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Detection of Plasmid Borne Extended Spectrum Beta Lactamase Enzymes from Blood and Urine Isolates of Gram-Negative Bacteria from a University Teaching Hospital in Nigeria

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Abstract: The present study investigated the occurrence of Extended Spectrum Beta Lactamase (ESBL) enzyme in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from Enugu, Nigeria. Ninety-nine clinical strains of gram-negative bacteria comprising of *E. coli* (69) and *K. pneumoniae* (30) were isolated from urine and blood samples from Enugu State University Teaching Hospital (ESUTH), Enugu, Nigeria, from April-July 2008. The presence of ESBL was determined using the Double Disc Synergy Test (DDST). To determine whether the ESBL present is plasmid or chromosomally mediated, positive ESBL isolates were grown in 0.1% acridine orange for 18-24 h. The transfer of resistance marker from ESBL-positive strains of Gram-negative organisms to a non-ESBL strain of *E. coli* was evaluated via conjugation. The ESBL production was detected in 17 (56.6%) isolates of *Klebsiella pneumoniae* and 41 (59.4%) isolates of *E. coli*. The results of our study showed that the prevalence of ESBL in ESUTH was high and was plasmid-mediated. Stable transfer of resistance marker of 8 (47%) ESBL-positive *E. coli* and 1 (14.3%) *K. pneumoniae* to the non-ESBL recipient strain was observed after trans-conjugation. There is apparently high prevalence of ESBL producing strains of *E. coli* and *K. pneumoniae* in present study environment and the enzyme genes are plasmid mediated.

Key words: Plasmid curing, resistant, enzyme, esbls, isolate, susceptibility, conjugation

INTRODUCTION

Extended Spectrum Beta Lactamases (ESBLs) are enzymes that are able to hydrolyze a broader spectrum of beta-lactam antibiotics than the simple parent beta-lactamases from which they were derived. They are chromosomally or plasmid-mediated β -lactamases that inactivate β -lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, ceftriaxone, cefotaxime as well as oxyimino-monobactam (aztreonam)) but are not active against cephamycins and carbapenems (Bradford, 2001). The ESBLs are produced by gram-negative rods and the vast majority of strains expressing these enzymes belong to the family Enterobacteriaceae (Paterson and Bonomo, 2005).

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Klebsiella pneumoniae and *Escherichia coli* are the two major ESBL producers from this family, although the growing incidence of ESBLs in *Salmonella* species and other organisms have been recorded (Winokur and Brueggemann-Desalvo, 2005).

The most prevalent ESBL types have evolved through point mutations of key amino acid substitution in the parent TEM and SHV enzymes (Giniadkowski, 2001). A number of amino acid residues are especially important for producing ESBL phenotypes when substitutions occur at that position. They include glutamate to lysine at position 104, arginine to either serine or histidine at position 164, glycine to serine at position 238 and glutamate to lysine at position 240. The selection pressure that drives the emergence of ESBLs has actually been attributed to the intense use of oxyimino beta-lactams mainly the third generation cephalosporins (Medeiros, 1997). However, the constant or fluctuating pressure of various β -lactam antibiotics including diverse oxyimino compounds as well as penicillin's and early generation cephalosporins has recently been proposed to affect ESBL variation (Blazques *et al.*, 2000). Therefore, ESBL is characterized by highly selective substrate preference. Prevalence of ESBL have been recorded in Western and Eastern part of Nigeria from tertiary and secondary hospital especially from septicemia patients (Iroha *et al.*, 2008; Aibinu *et al.*, 2003; Olusegun *et al.*, 2006a, b). Despite the studies carried out in different parts of Nigeria, information on ESBL types and prevalence in Nigeria is still scarce. The objective of this study was to detect plasmid borne ESBL producing organisms from urine and blood samples of Gram-negative organisms from a University Teaching Hospital in Enugu State, Nigeria.

MATERIALS AND METHODS

Bacterial Isolates

Ninety-nine clinical isolates of Gram-negative bacteria comprising *Escherichia coli* (69) and *Klebsiella pneumoniae* (30) was isolated from blood (52) and urine (47) samples of patients attending the Enugu State University Teaching Hospital in Southeastern Nigeria, from April-July 2008. Blood and urine samples were analyzed using standard microbiological techniques (Tison *et al.*, 1999). *Escherichia coli* and *Klebsiella pneumoniae* strains were also purified and characterized using standard Microbiological techniques (Chessbrough, 2002).

Susceptibility Studies

Disc diffusion test was performed using the Kirby- Bauer disc diffusion method to evaluate the sensitivity of test organisms to beta-lactam and non beta-lactam antibiotics which includes ceftazidime (30 μ g), cefepime (30 μ g), Augmentin (30 μ g) (Amoxicillin 20 μ g/clavulanic acid combination 10 μ g), ciprofloxacin (5 μ g), ofloxacin (5 μ g), ceftriaxone (30 μ g), ampicillin (10 μ g), cefotaxime (30 μ g), gentamicin (10 μ g), sulphamethoxazole/trimethoprim (25 μ g) (Oxoid, Uk). Any of the isolates that showed reduced susceptibility to any of the 2nd and 3rd generation cephalosporins were subjected to ESBL screening according to the guidelines of the National Committee for Clinical Laboratory Standard (NCCLS Document M2-A7, 2000).

ESBL Screening

All isolates that showed reduced susceptibility to 2nd and 3rd generation cephalosporins were screened for ESBL production using Double Disc Synergy Test Method (DDST) (Sorlozano *et al.*, 2004). An aliquot of a 0.5 MacFarland equivalent standard of the test organisms were streaked on the surface of a sterile Mueller Hinton agar plate using a sterile swab stick. After 20 min of pre-incubation, a combination disc Augmentin (30 μ g)

(Amoxicillin 20 µg/clavulanic acid combination 10 µg), was placed at the center of the Petri dish and ceftazidime (30 µg) and cefotaxime (30 µg) were placed 15 mm apart from the center disc. This was incubated for 18-24 h at 37°C.

Plasmid Curing

Seventeen clinical isolates of *K. pneumoniae* and 41 clinical isolates of *E. coli* that were positive for ESBL were selected and subjected to acridine orange (Merck) mediated plasmid elimination using the method of Stanisich (1988). Each of the 58 isolates of positive ESBL were grown in 5 mL of double strength Mueller Hinton broth supplemented with 0.1 mg mL⁻¹ of acridine orange and incubated at 37°C for 18-24 h. After incubation the test organisms were re-tested for ESBL production using DDST.

Conjugation Studies

Seven clinical isolates of *K. pneumoniae* and 17 clinical isolates of *E. coli* that were positive for ESBL enzymes and resistant or had decreased susceptibility to extended spectrum cephalosporins which were positive lactose fermenters were selected for bacteria conjugation. These were transconjugated with non-ESBL producing and non-lactose fermenting *E. coli* that were susceptible to extended spectrum cephalosporins. The ESBL producing *E. coli* and *K. pneumoniae* serves as the donor strain while the non-ESBL producing *E. coli* serves as the recipient strain. The recipient and donor strains were grown separately in 5 mL of a double strength nutrient broth at 37°C for 18-24 h. They were mixed together at the ratio of 1:10 (donor to recipient) and incubated for 3 h at 37°C. Samples of this mixture were spread plated on the surface of a MacConkey agar plate and incubated for 18- 24 h at 37°C. Samples from donor strains and recipient strains were used as control. Transconjugants growing in the selection plate were subjected to DDST to confirm the presence of ESBL in the recipient strain. The DDST patterns of 8 positive ESBL *E. coli* out of 17 and 1 *K. pneumoniae* out of 7 transconjugants mimicked DDST patterns of donor's strains.

RESULTS

The result of the present study showed that out of 99 clinical isolates, including *E. coli* (69) and *K. pneumoniae* (30) screened for ESBL production, 58 (58.6%) were positive ESBL producers. Of this, 41(59.4%) positive ESBL isolates were *E. coli* while 17 (56.6%) were *K. pneumoniae* (Table 1).

The result of the plasmid curing with acridine orange revealed that all the ESBL positive clinical isolates of *E. coli* (41) and *K. pneumoniae* (17) could not express ESBL enzymes after their incubation with acridine orange at 0.1% concentration. This indicates that they are most probably plasmid-mediated (Table 2). Furthermore, transconjugation studies between positive ESBL *E. coli* (17) and *K. pneumoniae* (7) with negative ESBL *E.coli*, showed that 8 (47%) ESBL positive *E. coli* and 1(14.3%) *K. pneumoniae* isolate transferred their resistant plasmid marker to recipient non-ESBL *E. coli* (Table 3).

Table 1: Prevalence of Gram-negative bacteria

Total No. of Gram-negative bacteria	No. of Gram-negative bacteria from each specimen		Percentage ESBL isolates from each specimen		Over all % Gram- negative ESBL isolates
	Urine	Blood	Urine	Blood	
<i>E. coli</i> = 69	<i>E. coli</i> = 22	<i>E. coli</i> = 20	35 (60.3%)	23(39.6%)	<i>E. coli</i> = 41 (59.4%)
<i>K. pneumoniae</i> = 30	<i>K. pneu.</i> = 25	<i>K. pneu.</i> = 32			<i>K. pneu.</i> = 17 (56.6%)
Total = 99					58 (58.6%)

Table 2: Effect of acridine orange mediated plasmid curing on positive ESBL isolated

No. of positive ESBL cured with acridine orange	No. of positive ESBL from each specimen cured with acridine orange		No. of plasmid ESBL cured
	Urine	Blood	
<i>E. coli</i> = 41	35	23	<i>E. coli</i> = 41
<i>K. pneumoniae</i> = 17			<i>K. pneumoniae</i> = 17
Total = 58			

Table 3: Antibiotic resistance transfer

No. of positive ESBL transconjugated based on selection	No. of successful transconjugants	No. of successful <i>K. pneumoniae</i> transconjugants from each specimen		No. of successful <i>E. coli</i> transconjugants from each specimen	
		Urine	Blood	Urine	Blood
<i>E. coli</i> = 17	<i>E. coli</i> = 8	1	None	7	1
<i>K. pneumoniae</i> = 7	<i>K. pneumoniae</i> = 1				
Total = 24					

DISCUSSION

The ESBL producing *E. coli* and *K. pneumoniae* has been reported in a community-acquired bacteremic infection and as a result of this there is a serious need to detect ESBL Producing Gram-negative bacteria in routine Microbiology Laboratory (Sorlozano *et al.*, 2004). Nosocomial septicemia infection continues to be a public health problem globally where *E. coli* strains have been isolated in an increasing number in bacteremic patients. The highest percentage of ESBL production was found in *K. pneumoniae* from a study carried out in China (Xiong *et al.*, 2002; Chiew, 2004). Present study revealed high prevalence of ESBL from Southeastern Nigerian Teaching Hospital. Out of 99 clinical isolates of Gram-negative bacteria comprising *E. coli* (69) *K. pneumoniae* (30), 58 (58.6%) of both strains were positive for ESBL enzymes. The 41 (59.4%) of *E. coli* out of 69 were positive for ESBL while 17 (56.6%) out of 30 *K. pneumoniae* were ESBL positive. Consequently, our data highlights an increased prevalence of ESBL in Enugu, Nigeria.

The ESBL prevalence varies from country to country and from institution to institution. A survey on 81,213 blood stream infectious pathogens during 1997-2002 showed that the *Klebsiella* sp. with an ESBL phenotype was isolated at a rate of 34.5 and 7% in Latin America, 21.7% in Europe and 5.8% in North America (Beidenbach *et al.*, 2004). The Pan European Antimicrobial Resistance Using Local Surveillance (PEARIS) study between (2001-2002) showed that the percentage of ESBL production among *E. coli* was 5.8%, *K. pneumoniae* 18.2% and enterobacter sp. 8.8%, respectively for all the study sites (Bouchillon *et al.*, 2004). In Egypt a high rate of 38.5% was observed, 27.4% from Greece and a very low percentage of 2% in the Netherlands and Germany 2.6%. In Japan, Korea and Hong Kong, the percentage of ESBL prevalence remains low (Ho *et al.*, 2000). In Southern and Eastern Nigeria the prevalence of ESBL from 2003-2007 was high 52.4% (Iroha *et al.*, 2008; Aibinu *et al.*, 2003).

Infection and colonization with ESBL producing organism are usually hospital-acquired especially in the intensive care units (Babini and Livermore, 2000). Other hospital units that are at increased risk include rehabilitation units, oncology wards, pediatrics and neonatology, surgical wards (Bermudes *et al.*, 1997; Martinez-Aguilar *et al.*, 2001). Some risk factors are responsible for infection or colonization with ESBL producing organisms which include: prior exposure to antibiotics, prolong stay in hospital or ICU, presence of vascular

or urinary catheters, hemodialysis, emergency of abdominal surgery and gut colonization (Hibbert-Rogers *et al.*, 1995; Lautenbach *et al.*, 2001; Patterson, 2001). It is interesting to note that specific ESBLs appear to be unique to certain country or region although, recent reports suggest worldwide dissemination (Ramphal and Ambrose, 2006; Pfaller and Segrti, 2006). The ESBL detected from our study reveals the presence of TEM, SHV and CTX-M ESBL enzymes.

The effect of plasmid curing studies in the present work with acridine orange shows that the plasmid resistant maker of positive ESBL isolates was eliminated showing that the ESBL genes of these organisms are plasmid mediated and not chromosomally mediated. The ESBLs are carried on bacterial chromosomes or plasmids and plasmid-mediated ESBLs can carry genes on them that have the ability to transfer a replica of themselves to other bacteria. They also carry genes conferring resistance to other classes of antibiotics that makes the recipient bacteria resistant to multiple antibiotics. Also, these plasmids can emerge on strains that do not cause human diseases but then the non-pathogenic strains could transfer their plasmids to strains that can cause human diseases. Plasmid conjugation is an important mechanism of disseminating drug resistance among bacteria population and in the present study, out of 24 positive Gram negative bacteria (*E. coli* (17) and *Kleb. Pneumoniae* (7)) transconjugated, 8 (47%) *E. coli* and 1 (14.3%) *K. pneumoniae* successfully transferred their resistant plasmid marker to recipient *E. coli*. Such a host transferable plasmids play an important role in the spread of antibiotic resistance. This also reveals that the *E. coli* and *K. pneumoniae* that transfer these resistance plasmid markers are plasmid mediated ESBL while those that fails to transfer are probably chromosomally mediated ESBL enzymes.

In conclusion the prevalence rate of ESBL producing organisms are high globally. The ESBL producing organisms are known to cause serious nosocomial infections, long term carriage in the community, community-acquired infections such as urinary tract infections, peritonitis, cholangitis and intra-abdominal abscess. The findings from the present study revealed high prevalence of ESBL from Enugu State University Teaching Hospital (ESUTH) where *E. coli* has a higher prevalence of 41 (59.4%) than *K. pneumoniae* 17 (56.6%). This certainly calls for urgent attention of the Ministries of Health and hospital administrators. This study thus, emphasizes the inclusion of ESBL detection in routine laboratory checks in hospitals and clinics, especially in the developing countries.

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