Original Article

Prevalence of Multidrug-resistant, Extensively Drug-resistant, and Pandrug-resistant Phenotypes among *Klebsiella pneumoniae* Collected from Referral Therapeutic Centers in Sari, North Iran

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Abstract

Aims: *Klebsiella pneumoniae* is an important cause of nosocomial infections. The present study sought to detect resistance status and the frequency of multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) *K. pneumoniae* strains isolated from hospitalized patients. **Materials and Methods:** Confirmation of *K. pneumoniae* isolates was performed by polymerase chain reaction (PCR) for *rpoB* fragment. Drug susceptibility was done by disk diffusion method based on the Clinical and Laboratory Standards Institute. According to the susceptibility pattern, the strains were categorized as MDR, XDR, and PDR. **Results:** On the basis of PCR results, *rpoB* gene was identified in all 100 *K. pneumoniae* isolates. Most *K. pneumoniae* strains showed a high percentage of resistance against ampicillin-sulbactam (93%). On the other hand, tigecycline and fosfomycin were active against 100% and 90% isolates, respectively. The multiple resistance analysis of the strains showed that 58% and 13% of isolates were identified as MDR and XDR, respectively. Overall, no PDR isolate was detected in any tested strains. **Conclusion:** Our results demonstrated that the vast majority of *K. pneumoniae* strains indicated the MDR phenotype including a high resistance rate to common antibiotics. Therefore, it is suggested to implement antimicrobial susceptibility testing before prescribing, to assist in selecting the most effective agents within the antimicrobial categories for the treatment of infections with multiple antibiotic-resistant *K. pneumoniae* strains in hospitalized patients.

Keywords: Extensively drug-resistant, fosfomycin, *Klebsiella pneumoniae,* multidrug-resistant, nosocomial infections

Introduction

The word "ESKAPE" covers six groups of bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.) with developing rate of resistance to available antimicrobial agents and pathogenicity. ESKAPE pathogens are responsible for most health-care-associated infections.[1,2] The *K. pneumoniae* belongs to the Enterobacterales family and is a Gram-negative opportunistic pathogen that is encapsulated and nonmotile.[3] The *K. pneumoniae* can cause pneumonia, urinary tract infections, bloodstream infections, and liver abscesses in hospitalized and/or immunocompromised patients.[4] *K. pneumoniae* uses diverse virulence factors during infection to avoid immune-mediated clearance.[5] A variety of virulence factors are found in this bacterium, including capsular antigen, lipopolysaccharide, adhesions,

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siderophores, biofilms, fibrils, and toxins. Increasing antibiotic resistance and the emergence of infection are closely linked to these factors.[1,4,5] Currently, *K. pneumoniae* displays a considerable resistance to a broad spectrum of antimicrobial agents, including beta-lactam, fluoroquinolones, and aminoglycosides.[6,7] Meanwhile, infections caused by multidrug-resistant *K. pneumoniae* (MDRKp) strains are getting increasingly difficult to treat worldwide as they become increasingly resistant to antibiotics.[7,8] Although several studies were performed in different parts of Iran on the drug resistance of this bacterium and the rate of MDR, extensively drug-resistant, (XDR), and pandrug-resistant (PDR) strains, there is a limited study on the frequency of this resistance phenotypes among *K. pneumoniae* strains, especially in northern Iran, Mazandaran.[9-11] Increasing the emergence of multiple drug resistance among *K. pneumoniae* strains has been a big challenge to clinicians. Moreover, limited treatment options are also associated with high morbidity and mortality for hospitalized patients. The characterization of drug susceptibility patterns will help clinicians make the best management decisions and help to prevent and control infections caused by resistant strains of *K. pneumoniae*. Considering this evidence, this research was designed to investigate antibiotic susceptibility pattern and the rate of MDR, XDR, and PDR *K. pneumoniae* isolated from clinical specimens of teaching hospitals of Mazandaran University of Medical Sciences (MAZUMS) located in Sari, Iran.

Materials and Methods

This project was approved by the Ethics Committee of MAZUMS (Approved Number: IR.MAZUMS.REC.1398.628). Although, we did not have a direct connection with the patients. We only obtained the clinical samples of the patients without their names from the hospital laboratories, and the data were kept secret by the authors. In this cross-sectional study, a total of 100 nonduplicate *K. pneumoniae* isolates from clinical samples were collected from inpatients, who were referred to three teaching hospitals of MAZUMS during November 2018 to October 2019. Clinical samples were obtained as portion of the common diagnostics from hospitalized patients and included samples from sterile and/or nonsterile body sites. The samples were cultured on blood agar and MacConkey agar (Merck, Darmstadt, Germany) and incubated at 37°C for 24 h. In order to identification of isolates, standard microbiological tests were conducted, including oxidase; citrate; triple sugar iron; urease; lysine decarboxylase; sulfide production, indole production, and motility; methyl red; and Voges–Proskauer tests.[12] Furthermore, the sex and age of the patients and the source of samples were documented. The *K. pneumoniae* isolates were stored at −70°C in trypticase soy broth (Merck, Darmstadt, Germany) plus 20% glycerol.

Confirmatory assessment of *K. pneumoniae* at species level was accomplished by polymerase chain reaction (PCR) of *rpoB* gene.[13] Briefly, a single colony of each strain was grown at 37°C in Mueller–Hinton agar plate. The total genomic DNA of tested isolates was extracted as previously described.[14] After extraction, NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) was used for assessment of the extracted DNA yield/purity. Amplification of *rpoB* gene among the tested strains was achieved with the following species/specific primers: (F: 5′-CAACGGTGTGGTTACTGACG-3′/R: 5′-TCTACGAAGTGGCCGTTTTC-3′) (BIONEER company).[15] In order to make sure of the primer specificity, its sequence is rechecked and confirmed in BLAST-NCBI primer program. *K. pneumoniae* American Type Culture Collection (ATCC) 700603 were used as positive control. Negative controls (all essential components of the amplification reaction except the template) were considered for every run of PCRs. Reactions were done in a thermal cycler system (Bio-Rad, USA). PCRs were carried out in 25-µl reaction volumes with 14 µL of ready-to-use 2X Master Mix (Amplicon), 6 µL of chromosomal DNA (50 ng), 1 µL (20 pM/µL) of each primer (forward/reverse), and 3 µl of sterile distilled water. PCR reactions were achieved by thermal cycler (Eppendorf, Germany) in the following steps: step 1 (initial denaturation): 95°C 10 min, step 2 (denature): 95°C 30 s, step 3 (anneal primers): 52°C 40 s, step 4 (extend DNA): 72°C 50 s, and step 5 (final extension): 72°C 5 min. Step 2/3/4 was repeated in 35 cycles. PCR products were electrophoresed in a 1% (w/v) agarose gel alongside a DNA size marker 100 bp (Cat No. DM003-R500).

According to the Clinical and Laboratory Standards Institute documents, disk diffusion was used to determine susceptibility and/or resistance to 18 antimicrobial agents.[16] A panel of 18 antimicrobial agents from different classes were tested, including trimethoprim-sulfamethoxazole (SXT, 1.25/23.75 µg), cefepime (FEP, 30 µg), amikacin (AN, 30 µg), imipenem (IPM, 10 µg), meropenem (MEM, 10 µg), cefotaxime (CTX, 30 µg), gentamicin (GM, 10 µg), ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30 µg), ciprofloxacin (CIP, 5 µg), levofloxacin (LEV, 5 µg), nitrofurantoin (NIT, 300 µg), ampicillin/sulbactam (SAM, 20 µg), ertapenem (ETP, 10 µg), cefoperazone (CEP, 75 µg), tetracycline (TE 30 µg), and fosfomycin (FOS, 200 µg) (MAST UK). In summary, a bacterial suspension equal to #0.5 McFarland standard (1.5×10^8 CFU/mL) was prepared from overnight cultures and then cultured on Mueller–Hinton agar (Merck, Darmstadt, Germany). Then, antibiotic disks were put in plate. After 24 h of incubation at 37°C, the zone of the inhibition was measured and the data were reported as susceptible (S), intermediate (I), and resistant. *Escherichia coli* ATCC 25922 was used as a quality control. In all analyzed strains, tigecycline (TGC, 15 µg) (HiMedia) disk was done to investigate the trend of TGC susceptibility.[17] In this study, MDR and XDR phenotypes among the strains were defined as an instruction previously described by Basak *et al*.[18]

The data derived from this study were analyzed by SPSS (Statistical Package for the Social Sciences, Chicago, IL, USA) statistical program (ver. 16) using Chi-square and Fisher's exact statistical tests at the confidence level of 95%.

Results

In this study, among the entire samples, 58% of the subjects were female and 42% were male. Based on Chi-square test, there was no significant relationship between relative antibiotic resistance and gender of patients ($P > 0.05$). In our study, *rpoB* gene was detected in all 100 *K. pneumoniae* isolates. Therefore, *rpoB* can be a good marker for identification of *K. pneumoniae* at the species level [Figure 1]. Of the 100 K. pneumoniae isolates, 64 (64%) were isolated from urine, 15 (15%) from tissue, 10 (10%) from blood, 7 (7%) from wound and 4 (4%) from sputum samples. The age range of the patients was 5–90 years. The isolates were obtained from patients in different age groups: 1–19 years (n = 5), 20–39 (n = 22), 40–59 years (n = 45), 60–79 years (n = 19), and 80–100 (n = 9). The overall susceptibility, intermediate, and resistance were determined, and the outcomes are displayed in Figure 2. Most *K. pneumoniae* isolates indicated a high ratio of resistance against studied antibiotics: SAM (93%), NIT (57%), CEP (52%), SXT (50%), CAZ (49%), CTX (48%), MEM (42%), CRO (42%), FEP (41%), CIP (34%), IPM (33%), LEV (29%), GM (23%), ETP (23%), TE (22%), AN (8%), FOS (2%), and TGC (0%). Table 1 shows the details of the antibiotic resistance profile of the strains based on antimicrobial class. Furthermore, in our study 13% of the strains were XDR and 58% were MDR. The antimicrobial resistance among MDR strains was found meaningfully higher than that of non-MDR strains ($P < 0.05$). It should be noted that no PDR isolate was detected in this study.

Discussion

K. pneumoniae is one of the major causes of community-and hospital-acquiring infections. It is thought that this bacterium is one of the most common pathogens that cause high rates of mortality in hospitals.[19-21] It is unfortunate that the increase in antibiotic resistance is becoming a serious threat to controlling infectious diseases caused by this pathogen.[22]

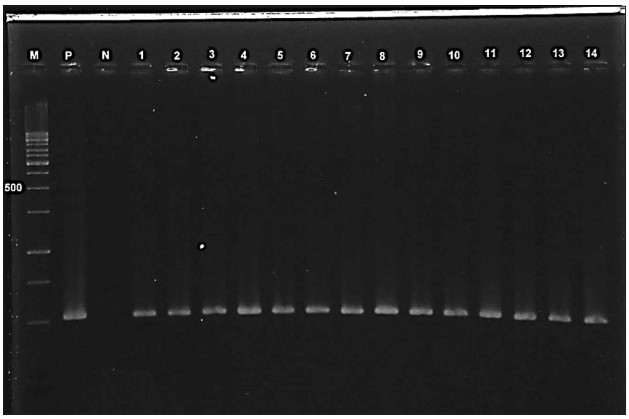

Figure 1: PCR amplification fragments for the detection of *rpoB* gene (108 bp) among *Klebsiella pneumoniae* isolates. Lane: M: 100 base-pairs (bp)-3K DNA size marker, Lane P: Positive control, lane N: Negative control, lanes 1–14: Tested strains, PCR: Polymerase chain reaction

Recent years have seen an increase in infections caused by MDR/XDR and PDR K. pneumoniae strains among critically ill patients, which are often associated with limited treatment options. The main subject of managing these infections is to find an effective antibiotic regimen or alternative treatment strategies.[18,23] Based on the results of the current study the highest rate of resistance was observed for SAM (93% resistant and 6% intermediate) and the lowest for FOS (2%). In addition, no resistant isolates against TGC were found. In our study, a small number of *K. pneumoniae* isolates (only 1%) were susceptible to SAM. Therefore, we concluded that SAM could not be a good choice for the initial empiric antimicrobial therapy in hospitalized patients infected with MDR *K. pneumoniae* strains. This result is in agreement with the outcomes stated by prior work conducted by Qadeer *et al*. in Pakistan.[24] Carbapenems were commonly used for treating infections caused by *K. pneumoniae*.[25] Three members of the carbapenem antibiotics were investigated in our study. The isolates' susceptibility rates to ETP (73%), IPM (65%), and MEM (56%) were consistent with those reported in another study in Tehran, Iran.[26] Gheitani *et al*., in their work which was directed in Isfahan, Iran, reported that 28%, 24%, and 44% of the studied *K. pneumoniae* isolates were susceptible against MEM, IPM, and ETP, respectively.[27] These inconsistent outcomes may be related to the variable sample sizes and the geographical areas. In this study, the resistance to LEV and CIP antibiotics has been achieved 34% and 29%, respectively. According to Akya et al.,[28] study in Kermanshah, Iran, CIP and LEV resistance rates are 28%, similar to our findings for quinolone antibiotics. These data are similar to Yedekci *et al*.[29] Another study showed that of the 142 K. pneumoniae

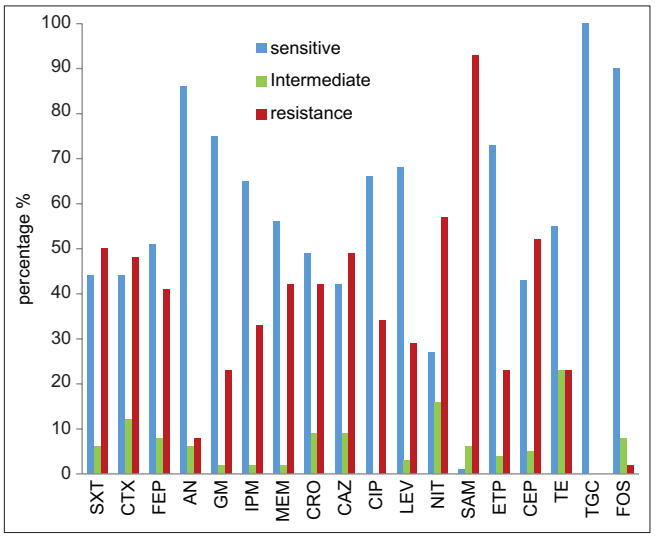

Figure 2: Antimicrobial resistance profiles of 100 *Klebsiella pneumoniae* isolates against 18 antibiotics. R: Resistance, S: Sensitive, and I: Intermediate. SXT: Trimethoprim-sulfamethoxazole, FEP: Cefepime, AN: Amikacin, IPM: Imipenem, MEM: Meropenem, CTX: Cefotaxime, GM: Gentamicin, CRO: Ceftriaxone, CAZ: Ceftazidime, CIP: Ciprofloxacin, LEV: Levofloxacin, NIT: Nitrofurantoin, SAM: Ampicillin/sulbactam, ETP: Ertapenem, TGC: Tigecycline, CEP: Cefoperazone, TE: tetracycline, FOS: Fosfomycin

Table 1: Susceptibility testing profile of 100 *Klebsiella pneumoniae* isolates based on antimicrobial class

Antimicrobial group	Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
β-lactam combination agents	Ampicillin/sulbactam	1	6	93
Carbapenems	Imipenem	65	2	33
	Meropenem	56	2	42
	Ertapenem	73	4	23
Cephems	Cefotaxime	40	12	48
	Ceftriaxone	49	9	42
	Cefepime	51	8	41
	Ceftazidime	42	9	49
	Cefoperazone	43	5	52
Aminoglycosides	Amikacin	86	6	8
	Gentamicin	75	2	23
Tetracyclines	Tetracycline	55	23	22
Folate pathway antagonists	Trimethoprim-sulfamethoxazole	44	6	50
Quinolones and fluoroquinolones	Ciprofloxacin	66	0	34
	Levofloxacin	68	3	29
Nitrofurans	Nitrofurantoin	27	16	57
Fosfomycins	Fosfomycin	90	8	2
Glycylcycline	Tigecycline	100	0	0

isolates analyzed, 76% were resistant to CAZ, and 73% were resistant to CTX and FEP.[30] In our study, the rate of third-generation cephalosporin resistance was 48%, 41%, 42%, and 49% against CTX, FEP, CRO, and CAZ, respectively. Various studies have shown controversial results with regard to the effectiveness of AN.[31,32] In a study from India, among 73 *K. pneumoniae* isolates, 74% were resistant to AN.[33] Furthermore, other studies from Iran such as Bayati *et al*.[34] and Azimi *et al*.,[35] the rate of susceptibility to AN was 48% and 79%, respectively. In the study conducted by Asadpour and Nahavandinejhad,[36] from northern of Iran in 2015, GM and AN were the most appropriate antimicrobial agents against *K. pneumoniae* isolates. The difference between the results is probably due to the difference in source of clinical samples, the quality of antimicrobial disks and/or culture medium, and geographical regions. The rates of MDR and XDR isolates detected in this study are worrying. The prevalence of MDR and XDR phenotypes among our isolates was found to be 58% and 13%, respectively. The rate of MDR and XDR strains observed in this study was similar to previously reported rates from Iran and China,[37,38] and it was higher than reported rates from a prospective surveillance study in 10 Asian countries.[39] This could be due to the difference on the study region population, geographical site, and type of antibiotics used in the treatment regimens. According to the results of the current study, all of collected *K. pneumoniae* were susceptible to TGC. Bokaeian *et al*. in 2018 determined that TGC is the most effective antibiotic for the treatment of *K. pneumoniae* infections.[40] Shokri from Iran stated that the resistance rate of the *K. pneumoniae* isolates to TGC in their study was 3.1%.[41] Fortunately, TGC also can be used in antibiotic therapy if MDR *K. pneumoniae* was isolated.[42,43] However, the use of it should be the main focus in hospital settings. This study has some limitations: results of this cross-sectional study were derived from the patients referred from three teaching health facilities, which do not represent the actual view of the Sari city in Mazandaran Province. There was a lack of adequate demographic information and underlying diseases about the studied patients. Furthermore, in order to reach better outcomes, detection of common antibiotic-resistance genes is also necessary.

Conclusion

In summary, the high prevalence of MDR strains of *K. pneumoniae* is alarming in our study. Therefore, it is suggested to implement antimicrobial susceptibility testing before prescribing, to assist in selecting the most effective antimicrobial agents within the antimicrobial categories for the treatment of infections caused by *K. pneumoniae* in hospitalized patients. In our work, TGC was the only antibacterial agent that inhibited 100% *K. pneumoniae* strains. Hence, the use of TGC and FOS in combination with other antibiotics are useful to treatment of infection caused by MDR *K. pneumoniae* isolates. Furthermore, timely identification and systematic monitoring and surveillance of highly resistant strains in hospital settings will help to prevent the spread of these strains. Multicenter research is essential to gaining a comprehensive understanding of antibiotic resistance in other Iranian regions.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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