Antibiotic Susceptibility Pattern and Distribution of Virulence Factors Among Klebsiella pneumoniae Isolated from Healthy Volunteers

Amine Dalil1,2, Shabnam Razavi1,2*, Malihe Talebi1,2, Faramarz Masjedian Jazi1,2, Maryam Mirshekar2, Vahid Lohrasbi2

1. Microbial Biotechnology Research Center, Iran University of Medical Sciences, Tehran, Iran
2. Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Tehran, Iran

10.30699/ijmm.15.6.676

ABSTRACT

Background and Aim: The healthy people’s fecal flora in the community represents a large potential reservoir. Therefore, the current study aimed to detect antibiotic resistance patterns and virulence factors in Klebsiella pneumoniae isolated from healthy volunteers’ feces.

Materials and Methods: Three hundred and fifty stool specimens were collected from sales rep healthy individuals referring to the Northwest Tehran Health Centers to get a health card. Bacterial isolation, identification, and antimicrobial susceptibility testing were conducted according to the routine instructions. In addition, polymerase chain reaction (PCR) was used to detect the genetic factors responsible for producing extended-spectrum β-lactamas (ESBLs: SHV, TEM, and CTX-M) blaKPC and other virulence genes.

Results: Among fecal samples analyzed, 60 (17.1%) K. pneumoniae were isolated. The results demonstrated that the highest resistance rate was related to piperacillin-tazobactam (n=25, 41.6%), followed by and meropenem (n=17, 28.8%) and co-trimoxazole (n=11, 18.3%), respectively. Also, all strains were susceptible to amikacin, gentamicin, and imipenem. The PCR results of the virulence gene showed that 95% (n=57) of isolates were positive for fimH gene, 93.33% (n=56) for βss5 gene, 27 (45%) for rmpA gene. The PCR results for antibiotic resistance genes showed that 41.66% (n=25) had blaTEM gene, 38.33% (n=23) blaCTX-M gene, 35% (n=21) blaSHV gene and 3.33% (n=2) isolates had blaKPC gene, and none of these isolates carried magA gene.

Conclusion: Antibiotic resistance was common among K. pneumoniae isolated from healthy volunteers’ feces who participated in this study. Transmission of resistant bacteria and plasmids through oral-fecal sources threatens to the public, which could complicate treatment options for community-acquired infections caused by K. pneumoniae.

Keywords: Antibiotic resistance, ESBL, Klebsiella pneumoniae, Virulence genes

1. Introduction

Klebsiella pneumoniae is an opportunistic Gram-negative bacterium associated with hospitals- and community-acquired infections. K. pneumoniae has evolved into two particular pathotypes: classical and hypervirulent K. pneumoniae (1). Classical K. pneumoniae has been called a superbug dependent on its acquisition of different antimicrobial resistance genes and is basically responsible for hospital-acquired infections (2). Hypervirulent strains are more virulent than classical strains and are initially described to cause community-acquired infections (3).

The increasing global prevalence of antimicrobial-resistant bacteria, both in the hospital and in the community, jeopardizes the ability to effectively treat patients (4). Because these bacteria often show
multidrug resistance (MDR) phenotypes, treatment options are severely limited (5). Therefore, it can be assumed that patients with serious infections will soon no longer be treatable with existing antimicrobial drugs. This resistance is mainly because of plasmids containing extended-spectrum β-lactamase (ESBL). ESBL enzymes in K. pneumoniae are mainly consisted of two types of enzymes SHV and TEM encoded by the plasmid, and were often associated with nosocomial outbreaks (6). Furthermore, there is an increasing rate of K. pneumoniae strains producing CTX-M type, and they are now considered to be the primary ESBL producers that are almost always associated with community-acquired infections (7, 8). Because the genes are simply spread via plasmid among the Enterobacteriaceae family, they can simplify the transmission of resistance to beta-lactams and other antibiotics (9). Moreover, the pathogenicity of K. pneumoniae is because of diverse virulence factors that empower the bacteria to conquer innate host immunity and to cause infection in a host (9, 10). K. pneumoniae virulence factors are lipopolysaccharides, capsule, the ability of biofilm formation, hyper-mucoviscosity, iron acquisition systems, serum resistance, and adhesins (11, 12).

K. pneumoniae with potency in acquiring antimicrobial resistance genes and pathogenicity can be asymptptomatically carried in the intestinal tract, nose, throat, and skin of healthy individuals. Studies of the gut flora of humans and animals give a more precise picture of the entire burden of antimicrobial resistance in society than data based on pathogenic isolates. As a matter of fact, the study of gut flora has shown that they may act as a reservoir for plasmids harboring antibiotic resistance genes, especially ESBL (13, 14). Moreover, drug resistance surveillance has reported that asymptomatic carriers in the population are mostly colonized with resistant bacteria, resulting in the infection (15, 16). So the study of gut flora is helpful in the understanding and monitoring of resistance mediated selection in the community (17). In addition, fecal carriage of antibiotic-resistant K. pneumoniae can be a community reservoir and lead to outbreaks. The current study was aimed to detect the antibiotic resistance pattern, the genetic determinants responsible for ESBL production (SHV, TEM, and CTX-M), carbapenem resistance (blaKPC), and other virulence genes [mqaA (Mucoviscosity associated gen), rpmA (hypermucoviscosity), fimH (fimbriae gene), BssS (biofilm formation)] in K. pneumoniae isolated from healthy volunteer’s feces.

2. Material and Methods

Sample Collection and Questionnaire

The cross-sectional descriptive study was conducted among 350 fresh stool samples randomly selected from healthy volunteers (healthy volunteers to get a health card) submitted to the Northwestern Tehran Health Center between February and October 2018. A questionnaire was prepared for every volunteer who participated in the study to collect data regarding name, gender, age, medical history (History of hospitalization or antacid and antibiotic intake), dietary habits (cheese, yogurt, milk, chicken, or meat consumption), and personal hygiene habits. People who had a history of using antibiotics, probiotics, and prebiotics and people with a history of chronic gastrointestinal infections were excluded from the research. The stool samples were cultured on MacConkey agar plates (Merck, Germany) and incubated at 37°C for 24 hours. The identifying process for K. pneumoniae isolates carried out using Gram staining and biochemical testing (methyl red, indole, citrate, vogus-prauskaur, urease, and motility).

Susceptibility Testing

An antimicrobial susceptibility testing was done for each pure isolation by Kirby-Bauer disc diffusion method following the Clinical and Laboratory Standard Institute guidelines (2018). The following antibiotics were used: ceftriaxone (30 μg), piperacillin tazobactam (100 μg), co-trimoxazole (1.25 μg), amikacin (30 μg), cefotaxime (30 μg), ciprofloxaxine (5μg), ceftazidime (30 μg), cefepime (30 μg), meropenem (10 μg), imipenem (10 μg), and gentamicin (10 μg). Escherichia coli ATCC 25922 was used as negative controls (18).

MIC was performed to detect susceptibility to imipenem, meropenem, ciprofloxaxine and ceftriaxone using E-test strip (Lioflichem, Italy), following the manufacture’s protocol. Briefly, Mueller-Hinton agar plates (Merck, Germany) were inoculated with K. pneumoniae strain suspensions adjusted to 0.5 McFarland standard turbidity and E-test strips were used. The MIC for each isolate was measured by reading the value at the point of intersection of the inhibition zone with the point on the scale of the E-test strip.

Analysis of Resistance and Virulence Genes using PCR Method

The extraction of bacterial DNA was performed by the boiling method (19). First, a colony of a bacterial isolate was added to 20 μL of tissue buffer consisting of 0.05 M NaOH + 0.25% SDS and incubated for 10 minutes at 95°C. After incubation, the mixture was centrifuged at 13,000 g for one minute, and 180 μL of Q water was added. The extracted DNA was measured by reading the point on the scale of the MIC was performed to detect susceptibility to imipenem, meropenem, ciprofloxaxine and ceftriaxone using E-test strip (Lioflichem, Italy), following the manufacture’s protocol. Briefly, Mueller-Hinton agar plates (Merck, Germany) were inoculated with K. pneumoniae strain suspensions adjusted to 0.5 McFarland standard turbidity and E-test strips were used. The MIC for each isolate was measured by reading the value at the point of intersection of the inhibition zone with the point on the scale of the E-test strip.

Table 1. Polymerase Chain Reaction (PCR) was performed in a total volume of 25 μL containing 12.5 μL of PCR Master Mix x2 (Amplicon, Denmark), 1 μL of each Forward and Reverse primer, 1 μL of DNA template, 9.5 μL sterile deionized distilled water, then DNA...
amplification was carried out with the thermocycler (Thermo Scientific, USA). *Escherichia coli* ATCC 25922, *K. pneumoniae* Strains ATCC 700603, and ATCC 7881 were used as controls.

Table 1. The sequences of primers used in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Sequence 5’ to 3’</th>
<th>Product size</th>
<th>Sources</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>magA</td>
<td>GCCAGTCCGAAGTGGAACGA</td>
<td>162</td>
<td>This study</td>
<td>56</td>
</tr>
<tr>
<td>RmpA</td>
<td>TTCAAGGGAAAGGGGAGGTT</td>
<td>250</td>
<td>This study</td>
<td>60</td>
</tr>
<tr>
<td>fimH</td>
<td>ATCGTTATCGCGGTGCGTA</td>
<td>274</td>
<td>This study</td>
<td>54</td>
</tr>
<tr>
<td>BssS</td>
<td>AGCTACGATGGCAGTGATGGTT</td>
<td>148</td>
<td>This study</td>
<td>58</td>
</tr>
<tr>
<td>Blakpc</td>
<td>GCCGTTACTTTGCTGCTGCT</td>
<td>141</td>
<td>This study</td>
<td>60</td>
</tr>
<tr>
<td>blaCTXM</td>
<td>CGCGTGATACCAAATCTCCCT</td>
<td>77</td>
<td>This study</td>
<td>60</td>
</tr>
<tr>
<td>blaSHV</td>
<td>ACAGCTGCGCTGCTTTGAGGG</td>
<td>85</td>
<td>This study</td>
<td>60</td>
</tr>
<tr>
<td>blaTEM</td>
<td>CGTGTCGCCCTTATTCCCTT</td>
<td>480</td>
<td>This study</td>
<td>60</td>
</tr>
</tbody>
</table>

The PCR amplification consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 40 s, annealing for 40 s at temperatures specified for each primer, and extension at 72°C for 30 s. This was followed by a final extension step at 72°C for 5 minutes. The PCR products were electrophoresed using 1% agarose gel and visualized by a gel documentation system.

3. Results

The stool samples of 350 healthy individuals were collected from Northwestern Tehran Health Centers during a period of 8 months, from February 2018 to October 2018. The specimens were examined for microbial and biochemical tests in the laboratory of the Medical School of Iran University of Medical Sciences for the presence of *K. pneumoniae*. Of the 350-stool sample, all belonging to male individuals, only 60 isolates were identified as *K. pneumoniae*. In this study, the age range was 20-42 years, 67% were under 30. About 49% of these people also had nutrition-related occupations (Figure 1). Among *K. pneumoniae* isolates, the highest resistance rate belonged to piperacillin-tazobactam (41.6%, n=25), meropenem (28.8%, n=17) and ceftriaxone (18.3%, n=11), followed by ceftazidime (8.3%, n=5), ceftazidime, ceftriaxone (6.6%, n=4), cefepime (6.6%, n=4) and ciprofloxacin (1.6%, n=1), respectively. The MIC of meropenem-resistant isolates was above 4 (μg/mL), and for ceftriaxone, resistance was above 12 (μg/mL), the only ciprofloxacin-resistant isolate was above 8 (μg/mL). Figure 2 shows the antibiotic susceptibility of the isolates.
Figure 1. Occupations of the study subjects

Figure 2. Percentage of antibiotic susceptibility in *Klebsiella pneumoniae* isolates
Among 60 isolates of *K. pneumoniae*, 95% (n=57) of isolates had *fimH*, 93.33% (n=56) had BssS, 45% (n=27) had *rmpA*, 41.66% (n=25) had *blaTEM*, 38.33% (n=23) had *blaCTX-M*, 35% (n=21) had *blaSHV* and 3% (n=2) had *blaKPC*, and none of these isolates carried magA gene (**Figure 3**). Out of 60 *K. pneumoniae*, 53 (88%) isolates had *fimH* and BssS genes simultaneously. MDR was observed in 4 (6.6%) cases of *K. pneumoniae* isolates. Out of MDR isolates, 3 had *fimH* and BssS genes.

![Gel electrophoresis results of virulence and antibiotic resistance genes](image)

**Figure 3.** Gel electrophoresis results of virulence and antibiotic resistance genes - well 1: 100 bp DNA molecular marker, well 2: *blaSHV*, well 3: *blaCTXM*, well 4: *blaKPC*, well 5 BssS, well 6: *rmpA*, well 7: *fimH*, well 8: *blaTEM* genes

### 4. Discussion

In this study, 60 *K. pneumoniae* isolates were identified in 350 stool samples obtained from comprehensive health examinations in Tehran, Iran. Our results confirm the high intestinal carriage of drug-resistant *K. pneumoniae*, in which the highest and lowest resistance rate belonged to piperacillin-tazobactam and ciprofloxacin, respectively. In addi-
tion, the results showed that among all *K. pneumoniae* isolates, more than 90% carried *fimH* and *BssS* genes.

The reported asymptomatic carriage rates of ESBL positive Enterobacteriaceae in the feces of community dwellers vary in Asian countries: 5.0% in Japan (20), 40% in Thailand (21), and 50.6% in China (22). Most carriers were positive for CTX-M-type ESBL, which has nearly displaced other enzymes in Enterobacteriaceae, including SHV and TEM variants. We showed that the prevalence of *blaTEM*, *blaCTX-M*, and *blaSHV* genes among the studied isolates were 35%, 38.33%, and 41.66%, respectively. However, Mansury et al. (2016) from Shiraz reported that the prevalence of *blaTEM*, *blaCTX-M*, and *blaSHV* genes among 38 ESBL producing *K. pneumoniae* were 16%, 19%, and 22.2%, respectively (23). In their study, the highest levels of resistance were related to amoxicillin (100%), cefotaxime (50%), and gentamicin (42.3%), and the lowest levels were related to meropenem (11.8%), amikacin and imipenem (both 15.9%). Asadpour et al., in 2016, determined the frequency of *blaTEM* and *blaSHV* genes in 34 broad-spectrum beta-lactamase producing *K. pneumoniae* by PCR (24). Of these, 40% of isolates carried *blaTEM* gene, and 23.07% had *blaSHV* gene. A Korean evaluation on stool samples of patients with comprehensive health examinations uncovered that from 109 fecal samples, only four *blaSHV* positive *K. pneumoniae* were identified (25). In another study carried out by Bing Lu et al., 22 *K. pneumoniae* isolates were detected from among 4340 stool samples collected from patients with diarrhea (26). Their results showed that all isolates were susceptible to ciprofloxacin, gentamicin, cefotaxime, ceftriaxone, nalidixic acid, imipenem, cefepime; the highest resistance rate belonged to amoxicillin-clavonate (100%) and ampicillin (72.7%) (26). The results showed that all strains were susceptible to imipenem, amikacin, and gentamicin, but little resistance was observed to ciprofloxacin, ceftriaxone and cefepime. In both studies, sensitivity to imipenem and gentamicin was observed, but cefalosporins may develop antibiotic resistance over time (8).

A study was performed by Candan and Aksöz (27) to evaluate the association among the genotypes of adhesins, mucoviscosity, capsules, carbapenem resistance, and other *K. pneumoniae* virulence factors. The OXA-48 and NDM-1 carbapenemase genes were detected in 33 and one from 50 *K. pneumoniae* strains, respectively. Also, KPC was not detected in *K. pneumoniae* isolates. Although no significant difference was detected between susceptible and resistant strains because of the presence of virulence gene regions (P<0.05), virulence factors were more diverse in carbapenem-resistant isolates (27). In research by Rodríguez-Navarro et al. (28), the genetic content of commensal and clinical strains was investigated. Their results indicated that the replicon profile was comparative in both populations, except for HI1 (only in commensal strains) and L, M, A/C, and N (detected only in clinical strains). Although F and 11 were the most common replicons, only Incl1, sequence type 12 (ST12) was related to *blaCMY-2* in both populations. Also, the discoveries from this investigation affirm that healthy people strains have lower virulence factors and antibiotic resistance and show a higher diversity of phylogenetic lineages (in *E. coli*) than infection-causing strains (28).

Biofilm formation is one of the reasons for the increasing antibiotic resistance. *fimH* and *BssS* genes are involved in biofilm formation and ultimately may have a role in increasing antibiotic resistance (29). The frequency of *fimH* and *rmpA* genes in a study by El Fertas-Aissani et al. in Algeria were 100% and 3.7%, respectively (9). Another study was performed to evaluate *fimH*, and *rmpA* genes among *K. pneumoniae* isolates in hospitalized patients from Babol, Iran, which their frequency was reported 86.1% and 10.8%, respectively (30). Also, similar to our study, none of the isolates showed the *magA* gene in the two recent reports.

5. Conclusion

In summary, although the sample was collected from healthy individuals, some antibiotic resistance genes were observed. We were able to isolate *K. pneumoniae* from 350 stool samples from adults that underwent a medical examination. Since the treatment of infections caused by this bacterium is associated with many limitations, there is a need for effective measures to prevent and control the infection and careful use of antibiotics to control the rise and spread of fecal carriage of antibiotic-resistant pathogens.

Acknowledgment

None.

Funding

None.

Conflict of Interest

There is no conflict of interest between the authors.
References


