PCR Detection of Extended Spectrum β-Lactamase from Some Gram Negative Bacteria of Clinical Source

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Abstract

Beta-lactamase-producing microorganisms present distinct challenges in health care systems around the world as a result of the extensive utilization of broad-spectrum antibiotics. The objective of this research endeavor was to ascertain the antibiotic resistance profile and presence of β-lactamase genes in Gram-negative bacteria that had been previously identified and collected from laboratory work benches at the Department of Microbiology and Parasitology, UCH, located in Ibadan, South-West Nigeria. The Kirby-Bauer disc diffusion method was used to assess antibiotic susceptibility on all the isolates, and the double disc synergy test was used to validate extended spectrum beta-lactamase (ESBL) production phenotypically. The PCR techniques were used to detect β-lactamase genes. The sample distribution of the forty clinical isolates were collected from the work benches and the distribution was: urine (17), blood (9), wound (6), sputum (4), amniotic fluid (3) and tracheal aspirate (1). Klebsiella pneumoniae was the most prevalent organism with 17 isolates (42.5%). Out of the 40 isolates that were obtained, 21 were ESBL producers and the distribution was as follows: Klebsiella pneumoniae (9), Escherichia coli (6), Enterobacter cloacae (2), Enterobacter aerogenes (1), Pseudomonas aeruginosa (1), Acinetobacter baumannii (1) and Hafnia alvei (1). The majority of the clinical isolates showed considerable resistance to antibiotic classes that were tested, with amoxicillin-clavulanate showing the highest resistance rate at 65%. Out of all the ESBL producers, 35.3% had blaTEM, 29.4% had blaCTX-M, 23.5% had blASHV, and 11.8% had blacMY.  

Keywords: AmpC β-lactamases, Antibiotic resistance, Disc diffusion, Gram-negative bacteria, Polymerase chain reaction.

INTRODUCTION

The development of effective antibiotics has been a game-changer in the fight against bacterial illnesses. Nevertheless, numerous bacterial species have developed antibiotic resistance or
multidrug resistance due to the extensive use of antibiotics. Unfortunately, drug-resistant diseases, such as multidrug-resistant gram-negative bacteria, are notoriously hard to detect with standard diagnostic procedures, and there is a severe shortage of novel antibiotics (Mutee et al., 2023). According to Hussain et al. (2021), the predominant mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria is by the synthesis of β-lactamases. The two most frequent types of β-lactamases are ESBLs and AmpC β-lactamases (Shaaban et al., 2022).

AmpC β-lactamases have gained importance for over than forty years since their discovery as one of the enzymes responsible for antibiotic resistance in Gram-negative bacteria (Zhou et al., 2022). AmpC β-lactamases are either plasmid or chromosomal mediated. The increased presence of plasmid mediated AmpC β-lactamases worldwide is becoming of great concern because most clinical laboratories and physicians remain unaware of their clinical importance, and therefore have been responsible for several nosocomial outbreaks (Xiong et al., 2021). Without accurate laboratory detection and reporting of such resistant phenotypes and strains producing plasmid-mediated AmpC, treatment of Gram-negative infection may remain suboptimal (Flannery et al., 2022).

ESBLs are most often a plasmid mediated heterogeneous group of β-lactamase enzymes that confer resistance to a wide range of commonly used β-lactam antibiotics (Bush and Bradford, 2020). TEM and SHV type ESBLs used to be the dominant ESBL genotypes (Dirar et al., 2020). However, in the past decade, the CTX-M type ESBLs have become the most widely distributed and globally dominant genotypes (Castanheira et al., 2021). Infections caused by ESBL-producing Gram negative bacteria are associated with increased morbidity and mortality. This scenario is linked to inappropriate, indiscriminate or delayed antimicrobial treatment (Husna et al., 2023).

In Nigeria, extended-spectrum cephalosporins and fluoroquinolones are widely used as broad-spectrum antibiotics and remain the drugs of choice to treat infections caused by various Gram-negative pathogens (Nwafuluaku et al., 2021). Therefore, this study is aimed to carry out phenotypic and genotypic characterization of β-lactamase genes and determine the antibiotic resistance pattern in Gram-negative bacteria obtained from University College Hospital, Ibadan, Oyo State, Nigeria.
MATERIALS AND METHODS

Study Area
The University of Ibadan (UI), situated in the vibrant city of Ibadan, the capital of Oyo State, Nigeria, serves as the primary research institution for this study. UI is located at approximately Latitude 7° 23′ 28.19″ N and Longitude 3° 54′ 59.99″ E.

Collection of samples
The isolates were obtained from different clinical samples such as sputum, amniotic fluid, urine, tracheal aspirate, wound and blood at the Department of Microbiology and Parasitology, University College Hospital (UCH), Ibadan, Oyo State. Ethical approval was sought before sample collection to adhere to ethical standards and institutional guidelines.

Sterilization of Media and Materials
Muller Hinton agar was prepared following the manufacturer's instructions and sterilized using autoclave at 121°C for 15 minutes. The media were purchased from Oxoid Limited. All glass wares were washed with detergent, rinsed, dried, wrapped in aluminum foil, and sterilized in a hot air oven at 180°C for 6 hours.

Identification of Isolates
The isolates were identified using the MicrobactTM Gram Negative System Identification Kit (Thermo Scientific-Oxoid, UK). The results were transcribed in to a code and organisms were identified using a computerized profile register.

Determination of Antibiotic Resistance
The experiment was conducted using the disc diffusion method (Krishnan et al., 2019), following the instructions provided by CLSI (2018) guidelines on Muller-Hinton agar after normalization of broth to 0.5 McFarland standard to test all the isolates against the panel of eleven different antibiotics. The antibiotics utilized were ciprofloxacin (5 µg), aztreonam (30 µg), gentamicin (10 µg), cefpodoxime (10 µg), Cefotaxime (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), azithromycin (15 µg), amoxicillin-clavulanate (30 µg), cefepime (30 µg), cefuroxime (30 µg) (Oxoid, UK).

Phenotypic Detection of ESBL
The clinical isolates were examined for potential production of extended-spectrum beta-lactamase (ESBL) using the double disc synergy test (DDST) in accordance with the standards set by the Clinical Laboratory Standards Institute (CLSI, 2018), Figure 2.
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**Figure 2**: Double Disc Synergy Test (DDST) for detection of ESBL production

**Note**: A larger area of inhibition or distortion surrounding ceftazidime, cefpodoxime, and cefotaxime when compared to amoxicillin clavulanate showed a positive result for extended-spectrum beta-lactamase (ESBL) synthesis.

Key: A = Cefotaxime (CTX); B = Ceftazidine (CAZ); C = Cefpodoxime (CPD); D = Amoxicillin Clavulanate (AMC)

**Identification of ESBL and AmpC genes in isolates that produce ESBLs**

The genomic DNA of the bacterial isolates that exhibited a positive phenotypic ESBL test was extracted using a Zymo Research Bacterial DNA MiniPrep™ Kit (Zymo Research Corporation, USA) according to the instructions provided by the manufacturer. The detection of genes was performed using the conventional PCR technique with specified primers. The nucleotide sequences of the target genes are displayed in Table 1.

**Table 1: Sequences of primers in detecting extended spectrum and AmpC$\beta$-lactamase genes**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Sequences (5’ – 3’)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| TEM Primers  | F-GCATCTTACGGATGGCATGA  
R-GTCCTCCGATCGTTCAGAA | Roschanski et al., 2014 |
| SHV Primers  | F-TCCTCATGATACACCTTTAAA  
R-TCTGTGGACATAGTGGAT | Roschanski et al., 2014 |
| #CTX-A Primers | F-GGCGCRATGGGCGCARAC  
R-GCRCCGGTGTATGGCC | Roschanski et al., 2014 |
| #CTX-B Primers | F-ACCGAGCCSACGCCTCAA  
R-CCGCTGCCGTTTTATC | Roschanski et al., 2014 |
| CMY Primers  | F-GCAAAACAGTGACAGGAT  
R-AAATGCAGGCTTAACAGG | Roschanski et al., 2014 |

**Note**:  
#CTX-A was designed for the detection $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CTX\text{-}M\text{-}9}$;  
#CTX-B was designed for the detection of $bla_{CTX\text{-}M\text{-}2}$ and $bla_{CTX\text{-}M\text{-}8}$ and $bla_{CTX\text{-}M\text{-}25}$
RESULTS
Among the 40 clinical isolates, 17 were identified as *Klebsiella pneumoniae*, 11 were *Escherichia coli*, 6 were *Pseudomonas aeruginosa*, 4 were *Enterobacter* spp., and there was 1 each of *Hafnia alvei* and *Acinetobacter baumannii*. The analysis of organisms obtained from different clinical samples in this study revealed that urine had the highest prevalence of clinical Gram-negative bacteria, accounting for (17/42.5%) The sequence of prevalence was followed in that order by blood (9/22.5%), wound (6/15%), sputum (4/10%), amniotic fluid (3/7.5%), and tracheal aspirate specimen (1/2.5%) had the lowest prevalence.

Figure 3 displays the resistance rates of Gram-negative bacteria derived from clinical sources against specific drugs. Among the 40 isolates, the highest resistance rates were observed for amoxicillin clavulanate, with 26 isolates (65%) being resistant. This was followed by cefepime, with 25 isolates (62.5%) showing resistance. Additionally, 22 isolates (55%) were resistant to gentamicin, while 21 isolates (52.5%) exhibited resistance to aztreonam, cefotixin, ceftazidime, cefotaxime, and ampicillin sulbactam. Out of the clinical Gram-negative bacteria, only 20 (50%) exhibited resistance to ciprofloxacin, cefpodoxime, and azithromycin, respectively.

Among the 40 isolates that were examined for ESBL production, 21 were found to be positive, resulting in an overall prevalence rate of 52.5%. The prevalence of ESBLs was highest in *Acinetobacter baumannii* (100%) and *Hafnia alvei* (100%) respectively, followed by *Enterobacter* spp. (75%), *Escherichia coli* (54.5%), *Klebsiella pneumoniae* (52.9%), and the lowest frequency of ESBL producing bacteria was observed in *Pseudomonas* spp. (16.7%).

The PCR technique was used for the molecular characterization of the isolates’ resistance genes. After screening for ESBL-associated genes (*bla*SHV, *bla*TEM, and *bla*CTX-M), only 18 of 21 phenotypic ESBL-positive isolates (85.7%) tested positive, suggesting that the phenotypic tests were quite sensitive. Among ESBL positive isolates, the *bla*TEM gene was the most common at 35.3%, followed by *bla*CTX-M at 29.4%, *bla*SHV at 23.5%, and *bla*CMY at 11.8%. Both *Klebsiella pneumoniae* 50 and *Escherichia coli* 192 showed a strong presence of all three gene groups. Only four (22.2%) of the isolates had the *bla*TEM gene alone; the rest, with the exception of one *Klebsiella* spp. isolate, had the *bla*CTX-M gene in conjunction with the *bla*TEM and/or SHV/CMY genes. Table 2 provides a summary of the β-lactamase gene distribution across ESBL producers.
Table 2: ESBL and AmpC β-lactamase gene profile of bacteria isolated from clinical samples

<table>
<thead>
<tr>
<th>Isolate identity</th>
<th>Source</th>
<th>Beta-lactamase genes detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pneumoniae 134</td>
<td>Urine</td>
<td>blαCTX-M, blαTEM</td>
</tr>
<tr>
<td>K. pneumoniae 131</td>
<td>Urine</td>
<td>blαTEM</td>
</tr>
<tr>
<td>K. pneumoniae 67</td>
<td>Urine</td>
<td>blαTEM</td>
</tr>
<tr>
<td>K. pneumoniae 129</td>
<td>Urine</td>
<td>blαCTX-M, blαSHV</td>
</tr>
<tr>
<td>K. pneumoniae 24</td>
<td>Sputum</td>
<td>blαCTX-M</td>
</tr>
<tr>
<td>K. pneumoniae 193</td>
<td>Sputum</td>
<td>blαTEM, blαSHV</td>
</tr>
<tr>
<td>K. pneumoniae 0001</td>
<td>Sputum</td>
<td>blαTEM, blαSHV, blαSHV</td>
</tr>
<tr>
<td>K. pneumoniae 50</td>
<td>Blood</td>
<td>blαCTX-M, blαTEM, blαSHV</td>
</tr>
<tr>
<td>K. pneumoniae 53</td>
<td>Blood</td>
<td>blαSHV</td>
</tr>
<tr>
<td>E. coli 12</td>
<td>Urine</td>
<td>blαTEM, blαCMY</td>
</tr>
<tr>
<td>E. coli 191</td>
<td>Urine</td>
<td>blαTEM</td>
</tr>
<tr>
<td>E. coli 17</td>
<td>Blood</td>
<td>blαCTX-M, blαSHV</td>
</tr>
<tr>
<td>E. coli 192</td>
<td>Blood</td>
<td>blαCTX-M, blαTEM, blαSHV, blαCMY</td>
</tr>
<tr>
<td>E. coli 13</td>
<td>Tracheal aspirate</td>
<td>blαCTX-M, blαTEM, blαSHV</td>
</tr>
<tr>
<td>E. coli 190</td>
<td>Amniotic fluid</td>
<td>None</td>
</tr>
<tr>
<td>E. cloacae 002</td>
<td>Wound</td>
<td>blαCTX-M, blαCMY</td>
</tr>
<tr>
<td>E. cloacae 195</td>
<td>Wound</td>
<td>blαCTX-M, blαCMY</td>
</tr>
<tr>
<td>E. aerogenes 196</td>
<td>Wound</td>
<td>blαCTX-M, blαTEM</td>
</tr>
<tr>
<td>Pseudomonas spp. 94</td>
<td>Urine</td>
<td>blαTEM</td>
</tr>
<tr>
<td>A. baumannii 56</td>
<td>Urine</td>
<td>None</td>
</tr>
<tr>
<td>H. alvei</td>
<td>Wound</td>
<td>None</td>
</tr>
</tbody>
</table>
Figure 3: Resistance of Clinical Gram-negative bacteria to selected classes of antibiotics

**Key:** AMC = Amoxicillin-clavulanate; FEP = Cefepime; CN = Gentamicin; ATM = Aztreonam; FOX = Cefotaxime; CTX = Cefazidime; CAZ = Ceftazidime; SAM = Ampicillin sulbactam; CIP = Ciprofloxacin; CPD = Cefpodoxime; AZM = Azithromycin

**DISCUSSION**

The rate of antibiotic resistance is increasing at a faster pace than the development of new treatments in clinical practice, which is causing a worldwide health burden (Salam *et al.*, 2023). Extended Spectrum Beta-Lactamase (ESBL) synthesis by bacteria is the predominant method that has rapidly disseminated globally and has been recently observed in clinical settings (Husna *et al.*, 2023).

The study revealed that *K. pneumoniae* accounted for 42.5% of the clinical isolates, making it the most frequently observed, followed by *E. coli* at 27.5%. This finding aligns with the study conducted by Wyres and Holt (2018), identified *K. pneumoniae* as the prevailing organism in clinical environments.

Clinical Gram-negative bacteria were most common in urine (42.5%) and blood samples (22.5%) among the clinical specimens. This finding is in agreement with that of Elbadawi *et al.* (2020), who also discovered that clinically-obtained Gram-negative bacteria were most common in blood (16.4% prevalence) and urine (61.2%) specimens, though there were differences in the percentages or values obtained due to variables such as the amount of samples analyzed and the duration of the research. Isolates of *Klebsiella pneumoniae* were the most common in the urine, which is in line with the findings of Hasan *et al.* (2021), who found that Klebsiella pneumoniae was the leading cause of UTIs in humans.

The prevalence of antibiotic resistance among the clinical isolates in this study was comparable to that reported by Ebrahim-Saraie *et al.* (2019). Out of all the isolates, most of them exhibited considerable resistance to certain classes of antibiotics. Additionally, a significant number of isolates had reduced sensitivity to potentially effective medicines such as amoxicillin clavulanate, cefepime, gentamicin, cefotaxime, and cefpodoxime.
The detection of Gram-negative bacteria in urine, blood, amniotic fluid, tracheal aspirate, sputum, and wound specimens, which have the capability to create ESBL, is consistent with the findings of Gharavi et al. (2021), Ionescu et al. (2022) and Isogami et al. (2023) observed comparable rates of ESBL generation in bacteria in their respective studies, similar to the findings of our study. ESBL-producing Gram-negative bacteria were primarily detected in urine samples (33%) and to a lesser extent in blood samples (23.8%). This could be attributed to the increased quantity of urine and blood samples incorporated in this investigation.

The study found that 52.5% of the bacteria analyzed were producing ESBL. Notably, Acinetobacter baumannii and Haemophilus alvei had the highest prevalence, with 100% of the isolates producing ESBL. This contradicts previous studies that identified Escherichia coli and Klebsiella species as the main ESBL producers (Naeem et al., 2021). It is worth noting that the number of isolates may have influenced these findings. The incidence of hospital-acquired infections caused by strains expressing blaTEM, blaCTX-M, and blaSHV has significantly risen in the past decade (Mohamed et al., 2020). Out of the ESBL phenotypic strains validated by PCR in this investigation, 21 strains showed positive genotypes for at least one of the ESBL genes investigated, accounting for 85.7% of the total. The Klebsiella isolates discovered in this investigation exhibited three forms of extended-spectrum beta-lactamases (ESBLs): SHV-type, CTX-M, and TEM.

The study identified blaTEM12 as the most prevalent gene (35.3%), followed by blaCTX-M10 (29.4%) and blaSHV8 (23.5%). This finding aligns with previous studies that have reported a high prevalence of ESBLs in hospital infections, with a significant proportion of these strains carrying the blaTEM gene (Ibrahim et al., 2021). Nevertheless, this contradicts the findings of Zeynudin et al. (2018) who identified the blaCTX-M gene as the most prevalent kind of extended-spectrum beta-lactamase (ESBL) in clinical settings worldwide. The discrepancy observed in this study, in relation to other studies on the prevalence rate of ESBLs, could be attributed to regional disparities. These disparities may arise from variations in the prevalence of TEM-type ESBLs across different geographic regions, differences in the level of healthcare facilities involved, variations in the type and quantity of antibiotics consumed, and disparities in the time period during which the isolates were collected (Mirkalantari et al., 2020).

**Conclusion**

ESBLs are regarded as one of the most significant mechanisms of antibiotic resistance. The high rates of resistance observed in this study are consistent with the prevalence of resistance genes, which greatly reduce the effectiveness of β-lactam antibiotics. Klebsiella pneumoniae was identified as the prevailing Gram-negative bacterium in this study. Most ESBL generating isolates exhibited resistance to drugs commonly employed for the treatment of various illnesses. This study demonstrated a significant occurrence of Extended-Spectrum Beta-Lactamase (ESBL) synthesis among Gram-negative bacteria found in clinical settings. This finding is supported by the identification of ESBL production in 21 out of 40 isolates, constituting 52.5% of the total clinical isolates. The prevalence of ESBL producers was highest in urine and blood samples. Beta-lactamase genes were absent in three of the ESBL-producing organisms, while AmpC genes were only found in isolates from urine and wound samples. This work presents additional evidence of the worldwide spread of blaTEM, blaCTX-M, and blaSHV genes, highlighting the importance of implementing effective epidemiological surveillance. The identification of ESBL production is crucial in both hospital and community isolates. Implementing effective infection-control measures and utilizing barriers are crucial considerations for limiting the transmission and occurrence of ESBL-producing bacteria. At the institutional level, it is necessary to adopt rapid diagnostic techniques for the effective monitoring and treatment of these strains in hospitals. Additionally, policies should be put in
place to restrict the indiscriminate use of broad-spectrum antibiotics, such as third- and fourth-generation cephalosporins and quinolones.

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