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Original Article

Prevalence of Metallo-β-Lactamase Genes in Clinical Isolates of *Klebsiella pneumoniae* in Health Care Centers in Mazandaran Province

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Abstract

Background: *Klebsiella pneumoniae* is one of the most important causes of opportunistic infections, including lobar pneumonia, urinary tract infection (UTIs), and wound infection. Treatment of disease caused by this bacterium has also become a challenge, as many strains are resistant to all β-lactam antibiotics. Metallo-β-lactamases hydrolyze most β-lactam antibiotics, especially carbapenems, but cannot inactivate monobactams. This study aimed to determine the prevalence of Metallo-β-lactamase genes (bla_{IMP}, bla_{VIM}, and bla_{NDM}) in *K. pneumoniae* isolated from clinical specimens.

Methods: Clinical samples were collected from hospitals of Mazandaran province. Among 500 clinical samples collected, only 40 *Klebsiella pneumoniae* isolates were detected by culture and biochemical tests. Antimicrobial susceptibility testing was performed on all isolates by the Kirby-Bauer method. The polymerase chain reaction (PCR) technique was used for the identification of bla_{IMP}, bla_{VIM}, and bla_{NDM} genes.

Results: Antibiogram by disk diffusion method showed that 21 (52%) and 19 (48%) isolates were classified as imipenem resistant and sensitive, respectively. Of all the samples, 30 (75%), 7 (17.5%), and 36 (90%) contained bla_{VIM}, bla_{IMP}, and bla_{NDM} genes, respectively. The co-existence of the bla_{VIM} and bla_{NDM} genes was observed in 22 (55%) isolates. The presence of both bla_{IMP} and bla_{NDM} genes was confirmed in 2 (5%) of the isolates. Four isolates (10%) had bla_{NDM}, bla_{IMP}, and bla_{VIM} genes simultaneously, but none of these genes were present in one isolate (2.5%).

Conclusions: This study showed that the prevalence of metallo-β-lactamase genes in *K. pneumoniae* isolated from clinical specimens is very high, so it is recommended that physicians treat patients based on phenotypic and genotypic characteristics.

Keywords: Metallo-β-lactamase, Resistance gene, *Klebsiella pneumoniae*, Clinical samples

Background

Klebsiella pneumoniae is a clinically significant pathogen that causes urinary tract infections (UTIs), sepsis, pneumonia, and other infections (1). The bacterium is a Gram-negative, immobile, encapsulated, lactose-fermenting, and facultative anaerobic bacillus (2). *Klebsiella pneumoniae* can asymptomatically colonize the skin, mouth, respiratory tract, and gastrointestinal tract, which may serve as a reservoir for human infections (3). The bacterium uses a variety of strategies to protect itself against antibiotics, one of which is the production of β-lactamase enzymes. This enzyme causes resistance by hydrolyzing and inactivating the β-lactam ring of beta-lactam antibiotics. Treatment of disease caused by *K. pneumoniae* has become a challenge because many strains are resistant to all β-lactam antibiotics through the production of carbapenemases (4). According to the Ambler classification, beta-lactamases are grouped into classes A, B, C, and D based on sequence similarity (5). Class B beta-lactamases require at least one or two zinc ions at their active site (6). Various beta-lactamases, including Verona integron-encoded *metallo-β-lactamase* (VIM), imipenemase (IMP), New Dehli metallo-β-lactamase (NDM-1), Sao Paulo metallo β-lactamase (SPM), and Seol imipenemase metallo-β-lactamase (SIM), have been detected in *K. pneumoniae* strains (7). Metallo-β-lactamases cause resistance to various antibiotics, including penicillins, cephalosporins, and carbapenems, except for monobactam (8,9). IMP is a carbapenemase that is resistant to almost all β-lactam antibiotics. One of the most common types of IMP is IMP-4, which was first identified in *Acinetobacter* spp. in Hong Kong in 2001 (10). VIM metallo-β-lactamases were first identified in Europe (11). The first case of VIM-4 in *K. pneumoniae* was reported in a recent study conducted in Tunisia (12). In 2009, NDM was first detected in a Swedish patient hospitalized in New Delhi, India (9). The genes encoding these carbapenemases are reported to be located on chromosomes or motile genetic elements such as plasmids, integrons, and transposons (13). Based on several reports on the presence of Metallo-β-lactamases genes in *K. pneumoniae* strains, the aim of the present study was to determine the prevalence of bla_{IMP}, bla_{VIM}, and bla_{NDM} genes in clinical isolates.

Methods

Isolation and Detection of Klebsiella pneumoniae

Clinical specimens were collected from wounds, burns, urine, and sputum. The study was conducted for seven months from June 2018 to December 2019. In total, 40 isolates of *K. pneumoniae* were transferred from the laboratories of Mazandaran province to the Microbiology Laboratory of Islamic Azad University, Babol branch. Isolates were inoculated on MacConkey agar plates and were incubated overnight at 37°C (Merck, Germany). Unique colonies of *K. pneumoniae* (lactose-fermenting mucoid colonies) were confirmed by standard biochemical tests, including fermentation of sugars in Triple Sugar Iron Agar medium (TSI) (Merck, Germany), fermentation of acidic or alcoholic in MRVP (Methyl Red, Voges–Proskauer) broth (Merck, Germany), growth in Simmons citrate medium (Merck, Germany), urea hydrolysis in urea medium, production of indole and hydrogen sulfide, and motility in sulfide indole motility (SIM) medium (Merck, Germany). According to biochemical tests, *K. pneumoniae* isolates were confirmed as indole negative, MR negative, VP positive, citrate positive, urease positive, and motility negative (14). All *K. pneumoniae* isolates were cultured in trypticase soy broth (TSB) (Merck, Germany). Then, 800 µL of bacterial suspension and 200 µL of glycerol were mixed in a 1.5 mL tube and were stored at -20°C for further studies (15,16).

Antibiotic Susceptibility Test

According to the Clinical and Laboratory Standards Institute (CLSI) recommendation, antibiotic susceptibility testing was performed by the disk diffusion method. A bacterial suspension with turbidity equal to 0.5 McFarland was cultured on the surface of Müller-Hinton agar medium. Then, the imipenem disc (10 µg) (Padtan Teb Co-Iran) was placed on the culture medium and was incubated at 37°C for 18 hours. The diameter of the growth inhibition zone was measured and the results were recorded as resistant, intermediate, and sensitive. Isolates with growth inhibition zones of ≥22 mm, 20-22 mm, and ≤19 mm were determined as sensitive, intermediate, and resistant, respectively (17).

Detection of Metallo-β-Lactamase Genes

The polymerase chain reaction (PCR) technique was used for the identification of metallo-beta-lactamase genes including bla_{NDM}, bla_{IMP}, and bla_{VIM} in *K. pneumoniae* isolates. DNA purification was performed by boiling method. The two colonies were transferred into a test tube containing 1 mL of distilled water, boiled in a water bath for 10 minutes, and centrifuged at 1000 rpm for 5 minutes. Then, 3 µL of supernatant was used in the PCR master mix (18). PCR amplification was performed in a final volume of 25 µL containing 12.5 µL of Master mix (CinnaGen Company, Iran), 3 µL of supernatant containing bacterial DNA, 1 µL of each primer (20 pmol), and 7.5 µL of sterile distilled water. All PCR assays were performed using an Eppendorf 5331 MasterCycler Gradient Thermal Cycler (Hamburg, Germany). The primers used for detection of these genes were listed in Table 1.

Thermal cycles of PCR were performed as follows. A cycle of initial denaturation was performed at 94°C for 3 minutes. Then, 35 cycles, including 94°C for 1 minute, annealing at a suitable temperature for 1 minute according to Table 1, and polymerization at 72°C for 1 minute were carried out. Final polymerization was done at 72°C for 10 minutes (19, 20). In the PCR reaction mixture, distilled water was used as the negative control. Finally, PCR products were electrophoresed in 2% agarose gel at 95 V for 100 minutes and were observed by UV transilluminator (Vilber Lourmat, Collégien, France).

Statistical Analysis

The results of this study were analyzed using SPSS (Statistical Package for Social Sciences) version 21.0.

Results

A total of 40 *K. pneumoniae* isolates were identified from clinical specimens. *K. pneumoniae* strains were isolated from 11 sputum, 21 urine, 3 blood, and 5 wound samples. The results of antimicrobial susceptibility testing performed on 40 isolates of *K. pneumoniae* showed that 21 (52%) and 19 (48%) isolates were resistant and sensitive to imipenem, respectively, and none of the samples were intermediate (Figure 1).

PCR technique was used to identify metallo-β-lactamase resistance genes. Agarose gel electrophoresis of PCR products produced special bands of 236, 587, and 383 bp for bla_{NDM}, $bla_{IMP,}$ and bla_{VIM}, respectively (Figures 2, 3, and 4).

The prevalence of metallo-β-lactamase genes is shown in Table 2.

Of the 40 *K. pneumoniae* isolates studied, the most common resistance gene was the *bla*NDM gene with a frequency of 36 (90%), but the bla_{IMP} gene had the lowest

Table 1. The Sequences of Primers Used to Identify NDM, IMP, and VIM Genes

Gene	Primers 5′→3′	Amplicon Base Pair (bp)	Annealing Temperature	References
bla_{NDM}	F-ACCGCCTGGACCGATGACCA R-GCCAAAGTTGGGCGCGGTTG	236	69 ° C	(18)
bla_{IMP}	F- GAAGGCGTTTATGTTCATAC R- GTATGTTTCAAGAGTGATGC	587	52.3 ° C	(18)
bla_{VIM}	F- GTTTGGTCGCATATCGCAAC R- AATGCGCAGCACCAGGATAG	382	63 ° C	(19)

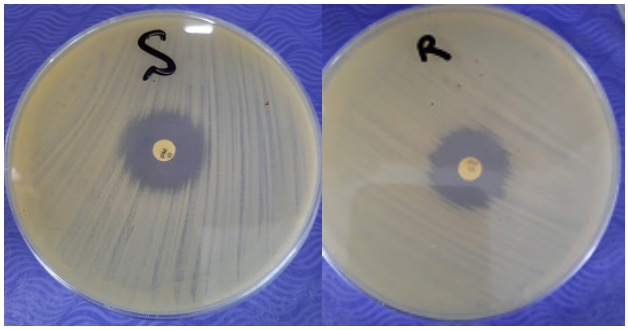

Figure 1. Antibiogram of *Klebsiella Pneumoniae* Strains to Imipenem by the Disk Diffusion Method R: resistant; S: sensitive

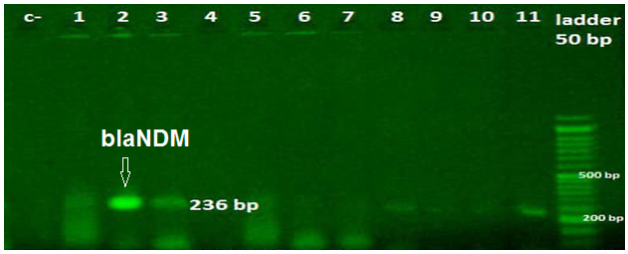

Figure 2. Agarose Gel Electrophoresis of the PCR Amplicon of *bla*$_{NDM}$. Ladder: (50 bp); Lanes 2,3,8 and 11: positive samples; Lanes c$^-$: negative control; Lane 1, 4, 6, 7, 9, and 10: negative samples.

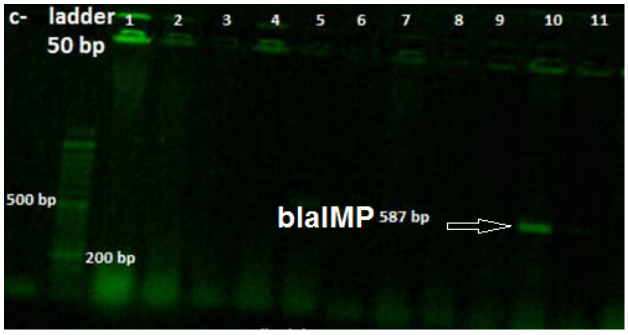

Figure 3. Agarose Gel Electrophoresis of the PCR Amplicon of *bla*$_{IMP}$. Ladder: (50 bp); Lanes 10: positive samples; Other lanes: negative samples; c$^-$: negative control.

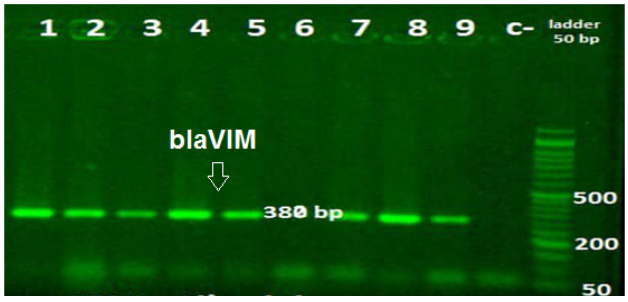

Figure 4. Agarose Gel Electrophoresis of the PCR Amplicon of *bla*$_{VIM}$. Ladder: (50 bp); Other lanes: positive samples; Lanes 6: negative samples; c$^-$: negative control.

prevalence (17.5%). The prevalence of the *bla*$_{VIM}$ gene was 75%. The coexistence of the *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$ was observed in 4 (10%) isolates of *K. pneumoniae*. The coexistence of *bla*$_{NDM}$ and *bla*$_{IMP}$ as well as *bla*$_{NDM}$ and *bla*$_{VIM}$ genes was detected in 2 (5%) and 22 (55%) isolates, respectively. The presence of both *bla*$_{IMP}$ and *bla*$_{VIM}$ genes was not detected in any of the isolates. Moreover, 2.5% of the isolates lacked beta-lactamase resistance genes.

Discussion

Klebsiella pneumoniae plays a clinically significant role in hospitalized infections. The bacterium causes many infections, such as UTIs, liver abscesses, pneumonia, and bacteremia (21). In our study, *bla*$_{NDM}$ gene was detected in 90% of the *K. pneumoniae* isolates, which differs from studies conducted in Northwest Pakistan (22.7%) (22), India (19%) (23), China (14.9%) (24), Egypt (26.32%) (25), and Bangladesh (22.4%) (26). In a study conducted by Saremi et al in Iran, the prevalence of *bla*$_{NDM}$-1 gene was 1.92% (7). In a study conducted in Peru, 85% of *Klebsiella pneumoniae* isolates were positive for *bla*$_{NDM}$ gene, which is consistent with our study (27). The prevalence of the *bla*$_{NDM}$ gene varies in different parts of the world depending on the level of health and overuse of antibiotics. In the current study, the *bla*$_{IMP}$ gene was observed in 17.5% of the isolates, which is not consistent with studies conducted in South India (8%) (28), China (36.5%) (29), and Egypt (15.2%) (30). In the present study, the prevalence of the *bla*$_{VIM}$ gene was 75%. In a study conducted by Ripabelli et al (31), *bla*$_{VIM}$ gene was detected in 76.5% of the isolates, which is consistent with our study. In our study, the coexistence of *bla*$_{NDM}$ and *bla*$_{IMP}$ as well as *bla*$_{NDM}$ and *bla*$_{VIM}$ genes was observed in 5% and 55% of the isolates, respectively. In a study conducted by Urmi et al (26), multiple Metallo-beta-lactamase genes were present simultaneously in one *K. pneumoniae* isolate . The results of a study conducted by Hussein showed the coexistence of both *bla*$_{NDM-1}$ and *bla*$_{IMP}$ genes in only three strains of *K. pneumoniae* (32). These studies show that the simultaneous presence of resistance genes varies in different geographical areas. In our study, 1 (2.5%) isolate did not contain any of the metallo-beta-lactamase resistance genes. In an investigation carried out by Pragasam et al (33), 4 isolates were negative for all the genes tested, which is consistent with our study. In the genotyping method, 97% of *K. pneumoniae* isolates contained Metallo-beta-lactamase resistance genes, but in the phenotyping method, 52% of isolates showed resistance to imipenem. Different phenotyping and genotyping results can be due to mutations in the promoter or coding sequence of metallo-beta-lactamase genes, which in turn prevent gene transcription and enzyme inefficiency, respectively. It is recommended that physicians use the

Table 2. Frequency of Metallo-β-Lactamase Resistance Genes

Gene	Number (%)
bla$_{NDM}$	36 (90)
bla$_{IMP}$	7 (17.5)
bla$_{VIM}$	30 (75)
bla$_{NDM+}$*bla*$_{IMP}$	2 (5)
bla$_{NDM+}$*bla*$_{VIM}$	22 (55)
bla$_{IMP+}$*bla*$_{VIM}$	0 (0)
bla$_{NDM+}$*bla*$_{IMP+}$*bla*$_{VIM}$	4 (10)
None	1 (2.5)
Total	40 (100)

results of this study to select the appropriate antibiotics to treat patients. Carbapenem-resistant *K. pneumoniae* strains are usually resistant to most beta-lactam antibiotics. Therefore, treatment options are limited to polymyxins, tigecycline, and aminoglycosides (34).

Conclusions

Due to the high prevalence of metallo-β-lactamase genes in this study and their roles in treatment failure, rapid detection of these strains in laboratories plays a more influential role in controlling the spread of these strains. Therefore, appropriate policies are needed to regulate the use of penicillins, cephalosporins, and carbapenems in patients. We recommend regular monitoring and screening for the emergence of *K. pneumoniae* strains containing the metallo-beta-lactamase genes.

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Authors' Contribution

Majid Alipour designed the study, analyzed data, wrote the manuscript, and carried out the interpretation of the results. Rougayeh Alizadeh and Fatereh Rezaee performed experimental tests and prepared the samples. All authors read and approved the final manuscript.

Conflict of Interests

There is no conflict of interest for authors.

Ethical Approval

There are no ethical issues for this article.

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