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Research Article

Phenotypic Detection of *Klebsiella pneumoniae* Strains - Producing Extended Spectrum β-Lactamase (ESBL) Enzymes

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Abstract: The primary objective of this study was to detect by *in vitro* phenotypic method the occurrence of extended spectrum β-lactamase (ESBL) enzymes from *Klebsiella pneumoniae* isolates, and to buttress the growing need to detect these all important pathogens in hospital laboratories across Nigeria. Our study included 50 clinical isolates of *K. pneumoniae* from urine specimens of patients that attended a tertiary hospital in Enugu state, Nigeria. A total of 21 strains of *K. pneumoniae* were isolated from male patients, while 29 strains were isolated from female patients. The antibiogram and the production of ESBL enzymes from the test organism were evaluated by the Kirby-Bauer disk diffusion method and double disk synergy test method respectively. ESBL was detected in 13 out of 50 *K. pneumoniae* isolates employed in our study. Of all the strains of *K. pneumoniae* isolated from the male patients, only 5 were confirmed to be ESBL positive, while 8 strains of *K. pneumoniae* from those isolated from female patients expressed ESBL. There was no significant difference (P>0.05) in the distribution of ESBL-producing and non ESBL-producing strains of *K. pneumoniae* among the male and female patients. *K. pneumoniae* isolates produced varied range of susceptibility and resistance towards the tested antibiotics with imipenem and meropenem showing the best antibacterial activity. Sulphamethoxazole-trimethoprim was the least active agent. Overall, ESBL production was detected in 26% of the *K. pneumoniae* isolates, and these were multidrug resistant. The presence of an ESBL complicates treatment option; hence their prompt detection is critical to patient's wellbeing.
Keywords: ESBL, *Klebsiella pneumoniae*, Antibiotics, Resistance, Nigeria

INTRODUCTION

The emergence and spread of bacterial pathogens producing ESBLs have been well noted [1, 2], and they have been observed in a number of *Enterobacteriaceae* as well as in non-enteric pathogens such as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [2, 3]. Extended spectrum β-lactamase (ESBL) is a β-lactamase enzyme produced by bacteria within the family *Enterobacteriaceae*, and which hydrolyze oxyimino 3rd-generation cephalosporins and aztreonam but are yet inhibited by clavulanic acid [3]. The first report of an ESBL-producing strain was in Germany in 1983 in a *Klebsiella* strain [3, 4]. ESBLs arise following mutation at the amino acid configuration of the earlier plasmid-mediated β-lactamase enzymes (e.g. TEM and SHV enzymes), and this increased the affinity and hydrolytic ability of these enzymes for extended spectrum oxyimino cephalosporins, a broader spectrum β-lactam antibiotics [4- 6]. Resistance of Gram-negative bacteria to 3rd generation cephalosporins is mediated by ESBLs, and they are an important cause of treatment failure in patients receiving cephalosporins [5, 6] because these agents are hydrolyzed *in vivo* by ESBLs when used for therapy. Some risk factors for acquisition of ESBLs includes prior antibiotic usage (especially the expanded spectrum antibiotics), use of catheter, long hospitalization and stay in intensive care units, and exposure to nosocomial isolates [2, 5]. Organisms producing ESBLs can be detected in the microbiology laboratory by phenotypic and genotypic methods. In view of the medical consequences of *K. pneumoniae* strains producing ESBLs, this study was undertaken to define the danger of ESBL in our hospitals and to determine their antibiotic sensitivity patterns, thus giving impetus to the lookout of pathogens that produce these enzymes in hospital environment across Nigeria.

MATERIALS AND METHODS

Clinical isolates and identification

Fifty non-duplicate clinical isolates of *Klebsiella pneumoniae* isolates were recruited for the present day study. A total of 21 strains of *K. pneumoniae* were isolated from male patients, while 29 strains were isolated from female patients. All isolates were subcultured, purified and identified by standard microbiology techniques [7].

Antibiogram

The susceptibility of all *Klebsiella pneumoniae* isolates were determined based on the Kirby-Bauer disk diffusion method in line with the clinical laboratory

standard institute (CLSI) guidelines by subjecting them to some conventional antibiotics including (drug concentrations in µg): sulphamethoxazole-trimethoprim (25 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftazidime (30 µg), cefotaxime (30 µg), imipenem (10 µg), meropenem (10 µg) and gentamicin (10 µg) on Mueller-Hinton agar plates. Incubation was at 37^{o}C for 18-24 hrs, and the inhibition zone diameters (IZDs) were read and recorded as was previously described [8, 9]. *Klebsiella pneumoniae* ATCC 700603 was used as positive control, and this was included in each run.

Screening for ESBL

ESBL screening was evaluated using a set of 3rd-generation cephalosporins including ceftazidime (30 µg), cefotaxime (30 µg), and aztreonam (30 µg) on Mueller-Hinton agar plates already inoculated with the test bacteria (adjusted to 0.5 McFarland turbidity standard) as recommended by the clinical laboratory standard institute, CLSI. Diameter of zones of inhibition (IZD) was carefully taken after incubation (at 37^{o}C for 18-24hrs), and isolates found to showed reduced susceptibility (or was resistant) to any of the corresponding cephalosporins (ceftazidime, cefotaxime, and aztreonam) according to the CLSI breakpoints was suspected to produce ESBL enzyme [10, 11].

Confirmatory test for ESBL production

ESBL production in suspect bacteria was confirmed by the double disk synergy test (DDST) method as was previously described [10, 11]. Briefly, a standardized inoculum of the test organism was swabbed aseptically on the surface of Mueller-Hinton (MH) agar plates. Single antibiotic disc of amoxycillin-clavulanic acid (30 µg) was placed at the center of the MH agar plate, and ceftazidime (30 µg) and cefotaxime (30 µg) discs were each place at a distance of 15 mm away from the central disc. The plates were incubated at 37^{o}C for 18-24hrs. An increase in zone diameter by ≥5mm for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with amoxycillin-clavulanic acid (30 µg) versus its zone when tested alone confirms ESBL production phenotypically [10, 11].

Susceptibility pattern of ESBL positive isolates

The susceptibility of all *K. pneumoniae* ESBL positive bacteria was determined by disk diffusion method in line with the CLSI guidelines [8]. They were subjected to some selected antibiotics including sulphamethoxazole-trimethoprim, ciprofloxacin, ofloxacin, imipenem, meropenem and gentamicin on Mueller-Hinton agar plates which were incubated 37^{o}C for 18-24hrs. Zones of inhibition were carefully taken and recorded using a meter rule.

Statistical analysis

The significance of demographic difference between ESBL and non-ESBL producing *K. pneumoniae* strains was assessed with the Statistical Package for Social Sciences (SPSS) software (version 17.0). The two-way ANOVA (without replication) were calculated.

RESULTS

The antimicrobial susceptibility profile of the *K. pneumoniae* isolates are shown in Table 1. Table 2 shows the frequency of ESBL production in all *K. pneumoniae* isolates. Also, the distributions of ESBL-producing and non ESBL-producing strains of *K. pneumoniae* among male and female patients are shown in Table 3. Table 4 shows the percentage susceptibility and resistance of the ESBL-producing strains of *K. pneumoniae* to some classes of antibiotics.

Table 1: Antibiogram

Antibiotic (µg)	Susceptible n(%)	Intermediate n(%)	Resistant n(%)
CTX	11(22)	5(10)	34(68)
CAZ	6(12)	12(24)	32(64)
SXT	5(10)	2(4)	43(86)
CN	18(36)	4(8)	28(56)
OFX	16(32)	2(4)	32(64)
CIP	15(30)	4(8)	31(62)
IPM	44(88)	0(0)	6(12)
MEM	46(92)	0(0)	4(8)

Key: CTX-Cefotaxime, CAZ-Ceftazidime, SXT-Sulphamethoxazole-Trimethoprim, CN-Gentamicin, OFX-Ofloxacin, CIP-Ciprofloxacin, IPM-Imipenem, MEM-Meropenem

Imipenem and meropenem were the most active agents tested, with percentage susceptibility of 88% and 92% respectively. *K. pneumoniae* isolate resistance to cefotaxime, ceftazidime, sulphamethoxazole-trimethoprim, gentamicin, ofloxacin and ciprofloxacin was 68%, 64%, 86%, 56%, 64% and 62% respectively.

Table 2: Occurrence of ESBL positive *Klebsiella pneumoniae* isolates

Organisms	ESBL screening	ESBL confirmation	Percentage of ESBL (+
K. pneumoniae (n=50)	32	13	26

Overall, ESBL was detected in 13 out of 50 *K. pneumoniae* isolates employed in our study. Also, as observed in Table 3, 21 strains of *K. pneumoniae* were isolated from male patients, while 29 strains were isolated from female patients. From Table 4, It can be observed that all *K. pneumoniae* ESBL positive isolates were completely resistant to sulphamethoxazole-trimethoprim. The percentage susceptibility of all ESBL positive *K. pneumoniae* isolates to gentamicin, ofloxacin, ciprofloxacin, imipenem and meropenem was 38.5%, 23.1%, 46.2%, 84.6% and 76.9% respectively.

Table 3: Distributions of ESBL-producing and non ESBL-producing isolates of *K. pneumoniae* among male and female patients

	Male	Female	p-value
ESBL-producing strains	5	8	0.156
Non-ESBL producing strains	16	21	
Total	21	29	

Table 4: Percentage susceptibility of ESBL positive *K. pneumoniae* isolates to some selected antibiotics

Antibiotics (µg)	Resistant n(%)	Susceptible n (%)
Gentamicin (10)	8(61.5)	5(38.5)
Ofloxacin (5)	10(76.9)	3(23.1)
Sulphamethoxazole-trimethoprim (25)	13(100)	0(0)
Ciprofloxacin (5)	7(53.8)	6(46.2)
Imipenem (10)	2(15.4)	11(84.6)
Meropenem (10)	3(23.1)	10(76.9)

DISCUSSIONS

Organisms producing ESBLs represent an important mechanism of antibiotic resistance in Gram negative bacteria. ESBL-producing pathogens are multidrug resistant, and they remain active and untouched by antimicrobial onslaught directed at them *in vivo* [1, 2]. It is in view of the underlying health and economic circumstances posed by multidrug resistant Gram-negative bacteria that the present day study detected ESBL production in 50 clinical isolates of *K. pneumoniae* from a tertiary hospital in Enugu State, Nigeria. All *K. pneumoniae* isolates was subjected to 8 different antibiotics in order to determine their susceptibility and resistance profiles. Our antibiogram result (as shown in Table 1) show that the percentage susceptibility of the *K. pneumoniae* isolates to cefotaxime, ceftazidime, sulphamethoxazole-trimethoprim, gentamicin, ofloxacin, ciprofloxacin, imipenem and meropenem were 22%, 12%, 10%, 36%, 32%, 30%, 88% and 92% respectively. Consistent antimicrobial susceptibility studies in the Microbiology laboratories are required to checkmate the emergence and spread of resistance strains of bacterial pathogens within the hospital environment. In the present study, we detected the production of ESBLs from clinical isolates of *K. pneumoniae* isolates (n=50). Out of the 50 *K. pneumoniae* isolates, only 13 were confirmed to produce ESBL phenotypically. Of all the strains of *K. pneumoniae* isolated from the male patients, only 5 were confirmed to be ESBL positive, while 8 strains from those isolated from female patients expressed the ESBL. There was no significant difference (P-value=0.156) in the distribution of ESBL-producing and non ESBL-producing strains of *K. pneumoniae* among the male and female patients in our study. The percentage prevalence of ESBL-producing *K. pneumoniae* isolates (26%) as shown in our study is high and worrisome. Pathogenic bacteria producing ESBLs have been considered as a serious therapeutic challenge because of limitations of therapeutic options for affected patients. Organisms producing ESBLs has steadily increased in the rate of its morbidity and mortality according to studies [1-3]. Ullah *et al.*, [9] reported 58.7% of *K. pneumoniae* clinical isolates to be ESBL producers, a result that is higher than ours. Elsewhere in Kano, Nigeria, a prevalence of 66.7% ESBL production was reported in *Enterobacteriaceae* [10]. However, our result of ESBL production of 26% *K. pneumoniae* was in contrast to a similar work done in Saudi Arabia where ESBL production was reported to be 4.4% in *K. pneumoniae* clinical isolates [11]. It is noteworthy that ESBL was first detected in a *K.*

pneumoniae strain (bearing SHV-2 gene) from Germany in 1983 [3, 4], and as such; *K. pneumoniae* isolates (including other enteric and non-enteric bacteria) found to show reduced susceptibility to any of the 3rd generation cephalosporins in the Microbiology laboratory should be subjected to ESBL screening in order to rule out or confirm ESBL production in them. It was also observed in our study that the *K. pneumoniae* producing isolates were resistant to 4 or more antibiotic classes. In all, only meropenem and imipenem was found to possess the best antibacterial activity on the ESBL positive *K. pneumoniae* clinical isolates. Sulphamethoxazole-trimethoprim was the least active antibiotic tested as all ESBL positive *K. pneumoniae* isolates was resistant to it. This was followed by gentamicin, ofloxacin, ciprofloxacin, imipenem and meropenem which showed percentage susceptibility of 38.5%, 23.1%, 46.2%, 84.6% and 76.9% against the ESBL positive *K. pneumoniae* isolates. Effective antibiotic policy and awareness on ESBLs in our hospitals are important factors to be considered in clinical practice in order to assuage disease outbreak due to ESBL organisms. Further molecular studies are required for the genotypic characterization and typing of our ESBL positive *K. pneumoniae* strains.

CONCLUSIONS

Our study shows that *Klebsiella pneumoniae* isolates of nosocomial origin in Enugu state, Nigeria produce extended spectrum β-lactamase (ESBL) enzymes in very high amount, and that these were multidrug resistant. Prompt and accurate detection and reporting of ESBL-producing organisms and other multidrug resistant strains of pathogenic bacteria in the microbiology laboratory is needed in our hospitals.

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