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## Research Paper

# PREVALENCE OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBLs) CARRYING GENES IN *KLEBSIELLA* SPP FROM CLINICAL SAMPLES AT ILE-IFE, SOUTH WESTERN NIGERIA

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Extended Spectrum Beta Lactamases (ESBLs) that mediate resistance to 3<sup>rd</sup> generation cephalosporins were investigated. The aim of this study was to determine the prevalence ESBLs genes among *Klebsiella pneumoniae* isolated from different clinical specimens of patients admitted in Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile –Ife, Osun State, from October 2010 to April 2011. All isolates were screened and confirmed for extended spectrum beta- lactamase (ESBL) production by Kirby-Bauer disk diffusion method on Mueller Hinton agar using CLSI guidelines. ESBL production was phenotypically determined using double disc synergy test and subjected to PCR for genotyping. Two hundred isolates were recovered, 66(33%) *K. pneumoniae*, 12(6%) *K. oxytoca*, 39(19.5%) *P. aeruginosa*, 33(16.5%) *Proteus species*, 15(7.5%) *Escherichia coli*, and 35(17.5%) *S. aureus*. ESBL producing *K. pneumoniae* were most frequently isolates from sputum 63(32%) followed by blood 43(21.5%), wound 39(19%), urine 32(16%), HVS 12(6%) and CSF with least incidence of 11(5.5%). By disk diffusion, the strains were resistant to almost antibiotics while Trimethoprim-Sulphamethoxazole showed highest resistance of (96.3%), while sensitivity to imipenem was 100%. The prevalence of 32% was recorded for *bla*TEM, while *bla*SHV and *bla*CTX-M, were 32% and 36% respectively.

**Keywords:** Extended Spectrum Beta-Lactamase (ESBL), *Klebsiella* species, Prevalence, Antimicrobial resistance, Double-disc method.

## INTRODUCTION

Extended Spectrum Beta-Lactamase (ESBL) producers have continued to draw attention

globally with their attendant clinical failure to new generation antibiotics and nosocomial spread.

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They were first reported in Germany in 1983 from *Klebsiella pneumonia* and later in *Escherichia coli*, *Pseudomonas aeruginosa* and other gram-negative bacilli (Kiratisin *et al.*, 2008). Bacteria belonging to the genus *Klebsiella* frequently cause human nosocomial infections in particular, the medically important species, *Klebsiella pneumoniae*, accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicemias, and soft tissue infections (Shawn, 2007). The principal pathogenic reservoirs for transmission of *Klebsiella* are the gastrointestinal tract and the hands of hospital personnel. These bacteria tend to cause nosocomial outbreaks because of their ability to spread rapidly in the hospital environment. These organisms have been isolated from abscesses, blood, catheter tips, lungs, peritoneal fluid, sputum, and throat cultures (Cheng *et al.*, 2008). ESBLs enzymes are capable of hydrolysing penicillins, broad-spectrum cephalosporins and monobactams, and are generally derived from TEM and SHV-type enzymes. (Feizabadi *et al.*, 2010) The genetic mutations that give rise to ESBLs broaden the parental resistance pattern to a phenotype that includes resistance to third-generation cephalosporins (Feizabadi *et al.*, 2010).

Hospital outbreaks of multidrug-resistant *Klebsiella* species, especially those in neonatal wards, are often caused by new types of strains, the so-called extended-spectrum-beta-lactamase (ESBL) producers (Jarlier, 1988). These organisms pose unique challenges to clinical microbiologists, clinicians, infection control professionals and antibacterial-discovery

scientists. This causes a major health problem because the incidence rate of infection is particularly high and delays in the prescription of appropriate antimicrobial drug therapy for these infections are a risk factor for poor prognosis and death (Coque *et al.*, 2009). *Klebsiella pneumonia* is an important nosocomial pathogen that has the potential to cause severe morbidity and mortality. In recent years following extensive use of the expanded spectrum cephalosporins, outbreaks of infection caused by extended spectrum beta lactamase producing *K. pneumoniae* have been widely reported throughout the world (Branger *et al.*, 1998). The production of ESBLs is a major threat to the use of new generation of cephalosporins (Mendes *et al.*, 2004). Long hospitalization, diabetes, age over 60 and previous antibiotic treatment have been reported as the risk factors to acquire infections with ESBL strains (Silva *et al.*, 2006).

According to a study carried out in Abeokuta, Nigeria by Olowe and Aboderin in 2010, evaluating ESBL producing pathogens of a hospital can help clinicians in deciding empirical treatment of high-risk patients with serious nosocomial infections. Therefore, the determination of CTX-M, TEM and SHV genes by molecular techniques in ESBL producing bacteria and their pattern of antimicrobial resistance can supply useful data about their epidemiology and risk factors associated with these infections (Bonnet, 2004). The aim of this study was therefore to identify the types of extended spectrum beta-lactamases (ESBL) produced by *Klebsiella* species isolated from various specimens and to determine the prevalence of antibiotic resistance genes SHV, CTX-M, and TEM among recovered isolates so as to establish a baseline data for epidemiological studies in Ile-Ife.

## MATERIALS AND METHODS

**Sources of samples:** Two hundred clinical isolates of *klebsiella species* were obtained from different clinical samples (urine, blood, sputum, wound, HVS, cerebrospinal fluid) at the Medical Microbiology Laboratory department of Obafemi Awolowo University Teaching Hospitals Complex, Ile –Ife, Osun State between October 2010- April 2011.

Ethical approval was obtained from the Hospital Ethical and Research Committee.

**Isolation and Characterization of the Organisms:** All isolates were identified by conventional bacteriological tests (Podschun and Ullaman, 1994). The swabs samples were cultured directly on Blood agar and MacConkey agar (Oxoid) and incubated overnight at 37°C. Bacterial colonies with characteristic mucous and pinkish colour were presumptively identified as *Klebsiella* spp. Further confirmation was done by microscopy and biochemical testing.

**Standardization of Inoculum:** The inoculum was standardized according to the method of Bauer *et al.*, (1979) where the turbidity of the broth was made equivalent to a 0.5 McFarland standard.

## ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antibiotic susceptibility testing was performed as CLSI (formerly NCCLS (2004) recommended using disk containing Ceftazidime (CAZ: 30 µg), Ceftriaxone (CRO: 30 µg), Cefotaxime (CTX: 30 µg), Ceftizoxime (ZOX: 30 µg), Gentamicin (GM: 10 µg), Amikacin (AN: 30 µg), Imipenem (IMP: 10 µg), Ciprofloxacin (CIP; 5 µg) (BBL, USA). *Escherichia coli* ATCC 25922 and *Pseudomonas*

*aeruginosa* ATCC 27853 were used as controls (NCCLS, 2004).

**ESBL Detection (Double Disk Diffusion technique):** A cotton swab was dipped into a standardized bacterial suspension and the surface of the sensitivity test agar was inoculated evenly. A Co-Amoxiclav disk was placed in the center of plate. Then Cefotaxime, Ceftriaxone, Ceftazidime and Cefopodoxime disks placed around the Co-Amoxiclav disk. The distance between satellite disks and the central disk was defined around 30mm. The plates were incubated aerobically. Results were evaluated and recorded after 24hr. If growth-free zones of satellite disks spread to the central disk microbe-free zone, the isolate was ESBL producing (Mahon, 2007).

**Gel Electrophoresis Procedure:** A little agarose was put into a flask and buffer solution was added. The solution was heated until agarose melted in the buffer; the gel was poured into the mold. The comb was placed in mold and carefully removed when the gel solidified. The buffer was poured into electrophoresis box and the gel mold in the electrophoresis box, barely submerged in the buffer. Loading buffer was added to the DNA sample. The sample was loaded into gel with the aid of a micropipettor. Injected DNA sample was mixed with the loading buffer into the first well of agarose gel. Micropipettor was used to load DNA size standard into the next well of the agarose gel. The electrophoresis box was connected to the electric current and turned on. The DNA strands moved away from the negative current. The short strands move through the gel more quickly than the long strands. This can be observed due to the blue dye of the loading buffer. The current was turned off and gel removed from

mold from the electrophoresis box. The gel was removed from mold and placed in DNA staining solution, ethidium bromide. The gel was in turn removed from ethidium bromide (after about 30 minutes) and place on UV light box. The DNA bands were visible for both the DNA sample and DNA size standard. Using the size standard, the lengths of the sample strands was estimated.

**Detection and Amplification of ESBL genes by PCR:** The specific primers used mainly, TEM, SHV, CTX-M-type  $\beta$  lactamase DNA sequences were obtained commercially from the manufacturer. ESBL producing isolates were amplified using *bla* TEM, SHV, CTX-M, specific primers listed in Table 5. Amplification reactions were carried out under the following conditions: initial pre-denaturation at 94°C for 2 min, followed by 30 cycle of denaturation at 94°C for 30s, annealing at 53°C for TEM, SHV and an extension at 72°C for 1 min, and a final extension at 72°C for 7 min. A molecular size marker (Fermentase SM0241 effective size range: 100 to 1000 kb) was used to assess PCR product size.

## RESULTS

Two hundred clinical samples were cultured from various sample sites from patients at Obafemi Awolowo University Teaching Hospitals Complex (OAUTHC), Ile -Ife. Table 1 showed the distribution of which 66 (33%) were *Klebsiella pneumoniae*, 12(6%) *Klebsiella oxytoca*, 39 (19.5%) *Pseudomonas aeruginosa*, 33 (16.5%) *Proteus species*, 15 (7.5%) *Escherichia coli*, and 35(17.5%) *Staphylococcus aureus*. Table 2 showed the distribution of ESBL positive *Klebsiella species*. Prevalence of ESBL positive of 51.3% (40 isolates) was recorded for *K. pneumonia* out of the 78 *Klebsiella species* isolated. There was no incidence of ESBL *K. oxytoca*. Table 3 shows

the antibiotic susceptibility pattern of ESBL positive and non-ESBL producers to different antibiotics. Level of resistance to the antibiotics was Amoxycilin 64%, Gentamicin 92.6%, Fusidic acid 90.0%, Erythromycin 82.3%, Trimethoprim-Sulpha-methoxazole 96.3%, Tetracycline 88.5%, Ciprofloxacin 31.2%, Nitrofurantoin 31.2%, Ceftazidime 69%, Cefotaxime 74%, Ceftriaxone 79.6% and Imipenem 0% as all the organisms showing 100% sensitivity to imipenem. The prevalence of *bla*SHV, *bla*TEM and *bla*CTX-M among these isolates were 9(22.5), 8(20%) and 8(20%) respectively as shown in Table 4. Table 5 shows Oligonucleotide primers used for detection of beta-lactamase genes.

**Table 1: Prevalence of Isolates from Clinical Samples**

| Organisms                    | No (%)    |
|------------------------------|-----------|
| <i>Klebsiella pneumoniae</i> | 66 (33.0) |
| <i>Klebsiella oxytoca</i>    | 12 (6.0)  |
| <i>Pseudomonas spp.</i>      | 39 (19.5) |
| <i>Proteus spp.</i>          | 33 (16.5) |
| <i>E.coli</i>                | 15 (7.5)  |
| <i>Staphylococcus aureus</i> | 35 (17.5) |
| Total                        | 200 (100) |

## DISCUSSION

*K. pneumoniae* is an important nosocomial pathogen that has the potential to cause severe morbidity and mortality. In recent years following extensive use of the expanded spectrum cephalosporins, outbreaks of infection caused by extended spectrum beta lactamase producing *K. Pneumoniae* has been widely reported throughout the world (El-Khizzim, 2002). The distribution of organisms isolated from the various clinical samples in the study were found to be 66 (33%)

**Table 2: Distribution of ESBL Positive *Klebsiella* Species from clinical samples**

| Clinical Samples | Organisms     |                 | Total          |
|------------------|---------------|-----------------|----------------|
|                  | ESBL +ve(%)   | ESBL -ve(%)     |                |
| Wound            | 4(5.1)        | 5(6.4)          | 9              |
| Blood            | 10(12.8)      | 5 (6.4)         | 15             |
| Urine            | 5(6.4)        | 6(7.7)          | 11             |
| Sputum           | 19(24.3)      | 4(5.1)          | 23             |
| CSF              | 2(2.5)        | 8(10.2)         | 10             |
| HVS              | 0(0)          | 10 (12.8)       | 10             |
| <b>Total</b>     | <b>(51.3)</b> | <b>38(48.7)</b> | <b>78(100)</b> |

**Table 3: Susceptibility Pattern of ESBL Positive and Non-ESBL Producers**

| Antimicrobial Agent | <i>Klebsiella pneumoniae</i> |             | <i>Klebsiella oxytoca</i> |             |
|---------------------|------------------------------|-------------|---------------------------|-------------|
|                     | % Susceptible                | % Resistant | % Susceptible             | % Resistant |
| Ciprofloxacin       | 72                           | 28          | 68                        | 32          |
| Ceftazidime         | 31                           | 69          | 41                        | 59          |
| Cefotaxime          | 26                           | 74          | 27                        | 73          |
| Ceftriaxone         | 21                           | 79          | 35                        | 65          |
| Sparfloxin          | 36                           | 64          | 47                        | 53          |
| Gentamicin          | 8.6                          | 91.4        | 20                        | 80          |
| Fusidic acid        | 9.1                          | 90.9        | 14                        | 86          |
| Erythromycin        | 17.7                         | 82.3        | 24                        | 76          |
| Trimethoprim        | 12                           | 88          | 14                        | 86          |
| Sulpamethazole      | 4                            | 96          | 23                        | 77          |
| Tetracycline        | 11.5                         | 88.5        | 22                        | 78          |
| Nitrofurantion      | 68.8                         | 31.2        | 55                        | 45          |
| Imipenem            | 100                          | 0           | 100                       | 0           |

*Klebsiella pneumoniae*, 12 (6%) *Klebsiella oxytoca*, 39 (19.5%) *Pseudomonas aeruginosa*, 33 (16.5%) *Proteus species*, while 15 (7.5%) *Escherichia coli*, and, 35 (17.5%) *Staphylococcus aureus* were also isolated. The detection

rate of ESBL producing *Klebsiella* isolates from clinical samples differ from each other however, from this study it was observed that an overall prevalence of 51.3% ESBL positive *K. pneumoniae* was recorded from the isolates.

**Table 4: Distribution of ESBL Genes Including *bla*TEM, *bla*SHV and *bla*CTX-M by PCR.**

| ESBL <i>K. pneumoniae</i> (n=40) | No (%)   |
|----------------------------------|----------|
| CTX-M                            | 9 (36)   |
| SHV                              | 8 (32)   |
| TEM                              | 8 (32)   |
| Total                            | 25 (100) |

The ESBL producing isolates were recovered mostly from sputum, followed by blood then urine and wound. We found a high percentage of resistance of the ESBL producing isolates to the tested antibiotics; Amoxicillin 64%, Gentamicin 92.6%, Fusidic acid 90.0%, Erythromycin 82.3%, Trimethoprim-Sulphamethoxazole 96.3% Tetracycline 88.5%, Ciprofloxacin 31.2% Nitrofurantoin 31.2%, Ceftazidime 69%, Cefotaxime 74%, Ceftriaxone 79.6%, however, none of the isolates were resistant to Imipenem.

The percentage distribution of isolated organisms was highest among age groups 1-10 (23%) and the lowest in age groups 41-50. This agrees with the work done by Olowe *et al.* (2010) at Abeokuta in Ogun State, Nigeria. The therapeutic use of all third generation cephalosporins should be discouraged in ESBL

producing *Klebsiella* spp. even if appears susceptible to any such compound. Since all the isolates were sensitive to imipenem, it is recommended as the drug of choice for treatment of infectious disease due to ESBL producing *K. pneumoniae* strains. The prevalence of antibiotic resistance to other groups of antibiotics among ESBL producing *E. coli* and *K. pneumoniae* has increased markedly in recent years and increases the possibility that traditional, empiric antimicrobial regimens may also become ineffective (Chlebicki, 2004). These enzymes are most commonly produced by *Klebsiella* spp. The production of ESBLs is a major threat to the use of new generation of cephalosporins (Kader, 2004). Long hospitalization, diabetes, age over 60 and previous antibiotic treatment have been reported as the risk factors to acquire infections with ESBL strains (Angel, 2009).

Extended Spectrum Beta- Lactamase producing organisms have become an important clinical problem due to their resistance to multiple antibiotics. Thus antibiotic options in the treatment of these organisms are extremely limited. Beta lactams are usually used for treatment of lower respiratory tract infections in children where gram negative bacteria are isolated. Detection of ESBL

**Table 5: Oligonucleotide Primers Used for Detection of Beta-Lactamase Genes**

| Primers   | (°C) | Nucleotide Sequences (5'to 3') | Ref (GenBank no) | Expected bp |
|-----------|------|--------------------------------|------------------|-------------|
| SHV-F     | 53   | GGG TTA TTC TTA TTT GTC GC     | S4114E09         | 500         |
| SHV-R     | 53   | TTA GCG TTG CCA AGT GCTC       | S4114E10         | 500         |
| TEM-F     | 53   | ATA AAA TTC TTG AAG AC         | S4114E07         | 400         |
| TEM-R     | 53   | TTA CCA ATG CTT AAT CA         | S4114E08         | 400         |
| CTX-M 15F | 35   | AGA ATA AGG AAT CCC ATG GTT    | S4114E11         | 373         |
| CTX-M 15R | 35   | ACC GTC GGT GAC GAT TTT AG     | S4124B04         | 373         |

production is important, because it is recommended that any organism that is confirmed as ESBL producing according to CLSI criteria should be reported as a resistant to all extended- spectrum beta lactam antibiotics, regardless of their susceptibility test results.

In Nigeria, Extended spectrum Beta-Lactamase producing organisms have been isolated with prevalence rate of 44.6% recorded in Enugu and 6.7% in Ebonyi Eastern Nigeria (Iroha *et al.*, 2009). In a study conducted at the Federal Medical Centre at Abeokuta, Nigeria by Olowe *et al.* (2010) and a work conducted by Aibinu *et al.* (2003), prevalence of 5% and 20.3% were recorded respectively. Globally, ESBL producing *K. pneumoniae* has emerged as one of the major multi-drug resistant organisms.

The incidence of ESBL- producing *Klebsiella* isolates in the United States has been reported to be 5%. In France and England 14 to 16% ESBL producers among *Klebsiella* isolates has been reported (Jacoby, 1991), however, the percentage of third generation cephalosporins resistant strains may be much higher because the conventional disc diffusion criteria used in the routine laboratory, underestimate the incidence of these isolates. This can increase the possibility that traditional, empiric antimicrobial regimens may also become ineffective (Chlebicki, 2004).

*Klebsiella* isolates identified from this study showed that three types of ESBLs were obtained namely: SHV-type, CTX-M and TEM. The prevalence of SHV, TEM and CTX-M genes in this study was 32%, 32% and 36% respectively which is higher than the result obtained in Iran in 2009 with 22.5% and 23% rates respectively. These imply that ESBL was detected in 34% of the isolates. This percentage was considered to be

a bit higher when compared to 27.3% in *K. pneumoniae* reported by Akram *et al.* (2007) who conducted a survey on the urinary tract isolates in India. The CTX-M beta lactamases are now widespread in both nosocomial and community acquired pathogens (Livermoore, 2005). The number of CTX-M type ESBLs is rapidly expanding. They have been detected in some geographical areas and are now the most frequent ESBL type worldwide (Paterson, 2005).

The prevalence of *bla*SHV and *bla*CTX-M genes in this study was 22.5% and 20% respectively which agrees with the work done in Iran in 2010 with 26% and 24.5% prevalence respectively (Naseli *et al.*, 2010).

## CONCLUSION

The emergence and spread of ESBL-producing *K. Pneumonia* strains is worrisome and usage of cephalosporins against these isolates is ineffective. As Imipenem is the drug of choice for serious infectious disease nowadays, extensive use of this drug in treatment of infection caused by resistant isolates will enhance. Because of this problem prudent use of  $\beta$ -lactam antibiotics containing an oxyimino group and consistent application of basic infection control procedures in treatment centers is necessary and care should be taken to use Imipenem when truly indicated. Due to importance of ESBL producing organisms and difficult treatment of infections caused by these bacteria, for rapid identification of ESBL producing isolates clinical laboratories should adopt simple test based on CLSI recommendation for confirming ESBL production. Laboratory services should be available to support every infection control program. We should always remember that effective treatment



of serious infections will only be achieved by close cooperation between clinicians and laboratory scientists.

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