Original Article

Role of efflux pumps inhibitor in decreasing antibiotic resistance of *Klebsiella pneumoniae* in a tertiary hospital in North India

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Abstract

Introduction: The contribution of efflux systems to drug resistance in *Enterobacteriaceae* is becoming increasingly appreciated. This study phenotypically analyzed the role of efflux mechanisms in resistance to ertapenem, doripenem, and tigecycline among clinical isolates of carbapenem-resistant *Klebsiella pneumoniae* (CRKP).

Methodology: Multidrug-resistant and carbapenem non-susceptible *K. pneumoniae* isolates were determined by disk diffusion test. Further susceptibility of these isolates to carbapenems, ceftriaxone, cefoperazone, ceftazidime, tigecycline, and colistin was determined by agar dilution assay, and CRKP was identified. While modified Hodge test was used to confirm carbapenemase production, the contribution of efflux mechanisms was determined by a minimum inhibitory concentration (MIC) reduction assay, and typing was done by enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR).

Results: Of the 238 isolates of *K. pneumoniae*, 174 were multidrug resistant and 74 were CRKP. Forty of the CRKP were positive for carbapenemase production, while 43, 11, and 2 of the CRKP isolates had elevated MIC of ≥ 32 µg/mL for ertapenem, doripenem, and tigecycline, respectively. Twofold or higher MIC reduction to ertapenem, doripenem, and tigecycline was observed in 6, 28, and 27 isolates, respectively; however, non-susceptibility to ertapenem, doripenem and tigecycline was abolished in 2, 11, and 18 *K. pneumoniae* isolates, respectively. Nine clones of CRKP widely distributed within the hospital were obtained from ERIC PCR.

Conclusions: Although colistin retained better activity against CRKP, efflux pumps contributed to increased MIC in ertapenem, doripenem, and tigecycline. Therefore, efflux systems are important aspects that should be explored in the fight against multidrug-resistant bacteria.

Key words: efflux mechanisms; MIC reduction assay; CCCP, carbapenems; tigecycline.


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Introduction

The increasing incidence of multidrug-resistant *Klebsiella pneumoniae* strains harboring carbapenemases has been reported across the globe [1]. They are frequent nosocomial pathogens that have a high propensity to acquire resistance genes and cause a wide variety of infections in humans. They have been shown to manifest all three broad mechanisms of drug resistance in Gram-negative bacteria: the acquisition of antibiotic catalytic gene, mutation of antibiotic target and membrane protein, and differential expression of specific genes such as those for efflux pumps, which mediate drug effects [2].

The balance of membrane permeability, which controls the traffic of various molecules, plays a key role in the influx and efflux of antibiotics [3]. In this circumstance, the intracellular antibiotic concentration is decreased, and bacteria become less susceptible to that compound [4]. Therefore, inhibiting the drug expulsion system offers a promising target, as it could increase intracellular drug concentration of antibiotics, restore drug susceptibility of resistant strains, and reduce capability for acquired additional resistance [3]. In this study, we analyzed phenotypically the contribution of efflux systems to doripenem, ertapenem, and tigecycline resistance in previously characterized carbapenem-resistant clinical isolates of *K. pneumoniae*.

Methodology

Isolation and characterization

Clinical isolates were collected and identified from various samples including urine, blood, sputum, endotracheal tube, pus aspirates, intravascular catheter tip, ascitic fluid, and wound swabs, received in the routine bacteriology section from in- and outpatients attending various departments of the university hospital. Samples were plated on cysteine lactose electrolyte deficient (CLED) agar or blood and MacConkey agar based on the type of specimen, and *K. pneumoniae* isolates were identified using standard bacteriological methods [5].
Susceptibility profile and screening for carbapenem-resistant
Klebsiella pneumoniae

The antibiotic susceptibility profiles of these isolates were determined by disk diffusion on Mueller-Hinton agar using Kirby-Bauer disk diffusion methods [6]. Briefly, two to three colonies of the test organism from an overnight culture were suspended in 2 mL of sterile normal saline and adjusted to match 0.5 McFarland turbidity standards. A sterile cotton swab was used to make a lawn of the test organism on Mueller-Hinton agar, and antibiotic disks were placed on the surface of the seeded plate with sterile forceps. The plate was incubated at 35°C for 16–18 hours, and *E. coli* ATCC 25922 was used as a control. The following disks were used: ampicillin (AMP, 10 µg), gentamicin (GEN, 10 µg), amikacin (AK, 30 µg), amoxicillin-clavulanic acid (AMC, 20 and 10 µg), piperacillin-tazobactam (PTZ, 100 and 10 µg), ceftriaxone (CTR, 30 µg), ceftazidime (CAZ, 30 µg), cefepime (CPM, 30 µg), cefotaxime (CTX, 30 µg), cefoperazone (CPZ, 75 µg), levofloxacin (LEV, 5 µg), ciprofloxacin (CIP, 5 µg), minocycline (MI, 30 µg), and aztreonam (ATM, 30 µg) (HiMedia, Mumbai, India). *Klebsiella pneumoniae* isolates showing nonsusceptibility to one or more antibiotic in three or more antimicrobial classes were considered multidrug-resistant *K. pneumoniae* [7]. Multidrug-resistant *K. pneumoniae* isolates that were not susceptible to any of the carbapenems by disk diffusion were further subjected to susceptibility analysis by determination of breakpoint minimum inhibitory concentration (MIC) by the agar dilution method to ertapenem (MSD-Chibret, Paris, France), meropenem (Zuventus, Maharashtra, India), imipenem (Ranbaxy, Delhi, India), doripenem (Aqua Vice Laboratory, Haryana, India), ceftazidime (GSK, Verona, Italy), ceftriaxone (Alkem, Mumbai, India), and cefotaxime (Akorn, Gurgaon, India) based on Clinical and Laboratory Standards (CLSI) guidelines and interpretative criteria [6]. Isolates not susceptible to any carbapenems and resistant to ceftazidime, ceftriaxone, and cefotaxime were considered carbapenem-resistant *K. pneumoniae* [8]. Furthermore, susceptibility of the carbapenem-resistant *K. pneumoniae* to ertapenem, doripenem, tigecycline (Gufic Bioscience, Gujarat, India), and colistin (Cipla, Mumbai, India) by the agar dilution method, at MIC beyond the resistance breakpoint, were determined using the CLSI 2013 interpretation criteria for carbapenems, the Food and Drug Administration (FDA) recommended guideline of susceptibility ≤ 2 and resistance ≥ 8, and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline of susceptibility ≤ 2 and resistance > 2 for tigecycline and colistin, respectively [9].

Determination of diffusible carbapenemase production

Evidence for carbapenemase production was analyzed using the modified Hodge test (MHT) based on the standard method. Briefly, a 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 mL of saline was prepared and 1:10 dilution was streaked as lawn onto a Mueller-Hinton agar plate. A 10 µg ertapenem susceptibility disk was placed in the center of the lawn made. The test organism was streaked in a straight line from the edge of the disk to the edge of the plate. The plate was incubated overnight at 35 ± 2°C for 16–24 hours [6].

Determination of efflux contribution to carbapenem and tigecycline resistance

To explore the effect of efflux mechanisms, an MIC reduction assay using carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) (HiMedia), a known efflux pump inhibitor, was used. In view of the bactericidal effect of CCCP at higher concentration, a predetermined concentration of 20 µg/mL that does not affect the growth of *K. pneumoniae* was used. The MIC of the test antibiotic to *K. pneumoniae* on Mueller-Hinton agar containing 20 µg/mL of CCCP was evaluated by the agar dilution method and then compared to the MIC without CCCP. A positive criterion for the contribution of efflux pumps to resistance was a two- to fourfold reduction in MIC of the antibiotic in the presence of the efflux inhibitor; however, fourfold MIC reduction represented significant efflux pump activity, indicating possible overexpression of the efflux pump gene [10].

Genotyping of carbapenem-resistant Klebsiella pneumoniae

Strain genotyping was done using enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR) and whole genomic DNA isolated by boiling and centrifugation. Briefly, a loopful of bacteria was suspended in 300 uL of double-distilled water and incubated in a water bath at 90°C for 15 minutes and then centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected and used for PCR amplification with ERIC1R (5’-ATGTAAGCTCCTGGG GATTCCAC-3’) and ERIC2F (5’-AAGTAAGTGACTGGGGTGAGCG-3’) primers as previously described [11]. The PCR reaction
condition included the initial denaturation at 94°C for 4 minutes followed by 36 cycles of amplification at 90°C denaturation for 1 minute, annealing at 49°C for 1 minute and extension at 70°C for 5 minutes, with a final extension at 72°C for 15 minutes [11]. The amplicons were electrophoresed on 2% agarose gel containing 0.5 mg/mL ethidium bromide alongside a 100 bp ladder [11]. The images were captured using a gel documentation system for further analysis.

Analysis of ERIC data

The ERIC banding patterns of the isolates were coded as 1 (band present) and 0 (band absent), and the binary data was statistically analyzed using NTSYS software. UPGMA clusters showing the genetic similarity of the isolates were plotted.

Results

A total of 238 isolates of K. pneumoniae, comprising 160 isolates from inpatient and 78 isolates from outpatient departments, were recovered during the study period. Urine samples accounted for the majority of the isolates (45.4%; 108/238), followed by blood (16.4%; 39/238), pus (14.7%; 35/238), sputum (6.7%; 16/238), and endotracheal tube (ETT) (5.5%; 13/238), while catheter tip, ascitic fluid, and swabs accounted for 11.3% (27/238) of the isolates.

The in vitro susceptibility data revealed minocycline as the most active agent, with the isolates showing a resistance rate of 17.2% (41/238), followed by piperacillin-tazobactam at 36.6% (87/213) and amikacin at 37.4% (89/238). Poor in vitro activity of cephalosporins (except cefepime) was observed (Figure 1). A total of 174 isolates were multidrug-resistant K. pneumoniae, of which 117 isolates were not susceptible to carbapenems, while 74 were carbapenem-resistant K. pneumoniae. Likely carbapenemase production among the carbapenem-resistant K. pneumoniae was observed in 40 isolates.

Level of resistance of the 75 carbapenem-resistant K. pneumoniae to doripenem, ertapenem, tigecycline, and colistin is shown in Table 1. A high MIC of ertapenem (> 16 µg/mL) was observed in 43 isolates, while 11 isolates had MIC > 32 µg/mL for doripenem, and only 2 isolates had MIC > 32 µg/mL for tigecycline (Table 1). On comparing the MIC of the isolates with and without CCCP, a twofold or higher MIC reduction in resistance to ertapenem, doripenem, and tigecycline was observed in 32% (24/75), 54.7% (41/75), and 61% (46/75) of isolates, respectively (Table 2).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>≤ 0.5 µg/mL</th>
<th>1 µg/mL</th>
<th>2 µg/mL</th>
<th>4 µg/mL</th>
<th>8 µg/mL</th>
<th>16 µg/mL</th>
<th>≥ 32 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ertapenem</td>
<td>0</td>
<td>9</td>
<td>5</td>
<td>6</td>
<td>0</td>
<td>12</td>
<td>43</td>
</tr>
<tr>
<td>Doripenem</td>
<td>16</td>
<td>5</td>
<td>13</td>
<td>15</td>
<td>3</td>
<td>6</td>
<td>17</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>2</td>
<td>9</td>
<td>11</td>
<td>24</td>
<td>20</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Colistin</td>
<td>1</td>
<td>70</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Twofold MIC reduction (%)</th>
<th>≥ Fourfold MIC reduction (%)</th>
<th>MIC reversed to susceptible breakpoint (%)</th>
<th>No change in MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ertapenem</td>
<td>11 (14.7)</td>
<td>13 (17.3)</td>
<td>18 (24)</td>
<td>51</td>
</tr>
<tr>
<td>Doripenem</td>
<td>25 (33.3)</td>
<td>16 (21.3)</td>
<td>28 (37.3)</td>
<td>34</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>28 (37.3)</td>
<td>18 (24)</td>
<td>36 (48)</td>
<td>29</td>
</tr>
</tbody>
</table>

Figure 1. Resistance of K. pneumoniae to commonly used antibiotics

PP: piperacillin
The *K. pneumoniae* isolates were distributed in several clusters comprising 9 clones of 39 isolates, with clones A and B being the most prevalent (Figure 2). In addition, a wide distribution of the prevalent clone over time and within the hospital was observed.

**Discussion**

Increasing evidence linking drug efflux mechanisms to resistance has been observed [3,12]. Although high-level resistance may not occur as a result of multidrug efflux alone, the contribution of this mechanism along with other resistant mechanisms in clinically resistant strains of *Enterobacteriaceae* cannot be overemphasized [12]. This study provided an insight into the current incidence of multidrug-resistant *K. pneumoniae* from our health center, and consequently the contribution of efflux pumps to increased MIC among resistant isolates.

In the present study, 67.2% of *K. pneumoniae* were isolated from inpatients. Urine samples accounted for the highest proportion of isolates in all the samples analyzed, followed by blood samples. This result highlighted the importance of *K. pneumoniae* in causing infection among patients housed within hospitals, and also the involvement of *K. pneumoniae* in urinary tract infections. Several studies around the globe have reported *K. pneumoniae* as an important pathogen in bloodstream, urinary tract, respiratory, and other infections. A recent study in the northern part of India reported 87.6% multidrug-resistant phenotype among clinical isolates of *K. pneumoniae* [13]. Similarly, reports from other parts of India have indicated a high prevalence of multidrug-resistant *K. pneumoniae* in clinical isolates [14,15]. In the same vein, a prevalence rate of 72.7% multidrug-resistant *K. pneumoniae* isolates was observed at our center. The variation in the prevalence of multidrug-resistant *K. pneumoniae* observed in our study compared to the studies from northern India underscore the need for continued local surveillance, as resistance patterns could vary between regions and even within different units in the same hospital.

The overall high rate of resistance to commonly used antibiotics (Figure 1), especially the poor performance of cephalosporin on *K. pneumoniae*, highlights the declining efficacy of these classes of antibiotics. Although carbapenems have been used as last-line drugs to tackle infection due to multidrug-resistant *Enterobacteriaceae*, the susceptibility rate of 67.3% to ertapenem, 50.4% to imipenem, 54.0% to meropenem, and 48.7% to doripenem, and occurrence of 42.8% carbapenem-resistant *K. pneumoniae* among the multidrug-resistant *K. pneumoniae* shows that the activity of this class of antibiotic has been compromised. Furthermore, the phenotypic expression of carbapenemase activity in 54.1% of the carbapenem-resistant *K. pneumoniae* indicates the presence of resistance mechanisms other than carbapenemases [16]. Earlier studies have reported that extended spectrum beta-lactamases (ESBLs) have reached an epidemic proportion and are widely distributed in India [17]. The ESBLs TEM, SHV, CTX-M, and over-expression of both acquired and constitutive AmpC, along with loss of outer membrane protein, could lead to increased non-susceptibility to carbapenems among the *Enterobacteriaceae*. These factors could have accounted for the level of carbapenemase resistance observed in our study, as 41.9% of the CRKP isolates did not show evidence for diffusible carbapenemase activity.

As therapy in clinical practice, tigecycline and colistin were intended to be used as reserve antibiotics in cases of treatment failure of the carbapenems. Even though initial successes were recorded, they were short lived; resistance to this group of antibiotic has been reported in several laboratories across the world [18-20]. A previous study conducted on 60 ertapenem-resistant *K. pneumoniae* revealed a susceptibility rate of 58.3% to colistin and 50.0% to tigecycline [21]. Similarly, a study in the United Kingdom on susceptibility of 81 CRE isolates by agar dilution showed that chloramphenicol, ciprofloxacin, and nitrofurantoin inhibited less than 25%, whereas colistin was active on 92.6% and tigecycline on 46.9% of the isolates [22]. The susceptibility rates of 75 CRKP to tigecycline and colistin were 29.3% and 94.7%, respectively, in this study. In view of the already escalated problem associated with multidrug-resistant MDR *K. pneumoniae* [23], and their ability to
express resistance to a last-resort drug, particularly tigecycline, is a source of great concern (Table 1).

Efflux pumps, which reduce drug effect, have been reported as one of the mechanisms of resistance in K. pneumoniae [3]. Studies have shown that targeting the efflux pump system could obviate other resistance mechanisms and reverse multidrug resistance, thus restoring the efficacy of antibiotics [24]. In this study, we observed that susceptibility to ertapenem, doripenem, and tigecycline was increased two- to fourfold in the presence of CCCP (Table 2); we also found that non-susceptibility to ertapenem, doripenem, and tigecycline was completely abolished in 24%, 37.3%, and 48% of the isolates, respectively. The complete reversion to susceptible breakpoint and reduction in MIC suggest the involvement of the drug efflux system in conferring resistance to these antibiotics. Interestingly, tigecycline, which is the most potent agent in this study, retained the highest activity in the presence of CCCP, followed by doripenem. This finding confirmed the involvement of efflux activity in contributing to resistance among the isolates, since when efflux activity was inhibited, inhibitory activity of the drug was observed based on the comparative potency of the antibiotics.

As bacteria continue to express multiple resistance mechanisms to antibiotics, there is a need to develop drugs that are capable of attacking resistance simultaneously at different fronts. The restoration of sensitivity and reduction of MIC to antibiotic by CCCP suggest that successfully designed efflux pump inhibitors for use in clinical practice could offer some hope. Recently, studies have shown that quorum sensing and biofilm production are intimately tied to proton motive force-dependent efflux systems of organisms [25,26]. Consequently, inhibiting the efflux pump system resulted in the inhibition of quorum sensing responsible for biofilm formation [24].

A diverse pattern of CRKP genotypes from our health center was observed. All the clones were widely distributed within the hospital. Isolates were mainly from inpatients, and were scattered throughout the eight-month period of isolation. This observation suggests the persistence and circulation of several clones of CRKP within the hospital environment, thus rendering them potential reservoirs for resistance gene transmission to other members of Enterobacteriaceae [27]. A and B clones were the major clones, comprising 13 and 9 isolates, respectively. They showed less variation with respect to carbapenem resistance; however, the MIC value for tigecycline in B clones was higher than in A clones. This may suggest the inability of our typing method to discriminate carbapenem resistance. While B clones were isolated from diverse samples from the intensive care unit (ICU) of the new medical and urology ward of our center, A clones were recovered from catheter tips and urine samples mainly from the urology ward. The recovery of isolates in A clones from urine samples mainly from the urology ward and B clones from the new medical and urology ward, which incidentally occupy the same floor, and their distribution within three months of isolation, suggests a possible clonal persistence within this unit. The isolation of a B clone from a sample from the ICU could have been from a patient sample transferred from any of the new medical or urology ward to the ICU, although we could not verify this claim. Therefore, great awareness, monitoring drug resistance patterns and sources of infection within clinical settings, coupled with observing a strict infection control policy, are needed to control the circulation of pathogen.

Conclusions

High prevalence of multidrug-resistant K. pneumoniae was observed. While other mechanisms could be involved in the resistance to carbapenems other than carbapenemases, we demonstrated the contribution of efflux systems in resistance to ertapenem, doripenem, and tigecycline in some isolates. This suggests a connection between antibiotic use in combination with efflux inhibitors, thus opening up an area for consideration in the fight against multidrug resistance in bacteria.

References


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**Conflict of interests:** No conflict of interests is declared.