INTRODUCTION

Hospital-acquired bacterial infections may dominate the headlines, but most infections occur in the community. Indeed, 80% of the antibiotic prescribing takes place in the community – in local practices, daycare centers and long-term care facilities such as nursing homes and rehabilitation centers. Most patients hospitalized in the Intensive Care Units after being discharged continue to carry Extended Spectrum \( \beta \)-lactamase (ESBL) producing Enterobacteriaceae over prolonged periods. Continued carriage of such strains may contribute to their extrahospital propagation.[1]

In recent years, resistant bacteria have been isolated from apparently non-selective environments, including plants, coastal and estuarine environments, deep ocean water and sediments and drinking water.[2] Organisms resistant to naturally occurring and human-modified antibiotics were detected in 22 rivers in the United States of America. A large proportion of the resistant organisms were ESBL producers, and many were found to contain plasmids with resistance traits.[3] Resistance to additional classes of antibiotics has also been noted among ESBL-producing Escherichia coli and Klebsiella species (ESBL-EK) as many of these additional resistance genes are encoded on the ESBL-associated plasmid. The acquisition of resistance genes has not decreased the pathogenicity or virulence of Klebsiella species and Escherichia coli. Multidrug-resistant (MDR) ESBL-EK isolates (i.e., those resistant to multiple other antibiotics or antibiotic classes in
addition to the oxyimino β-lactams) thus pose significant therapeutic challenges, even greater than those of ESBL-EK, further curtailing the number of drugs useful against these bacteria. Additionally, recently reported carbapenem-resistant ESBL-EK isolates are of paramount concern because of the scarcity of effective therapies for infections with such organisms.\textsuperscript{[4]}

Resistance genes can be spread far wider than once believed by horizontal gene transfer mechanisms like conjugation, transformation and transduction. Such gene transfer mechanisms allow mobilization of specific DNA fragments from one region to another, from plasmids to plasmids, from chromosome to chromosome and between plasmids and chromosomes. Plasmid-mediated diffusion of β-lactamases is of great concern and contributes to the enormous spread of this kind of enzyme throughout the microbial world.\textsuperscript{[5]} Considering the co-resistance of ESBLs with various other antibiotics and the extraordinary propensity of ESBL producers for horizontal gene transfer, the present work was aimed at studying the transfer of resistance among Gram negative bacteria like \textit{Escherichia coli} and \textit{Klebsiella pneumoniae} under laboratory as well as under simulated environmental conditions.

\section*{MATERIALS AND METHODS}

\subsection*{Clinical isolates and antimicrobial susceptibility testing}

A retrospective analysis of susceptibility patterns of 120 consecutive, non-repetitive ESBL screen-positive clinical isolates of \textit{Escherichia coli} (\(n = 70\)) and \textit{Klebsiella pneumoniae} (\(n = 50\)) was carried out. These isolates were obtained from a 100-bedded tertiary care hospital in Mumbai, India, during January 2007 to January 2008. The organisms were subjected to antimicrobial susceptibility testing by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines on Mueller Hinton agar plates by using commercially available discs (Hi Media Laboratories, Mumbai, India) of amoxicillin (30 µg), amikacin (30 µg), gentamicin (30 µg), trimethoprim (30 µg), nalidixic acid (30 µg), norfloxacin (10 µg), ciprofloxacin (30 µg), cefuroxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg) and nitrofurantoin (300 µg). The diameter of the zone of inhibition for each antibiotic was measured and interpreted as per the CLSI recommendations. The ESBL screen breakpoints criteria used were \(\leq 22\) mm for ceftazidime, \(\leq 27\) mm for cefotaxime and \(\leq 25\) mm for ceftriaxone. \textit{Escherichia coli} ATCC 25922 as a negative quality control strain and \textit{Klebsiella pneumoniae} ATCC 700603 as a positive ESBL control (known to contain an SHV-type ESBL) were used for all the susceptibility testing studies as well as for the phenotypic confirmatory methods carried out for the detection of the ESBL producers.\textsuperscript{[6]}

\subsection*{Double disc synergy test}

The Double disc synergy test (DDST) was performed to determine the synergy between a disc of amoxicillin/clavulanic acid (CA) (20/10 µg) and 30 µg discs of each ceftazidime and cefotaxime placed at a distance of 15 mm (center-to-center) from the amoxicillin/CA disc. Inoculated plates were incubated overnight at 36 ± 1ºC for 24 h. Enhancement of the zone of inhibition between the clavulanate disc and any one of the β-lactam discs towards the amoxicillin/CA disc or lack of inhibition by either discs alone but inhibition of growth where the two antibiotics diffused together indicated the presence of ESBL.\textsuperscript{[6,7]}

\subsection*{ESBL confirmation by inhibitor-potentiation disc diffusion method}

Inhibitor-potentiation disc diffusion (IPDD) was used to confirm the presence of ESBL-positive isolates by placing a disc of ceftazidime (30 µg) and cefotaxime (30 µg) alone and ceftazidime (30 µg) and cefotaxime (30 µg) in combination with CA (10 µg), at least 20 mm apart from each other, on the Mueller Hinton agar plates. A 5-mm increase in zone diameter for either antimicrobial agent tested in combination with CA versus its zone when tested alone was taken as an indication of ESBL-producing isolates.\textsuperscript{[6]}

\subsection*{Intra-genus genus transfer of antibiotic resistance by conjugation}

Intra-genus transfer of antibiotic resistance was studied using conjugation for all the ESBL-producing \textit{Escherichia coli} isolates. A plasmid-free, streptomycin-resistant (F, Str) auxotrophic strain of \textit{Escherichia coli} (E. coli AB 1157), showing sensitivity to all the antibiotics under study, was used as a recipient, while all the ESBL-producing \textit{Escherichia coli} served as the donors. Overnight cultures were grown to an A\textsubscript{540} of 0.1 (approximately 10\textsuperscript{8} cells/mL). Five milliliters of each participating bacterial culture was mixed (1:1) in a test tube containing sterile Luria Bertani (LB) broth and incubated without shaking for 24 h at 36 ± 1ºC. The transconjugants were selected on the LB agar plates supplemented with streptomycin (100 µg/mL) in addition to at least one of the following antibiotics, viz. amoxicillin, amikacin, gentamicin, cefotaxime and
In the present study, the overall prevalence of ESBL-producing *Escherichia coli* (14/70) and *Klebsiella pneumoniae* (10/50) was 20% each by the DDST and as confirmed by the IPDD method. Ceftazidime detected 64.29% (9/14) ESBL-producing *Escherichia coli* and 70% (7/10) ESBL-producing *Klebsiella pneumoniae* while cefotaxime detected 71.43% (10/14) and 70.0% (7/10) ESBL-producing *Escherichia coli* and *Klebsiella pneumoniae*, respectively, by IPDD. Both the cephalosporins together identified 35.71% *Escherichia coli* and 40.0% *Klebsiella pneumoniae*.

The present investigation showed multidrug resistance among all the isolates used under the study (resistance to ≥ three antibiotics). ESBL-producing *Escherichia coli* showed resistance to eight to 13 antibiotics tested while ESBL-producing *Klebsiella pneumoniae* showed resistance to six to 10 antibiotics tested. On the other hand, non-ESBL producers showed resistance to three to 11 antibiotics tested among both the isolates. All the isolates in the study, however, were sensitive to both the carbapenems tested. ESBL-producing *Escherichia coli* showed 100% resistance to amoxicillin, amikacin and nalidixic acid as seen in Table 1. Resistance of ESBL-producing *Escherichia coli* to third-generation cephalosporins used under study, viz. ceftazidime (85.7%), cefotaxime (71.4%) and ceftriaxone (78.6%), was notable. Resistance to gentamicin (92.9%) and nitrofurantoin (42.9%) was equivalent in ESBL and non-ESBL *Escherichia coli*. Eighty percent of the ESBL-producing isolates of *Klebsiella pneumoniae* showed resistance to amoxicillin, nalidixic acid, ciprofloxacin and ceftazidime, 50% to cefotaxime, 70% to ceftriaxone and 90% exhibited resistance to nitrofurantoin. However, non-ESBL-producing *Klebsiella pneumoniae* showed a higher resistance (75%) to cefepime compared with ESBL *Klebsiella pneumoniae* (20%).

Conjugation was used to elucidate intra- and inter-genus transfer of plasmid-borne resistance to a susceptible strain of *Escherichia coli* sensitive to all the antibiotics used in the study. The resistance was transferred to the recipient cells of *Escherichia coli* AB 1157 sensitive to the individual markers, i.e. amoxicillin, amikacin, gentamicin, cefotaxime and ceftriaxone, at a frequency of 3–4 × 10⁻⁵. As the number of markers transferred increased from two to five, the frequency of transfer decreased from 10⁻⁵ to 10⁻⁷. All the pairs of isolates assessed for the inter-genus transfer of the antibiotic resistance showed transfer of resistance. Among the four mating pairs tested, three pairs achieved two-way transfer of antibiotic-resistance markers under laboratory (LB broth) as well as under simulated environmental conditions (sewage and sea water), as shown in Table 2.

In the present study, plate assay was carried out to demonstrate the natural transformability of all the ten
ESBL-producing *Klebsiella pneumoniae* isolates in vitro. In all the cases, transformants were obtained on LB agar plates, indicating the development of the competence and uptake of DNA, leading to recombination and thus, transformation. Both the markers were transferred at a low frequency of about 1–5 x 10⁻⁷. Cefotaxime resistance was transferred in all the isolates while only three isolates showed transfer of gentamicin resistance. Two isolates showed simultaneous transfer of both the markers at a frequency of 1–3 x 10⁻⁷.

**DISCUSSION**

In India, ESBL-producing strains of *Enterobacteriaceae* have emerged as a challenge in the hospitalized as well as in the community-based patients. In 2002, 68% of the Gram negative bacteria were found to be ESBL producers in a study from New Delhi, with 80% of *Klebsiella* being ESBL-producers.[9] In the present study, the incidence of ESBL was, however, much lower, at 20%. Distinct regional variations have been detected in the incidences of ESBL-producing isolates, which can be attributed to the different patterns of antibiotic use.

Although the phenotypic confirmatory tests for the detection of ESBLs have proven reliable over many years at detecting the great majority of conventional ESBLs, the strains that are positive on the screening test but negative on the confirmation test (false-positive) or isolates that fail to indicate the presence of ESBL in the screen test but are detected in the combination test (false-negative) need attention. In the present work, 56 isolates of *Escherichia coli* and 40 isolates of *Klebsiella pneumoniae* were screen positive but failed to show clavulanic acid inhibition effect (CAIE). This can be attributed to the additional resistance mechanisms possessed by the organisms that can mask the presence of ESBL activity. In particular, the emergence of plasmid-borne AmpC β-lactamases, which are not inhibited by CA in the members of the *Enterobacteriaceae*, is likely to explain at least some of the strains that have a positive screening test but a negative confirmation test. Other mechanisms include possible co-existence of AmpC with ESBLs, hyperproduction of ESBL, modification of outer membrane proteins, TEM and SHV β-lactamases that are no longer inhibited by CA due to mutations in the coding sequences, etc.[10]

Outbreaks of ESBL-producing *Enterobacteriaceae* strains, often characterized by resistance to multiple drugs, including ciprofloxacin, gentamicin and aminoglycosides, have been reported. A study in 2005 reported an occurrence of 18.8% of MDR ESBL-EK isolates, demonstrating variable resistance to other antibiotics and antibiotic classes except imipenem. There was a high prevalence of resistance to fluoroquinolone, cefepime and piperacillin–tazobactam. They found no association between MDR ESBL-EK and prior antibiotic exposure.[11] A study in 2008 in Chandigarh with Gram negative ESBL-positive uropathogenic bacteria showed a high

### Table 1: Resistance of ESBL-producing and non-producing *Escherichia coli* and *Klebsiella pneumoniae* to various antibiotics

|                               | Am | Ak | G  | Tr | Na | Nx | Cf | Cu | Cn | Caz | Ce | Ci | Cpm | NF |
|-------------------------------|----|----|----|----|----|----|----|----|----|----|----|----|-----|----|
| **Escherichia coli**           |    |    |    |    |    |    |    |    |    |    |    |    |     |    |
| ESBL (n = 14)                 | 14 | 14 | 13 | 11 | 14 | 11 | 12 | 12 | 12 | 11 | 17 | 17 | 20  | 20 |
| Non-ESBL (n = 56)             | 52 | 52 | 52 | 32 | 50 | 38 | 50 | 50 | 33 | 44 | 20 | 25 | 30  | 64 |
| **Klebsiella pneumoniae**     |    |    |    |    |    |    |    |    |    |    |    |    |     |    |
| ESBL (n = 10)                 | 8  | 7  | 7  | 6  | 8  | 5  | 8  | 7  | 5  | 5  | 7  | 5  | 2   | 9  |
| Non-ESBL (n = 40)             | 30 | 22 | 25 | 20 | 20 | 17 | 20 | 15 | 15 | 16 | 26 | 24  | 30  | 21 |

Values in parentheses indicate percentage; Am – amoxicillin; Ak – amikacin; G – gentamicin; Tr – Trimethoprim; Na – nalidixic acid; Nx – norfloxacin; Cf – ciprofloxacin; Cu – cefuroxime; Cn – cefoxitin; Caz – ceftazidime; Ce – cefotaxime; Cpm – ceftepime; NF – nitrofurantoin

### Table 2: Intergenus transfer of resistance by conjugation

| Mating pair | Before co incubation | Resistance pattern | Type of resistance transfer |
|-------------|----------------------|-------------------|----------------------------|
|             | Am | Ak | G  | Ce | Cl | Am | Ak | G  | Ce | Cl |
| *E. coli* 64 + *K. pneumoniae* 35 | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  |
| *E. coli* 43 + *K. pneumoniae* 13 | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  |
| *E. coli* 26 + *K. pneumoniae* 11 | R  | R  | R  | S  | R  | R  | R  | R  | R  | R  | R  |
| Resistance for Ce transferred from *K. pneumoniae* 35 to *E. coli* 64 | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  |
| Resistance for G and Ci transferred from *E. coli* 43 to *K. pneumoniae* 13 | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  | R  |
| Resistance for G transferred from *E. coli* 26 to *K. pneumoniae* 11 | R  | R  | R  | S  | R  | R  | R  | R  | R  | R  | R  |

Am – amoxicillin; Ak – amikacin; G – gentamicin; Ce – cefotaxime; Cpm – ceftepime; NF – nitrofurantoin
The ability of the plasmid DNA to transfer or be mobilized between different strains of the same species or between bacterial species has been demonstrated repeatedly. As more and more genome sequences are determined, it is becoming clear that inter-species transmission of the genetic information is pervasive among microorganisms and that it may occur at vast phylogenetic distances and change the ecological and pathogenic character of bacterial species. A study demonstrated transfer of ESBL-mediated resistance to cefotaxime along with transfer of gentamicin and other beta-lactam antibiotics.\[7\] In a study conducted in France in 1996, mating between exponentially growing cultures of Klebsiella pneumoniae RIC and Escherichia coli K12 J53-2 resistant to rifampin or Escherichia coli K12 C600 resistant to nalidixic acid was performed. The transfer of \(\beta\)-lactam resistance determinants from donor cells was obtained at a frequency of \(10^7\). Resistance markers to aminoglycosides, tetracyclines, chloramphenicol, trimethoprim and sulfonamides were co-transferred.\[7\]

In a study in China, transfer of resistance to ceftazidime, cefotaxime, ceftriaxone, gentamicin, amikacin, ciprofloxacin, aztreonam, cefoxitin and ticarcillin/CA and intermediate resistance to piperacillin/tazobactam, cefoperazone/sulbactam and cefepime was achieved from the clinical isolates of Klebsiella pneumoniae to Escherichia coli C600 by conjugation with a transfer frequency of \(10^6\) to \(10^7\).\[8\] The results of this study also reiterated the fact that difference in the genera did not affect the transfer of the resistance in any way.

A pool of resistance is developing in the non-pathogenic organisms found in humans, animals and the environment. In recent years, resistant bacteria have been isolated from apparently non-selective environments, including plants, coastal and estuarine environments, deep ocean water and sediments and drinking water. The release of the non-disinfected wastewaters into the marine/aquatic environment, a common worldwide practice, in underdeveloped as well as in highly developed countries, exerts enormous pressure on these environments. Both the resistant microorganisms and the residues of antibiotics administered to humans and animals reach the sewage systems in urine or feces in the form of either the parent compound or the degraded metabolites depending on the pharmacology of the specific antibiotic. Waste effluent from hospitals contains high numbers of resistant bacteria and antibiotic residues between a concentration of 1 and 100 \(\mu\)g/L, which has a potential to select for antibiotic resistance. The pathogenic organisms serve as a source from which non-pathogens can acquire genes conferring resistance and in turn, they can become resistant by acquiring the genes from the pathogens discharged into the environment. Thus, dissemination of the resistant bacteria...
An increasing body of evidence points at natural genetic transformation as an important mechanism of the horizontal exchange of genes. Natural genetic transformation is a gene transfer process where the bacteria can pick up the naked DNA from their environment. The DNA may come from a variety of sources, but the most frequent is remnants from dead bacterial cells. The process involves the binding of the exogenous DNA to specific cell surface receptors, then the DNA is transported across the membrane and one strand of the DNA is digested away. The DNA that enters the cell is thus single stranded. As long as the incorporated DNA is sufficiently homologous to the host DNA, recombination occurs and the new DNA replaces a strand of the host DNA. If the new DNA is of a different allelic nature than the host DNA, a gene conversion event can occur.[1] A study carried out on isolates of Enterobacteriaceae from 43 hospitals in the UK during 1990–1991 demonstrated transfer of resistance from the plasmid DNA extracted from three isolates of Klebsiella pneumoniae by transformation.[2] Another study in France also showed co-transfer of chloramphenicol, tetracycline and sulfonamide resistance with the CTX-M-1 enzyme using transformation experiments.[3]

The results of the study reiterated the fact that the well-established clones enhance their resistance phenotype by the acquisition of the new resistant genes via gene capture genetic units such as plasmids. The intra- and inter-generic transfer of resistant markers in the laboratory as well as in the environment points towards the availability of a large pool of resistance genes in the hospital setting as well as in the environment thus facilitating the long-term persistence of organisms in the selective environments. In this scenario, a multiclonal population structure of bacteria corresponds to a collection of different strains sharing resistance genes carried by horizontally transferred genetic structures.

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