



SJMLS - 4 (3) - 006

Extended Spectrum Beta-Lactamases status among Gram-Negative Bacterial Isolates from Clinical Specimens in four Major Hospitals in Southern NigeriaOdumosu, B.T.¹, Dada-Adegbola, H.², Iroagbalachi, J.Z.¹*Department of Microbiology, University of Lagos, Akoka-Yaba Nigeria¹, Department of Medical Microbiology and Parasitology, University of Ibadan, Nigeria².**Author for Correspondence: bodumosu@unilag.edu.ng/deliniz@yahoo.com /+234-803-451-5048***Abstract**

Antibiotic resistance among clinically important bacteria is a global health challenge. Strains that have the potentials of producing Extended-Spectrum Beta-lactamase (ESBL) have become more prevalent. The aim of this study was to determine the antibiotic susceptibility and ESBL production among Gram-negative rods isolated from four hospitals in Nigeria. A total of 315 Gram-negative rods were isolated from various clinical samples. Standard identification of the isolates was carried out using Vitek 2 compact. Susceptibilities to antibiotics and production of ESBL was carried out by disk diffusion and double-disk synergy test methods respectively. Ten genera of Gram-negative rods were identified which includes *Klebsiella pneumoniae*, *Escherichia coli*. All the isolates were resistant to ampicillin followed by high resistance to amoxicillin/clavulanic acid, while imipenem had the highest number of susceptibilities. ESBL result was positive for 168/315 (53.3%) of which *K. pneumoniae* and *E. coli* isolates were the highest producers with 86/168 (51.1%) and 61/168 (36.3%) respectively. The results of this study underscore the significance *K. pneumoniae* and *E. coli* among clinical isolates with potentials for ESBL production. However, the detection of ESBL producing bacteria is of uttermost importance for effective infection control and epidemiological surveillance.

Keywords: Antibiotic-resistance Gram negative rods, ESBL,

Introduction

Antimicrobial resistance (AMR) is an increasing

global threat of significant interest especially among Gram-negative bacteria. Of particular interest are the isolates of clinical origins which are believed to possess multiple mechanisms of antibiotic resistance that often lead to therapeutic failures, longer duration in the hospital as well as high cost of treatments. Extended-Spectrum β -lactamases (ESBL) has been widely reported as a major antibiotic resistance mechanism among Gram-negative bacteria. ESBL are enzymes produced by bacteria that affords them the ability to render β -lactam antibiotics such as the third-generation cephalosporin and monobactams inefficient (Paterson and Bonomo, 2005).

Overuse and misuse of antibiotics in the hospital settings as well as in the agriculture has been globally reported as primary driver leading to the development of resistance. Resistance to β -lactam antibiotics may occur by mutations or by inactivating enzymes mediating bacteria resistance and its dissemination via mobile genetic elements (Odumosu *et al.*, 2013). Beta-lactam antibiotics are being used in the empirical treatment of infections with Gram-negative bacteria partly because of its affordability and cost since most patients in this part of the world have to bear the cost of their own healthcare and partly because of their low toxicity hence most clinicians prescribe it often (authors's personal observation). Excessive use of the beta-lactam antibiotics has led to more production of ESBL and its dissemination among the bacteria population (Michael *et al.*, 2014).

The first report of mobilized β -lactamases was described in 1983 and have since been detected

in several members of the family *Enterobacteriaceae* and *Pseudomonadaceae* from around the world, they are commonly detected in *Klebsiella pneumoniae* and *Escherichia coli* (Knothe *et al.*, 1983). Failure to constantly identify the state of the ESBL producing organisms and prevention may also contribute largely to their unabated spread in the hospital and even to the communities. Therefore, assessment of the current state of ESBL producing clinical isolates, where excessive use of antibiotics abounds and where adequate antimicrobial resistance surveillance is deficient, can never be overemphasized. This study was carried out with the purpose of determining the antibiotic susceptibilities profiles and ESBL producers among Gram-negative rods isolated from four major hospitals in Lagos and Oyo states in Nigeria.

Materials and Method

Test bacteria

A total of 315 consecutive, non-repetitive Gram-negative bacterial isolates recovered from various clinical samples were collected from 4 hospitals in Southwestern Nigeria between August 2015 and September 2017. These hospitals include McCure HealthCare Limited Lagos, University of Lagos Teaching Hospital, University College Hospital Ibadan and Adeoyo Hospital Ibadan. Complete data of all the isolates such as sample source, patient age, and gender were also collected and documented for the purpose of this study.

The study does not deal directly with the patients hence no informed consent was required as there was no participant, only bacteria isolates from different samples were used for this study.

Bacterial Identification

Primary identification of the isolates were carried out according to standard laboratory procedure (Cheesbrough, 2000) their genus and species identities were further confirmed by using Microbact™ and Vitek 2 (BioMerieux Inc. USA System). All the test procedures were carried out according to the manufacturer's instructions and guidelines. Identified isolates were stored on agar slant and kept at -20°C until required for the further assay.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing (AST) of the isolated bacteria using the modified agar disk diffusion method on Mueller Hinton agar (MHA) (Lab M) were determined and interpreted according to Clinical and Laboratory Standard Institute guidelines (CLSI, 2016). The following antibiotics disk (Oxoid Ltd, Basingstoke, UK) were used; beta-lactam: ampicillin (10 µg), ceftazidime (30 µg), cefotaxime (30 µg) cefuroxime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), beta-lactamase inhibitors: amoxicillin/clavulanic acid (20/10 µg) piperacillin/tazobactam (100/10 µg), fluoroquinolones: ciprofloxacin (5 µg) and ofloxacin (5 µg), diameter of zone of growth inhibition were measured in millimeters after 24 h of incubation at 37°C. *E. coli* ATCC 25922 was used as control in this study.

Detection of ESBL Production among the isolates

In order to detect the presence of ESBL production among the test bacteria, standardized fresh cultures (0.5 McFarland) of all the test bacteria were prepared and evenly inoculated on MHA using sterile swab sticks and by adopting the double-disk synergy test and using piperacillin/tazobactam (110 µg) (TZP) as beta-lactamase inhibitor. TZP was firmly placed at the center of the MHA plate using sterile forceps while cefotaxime, ceftazidime, and cefepime all of 30 µg concentration were placed at 25 mm equidistance away from the inhibitor. For *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, the cephalosporins were placed at 20 mm equidistance from the inhibitor. All plates were incubated at 37°C for 24 h. For quality control *E. coli* ATCC 35218 was included in the study.

Statistical Analysis

Dataset was analyzed using PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Values of $x < 0.05$ were considered statistical significance.

Results

Three hundred and fifteen (315) isolates were collected and were further screened for

confirmation of identities. The samples data such as age and gender of the patients were taken into account as shown in Table 1. A total of 141 male and 174 female samples were collected from patients with age range of between 0 – 20 and > 60 years (Table 1). Eleven different types of clinical samples were investigated in the present study. Urine as a source of bacteria isolates dominated the samples collected with 158/315 (50%) of the total isolates followed by swabs from various clinical sites 75/315 (24%), tracheal aspirate 31/315 (9.8%), blood culture 24/315 (7.6%), sputum 11/315 (4%) Table 2. The isolates recovered after screening for purity and phenotypic characterization according to morphology and biochemical characteristics were identified as described in the previous section.

Identification of isolates

Ten genera of Gram-negative rods isolates were identified as shown in Table 3.

The genus *Klebsiella* was the most commonly isolated bacteria from the samples comprising of 121/315 (38 %) of the total Gram-negative bacteria in this study. *K. pneumoniae* was isolated from urine samples representing 68/158 (43%), 15/31 (48%) of tracheal aspirate, 8/26 (31%) wound swab, and 15/ 26 (58.0 %) of HVS were recovered from the 4 hospitals.

E. coli ranked second among the isolated pathogens with the total population of 119/315 (37.7%) of the total isolates and were distributed in the following samples 58/158 (36.7%) from urine, 14/31 (45.1 %) from tracheal aspirate, and 11/26 (42.3 %) from HVS Table 4.

P. aeruginosa population in the total isolates was 20/315 (6.3 %) while the majority of the isolates were from the wound swab and urine sample at 10/26 and 8/ 158 respectively. *Proteus mirabilis* isolates represented a total of 12/ 315 (3.8 %) of all the isolated bacteria. A total of 6/158 (3.7 %) was isolated from urine, 3/7 from eye swab while others were from the catheter and wound swabs Table 4.

A. baumannii constituted 13/315 of which majority were found in urine 9/158, wound swab

2/26, sputum 2/11. The genus *Enterobacter* was represented by two species namely *E. cloacae* and *E. aerogenes*. *Enterobacter cloacae* were found majorly in sputum while *E. aerogenes* were isolated majorly from ear swab Table 1. *Morganella morganii* constituted 6/315 (1.9 %) of the total isolate and were distributed in urine, CSF and sputum Table 4. Other Gram-negative bacteria isolated were also represented according to their source in Table 4.

Antimicrobial susceptibility testing

All the Gram-negative bacteria investigated were resistant to ampicillin (100%) without any zones of inhibitions or zones below susceptible range. Of the two beta-lactamase inhibitors assayed in this study, high-level resistance rate 273/315 (86.7 %) was observed for amoxicillin/clavulanic acid while piperacillin/tazobactam recorded a higher number of susceptibilities 205/315 (65%) Table 5. Among the cephalosporin, high-level resistance rate was recorded for cefuroxime 262/315 (83.2%) followed by cefotaxime 204/315 (64.8%), and ceftazidime 126/315 (40.0 %) Table 5. Imipenem and meropenem belonging to carbapenem class of beta-lactam antibiotics recorded the highest number of susceptibilities of 290 (92 %) and 205 (65%) susceptibility rates respectively, while aztreonam a monobactam had 165/315 (52.4%) susceptible rate Table 5.

Detection of ESBL by double disk

ESBL was detected in only 168 (53.3%) out of 315 investigated while 105 (33.3 %) isolates suspected to be ESBL producers did not show any synergy with the tazobactam. ESBL was detected at the 25 mm distance in between tazobactam and the cephalosporin disk for the members of *Enterobacteriaceae* while 20 mm distance was for *P. aeruginosa* and *A. baumannii*. The results revealed the presence of ESBL in 7 species of bacteria of which *K. pneumoniae* was the highest with 86/168 (51.1 %) followed by *E. coli* with 61/168 (36.3%), *Enterobacter* spp 7/168 (4.2%) *P. mirabilis* 4/168 (2.3%), *P. aeruginosa* 4/168 (2.3%), *A. baumannii* 3/168 (1.7%) and *M. morganii* 3/168 (1.7%). The overall percentage of *K. pneumoniae* with ESBL is 71. % at (86/121) of which majority of the isolates were from urine, *E. coli* 51% at (61/119), *Enterobacter* spp 43% at (7/16). *P. mirabilis* 33%

at (4/12) and *P. aeruginosa* 33% at (4/12), *A. baumannii* 23% at (3/13) and *M. morgani* 23% at (3/13).

Discussion

The present study investigated the prevalence, susceptibilities and ESBL production among the family *Enterobacteriaceae* and other Gram-negative rods isolated from a total of 315 clinical samples for a period of 25 months from four hospitals in southwest Nigeria.

The age group bracket of patients with the highest number samples recovered was between 41 and 50 years (Table 1). Although there are more females' patients 55% than male 45%, there was no significant difference in the patient based on gender as samples were collected from both genders except HVS that were strictly from females. In the present study, the highest number of isolates recovered were from urine suggesting a high prevalence of urinary tract infections among the hospital attendees (Table 2). The result of this study is consistent with previous studies from India, Jordan, and Nigeria where urine isolates dominated the total number of isolates recovered (Ogefere *et al.*, 2015; Dalela, 2012; Batchoun *et al.*, 2009).

Among the isolated Gram-negative rods in this study, *K. pneumoniae* (38.0%) and *E. coli* (37.7%) ranked highest among the total isolate recovered in the present study. Both organisms were found in all the samples recovered except *E. coli* that was never isolated from sputum (Table 4). Prevalence of both genera in clinical samples is consistent with a previous study carried out in Jordan where *E. coli* and *Klebsiella* were among the most frequently isolated genera in their study (Hayajneh *et al.*, 2015). In another similar study carried out in Nigeria (Ogefere *et al.*, 2015), *E. coli* and *Klebsiella* spp ranked highest among the isolates investigated. *P. aeruginosa* and *A. baumannii* ranked low among the isolates in this present study even though both organisms are reportedly frequently isolated from clinical samples. The result of our study further suggests *E. coli* and *K. pneumoniae* are leading causes of bacterial infections in developing countries and are frequently isolated from various clinical samples.

The result of the antibiotic susceptibility of the isolates in this study (Table 5), revealed complete resistance to ampicillin by all the 315 Gram-negative rods tested suggesting all the investigated bacteria possess one or mechanisms of resistance to ampicillin. Consistent with the present results, a recent report by Olowookere *et al.* (2018) also reported a complete resistance (100%) to ampicillin by gram-negative rods isolated from wound samples (Olowo-okere *et al.*, 2018). Similarly in another South African study, high level resistance (91%) to ampicillin was also reported for Gram-negative bacteria (Mthembu *et al.*, 2017). The increasing level of resistance to ampicillin by the gram-negative rod is an indication of loss of potency by this beta-lactam antibiotic and should be discouraged from prescription for treatment any longer due to reported resistance by microorganisms. Among the beta-lactamase inhibitors assayed in this study, a higher level of resistance to amoxicillin/clavulanic acid was observed. This beta-lactamase inhibitor popularly known as augmentin or amoxycylav has overtime experiencing increasing resistance among gram-negative bacteria. In consistent with previous reports and observations (Odumosun *et al.*, 2016; Bush *et al.*, 1993), piperacillin/tazobactam activities as a beta-lactamase inhibitor in this study were 7 times higher than amoxycillin/clavulanic acid.

Among the cephalosporin assayed in this study, high-level resistance was observed for cefuroxime and cefotaxime, 83.2% and 64.8% of the isolates showed resistance to cefuroxime and cefotaxime antibiotics respectively. It will be interesting to note that cefuroxime is a second-generation cephalosporin while cefotaxime is the third generation. Ceftazidime which is also a third-generation cephalosporin showed better activities than cefotaxime in this study having 55.6% susceptibility rates (Table 5). Ceftazidime overtime has been shown to be more potent than cefotaxime which is usually susceptible to ESBL and the latter is no longer listed among antipseudomonal antibiotics by the CLSI. Carbapenem antibiotics used in this study, imipenem and meropenem have consistently proved their efficacies against clinical isolates of bacteria by having a high level of susceptibilities of 88.8 % and 80.9 % respectively.

Detection of ESBL in this study was carried out by using tazobactam as the beta-lactamase inhibitor along with piperacillin a ureidopenicillin against the conventional amoxicillin and clavulanic acid combination that was recommended previously (Drieux *et al.*, 2008). The reason for this choice was the observed higher potency of tazobactam over clavulanic acid. There are three β -lactamase inhibitors used clinically; sulbactam, clavulanic acid, and tazobactam, of the three, tazobactam activities against ESBL have been shown to be higher (Bush *et al.*, 1993). The occurrence of ESBL producers among the investigated Gram-negative bacilli investigated in this study was 168/315 (53.3%) while 51.1% *K. pneumoniae*, 36.3% *E. coli*, 4.2% *Enterobacter* spp, 2.3% *P. mirabilis*, 2.3% *P. aeruginosa*, 1.7% *A. baumannii* and 1.7% *M. morgani* were all positive for the production of ESBL. The overall ESBL production of 53.3% in this study is higher than the 44.3% reported from a study in Nigeria (Ogefere *et al.*, 2015), but lower than 64.29% reported in Bangladesh (Hena and Karim, 2015) and from another recent study (Olowo-okere *et al.*, 2018). In agreement with most studies on ESBL production, higher percentage of *K. pneumoniae* found with ESBL in this study showed that this organism has an extensive capacity for ESBL production, especially among the clinical isolates. The prevalence of *K. pneumoniae* producing ESBL among Gram-negative bacilli in this present study is consistent with many studies while the percentage detected (51.1%) corroborated with a Saudi Arabian study where 53.84% ESBL prevalence was reported. Globally speaking *K. pneumoniae* has been reported widely for the production of ESBL and this calls for urgent surveillance on this pathogen especially in the hospital. *E. coli* ranks second in this study for both prevalence of isolates and ESBL production and our result is consistent with many other previous studies which reflect the abundance of *E. coli* among the implicated bacteria in the hospital, although the current record did not show whether these samples were collected from outpatient or inpatient.

Detection of ESBL from organisms such *P. aeruginosa* and *A. baumannii* could be very difficult due to an interplay of multiple mechanisms of resistance hence could affect the

detection ESBL amongst them as well as isolation because in most cases, these organisms were regarded as contaminants whereas they also play an important role as nosocomial agents.

Conclusion and Recommendation

In conclusion, this study describes the prevalence of gram-negative rods implicated in several clinical samples. The ESBL rate detected is alarming considering the species that dominated the positive producers especially among UTI. There was a massive resistance to beta-lactam antibiotics investigated especially ampicillin, amoxicillin/clavulanate, cefuroxime, and the third-generation cephalosporin cefotaxime. Increased resistance to these third generation cephalosporins is worrisome and can be attributed to the presence of ESBL or other resistance mechanisms that was not in the scope of the present study. Susceptibilities of the isolates to carbapenem is an indication that these class of beta-lactam still remain the last resort for ESBL producing bacteria with an extensive range of activities. We recommend the discontinued use of ampicillin for treatment of infections associated with Gram-negative bacteria because all the bacteria in this study showed no sensitivity towards it except when used in combination with other antibiotics or a strong beta-lactamase inhibitor other than sulbactam. The use of clavulanic acid as a beta-lactamase inhibitor should be properly reviewed especially among Gram-negative bacteria, almost all the isolates in this study were resistant to it.

This study, therefore, reiterates the need for continued hospital surveillance of resistant bacteria and their susceptibility profile to commonly prescribe antibiotics especially the clinical isolates and the status of the ESBL production must be known from time to time. We also use this opportunity to reiterate the call for the development of antimicrobial stewardship initiatives and implementation of infection control programmes so as to reduce the spread of AMR both locally and globally.

Source of funding /Acknowledgment

None.

Conflict of Interest

Authors declare no conflict of interest.

Table 1: Age and Gender distribution of isolates from clinical sample

Age group (years)	Male	Female	Total No. (%)
0-20	20	12	32 (10.0)
21-30	29	33	62 (20.0)
31-40	22	19	41 (13.0)
41-50	23	48	71 (22.5)
51-60	29	41	70 (22.2)
> 60	18	21	39 (12.3)
Total	141	174	315 (100%)

Table 2: Rate of isolates from clinical sample

S/N	Sample	No of isolates	% Occurrence
1	Urine	158	50
2	Tracheal aspirate	31	9.8
3	High vaginal swab	26	8.2
4	Wound swab	26	8.2
5	Blood culture	24	7.6
6	Sputum	11	4.0
7	cerebrospinal fluid	9	2.8
8	Ear swab	8	2.5
9	Endocervical swab	8	2.5
10	eye swab	7	2.2
11	catheter tip	7	2.2
	Total	315	100

Table 3: Prevalence of bacterial species among the isolates (n = 315).

S/No	Bacteria Genera	No of isolates	% Occurrence
1	<i>Klebsiella</i>	121	38.0
2	<i>Escherichia</i>	119	37.7
3	<i>Pseudomonas</i>	20	6.3
4	<i>Acinetobacter</i>	13	4.1
5	<i>Proteus</i>	12	4.0
6	<i>Enterobacter</i>	16	5.2
8	<i>Morganella</i>	6	2.2
9	<i>Citrobacter</i>	5	1.6
11	<i>Serratia</i>	2	0.6
13	<i>Raoutella</i>	1	0.3
	Total	315	100

Table 4. Distribution of bacterial species from clinical samples

Bacterial species	Blood culture	Catheter tip	CS F	Sputum	swabs	Tracheal Aspirates	Urine	Total
<i>Acinetobacter baumannii</i>				2	2		9	13
<i>Citrobacter freundii</i>				2			1	3
<i>Citrobacter koseri</i>				1			1	2
<i>Enterobacter aerogenes</i>					4		3	7
<i>Enterobacter cloacae</i>		1		4	3		1	9
<i>Escherichia coli</i>	12	3	6		26	14	58	119
<i>Klebsiella pneumoniae</i>	12	1	1	1	23	15	68	121
<i>Morganella morganii</i>			2	1			3	6
<i>Proteus mirabilis</i>		2			4		6	12
<i>Pseudomonas aeruginosa</i>					10	2	8	20
<i>Raoutella ornithinolytica</i>							1	1
<i>Serratia fonticola</i>					1			1
<i>Serratia marcescens</i>					1			1
Total	24	7	9	11	74	31	159	315

Table 5: Sensitivity pattern of the isolates to conventional antibiotics

Antibiotics	S (%)	I (%)	R (%)
Amoxycillin/clavulanic	29 (9.2)	13 (4.1)	273 (86.7)
Ampicillin	0	-	315 (100)
Aztreonam	165 (52.4)	5 (1.6)	145 (46.0)
Cefotaxime	100 (32.0)	11 (3.5)	204 (64.8)
Ceftazidime	175 (55.6)	14 (4.4)	126 (40.0)
Cefuroxime	44 (14.0)	9 (2.8)	262 (83.2)
Ciprofloxacin	127 (40.3)	-	188 (59.7)
Imipenem	290 (92.0)	-	25 (8.0)
Meropenem	270 (85.7)	-	45 (14.3)
Ofloxacin	126 (40)	-	189 (60)
Piperacillin/Tazobactam	205 (65)	-	110 (35.0)

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Citation: Odumosu, B.T., Dada-Adegbola, H., Iroagbalachi, J.Z. Extended Spectrum Beta-Lactamases status among Gram-Negative Bacterial Isolates from Clinical Specimens in four Major Hospitals in Southern Nigeria. *Sokoto Journal of Medical Laboratory Science*;4(3): 45 - 53.

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