Molecular epidemiology of various antibiotics resistance genes, including $\text{bla}_{\text{NDM-1}}$, in Indian environmental and clinical bacterial isolates: a comparative study


Abstract

Objectives: There are concerns regarding the emergence of NDM-1 metallo-beta-lactamase in clinical and environmental bacteria; however, no attempts have yet been made for their molecular correlation. We analyzed antibiotics-resistant environmental and clinical isolates for $\text{bla}$ genes, mobile genetic elements and for the epidemiological relationship based on molecular results.

Methods: The clinical and environmental bacterial isolates were collected from Aligarh, a north-Indian city. Antibiotics susceptibility was performed and the isolates were investigated for the presence of $\text{bla}_{\text{NDM-1}}$, $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{ampC}}$ and also for the mobile genetic elements (IS26, ORF513, ISEcp1, Sul-1-type integrons) by PCR. The bacterial isolates were typed by Random Amplified Polymorphic DNA (RAPD)-typing. Statistical analyses of the molecular results pertaining to genetic environment of clinical vs. environmental isolates were performed to assess any genetic relatedness or diversity.

Results: A total of 149 clinical- and 27 environmental-bacterial isolates were studied. Significant difference in the antibiotics resistance profiles between clinical and environmental bacterial population was noticed. $\text{bla}_{\text{CTX-M}}$ and $\text{bla}_{\text{ampC}}$ were noticed as the most prevalent antibiotics-resistance genes in clinical and environmental bacteria, respectively. $\text{bla}_{\text{NDM-1}}$ was detected in two clinical (1 Escherichia coli; 1 Klebsiella pneumoniae) and one environmental E. coli isolate. Both clinical as well as environmental isolates possessed mobile genetic elements; however, their frequency of occurrences in the two groups was significantly different, so was the case for their occurrence in various combinations. Moreover, the clinical and environmental isolates were not found genetically related, based on the RAPD -typing.

Conclusion: It is evident that the genetic environment of clinical and environmental bacterial isolates is entirely different and suggests their unique evolution; that could really be a tormenting public health problem.

Key words: NDM-1, clinical isolates, environmental isolates, class A ESBLs, class C beta-lactamases, genetic relatedness, India.

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Introduction

NDM-1 is a recently described resistance mechanism that confers resistance to almost all the beta-lactam antibiotics (including carbapenems), except aztreonam (1). Not only to beta-lactam antibiotics, the isolates harbouring NDM-1 are also reported resistant to other antibiotics classes such as fluoroquinolones and aminoglycosides, mainly used in the treatment of Gram-negative bacterial infections (2). However, most of the isolates remain susceptible to colistin and tigecycline.

NDM-1 was first reported in a Klebsiella pneumoniae strain (05-506) from a Swedish patient of Indian origin in January 2008 (1). Since then, it has spread to numerous other countries such as United States, Canada, Australia, Germany, Japan and Hong Kong (3). Recently, Walsh et al. described the appearance of $\text{bla}_{\text{NDM-1}}$ in the Indian environmental isolates recovered from seepage and drinking water samples and thus suggested grave public health concerns (4). However, no attempts were taken to analyze the genetic relatedness of these environmental isolates with clinical isolates despite the fact that a subset of clinical isolates was previously collected from the same geographic area (New Delhi, India). Moreover, no further studies were conducted from other cities in India to look for the prevailing situation, especially the presence of $\text{bla}_{\text{NDM-1}}$ in the environment.

In the present study, we analyzed the clinical and environmental bacterial isolates collected from Aligarh; a nearby city to New Delhi (132 km away) from where the first and subsequent reports of occurrence of $\text{bla}_{\text{NDM-1}}$ were published. We investigated our bacterial cohort for the presence of $\text{bla}_{\text{NDM-1}}$, $\text{bla}_{\text{CTX-M}}$, $\text{bla}_{\text{TEM}}$, $\text{bla}_{\text{SHV}}$, $\text{bla}_{\text{ampC}}$ and also for the mobile genetic elements (ISEcp1, IS26, ORF513, Sul-1-type integrons) by PCR. We also statistically analyzed the isolates for any epidemiological relationship based on our molecular results.

Methods

Bacterial isolates

During 2009-10, a total of 14,129 clinical specimens were received in the Department of Microbiology of J. N. Medical College & Hospital, Aligarh, India for routine culture and susceptibility testing. Of these, 2,065 yielded growth of Gram-negative bacterial species. During that period, a random collection of 145 Gram-negative bacterial isolates found resistant to any of the third-generation cephalosporins (3GCs) and four isolates found resistant to imipenem (in addition to 3GCs) were selected for this study; thus the clinical cohort comprised of 149 isolates. The environmental cohort comprised of 27 third-generation cephalosporin-resistant (3GCR) bacterial isolates that were obtained from the culture of 64 environmental samples (drinking water, drain, sewage).

Procedures

The bacterial isolates were identified according to standard procedures (5) and antibiotics susceptibility was performed by disc diffusion method using Clinical and Laboratory Standards Institute (CLSI) break points (6). For detection of class A ESBLs, $\text{bla}_{\text{CTX-M}}, \text{bla}_{\text{TEM}}$ and $\text{bla}_{\text{SHV}}$ were detected by monoplex-PCRs (7) and further characterization of the $\text{bla}_{\text{CTX-M}}$ genegroups was performed by multiplex-PCR (8). For class C beta-lactamases (AmPC), the screening was done by monoplex-PCR (9) and further characterization of the $\text{bla}_{\text{ampC}}$-families was done by multiplex-PCR (10).
The presence of blaNDM-1 was detected by PCR using specific primers targeting the said gene (11). Sequencing was performed to identify the resistance genes (blaCTX-M, blaBPMC and blaNDM-1). Detection of mobile genetic elements (ISEcp1, IS26, ORF513, Sul1-type integrons) was performed by PCR as described previously (7,12). RAPD was performed to analyze any genetic relatedness/diversity in the clinical and environmental isolates (7).

Statistical analysis
Chi-square test (using the GraphPad software: http://graphpad.com/quickcalcs/contingency2/) was applied to analyze the significant difference in occurrence of bla genes in clinical vs. environmental isolates. The logistic model (http://statpages.org/logistic.html) was applied for evaluating the occurrence of mobile genetic elements in clinical vs. environmental isolates and p value and odds ratios were calculated.

Results
Clinical bacterial cohort
The clinical bacterial cohort comprised of 149 bacterial isolates, namely Escherichia coli (n=125), Klebsiella pneumoniae (n=22) and Pseudomonas aeruginosa (n=2); of which four isolates (1 E. coli, 1 K. pneumoniae, and 2 P. aeruginosa) were carbapenem-resistant. These isolates were obtained from pus (n=82), urine (n=53), semen (n=3), cervical swab (n=4), CSF (n=1), nasopharyngeal aspirate (n=2), sputum (n=1) and peritoneal fluid (n=3).

Environmental bacterial cohort
After culturing 64 environmental samples (drinking water, drain, and sewage), 27 samples yielded growth of a total of 50 Gram-negative organisms (mixed culture growth was noticed) (Figure 1a). These isolates were obtained after culturing the following samples: 22 tap water, 9 bucket water, 15 drain water, four sewage samples, three hand pump water, and 11 filtered water samples (taken from aquaguard/ reverse osmosis (RO) water purification system used in homes). Maximum occurrence was noticed for E. coli (25/50), followed by Citrobacter spp. (7/50). The least occurrence was noticed for K. pneumoniae and Acinetobacter spp. (4/50 each).

Figure 1a. Water, drain and sewage cultures, showing presence/ absence of bacterial growth, obtained from urban and rural areas of Aligrah city, India.

Of these 50 Gram-negative isolates, 27 were found resistant to any of the 3GCs tested (ceftazidime was tested as a 3GC in case of P. aeruginosa). None of the isolates was resistant to the carbapenem tested (imipenem). These 27 3GCR-resistant isolates formed the environmental cohort for further study. This environmental cohort comprised of E. coli (n=12), P. aeruginosa (n=5), Citrobacter spp. (n=6), Acinetobacter spp. (n=3) and K. pneumoniae (n=1). These 3GCR environmental isolates (n=27) were obtained from the drain (n=15), tap water (n=4), sewage (n=5), and bucket water (n=3). The detailed results are described in Figure 1b.

Environmental cohort antibiotic resistance
Antibiotic susceptibility to various classes of antibiotics (shown in Figure 2) was performed on all the 50 environmental isolates. Amongst 3GCs, the maximum resistance was noticed for ceftazidime (52% / 26/50). On cumulative analyses, a total of 27 isolates were noticed as 3GCR (one isolate showed resistance to cefotaxime while that was sensitive to ceftazidime). Resistance to a 4th generation-cephalosporin, cefepime, was noticed in 24% (12) isolates. Aminoglycoside-resistance was low (amikacin resistance in 6% isolates only) while the resistance to a fluoroquinolone (ofloxacin) was noted in 12% (6) isolates. None of the environmental isolates was found resistant to imipenem (Figure 2, detailed results).

Figure 1b. Water, drain and sewage cultures and bacteria obtained from urban and rural areas of Aligrah city, India. 3GCR= third-generation cephalosporin-resistant. 3GCS= third-generation cephalosporin-sensitive.

Clinical cohort antibiotic resistance
Among clinical isolates, maximum resistance was noticed for cefotaxime (95.30%), followed by ceftriaxone (93.55%), cefixime (88.55%), and then ceftazidime (83.22%). Resistance to cefepime was observed in 64.43% of clinical isolates. Resistance to aminoglycosides (amikacin) was noticed in 42.36% isolates, while to that of fluoroquinolones (ofloxacin) was observed in 90.27% isolates. Moreover, imipenem resistance was observed in 2.68 % isolates.
Environmental cohort antibiotic resistance genes

*bla*ampC was observed in 48.15% (13/27) isolates, while among *bla*ESBLs, *bla*SHV was observed in 18.52% (5/27), and *bla*CTX-M and *bla*TEM were observed in 11.11% (3/27) isolates, each. In multiplex-PCR for *bla*CTX-M*+* genotype groups, all the isolates were noticed belonging to the CTX-M-group-1. Irrespective of the phenotypic results for carbapenem-resistance, we screened all our 3GCR environmental isolates for *blaNDM-1*, and only one isolate was found to harbour *blaNDM-1*.

Clinical cohort antibiotic resistance genes

When clinical samples were analyzed for the respective *bla* genes, a somewhat different pattern was observed. 73.82% (110/149) isolates were found to harbour *bla*ampC. Among *bla*ESBLs, *bla*CTX-M was present in maximum number of clinical isolates (121/149; 81.20%) followed by *bla*SHV (72/149; 48.32%) and then *bla*TEM (63/149; 42.28%). Moreover, out of four imipenem-resistant isolates, two were found to harbour *blaNDM-1*. Since we noticed the presence of *blaNDM-1* in environmental isolates that were sensitive to imipenem, we randomly selected 20 imipenem-sensitive clinical isolates for *blaNDM-1* screening and found all of them negative for *blaNDM-1*.

Occurrence of *bla*ampC families in the environmental cohort

Out of 27 3GCR environmental isolates, 13 showed amplification for *bla*ampC in monoplex-PCR. On multiplex-PCR, only eleven of them demonstrated the presence of plasmid-mediated AmpC-genes (pMAmpCs). Maximum number of isolates were found to possess *bla*ACC (52/149; 35.38%) followed by *bla*FOX (42/149; 28.53%), and then *bla*TEM (63/149; 42.28%). Moreover, out of four imipenem-resistant isolates, two were found to harbour *bla*NDM-1*. Since we noticed the presence of *blaNDM-1* in environmental isolates that were sensitive to imipenem, we randomly selected 20 imipenem-sensitive clinical isolates for *blaNDM-1* screening and found all of them negative for *blaNDM-1*.

Table 1. Occurrence of mobilizing genetic elements in various combinations of *bla* genes in clinical and environmental isolates.

<table>
<thead>
<tr>
<th>Combinations of <em>bla</em> genes</th>
<th>Clinical Isolates</th>
<th>Environmental Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Isolates</td>
<td>ISEcp1</td>
</tr>
<tr>
<td><em>bla</em>ampC</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>CTX-M</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>SHV</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>TEM</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>FOX</td>
<td>30</td>
<td>22</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>FOX + <em>bla</em>CTX-M</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>FOX + <em>bla</em>SHV</td>
<td>20</td>
<td>15</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>TEM + <em>bla</em>FOX</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>TEM + <em>bla</em>SHV</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>TEM + <em>bla</em>FOX</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>TEM + <em>bla</em>SHV</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>FOX + <em>bla</em>SHV</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>bla</em>ampC + <em>bla</em>FOX + <em>bla</em>TEM + <em>bla</em>SHV</td>
<td>33</td>
<td>24</td>
</tr>
<tr>
<td>No antibiotic resistance genes</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>149</td>
<td>88</td>
</tr>
</tbody>
</table>

Clinical vs. environmental isolates: comparative analyses

The antibiotic resistance profile of the 3GCR-clinical isolates was compared with that of 3GCR-environmental isolates. Resistance to cefepime was 64.43% in clinical isolates while it was 44.44% in the environmental samples. Resistance to cefpirome was found almost comparable (88.55% in clinical isolates and 77.78% in environmental isolates) but fair difference was noticed in the resistance rate for cefotaxime and ceftriaxone. Resistance to cefotaxime was observed in 95.30% of clinical isolates while only 25.93% environmental isolates were found as cefotaxime-resistant. Similarly, ceftriaxone-resistance was noticed in 93.55% clinical isolates and it was observed in 18.52% environmental isolates only. Resistance to cefazidime was observed higher in environmental isolates (96.30%) as compared to that in the clinical isolates (83.22%). Resistance to ofloxacin and amikacin was found higher in clinical isolates than environmental isolates. 3.10% clinical isolates were found resistant to imipenem while none of the environmental isolates was found resistant to imipenem based on the antibiotics susceptibilities results (Figure 3).
As far as bla genes were concerned, bla$_{CTX-M}$ was found to be the most frequent (81.20%) among clinical isolates but the situation noticed in the environmental isolates was unlike as the occurrence of bla$_{ampC}$ was noticed in maximum number of isolates (48.15%). Among bla$_{ESBLs}$, bla$_{CTX-M}$ was observed in maximum number of clinical isolates, followed by bla$_{SHV}$ (48.32%) and the least occurrence was observed for bla$_{TEM}$ (42.28%). While, different pattern of occurrence of bla$_{ESBLs}$ was noted in environmental isolates where bla$_{ampC}$ was observed in maximum number of isolates (48.15%) and bla$_{CTX-M}$ and bla$_{TEM}$ were present in equal number of isolates (11.11%). Two clinical (imipenem resistant) isolates showed the presence of bla$_{NDM-1}$ while only one environmental isolate that was imipenem-sensitive showed the presence of NDM-1 gene. Occurrences of bla$_{ampC}$ and bla$_{ESBLs}$ in clinical and environmental isolates are shown in Figure 4. Statistical analyses (Chi-square test) demonstrated significant differences in the occurrence of these bla genes in clinical vs. environmental isolates (bla$_{CTX-M}$ p <0.0001; bla$_{ampC}$ p =0.0075; bla$_{TEM}$ p =0.0021; bla$_{SHV}$ p =0.0041).

Sul-1 type integrons demonstrated the most frequent occurrence (among the mobile genetic elements) in clinical and environmental isolates (87/149 and 11/27, respectively). The least occurrence was noticed for IS26 in clinical isolates (48/149; 32.21%) while it was observed for IS£cp1 in the environmental isolates (3/27; 11.11%). Occurrence of ORF513 (82/149; 55.03%) and IS£cp1 (86/149; 57.72%) was noticed almost comparable in clinical isolates, while equal number of environmental isolates were found to harbour ORF513 and IS26. By applying the logistic model for analyses of occurrence of these mobile genetic elements in clinical vs. environmental isolates, the occurrence of IS£cp1 and ORF513 was found statistically significant (p= 0.0002; OR= 10.92 and p= 0.0184; OR= 2.91, respectively). However, it was statistically insignificant for IS26 and Sul-1 (p= 0.7908; OR= 1.13 and p= 0.0903; OR= 2.04, respectively). Details of the association of MGEs with various combinations of bla genes in clinical and environmental isolates are shown in Table 1.
Randomly amplified polymorphic DNA (RAPD) - typing
The 3GCR environmental isolates were typed by RAPD in order to determine whether any predominant clone was circulating in environmental isolates. Eight *E. coli* (out of 18) isolates did not produce any amplified product in the repetitive experimentations and were categorized as un-typable isolates. Only four of them demonstrated the banding patterns that differed from each other and also from the clinical *E. coli* isolates. RAPD profile of environmental vs. clinical *E. coli* isolates is shown in Figure 5a. Out of six 3GCR *Citrobacter* isolates (obtained from the environmental samples), four could be grouped in 2 clusters and 1 isolate showed a unique banding pattern. The remaining one isolate was un-typable. Out of 5 *Pseudomonas* isolates that were 3GCR, two isolates showed similar banding patterns and the remaining three showed their unique banding patterns. None of the *Acinetobacter* isolates showed a common RAPD pattern. The RAPD profiles of *Citrobacter* sp., *Pseudomonas* sp., and *Acinetobacter* isolates are shown in Figure 5b-d. The only environmental *K. pneumoniae* isolate that was 3GCR did not produce any band in the RAPD and thus was categorized as un-typable.

**Discussion**
Recent surveys have shown a high frequency of multidrug-resistant Enterobacteriaceae isolates making the use of reserved antibiotics such as carbapenems necessary. If adopted as a policy, in turn cause the emergence of newer antibiotics resistance pathways, such as production of NDM-1 enzymes. The NDM-1 harboring isolate was in fact recovered for the first time in 2008 from a Swedish resident of Indian origin who had a history of recent hospitalization in India (1), and subsequently, by other workers, who examined the Indian clinical bacterial isolates (2,13,14). Subsequently, the presence of NDM-1 has been reported around the world and thus raise concerns regarding their wide and rapid dissemination (4). Very recently, in an interesting study by Walsh et al, the presence of *blaNDM-1* was reported in the environmental bacterial isolates obtained from the water samples collected from a localized region of New Delhi, India (4). However, those environmental isolates could not be analyzed for the genetic relatedness (if any) with the clinical isolates previously characterized (for the presence of *blaNDM-1*) from the same place (2). To date, no research group has yet analyzed the genetic relationship between the environmental and clinical isolates and also did not compare their genetic environment, especially the frequencies of association of mobile genetic elements. Lack of robust data itself adds to the looming threat of new forms and pathways of antibiotic resistance world-wide and their wider dissemination.

In the light of these facts, we planned this study to analyze the molecular epidemiology and genetic relatedness (if any) between the environmental and clinical isolates found resistant to third-generation cephalosporins/carbapenems in our area. We planned this study to extensively look for the class A (IMI, *blaTEM*, *blaSHV*), class B (*blaNDM-1*) and class C (*blaIMP*) β-lactamases as well as the mobile genetic elements (IS*Ecp*1, IS*CR1* (ORF513), IS26 and * Sul-1* type integrons).

We observed *blaCTX-M* as the predominant ESBL in clinical isolates and combination of *blaCIT* + *blaREC* was noticed in the maximum number of isolates harboring class C (AmpC) β-lactamases. We also observed a clinical *E. coli* isolate to harbor *blaESBL* (*blaCTX-M*, *blaSHV*), *blaNDM-1*, and *blaCIT*, while another imipenem-resistant clinical *K. pneumoniae* isolate was noticed to harbour Class A (*blaTEM*), Class B (*blaNDM-1*; GenBank Accession: JN 157767) and Class C (*blaCIT*). Similar types of findings were reported by other workers who described a single isolate harbouring multiple types of β-lactamases such as Class A (*blaSHV-12*), Class B (*blaIMP-1*), and Class C (*blaCIT* (15)). Moreover, the NDM-1-harbouring clinical *E. coli* isolate was carrying *ISCR1*, *Sul-1*, *ISEcp1*, and IS26 and the other clinical NDM-harbouring *K. pneumoniae* isolate was carrying *ISCR1*, *Sul-1* and *ISEcp1*. On contrary to this, the only environmental NDM-1 harboring *E. coli* isolate did not have any of the class A and class C beta-lactamases tested, and was only carrying the mobile genetic elements, *ISCR1* and *Sul-1*. We noticed the presence of *ISEcp1*, *ISCR1* (ORF513), and *Sul-1* type integrin in both of the *blaNDM-1*-harbouring clinical isolates but the IS26 was noticed in only one of them. Similar types of findings have been described in previous studies where genetic environment of *blaNDM-1* was described by Yong et al who reported the presence of intact *ISCR1* followed by *Sul-1*, *blaNDC1*-flanked by *ISEcp1* and *blePS1*. It was reported to be flanked by truncated *Sul-1* on one side (1). The plasmid carrying *blaNDM-1* also carried *blaIMP-1*, and the complex class 1 integron (16). A unique approach in our study was the analyses of occurrence of mobile genetic elements, *ISEcp1*, *ISCR1* (ORF513), IS26 and *Sul-1* type integrons in clinical vs. environmental isolates and we found it statistically significant for *ISEcp1* and ORF513 while it was statistically insignificant for IS26 and *Sul-1*.

Walsh et al, in their study conducted on Indian environmental isolates, showed the presence of *blaNDM-1* in 50/171 seepage samples and 2/50 drinking water samples (4). However, in the present study we found only one environmental isolate to possess *blaNDM-1*. Though in a lesser frequency, it is quite alarming that the NDM-1 is detected in the environment of another Indian city. More worrying is that the NDM-1 harboring isolate did not show resistance to a carbapenem in the susceptibility testing and suggested that the said gene was lying dormant as an unexpressed gene. If provided with selection pressure to the environment, such bacteria could start expressing the genes and could disseminate widely. Moreover, the presence of mobile genetic elements in our environmental isolates is quite alarming since these elements could trap novel genes to help their dissemination. More important is that the genetic environment of these resistant environmental bacteria is entirely different from the clinical counterpart and thus suggests their unique evolution. Therefore, if these environmental bacteria have a chance to enter clinics/hospitals, they may further complicate the existing problem of antibiotics resistance. We, therefore, suggest that further large scale studies on similar aspect are urgently needed so as to combat this emerging threat.

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