RESEARCH ARTICLE

PREVALENCE AND MOLECULAR CHARACTERIZATION OF EXTENDED SPECTRUM BETA-LACTAMASES (ESBLs) PRODUCING ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE

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ABSTRACT

The aim of this study was to determine the prevalence of ESBL-producing Escherichia coli and Klebsiella pneumoniae as well as genes encoding ESBLs. During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. Escherichia coli was the most prevalent 25.58% followed by Klebsiella pneumoniae (13.65%). Out of 296 E. coli isolates and 158 K. pneumoniae isolates, 247 (83.44%) and 128 (81.01%) isolates were ESBL producers, respectively. For testing with getotyping of isolates by Multiplex PCR detection using TEM, SHV, CTX-M genes, among the isolates harbouring single ESBL gene (61.53%), blaSHV, blaTEM and blaCTX-M were present in 42.30%, 19.23% and 11.53% strains of E. coli and K. pneumoniae, respectively. The 22 strains of E. coli and 16 strains of K. Pneumoniae had a single ESBL gene, although TEM and SHV types of ESBL were frequently found in E. coli (12/6) and K. pneumoniae (10/4), respectively. Two or more genes for ESBL were present in 14 (26.92%) of the 52 ESBL typeable isolates, blaTEM + blaSHV being the most common combination (9.61%), followed by blaTEM + blaCTX-M and bla SHV + blaCTX-M (5.76%). One strains of both E. coli and K. pneumoniae harbour 3 genes for ESBL. The majority of strains harbour two or more ESBL genes and the most common phenotypes were TEM, SHV and CTX-M. Identification of the genes is necessary for the surveillance of their transmission in hospitals.

INTRODUCTION

β-Lactam antibiotics are commonly used to treat bacterial infections. The groups of antibiotics in this catagory include penicillins, cephalosporins, carbapenems & monobactams. Increased use of antibiotics, particularly the third generation of cephalosporins, has been associated with the emergence of β-Lactamases mediated bacterial resistance, which subsequently led to the development of ESBL producing bacteria. ESBLs are enzymes that mediate resistance to extended spectrum e.g., third generation cephalosporins as well as monobactams such as aztreonam (CLSI, 2010). These enzymes catalyze the hydrolysis of the β-lactam ring of antibiotic, thereby destroying the antimicrobial activity. ESBLs have been reported worldwide in many different genera of enterobactericeae and Pseudomonas aeruginosa (Friedman et al., 2005). The development of extended spectrum cephalosporins in the early 1980s was regarded as a major addition to our therapeutic armamentarium in the fight against beta-lactamase mediated bacterial resistance.

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The emergence of Escherichia coli and Klebsiella pneumoniae resistant to cefazidime & other cephalosphorins seriously compromised the efficacy of these life saving antibiotics (Perez et al., 2007). The new bacterial beta-lactamases present in these common enteric bacilli (the parent TEM -1 and SHV-1 enzymes) demonstrated unique hydrolytic properties (Shobha et al., 2007). ESBLs are inhibited in vitro by, β-lactamase inhibitors such as clavulanic acid and tazobactam. Some ESBLs are derived from earlier, broad-spectrum, β-lactamases (e.g., the TEM, SHV and OXA enzyme families) and differ from the parent enzyme by a few point mutations, which confer an extended spectrum of activity (Hawkey 2008). Point mutations in the SHV and TEM genes that resulted in single amino acid changes, (Gly 238 ñser, Glu 240-Lys arg 164-ser, arg164- His, Asp 179- Asn & Gul (Asp) 104- Lys) (Perez et al. 2007). More recently another family of ESBLs, the CTX-M types, has emerged and these ESBLs are becoming increasingly common (Hawkey, 2008). The TEM -1 enzyme was first reported from an E.coli isolate in 1965 and is now the commonest beta lactamase found in Enterobactericeae (Onze et al., 1995) and the older TEM is derived from Temoniera, a patient from whom the strain was first isolated in Greece (Turner 2005).
The SHV-1 beta-lactamase is most commonly found in _K. pneumoniae_ and is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species. Among the SHV type of β-lactamases, SHV-5 was found to be responsible for outbreaks of nosocomial infection in several countries. Acquired resistance to beta-lactams is mainly mediated by extended-spectrum beta-lactamases (ESBLs) that confer bacterial resistance to all beta-lactams except carbapenems and cephemycins, which are inhibited by other beta-lactamase inhibitors such as clavulanic acid. A shift in the distribution of different ESBLs has recently occurred in Europe, with a dramatic increase of CTX-M enzymes over TEM and SHV variants (Coque et al. 2008; Livermore and Canton 2007). The continuous pressure exerted by the use of newer expanded-spectrum beta-lactams promoted the development of new TEM and SHV derivatives. There are so many types of ESBLs like TEM, SHV, CTX-M, OXA, AmpC but majority of the ESBLs are derivatives of TEM or SHV enzymes and these enzymes are most commonly found in _E. coli_ and _K. pneumoniae_ (Sharma et al., 2010) and studies showed that new ones are being found every week. The rapid emergence of the ESBL-production among _enterobacteriaceae_ has already had serious clinical implications.

**MATERIALS AND METHODS**

**Bacterial isolates**

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. During this study, 1465 different clinical samples were collected for isolation of _E. coli_ and _Klebsiella pneumoniae_. These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics.

**Antimicrobial susceptibility testing**

Susceptibility of all isolates was determined by the disk diffusion method on Mueller-Hinton agar following the zone size criteria as recommended by the Clinical and Laboratory Standards Institute (CLSI). The antibiotics that were tested included: Amikacin (30 μg/disc), Ampicillin (10 μg/disc), Penicillin (10 μg/disc), Cloxacillin (5 μg/disc), Erythromycin (15 μg/disc), Tetracycline (30 μg/disc), Gentamicin (10 μg/disc), Cotrimoxazole (25 μg/disc), Chloramphenicol (30 μg/disc), and some of the newer generation antibiotics including Cefixime (30 μg/disc), Cefuroxime (30 μg/disc), and Cefotaxime (30 μg/disc). The antibiotic susceptibility tests on the isolates were done according to the guideline set by the Clinical and Laboratory Standards Institute (CLSI, 2010).

**E- strip method**

Tested colonies from overnight culture were suspended with 0.85% of normal saline (NaCl) to a turbidity of 0.5 McFarland’s. A sterile cotton swab was used to produce a uniform layer on a Mueller-Hinton agar plate and the excess moisture was allowed to be absorbed for about 15 min before the E-test strip was applied. The plate was incubated for 16 to 18 h at 37°C and the MIC end points were read where the inhibition ellipses intersected the strip (Bashir et al., 2011).

**Multiplex PCR for detecting TEM, SHV and CTX-M genes**

**DNA extraction from colony was done by alkaline lysis method**

A single colony of each organism was inoculated from MacConkey agar into 5ml of Luria-Bertani broth (LB) and incubated for 20 h at 37°C. Cells from 1.5ml of the overnight culture was harvested by centrifugation at 12,000 rpm for 5 min. 1.5 ml from LB media containing cells was taken appendrof tube, than 100 μl TNE buffer was mixed. The mixture was centrifuged for 1 min at 10000 rpm and supernatant was discarded. Again 100 μl NaOH (50 mM) was added to pellet. After heating at 40°C in water bath for 1 min, 60 μl of IM Tris HCl (PH 6.7) was added. Vortex, centrifuge at 10000 rpm for1 min was done. Then supernatant was used as template (1μl) (Medici et al. 2003).

**DNA amplification in thermal cycler**

PCR analysis for beta lactamase genes of the family TEM, SHV, CTX-M were carried out.

- **Preparation of reaction mixture**

  For PCR amplification, 1 μl of template DNA was added to 50 μl of master mixture containing 4 μl of dNTPs mixture (2.5mM of each), 10X PCR buffer 5 μl (Ex Taq),0.5 μl of _Taq_ polymerase (250 U), 1 μl of each primer stock solution (50pmol/μl), and remaining 38.5 μl volume was fulfilled by nuclease free water.

- **Amplification**

  The prepared PCR tubes with master mixture were placed in the eppendorf off thermal cycler. Amplification was carried out according to the following thermal and cycling condition:

  **For TEM, SHV gene**

  ![Initial Denaturation](image)

  ![Denaturation](image)

  ![Annealing](image)

  ![Extension](image)

  ![Final extension](image)

  35 cycles

  **For CTX-M gene**

  ![Initial Denaturation](image)

  ![Denaturation](image)

  ![Annealing](image)

  ![Extension](image)

  ![Final extension](image)

  30 cycles

The PCR products were analyzed after electrophoresis in 1.0% agarose gel to detect specific amplified product by comparing with standard molecular weight marker. One percent agarose gel was prepared by melting 2.0 gm agarose in 200 ml of diluted TBE Buffer using a microwave woven. The melted agarose was allowed to cool to about 50°C and 20 μl ethidium bromide was mixed and shacked and was poured into gel tray and combs were placed. After solidification of the gel, the comb was removed. During electrophoresis, the gel was placed in a Horizontal electrophoresis apparatus containing TBE buffer and ethidium bromide.
Loading and electrophoresis of the sample

Five µl of amplified PCR product was mixed with 2.0 µl of loading buffer. The mixture was slowly loaded into the well using disposable micropipette tips. Hundred bp molecular weight marker was loaded in one well to determine the size of the amplified PCR products. Electrophoresis was carried out at 100 volts for 35 minutes. The amplified products of the study samples were visualized by Gel-DOC system (Sharma et al., 2010).

RESULTS AND DISCUSSION

In this study, bacteria were isolated from different clinical samples such as urine, blood, sputum, pus/wound swab and cerebrospinal fluid (CSF). The clinical samples were collected from different pathology laboratories of Nagpur City. These samples were screened on different bacteriological media and identified on the basis of their morphological, cultural and biochemical characteristics. During this study, 1465 different clinical samples were tested in which 1255 (85.66%) samples showed growth of bacteria. Out of 1255 clinical samples, 1157 (92.19%) different strains of bacteria were isolated. Some clinical samples showed more than one bacterial colonies. Among the isolated organisms *Escherichia coli* was the most prevalent 25.58% followed by *Klebsiella pneumoniae* (13.65%). Out of 296 *E. coli* isolates and 158 *K. pneumoniae* isolates, 247 (83.44%) and 128 (81.01%) isolates were ESBL producers, respectively. In the present study, antibiotic susceptibility was tested against *E. coli* was found to be highly resistant to Gentamicin (97.29%) followed by Tetracyclin (95.94%), Amikacin (93.91%), Ampicillin (90.54%), Cefepime (87.83%), Ciprofloxacin (87.50%), and Co-trimazol (84.79%) (Wasnik et al., 2013).

*Figure 1* Gentamicin and amikacin showed high resistant towards *E. coli*, the similar studies indicating relative efficacy of gentamicin and amikacin have been reported from Pakistan (Farooqi et al., 2000). High resistance of *E. coli* to antimicrobial agents tested was observed in this study. Amikacin and ampicillin showed 93.91% and 90.54% resistant against *E. coli* respectively. This is similar to what was observed by Aibinu et al. (2004) who reported 100% resistance of their *E. coli* isolates to ampicillin and amikacin. The present studies also agree with other studies (Sasirekha et al., 2010).

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer name</th>
<th>Primer sequence (5′ → 3′)</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>BlaTEM</em></td>
<td>TEM-F</td>
<td>TCGCCOCATACACTTCTCAGAATG</td>
<td>445</td>
<td>Monstein et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>TEM-R</td>
<td>TTGGTTCTGAGTTACCAATGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>BlaSHV</em></td>
<td>SHV-F</td>
<td>TGTTGTGTTGGGGCCA</td>
<td>747</td>
<td>Monstein et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>SHV-R</td>
<td>ATGCCTTATTCGGCCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>BlaCTX-M</em></td>
<td>CTX-F</td>
<td>TGGTTAATAGTACCGAGGCCG</td>
<td>593</td>
<td>Monstein et al. (2005)</td>
</tr>
<tr>
<td></td>
<td>CTX-R</td>
<td>ATGTCAGCAGATTACGTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Primers used for detection and sequencing of resistant genes

*Figure 1. Antibiotic susceptibility testing of E.coli*

*Figure 2. Antibiotic susceptibility testing of K. pneumoniae*
All isolates were highly resistant to ampicillin, which is in agreement to the findings of (Nagoba et al., 2008). Densenclos et al. (1998) reported 53% of their E. coli isolates were resistant cotrimoxazole and 67% to tetracycline. Their finding is in harmony with the report of this study, showing 84.79% and 95.94% resistance to co-trimoxazole and tetracycline respectively. Similarly, 90% isolates were resistant to tetracycline by Maynard et al. (2004) also reported an increase in the tetracycline resistance in human isolates. The antibiotic susceptibility testing showed that Ceftriaxone, Tetracycllin and Ciprofloxacin had the highest resistant of 61.48% 95.94% & 87.5% respectively (Figure 1). This results completely agree with a study done by P. L. Winokur et. al. (2001) which reported that ESBL strains show high level of co-resistance to ciprofloxacin, aminoglycosides, tetracycline, and sulfanethoxazole/Trimethoprim.

It was also similar in Farooqui et al. (2001) who reported a relatively high resistance of 25% to quinolones and Khan and Ahmed (2001) who reported 46% resistance in Pakistan. In this study, out of 296 E. coli isolates, 247 isolates or 83.44% exhibited multiple drug resistance. The similar result was found in many studies multi drug resistance has serious implications for the empiric therapy of infections caused by E. coli and for the possible co-selection of antimicrobial resistance mediated by multi drug resistance plasmids (Sherley et al., 2004). The multidrug resistant E. coli, i.e. isolates resistant to fifteen antibiotics, were observed to be very common in this study. The growing antimicrobial resistance may be due to un rational use of antibiotics and the transfer of resistance genes by transport means including antibiotic resistant plasmids, bacterio-phages, transposons and integrons. Since a plasmid or transposon can carry several resistance indexes, simultaneous resistance to multiple antimicrobial agents may be developed and the result would be MDR organisms. For example, resistance to co-trimoxazole is usually accompanied by resistance to Ampicillin, cephalothin and Tetracycllin. Chloramphenicol, Cephotaxime, amikacin and imipenem can be still prescribed for UTI as their resistance rate still under control. The high level of antibiotic resistance among E. coli isolates may be due to self prescription policy, comparatively cheaper antibiotics intake, lack of dependency on laboratory guidance and in adequate doses of antibiotics intake. In many areas of India antibiotics can be easily available over the counter without prescription of registered medical practitioner. This is the main cause of misuse of antibiotics responsible for the increasing emergences of MDR. Antibiotic resistance is a major clinical problem in treating infections caused by microorganisms. The resistance to the antimicrobials has increased over the years. Resistance rates vary from country to country (Kahan et al., 2006). In Pattukkottai, there is an evidence for increase in antibiotic resistance. In this study, higher percentage of resistance was noted against Gentamicin (98.10%) followed by Ampicillin (87.97%), Amikacin (86.70%), Tetracycllin (86.07%), Ciprofloxacin (81.01%), Meropenem (81.64%), Amoxicillin (72.78%) and Co-trimaxazole (71.51%). (Figure No. 2) The comparative similar result was previously reported in other hospital as well as in other institutions in the various country (Daoud et al., 2006). Amoxicillin resistance (72.78%) observed in the present study was lower than the reports of Sonavane et al. (2008) was found (97.7%). The resistance of Klebsiella spp. to cotrimaxazole (71.51%) was high, compared to most Indian studies (Biswa et al., 2006).

Table 2. Extended spectrum β-lactamase (ESBL) genotypes in E. coli and K. pneumoniae strains

<table>
<thead>
<tr>
<th>Positive by PCR for ESBL genes</th>
<th>E. coli (n = 30)</th>
<th>K. pneumoniae (n = 22)</th>
<th>Total N = 52 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Single ESBL gene</td>
<td>22</td>
<td>16</td>
<td>38 (61.53)</td>
</tr>
<tr>
<td>blaTEM only</td>
<td>12</td>
<td>10</td>
<td>22 (42.30)</td>
</tr>
<tr>
<td>blaSHV only</td>
<td>06</td>
<td>04</td>
<td>10 (19.23)</td>
</tr>
<tr>
<td>blaCTX-M only</td>
<td>04</td>
<td>02</td>
<td>06 (11.53)</td>
</tr>
<tr>
<td>B. Two or more ESBL genes</td>
<td>08</td>
<td>06</td>
<td>14 (26.92)</td>
</tr>
<tr>
<td>blaTEM + blaSHV</td>
<td>03</td>
<td>02</td>
<td>05 (9.61)</td>
</tr>
<tr>
<td>blaTEM + blaCTX-M</td>
<td>01</td>
<td>02</td>
<td>03 (5.76)</td>
</tr>
<tr>
<td>blaSHV + blaCTX-M</td>
<td>02</td>
<td>01</td>
<td>03 (5.76)</td>
</tr>
<tr>
<td>blaTEM + blaSHV + blaCTX-M</td>
<td>01</td>
<td>01</td>
<td>02 (3.84)</td>
</tr>
</tbody>
</table>

Figure 3. Multiplex PCR detection of TEM, SHV, CTX-M in Gram negative bacteria. M : Marker; Lane 1 – 3 : E1 – E3 (E.coli isolate); Lane 4 -6 : K1 – K3 (K. pneumoniae isolate)
Characterization of Multiple Drug Resistant (MDR) strains using molecular method: (Genotyping Resistance Testing)

The characterization of presence of multiple drug resistant (MDR) strains using molecular method can be done by selecting the bacteria on the basis of their highest resistant pattern against the antibiotic tested. Those bacteria having high resistant pattern were selected for the presence of resistant gene. blaTEM, blaSHV and blaCTX-M among the total Enterobacteriaceae in this study were recorded while compare with earlier studies done for the presence of blaTEM, blaSHV and blaCTX-M (Ahmed et al., 2009). The rate of co-existence of two different β-lactamase encoding genes among Enterobacteriaceae in this study was also reported by Ahmed et al. (2009). The dominance of blaCTX-M gene either alone or in association with blaTEM among E. coli isolates was reported previously in Egyptian clinical setting (Fam and El-Damaryaw, 2008). The previous findings demonstrate the increasing tendency of K. pneumoniae and E. coli to accumulate β-lactamase resistance determinants which is higher among K. pneumoniae isolates than E. coli isolates. The previous finding ascertain also the predilection of E. coli to aggregate blaCTX-M and blaTEM genes together as well as the predilection of K. pneumoniae to associate either blaCTX-M together with blaTEM or blaTEM together with blaSHV. Existence of blaSHV among E. coli isolates is seldom encountered. (Figure No. 3)

Genomic DNA isolated from 52 phenotypic confirmed ESBL-producing organisms (E. coli-30 and K. pneumoniae-22) were subjected to PCR using pairs of primers. All isolates was found to be ESBL positive and these isolates could be typed for one or more genes. Among the isolates harbouring single ESBL gene (61.53%), blaSHV, blaTEM and blaCTX-M were present in 42.30%, 19.23% and 11.53% strains of E. coli and K. pneumoniae, respectively. The 22 strains of E. coli and 16 strains of K. Pneumoniae had a single ESBL gene, although TEM and SHV types of ESBL were frequently found in E. coli (12/6) and K. pneumoniae (10/4), respectively. Two or more genes for ESBL were present in 14 (26.92%) of the 52 ESBL typeable isolates, blaTEM + blaSHV being the most common combination (9.61%), followed by blaTEM + blaCTX-M and blaSHV + blaCTX-M (5.76%). One strains of E. coli and one strains of K. pneumoniae harbour 3 genes for ESBL. The most common combination was blaTEM + blaSHV + blaCTX-M (3.84%). There is a difference between strains harbouring a single ESBL gene (61.53%) compared to those with two or more genes (26.92%) (Table 2). ESBLs have been reported from all parts of the world. However, prevalence varies widely even in closely related regions. The true incidence is difficult to determine because of the difficulty in detecting ESBL production & due to inconsistencies in testing & reporting
Extended spectrum \( \beta \)-lactamase (ESBL) genotypes in \textit{E. coli} and \textit{K. pneumoniae} strains

To conclude, phenotypic methods are only screening methods for detection of ESBLs in a routine laboratory. The prevalence of ESBL-producing \textit{E. coli} and \textit{K. pneumoniae} had means of 61.53% and 26.92%, respectively. The prevalence of ESBL-producing \textit{E. coli} and \textit{K. pneumoniae} in surgical clinics was higher compared to that in clinics of internal medicine. The genotypic methods help us to confirm the genes responsible for ESBL production. The majority of our strains harboured two or more ESBL genes and the most common phenotypes were TEM, SHV and CTX-M. In this study used a multiplex PCR for the detection of \textit{blaTEM}, \textit{blaSHV} and \textit{blaCTX-M} genes in ESBL-producing \textit{E. coli} and \textit{K. pneumoniae}. This method provided an efficient, rapid differentiation of ESBLs. Appropriate antibiotic policy and infection control measures in hospital settings are crucial to overcome the problems associated with infections by ESBL-producing strains.

REFERENCES


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