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Original Article

Association between Presence of *RmpA, MrkA and MrkD* Genes and Antibiotic Resistance in Clinical *Klebsiella pneumoniae* Isolates from Hospitals in Tehran, Iran

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Abstract

Background: *Klebsiella pneumoniae* is an opportunistic pathogen causing nosocomial infection in human. This study aimed to investigate the relationship between the presence of genes involved in biofilm formation in *K. pneumoniae* isolated from patients and the presence of antibiotic resistance genes.

Methods: Biochemical tests were used for the identification of *K. pneumonia* isolated from urine samples referred to hospitals in Tehran, Iran, from Sep 2018 to Jan 2020. The antibiotic resistance pattern was performed and biofilm formation was assessed phenotypically. Finally, β-lactamase genes and adhesion genes were detected by the PCR method.

Results: We collected 457 *K. pneumoniae* isolates from hospitals in Tehran, Iran. 110 isolates were resistant to imipenem. Fifty isolates were positive for metallo-β-lactamases that thirty-nine isolates (35.45%) has *blaKPC* gene, 18 isolates (16.36%) had blaVIM-1 gene and 9 isolates (8.18%) had blaIMP-1 gene detected by PCR. Sixty isolates (54.54%) had strong biofilm, 35 isolates (31.81%) had moderate biofilm and 15 isolates (13.63%) had weak biofilm. The presence of adhesion genes in *K. pneumoniae* isolates significantly correlated with resistance genes ($P<0.001$).

Conclusion: It is clear antibacterial resistance has been significant association with biofilm formation in *K. pneumoniae* isolates. Therefore, understanding resistance pattern and mechanisms leading to biofilm formation can facilitate efficient treatment of infections caused by *K. pneumoniae*.

Keywords: *Klebsiella pneumonia;* Adhesion genes; Biofilm; Drug resistance; Iran

Introduction

Klebsiella pneumoniae is an opportunistic pathogen and can cause a wide range of infections in humans. About 8% of nosocomial infections in the United States and Europe are due to the bacte-

rium (1). Most *Klebsiella* genera are isolated from urine, pneumonia, bacteremia and wound infections. The presence of beta-lactamase genes such as *TEM, SHV*, and *CTX* in *K. pneumoniae* has caused its resistance to beta-lactams (penicillins and cephalosporins). The first *K. pneumoniae* carbapenemase (KPC) gene was introduced in *K. pneumoniae* in 1999. In 2007, 8% of Klebsiella isolates were reported to be resistant to carbapenems (2). This type of resistance is caused by the production of enzymes called beta-lactamases, which cause resistance beta-lactam agent. These enzymes were classified into four groups A, B, C and D based on their molecular structure. Types A, C, and D are serine beta-lactamase and class B metallo-β-lactamases that require zinc for their activity. Imipenem (*IMP*) and Verona integron-encoded metallo-β-lactamase (*VIM*) classified in Class B and *K. Pneumoniae* Carbapenemase (*KPC*) classified in A class. *IMP* and *VIM* genes induce resistance to carbapenem antibiotics (imipenem, meropenem, doripenem and ertapenem). *KPC* serine beta-lactamase gene induces resistance to third-generation beta-lactams, carbapenems and cephalosporins (3).

Other problems make *Klebsiella* as important urinary pathogen is biofilm formation in this bacterium. Biofilm is a group of bacteria that enables the aggregation and attachment to surface, and then the low permeability of the drugs to the biofilm layer makes the drugs ineffective (4, 5). *Klebsiella* generates fimbriae that facilitate bacterial attachment to host mucosal surfaces, that most strains of *Klebsiella* producing the type 3 fimbriae (6). The MrkA protein is the major structural component of type 3 fimbriae, whereas binding to collagen molecules is determined by the presence of the MrkD adhesion. Three allelic variants of the mrkD gene of *K. pneumoniae* have been reported (7). Capsule has anti-phagocytosis activity. Klebsiella 1 and 3 types of pili and bacterial capsule are involved in its colonization in urinary tract infection. The ability of biofilm formation in these Klebsiella has led to chronic urinary tract infections (8).

In this study, we investigated the relationship between the presence of the rmpA capsular genes and the MrkA, MrkD adhesion fimbriae gene with Antibiotic resistance genes such as *KPC, VIM, IMP* in *K.pneumoniae*

Materials and Methods

Sample collection

The Ethics Committee of the Tehran University of Medical Sciences, Tehran, Iran, approved this study (Ethic of Number: IR.TUMS.SPH.REC.1397.152).

In thiscross-sectional descriptive-analytic study, patients with symptoms of dysuria and urinary frequency were to be aware of the inclusion and exclusion criteria for this study. We collected 457 nonduplicate *K. pneumoniae* isolates from urine samples in 2 hospitals of Tehran city from Sep 2018 and continue until Feb 2020.

Bacterial Identification

These isolates were mostly isolated from urine and identified as *K. pneumoniae* using biochemical tests for identification of Enterobacteriaceae. Based on microscopic methods to identify typical bacterial morphology (8). Gram stain, culture media (in MacConkey agar producing pink mucoid colony). Biochemical tests, positive catalase test, negative oxidase test, gas production from glucose, Methyl red test, FeS production, motility, indole production, sodium citrate utilization, and urea utilization were carried out (8).

Antibiotic resistance pattern

We determined antibiotic susceptibilities via the disk diffusion method on Mueller-Hinton agar plates (Merck, Germany) and Used 10 µg imipenem discs (MAST, England) as recommended by the Clinical and Laboratory Standards Institute (CLSI). We used *Escherichia coli* ATCC 25922 for quality control of antimicrobial susceptibility.

Phenotypic identification of metallo-β-lactamase producing isolates

Double disk synergy test (DDS) was used to identification metallo-beta-lactamase producing isolates (class B beta-lactamase) used imipenem alone and imipenem with EDTA Combination disc and class A beta-lactamase such as *KPC* positive bacteria used imipenem alone and imipenem with boronic acid Combined disk synergy test. From 10 µl boronic acid and 10 µl EDTA solution (0.5M) was dispensed onto imipenem disk. The tests were performed on Mueller-Hinton agar by the standard diffusion method. The plates were incubated at overnight in 37 °C. increase in the diameter of the inhibition zone more than or equal to 7 mm around the imipenem-EDTA disc relative to the imipenem disc alone indicates the production of metallobetalactamase. Increasing the diameter of the inhibition zone around the imipenem + bronchic acid composite disc by 5 mm and larger than the imipenem alone disc is considered as a positive carbapenemase isolate (9).

DNA extraction

The DNA of the bacterial isolates were extracted using a DNA extraction kit (Bioneer, Korea, Cat. No. K-3032-2).

Detection of Resistant genes and adhesion genes using PCR

Identification of plasmid-mediated carbapenems resistance genes or chromosomal genes was performed by PCR. Bioneer master mix PCR (Korea) used for PCR reactions. Specific primers, PCR product and PCR conditions for detection of carbapenems resistance genes are shown in Table 1. The PCR products were analysed by 1% agarose gel electrophoresis with in 1X Tris-Acetate-EDTA buffer. The gels were stained with safe stain and the PCR products were visualized under UV light.

Table 1: The primers and PCR condition was used in this study

GENES	Primers	PCR Condition	PCR Product (bp)
Kpc(1)	CTGAACTCCGCCATCCCAAG AGGCGCCCGGGTGTAGAC	95 °C, 30 sec, 58 °C, 30 sec, 72 °C, 30 sec (33 cycles)	297
VIM-1	CTGAACTCCGCCATCCCAAG AGGCGCCCGGGTGTAGAC	95 °C, 30 sec,58 °C,30 sec,72 °C,60 sec (33 cycles)	539
IMP-1	AAAAAAGACGGTAAGGTTCAAGC ACCAGTTTTGCCTTACCATATTTG	95 °C, 30 sec,56 °C,30 sec,72 °C,30 sec, (33 cycles)	260
Rmpa	ACTGGGCTACCTCTGCTTCA CTTGCATGAGCCATCTTTCA	95 °C, 30 sec,55 °C,30 sec,72 °C,30 sec (33 cycles)	535
Mrkd	GTCTTTTCGTCCCGGGTATATAAC CCACATCGACATTCATATTTTTCC	95 °C, 30 sec,58 °C,30 sec,72 °C,30 sec (33 cycles)	244
Mrka	ATGCGAACGTTTACCTGTCTCC CCCGGGATGATTTTGTTGG	95 °C, 30 sec,58 °C,30 sec,72 °C,30 sec (33 cycles)	298

Biofilm formation

To biofilm formation used microtiter plate assay using 96 well plate. Each isolate was cultured in Luria broth for 18h in 37C, then 200 µl of tryptic case soy broth (TSB) was transferred to each well. Afterwards, 10 microliters of the microbial suspension of the isolates from the 24-hour cultured reached to 0.5 McFarland turbidity was added to the wells, and incubated at 37° C for 24 h, and then, each well was emptied and washed 3 times with saline. Then, 200 µL of 10% crystal violet was poured into wells incubated for 20 min in room temperature. After that, we washed and dried 3 times with normal saline, 200 µL of dimethyl sulfoxide (DMSO) was added to each well and the plate was assessed at 630 nm by ELISA

reader. For measurement of biofilm formation, the test OD was compared with control OD: that OD>0.65 means strong biofilm, 0.5>OD>0.3 means moderate biofilm, OD<0.3 means weak biofilm, OD> 0.15 means no biofilm formation.

Statistical analysis

The analysis of data was performed by SPSS software version 18 (Chicago, IL, USA). Confidence interval test was used to assess the statistical significance with confidence level of 95% (α=0.05).

Results

Detection of K. pneumonia

This descriptive cross-sectional study was performed on urine samples suspected of *K. pneumoniae* from hospitalized patients as well as outpatient in Tehran hospitals during a one-year period. Overall, 259 samples (56.67%) were admitted and 198 samples (43.33%) were outpatient. Its frequency of *K. pneumoniae* isolates in women and men, respectively 37.41% for man and 62.59% for women. Frequency of metallo-beta-lactamase enzymes of *K. pneumoniae* isolates by phenotypic method, from 457 *K. pneumoniae* isolates, 110 isolates were resistant to imipenem, which 97 isolates with double disk diffusion method (imipenem-boronic acid disk and imipenem disk alone) were positive for carbapenems enzymes Fig. 1. 50 isolates were positive for metallo-beta-lactamase by dual disk diffusion method (imipenem-EDTA disk and imipenem disk alone).

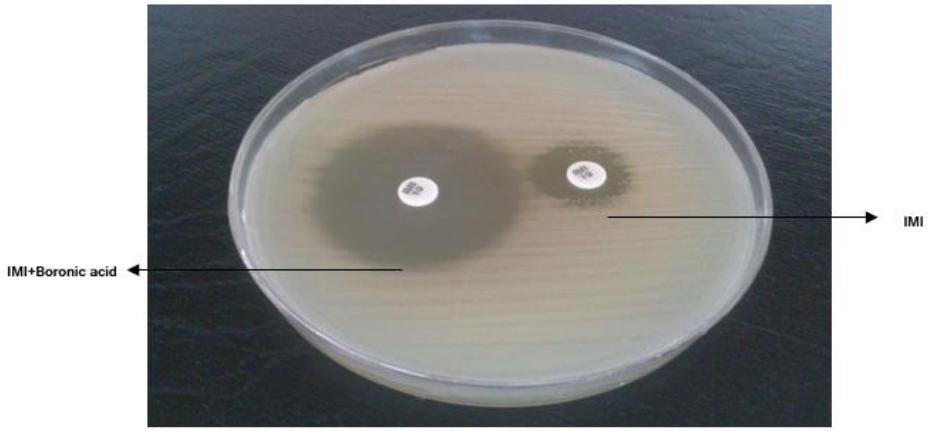

Fig. 1: Representative results using the boronic acid-based method for isolates possessing KPC

Presence of KPC, IMP, VIM gens and adhesion genes (mrkA mrkD, rmpA)

PCR was used to control the presence of target enzymes gene for each of 110 Imipenem resistant *K. pneumoniae* isolates to evaluate the presence of *blaKPC, blaIMP, blaVIM* genes Fig. 2. Thirty-nine isolates (35.45%) has blaKPC gene, 18 isolates (16.36%) had *blaVIM*-1 gene and 9 isolates (8.18%) had *blaIMP*-1 gene Imipenem resistant isolates of *K. pneumoniae* evaluated for presence of *mrkA, mrkD rmpA* genes. Ninty-isolates had mrkA gene (81.81%), 76 isolates had mrkD gene (69.09%) and 30 isolates had rmpA gene (27.27%) Fig. 3.

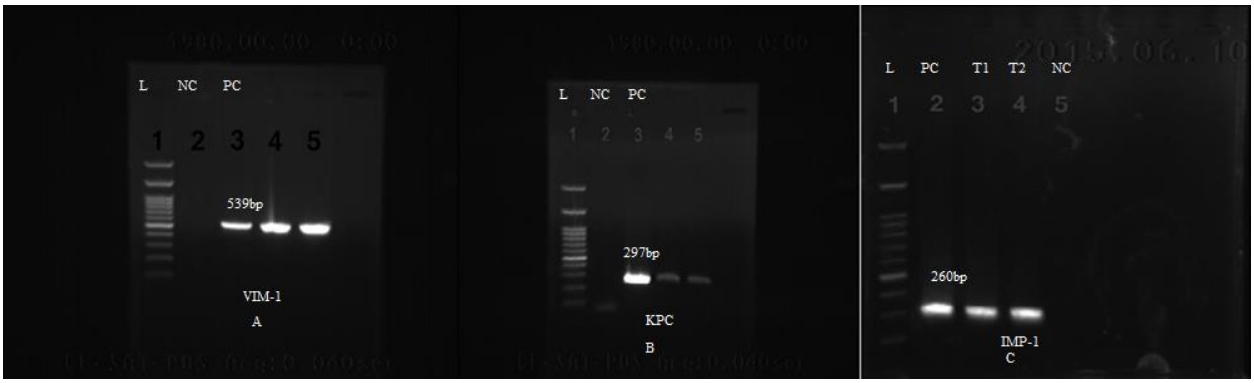

Fig. 2: Polymerase chain reaction (PCR) for detection of *Vim*-1(A), *KPC*(B) and *IMP*-1(C) genes. L.: 100 bp plus ladder; NC: Negative control; PC: Positive control; T: Clinical isolates

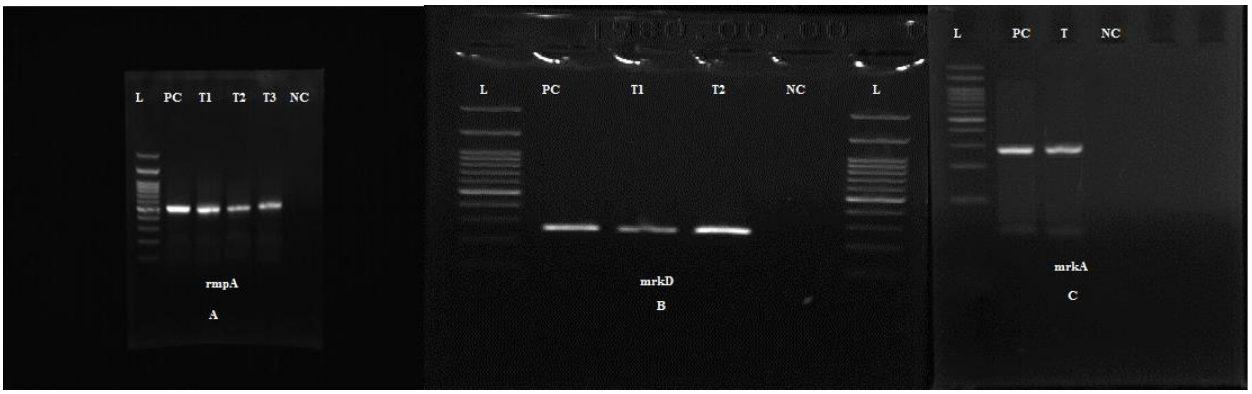

Fig. 3: Polymerase chain reaction (PCR) for detection of rmpA(A) and *mrkD*(B) and *mrkA*(B) genes. L.: 100 bp plus ladder; NC: Negative control; PC: Positive control ; T: Clinical isolates

Biofilm forming ability

All 110 imipenem resistant *K. pneumoniae* isolates were carried out phenotypically for evaluation of biofilm formation ability. Sixty-isolates (54.54%) had strong biofilm, 35 isolates (31.81%) had moderate biofilm and 15 isolates (13.63%) had weak biofilm. All isolates containing mrkA, mrkD rmpA had strong or moderate biofilm and no weak biofilm was found. The presence of these adhesion genes were significantly correlated with biofilm. Most isolates containing these genes had strong biofilms. Fifty isolates with mrkA gene had strong biofilm and 40 isolates had moderate biofilm. Forty-six isolates containing mrkD gene has strong biofilm and 30 isolates had moderate biofilm gene. In the case of rmpA gene isolates, 24 isolates had strong biofilm and 6 isolates had moderate biofilm Fig. 4.

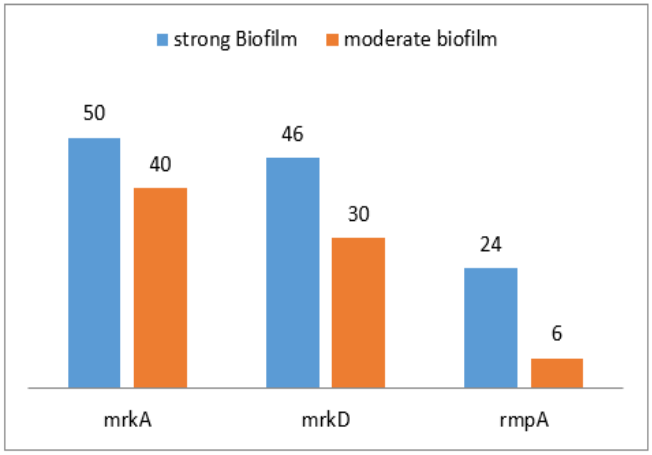

Fig. 4: Number of isolates with strong and medium biofilm in isolates with adhesen gene

The relationship between the adhesion genes and resistance genes

Distribution of adhesion genes in *VIM, IMP and KPC* resistance isolates as shown in Fig. 5. The presence of adhesion genes in these *K. pneumoniae* isolates significantly correlated with resistance genes.

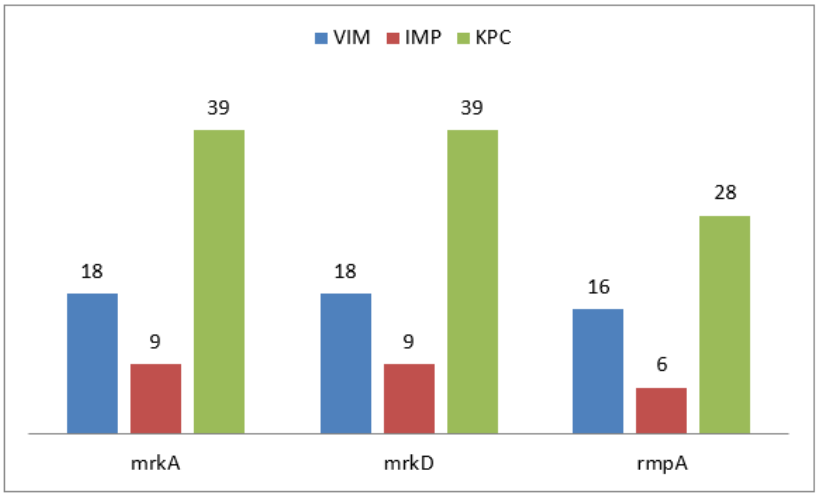

Fig. 5: Distribution of adhesion genes in *VIM, IMP and KPC* resistance isolates

Discussion

K. pneumoniae is an important human pathogen received much attention in recent years due to the development of resistant nosocomial infections, especially in patients with urinary tract infections or in specialized care(9, 10).In this study, analysed the prevalence of antibiotic resistant genes in patients hospitalized and significantly related with presenting adhesion genes.

In some study, from the clinical sample set, 56.4% were positive for *K. pneumoniae*, which consistent with this study (57.09 %), as well as imipenem resistance was reported to be about 10.10 % in India, but in this study, imipenem resistance was reported 24.07%, which is higher than Indian report. Differences in geographical area and antibiotic use pattern can be the reason for this difference prevalence (11). However, the present study was consistent with the study from Iran that the reported 22.8% for present of Imipenem resistant gene (12). The prevalence of carbapenems resistance gene in Iranian study was 4.9%. In a Brazilian study on urine samples, the prevalence of the carbapenems resistance gene was 20.07%, that consistent with the present study but the prevalence of *Klebsiella* was lower than our study (13).

KPC frequency was significantly higher than 2.18% reported (14) and in another study were not deleted (15). *VIM* frequency was higher than 10.3% reported (15) but not significant, and significantly lower than 26.7% reported by Bahmani (15). Frequency of *blaMIP* was significantly lower than 15. reported (16).

The ability to produce biofilm isolates results in increased resistance to antibiotics, as a result, treatment failure, increasing treatment costs and increasing mortality (16-18). In *K. pneumoniae*, the genes *mrkA* (main-unit) and *mrkD* (sub-unit) encode adhesions of type 3 fimbriae, which mediate binding to the extracellular matrix, that necessary for biofilm formation. About 80% of *K. pneumoniae* isolates were capable of biofilm formation (19) which is in agreement with the results of this study. In the present study, among 110 imipenem resistant gene isolates, 60 isolates had strong biofilm, 35 isolates had moderate biofilm, and 15 isolates had weak biofilm, as well as 90 isolates had mrkA gene, 76 isolates had *mrkD* and 30 iso-

lates had rmpA gene. In Finland (20), from 40 isolates of *K. pneumoniae*, 6 (15%) isolates had *mrkD* gene and 34 (85%) isolates had mrkA genes, that consistent with our study. In our study, strong biofilm producing isolates had significantly higher abundance of antibiotic resistance genes. Strong biofilm producing isolates had higher resistance to beta lactam antibiotic compared to low producing biofilm isolates (18). The results of PCR in this study showed that all isolates with *mrkA, mrkD, rmpA* genes had strong or moderate biofilm. Among the 69 isolates of *K. pneumoniae*, 55 isolates were capable of producing biofilm and in all isolates with strong and moderate producing biofilm had mrkA gene, then 57% had *mrkD* gene, that consistent our study (17). The results of this study showed that the presence of adhesion genes was significantly correlated with the presence of resistance genes. Of 200 *K. pneumoniae* isolates, 115 (57.5%) were ESBL producers; 74.0% carried the class 1 integron, and 1.0% carried the class 2 integron. The gene *rmpA* was detected in 7% of isolates and the gene *wcaG* in 23.5% of isolates. Integron-positive isolates showed a higher prevalence of *wcaG* compared with to integron-negative isolates (21). In another study, 36 types of *K. pneumoniae* were identified. CTX-M-15-producing *K. pneumoniae* isolates were grouped into 5 clonal complexes (22). Of these isolates, *mrkD* was the most prevalent virulence gene (95%), followed by *kpn* (60%), *rmpA* (37.5%), *irp* (35%), and *magA* (2.5%). Results of this study showed that biofilm formation could be dependent on the presence of drug resistant genes and adrenergic genes. The simultaneous presence of both sets of genes can lead to stronger biofilm formation.

Conclusion

Resistance to antibiotics associated with metallo lactamase genes widely increasing. One of the reasons for this increasing resistance could be the formation of biofilms. Biofilm formation in the isolates causes antibiotic to not have access to bacteria, as a result, increased antibiotic resistance will prolong the course of treatment. Moreover, increased biofilm production seems to be associated with the appearance of multidrug resistance. Therefore, further study be done in Iran to find out the resistance pattern of other antibiotics related with formation of *K. pneumonia* biofilm.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

All authors declare that they have no conflict of interest.

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