Full Length Research Paper

Detection and antimicrobial susceptibility of some gram negative bacteria producing carbapenemases and extended spectrum β-Lactamases

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The objective of this research was to determine phenotypically the occurrence of carbapenemase enzymes and extended spectrum β-lactamase (ESBL) producing Gram negative bacteria from some clinically important bacteria. Ninety nine clinical isolates comprising of Escherichia coli (n=40), Klebsiella pneumoniae (39) and Pseudomonas aeruginosa (n=20) were used for this study. Susceptibility testing for all isolates was performed by Kirby-Bauer disk diffusion method. Imipenem and meropenem was the most effective antibiotic while sulphamethoxazole showed least antibacterial activity. ESBL was detected in 7.5 % E. coli and 33.3 % K. pneumoniae. None of the P. aeruginosa isolates in our study showed ESBL production. Carpenemase production was detected in 12.5 % E. coli, 7.7 % K. pneumoniae and 15 % P. aeruginosa isolates. Plasmid curing experiment showed that the ESBL phenotypes in our study were both plasmid-born and chromosomally-mediated. Five (5) out of the eight (8) positive ESBL isolates were successfully transconjugated. Our study reveals the prevalence of ESBL and carbapenemase phenotypes in this environment, and these were resistant to some conventional antibiotics. Awareness, proper infection control measures, and prompt and accurate detection of ESBLs and carbapenemases from clinically important microbes are required for optimal care of infected patients and affected population.

Key words: Carbapenemases, ESBLs, Gram negative bacteria, Antimicrobial Resistance

INTRODUCTION

The existence of new β-lactamases including carbapenemases and extended spectrum enzymes amongst clinically important pathogens is an important mechanism by which bacteria develop resistance to available antibiotics. Their increasing prevalence is of global concern as they are known to make the treatment of bacterial related infections difficult (Jacoby et al., 2005). This puts the efficacy of available antibiotics into risk, thus worsening patient’s health condition. Extended spectrum β-lactamases (ESBLs) are plasmid-mediated β-lactamases capable of hydrolyzing β-lactam antibiotics including 3rd-generation cephalosporins and monobactams (SCIEH, 2004; Abhilash et al., 2010), but are yet inhibited by clavulanic acid, a β-lactamase inhibitor (Bonnet, 2004). ESBLs arise by mutations in genes for common plasmid-mediated β-lactamases (especially TEM and SHV enzymes) that alter the amino acid configuration of the enzyme near its active site to increase the affinity and hydrolytic ability of the β-lactamases for oxyimino cephalosporins (Jacoby et al., 1996). Their prevalence has also been noted worldwide in the community and hospital environments (Jacoby et al., 2005; Abhilash et al., 2010; Bonnet, 2004; Spanu et

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ESBL production was confirmed phenotypically in the Double Disk Synergy Test (DDST) strains for susceptibility testing (CLSI, 2009). Twenty-seven (27) standard organisms were used as the control: *Escherichia coli*, amoxicillin-clavulanic acid (20/10), and gentamicin (10), ofloxacin (5), ceftazidime (30), cefotaxime (30), sulfamethoxazole-trimethoprim (25), ciprofloxacin (5), and antibiotics tested included (drug concentrations in µg): amoxicillin-clavulanic acid, ceftazidime, cefotaxime, sulfamethoxazole-trimethoprim, ciprofloxacin, and antibiotics tested included (drug concentrations in µg): amoxicillin-clavulanic acid, ceftazidime, cefotaxime, sulfamethoxazole-trimethoprim, ciprofloxacin, and antimicrobial susceptibility testing according to CLSI (15) to determine the location (plasmid or chromosomal) of the drug resistance determinants, and carbapenemases have serious clinical implications as they are usually associated with high rate of morbidity, mortality, increased length of hospital stay and high treatment costs (Thompson, 2010; Spanu et al., 2002; Bradford, 2001; Bashir et al., 2011). In the present study, we sought to investigate presumptively, the production of carbapenemases and ESBLs from clinically important Gram negative bacteria from a tertiary hospital in Enugu, Nigeria.

**MATERIALS AND METHODS**

**Bacterial Isolates**

Ninety nine (99) bacterial isolates comprising of *Escherichia coli* (n=40), *Klebsiella pneumoniae* (n=39), and *Pseudomonas aeruginosa* (n=20) were isolated over a seven month period (May-November, 2011). All bacterial isolates were subcultured onto fresh growth medium, purified and confirmed by conventional microbiological tests (Cheesbrough, 2000).

**Susceptibility studies**

The antibiograms were tested by the Kirby-Bauer disk diffusion method after overnight incubation at 37°C on Mueller-Hinton agar plates as recommended by the clinical laboratory standard institute, CLSI (15). The antibiotics tested included (drug concentrations in µg): sulphonmethoxazole-trimethoprim (25), ciprofloxacin (5), ofloxacin (5), ceftazidime (30), cefotaxime (30), amoxicillin-clavulanic acid (20/10), and gentamicin (10) [Oxoid, UK]. *Escherichia coli* ATCC 25922, *K. pneumoniae* ATCC 700603 and *P. aeruginosa* ATCC 27853 standard organisms were used as the control strains for susceptibility testing (CLSI, 2009).

**Double Disk Synergy Test (DDST)**

ESBL production was confirmed phenotypically in the *E. coli*, *K. pneumoniae* and *P. aeruginosa* clinical isolates that showed reduced susceptibility to any of the screening cephalosporins as per the CLSI guidelines by double disk synergy test method as previously described (Aibinu et al., 2007; Bradford, 2001; CLSI, 2009). ESBL production was confirmed when there is an increase of ≥5mm in inhibition zone diameter for either of the cephalosporins (ceftazidime and cefotaxime) tested in combination with amoxycillin-clavulanic acid versus its zone when tested alone.

**Carbapenemase detection**

The Modified Hodges Test (MHT) was performed according to a previously described method (Aibinu et al., 2007; Thompson, 2010; Bashir et al., 2011). A standard strain of *E. coli* ATCC 25922 adjusted to 0.5 McFarland turbidity standard was inoculated on a Mueller-Hinton (MH) agar (Oxoid, UK) plate, and the plate was allowed to dry for 10 mins. One imipenem disk (10µg) (Oxoid, UK) was applied aseptically at the center of the inoculated MH agar plate, and a suspension of the test bacterium was heavily streaked from the edge of the imipenem disk (10µg) to the edge of the MH agar plate. After incubation (at 37°C for 18-24hrs), the MH agar plates were observed for Cloverleaf effect (symbol that is typical of carbapenemase production) at the intersection of the test bacterium and the *E. coli* ATCC 25922 standard organism, within the inhibition zone of the imipenem disk (10µg). The presence of growth of the test bacterium towards the imipenem disk (10µg) is considered and interpreted as a positive result for MBL production phenotypically (Bashir et al., 2011).

**Plasmid curing**

Plasmid curing experiment was undertaken for all ESBL positive isolates to determine the location (plasmid or chromosomal) of the drug resistance determinants, and this was performed according to a previously described method (Iroha et al., 2008).

**Conjugation studies**

Conjugation studies was undertaken to determine the transferability of resistance plasmids from ESBL positive organisms to non-ESBL producing bacteria. A previously described method was used to undertake this experiment (Iroha et al., 2008).

**RESULTS**

The clinical samples from which these isolates were isolated from included: urine (56), sputum (19), ear swab...
Table 1. Distribution of the clinical isolates by hospital location/sites.

<table>
<thead>
<tr>
<th>Clinical isolates</th>
<th>GOPD</th>
<th>CAS</th>
<th>SOP</th>
<th>GYNAE</th>
<th>MOP</th>
<th>AE</th>
<th>ENT</th>
<th>MMW</th>
<th>CHER</th>
<th>CHOP</th>
<th>EYE</th>
<th>WARD</th>
<th>FMW</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>28</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>29</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>7</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>1</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

Key: GOPD-General out-patient department, CAS-Casualty, SOP-Surgical out-patient, GYNAE-Gynecology ward, MOP-Medical out-patient, A/E-Accident & emergency, ENT-Ear, nose & throat, MMW-Male medical ward, CHER-Children emergency, CHOP-Children out-patient, FMW-Female medical ward

Figure 1. Percentage Susceptibility of the *E. coli* (n=40), *K. pneumoniae* (n=39) and *P. aeruginosa* (20) Clinical Isolates.

(7), pleural aspirate (1), conjunctival swab (1), wound swab (14), and High Vaginal swab, HVS (1). The frequency of isolation of the Gram – negative bacilli (*E. coli*, *K. pneumoniae*, and *P. aeruginosa*) employed in this study from various hospital sites/location is illustrated in Table 1. There was a high percentage of isolates from general out-going patients department (GOPD) when compared to isolates from other hospital sites. The reason for this high level of isolates from the GOPD might be in part to the high influx of patients to the GOPD. The result of susceptibility studies is shown in Figure 1, and the percentage susceptibility to tested agents are: CTX (*E. coli* 45%, *K. pneumoniae* 25.6%, *P. aeruginosa* 25%), CAZ (*E. coli* 47.5%, *K. pneumoniae*...
Table 2. Frequency of ESBL and Carbapenemase producers.

<table>
<thead>
<tr>
<th>Clinical isolates</th>
<th>No of isolates</th>
<th>ESBL positive n (%)</th>
<th>Carbapenemase positive n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>40</td>
<td>3 (7.5)</td>
<td>5 (12.5)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>39</td>
<td>13 (33.3)</td>
<td>3 (7.7)</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>20</td>
<td>0 (0)</td>
<td>3 (15)</td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>16 (16.2)</td>
<td>11 (11.1)</td>
</tr>
</tbody>
</table>

Table 3. Plasmid Curing Analysis of ESBL Positive isolates with acridine orange (0.1mg/ml).

<table>
<thead>
<tr>
<th>Clinical isolate</th>
<th>Pre-curing (n)</th>
<th>Cured (Plasmid-borne) n (%)</th>
<th>Not cured (chromosomal) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>3</td>
<td>2 (12.5)</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>13</td>
<td>5 (31.3)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>7 (43.8)</td>
<td>9 (56.3)</td>
</tr>
</tbody>
</table>

Key: n=number

Table 4. Antibiotic susceptibility of ESBL and Carbapenemase phenotypes to some selected non-β-lactam antibiotics.

<table>
<thead>
<tr>
<th>Antibiotics (µg)</th>
<th>ESBL phenotypes (n=16)</th>
<th>Carbapenemase phenotypes (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Gentamicin (10)</td>
<td>3</td>
<td>18.8</td>
</tr>
<tr>
<td>Ofloxacin (5)</td>
<td>4</td>
<td>25</td>
</tr>
<tr>
<td>Sulphamethoxazole- Trimethoprim (25)</td>
<td>1</td>
<td>6.3</td>
</tr>
<tr>
<td>Ciprofloxacin (5)</td>
<td>4</td>
<td>25</td>
</tr>
</tbody>
</table>

25.6%, P. aeruginosa 50%), SXT (E. coli 2.5%, K. pneumoniae 12.8%, P. aeruginosa 0%), CN (E. coli 45%, K. pneumoniae 46.2%, P. aeruginosa 55%), OFX (E. coli 25%, K. pneumoniae 41%, P. aeruginosa 30%), CIP (E. coli 35%, K. pneumoniae 30.8%, P. aeruginosa 45%), IPM (E. coli 95%, K. pneumoniae 87.2%, P. aeruginosa 100%) and MEM (E. coli 92.5%, K. pneumoniae 92.3%, P. aeruginosa 100%). The production of extended spectrum β-lactamase (ESBL) and carbapenemases was phenotypically detected by the double disk synergy test (DDST) and modified Hodges test (MHT) methods respectively, and the results are as shown in Table 2. Plasmid curing experiments was carried out on all the ESBL positive bacteria (n=16) using acridine orange (0.1mg/ml), and the result is illustrated in Table 3. Table 4 shows the antimicrobial susceptibility of ESBL and Carbapenemase phenotypes to some selected non-β-lactam antibiotics.

**DISCUSSION**

One of the major problems facing the health sector today is the rapid emergence and spread in the resistance of multidrug resistant (MDR) pathogenic bacteria to readily available antibiotics (especially the beta-lactams). This growing resistance of pathogens to antibiotics is a challenge to medical health practitioners when it comes to treating and managing most infections caused by MDR organisms (Jacoby et al., 2005; Walsh et al., 2005). All test isolates showed varied rates of susceptibility to the test antibiotics (Figure 1). The carbapenems (imipenem and meropenem) were the most effective agent with substantial antibacterial activity. This was followed by gentamicin, ofloxacin, ceftazidime and cefotaxime. Sulphamethoxazole-trimethoprim was the least active agent tested (Figure 1). Overall, the ESBL production in our study was 7.5 % and 33.3 % in E. coli and K. pneumoniae isolates respectively. Notably, none of the P. aeruginosa isolates in our study showed ESBL production (Table 2). The prevalence of ESBL production of 16.2 % among the tested Enterobacteriaceae in our study (33.3 % K. pneumoniae and 7.5 % E. coli) is consistent with data from Singapore (44 % Klebsiella, 16.1 % E. coli), Pakistan (58.7 % K. pneumoniae) and Kano in Northern Nigeria where ESBL production was 66.7 % in Enterobacteriaceae (Chlebicki et al., 2004; Chakraborty et al., 2010; Yusha’u et al., 2010). However, our results of ESBL production of 7.5 % E. coli and 33.3 % K. pneumoniae was in contrast to a similar work done...
in Saudi Arabia where ESBL production was 4.4% and 95.6% in K. pneumoniae and E. coli respectively (Kader et al., 2009). The unanticipated non-production of ESBL in P. aeruginosa isolates in this study is in contrast to a similar work carried out in Pakistan and Nigeria where ESBL was detected in P. aeruginosa isolates from blood specimens (Mumtaz et al., 2008; Aibinu et al., 2007). A possible reason for this development in our study (i.e. the non-production of ESBLs by P. aeruginosa isolates) could be due to the non-transferability of ESBL genes (or resistance plasmids) from ESBL-producing Enterobacteriaceae to non-enteric bacteria (in this case P. aeruginosa) in this environment. Nevertheless, it is still important to screen clinically important bacteria that are multiply resistant for possible production of ESBLs in order to forestall any outbreak due to their infections. Carbapenemase enzyme production was detected in 12.5%, 7.7% and 15% of E. coli, K. pneumoniae and P. aeruginosa isolates respectively (Table 2). This observed prevalence of carbapenemase production in our study is high, and in contrasts to the results obtained in India (Chakraborty et al., 2010). However, our result of carbapenemase production corresponds to those obtainable in Kashmir (Bashir et al., 2011). Carbapenemase production according to reports is found to be more prevalent in P. aeruginosa isolates than the Enterobacteriaceae (Walsh et al., 2005; Chakraborty et al., 2010; Franklin et al., 2006). Our study nevertheless, has presumptively shown that Enterobacteriaceae and P. aeruginosa isolates producing carbapenemase enzymes exist in southeastern Nigeria. The ESBL phenotypes in our study were both plasmid-mediated and chromosomally-mediated. Particularly, 2 (12.5%) out of the 3 ESBL positive E. coli isolates lost their plasmids while 5 (31.3%) of the 13 ESBL positive K. pneumoniae isolates lost their plasmid following curing by acridine orange (at a sub-inhibitory concentration of 0.1 mg/ml). Bacteria Population containing plasmids when subjected to Curing agents such as acridine orange will become more and more dominated by plasmid-free cells with time (Iroha et al., 2008). Bacterial plasmids play a very important role in the spread of antibiotic resistance traits (such as ESBLs) amongst bacterial population through various means of genetic transfers including conjugation, transformation and transduction (Jacoby et al., 2005; Thompson, 2010). In our study, the antibiogram of our ESBL and carbapenemase phenotypes as illustrated in Table 4 shows that sulphonamethoxazole-trimethoprim had no antibacterial activity on the tested bacteria while gentamicin, ofloxacin and ciprofloxacin were moderately active. Having in mind the antibiotic susceptibility patterns of ESBL- and carbapenemase-producing organisms is useful in choosing the right antibiotic therapy in the face of an infection. Our conjugation experiment show some evidence of transfer of plasmid-mediated resistance in some of the transconjugated isolates. It was noted from our study that more than half of the recipient strain was successfully transconjugated as the transconjugants were observed to be resistant to virtually all the antibiotics (CAZ, CTX, CN, SXT, OFX, CIP, MEM and IPM) tested. Particularly, 5 out of the 8 ESBL positive bacteria isolates transconjugated successfully transferred their antibiotic resistant determinants to the recipient strain (E. coli ATCC 25922). The ability of antibiotic resistant bacteria to successfully transfer their resistant plasmids to susceptible population of bacteria is the single most popular reason (amongst other criteria) by which multidrug resistant traits such as ESBLs can be spread amongst bacterial population in a given hospital environment. From our study, those bacterial isolates that successfully transferred their plasmids have their resistance genes in their plasmids, and those that failed to successfully transfer theirs were inferred to be chromosomally-borne.

Conclusion

Conclusively, our study has clearly shown that ESBL- and carbapenem-producing pathogens occur in Enugu, southeastern Nigeria; and that these pathogens are resistant to some readily available drugs. ESBLs and carbapenemases now exist worldwide, and they hydrolyze and confer resistance to the extended spectrum cephalosporins and the carbapenems respectively. It is trendier in Nigeria for people to obtain antibiotics over-the-counter (OTC), a practice that allows microbes to develop resistance through selective pressure. Antibiotic resistant bacteria lead to increase in the length of hospitalization of a patient, severity of illness and wastage of resources. There prompt and accurate detection in this region will help to assuage any damage due to them.

REFERENCES


