Phenotypic and Molecular Characterization of CTX-M Type B-Lactamases in Gram Negative Bacterial Strains Isolated from Hospitals, Lahore, Pakistan

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Abstract: One of the principal mechanisms that contribute resistance to antibiotics is the production of extended spectrum beta lactamase (ESBL) in Gram negative bacteria. In the present study, molecular methods were used to evaluate the prevalence of the extended-spectrum beta-lactamase (ESBL)-encoding CTX-M gene among Gram negative bacterial strains. In total, 148 clinical samples were collected from different tertiary care hospitals of Lahore, Pakistan. Disc synergy diffusion method was used to detect the presence of ESBL production. Moreover, antibiotic resistance patterns and molecular detection of \(\text{bla}_{\text{CTX-M}}\) ESBLs, were also studied. The pathogens isolated from the 148 samples included \(\text{Escherichia coli}\) (43%) followed by \(\text{Klebsiella}\) sp. (28%), \(\text{Proteus}\) sp. (18%) and \(\text{Pseudomonas}\) sp. (11%). In all 148 strains, 95 (64%) were ESBL producers while 53 (36%) were non ESBL producers. The strains which were phenotypically ESBL producers, \(\text{bla}_{\text{CTX-M}}\) were found in 46% \(\text{E. coli}\) strains, while 50% \(\text{Klebsiella}\) sp. were harboring the gene. A high resistance rate was observed against cephalexin 63% sparaxin 61%). Lower resistance was observed against meropenem among all isolated bacterial strains. Genotypic detection of \(\text{bla}_{\text{CTX-M}}\) genes by PCR revealed 46% of \(\text{E. coli}\) and 50% of \(\text{Klebsiella}\) strains harbored \(\text{bla}_{\text{CTX-M}}\) gene. The present study showed that ESBLs producers were resistant to commonly used antibiotics. Similarly, \(\text{bla}_{\text{CTX-M}}\) ESBL production is more prevalent in our clinical isolates.

Key words: antibiotic resistance, ESBL, CTX-M, PCR, Pakistan

1 Introduction

Bacterial resistance to the beta-lactams such as penicillins, cephalexins and carbapenems is due to the Beta-lactamase enzymes and is the major cause of resistance. The use of extended-spectrum beta-lactam antibiotics for treatment of serious Gram-negative infections has been very common. This has led to the emergence of bacterial resistance mediated by the production of extended-spectrum beta-lactamases (ESBLs). These enzymes are capable of hydrolyzing beta-lactam antibiotics such as penicillins, cephalexins or cephalosporins along with a monobactam (aztreonam). However, they are inhibited by inhibitors such as clavulanic acid, sulbactam, and tazobactam\(^1\).

Mutation is a common phenomenon in the genes for the narrow-spectrum beta-lactamases (TEM-1, TEM-2, or SHV-1) that are mostly encoded by plasmids. These plasmids have the capability to be transferred readily between bacterial species and are most commonly produced by Escherichia coli, Klebsiella and other members of enterobacteraceae. ESBLs enzymes are classified into several main groups like, sulphydryl variable (SHV), temeirena (TEM) and cefotaximases (CTX-M)\(^1\)–\(^3\). Among ESBLs, CTX-M types are more effective against cefotaxime and ceftriaxone rather than ceftazidime\(^3\) and are reported to have emerged as new forms of ESBL. CTX-M type ESBLs producing strains have been shown in numerous reports to

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Accepted February 9, 2022 (received for review January 27, 2022)
Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online
http://www.jstage.jst.go.jp/browse/jos/ http://mc.manuscriptcentral.com/jjocs
be involved in serious infections of hospitalized and non-hospitalized patients. The frequency of CTX-M type group of enzymes has been reported to be increasing, especially in E. coli. Moreover, emergence of ESBLs of the CTX-M type has emerged in many countries around the world during the past decade. In Pakistan, very limited data regarding the prevalence of genes responsible for beta-lactam resistance have been documented.

The present study was designed to determine the prevalence of ESBL genes in our geographical region. Significant proportion of laboratories in Pakistan does not perform tests to detect ESBL producers. Therefore, this issue is of particular concern as it poses a great challenge due to growing proportion of ESBL producing Enterobacteriaceae worldwide. Double-disc synergy test with expanded-spectrum cephalosporins and ticarcillin-clavulanic acid discs is the primary phenotypic test used for the detection of ESBLs. However, other alternative strategies have emerged over the last 20 years that are replacing or complementing traditional phenotypic methods. Among these, the most widely used techniques are standard PCR, gene sequencing and nanotechnology. The aim of the present study was to find the prevalence of ESBL producing and drug resistance in Gram negative bacterial isolates phenotypically and to detect blaCTX-M gene in such organisms at molecular level.

2 Materials and Methods
2.1 Bacterial isolates
One hundred and fifty clinical samples (pus, urine, blood) were collected from Sir Ganga Ram Hospital, Services Hospital, Sheikh Zayed Hospital and Jinnah Hospital, Lahore during the period of June 2018 to January 2019. Bacterial isolates were identified by conventional microbiological methods based on colony characteristics on MacConkey agar plates and biochemical characteristics of the organisms shown on different media and tests. All isolated strains were stored at -70°C for further usage in the tests.

2.2 Antibiotic Susceptibility Testing and ESBL identification
Antibiotic sensitivity was tested by Kirby Bauer disk diffusion method according to the Clinical laboratory Standards Institute (CLSI) guidelines. The antibiotic disks used include imipenem (10 µg), meropenem (10 µg), ceftazidime (30 µg), cefepime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg). The results were interpreted by measuring inhibition zone diameters after overnight incubation at 37°C. The measured inhibition zone diameter was compared according to the interpretive criteria of CLSI guidelines.

2.3 Screening test for ESBLs
2.3.1 Phenotypic confirmatory test
The production of ESBL production in all bacterial isolates was determined by double disk synergy test (DDST) as illustrated elsewhere. Synergy was determined between a third-generation cephalosporin antibiotic disk ceftazidime (30 µg) alone and in combination with ceftazidime + clavulanic acid disk (30 µg) in this test. Both the disks were placed at center, approximately 25 mm apart, on a Mueller-Hinton agar plate with bacterial culture. Difference in the diameter of zones of inhibition with and without clavulanic acid was measured after 24 hrs incubation at 37°C.

The test strains were considered to produce ESBL if the diameter of inhibition zone around the ceftazidime + clavulanic acid combination disk was ≥ 5 mm versus the zone size of ceftazidime disk alone. Results were recorded in relation to the CLSI guidelines.

2.3.2 Molecular characterization
The presence of blaCTX-M was determined in 98 ESBL-positive isolates by using polymerase chain reaction (PCR) with previously published primers and methods. The PCR was done to identify blaCTX-M gene, the sequence of primers were used 5’-GACGATGTCACTGGCTGAGC3’ as a forward primer and 5’-AGCCGCCGACGCTAATACAG3’ as a reverse primer. Template DNA was isolated from ESBL producing Gram negative bacterial strains by standard alkaline lysis method. The cycling conditions was carried out in a reaction mixture volume of 50 µl. Initial denaturation was done at 94°C for 5 minutes followed by annealing step at 48°C followed by 72°C for 1 minute each and final extension at 72°C for 10 minutes for 35 reaction cycles. Amplified product (499 bp fragment) was resolved by gel electrophoresis on 1% agarose gel.

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>Total Number</th>
<th>E. coli</th>
<th>Klebsiella sp.</th>
<th>Proteus sp.</th>
<th>Pseudomonas sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N=63</td>
<td>N=42</td>
<td>N=27</td>
<td>N=16</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>148</td>
<td>(43%)</td>
<td>(28%)</td>
<td>(18%)</td>
<td>(11%)</td>
</tr>
<tr>
<td>Pus</td>
<td>33</td>
<td>14</td>
<td>9</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Blood</td>
<td>33</td>
<td>8</td>
<td>18</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1: Bacterial strains isolated from different clinical specimens.
3 Results

In this study, antibiotic susceptibility profile of 148 Gram negative strains against third generation cephalosporins were obtained. These Gram-negative strains were isolated from urine (56%), pus (22%) and blood (22%) samples (Table 1). The most commonly isolated bacteria were *Escherichia coli* (43%) followed by *Klebsiella* spp. (28%), *Proteus* spp. (18%) and *Pseudomonas* spp. (11%) respectively (Table 1).

Most of bacterial strains isolated were highly resistant against cephalosporins (cefopodoxime 67%, cefoperazone 73%, cephalexin 63% and sparaxin 61%). Lower resistance was observed against meropenem among all isolated bacterial strains. However, high resistance was also observed against tazobactam (69%) as shown in Figs. 1a and 1b.

In total, among 148 strains, 90 (61%) were ESBL producers while 58 (39%) were non-ESBL producers. The frequency of ESBL producers found among Gram negative bacterial strains was shown in Table 2. The high prevalence of ESBL producers was found in urine (49%), pus (15%) and blood (22%) samples, respectively (Table 3).

Genotypic detection of bla\textsubscript{CTX-M} genes in ESBL producing *E. coli* (n = 63) and *Klebsiella* (n = 42) strains was further characterized by PCR. Among the *E. coli* strains, 29 (46%) while 21 (50%) *Klebsiella* spp. were found harboring bla\textsubscript{CTX-M} gene. Figure 2 showed the bla\textsubscript{CTX-M} gene amplification in 7 *E. coli* isolates. Highest number of bacterial strains (100%) that were isolated from pus were found harboring bla\textsubscript{CTX-M} gene followed by urine samples (Table 3).

4 Discussion

Gram negative bacterial resistance to beta-lactam antibiotics has been on rise worldwide\cite{16}. The objective of this study was to determine the prevalence of ESBL producing and presence of CTX-M genes among these ESBL producing Gram negative bacterial isolates. In the present study, ESBL production was detected by DDST method. High prevalence of ESBL producing *E. coli* and *K. pneumoniae*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Number</th>
<th>ESBL(+)</th>
<th>bla\textsubscript{CTX-M}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>56</td>
<td>49</td>
<td>0 (61%)</td>
</tr>
<tr>
<td>Pus</td>
<td>23</td>
<td>15</td>
<td>15 (100%)</td>
</tr>
<tr>
<td>Blood</td>
<td>26</td>
<td>12</td>
<td>05 (42%)</td>
</tr>
<tr>
<td>Total</td>
<td>105</td>
<td>75</td>
<td>50 (67%)</td>
</tr>
</tbody>
</table>

ESBL: Extended-spectrum beta-lactamase. CTX-M: cefotaximases

Table 2  Phenotypic screening and prevalence of ESBL bacterial isolates.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Number of isolates screened</th>
<th>ESBL producers (%)</th>
<th>ESBL Non producers (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>63</td>
<td>45 (71)</td>
<td>18 (29)</td>
</tr>
<tr>
<td><em>Klebsiella</em> sp.</td>
<td>42</td>
<td>25 (60)</td>
<td>17 (40)</td>
</tr>
<tr>
<td><em>Proteus</em> sp.</td>
<td>27</td>
<td>10 (37)</td>
<td>17 (63)</td>
</tr>
<tr>
<td><em>Pseudomonas</em> sp.</td>
<td>16</td>
<td>10 (63)</td>
<td>6 (37)</td>
</tr>
<tr>
<td>Total</td>
<td>148</td>
<td>90 (61)</td>
<td>58 (39)</td>
</tr>
</tbody>
</table>

ESBL: Extended-spectrum beta-lactamase. CTX-M: cefotaximases

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was observed (71% and 60%, respectively) in this study. A number of previous reports have shown 46.51% E. coli and 44.44% K. pneumoniae isolates as ESBL producers\(^7\). Similarly high prevalence of ESBL producers among Gram negative bacteria have been reported in studies from other geographical regions\(^1\). In Pakistan, the prevalence of the ESBLs producing E. coli\(^{2,10,18,19}\) was reported\(^2\). Similarly prevalence of the ESBLs producing E. coli isolates was reported as 56.9% in another study from Pakistan\(^19\). A 40% ESBL production rate was reported in several studies from Pakistan in 2005 while this rate has increased to 43%, 58.7% respectively in 2009 and 64% in 2011 as reported in other studies\(^1,18\) in 2011. The high occurrence of ESBL producing E. coli is alarming and may be the result of the overuse of third generation cephalosporins and monobactams\(^19\). In Pakistan, the differences in prevalence and distribution of ESBLs may be due to different geographical distributions and other contributing factors like overuse of antibiotics in hospital settings and poor infection control policies. The results of the study warrant use of improved detection methods of ESBL. It is now need of time that these improved methods be incorporated into routine laboratory procedures.

It has been observed that high resistance among ESBL-producing E. coli to first line antimicrobial therapy such as cephalosporins is common. These findings have also been reported in developing countries\(^2,9,10,18,19\) as well as in developed countries\(^5,8\). The significantly high rates of resistance to commonly used oral antimicrobials reported worldwide have rendered these agents not effective in hospitals for treatment of infections caused by ESBL-producing bacteria\(^17–19\). In the study, ESBL-producing isolates also exhibited high resistance against non-β-lactamase antimicrobials agents including fluoroquinolones, aminoglycosides. Higher resistance observed in non-β-lactamase antimicrobials may be due to the presence of resistance genes encoded on plasmids. The plasmids are usually mobile and are easily transmissible as resistance gene elements from one organism to another\(^4\). In this study, E. coli isolates were found more susceptible to Meropenem than cephalosporins. While cefotaxime and ceftriaxone are most commonly used for treating nosocomial and community acquired infections in our hospitals, results of this study suggested ESBL-producing strains have become strongly resistant to cephalosporins and fluoroquinolones in our hospital settings. Nevertheless, meropenem remains drug of choice that could still be prescribed to treat infections caused by E. coli. A high prevalence of Gram negative strains (74%) harboring the bla\(_{CTX-M}\) gene was observed as examined by amplifying bla\(_{CTX-M}\) gene by PCR. The results of this study is consistent with some surveillance studies conducted in South America, Asia and Europe showing high rates of CTX-M enzymes among ESBL-producing E. coli and K. pneumoniae isolates. While in Canada lower rate was observed\(^7\).

5 Conclusion

In conclusion, significant differences in susceptibility to various antibiotics among CTX-M-producing Gram negative strains have been observed in this study. Similarly, a high prevalence of bla\(_{CTX-M}\) type genes was observed in ESBL producing E. coli and Klebsiella strains. Isolates had high resistance towards cephalosporins. Strict measures like antibiotic control policies together with the implementation of infection control policy may be adopted in our health care settings to address the limited available treatment options. Moreover, there is dire need to develop effective phenotypic and molecular diagnostic tools for routine detection of β-lactamases genes at laboratory level.

Acknowledgements

The authors like to thank Director, INMOL for collection of data and use of research facilities.

Contribution of Authors

IL, FR conceived and designed the study, MS collected samples and performed experimental work, IL, MU, MF, SE and AB analyzed the data and provided technical expertise. IL and FR wrote the manuscript. All authors read and approved the manuscript for publication.

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