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Occurrence of Beta-Lactamases and the Antibiogram Pattern of Clinical Isolates of *Escherichia coli* and *Klebsiella* Species in Nsukka Metropolis

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Abstract
Infections due to β-Lactamases producing *Escherichia coli* and *Klebsiella* species are increasingly recognized in recent years. They are creating clinical concerns. This is because few antibiotics are available as therapeutic options. A total of 113 screened isolates of *E.coli* and *Klebsiella* spp obtained from November 2013 to December 2014 from various clinical specimens such as urine, stool, sputum, wound swab and high vaginal swab (HVS) were included in this study. All isolates were identified and confirmed by standard methods. Isolates were screened for β- lactamase and extended spectrum beta- lactamases (ESBLs) using Nitrocefin test and Double disk synergy detection test. All confirmed β-lactamases producing isolates were tested for susceptibility to ten different antibiotics by disk diffusion method. The overall prevalence of 51(45.1%) and 33(27.2%) were recorded for β-lactamases and ESBLs production respectively. *E.coli* showed higher prevalence (47%, 30.3%) followed by *Klebseilla* species (42.7%, 27.7%) for β-lactamase and ESBLs respectively. Age distribution of β-lactamse producing isolates among patients showed highest prevalence (68.4%) among 41-50 years age group. ESBL producers were 50.0% of the organisms isolated from individuals 51years and above. Both β- lactamase and ESBLs producers were susceptible to Imipenem and Nitrofuration and resistant to Ampicilin, Augmentin and the cephalosporins. This study indicates that β-lactamase producers are widely distributed in the study area. This poses public health challenges.

1. Introduction

Beta-lactamases are enzymes produced mostly by Gram-negative bacteria. They are often responsible for resistance to β-lactam antibiotics by organisms possessing them (Bush *et al.,* 1995). ESBLs are chromosomal or plasmid mediated and occur as a result of spontaneous mutations that take place in the serine active site of the old beta-lactamase enzyme, adding 4-6 new amino acids that extend their hydrolytic substrate (Steward *et al*., 2001). These enzymes mediate resistance to oxymino-cephalosporins (ceftriaxone, ceftazidime, cefotaxime, cefepime) and monobactams (aztreonam) with the exception of carbapenems. The first β-lactamase with extended spectrum was detected in *Klebsiella pneumoniae* in Garmany in 1983(Knothe *et al.,* 1983) and later in the western parts of Europe, probably because extended-spectrum β-lactam antibiotics were first

used there clinically. They occur predominantly in members of the enterobacteriaceae with *Klebsiella pneumoniae* and *Escherichia coli* being the most commonly reported worldwide. They are responsible for 5-20% of outbreak of nosocomial infections in intensive care unit, burn, oncology and neonatal units (*Kotra et al.,* 2002). Poor hygienic practices, indiscriminate use of antibiotics as well as lack of monitoring of microbial drug resistance has created suitable condition for the emergence and controllable spread of these enzymes in Nigeria (Arzai and Adamu 2008).

Laboratory detection of these enzymes, proper reporting, awareness creation and necessary precautions to avoid their spread is lacking in our area, despite the fact that it is a significant health problem. In view of this, this research was conducted to determine the incidence, distribution and antibiotic susceptibility of β-lactamase and ESBLs producing clinical isolates of *Escherichia coli* and *Klebsiella* spp with a view to creating proper awareness of the status of these enzymes in Nsukka Metropolis.

2. Materials and Methods

2.1. Study Area

The study area is Nsukka metropolis, Enugu state, Nigeria. The sites for sample collection were University of Nigeria Nsukka Medical center and Bishop Shanahan hospital Nsukka. These are tertiary health care facilities patronized by people with different socioeconomic backgrounds from within and outside Nsukka metropolis. They serve as referral centers for Nsukka and neighboring villages. This informed the choice of these hospitals as the study sites as nutrition is said to be among the predisposing factors for infection with ESBLs producing organisms (Paterson and Bonomo 2005).

2.2. Sample and Sampling Techniques

One hundred and thirteen isolates of Gram-negative bacteria comprising *E.coli* (n=66) and *Klebsiella* species (n=47) recovered from 165 specimens collected from both inpatients and outpatients attending University of Nigeria, Nsukka (UNN) Medical Center and Bishop Shanahan hospital, Nsukka were used. All clinical specimens were processed according to standard operating procedure. All samples were inoculated on Blood agar and MacConkey agar. Identification and confirmation were performed using biochemical tests and chromogenic agar orientation (France). The study was done between October 2013 and December 2014.

2.3. Isolation of Samples

The samples collected from each patient were inoculated onto MacConkey and Blood agar by spreading and the plates were incubated for 24hours at 36^0C. The colonies were further subcultured to obtain pure culture as described by Chesbrough 2006

2.4 Antimicrobial Sensitivity Testing

Susceptibility was determined by the Kirby Bauer disk diffusion method as described by Clinical Laboratory Standards Institute (CLSI, 2006). Bacteria were grown on nutrient broth at 37^0C overnight. The suspension was visually adjusted with normal saline to 0.5 Macfarland turbidity standard. Each inoculum was separately swabbed across the entire surface of Muller Hinton agar plate (Biotech) using sterile swab stick and the plate was rotated approximately 60^0C between streaking to ensure even distribution.

Inoculated plates were left to stand for at least 3 minutes before the disks were applied. Commercial antibiotics disks (Abtex Biological Ltd) used include: Ceftazidime (30µg), Cefuroxime (30µg), Cefotaxime(30µg), Gentamicin (10µg), Ciprofloxacin (5µg), Ofloxacin(5µg), Augumentin(30µg), Nitrofuratoin(300) µg, Ampicilin(10µg) and Imipenem (10µg). The plates were incubated within 15 minutes of the application of the disks at 37^0C for 24 hours. The inhibition zone diameters around the disks were measured and interpreted according to the CLSI guideline.

2.5. Tests for β-lactamase production

Nitrocefin Test

Nitrocefin is chromogenic cephalosporin that changes from yellow to red on hydrolysis. The experiment was done according to O'Callaghan and Cynthia, (1972) and Parr (1984). Briefly, 1.0 mg/ml solution was prepared by dissolving 10.0 mg of Nitrocefin powder (TOKU-E, USA) in 1mL of dimethylsulphoxide(DMSO), vortexed appropriately and diluted with 9.0mL phosphate buffer saline (PBS) buffer (100mM, neutral pH) (this solution remains stable for 14days at 4^0C). Colonies of the test isolates were scraped from nutrient agar plates and suspended in 1ml of broth, 3-5 drops of 1.0mg/ml of nitrocefin solution were added. Appearance of red colour within 20-30 minutes indicates β-lactamase activity. *Escherichia coli* (ATCC 25922) and *Pseudomonas aeroginosa* (ATCC 27853) were used as reference strains throughout the study.

2.6. Screening for Extended Spectrum Beta-Lactamases

The double disc synergy test (DDST) method described by CLSI (2006) was employed. Standardized inocula of the test organisms were inoculated on Muellar Hinton Agar (MHA) using sterile swab sticks as previously described. Amoxicillin/clavulanic acid disc (20/10µg,) was placed at the center of each inoculated MHA. Ceftazidime (30µg) and Cefotaxime (30µg) were placed 15 mm center to center from the Amoxycillin/clavulanic acid disc. The plates were incubated at 37^oC for 24 hours. After incubation, enhancement of zone of inhibition of either or both the Ceftazidime and Cefotaxime discs towards the Amoxycillin/Clavulanic acid discs is indicative of ESBL production.

3. Results

Table 1. *Occurrence of β-lactamase and ESBLs in test isolates.*

Isolates	No screened	No(%)B-lactamase producers	No(%)ESBLs producers
Escherichia coli	66	31(47%)	20(30.3%)
Klebsiella species	47	20(42.7%)	13(27.7%)
Total	113	51(45.1%)	33(29.2%)

A total of 113 clinical isolates of Gram- negative bacteria obtained from urine, stool, sputum, wound swabs and HVS were tested. Forty five isolates were from urine, 26 from stool, 32 from sputum, 9 from wounds and 1 from HVS. Out of the 113 isolates screened for β-lactamase and ESBL production, 51 and 33 were confirmed to produce β-lactamase and ESBL respectively, giving an overall prevalence of 45.1% and 29.2%. The highest prevalence of β-lactamase (47.0%) and ESBLs (30.3%) were found in *E.coli* while *Klebsiella* species had 42.7% β-lactamase and 27.7% ESBLs (Table 1).

3.1. Distribution of β-lactamase and ESBL Producers

The distribution of β-lactamse and ESBLs producers according to clinical samples, sexes and age are presented in Tables 2, 3 and 4 respectively. β-lactamse producers were most prevalent in urine (84.4%) while ESBLs producers were most prevalent in stool with an overall prevalence of 76.9%. The distribution of β-lactamase and ESBLs producers based on gender indicates that males had a higher prevalence rate of β-lactamase (55.3%) and ESBLs (36.8%) than females (40% and 25.3%) respectively (Table 3).The age distribution of the β-lactamase producers among the patients showed highest prevalence among the 40-51years age group (63.2%) while ESBL producers from 51-above year age group stood at 50% (Table 4).

Table 2. *Distribution of β-lactamase and ESBLs among isolates.*

SAMPLE	TOTAL NO SCREENED	NO AND (%) OF ISOLATES POSITIVE FOR β-LACTAMASE	NO AND (%)OF ISOLATES POSITIVE FOR ESBLs	Total
	E.coli	***E.coli***	***E.coli***	
	***K.*spp**	***K.*spp**	***K.*spp**	
Urine	22	13(59.1)	9(40.9)	38(84.4%)
	23	10(43.5)	6 (26.1)	
Stool	20	8(40)	5(25)	20(76.9%)
	6	4(66.7)	3(50)	
Sputum	16	8(50)	5(31.3)	22(68.8%)
	16	5(31.1)	4(25)	
Wound Swab	7	2(28.6)	1(14.3)	4(44.4%)
	2	1(50)	0(0)	
HVS	1	0(0)	0(0)	0(0%)
	0	0(0)	0(0)	
Total	66	31(46.9)	20(30.3)	84(74.3%)
	47	20(42.6)	13(27.7)	

Legend: K.spp= *Klebsiella* species

Table 3. *Sex distribution of patients habouring β-lactamase and ESBLs positive isolates.*

S N	CLINICAL ISOLATES	NO AND % SCREEENED		NO AND % OF ISOLATES POSITIVE FOR β LACTAMASE		NO AND % OF ISOLATES POSITIVE FOR ESBLs	
		MALE	FEMALE	MALE	FEMALE	MALE	FEMALE
1	*E.coli*	30(78.9)	36(48)	16(53.3)	15(41.7)	11(36.7)	9(25)
2	*Klebspp*	8(21.1)	39(52)	5(62.5)	15(54)	3(37.5)	10(25.6)
TOTAL		38(100)	75(100)	21(55.3)	30(40)	14(36.8)	19(25.3)

Table 4. *Age distribution of patients habouring β-lactamase and ESBLs positive isolates.*

Age	Frequency of occurrence	No(%) β-lactamase positive isolates	No(%)ESBLs positive isolates
1-10	5	1(20%)	1(20%)
11-20	9	4(44.4%)	3(33.3%)
21-30	43	16(37.2%)	9(20.9%)
31-40	31	15(48.3%)	8(25.8%)
41-50	19	12(63.2%)	9(47.4%)
51-above	6	3(50%)	3(50%)
Total	113	51(45.1%)	33(29.2%)

3.2. Antimicrobial Susceptibility Profile

Antimicrobial susceptibility of β-lactamase producers to the selected antibiotics showed that both the β-lactamase and ESBL producing isolates exhibited appreciable susceptibility to carbapenem: IMI (Imipenem) and flouroquinolones. β-lactamase positive *E.coli* were 6.5% susceptible to IMI while ESBLs producers were 10% susceptible. Resistance to Ampicillin, Augmentin, and 2nd and 3rd generation cephalosporin was high. (Figures 1 and 2).

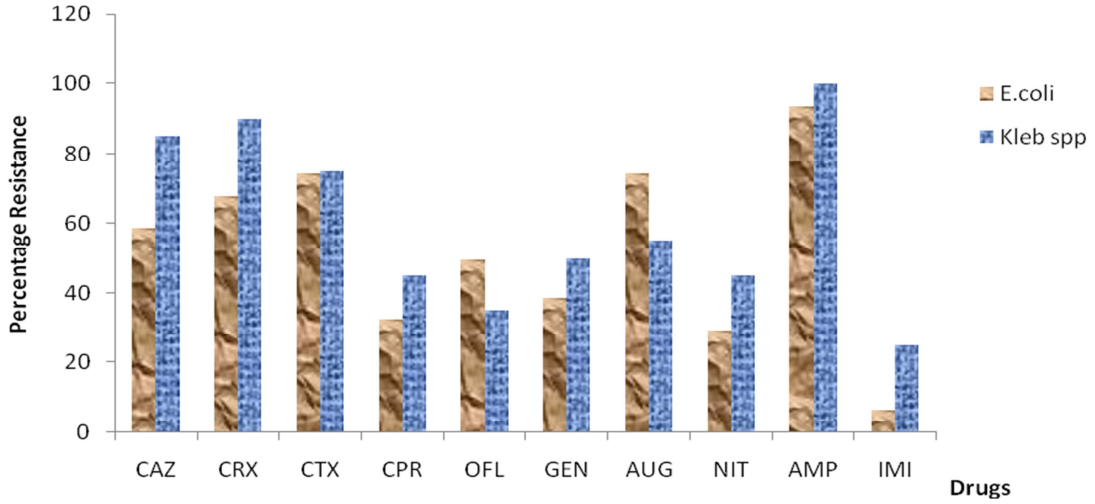

Fig. 1. *Antibiotics susceptibility profiles of β-lactamase producing E .coli (n=31) and Klebsiella spp (n=20) from clinical isolates.*

Key: CAZ= Ceftazidime, CEF=Ceftazidime; CRX= Cefuroxime; CTX= Cefotaxime; CPR= Ciprofloxacin; OFL= Ofloxacin; GEN= Gentamycin; AUG = Augumentin; NIT= Nitrofuratoin; AMP= Ampicillin; IMI= Imipenem.

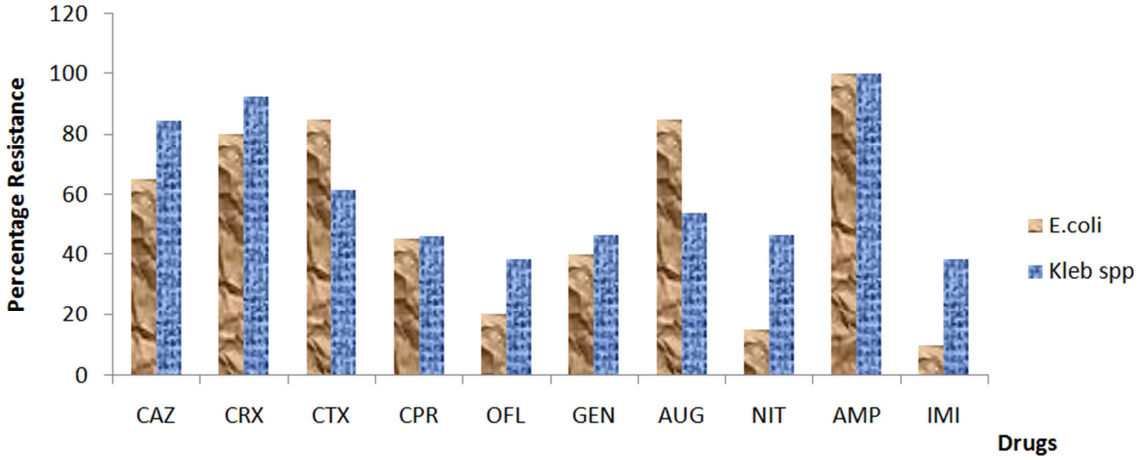

Fig. 2. *Antibiotics susceptibility profiles of ESBLs producing E.coli (n=20) and Klebsiella spp (n=13) from clinical isolates.*

Key: CAZ= Ceftazidime, CEF=Ceftazidime; CRX= Cefuroxime; CTX= Cefotaxime; CPR= Ciprofloxacin; OFL= Ofloxacin; GEN= Gentamycin; AUG = Augumentin; NIT= Nitrofuratoin; AMP= Ampicillin; IMI= Imipenem.

4. Discussion

Multi drug-resistant (MDR) Gram negative bacteria induced infections have been reported with an increasing frequency in tertiary health care facilities in Nigeria and they have been found to be associated with significant morbidity and mortality (Yusuf *et al*., 2012). Members of *Enterobacteriaceae* are the most common causative agents of nosocomial and community bacteria acquired infections (Coque *et al.,* 2008). The overall prevalence of β-lactamase and ESBLs producers in this study was 45.1% and 29.2% respectively. Although an increase in β-lactamase producers has been reported in a previous study (Dougharia and Akafam 2009), our result shows comparatively lower overall level of β-lactamase occurrence. Similarly, a comparison of

the overall prevalence of ESBLs (29.2%) found in this study with the findings of similar studies from the same country, indicates that the prevalence rate in this study is lower when compared to previous studies by Iroha *et al.,* (2010) and Yusuf *et al.,* (2011) who reported prevalence of 58.6%and 37.1% in Enugu and Kano respectively. Similar observation was shown by Aibinu *et al.,* (2003) who reported ESBLs production of 20.8% in *E.coli* and *Klebsiella* spp in Lagos Nigeria. When compared to the other countries, the prevalence in the present study is much lower than those reported by Bouchillion *et al,* (2002) from Egypt showing ESBLs production among *E. coli* and *Klebsiella* spp to be 40.9%. These variations could be due to differences in antibiotic selection pressure, local antibiotics and prescribing habits, which differ from state to state, institution to institution and from country to country.

Distribution of β-lactamase and ESBLs among clinical specimens was also determined. Urine had the highest prevalence of 86.7% followed by stool with the prevalence of 84.6%. The least prevalence was observed in wound swab (44.4%). This finding is in agreement with Doughari and Akafa (2009), who reported a higher prevalence rate of 91% β-lactamase in urine, Iroha *et al*., (2010) who reported high prevalence of 60.3% and 59.6% ESBLs in urine and blood isolates respectively and Osazuwa and Osazuwa (2011) who also found that ESBLs prevalence was high in urine (61.4%) and blood (61.2%). The high prevalence of β- lactamse and ESBLs in urine may be attributed to factors like extreme age, female gender, sexual activity, contraception, pregnancy, urinary tract obstruction, neurological dysfunction, antimicrobial use and poor hand washing techniques among health care practitioners, which are some of the factors that can predispose one to urinary tract infection (UTI) development.

A high prevalence of β-lactamase and ESBLs was also observed among males. This finding is in consonance with the finding of Yusuf *et al,* (2011) who also revealed a slightly higher recovery in males (*E.coli* 38.9% *K. pnemoniae* 40%) than in females (*E.coli* 36.8%, *K. pneumoniae* 33.3%).

The age distribution of β-lactamase and ESBLs producers among the patients showed high prevalence among 41-51years age group. This may bethat in the old people often become immune-compromised and stand greater chances of coming down with infections. Such infections are of many types but in patients with low immunity they are more difficult to treat (Muratani and Matsumoto, 2006). This may explain the relatively high presence of extended spectrum beta-lactamase producers among the aged as seen in this report. This result is in consonance with the finding of Yusuf *et al*, (2011) and Kiratisin *et al,* (2008) that showed high prevalence rate of 33.3% each for ESBLs among 51years and above group. Riaz *et al* 2012 also reported similar results in Pakistan. High resistance to β-lactam was shown in β-lactamase and ESBLs producers in clinical isolates. This is because β-lactamase producers have enzymes that relax the active site of the antibiotics. Increase in resistance to ciprofloxacin had been reported earlier in Nigeria by Aibinu *et al.,* (2003) who discovered that 18% of all ESBLs producing *Enterobacter* spp were resistant to ciprofloxacin. Paterson *et al.,* (2000) had reported that globally, 18% of all ESBLs producers were resistant to ciprofloxacin. Today, 14 years after, this study found 45% and 46.1% of ESBLs producing *E.coli* and *Klebsiella* spp in Nsukka, Enugu State resistant to ciprofloxacin. This means that the resistance phenomenon is on the increase. This increasing resistance to several antimicrobial drugs may be due to inappropriate use of antimicrobial drugs (over use, misuse, suboptimal dosage and non- compliance with the treatment duration) which leads to selection pressure. Mohammad *et al*, (2010) reported that abuse and misuse of antimicrobial agents for growth promotion and prevention of diseases have impressed a selective pressure that causes discovery of more resistant bacteria. The overall least resistance by β-lactamase and ESBLs producers (both *E.coli* and *Klebsiella* spp) was shown against imipenem. This is in agreement with the work done by Asma *et al,* (2014).

In conclusion, there is appreciable presence of β-lactamase and ESBLs producing Gram negative potential and overt pathogenic bacteria in Nsukka, Enugu State, Nigeria. This research report is therefore a clarion call on health care practitioners to indulge in public health campaigns and prudent use of antimicrobials to minimize the spread.

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