Phenotypic and Molecular Characterization of Plasmid-Encoded Extended Spectrum Beta-Lactamases Produced by Escherichia coli and Klebsiella spp from Lahore, Pakistan

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Received: 14 May 2015
Revised accepted: 8 August 2015

Abstract

Purpose: To investigate the distribution of plasmid-encoded extended spectrum beta-lactamases (ESBLs) in Lahore, Pakistan using different phenotypic and molecular methods.

Methods: Escherichia coli and Klebsiella spp were obtained over a period of nineteen months (June 2007 to December 2008). Both were tested by the double disk synergy test, combined disk test and Epsiometer-test (E-test) to evaluate their ability to detect ESBLs. The genotypes of ESBLs were analyzed by monoplex polymerase chain reaction (PCR), multiplex PCR, DNA sequencing and isoelectric focusing.

Results: 662 E. coli and 153 Klebsiella spp were analyzed. Among these isolates, 39.3 % E. coli and 26.1 % Klebsiella spp were positive for extended spectrum beta-lactamases (ESBLs).71.9 % E. coli and 79.6 % Klebsiella spp showed minimum inhibitory concentration (MIC) in the range > 32/0.064 = 500 μl/mL for cetatzidime/cetatzidime + clavulanic acid, while 66.5 % E. coli and 69.1 % Klebsiella spp revealed MIC in the range of > 16/0.016 = 1000 μl/mL for cefotaxime/cefotaxime + clavulanic acid. Antibiotic susceptibility testing revealed that imipemem, meropenem and tazocine were the most effective in the management of such infections. The most frequent genotype of ESBL was OXA (19.2 %) for E. coli and SHV (92.5 %) for Klebsiella spp. The highest genotypic combination found was the combination of TEM/OXA (44.2 %) for E. coli.

Conclusion: The resistance of E. coli and Klebsiella spp-producing ESBLs in Pakistan is a serious issue, and TEM, OXA and SHV type ESBL were the most common genotypes. Some isolates produced two or three genotypes at a time. Multiplex PCR of ESBL may help in early detection as well as phenotypic antibiotic therapy of these infections.

Keywords: Beta-lactamases, Escherichia coli, Klebsiella spp, Antibiotic susceptibility, Plasmid-encoded, Structural genes, Imipemem, Meropenem, Tazocine

INTRODUCTION

Antimicrobial resistance is a very serious issue all over the world. Pathogenic and opportunistic bacteria, which become highly resistant to drugs are causing untreatable infections [1]. Such bacteria are frequently reported as multidrug resistant bacteria (MDR) and some of the MDRs produce ESBL. The incidence and type of ESBL producer strains varies according to different geographical locations [2,3].
Plasmid-mediated extended-spectrum beta-lactamase (ESBL) confers resistance to cephalosporin group, such as ceftazidime, ceftriaxone, and others. These enzymes occur in genera of the family Enterobacteriaceae for example Citrobacter, Enterobacter but predominantly present in E. coli and Klebsiella species [4]. The successful spread of ESBLs can be attributed to the fact that genes encoding for ESBLs are often located on self-transmissible or mobilizable broad range plasmids [5]. Change in the type of ESBL gene determines the activity of enzyme, for example if organisms producing TEM and SHV type ESBLs, apparent in vitro sensitivity to cefepime and to tazobactum is changed [6]. These drugs show an inoculum effect, with diminished susceptibility as the size of the inoculum was increased from 10^5 to 10^7 organisms. Strains having CTX-M-type and OXA-type ESBL are resistant to cefepime despite the use of standard inoculums [7].

Beta-lactams are important antibiotics in this part of world. Only few studies analyzing the extent of beta-lactam resistance based on their phenotypic features have been conducted. Very little is known about the molecular genotyping of extended spectrum beta-lactamases. The main objective of this work, therefore, was to determine beta-lactam resistant phenotypes and genotypes of E. coli and K. pneumoniae isolates from Lahore, Pakistan.

**EXPERIMENTAL**

**Bacterial isolation and identification**

A total of 1018 consecutive and non-duplicate lactose fermenting Enterobacteriaceae members were collected from Citi lab and Research Center during June 2007 to December 2008. All clinical specimens were processed according to standard operating procedures [8]. Lactose fermenting Gram-negative bacteria were characterized by colony morphology and biochemical tests. Confirmation of identification was performed by using API 20E (BioMerieux). Data were reported according to guidelines of Heath protection agency [9].

**Phenotypic detections tests**

Phenotypic test includes, double disk synergism augmentin (amoxicillin-clavulanate) was used to read the synergism with ceftriaxone (30 μg), ceftazidime (30 μg), aztreonam (30 μg), cefotaxime (30 μg). Combination disc (CD) test was performed using ceftazidime/ceftazidime+ clavulanic acid and cefotaxime/cefotaxime+ clavulanic acid. Combination disk test was considered positive when there was 5 mm increase in zone of inhibition as compared to non-combination disc. E-test was performed as final phenotypic confirmatory test. Detection of ESBL was determined based on ratio between MIC of ceftazidime and cefotaxime alone or in combination with clavulanic acid using E-test strips.

**Confirmation by using ribotyping**

Isolates were also confirmed using 16SrRNA sequences. The PCR products were subsequently sequenced by Macrogen Bioengineering Co., Ltd. The nucleotide sequences were analyzed using BLAST software, available from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Phylogenetic analysis was performed using software seaview. Confirmed isolates were stored in tryplycase soy broth containing 20 % glycerol at -80 °C until use.

**Antibiotics susceptibility testing**

The antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-la-Coquette, France). Multiple antibiotic resistances (MAR) of ESBL producing isolates according to gender and age groups of patients were calculated. The panel used in this study consists of 21 antibiotics that belong to three major groups of antibiotics according to mechanism of action. Such as antibiotic disks containing amikacin 30 μg, ampicillin 10 μg , amoxicillin clavulanic acid (20 μg/10 μg) 30 μg, carbcincilin 100 μg, cephradine 30 μg, cefapazone 75 μg, ciprofloxacin 5 μg, gentamicin 10 μg, nitrofurantoin 300 μg, impenem 10 μg, meropenem 10 μg, norfloxacin 10 μg, cotrimoxazole 25 μg, cefizoxime 30 μg, cefotaxime 30 μg, cefoxitin 30 μg, cetalazidine 30 μg, ceftriaxone 30 μg, cefixime 5 μg, aztreonam 10 μg, and pipracilin-tazobactam 100/10 μg were tested.

**Genotyping using PCR and sequence data analysis**

Colon PCR was performed following Jemima [10]. To differentiate blaSHV, blaTEM, blaOXA, the published primers were used [11]. Initially, singleplex PCR was optimized and then multiplex PCR assay of three-primer set was optimized (OXA, TEM and SHV genes) and performed. ATCC 25922 E. coli (SHV-negative), ATCC 700603 K. pneumoniae (SHV-positive), ATCC
35218 E. coli (TEM-positive) and a strain of E. coli (OXA-positive) were used as control strains for PCR. Multiplex PCR was developed using primer set (OXA, TEM and SHV) in single reaction with maximum 25 µL volume of PCR mixture. The PCR products were purified using Qiagen kit Agarose Gel DNA Purification Kit Version 2.0. Subsequently, the amplicons were sequenced by Macrogen Bioengineering Co., Ltd. The nucleotide and deduced protein sequences were compared to those available in GenBank.

Isoelectric focusing (IEF)
Beta-lactamase was isolated using Massidda protocol [12]. Isoelectric focusing was performed using 2D gel electrophoresis (BioRad) following manufacturer instructions. Spot editing, scanning, quantification and evaluation of isoelectric points (pI) was calculated by ImageMaster 2D Elite v3.1 software (Amersham Biosciences).

RESULTS

Bacterial isolates
Out of 1018 Enterobacteriaceae members, 662 E. coli, 153 Klebsiella spp, 122 Enterobacter and 81 Citrobacter were isolated. Clinical samples positive for ESBL were as: urine (53 %), blood (7.3 %), wound swabs (20.6 %), Foley catheter tip (10.3 %) and others (8.6 %). Total 300 bacterial strains were positive for beta-lactamases, 39.2 % E. coli and 26.10 % Klebsiella species were found to express ESBLs. Regarding gender classification in the case of beta-lactamase producing E. coli, females (60 %) had a higher incidence as compared to males (40 %). Similarly, in case of Klebsiella, more females (55 %) than males (45 %) were affected. Comparison of hospitalized and community acquired infections revealed that in both cases > 55 % E. coli caused infection following Klebsiella > 45 %. Here data of E. coli and Klebsiella was arranged according to source of specimens. However, in the case of pus swabs, males were more infected by Klebsiella (71 %) than E. coli (69 %). In blood samples, E. coli showed higher infection rates (60 %) as compared to Klebsiella (40 %) for male patients. Frequency of infection of E. coli from Foley tip was less in males (29 %) than females (71 %). In Klebsiella, Foley tip infections revealed more for females (62 %) than males (38 %). Other specimens had also high infection rate in females as compared to males.

Phenotypic characteristics
The isolates were further selected based on phenotypic detection test from hospitalized and community patients following Clinical and Laboratory Standard Institute (CLSI) guidelines [8]. Ceftazidime had high rate of synergism (93.4, 95 %) followed by cefotaxime (88.9, 88.7 %) aztreonam (86.2, 83.5 %) and ceftriaxone (84.5, 83.1 %). In Klebsiella spp highest synergism was found with ceftazidime (88.4, 89.1 %) followed by aztreonam (88.6, 86.2 %), cefotaxime (86.7, 89.6 %) and ceftriaxone (84.7, 88.5 %). Cephalosporin antibiotic ceftazidime, cefotaxime alone and combination disc were selected for further confirmation. E. coli was 99.2 % resistant to ceftazidime but was found 98 % sensitive, 1.34 % resistant and 0.74 % intermediate to ceftazidime-clavulanic acid (disc). E. coli was more resistant to cefotaxime (99.5 %) and less resistant to cefotaxime+clavulanic acid (disc) (3.84 %).

Cefotaxime+clavulanic acid (disc) increased E. coli sensitivity to 96.12 %. Klebsiella spp was 99.5 % and 99.4 % resistance to ceftazidime and cefotaxime, respectively. Combination discs of ceftazidime+clavulanic acid and cefotaxime+clavulanic acid altered resistance against ESBL to 1.36 and 2.12 %, respectively. It was observed that 2.8 % E. coli and 1.4 % Klebsiella spp had non-determinable > 32/> 4 (ND), while a majority of ESBL E. coli (71.9 %) and Klebsiella (79.6 %) groups fell in the range of MIC > 32/0.064 = 500 µl/mL for ceftazidime/ceftazidime+clavulanic. For cefotaxime/ceftotaxime+clavulanic (CT/CTL), maximum ESBL E.coli (66.5 %) and Klebsiella (69.1 %) isolates were in the MIC range of > 16/0.016 = 1000 µl/mL (Table 1).

Susceptibility of isolates
This panel is designed according to the CLSI guidelines [8]. This antibiotic panel is generally used in our hospitals and diagnostics labs. Out of 260 E. coli isolates > 97 % were resistant to ampicillin, carbenicillin, cephradine, cefuroxime, ceftriaxone and ceftazidime. E. coli isolates were > 80 % and > 60 % resistant to cefoperazone and cefotaxime respectively. E. coli isolates were 50 % resistant to amoxyccilin/clavulanic acid.

Out of 40 Klebsiella isolates, 100 % were resistant to carbenicillin, cephradine, cefuroxime and ceftriaxone. Klebsiella isolates > 90 %
Table 1: E-test with cetzidime/cetzidime+clavulanic acid (TZ/TZL) and cefotaxime/cefotaxime+clavulanic acid (CT/CTL) MIC ratio

<table>
<thead>
<tr>
<th>Cetazidime/ cetazidime + clavulanic acid (TZ/TZL) MIC(µl/ml)</th>
<th>Cefotaxime/ Cefotaxime + clavulanic acid (CT/CTL) MIC(µl/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MIC Ratio</strong></td>
<td><strong>MIC Ratio</strong></td>
</tr>
<tr>
<td>&gt;32/&gt;4 (ND)</td>
<td>&gt;16/&gt;1 (ND)</td>
</tr>
<tr>
<td>&gt;32/0.064=5</td>
<td>&gt;16/0.016=1</td>
</tr>
<tr>
<td>&gt;32/0.125=2</td>
<td>12/0.023=52</td>
</tr>
<tr>
<td>24/0.19=126</td>
<td>3/0.023=130</td>
</tr>
<tr>
<td>16/0.38=4</td>
<td>8/0.125=6</td>
</tr>
<tr>
<td>4/0.25=16</td>
<td>4/0.094=4</td>
</tr>
</tbody>
</table>

- **E. coli**
  - Cetazidime: 2.8, 71.9, 14.6, 7.4, 1.3, 2.0
  - Cefotaxime: 5.1, 66.5, 17.6, 4.5, 2.8, 3.5

- **Klebsiella spp**
  - Cetazidime: 1.4, 79.6, 9.3, 4.5, 5.2, 0.0
  - Cefotaxime: 1.5, 69.1, 14.8, 8.1, 5.3, 1.2

Resistant to ceftizoxime whereas > 80% were resistant to cefoperazone. > 60% resistance and 20% intermediate sensitivity to amoxycillin/ clavulanic acid were seen in *Klebsiella* isolates. *E. coli* and *Klebsiella* isolates showed highest susceptibility to imipenem followed by meropenem and tazocine. There was very rare resistance found to meropenem and tazocine (< 2%), but no resistance was found to imipenem in both types of isolates. Hence, the most effective antibiotics that can be used as a therapy of choice in both types of isolates were imipenem followed by meropenem and tazocine. Protein inhibiting drugs, gentamycin and amikacin were also used. *Klebsiella* isolates > 80% and *E. coli* isolates > 60% were resistant to gentamycin. On the other hand amikacin responded well and showed highest activity for *E. coli* and *Klebsiella* spp. In nucleic acid inhibiting drugs *E. coli* showed > 80% resistance to ciprofloxacin and trimethoprim/sulfamethoxazole, while > 90% resistance to nitrofurantoin and norfloxacin. *Klebsiella* isolates had > 70% resistance to ciprofloxacin but > 90% resistance to trimethoprim/sulfamethoxazole, nitrofurantoin, and norfloxacin.

**Phylogenetic data**

Phylogenetic relation was developed using neighbor joining method (NJ method). The tree length (sum of branch lengths) was 0.01111 in the case of *E. coli* (Figure 1) and 0.02171 in *Klebsiella* spp (Figure 2); branch lengths are proportional to the number of substitutions per site. Comparison of two bacterial groups was done using Parsimony analysis bootstrap 100 replicates along with control strains. K-TJW and K-ANT used as control *Klebsiella* spp while K12 that is used as control *E. coli*. Sequences were submitted to GenBank nucleotide database under accession numbers GU594294-GU594311.
Molecular phylogeny of the \( \text{bla}_{\text{SHV}} \), \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{OXA}} \) beta-lactamase genes

Distributions of TEM, OXA and SHV in bacterial isolates were observed (Table 2). Of the 260 clinical isolates of \( E. \) coli suspected to be ESBL producers 13.2 %, 19.2 % and 7.6 % demonstrated TEM, OXA or SHV-specific PCR products respectively. Combination of ESBL showed 44.2 %, 3.8 %, 1.5 %, 1.9 % of TEM/OXA, TEM/SHV, OXA/SHV, TEM/SHV/OXA-specific products respectively. Sequence determination of PCR products obtained from \( E. \) coli and \( K. \) lebsiella spp confirmed the identity of the genes (Figure 3). Of the 40 clinical isolates of \( K. \) lebsiella spp 92.5 % demonstrated SHV-specific products, 5 % TEM/OXA and 2.5 % TEM/SHV (Table 2). The nucleotide sequences reported in this paper have been submitted to the GenBank nucleotide database under accession numbers HM063036-HM063040 = SHV, HM063041-HM063050 = TEM, HM063051-HM063064 = OXA.

**IEF data**

Amino acids calculation showed that in SHV enzyme most frequent amino acid was Arginine following glutamine with average pl 8.11, whereas in TEM beta-lactamase most frequent amino acid was Glutamine following Arginine with 4.47 pl. In OXA beta-lactamase most frequent amino acid was lysine, glutamine, aspartic acid following other amino acids. Average pl of OXA beta-lactamase calculated was 9.03.

**Table 2**: Distribution of TEM, SHV and OXA in bacterial isolates \((n = 300): \) **Note**: Data in parenthesis indicate percent

<table>
<thead>
<tr>
<th>Bacterial isolate</th>
<th>No</th>
<th>TEM</th>
<th>OXA</th>
<th>SHV</th>
<th>TEM/OXA</th>
<th>TEM/SHV</th>
<th>SHV/OXA</th>
<th>TEM/SHV/OXA</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>( E. ) coli</td>
<td>260</td>
<td>36(13.8)</td>
<td>50(19.2)</td>
<td>20(7.6)</td>
<td>115(44.2)</td>
<td>10(3.8)</td>
<td>4(1.5)</td>
<td>5(1.9)</td>
<td>0(0)</td>
</tr>
<tr>
<td>( K. ) lebsiella spp</td>
<td>40</td>
<td>0(0)</td>
<td>0(0)</td>
<td>37(92.5)</td>
<td>2(5)</td>
<td>1(2.5)</td>
<td>0(0)</td>
<td>0(0)</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

**Figure 3**: Parsimony analysis of OXA, TEM and SHV sequences. Analysis of OXA, TEM and SHV aligned sequences with bootstrap100 replicates, randomize sequence order 5

**DISCUSSION**

This research work was conducted because there was little available information regarding antimicrobial susceptibility and molecular characterization of ESBL genotypes of \( E. \) coli and \( K. \) lebsiella spp in Pakistan. These members of Enterobacteriaceae are well known ESBLs producers and are responsible for nosocomial and community acquired infections [13]. Depending upon the rate of infecting isolates, \( E. \) coli and \( K. \) lebsiella were considered as important research work. There are many reasons for the spread of infections, and these include misuse of...
antibiotics, animals and hospitals cross infection [4]. The prevalence of ESBLs can be different based on geographical areas. In Europe, ESBLs positivity was 1.3 % of _Escherichia coli_ and 18.4 % for _Klebsiella pneumonia_ [14]. Several other reports are available for other areas like Latin America, Asia and also North America [15]. In 2003 about 30 % ESBL producing _E. coli_ were found in Aga Khan University research [12]. ESBLs producers were most commonly isolated from urinary tract infections (UTI) both in hospital and community-acquired infections [16]. In Iran, UTI causing ESBL producers were in high frequency [17]. Similarly in Japan the increased frequency of UTI caused by ESBL producers have been reported [18].

When data were analyzed according to gender and age groups it depicts that females were more infected. This complexity is because of lack of medical facilities in rural areas. High frequency of infected patients were observed either from young or old age group, this might be because of low immunity [13]. Data revealed that in Pakistan multidrug resistance was more common in the age group of 50+ in both males and females [19]. Resistance appeared to be correlated with the old age as observed in another report [20]. In susceptibility profile imipenem, meropenem and tazocine were effective against ESBL isolates. Meropenem was considered as best option for therapy against gram-negative bacilli especially for cephalosporin resistant gram-negative bacilli [21]. The good activity of imipenem, meropenem and tazocine was also previously known [22]. Another work that supports this was conducted at King Fahd Hospital, Saudi Arabia. However, variability with other drugs such as amoxiclar augmentin, cefoperazone, cefotizoxime, gentamycin and ciprofloxacin was also observed [23].

Our data also suggest that majority of cases are positive for ESBLs as earlier reported [24]. Overall, phenotypic detection of clinical ESBL was 39 % in _E. coli_ and 26 % in _Klebsiella spp_. In previous report, 53 % _K. pneumoniae_ and 44 % _E. coli_ were identified as suspected ESBL producer [25]. Comparative study of different phenotypic techniques revealed that double disc synergism (DDS) test was the most economical method of detection.

In current study multiplex PCR was developed for _TEM, OXA_ and _SHV_ genes. This method of diagnosing ESBL is a one-step procedure, which is very significant in diagnostics. There are a number of studies that showed the significance of using multiplex PCR in ESBL. Multiplex PCR for _SHV_ and _CTXM_ was developed in another study [26]. Multiplex PCR for detection of plasmid mediated quinolone resistance ESBL has also been developed [27]. The change in the antibiotic phenotype pattern in different ESBL clearly differentiated the resistance mechanism of beta-alactamase producing isolates. This was solved by PCR, which accurately distinguished between different types of ESBL genes [28]. On sequence similarity analysis, it was found that tested isolates had 96 to 100 % similarity to sequences of ESBL isolates from China. _Klebsiella pneumoniae_ strain B27 (HM063040) _SHV_ beta-lactamase producer had 98 % similar sequence to clinical _Klebsiella pneumoniae_ strain HS94 beta-lactamase [28]. _Escherichia coli_ strain B1 (HM063048) _TEM-beta-lactamase_ was 100 % similar to _Escherichia coli_ strain SA-Y1-39 [29].

**CONCLUSION**

ESBLs are an increasing problem in healthcare facilities, especially in rural areas. Imepemem, meropenem and tazocine remain good choices for treating infections. Addition of a combination disc in routine susceptibility testing gives satisfactory results for ESBLs in less time. _TEM, OXA_ and _SHV_ type ESBLs were the most common genotype in this region.

**ACKNOWLEDGEMENT**

This study was supported a Research Project (2007-2010) funded by University of the Punjab. Part of this work was presented at International Congress on Infectious Diseases, Bangkok, Thailand. June 13 - 16, 2012.

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Trop J Pharm Res, September 2015; 14(9): 1603