

# High Frequency of Multidrug-resistant (Mdr) *Klebsiella Pneumoniae* Harboring Several $\beta$ -lactamase and Integron Genes Collected From Several Hospitals in the North of Iran

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## Research

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# Abstract

**Background:** *Klebsiella pneumoniae* is one of the leading causes of hospital outbreaks worldwide. Also, antibiotic-resistant *K. pneumoniae* is progressively being involved in invasive infections with high morbidity and mortality. The aim of the current study was to determine antimicrobial susceptibility patterns and the incidences of resistance genes (integron types and  $\beta$ -lactamase-encoded genes) among clinical isolates of *K. pneumoniae*.

**Methods:** In this cross-sectional study, a total of 100 clinical samples were obtained from hospitalized patients in three teaching hospitals in the north of Iran, from November 2018 and October 2019. Antimicrobial susceptibility testing was performed using disk agar diffusion test in line with CLSI recommendation. For colistin, minimum inhibitory concentration (MIC) was determined using broth microdilution. Based on antibiogram, multi-drug resistant (MDR) and extensive-drug resistant (XDR) strains were detected. Finally, integron types and  $\beta$ -lactamase resistance genes were identified using polymerase chain reaction technique.

**Results.** The most and least clinical samples were related to the urine and bronchoalveolar lavage, respectively. Based on the antibiogram results, amikacin and gentamicin exhibited good activity against *K. pneumoniae* strains in vitro. High resistance rate (93%) to ampicillin/sulbactam also predict the limited efficacy of this antibiotic. Among all the 100 isolates, the frequency of MDR and XDR strains were 58% and 13%, respectively, while no pan-drug resistant (PDR) isolates were found. The prevalence of *bla*<sub>SHV</sub>, *bla*<sub>TEM</sub>, *bla*<sub>CTX-M-15</sub>, *bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub>, *bla*<sub>NDM</sub>  $\beta$ -lactamase genes were 91.4%, 82.7%, 79.3%, 29.3%, 36.2% and 6.9%, respectively, however 58% of the isolates were carrying *intl* gene. Class II and III integrons were not detected in any isolates.

**Conclusion:** The MDR *K. pneumoniae* is becoming a serious problem in hospitals, with many strains developing resistance to most available antimicrobials. Our results indicate co-presence of a series of  $\beta$ -lactamase and integron types on the MDR strains recovered from hospitalized patients. The increasing rate of these isolates emphasizes the importance of choosing an appropriate antimicrobial regimen based on antibiotic susceptibility pattern.

## Background

Among the *Klebsiella* spp, *Klebsiella pneumoniae* (*K. pneumoniae* or KP) a gram- negative encapsulated bacterium, is responsible for up to 10% of the nosocomial infections [1]. This organism causes a wide range of infections such as, pneumonia, burn and urinary tract infections (UTIs), septicemia, and meningitis [2]. Management of the infections caused by antibiotic-resistant *K. pneumoniae* is problematic due to its intrinsic and acquired resistance to a broad spectrum of the drugs, such as  $\beta$ -lactams. The MDR strains can be fairly challenging, especially for elderly or immunosuppressed individuals and infants with immature immunity [3]. The  $\beta$ -lactamase-producing *K. pneumoniae* can destroy a varied range of  $\beta$ -lactams such as penicillins, carbapenems, and cephalosporins [4]. The key mechanisms of

resistance to these antimicrobials are hyperexpression of chromosomal cephalosporinases and production of plasmid-encoded Ambler class A [Extended spectrum  $\beta$ -lactamases (ESBLs)], B (Metallo- $\beta$ -lactamases) and D (oxacillinases)  $\beta$ -lactamases [5]. ESBLs are plasmid-borne enzymes that hydrolyze the oxyimino  $\beta$ -lactam ring such as 3<sup>rd</sup> generation cephalosporins and aztreonam. The extensive use of numerous  $\beta$ -lactam agents in recent decades has led to the appearance of ESBLs, which are frequently derivatives of TEM-1 and SHV-1 enzymes [6]. Carbapenems are the  $\beta$ -lactams of choice for the treatment of infections caused by ESBL-producing *K. pneumoniae*. Ambler class B enzymes which play a critical role in drug resistance against carbapenems, are zinc dependent and inhibited by EDTA [7]. Resistance genes have a high ability to spread, because the genes have commonly found on the transferable elements such as integrons, insertion sequences (IS) and transposons [8, 9]. Integrons, a segment of double-strand DNA sequence, are immobilized, but contain an integrase (*intI*)-encoding gene that allows the insertion of the resistance gene cassettes between highly conserved nucleotide sequences. Although several types of integrons have been identified, but class I, II and III integrons are the most common types in the clinical settings [10, 11]. In recent years, MDR *K. pneumoniae* strains producing ESBLs-, MBLs, and KPC resistance genes have been progressively found in many regions of Iran [12-14]. Despite the high importance of this issue, only restricted numbers of works have been reported from the north of Iran addressing the frequency and co-existence of resistance genes among the clinical isolates of *K. pneumoniae*. Therefore, this study was performed to determine the antibiotic resistance profiles, incidences of MDR, XDR and PDR and also prevalence of  $\beta$ -lactamase and integron resistance genes among *K. pneumoniae* strains isolated from hospitalized patients in the north of Iran.

## Methods

### Study Design and Sampling

In this descriptive cross-sectional study, based on the previous studies, confidence interval 95% and by equation  $n = z^2P(1 - P)/d^2$ , a total of 100 non-duplicated samples were obtained from the one-year from November 2018 and October 2019. Several clinical specimens from hospitalized patients such as blood, sputum, bronchoalveolar lavage (BAL), throat and intratracheal tube (ITT), wound exudates, urine, cerebrospinal fluid (CSF) and synovial fluid included in this study. In this study, all clinical specimens from any human source which contain *K. pneumoniae*, including both gender, and from all age groups including infants to elderly were included. Hence, *K. pneumoniae* strains isolated from out-patients, other species of *klebsiella* and mixed and/or contaminated plates were excluded.

### Microbiological method

All isolates were identified using conventional biochemical and microbiological methods [15] and confirmed by the API 20E (bioMérieux, La-Balme-les-Grottes, France). All isolates were preserved in the Luria–Bertani (LB) broth (Merck, Co., Germany) containing 20% glycerol at -80 °C for further used.

### Susceptibility testing

In concordance with the Clinical and Laboratory Standards Institute; CLSI. 2018 [16], antimicrobial susceptibility testing was done on the Mueller-Hinton agar plates (Merck Co., Germany) by disk agar diffusion (DD) method against 16 following antimicrobials: levofloxacin (LEV; 5 µg); ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), cefepime (FEP; 30 µg), ertapenem (ETP; 10 µg), amikacin (AK; 30 µg), meropenem (MER; 10 µg), ceftriaxone (CRO; 30 µg), ampicillin/sulbactam (SAM; 10/10 µg), cefoperazone (CFP; 75 µg), imipenem (IPM; 10 µg), nitrofurantoin (NIT; 300 µg), gentamicin (GM; 10 µg), ciprofloxacin (CIP; 5 µg), tetracycline (TET; 30 µg), and trimethoprim-sulfamethoxazole (SXT; 5 µg) (MAST Diagnostics, Merseyside, UK). The MDR and possible XDR/PDR strains were recognized according to the guidelines suggested by the European Center for Disease Control and Prevention (ECDC) (17). *E. coli* ATCC 25922 was used as a quality control (QC) organism. Also, colistin susceptibility assay was performed for carbapenems-resistance isolates by broth microdilution method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. The *E. coli* NCTC 13846 (a colistin resistant strain) was used as QC in colistin minimum inhibitory concentration (MIC) determination.

### Genomic DNA extraction

Total genomic DNA of every isolate was obtained from the colonies grown on the Luria-Bertani medium (Merck, Darmstadt, Germany) using a High Pure PCR Template Preparation Kit (Roche, Germany), based on the manufacturer's instruction. The extracted DNAs were quantified by measuring the absorbance at A260/A280 with Nanodrop spectrophotometer (ND-1000; Thermo Scientific; Wilmington, DE, USA), to evaluate the DNA purity. Purified DNA was kept at -20°C until further use.

### Molecular method

The PCRs were used for the detection of *int* genes (*intl*, *intlI*, and *intlII*), and resistance elements such as *bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>KPC</sub>, *bla*<sub>OXA-48</sub> and *bla*<sub>NDM</sub> genes. The primer sequences are listed in Table 1. Amplification reactions were performed in a final volume of 25 µl, contained of 0.8 µl of DNA extracted, 2.0 µl of 10× PCR buffer, 1.2 mmol/l MgCl<sub>2</sub>, 0.6 µl (each) dATP, dGTP, dCTP, and dTTP, 0.7 µl of each primer, 0.9 µl of Taq DNA polymerase (5 U/µl) (Amplicon Co., Denmark) and 18.1 µl ddH<sub>2</sub>O. The samples were amplified in a Techne TC-512 thermal cycler (Eppendorf, Hamburg, Germany) as follows: initial denaturation at 95 °C for 1min, 32 cycles of denaturation for 30 s at 94 °C, annealing for 30s at 60 °C and extension for 1min at 72 °C, and a final extension for 5 min at 72 °C. PCR amplicon products were subjected to electrophoresis in a 1.0% agarose gel, stained with Gel Red™ (Biotium, USA) and photographed with ultraviolet illumination (Bio-rad, Hercules, USA). Both positive and negative controls were included in each run. The positive PCR products were sequenced by Bioneer Company (Korea). The nucleotide sequences alignments were analyzed with running Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI) database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### Statistical Analysis

After collection of the data, statistical analysis was performed with the IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL) and a *p-value* less than 0.05 was considered as statistically significant.

## Results

A total of 100 non-duplicated *K. pneumoniae* were obtained from blood (n=15), sputum (n= 5), BAL (n= 2), wound exudates (n= 10), urine (n= 65) and CSF (n= 3). The frequency of isolates in hospital wards were as follows: Intensive Care Units (ICUs) (n= 27), burn (n= 19), dialysis (n= 17), hematology-oncology (n= 12), respiratory care (n= 9), internal medicine (n= 8), neonatal intensive care unit (NICU) (n= 5), and surgery (n= 3). The mean age of the patients was 51.7 years (ranging from 15 to 91 years), where 58% (n= 58) of the patients were female and 42% (n= 42) of them were male. In our studied therapeutic centers, the prevalence of *K. pneumoniae* were as follows: 41% in Imam Khomeini, 45% in Zare and 14% from Razi hospitals affiliated to the Mazandaran University of Medical sciences (north of Iran). Based on the acquired antibiotic resistance pattern, the highest and lowest resistance rate were related to SAM (93%) and AK (8%), respectively (Table 2). Also, 58% (58/100) of the isolates were resistant to three or more antimicrobials (MDR), and 13% (13/100) isolates were XDR. No PDR isolates were found. No non-MDR strains were resistant to AK. Altogether, the frequency of resistance genes among MDR strains was significantly higher than in non-MDR strains ( $p < 0.05$ ). Overall, among 45 carbapenem-resistant *K. pneumoniae* (CRKP) strains, 28 cases were resistant to colistin antibiotic with a MIC >2. Molecular analysis results showed that class I integrons were the predominant resistance transferable elements in our isolates. However, 91.4% and 11.9% of the MDR and non-MDR isolates were carrying *intI* gene, respectively. Class II and III integrons were not detected in any isolates. As shown in table 3, the frequency of resistance genes in MDR isolates was higher than non-MDR. The prevalence of *bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>CTX-M-15</sub>*, *bla<sub>KPC</sub>*, *bla<sub>OXA-48</sub>*, *bla<sub>NDM</sub>* were 91.4%, 82.7%, 79.3%, 29.3%, 36.2% and 6.9%, respectively. The co-existence of the *bla<sub>SHV</sub>/bla<sub>TEM</sub>*, *bla<sub>TEM</sub>/bla<sub>CTX-M-15</sub>*, *bla<sub>SHV</sub>/bla<sub>CTX-M-15</sub>*, *bla<sub>CTX-M-15</sub>/bla<sub>OXA-48</sub>*, *bla<sub>SHV</sub>/bla<sub>OXA-48</sub>*, *bla<sub>TEM</sub>/bla<sub>OXA-48</sub>*, and *bla<sub>SHV</sub>/bla<sub>KPC</sub>*, were 21%, 18%, 13%, 8%, 5%, 4%, and 3%, correspondingly. Only one isolate was carrying *bla<sub>SHV</sub>/bla<sub>TEM</sub>/bla<sub>CTX-M-15</sub>/bla<sub>KPC</sub>/bla<sub>OXA-48</sub>* gene.

## Discussion

The increasing rate of *K. pneumoniae* resistant strains against multiple antimicrobials is a main challenge in medical centers [27]. In a systematic review and meta-analysis article, Vaez et al, (2019), declared that there is a relatively high frequent antibiotic resistant *K. pneumoniae* in Iran [28]. In the present study, the highest and lowest resistance rate were related to SAM (93%) and AK (8%), respectively. In this study, 33% of the isolates were considered as an IMP-resistant *K. pneumoniae* (IRKP); 30% of the MDR and 3% of the non-MDR strains, while no non-MDR strains were resistant to AK. In total, only 8 isolates were resistant to AK, therefore, this antimicrobial was the best choice against the tested strains. The frequency of MDR and XDR isolates in our hospitals were 58% and 13%, respectively. In comparison with our data, Moghadas et al. (2018), reported that the 7.5%, 16.1%, 32.9%, 34.1%, 36.4% and 42.7% of their isolates were resistant to IPM, CP, SXT, FEP, AN, and CAZ, respectively [29]. In difference with our

data, 89.5% of the strains were MDR in Hou et al, study in 2015 [30]. This percentage was far higher than those reported in the current work. Geographic distance, level of hygiene, type of samples, date of study, sample size, and restriction on antibiotic usage may be the reasons for this inconsistency. Colistin resistance was found in 62.22% CRKP isolates. We detect a surprisingly high level of colistin resistance among CRKP strains. Possibly the augmented usage of the antimicrobial during recent years in treatment of infections caused by organisms resistant to less toxic antibiotics, could be the cause of this. Molecular analysis showed that the frequency of resistance genes in MDR isolates was higher than non-MDR ones. Also, 74%, 66%, 51%, 20%, 28% of the isolates were harboring *bla<sub>SHV</sub>*, *bla<sub>TEM</sub>*, *bla<sub>CTX-M-15</sub>*, *bla<sub>KPC</sub>* and *bla<sub>OXA-48</sub>* genes, respectively. Mahmoudi et al, (2019) showed that out of 30 *K. pneumoniae* isolates, the frequency of *bla<sub>SHV</sub>*, *bla<sub>CTX-M-15</sub>* and *bla<sub>TEM</sub>* genes were 83% (n=25), 70% (n=21) and 57% (n=17), respectively [31]. In other study directed by Ghafourian et al, (2012), in total, 36.1% (n; 104/288), 7.6% (n; 22/288) and 5.9% (n; 17/288) of the isolates were positive for *bla<sub>SHV</sub>*, *bla<sub>CTX-M-15</sub>* and *bla<sub>TEM</sub>* genes, respectively [32]. Furthermore, 20% of the isolates were carrying *bla<sub>KPC</sub>* gene. In contrast with Bina et al, (2015), *bla<sub>KPC</sub>* gene was not observed in any of the 41 strains, but 80.5% (n; 33) of the isolates were positive in phenotypic modified Hodge test [33]. According to the researchers, the discrepancy could be due to at least one extended-spectrum cephalosporin and another mechanism such as an ESBL or AmpC-type enzyme with porin loss [34,35]. An interesting point in this study is the low prevalence of *bla<sub>NDM</sub>* gene. According to the Fallah et al, (2014), the close distance of India and Pakistan to Iran and large number of journeys between the countries on one side and the ease of resistance transfer among microorganisms on the other hand led us to think that it may be likely for our strains to have the same gene [36]. PCR test results showed that *intI* gene was detected in 58% of the isolates (53 MDR and 5 non-MDR). In a study similar to ours, Derakhshan et al, (2014) showed that 25.8% (n; 8/31) of their isolates were carried *intI* [37]. In addition, they did not find class II and III integrons. In contrast with Haddadi et al (2019), out of 54 MDR *K. pneumoniae* isolates in our study, 27% of them were harbored class I integron [38]. So, Firoozeh et al, (2019) showed that 100% (n; 150) and 36.7% (n; 55) of their MDR *K. pneumoniae* carried *intI1* and *intI2* genes, respectively, but we did not find any class II and III integrons [39]. This discrepancy could be due to the source of samples, microbial genetic diversity and level of hygiene.

## Conclusion

This study has the potential to add to the body of literature regarding to MDR or XDR in Iran. MDR *K. pneumoniae* is becoming a serious problem in hospitals, as many strains are developing resistance to most available antimicrobials. The increasing rate of these isolates emphasizes the importance of choosing an appropriate antimicrobial regimen based on antibiotic susceptibility pattern. The finding of the present study exposed a high prevalence of class I integron among MDR *K. pneumoniae* isolates and resistance genes especially *bla<sub>SHV</sub>* and *bla<sub>CTX-M-15</sub>* from Sari (north of Iran), which led to more attention to MDR strains.

## Abbreviations

BAL; bronchoalveolar lavage, UTIs; urinary tract infections, MDR; multidrug-resistant, XDR; extensively drug-resistant, DD; disk agar diffusion, PCR; polymerase chain reaction, CLSI; clinical laboratory standards institute, SDS; Sodium Dodecyl sulfate, QC; quality control, ITT; intratracheal tube, CSF; cerebrospinal fluid, BLAST; Basic Local Alignment Search Tool, NCBI; National Center for Biotechnology Information, EUCAST; European Committee on Antimicrobial Susceptibility Testing, LB; Luria–Bertani, ECDC; European Center for Disease Control and Prevention, ESBLs; Extended spectrum  $\beta$ -lactamases, IRKP; IMP-resistant *K. pneumoniae*, CRKP; carbapenem-resistant *K. pneumoniae*, MBLs; metallo- $\beta$ -lactamases,, KPC; *K. pneumoniae* carbapenemase, NICU; neonatal intensive care unit, ICUs; Intensive Care Units,

## Declarations

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### Authors' contributions

MG: Design of the study and supervision. MF: collected the data, cultured the samples and performed experiments. MA Advisor in the study and contributed to the analysis of the data in collaboration with HG. MH: Assisted in molecular examinations and edited the manuscript. MG drafting of the manuscript in collaboration with MF. All authors read and approved the final manuscript.

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### Availability of data and materials

All data generated or analyzed during this work are included in this published article. Also. the all data used to support the findings of this study are available from the corresponding author upon request.

### Ethics approval and consent to participate

The study was approved by the ethical committee of Mazandaran University of Medical Sciences, Sai, Iran (IR.MAZUMS.REC.1398.628).

### Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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## Tables

**Table 1.** Primers used for PCR amplification in this study

Reference	Product size (bp)	Primer sequence (5'→3')	Target genes
18	280	F=5'- CCTCCCGCACGATGATC-3' R=5'- TCCACGCATCGTCAGGC-3'	<i>intl</i>
19	233	F=5'- TTATTGCTGGGATTAGGC-3' R =5'- ACGGCTACCCTCTGTTATC-3'	<i>intlI</i>
20	300	F=5'- AGTGGGTGGCGAATGAGTG-3' R =5'- TGTTCTTGTATCGGCAGGTG-3'	<i>intlII</i>
21	996	F=5'- CACACGTGGAATTTAGGGACT-3' R =5'- GCCGTCTAAGGCGATAAACA-3'	<i>bla<sub>CTX-M-15</sub></i>
22	972	F=5'- TCGGGGAAATGTGCGCG -3' R =5'- TGCTTAATCAGTGAGGCACC -3'	<i>bla<sub>TEM</sub></i>
23	231	F=5'- AAGATCCACTATCGCCAGCAG-3' R=5'- ATTCAGTTCCGTTTCCCAGCGG-3'	<i>bla<sub>SHV</sub></i>
24	489	F=5'- CTTGCTGCCGCTGTGCTG-3' R=5' GCAGGTTCCGTTTTGTCTC-3'	<i>bla<sub>KPC</sub></i>
25	900	F=5' -GGGGACGTTATGCGTGTATT-3' R=5' -GAGCACTTCTTTTGTGATGGC-3'	<i>bla<sub>OXA-48</sub></i>
26	621	F=5' - GGTGGCGATCTGGTTTTTC-3' R=5' - CGGAATGGCTCATCACGATC-3'	<i>bla<sub>NDM</sub></i>

**Table 2.** Antimicrobial susceptibility pattern in MDR and non-MDR *K. pneumoniae* isolates

Antimicrobial agents	No. (%) in MDR-isolates			No. (%) in Non-MDR isolates		
	S	I	R	S	I	R
<b>LEV</b>	36 (36%)	3 (3%)	19 (19%)	38 (38%)	0 (0%)	4 (4%)
<b>CAZ</b>	10 (10%)	7 (7%)	41 (41%)	32 (32%)	2 (2%)	8 (8%)
<b>CTX</b>	11 (11%)	8 (8%)	39 (39%)	29 (29%)	4 (4%)	9 (9%)
<b>FEP</b>	15 (15%)	5 (5%)	38 (38%)	36 (36%)	3 (3%)	3 (3%)
<b>ETP</b>	35 (35%)	3 (3%)	20 (20%)	38 (38%)	1 (1%)	3 (3%)
<b>AK</b>	45 (45%)	5 (5%)	8 (8%)	41 (41%)	1 (1%)	0 (0%)
<b>MER</b>	17 (17%)	2 (2%)	39 (39%)	39 (39%)	0 (0%)	3 (3%)
<b>CRO</b>	14 (14%)	6 (6%)	38 (38%)	35 (35%)	3 (3%)	4 (4%)
<b>SAM</b>	1 (1%)	1 (1%)	56 (56%)	0 (0%)	5 (5%)	37 (37%)
<b>CFP</b>	11 (11%)	4 (4%)	43 (43%)	32 (32%)	1 (1%)	9 (9%)
<b>IPM</b>	26 (26%)	2 (2%)	30 (30%)	39 (39%)	0 (0%)	3 (3%)
<b>NIT</b>	2 (2%)	3 (3%)	53 (53%)	25 (25%)	13 (13%)	4 (4%)
<b>GM</b>	40 (40%)	1 (1%)	17 (17%)	35 (35%)	1 (1%)	6 (6%)
<b>CIP</b>	31 (31%)	0 (0%)	27 (27%)	65 (65%)	0 (0%)	7 (7%)

<b>TET</b>	22 (22%)	17 (17%)	19 (19%)	33 (33%)	6 (6%)	3 (3%)
<b>SXT</b>	13 (13%)	4 (4%)	41 (41%)	31 (31%)	2 (2%)	9 (9%)

MDR; multidrug resistant, S; susceptible, I; intermediate, R; resistant, LEV; levofloxacin, CAZ; ceftazidime, CTX; cefotaxime, FEP; cefepime, ETP; ertapenem, AK; amikacin, MER; meropenem, CRO; ceftriaxone, SAM; ampicillin/sulbactam, CFP; cefoperazone, IPM; imipenem, NIT; nitrofurantoin, GM; gentamicin, CIP; ciprofloxacin, TET; tetracycline, SXT; trimethoprim-sulfamethoxazole

**Table 3.** Antimicrobial resistance genes and integron types in MDR and non-MDR *K. pneumoniae* isolates

Tested isolates	Resistance-encoding genes						Integron types		
	<i>bla<sub>SHV</sub></i>	<i>bla<sub>TEM</sub></i>	<i>bla<sub>CTX-M-15</sub></i>	<i>bla<sub>KPC</sub></i>	<i>bla<sub>OXA-48</sub></i>	<i>bla<sub>NDM</sub></i>	intI	intII	intIII
<b>MDR</b>	53 (91.4%)	48 (82.7%)	46 (79.3%)	17 (29.3%)	21 (36.2%)	4 (6.9%)	53 (91.4%)	0 (0%)	0 (0%)
<b>Non-MDR</b>	31 (73.8%)	18 (42.6%)	5 (11.9%)	3 (7.4%)	7 (16.6%)	0 (0%)	5 (11.9%)	0 (0%)	0 (0%)