

Strains of *Klebsiella Pneumoniae* from Intensive Care Unit Producing CTX-M-15 Extended Spectrum Beta Lactamases

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Abstract: Problem statement: Resistance to third generation cephalosporins due to acquisition and expression of Extended Spectrum β -Lactamase (ESBL) among Gram-negative bacteria is on the increase. Infections involving extended spectrum beta lactamase bacteria are associated with significant morbidity and mortality. Therefore, infections due to ESBL isolates continue to pose a serious challenge to infection management worldwide. Since screening for ESBL is not a common practice in hospitals in Enugu state, this study was undertaken to characterize ESBL genes in *K. pneumoniae* strains from intensive care unit of the University of Nigeria Teaching Hospital (UNTH) Enugu. **Approach:** Over a period of 29 months, 57 patients out of 140 receiving treatment in the intensive care unit of the University of Nigeria Teaching Hospitals Enugu were found to be infected with extended-spectrum β -lactamase-producing strains of *K. pneumoniae*. Species identification of *K. pneumoniae* strains was performed by Standard Microbiology methods and re-confirmed by MALDI-TOF technology. Phenotypic characterization of Extended Spectrum Beta Lactamase (ESBL) was determined by double disc synergy test and presence of ESBL genes was determined by specific PCR. **Results:** All ESBL producers were positive in a PCR for *bla*_{CTX-M-1} cluster and on sequencing, *bla*_{CTX-M-15} were found to be present. Genotypic characterization of extended spectrum beta-lactamase producing *K. pneumoniae* showed that all isolates carried CTX-M-15 and SHV genes, 41(71.9%) carried *aac* (6')-Ib-cr and *bla*_{OXA-1}, 19(33%) carried *bla*_{TEM}. *ISEcp1* was found upstream and ORF 477 downstream of *bla*_{CTX-M} in all strains. Random amplified polymorphic DNA analysis grouped the strains into two clonal groups- A and B and majority of the strains belong to clonal group A (n = 42). **Conclusion:** This study shows for the first time the presence of ESBL genes in *K. pneumoniae* from the ICU of UNTH Enugu and therefore strongly buttresses the need for regular screening for ESBL-producing bacteria in clinical specimen in ICU and other wards in Nigerian hospitals.

Key words: Extended Spectrum β -Lactamase (ESBL), University of Nigeria Teaching Hospital (UNTH), *Klebsiella pneumoniae*, CTX-M-15, Intensive care unit

INTRODUCTION

Resistance to oxyimino-cephalosporins such as cefotaxime, ceftriaxone or ceftazidime is a growing problem in the treatment of nosocomial infections and is often caused by the production of Extended Spectrum β -Lactamases (ESBLs) (Paterson *et al.*, 2003). ESBLs are frequently identified in *K. pneumoniae* but also in other species such as *E. coli*, *Citrobacter* spp.,

Enterobacter spp. and *Pseudomonas aeruginosa* (Gniadkowski *et al.*, 1998). ESBLs of the CTX-M-type have less than 40% homology with TEM and SHV (Carton and Coque, 2006). CTX-M- β -lactamases are rapidly disseminating to different geographical areas including many parts of Europe, Asia, Africa and the Americas (Al-Agamy *et al.*, 2006). Epidemiological reports demonstrate that some enzymes are more frequent than others and the predominant enzyme types

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vary with country and the diverse CTX-M-types often exist within a single country (Livermore *et al.*, 2007; Coque *et al.*, 2002). *Klebsiella* spp. particularly *K. pneumoniae* is an important cause of nosocomial infections and the main population at risk are neonates and immunocompromised hosts. An outbreak of multiple resistant *Klebsiella*, which causes systemic infections and death has been widely reported (Podschum and Ullmann, 1998). *Klebsiella* became resistant to broad-spectrum β -lactam antibiotics due to the emergence and spread of plasmid-mediated β -lactamases such as Extended Spectrum β -Lactamases (ESBLs) and has been associated with infection acquired in the Intensive Care Units (ICU) (Tenover and Hughes, 1996). The frequent use of antimicrobial agents in ICUs increases the likelihood of infection with resistant strains (John *et al.*, 1983; Pessoa-Silva *et al.*, 2003). An increasing emergence of multidrug resistance among *Klebsiella pneumoniae* nosocomial isolates has limited the therapeutic options for the treatment of the intra-hospital infections caused by this opportunistic pathogen. A multidrug resistance phenotype results from the progressive accumulation of different mechanism of resistance in the same microorganism, including high-level production of Extended-Spectrum β -Lactamases (ESBLs). No data on genotype of ESBL from *K. pneumoniae* is available in this region of Nigeria. Therefore, this study was undertaken to characterize ESBL genes in *K. pneumoniae* strains from intensive care unit of University of Nigeria Teaching Hospital (UNTH) Enugu.

MATERIALS AND METHODS

Collection of bacteria strains: Fifty-seven isolates of ESBL producing *K. pneumoniae* were isolated from 140 patients receiving treatment in the ICU of UNTH Enugu over a period of 29 months. ESBL producing *K. pneumoniae* was recovered from urine (39) and sputum (18). These clinical samples were collected from male and female patients within the age range of 9-97 years and were characterized using standard microbiology techniques and re-confirmed by MALDI-TOF (NCCLS, 1997; Laura *et al.*, 2010).

Phenotypic characterization of ESBLs: Extended spectrum beta lactamases were determined by the Double Disc Synergy Test (DDST) method. This was performed by placing disks of ceftazidime, cefotaxime, ceftriaxone, cefepime and aztreonam (30 μ g each) at a distance of 15 mm (center to center) from a disk containing amoxicillin plus clavulanic acid (20 and 10 μ g) respectively (Pitout *et al.*, 2004).

Isolation of genomic DNA: Genomic DNA of the ESBL producing *K. pneumoniae* was prepared using the Nucleospin Kit (Macherey and Nagel, Germany) following manufacturer's instructions.

Detection of ESBL gene by PCR: Genes for TEM, SHV, CTX-M, OXA-1, aac (6')-Ib-cr, ISEcp-1 and ORF 477 beta lactamases was determined by PCR using specific primers as described by (Schlesinger *et al.*, 2005; Karisik *et al.*, 2006; Park *et al.*, 2006; Eckert *et al.*, 2006) with primers shown in Table 1.

Table 1: Primers used in this study

Primer	Sequence	Amplicon size	Source
TEM-seq	ATAAAATCTTGA	860 bp	Yun-Tae <i>et al.</i> (2006).
TEM-rev SHV-seq	GACAGTTACCAATGCTTAATC TCGTTATGCGT TATATTCGCC	776 bp	Yun-Tae <i>et al.</i> (2006).
SHV-rev CTX-M-seq	GGTTAGCGTTGCCAGTGCT CGCTTTGCG ATGTGCAG	730 bp	Yun-Tae <i>et al.</i> (2006).
CTX-M-rev OXA-1-fw	ACCGCGATATCGTTGGT GGATAAAACC CCCAAAGGAA	369 bp	Karisik <i>et al.</i> (2006).
OXA-1-rev ISEcp1-5	TGCACCAGTTTTCCCATACA TTCAAAAAGC ATAATCAAAGCC	Variable	Eckert <i>et al.</i> (2006).
MA1-rev CTX-M-1-540 orf477-rev	ACYTTACTGGTRCTGCACAT GCGTGATACCACTTCACCTC GAAGGAGAACC AGGAACCAC	460 bp 482 bp	This study This study
aac6-Ib-fw	TTGCGATGCTCTA TGAGTGCTA		Park <i>et al.</i> (2006).
aac6-Ib-rev RAPD-1290	CTCGAATGCCTGGCGTGTTT GTGGATGCGA		Appleford and Denman (1979).

Amplified PCR products were separated on 0.8% agarose gels at 100 Volts, stained with ethidium bromide and visualized under UV illumination. Appropriate positive and negative controls were used in all cases.

Randomly Amplified Polymorphic DNA (RAPD) analysis: RAPD was performed with all positive ESBL *K. pneumoniae* strains with a single primer 5'-GTGGATGCGA-3' (Appleford and Denman, 1979). Amplified PCR products were separated on 1.5% agarose gels at 75 Volts.

RESULTS

As shown in Table 2 57 *K. pneumoniae* strains were ESBL positive, 39(68.4%) were samples from urine and 18(31.6%) were from sputum. 24(42.2%) were from male patients while 33(57.8%) were from female. Greater proportion of ESBL was isolated from patients between the ages of 22-30 years 36(63.1%). Also all the 57 strains of *K. pneumoniae* gave an amplicon using primers that amplified all currently known CTX-M-genes. Subsequently a PCR specific for CTX-M-1 cluster gene was performed and was positive in all strains.

Table 2: Demographic AND Patients DATA

Nos of positive ESBL	57.00
Nos of female patients	33.00
Nos of male patients	24.00
Age range of patients	9-79
Patients below 30 years of age	36.00
Mean age of patients	27.20
Isolates from Urine	39.00
Isolates from sputum	18.00

Table 3: Frequency of occurrence of β - lactamase genes

β - lactamase genes	Number positive
CTX-M-1	57
ISEcp 1	57
ORF 477	57
SHV	57
TEM	30
OXA-1-	57
aac(6)-Ib-cr	41

Table 4: Clonal classification of *K. pneumoniae* ESBL

RAPD Clonal group	<i>K. pneumoniae</i>
A	42
B	15

Table 5: PCR results in relation to RAPD groups

PCR tests	Clone A	Clone B
CTX-M-1	45	12
ISEcp 1	45	12
ORF 477	45	12
SHV	45	12
TEM	22	8
OXA-1-	45	12
aac(6)-Ib-cr	50	04

PCRs for ISEcp1 upstream and ORF 477 downstream were positive in all strains Table 3. PCRs for TEM were positive in 19(33%) of the isolates while OXA-1- and aac (6') Ib-cr β -lactamase genes were positive in the majority of ESBL isolates 41(71.9%).

SHV beta lactamases was found to be present in all the strains as expected. RAPD analysis of positive ESBL strains for clonal relatedness grouped our strains into two clonal groups (A and B). Clone A was by far the most frequent clone and found in 42(73.6%). PCR results in relation to clonal type are presented in Table 4. The association of CTX-M-1 cluster genes to TEM, OXA-1 and aac (6')-Ib-cr is stronger in Clone A ESBLs than in other clonal types (Table 5).

DISCUSSION

In the present study, we characterized strains of *K. pneumoniae* harbouring CTX-M-15 ESBL in the intensive care unit of UNTH in Southeastern, Nigeria where there is no record of investigation of molecular epidemiology of ESBL *K. pneumoniae*. Nosocomial bacterial infections constitute a substantial cause of morbidity and mortality in developing countries such as Nigeria. *K. pneumoniae* is known to be one of the major organisms causing nosocomial infections within the hospital and has also been implicated in community acquired ESBL. The observation of ESBL producing *K. pneumoniae* strains in this study is very alarming and this could be attributed to the indiscriminate and widespread use of antibiotics, particularly beta lactam antibiotics in this unit of the hospital.

This misuse of antibiotics might have contributed to the emergence of ESBL. Fifty-seven multiple resistant *K. pneumoniae* strains were isolated in the intensive care unit of UNTH Enugu; all strains of *K. pneumoniae* were found to be Producing Extended Spectrum Beta Lactamases (ESBLs), which harbored ESBLs of the CTX-M-1 cluster. These results further emphasize that this enzyme is now one of the most common CTX-M β -lactamases worldwide. One finding that is worthy of noting in our study is that most ESBL isolates were detected in urine from young patients between the ages of 22-30.

We observed a high association of CTX-M-1 cluster ESBLs with ISEcp1 upstream and ORF 477 downstream of the gene, with the presence of TEM and OXA-1 beta lactamases and with the detection of aac (6')-Ib-cr. Bla_{SHV} was found to be present in all the strains as expected.

The presence of ESBL- producing *K. pneumoniae* especially in the ICU of this hospital could lead to

major therapeutic and epidemiological consequences if not taken care of. Multiple outbreaks of ESBL-producing Enterobacteriaceae have been reported over the past decades and *K. pneumoniae* has been shown to be the most frequently involved organism (Rebuck *et al.*, 2000; Quale *et al.*, 2002). Over the past decades, CTX-M genes have become the most common ESBLs in many countries all over the Europe, Asia, Africa and America (Livermore *et al.*, 2007). Random amplified polymorphic DNA result indicates that majority of the ESBL strains belongs to clonal group A 42(71.9%) showing that ESBL outbreaks from this unit may be due to a single clone. Evidence for nosocomial transmission of a single strain can be inferred because of the fact that most of the strains belong to one clone. The rapid and massive spread of CTX-M- type of ESBLs is rapidly changing the ESBL epidemiology and in some geographical areas, these enzymes are now the most prevalent ESBLs in Enterobacteriaceae. Outbreaks of CTX-M- 15- producing Enterobacteriaceae have also been reported in France, Italy, Spain, Portugal, Austria, Norway, the United Kingdom, Tunisia, South Korea, Canada, Egypt, Nigeria and Kuwait (Al-Agamy *et al.*, 2006; Livermore *et al.*, 2007; Ensor *et al.*, 2009; Conceicao *et al.*, 2005; Soge *et al.*, 2006; Abbassi *et al.*, 2006; Schleslinger *et al.*, 2005). The rate of ESBL expression from our study was quite high compared to other geographical areas of the world. The high rate of ESBLs among hospitalized patients is a global problem as reported in our findings and it is generally thought that patients infected by an ESBL-producing organism are at an increased risk of treatment failure with extended spectrum beta lactam drug. This has posed a very serious problem in this part of Nigeria because alternative antibiotics therapy is difficult to find and as a result patients infected with an ESBL producing organism most often do not survive.

CONCLUSION

In conclusion, our study revealed for the first time the presence of *K. pneumoniae* producing CTX-M-15 ESBL in the intensive care unit of UNTH, which is an evidence of its increase detection in patients in this unit. More studies on the cefotaxime-resistant *Klebsiella* sp. and other enterobacteriaceae need to be carried out in this region of Nigeria not only in the ICU but also in other wards of the hospital.

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