Original Research Article

Evaluation of CHROMagar ESBL and Double Disk Synergy Test (DDST) for screening of Extended Spectrum Beta-lactamase producing Uropathogens in South-South Nigeria.

Abstract

Background/purpose: The aim of this study was to evaluate the effectiveness of CHROMagar ESBL in comparison with Double Disc Synergy Test (DDST) for the detection of ESBL producing uropathogens.

Method: Six hundred and sixty urine samples were collected from pregnant women attending antenatal at General hospital Ikot Ekpene, Eket and Oron. Two hundred and fifty eight isolates were obtained while two hundred and thirty one isolates were ESBL producers. Microbact 24E(Oxoid, UK) was used in the identification of bacterial isolates, antibiotic susceptibility test was done using Kirby-Bauer disk diffusion method following CLSI guidelines using commercially available disc (Oxoid Ltd). Double disk synergy test was carried out on the isolates and inoculation was done using CHROMagar ESBL (France).

Results: The prevalence of ESBL was 35% was recorded. The sensitivity and specificity of DDST was 88% and 89%, respectively. CHROMagar showed an increase in sensitivity and specificity at 48 h with 98% and 99.0%, respectively. 80% of the ESBL producing isolates were multi drugs resistant. The predominant bacterial pathogens were *Enterobacter cloacae* (23%), *Proteus mirabilis* (14%) and *Acinetobacter baumanii* (13.4%).The ESBL producing isolates showed maximum resistance against Ceftazidime (90%), Cefotaxime (91%), Azetronam (95%), Amikacin (68.2%) followed by ofloxacin (70%) while maximum sensitivity was seen for imipenem (90%) and Augumentin (80%). The study demonstrated that CHROMagar was superior and more sensitive than DDST.

Conclusion: It can therefore be recommended for use to detect ESBL production in the absence of PCR.

Key Words: Antibiotic resistance, Extended spectrum beta-lactamase, CHROMagar, DDST

Introduction

Extended spectrum beta-lactamases(ESBLs) are typically plasmid-mediated enzymes that confers resistance to Extended-spectrum beta-lactam antibiotics such as Ceftazidime, Cefotaxime or Azetronam. Extended-spectrum β-lactamases are a subset of beta lactamases that confer resistance to penicillin, cephalosporins and monobactams and are less efficiently antagonized by beta-lactamase inhibitors such as clavulanate, sulbactam and tazobactam [3]. ESBLs enzymes are produced by both Gram positive and Gram negative bacteria but occur predominantly in the family Enterobacteriaceae. Strains resistant to a variety of commonly used antimicrobials produce ESBLs. This implies that their proliferation pose serious global health problem if not checked. Betalactamases has the ability to open the beta-lactam ring and inactivate the antibiotics, and render them ineffective for treatment [2]. 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 [37] .The recent resurgence of another group of beta-lactamases, carbapenemase producing bacteria has raised a major public health concern.New Delhi Metallo beta-lactamase (NDM-1) hydrolyses a wide range of beta-lactam antibiotics including carbapenems, which are the last resort of antibiotics for the treatment of infections caused by resistant strain of bacteria [36]. Due to the rising incidence of ESBL harboring microorganisms, there has been a worrisome increase in the use of carbapenems and this can result in pan-resistant organisms [11].

Hospital and community acquired ESBL producing uropathogens are prevalent worldwide, due to inappropriate use of beta-lactam antibiotics, poor sanitation in hospitals, and unhealthy lifestyles leading to serious infections and raising therapeutic

problems [1]. Beta-lactamase may be chromosomal or plasmid borne, inducible or constitutive (Hugo and Russell's, 2013). ESBLs are often located on plasmids harbouring resistance gene to other antimicrobial classes, resulting in multidrug resistant isolates [2,31].

Extended spectrum beta-lactamases can be readily detected by iodometric, colometric and chromogenic methods [7]. Invitro detection of ESBL has faced many challenges due to the fact that many strains are susceptible to broad spectrum beta-lactam such as Cefotaxime and Ceftriazone [8, 9].

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). *E .coli* ESBL produces dark pink to reddish colouration, *Klebsiella, Enterobacter* and *Citrobacter* produces a metallic blue colouration while *Proteus* produces a brown halo colour. According to the instruction of the instructions of the manufacturer, any coloured oxidase negative colonies growing on the chromogenic media, should be regarded as presumptive ESBL producing isolates.

In the study described here, we evaluate the effectiveness of CHROMagar ESBL in comparison with Double Disc Synergy Test (DDST) for the detection of ESBL producing uropathogens from pregnant women attending antenatal at general hospital Ikot Ekpene, Eket and Oron, Akwa Ibom State, Nigeria.

Materials and Methods

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. Ethical approval was obtained from Ministry of health, Akwa Ibom State. 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 Mackonkey 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 [32] *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.,* [10] 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. An extension of inhibition of the zone of inhibition around the peripheral disk towards the centrally placed amoxicillin-clavulanate disk indicates ESBL production. This extension occurred because the clavulanic acid present in the Augmentin disc inactivated the ESBL produced by the test 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) [38] 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.Ref:MH/PRS/99/VOL.IV/200

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.

Statistical Analysis

The SPSS statistical package version (18.0) was used for statistical analysis. A p-value <0.05 was considered as statistically significant.

Results

Description of the Bacterial Isolates

During the study period, a total of 660 urine samples from pregnant women were processed. Out of the 660 samples, 230(35%) were identified as ESBL producers Among these isolates, the predominant bacterial pathogens were *Enterobacter cloacae* (23%), *Proteus mirabilis (*14%) and *Acinetobacter baumanii* (13.4%).Followed by *Hafnia alvei* (7.3%), *Xanthomonas maltophilia* (4.8%) and *Enterobacter agglomerans* (4.8%).Antimicrobial susceptibility analyses showed that thirty two ESBL producing *A. baumanii* isolates(12.5%) were resistant to quinolone (Ofloxacin 70%) and third generation Cephalosporins (Cefotaxime, 62.5%, Ceftazidime, 90%, and Azetronam and 95%).

Most importantly most isolates were susceptible to carbapenem (Imipenem, 90%,) and Augumentin, 80%, aminoglycoside (amikacin) showed 68.2%. 231(35%) isolates were ESBL producing, double disk synergy test detected 143(55%) isolates while CHROM agar ESBL detected 214(82%) isolates.32.4%(n=214) of isolated Gram negative bacilli were ESBL positive while 2.6% (n=17) of isolated Gram positive bacteria were ESBL positive.

Table 1: Result of Screening And Detection Of ESBL By DDST And ChromAgar ESBL From Oron General Hospital

Bacterial isolates	CHROMagar ESBL **N%**	DDST **N%**	p-value
E. cloacae	40(41.2)	15(39)	0.5195
E. hormaechei	3(3.1)	3(77)	
E. agglomerans	2(2.1)	2(5.1)	
Proteus mirabilis	11(11.3)	3(77)	
Morganella morganii	3(3.1)	2(5.1)	
A. baumamii	9(9.3)	2(5.1)	
Xanthomonas maltophilia	2(2.1)	1(2.6)	
Hafnia alvei	6(6.2)	2(5.1)	
Staphylococcus aureus	-	2(2.6)	
Serratia marcescens	7(7.2)	2(5.1)	
Serratia luquefaciens	1(1)	1(2.6)	
Citrobacter sakazakii	1(1)	1(2.6)	
Salmonella subspecies	6(6.2)	2(5.1)	
E. coli	4(4.1)	1(2.6)	
TOTAL	**97(100)**	**39(100**	

Table 2: Result of Screening And Detection Of ESBL By DDST And ChromAgar ESBL From Ikot Ekpene General Hospital

Bacterial isolates	CHROMagar ESBL **N%**	DDST **N%**	p value
Acinetobacter baumanii	20(34.4)	15(25)	0.5962
Acnetobacter haemolyticus	**5(7)**	**5(8.3)**	
A. iwoffi	**2(3.4)**	**2(3.3)**	
E. coli	**6(10.3)**	**5(8.2)**	
Citrobacter youngae	**1(1.7)**	**1(1.7)**	
Citrobacter freundii	**1(1.7)**	**1(1.7)**	
Citrobacter diversus	**1(1.7)**	**3(5)**	
Hafnia alvei	**4(6.9)**	**3(5)**	
Staphyloccus aureus	**-**	**15(25)**	
Enteobacter agglomeran	**1(1.7)**	**1(1.7)**	
Enterobacter clocae	**2(3.4)**	**3(5)**	
S. maltophilia	**6 (10.3)**	**3(5)**	
Proteus mirabilis	**5(8.6)**	**3(5)**	

Salmonella subspecies	**1(1.4)**	**3(5)**
Klebsiella pneuimoniae	**2(3.4)**	**1(1.7)**
TOTAL	**73(100)**	**51 (100)**

Table 3: Result of Screening And Detection Of ESBL By DDST And ChromAgar ESBL From Eket General Hospital

Bacterial isolates	**CHROMagar ESBL**	**DDST**	**p-value**
	N%	**N%**	
E. clocae	15(25)	14(32)	0.5962
A. baumanii	2(3)	1(2.3)	
A. haemolyticus	1(1.6)	1(2.3)	
A. iwoffii	1(1.7)	1(2.3)	
E. agglomeran	8(13.3)	7(16)	
E. hormaechei	1(1.7)	1(2.3)	
P. gresoviae	1(1.7)	1(2.3)	
P. mirabilis	16(27)	8(18)	
Morganella morganii	2(3.3)	1(2.3)	
S. maltophilia	4(6.7)	3(6.8)	
Hafnia alvei	7(11.6)	4(9.1)	
Staphyloccus aureus	**-**	1(2)	
TOTAL	**60(100)**	**44(100)**	

Statistical Analysis

Chi-square was used to determine if a significant difference existed between results from both procedures. Where a significant difference exists it was interpreted as $P < 0.05$.

Sensitivity = (TP/TP + FN) × 100; Specificity = (TN/TN + FP) × 100.

Discussion

In this study, we present ESBLs producing isolates from Akwa Ibom State, Nigeria. The isolates showed resistance to third generation cephalosporins, quinolone and aminoglygoside and showed sensitivity to Imipenem and Augmentin.

The study was carried out to evaluate the performance of Double Disc Synergy Test (DDST) and CHROMagar ESBL) in screening for ESBL among isolates from urine samples of pregnant women attending antenatal in the three study areas. Among clinical isolates, the prevalence of ESBL greatly varies in geographical areas and worldwide.

The prevalence of ESBL in this research was 35%. Much higher (58%) prevalence of ESBL producers in urinary isolates of gram negative bacilli was observed in India (Grude, Tveta and Kristiansen, 2001; Bell *et al*., 2002). Also, Ezeanya *et al.,* reported a higher prevalence of 61% from their studies (Ezeanya *et al.,* 2017).

In our study, 32.4 %(n=214) of isolated Gram negative bacilli were ESBL positive while 2.6% (n=17) of isolated Gram positive bacteria were ESBL positive. The predominant bacterial pathogens were *Enterobacter cloacae* (23%), *Proteus mirabilis (*14%) and *Acinetobacter baumanii* (13.4%).Followed by *Hafnia alvei* (7.3%), *Xanthomonas maltophilia* (4.8%) and *Enterobacter agglomerans* (4.8%). In contrast to our results, in the study of Hosain Zadegan *et al*. 23.5% of isolated Gram-negative microorganisms (53 of 222 isolates) were ESBL producers with the most frequent isolates being *K. pneumoniae* (8.9%), *E. coli* (4.4%), and *P. aeruginosa* (4.4%); also, of nine isolated *Acinetobacter* spp. strains, 2 (0.9%) were ESBL-positive [19].These values are lower than the rates in our study. The frequency of ESBL in this study agrees with the study of Adham *et al*., who reported a sensitivity of (38.4%) [21].

Increasing resistance to broad spectrum cephalosporins due to the production of β-lactamases have been reported from different countries (Bouchillon *et al*., 2002; Khanfar *et al*., 2009). The development and use of simple screening tests that are suitable for routine use in the clinical microbiology laboratory is a critical step towards large-scale monitoring of these enzymes (Migliavacca *et al*., 2002). Due to the outcome of the antibiotic Susceptibility test result, the ESBL producing isolates were subjected to ESBL screening using Double Disc Synergy Test (DDST) and CHROMagar ESBL. DDST is described as a reliable technique for ESBL detection [12]. The differences in sensitivity results in DDST be due to the fact that optimal substrate profile varies from one ESBL enzyme to another [35]. DDST an easy procedure with subjective interpretation of result [39].

The distance between antibiotic discs affects the sensitivity of DDST. Studies by Ho *et al*. [16] revealed the sensitivity of DDST to be 83.8% at a single interdisc width of 30mm. Their study also showed an increase in sensitivity to 97.9% by

decrease in the interdisc width to 20 mm. In this study, sensitivity and specificity of DDST was 91.3% and 89.5% respectively at 24 h which was the same at 48h at a single interdisc width of 15mm. DDST can detect both Gram positive and Gram negative ESBL producing bacteria while CHROMagar is only limited to Gram negative organisms.

There were significant differences (P > 0.001) among ESBL-producing isolates that emerged from the three general hospitals. The differences could be attributed to the fact that DDST is a technically easy procedure with subjective interpretation of result [39]. While CHROMagar ESBL Agar gives an advantage of easier detection of ESBL-producing Gram negative as well as other members of the Enterobacteriaceae family due to its chromogenic properties. Results are easier to interpret as it employs colony coloration technique [13,14].

In this study, it was observed that CHROMagar ESBL had 99% specificity which is significantly higher than that of DDST with specificity of 85%. The specificity of CHROMagar ESBL in this study was higher than previous studies whose studies revealed a specificity of 95.7% and 93.0% respectively [33,34]. The sensitivity of CHROMagar in this study correlates with the study Ezeanya, *et al.* Who reported a sensitivity of 97.8% [29] Vercauteren *et al.*(Vercauteren *et al.*,1999) reported a sensitivity of 96.9% . Also Ravi et al. reported 94.89% sensitivity and 75.91% specificity for DDST [39].

The inclusion of Cefpodoxime in CHROMagar ESBL rather than Cefotaxime and Ceftazidime could attribute for its higher sensitivity over DDST. Thus, performance of CHROMagar ESBL agar in this study justifies claims that Cefpodoxime is the best substrate for screening all ESBL types in clinical specimens[17].

The carbapenems (Imipenem, Ertapenem and Meropenem) are still the first line agents in treatment of serious infection with ESBL-producing organisms as >98% of ESBL-producing organisms stillsusceptible to these drugs [22] Adham *et al.,* observed 98.8% susceptibility for Imipenem while in our study, we observed 90% susceptibility for Imipenem.

In this study, we found that about 68.2%of ESBL producing uropathogens were susceptible to amikacin, however, a poor susceptibility for cefotaxime(90%), ceftazidime (91%), Ofloxacin (70%) was observed. Also, 70% of ESBL-producing uropathogens were sensitive to Augmentin. Aninoglycosides displays bactericidal concentration-dependent killing action and are active against ESBL producing bacteria.

In a Spanish study published in 2014,aminoglycosides were used in the treatment of carbapenems-resistant Klebsiella infection showing a statistically significant reduction in mortality [22,25].

Amoxicillin/clavulanic acid as an alternative treatment to carbapenems for infections involving ESBL-producing organisms remains debated [23].

In this study, a high rate of susceptibility (80%) was observed for Amoxicillin/clavulanic acid. In a Randomized controlled trial conducted by Yu Bin Seo, it was reported that Piperacillin/tazobactam(TZP) which is also a beta-lactamse inhibitor like Amoxicilin/clavulanic acid is effective in the treatment of UTI caused by ESBL-*E. coli* when the in vitro test indicates susceptibility [24] Hence this antibiotic may be used as an alternative treatment to carbapenems for pyelonephritis caused by ESBL-producing uropathogens.

The variations of resistance to antibiotics can be explained in part based on different local antibiotic practices [5]. Differences insusceptibility patterns of organisms and frequency of infection between hospitals and communities make knowledge of local prevalence and resistance data extremely important [30]. This has direct bearing on choice of empiric therapy. Our research showed that large numbers of Gram-negative bacteria causing community acquired UTIs produce ESBL with most being multi-drug resistant (MDR). Therefore, routine ESBL detection testing and subsequent antibiogram with disk diffusion method could be useful to determine the best treatments for UTI.

ESBL continues to pose a serious public health threat as it receives attention from the general public, policy makers and clinical microbiologist. Results from our study revealed that CHROMagar ESBL has a high sensitivity and specificity making it reliable for ESBL detection. This medium allows for easy differentiation of different bacteria based on colony colouration.

Table 4Antibiotic Susceptibility profile of ESBL producing *Acinetobacter baumanii* from Ikot Ekpene, Eket and Oron General Hospital

Antimicrobials	Ikot Ekpene n=20		Eket n=3		Oron =9	
µg	S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
CTX(30)	2(10)	18(90)	0	3(100)	9(100)	0

OFX (5)	19(95)	1(5)	1(33)	2(67)	7(78)	2(22)
CAZ(30)	0	20(100)	1(33)	2(67)	5(56)	4(44)
ATM (30)	0	20(100)	0	3(100)	6(67)	3(33)
IPM (30)	19(95)	1(5)	1(33)	2(67)	9(100)	0
AUG (30)	20(100)	0	1(33)	2(67)	9(100)	0
AK(30)	12(60)	8(40)	1(33)	2(67)	5(56)	4(44)

Key:CTX- cefotaxime, OFX-oflxacin, CAZ-Ceftazidime, ATM-Azetronam, IPM-Imipenem, Aug-Augumentin, AK-Amikacin

Table 5:Antibiotic susceptibility profile of *Enterobacter cloacae* from Ikot Ekpene, Eket and Oron General Hospital.

Antimicrobials µg	n=65	
	S (%)	R(%)
CTX(30)	16(25)	49(75)
OFX (5)	18(28)	47(72)
CAZ(30)	16(25)	49(75)
ATM (30)	15(23)	50(77)
IPM (30)	56(74)	9 (26)
AUG (30)	48 (74)	17(26)
AK(30)	8(12)	57 (87)

Key:CTX- cefotaxime, OFX-oflxacin, CAZ-Ceftazidime, ATM-Azetronam, IPM-Imipenem, Aug-Augumentin, AK-Amikacin

Table6:Antibiotic susceptibility profile of ESBL producing *Proteus mirabilis* the three study Area

Antimicrobials µg			Ikot Ekpene n=11		Eket n=16	
	n=5 S(%)	R(%)	S(%)	R(%)	S(%)	R(%)
CTX(30)	0	5(100)	10(91)	1(9)	8(50)	8(50)
OFX (5)	5(100)	0	10(91)	1(9)	9(56)	7(44)
CAZ(30)	0	5(100)	6(55)	5 (45)	4 (25)	12(75)
ATM (30)	0	5(100)	1(9)	10(91)	10(62)	6(38)
IPM (30)	4(80)	1(20)	10(91)	1(9)	10(62)	6(38)
AUG (30)	4(80)	1(20)	5(45)	6(55)	5(31)	11(69)
AK(30)	2(40)	3(60)	2(18)	8(72)	11(69)	5(31)

Key:CTX- cefotaxime, OFX-oflxacin, CAZ-Ceftazidime, ATM-Azetronam, IPM-Imipenem, Aug-Augmentin, AK-Amikacin

Fig. 1. Culture plate of showing a clear extension of the edge of the inhibition zone of cephalosporin using Co-amoxiclav Disc on Mueller-Hinton agar was interpreted as positive for ESBL production

Fig. 2 Culture plate of clinical isolate of *Escherichia coli* from urine showing distinctive pink colony colouration on CHROMagar ESBL agar was interpreted as positive for ESBL production

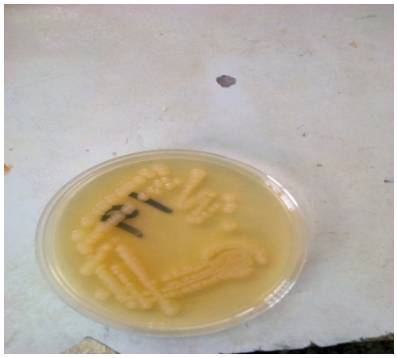

Fig. 3 Culture plate of clinical isolate of *Acinetobacter baumanii* from urine showing distinctive colourless to cream colony colouration on CHROMagar ESBL agar was interpreted as positive for ESBL production

Fig 4: Culture plate of clinical isolate of *E. clocae* from urine showing blue colonies on CHROMagar ESBL

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