Detection of SHV and TEM-type extended spectrum β-lactamase in bacterial isolates in military hospitals

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ABSTRACT

BACKGROUND
Multi-drug resistant bacterial strains have been increasingly implicated in clinical infections worldwide and beta-lactamase production is one of the commonest mechanisms of resistance in these strains. This study investigated the prevalence of extended spectrum β-lactamase (ESBL)-producing isolates and determined the temoneira (TEM) and sulfhydryl variable (SHV) types implicated in two military hospitals in South-South Nigeria.

METHODS
Three-hundred and eighty (380) consecutive non-duplicate bacterial isolates (Gram negative bacilli) recovered from clinical samples were identified following standard techniques. Antimicrobial susceptibility tests were performed for each isolate following the Clinical Laboratory Standards Institute guidelines. Bacterial isolates recovered which comprised Escherichia coli, Klebsiella spp, Proteus spp and Pseudomonas aeruginosa were screened for ESBL using a phenotypic method (double disc synergy test). All positive isolates were screened for TEM and SHV genes by PCR method.

RESULTS
Sixty-five isolates (17.1%) were ESBL producing using phenotypic method, E. coli showed the highest ESBL prevalence (24.3%). One isolate was SHV positive (1.5%), 8 (12.3%) were TEM positive while 3 (4.6%) isolates harbored both SHV and TEM genes. Fluoroquinolone - ofloxacin showed marked activity against ESBL-producing isolates (90.8%) while the least active were ceftriaxone (9.2%), ceftazidime (3.1%) and ampicillin (1.5%).

CONCLUSION
This study demonstrated that 17.1% of Gram-negative bacilli were ESBL producers. Screening of clinical isolates for ESBL should be implemented. The findings of this study suggest the need for caution in the use of antimicrobial agents in order to curb the incidence of antimicrobial resistance.

Keywords: Extended spectrum β-lactamase, resistance, Gram negative bacilli, antimicrobial
INTRODUCTION

The rising prevalence of drug-resistant bacteria causing clinical infections is an alarm suggesting that therapeutic options for patients are constantly being reduced. Extended spectrum beta-lactamase (ESBL), a β-lactamase enzyme type produced by some bacteria, is capable of hydrolyzing the amide bond of the four-membered β-lactam ring of the penicillins, the first, second and third generation cephalosporins, and aztreonam, thereby inactivating them.\(^1\) Its genetic determinants are largely plasmid-borne and highly transmissible between bacterial strains, being of the sulphydryl variable (SHV), temoneira (TEM) and cefotaxime-Munich (CTX-M) types, with oxacillin-hydrolyzing (OXA), Vietnamese ESBL (VEB) and Pseudomonas extended resistance (PER) types being increasingly implicated worldwide.\(^1-3\)

Although SHV variants have not been as rapidly disseminated worldwide as the CTX-M variants, they have been increasingly detected in Enterobacteriaceae strains besides Escherichia coli and Klebsiella pneumoniae in which they were first detected in the 1970s and 1980s.\(^1,3\) Indeed, outbreaks of SHV-producing Acinetobacter spp and Pseudomonas aeruginosa have also been reported worldwide from clinical specimens and hospital environments.\(^3\) Similarly, over 100 variants of TEM have been detected in several Enterobacteriaceae since their first detection in K. pneumoniae and K. oxytoca in the 1970s.\(^4\) In Nigeria, literature is growing on the increased association between multidrug resistance and ESBL production among Enterobacteriaceae causing clinical infections.\(^5-7\) Previous studies in South-western Nigeria reported a high prevalence of SHV, TEM and CTX-M genes among Gram negative bacterial isolates causing clinical infections from tertiary hospitals.\(^8\) Another study in Benin City, South-South Nigeria reported an ESBL prevalence of 41.7% among clinical isolates.\(^8\)

Military hospitals are secondary hospitals tending to the health needs of military personnel and community dwellers where they are located. To the best of our knowledge, no previous Nigerian study has explored ESBL prevalence among clinical isolates from military hospitals. This study therefore sought to determine the prevalence of ESBL-producing isolates among clinical isolates from two military hospitals in South-South Nigeria as well as detect the TEM and SHV types implicated by using polymerase chain reaction method (PCR).

METHODS

Study area

This study was carried out in the metropolitan cities of Benin City, Edo State and Port-Harcourt in Rivers State South-South Nigeria.

Research Design

A hospital based cross-sectional study was conducted in the Microbiology section of Military Hospital, Benin City and Military Hospital, Port-Harcourt in Edo and Rivers States, Nigeria, respectively from 5\(^{th}\) June 2019 to 4\(^{th}\) September, 2019. Both hospitals are a 150 bed secondary care hospital and serve as referral center for primary health care with outpatient clinics, medical, surgical, gynecological, pediatric and geriatric wards.

Bacterial isolates

Consecutive non-duplicate bacterial isolates recovered from clinical samples were Gram stained. Gram negative bacilli were identified following standard techniques.\(^9\) These were thereafter preserved on nutrient agar slants at 4°C for further tests.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were performed for each isolate following the Clinical Laboratory Standards Institute (CLSI) guidelines.\(^10\) Briefly, test organisms were
emulsified in sterile water and the turbidity matched with 0.5 McFarland standard. Once matched, a sterile cotton-wool swab was dipped in the organism suspension and excess liquid was removed by turning the swab on the side of the test tube. The entire surface of Mueller-Hinton agar plate was seeded by swabbing in three directions with the swab. The antibiotics included; ofloxacin (5 µg), ceftriaxone (30 µg), gentamicin (10 µg), amoxicillin-clavulanate (30 µg), ampicillin (30 µg), cefprozil (30 µg), imipenem (10 µg), nitrofurantoin (100 µg) and ceftazidime (30 µg) (all from Abtek Biologicals Limited, U.K). The control strain Escherichia coli ATCC 25922 was also included.

**Phenotypic detection of ESBL**

All gram negative bacilli isolates were screened for ESBL. The double disc synergy test was used in detecting the presence of ESBL. Briefly, the test organisms were emulsified in sterile distilled water and the turbidity matched with 0.5 McFarland standard. A sterile cotton wool swab was then dipped in the organism suspension and excess liquid was drained off by turning the swab on the side of the test tube. The entire surface of Mueller–Hinton agar (MHA) plate was then seeded by swabbing in three directions and allowed to dry for 3-10 mins. A 30 µg amoxicillin–clavulanate (Oxoid, U.K) disc was placed at the center of the agar plate. This was flanked at opposite sides by 30 µg ceftazidime and cefotaxime discs (Abtek Biologicals Ltd, Liverpool, U.K) at a distance of 25mm from the amoxicillin-clavulanate disc. The plates were thereafter incubated at 37°C for 18-24 hrs. ESBL production was inferred as positive if there was an expansion of the zone of inhibition between amoxicillin–clavulanate disc and any of the indicator cephalosporins (ceftazidime and cefotaxime) or both.

**DNA extraction and amplification**

The DNA used for PCR was extracted from pure isolates grown overnight on nutrient agar using the Quick-DNA™ Fungal/Bacterial Miniprep Kit according to the manufacturer’s protocol. The concentration and purity of the extracted DNA was estimated using a nanodrop spectrophotometer. The gene primers which included TEM (forward primer) 5‘-ATGAGTATTCAACATTTCCGC-3’; reverse primer 5‘-TGACAGTTACCAATGCT-3’ and SHV (forward primer) 5‘-CCGCAGCCGCTTG AGCAAA-3’; reverse primer 5‘-GCTGGCCGGG GGTAGTGGTGTC-3’ were used for PCR amplifications as previously described. A molecular size marker (Thermo Scientific™ SM0241 effective size range: 100 to 1000 kb) was used to assess PCR product size. Then, PCR products were thereafter analyzed by agarose gel electrophoresis and photographed using an ultraviolet illuminator. The PCR products for TEM and SHV genes were detected at 867bp and 477bp respectively. ESBL-positive Klebsiella pneumoniae ATCC 700603 and ESBL-negative E. coli ATCC 25922 were included as control strains.

**Statistical analysis**

The data obtained were analyzed with Chi ($X^2$) square test or Fischer’s exact test where appropriate using INSTAT® software. A p value of < 0.05 was deemed statistically significant.

**Ethical clearance**

Ethical approval was sought from the Ministry of Health, Edo State. This was approved with protocol number HA.577/Vol.11/18/201. Strict patient confidentiality was observed throughout the study and patients that declined were not included.

**RESULTS**

A total of 380 bacterial isolates (Gram negative bacilli) were recovered from various clinical specimens during the study period. Sixty-five isolates (17.1%) were ESBL producing using phenotypic method, while E. coli showed the highest ESBL prevalence (24.3%) and
showed statistical significance (p=0.0016). Similarly, among 65 ESBL producing isolates which were screened using PCR technique to detect SHV and TEM genes, one was SHV positive (1.5%), a total of 8 (12.3%) were TEM positive while 3 (4.6%) isolates harbored both SHV and TEM genes (Figure 1 and 2) (Table 1).

In relation to clinical specimens, isolates recovered from urine specimens showed the highest ESBL prevalence with 29 (23.5%), 4 of which harbored TEM gene and one isolate being positive for SHV + TEM. The findings were however not statistically significant (p=0.2335). All gram negative bacteria recovered from tongue swabs showed absence of ESBL activity (Table 2).

The fluoroquinolone - ofloxacin showed marked activity against ESBL-producing isolates of *E. coli* (86.5%), *Klebsiella* spp (100%), *Proteus* spp (100%) and *P. aeruginosa* (92.3) in comparison with other antibiotics while the least active were ceftriaxone (9.2%), ceftazidine (3.1%) and ampicillin (1.5%). Moderate activity was observed for imipenem (58.5%) and ciprofloxacin (58.5%) (Table 3).

**DISCUSSION**

For this study, the prevalence of ESBL producing bacteria causing infection in military hospitals in South-southern Nigeria was 17.1%. This value is lower than that of previous studies in a tertiary hospital in Benin City, Nigeria.
(41.7%) and a Chadian hospital, where 47.42% prevalence was observed.\textsuperscript{(7,13)} It is however similar to that of another study in a tertiary hospital in Kano, Nigeria (15.0%).\textsuperscript{(14)} The finding is also strikingly lower when compared with a Ugandan study which used the same phenotypic method where a prevalence of 89.0% was observed.\textsuperscript{(15)} The prevalence of ESBL has been shown to vary according to geographical location and over time.\textsuperscript{(1,3)} Our study thus shows the existence of ESBL phenotypes among bacterial isolates causing clinical infections at the secondary healthcare level. It is noteworthy however that isolates recovered are not routinely screened for this enzyme despite the simplicity of the double disk diffusion technique and the implication of such results for patient management.

\textit{Escherichia coli} showed the highest prevalence (24.3%) in this study. This finding differs with earlier mentioned studies in Nigeria where \textit{Providencia} spp and \textit{Klebsiella} spp ranked highest in Benin and Kano respectively.\textsuperscript{(7,14)} \textit{Escherichia coli} however is of great interest to researchers because of its ability to cause opportunistic infections, nosocomial infections as well as acquire plasmids which confer antimicrobial resistance or may harbor virulence determinants to the detriment of their host. Moreover, ESBL-producing \textit{E. coli} has been implicated in clinical infections in several studies.\textsuperscript{(5-7)}

The prevalence of SHV and TEM genes among ESBL producing isolates was 1.5% and 12.3% respectively, while 4.6% of isolates showed positivity for both genes. This finding suggests that some other genetic determinants for ESBL are at play besides the aforementioned two. Recent studies have documented the role of CTX-M ESBL variants in clinical infections in Nigeria.\textsuperscript{(5,16)} Previous studies in South-

<table>
<thead>
<tr>
<th>Organisms</th>
<th>No. Tested</th>
<th>ESBL positive (Phenotypic detection)</th>
<th>PCR Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SHV</td>
</tr>
<tr>
<td>\textit{E. coli}</td>
<td>152 (40)</td>
<td>37 (24.3)</td>
<td>1 (2.7)</td>
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<tr>
<td>\textit{Klebsiella spp}</td>
<td>95 (25)</td>
<td>8 (8.4)</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Proteus spp}</td>
<td>76 (20)</td>
<td>7 (9.2)</td>
<td>-</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>57 (15)</td>
<td>13 (22.8)</td>
<td>-</td>
</tr>
<tr>
<td>\textbf{Total}</td>
<td>380</td>
<td>65 (17.1)</td>
<td>1 (1.5)</td>
</tr>
</tbody>
</table>

Isolates vs Phenotypic ESBL; p=0.0016; Abbreviations: PCR-Polymerase chain reaction, ESBL-Extended spectrum beta-lactamase, number in brackets = value in percentage

<table>
<thead>
<tr>
<th>Specimen</th>
<th>No. of isolates</th>
<th>ESBL positive (Phenotypic method)</th>
<th>PCR Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TEM</td>
</tr>
<tr>
<td>Urine</td>
<td>123</td>
<td>29 (23.5)</td>
<td>4 (13.8)</td>
</tr>
<tr>
<td>Wound</td>
<td>99</td>
<td>15 (15.2)</td>
<td>-</td>
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<tr>
<td>Sputum</td>
<td>68</td>
<td>11 (16.2)</td>
<td>2 (18.2)</td>
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<tr>
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<td>6 (14.3)</td>
<td>1 (16.7)</td>
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<tr>
<td>HVS</td>
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<td>-</td>
</tr>
<tr>
<td>Tongue Swab</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Semen</td>
<td>12</td>
<td>2 (16.7)</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

Specimen vs Phenotypic ESBL; p = 0.2335; HVS-High vaginal swab, DDST-Double disk synergy test, PCR-Polymerase chain reaction, number in brackets = value in percentage
Southern Nigeria. The researchers also showed that a high percentage of isolates causing clinical infections were ESBL-producing and of the CTX-M type.\(^5,16\)

Also, novel genetic determinants of ESBL namely VEB and OXA types had been detected among Providencia spp in a tertiary hospital in Lagos; the VEB and CTX-M-15 variants have also been markedly detected among isolates causing urinary tract infections in southwestern Nigeria.\(^5,16\) Thus, we can surmise that the low detection of SHV and TEM genes in known ESBL strains points to the fact that other ESBL genetic determinants are at play. Further studies exploring the role of the CTX-M, VEB, OXA, PER and other novel genes among ESBL-producing isolates in our region would give clarity to this assertion as well as aid epidemiological surveys.

Interestingly, isolates recovered from urine of patients with significant bacteriuria had a higher ESBL prevalence when compared with any other sample. This finding is similar to two previous Nigerian studies and one Ugandan study.\(^7,12,15\) However, it differs from another study in Rajasthan, India in which respiratory specimens had the highest prevalence.\(^1\) Urine specimens are one of the most frequently received specimens in diagnostic microbiology laboratories.\(^7\) Their connection with high prevalence of ESBL-producing bacterial strains may be due to the widespread availability of antibacterial agents notably the third generation cephalosporins in hospital and community settings where blind prescription and abuse is rife.\(^6,7,16\) Such practices exert selective pressure, induce resistance mechanisms notably ESBL production and ensure survival and subsequent spread of these resistant bacterial strains.\(^5-7\)

The fluoroquinolone-ofloxacin showed high activity against ESBL-producing strains in comparison with any other antibacterial agent. The fluoroquinolones are however contraindicated in children and pregnant women and their use in various formulations has been restricted in European countries owing their serious, disabling and potentially permanent side effects.\(^19\) Bacterial resistance to fluoroquinolones increased drastically not long after the introduction of ofloxacin and ciprofloxacin.\(^20\) Other antibacterial agents used in this study were also poor against ESBL-producing bacterial strains. This therefore highlights the limited therapeutic options faced by clinicians in patient management as it is an established fact that ESBL enzymes inactivate the penicillins and the second and third generation cephalosporins, some of which were used in this study.\(^1\) Similarly, plasmids harboring ESBL determinants have been known to harbor genes.
confering resistance to other antibacterial classes. These reasons may explain our finding. Although ofloxacin was the most active antibacterial agent against ESBL-producing bacteria, considering the brewing resistance to the carbapenems, our study harps on prudence in the use of antimicrobial agents. There is therefore need for institutional, local and national surveillance in order to monitor and curb the menace of antimicrobial resistance.

CONCLUSION

Our study documented a comparatively high prevalence of ESBL genes (SHV and TEM) among *E. coli* isolates in comparison to other Gram-negative bacterial isolates in military hospitals in South-South Nigeria. The relatively low prevalence of the aforementioned genes among ESBL-producing isolates suggests the need to determine the prevalence of a wider array of genes implicated in ESBL production.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

CONTRIBUTORS

HOO, ISE and EEI conceived the study. ISE and EEI contributed to data collection and data analysis. HOO assisted in data interpretation. All authors participated in writing and approved the final draft of the manuscript.

REFERENCES


