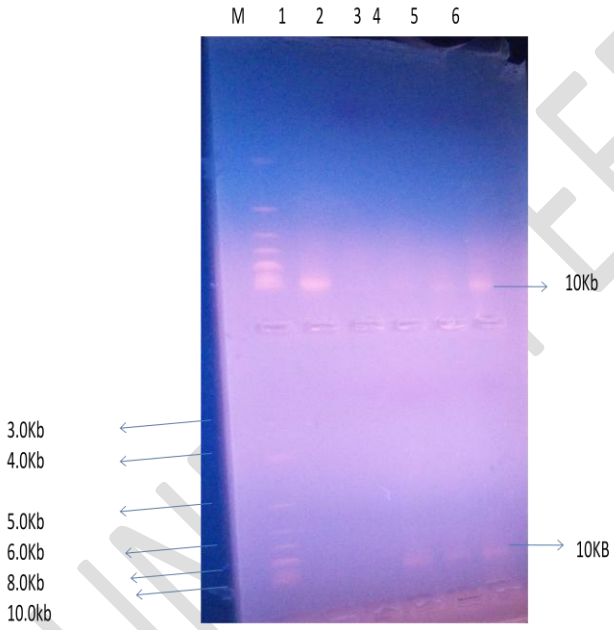


Figure 4:Agarose gel Electrophoresis of plasmids recovered from ESBL producing bacterial isolates. Lane M: 10kb DNA ladder, lanes 1=*P. mirabilis* ,Lanes 5, 6-*A. baumanii* (Upper gel) Lanes 4,5,6 =*E. clocae*

Conclusion

In the present study *bla*$_{CTX\text{-}M\text{-}15}$ had the highest frequency of 58.3% obtained from pregnant women attending antenatal at the three study areas. The biological characteristics of ESBL isolates suggest that the predominant *bla*CTX-M-15 is carried by plasmids. Antibiotic use,

poverty, hygiene failures has enhance the high increment in ESBL producing Gram negative organism disseminating in African continent [31].

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