Faecal carriage of CTX-M-15-producing *Klebsiella pneumoniae* in patients with acute gastroenteritis


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Background & objectives: Data on extended-spectrum β-lactamases (ESBLs) produced by Gram-negative bacteria including *Klebsiella pneumoniae* especially molecular types of ESBL genes from India are limited. The present study was conducted to investigate the carriage and ESBL contents of multidrug-resistant *K. pneumoniae* recovered from patients with gastroenteritis in a rural village in southern India.

Methods: Nine *K. pneumoniae* isolates obtained from 45 stool samples from patients with gastroenteritis from one rural and two urban sites, in southern India were included in the study. Antibiotic susceptibility testing, PCR analysis and sequencing were conducted to characterize the ESBL genes. Clonal relatedness was assessed by pulsed-field gel electrophoresis (PFGE).

Results: All the isolates were found to be resistant to at least one of the third generation cephalosporins tested. All the study isolates were confirmed to produce ESBLs. PCR and sequencing revealed the responsible gene to be *bla_{CTX-M-15}, bla_{TEM}* and *bla_{SHV}* were absent. PFGE indicated that five of seven isolates from villagers were genetically closely related, and in turn were related to isolates from patients in two urban areas in this region.

Interpretation & conclusions: Our findings showed that genetically-related isolates of *K. pneumoniae* producing CTX-M-15 were present in multiple areas in southern India. Larger studies need to be done in various geographical regions of the country to better define the molecular epidemiology of ESBL-producing *K. pneumoniae* and its clinical implications.

Key words: CTX-M-15 - extended-spectrum beta-lactamase - *Klebsiella pneumoniae*
often ineffective. Second, these organisms tend to be also resistant to other classes of antimicrobials including fluoroquinolones and aminoglycosides. CTX-M-type β-lactamases are increasingly becoming the predominant ESBLs globally in recent years, as opposed to the conventional TEM and SHV-type ESBLs. Studies have reported high prevalence of ESBL-producing Enterobacteriaceae in India, where use of antimicrobials is relatively unrestricted. A study from northern India showed that 26 to 48 per cent of uropathogens belonging to Enterobacteriaceae were ESBL producers. Some reports have described the molecular epidemiology of ESBL producers, from the northern parts of the country.

Though presence of ESBLs in K. pneumoniae has been reported from India, there is scarce information on the molecular types from southern India. From the limited data available, CTX-M-15 appears to be the predominant ESBL in northern India. In the present study, we investigated the occurrence of ESBL-producing K. pneumoniae carried by patients presenting with gastroenteritis in southern India.

**Material & Methods**

**Bacterial isolates:** A total of 45 stool samples were collected randomly from patients with gastroenteritis at three sites in Karnataka, southern India. Out of 45 samples collected, 43 were from Sedum, the other one each from Gulbarga and Raichur cities. K. pneumoniae was isolated from nine of these samples and included in the study. Seven were isolated from inpatients with diarrhea in Sedum village in south India in August, 2005. The other two were isolated from patients with diarrhea in the cities of Raichur and Gulbarga in August, 2005 and February, 2006, respectively. Basic microbiology work was done in Department of Microbiology, Gulbarga University, Gulbarga and molecular work was performed in Dr David L. Paterson Laboratory, Pittsburgh, USA. The susceptibility profiles of each study isolate were not known when the study was initiated. The village of Sedam is located in the northern part of Karnataka, 52 km away from the large regional center, Gulbarga. Raichur is 147 km away from Gulbarga.

**Antibiotic susceptibility testing:** The antibiotic susceptibilities were determined by disk diffusion method on Mueller-Hinton agar plates (Beckton Dickinson, Sparks, MD, USA) as recommended by the Clinical Laboratory Standards Institute (CLSI). The disks containing the following antibiotics (µg) were used (Beckton Dickinson): cefoxitin (30), cefepime (30), ceftazidime (30), cefotaxime (30), ertapenem (10), imipenem (10), amikacin (10), gentamicin (10), ciprofloxacin (5) and chloramphenicol (30).

**ESBL screening and confirmation by phenotypic methods:** The isolates showing reduced susceptibility to ceftazidime or cefotaxime or both were tested for ESBL production by double disk-diffusion test (DDDT) using four disks (µg): cefotaxime (CTX), cefotaxime + clavulanic acid (10), ceftazidime (CAZ) (30), and ceftazidime + clavulanic acid (10). The inoculum and incubation conditions were the same as for standard disk diffusion recommendations. A ≥5 mm increase in zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone was designated as ESBL-positive. Escherichia coli ATCC 25922 was used as the control strain.

**PCR detection and sequencing of ESBLs:** Genomic DNA was prepared by boiling the isolates and used as the templates for PCR reactions. Amplification of TEM, SHV and CTX-M-type ESBL genes was performed on GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with primers and cycling conditions given in Table I. The PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla_shv</td>
<td>SHV-F-S1</td>
<td>5'-ATTGTGCTGCTTCTTTACCTGC-3'</td>
<td>60</td>
<td>1051</td>
</tr>
<tr>
<td></td>
<td>SHV-R-S2</td>
<td>5'-TTATGCGATTACCTTAAAAAC-3'</td>
<td></td>
<td></td>
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<tr>
<td>bla_tem</td>
<td>TEM-F</td>
<td>5'-ATGAGTAATTCAACATTTCCGTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TEM-R</td>
<td>5'-TTACCAATGCTTAATCAGTGAG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaCTX-M</td>
<td>CXT-M/F'</td>
<td>5'-TTTGCCATGTTGCACTTCCGTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXT-M/R'</td>
<td>5'-CGATATCGTTGCACTTCCGTG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaCTX-M-15</td>
<td>CXTM15F</td>
<td>5'-CAACACGGGATTAAGGCGAT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CXTM15R</td>
<td>5'- GCCGTCTAAGGCAGAAACA-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Source: Ref. 10
The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) and were sequenced on an ABI Prism 3100 automated sequencer (Applied Biosystems).

**Genomic fingerprinting by pulsed field gel electrophoresis (PFGE)**: Agarose plugs containing chromosomal DNA were prepared as described by Yuan et al.\(^{11}\). The chromosomal DNA was digested overnight with 30 U of Xbal (New England Biolabs, Ipswich, MA, USA). PFGE was run in a CHEF-DRIII apparatus (Bio-Rad, Hercules, CA, USA) at 6V/cm for 22 h. The pulse times were 5-40s. The banding patterns were interpreted according to the criteria of Tenover et al.\(^{12}\).

**Results**

The results of antibiotic susceptibility tests indicated that all nine isolates in the study were resistant to cefotaxime and ciprofloxacin and intermediate or resistant to ceftazidime (Table II). Susceptibility to cefepime, chloramphenicol, gentamicin and amikacin was variable. All were susceptible to cefoxitin, ertapenem and imipenem. The inhibitory zones of cefotaxime and ceftazidime were increased in the presence of clavulanic acid by more than 5 mm in all isolates, which confirmed ESBL production. PCR analysis was positive for CTX-M-type ESBL gene in all nine isolates but negative for TEM and SHV-type β-lactamase genes. Upon sequencing, the deduced amino acid sequences of representative amplicons for CTX-M-type ESBL gene matched that of CTX-M-15. Macrogenetic polymorphism of genomic DNA determined by PFGE revealed the presence of two pulsotypes among the nine study isolates. Seven belonged to one and two the other (Fig.). The two isolates from Raichur and Gulbarga showed the same pulsotype with five of the seven isolates from Sedam (Table II, Fig.).

**Discussion**

Asymptomatic carriage of ESBL-producing *K. pneumoniae* in the gastrointestinal tract may serve as the source of subsequent infections such as urinary tract infection\(^{13}\). Although the sample size was small in the study, isolates originating from stool were confirmed as ESBL producers. The results of our
study revealed that \textit{K. pneumoniae} producing CTX-M-15 is already present in various geographic areas in southern India, as it has been observed in northern India\textsuperscript{6}. CTX-M-15 is the predominant ESBL among \textit{E. coli} causing community-associated infections worldwide\textsuperscript{1}. It differs from CTX-M-3 by one amino acid substitution (Asp240 to Gly), which is known to extend the spectrum of activity to ceftazidime in addition to cefotaxime\textsuperscript{14}. The isolates in the present study were all intermediate or resistant to ceftazidime, which is consistent with this enzymatic property of CTX-M-15. In addition to the cephalosporins all isolates were resistant to ciprofloxacin as well. Orally administered antimicrobials including ciprofloxacin are available over-the-counter and frequently used for the purpose of self-medication in India, which may account for the accumulation of multi-drug resistance among ESBL producing organisms\textsuperscript{15}. Investigation of the mechanisms of ciprofloxacin resistance among the study isolates is underway.

Of note, study isolates recovered from patients at three geographically distant sites shared identical macrodigestion patterns by PFGE. This finding suggests that a particular clone of \textit{K. pneumoniae} may be associated with the spread of CTX-M-15 in southern India. Further studies are needed to determine the potential of this particular clone of CTX-M-15-producing \textit{K. pneumoniae} in causing community-associated infections, as has happened with CTX-M-15-producing \textit{E. coli}.

In conclusion, CTX-M-15-producing \textit{K. pneumoniae} isolates from stool samples of patients with gastroenteritis in rural and urban areas in southern India were found, many of which belonged to a single clone. Hence routine diagnosis of ESBL-producing \textit{K. pneumoniae} should be done in hospitals, coupled with prudent use of antimicrobials to reduce propagation of multidrug-resistant and ESBL-producing organisms.

References


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