Detection and Molecular Typing of Extended Spectrum Beta Lactamases (ESBLs) Producing Gram-Negative Bacteria from Wound Infections

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ABSTRACT: Extended spectrum β-lactamases (ESBLs) producing bacteria, which are mainly Gram-negative, have emerged as a major threat in recent years as they are resistant to most antimicrobials. This study was conducted to investigate the prevalence of ESBL producing Gram-negative bacteria in wound infections and to map their antimicrobial resistance profile. A total of 100 samples were collected and identified from infected wounds. The Gram-negative bacteria were checked for identification as ESBL producers by using double disc diffusion test. The identified ESBL producers were subjected to PCR based typing of ESBLs. Out of 100 collected infected wound samples, 69 Gram-negative bacteria were isolated. The isolated pathogens were: Klebsiella pneumoniae (45%), Escherichia coli (29%), Enterobacter (10%), Proteus spp (9%) and Pseudomonas aeruginosa (7%). Twenty-eight (40%) isolates were detected as ESBL producers. The ESBL producers were subjected to molecular analysis that showed CTX-M (82%) as most prevalent enzyme type responsible for ESBL production. One E. coli and one Proteus isolate was identified as GES and PER type ESBL producer respectively, where as one of the E. coli isolates was found to harbor both CTX-M and GES type ESBLs. TEM and OXA types were not found in any of the isolates. All ESBL producers showed multidrug resistance for eleven antimicrobials. Klebsiella (40%) was identified as the most prevalent ESBL producer followed by E. coli (35%). Most dominant ESBL type identified by PCR was CTX-M and Klebsiella pneumoniae (83.3%) and E. coli (100%) were the dominant CTX-M type ESBL producers.

Key words: antimicrobial resistance, wound infection, extended spectrum beta lactamases (ESBL)

INTRODUCTION

Bacterial wound infections occur everywhere, and like other infections face the challenge of ever evolving antimicrobial resistance in bacteria (Mirza et al., 2006; Hassan et al., 2011; Koczura et al., 2011). Among these mechanisms, the production of β-lactamases is one of the most prominent and prevailing mechanism. Gram-negative bacteria are rapidly increasing their array of extended spectrum beta lactamases (ESBLs). ESBLs are a group of plasmid-borne enzymes with the ability to hydrolyze third generation cephalosporins and monobactams. ESBLs
evolve from TEM and SHV enzymes, mainly due to the structural mutations (Livermore, 1995a&b; Livermore and Hawkey, 2005).

Limited data is available from the developing countries like Pakistan regarding the causative microbes and their mechanisms of acquiring resistance specifically in wound infections. There is no specific report on ESBL producers involved in wound infections in Pakistan, although work has been reported from urinary tract infections (UTI), respiratory infections, ventilator associated pneumonia (VAP), bloodstream infections, gastrointestinal and soft tissue infections (Kusum et al., 2004; Shaikh et al., 2008; Jabalameli et al., 2011). This study was planned to investigate the present status of ESBL production and antimicrobial resistance in Gram-negative bacteria isolated from locally collected samples from wound infections by disc diffusion method and classification of ESBL producing isolates by identification of various genes related to the ESBLs production.

**METHODS**

**Sample Collection**

Hundred (100) samples of infected wounds were collected from different regions of Punjab including Multan, Shujabad, Dera Ghazi Khan and Faisalabad during the period November 2013 to February 2014. The wound sites included infections after surgery, burns, diabetic lesions, abscesses, ear infections and animal bite wounds. The patients represented both sexes and all ages. Tryptic soya broth (Merck, Germany) with 0.8% agar (3 ml per tube) was used to store swabs taken from site of wound. Samples were kept at 4°C till transport to the laboratory.

**Bacterial Isolation**

Wound sample swabs were placed in the incubator overnight at 37°C. The swabs were streaked on nutrient and MacConkey agar (Merck, Germany) plates. Nutrient agar was used to exclude samples with Gram-positive infection. MacConkey agar plates facilitated the isolation and purification of Gram-negative bacteria. Sub-culturing was carried out for isolation of single bacterial colonies.

**Biochemical Identification**

Preliminary biochemical identification of the isolates was based on Triple Sugar Iron (TSI) agar (Merck, Germany) reactions. The TSI slants were prepared according to the manufacturer instructions. A single isolated colony selected from Mac Conkey agar plate was inoculated on TSI agar slant by stab and streak method. After overnight incubation at 37°C, the isolates were identified.

The isolates were further identified by Remel Rap ID ONE system (Thermo Fisher Scientific, Lenexa, USA) specific for Gram-negative bacteria. It was used according to manufacturer’s instruction and results were interpreted using ERIC software (Electronic Rap ID Compendium), (Thermo Fisher Scientific, Lenexa, USA).
Molecular identification by PCR:

Total genomic DNA from bacterial isolates was extracted from overnight grown culture in 3 ml of TSB using chloroform extraction method (Sambrook et al., 1989). The PCR was carried out for the confirmation of isolates using different sets of primers targeting specific genes (Table 1).

Table 1. Primers for PCR based identification of isolates

<table>
<thead>
<tr>
<th>Organism</th>
<th>Primer</th>
<th>Oligonucleotide sequences (5’-3’)</th>
<th>Targeted gene</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>EC-F</td>
<td>ATCACCGTGTTGACGATGTCGC</td>
<td><em>uid</em> A</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td>EC-R</td>
<td>CACCCAGTGCATGTCCTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>KI-F</td>
<td>GACGCAAGCAGAAATCGAAT</td>
<td><em>gnd</em></td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>KI-R</td>
<td>CGTTACGCCGATGGAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PsA-F</td>
<td>GGGGATCTTCGACCTCA</td>
<td><em>alg</em> D GDPmannose</td>
<td>956</td>
</tr>
<tr>
<td></td>
<td>PsA-R</td>
<td>TCCTTAGAGTGCCACCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella sonnet</em></td>
<td>EC-F</td>
<td>ATCACCGTGTTGACGATGTCGC</td>
<td><em>uid</em> A</td>
<td>486</td>
</tr>
<tr>
<td></td>
<td>EC-R</td>
<td>CACCCAGTGCATGTCCTGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Detection of ESBL producing isolates by double disc diffusion method

The isolates were subjected to ESBL detection by double disc diffusion method (Clinical and Laboratory Standards Institute, 2005). Mueller Hinton agar plates were used with the following combination: Ceftazidime (30µg), ceftazidime + clavulanic acid (40µg) and cefotaxime (30µg), cefotaxime + clavulanic acid (40µg). If the zone of inhibition of any of the combination disc was increased by ≥5 mm compared to the disc without clavulanic acid, the particular isolate was considered as ESBL producer.

Molecular typing of ESBL producers

Molecular typing was done on the basis of already reported types of ESBL-coding *bla* genes using different primer sets (Table 2). In addition to total genomic DNA, plasmid DNA was also extracted by miniprep method (Sambrook et al., 2001). PCR condi-
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tions for 35 cycles for amplification of CTX-M genes were as follows: denaturation at 94°C for 1 minute, annealing at 55°C for 40 seconds, extension at 72°C for 1 minute followed by final extension at 72°C for 5 minutes. The PCR conditions were same for GES and PER except the annealing temperature that was 50°C for 45 seconds for GES and 47°C for 30 seconds in case of PER.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaCTX-M</td>
<td>CTX-F</td>
<td>ATGTGCAGYACCAGTAARGTKATGGC</td>
<td>593</td>
</tr>
<tr>
<td></td>
<td>CTX-R</td>
<td>TGGGTRAARTARGTSACCAGAAACGAGGC</td>
<td></td>
</tr>
<tr>
<td>blaOXA-2</td>
<td>OXA-2-F</td>
<td>AAGAAACGCTACTCGCCTGC</td>
<td>478</td>
</tr>
<tr>
<td></td>
<td>OXA-2-R</td>
<td>CCACTCAACCATCCTACCC</td>
<td></td>
</tr>
<tr>
<td>blaPER</td>
<td>PER-F</td>
<td>ATGAATGTCACTCAAAATG</td>
<td>927</td>
</tr>
<tr>
<td></td>
<td>PER-R</td>
<td>TCAATCCGACTCANT</td>
<td></td>
</tr>
<tr>
<td>blaGES</td>
<td>GES-F</td>
<td>ATGCAGTCATTACCAGCAG</td>
<td>864</td>
</tr>
<tr>
<td></td>
<td>GES-R</td>
<td>CTATGGTGCGTGCTACGG</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Oligonucleotide Primers for PCR Based Identification of ESBL Genes

Drug Susceptibility testing

Antimicrobial susceptibility test was performed by disc diffusion method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI 2011). Eleven drugs representing all major groups were used. These included cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), ciprofloxacin (5µg), gentamicin (10µg), nalidixic acid (30µg), cefixime (5µg), colistin (10µg), ticarcillin-clavulanic acid (75 µg), fosfomycin (200 µg) and imipenem (10 µg). The results were interpreted by following CLSI (2011) guidelines.

RESULTS

From 100 samples, 69 Gram-negative bacteria were identified on MacConkey agar plates and by using Remel RapID ONE system. All seven types of wound infections showed the presence of Gram-negative bacteria. These included burns (30), surgical wounds (24), orthopedic wounds (14), abscesses (13), ear infection (10), diabetic wounds (7), and animal bites (2). Twenty isolates of E. coli, 31 of Klebsiella pneumoniae, 6 of Proteus spp, 5 of Pseudomonas aeruginosa and 7 of Enterobacter spp. were identified. Out of sixty nine (69) Gram-negative isolates, twenty eight (28) were found to
be ESBL producers on the basis of disk diffusion method. These included 9 *E. coli*, 12 *K. pneumoniae*, 2 *Enterobacter* spp., 4 *Proteus* spp., and 1 *Pseudomonas aeruginosa*. The distribution of ESBL producing bacteria in terms of various wound types is described in

Table 3. Highest occurrence of ESBL producers was seen in burn infections (25%), followed by surgical wounds and abscesses (21%), ear infections and diabetes wounds (14%), and animal bites (3.5%).

<table>
<thead>
<tr>
<th>Wound Type</th>
<th>Number</th>
<th><em>Escherichia coli</em></th>
<th><em>Klebsiella pneumoniae</em></th>
<th><em>Proteus</em> spp.</th>
<th><em>Pseudomonas aeruginosa</em></th>
<th><em>Enterobacter</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burn</td>
<td>7</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Surgical</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Abscesses</td>
<td>6</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Ear Infection</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetics</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Animal bite</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>% Contribution</td>
<td>28</td>
<td>32.14</td>
<td>42.85</td>
<td>14.28</td>
<td>3.57</td>
<td>7.14</td>
</tr>
</tbody>
</table>

Among 28 ESBL producers, 23 were found to be positive for CTX-M type and one for each of the GES and PER types; no isolate was found positive for SHV, TEM, OXA-2 and OXA-10 types. *K. pneumonia* (42.85%) was the most frequent ESBL producing spp. followed by *E. coli* (32.14%), *Proteus* (14.28%), *P. aeruginosa* (3.57%) and *Enterobacter* spp. (7.14%).

All ESBL producers showed multidrug resistance towards currently used antimicrobials by disc diffusion method. High level of resistance was observed towards imipenem (89%), ticarcilnine, clavulanic acid (100%), ceftazidime (92%), cefotaxime (96%), ciprofloxacin (98%), and gentamicin (99%), but fosfomycin (25%) and colistin (36%) showed better results. The antimicrobial resistance profile is given in Table 4.

**DISCUSSION**

Gram-negative bacteria are one of the main reservoirs of drug resistance genes particularly those related to ESBL production that confers resistance against all beta-lactam antimicrobials. Gram-negative bacteria are also a major cause of acute and chronic wound infections.

In Asia, the percentage of ESBL producers in different types of infections is variably reported; 53% in India (Rodrigues et al., 2004), 26% in Thailand (Kusum et al., 2004), >40% in Japan (Shibata et al., 2006), 16% in South Korea (Ryoo et al., 2005).
China and India represent the largest reservoirs of CTX-M type ESBL genes in the world (Hawkey, 2008). In none of these reports the prevalence of ESBL producing bacteria has been addressed specifically with reference to wound infections. In current study, we found high (40%) proportion of ESBL producers in wound infections.

In a study, the prevalence of ESBL production among Enterobacteriaceae has been reported to be 37.5% in Pakistan (Shah et al., 2004). Another study showed the prevalence of ESBL producers in clinical isolates collected from blood stream infection to be 32.6%. Among these isolates, E. coli was the most prevalent (53.3%) followed by K. pneumoniae (25%) (Mumtaz et al., 2008). But in current study, among ESBL producers, K. pneumonia prevalence was higher (40%) as compared with E. coli (35%). Our results are in accordance with various other global studies which have reported K. pneumoniae as the most prevalent ESBL producers (Sattar et al., 2009; Khan et al., 2010a; Idowu et al., 2011).

Most of the studies conducted in Pakistan are based on phenotypic screening of ESBL producing pathogens but very limited data are available on genetic characterization of ESBL producing bacteria. Studies carried out elsewhere show CTX-M types as the most dominant ESBL types. These are not related to TEM and SHV types. The CTX-M type β-lactamases are replacing SHV and TEM enzymes as the prevalent type of ESBLs (Falagas and Karageorgopoulos, 2009). These CTX-M types preferably hydrolyze cefotaxime but variants have emerged having additional capability of hydrolyzing ceftazidime as well (Poirel et al., 2001a; Bonnet, 2004). Prevalence of CTX-M phylogroup 1 and 9, and SHV and TEM genes encoding ESBLs among E. coli and K. pneumoniae has been stated in some previous

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Escherichia coli</th>
<th>Klebsiella pneumoniae</th>
<th>Proteus</th>
<th>Enterobacter</th>
<th>Pseudomonas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>25(89%)</td>
<td>10(100%)</td>
<td>10(83%)</td>
<td>2(66%)</td>
<td>2(100%)</td>
<td>0(0%)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>7(25%)</td>
<td>3(30%)</td>
<td>2(16.6%)</td>
<td>1(33%)</td>
<td>0(0%)</td>
<td>1(100%)</td>
</tr>
<tr>
<td>Colistine</td>
<td>9(36%)</td>
<td>1(10%)</td>
<td>4(33%)</td>
<td>3(100%)</td>
<td>1(50%)</td>
<td>1(100%)</td>
</tr>
<tr>
<td>Ticarcillin clavulanic acid</td>
<td>28(100%)</td>
<td>10(100%)</td>
<td>12(100%)</td>
<td>3(100%)</td>
<td>2(100%)</td>
<td>1(100%)</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>26(92%)</td>
<td>9(34%)</td>
<td>12(100%)</td>
<td>2(66%)</td>
<td>2(100%)</td>
<td>1(100%)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>27(96%)</td>
<td>9(33%)</td>
<td>12(100%)</td>
<td>3(100%)</td>
<td>2(100%)</td>
<td>1(100%)</td>
</tr>
</tbody>
</table>
The prevalence rate of CTX-M types (57%) ESBL producing bacteria in current study was also higher than that reported from Japan (32%), India (32%), Italy (32%), Thailand (26%), Tanzania (24%), Brazil (17%), South Korea (16%), Eastern Europe (10%), Western Europe (6%), Latin America (6%) and North America (3%) (Kusum et al., 2004; Rodrigues et al., 2004; Shiraki et al., 2004; Ryoo et al., 2005; Jones et al., 2009; Luzzaro et al., 2009; Mshana et al., 2009; Abreu et al., 2011).

The widespread production of CTX-M type ESBL in Enterobacteriaceae strains which are resistant to extended-spectrum cephalosporins is the most remarkable example of rapid and global spread of plasmid mediated antimicrobial resistance in bacteria (Bayraktar, Toksoy, and Bulut, 2010). It seems that the bacterial pathogens have found CTX-M type ESBLs as better tools for survival as compared with SHV and TEM and therefore acquiring them gradually to replace others. However, detailed molecular studies are required to confirm this hypothesis.

The antibiotic resistance profile of ESBL producing Gram-negative bacterial strains against nine antimicrobials representing the major groups of antibiotics was very alarming. High resistance was observed towards imipenem (85%), ticarcilene, clavulanic acid (100%), ceftazidime (92%) and cefotaxime (96%), ciprofloxacin (92%), gentamicin (100%), but fosfomycin (25%) and colisitin (36%) showed better results. Similar results on colisitin resistance (36%) in ESBLs producers has been reported elsewhere (Idowu et al., 2011). Similarly resistance towards imipenem is shown to be 47.8% in one of the reports from Pakistan (Khan et al., 2010b) which is comparatively lower than our study. One study showed 50% resistance against ceftazidime in K. pneumoniae.
while in our study 100% resistance was found against ceftazidime. Moreover, resistance against ciprofloxacin (92%) and gentamicin (100%) was found to be higher as compared to previous reports which showed 78% and 85% resistance towards ciprofloxacin and gentamicin respectively in nosocomial infections (Ramphal and Ambrose, 2006; Umadevi et al., 2011). None of the referred studies addressed wound infections. This may be a factor responsible for higher level of resistance found in this study. It highlights the greater gravity of emergence of MDR and XDR isolates of these common pathogens isolated from wound infections.

In conclusion it can be said that Gram-negative pathogens which dominate wound infections as shown in this study, have become very sophisticated and found tools to neutralize traditional antimicrobials. They are rapidly acquiring various types of ESBLs to strengthen their armory. They pose a great challenge as they are resistant even to third generation cephalosporins. The threat is manifold greater in Pakistan as we have found much higher prevalence of ESBL producing bacteria as compared to reports from other regions. There is a need to characterize ESBLs, especially the CTX-M types, on molecular level to develop better antimicrobial drugs.

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Competing interests

All authors declare that they have no competing interest.

Authors contribution

This manuscript article represents original, unpublished material that is not under editorial consideration elsewhere, in whole or in part, and that each author has participated sufficiently in the intellectual content, analysis of data (if applicable) and writing of the manuscript to take public responsibility for it.

REFERENCES


