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Investigating Class I Integron and Antimicrobial Resistance Profile of *Klebsiella pneumonia* isolates in Babol, North of Iran

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ABSTRACT

Introduction: Integronsare are mobile genetic elements which play an essential role in the distribution of antibiotic-resistant genes among bacteria. This study aimed to investigate the Class I integron in *Klebsiella pneumoniae* clinical isolates and its association with multiple drug resistance (MDR). **Methods**: We obtained 30 *K. pneumoniae* isolates from patients admitted to the ICU at Shahid Beheshti Hospital in Babol City, Mazandaran province, Iran. Different classes of antimicrobials were used to determine the resistance pattern. A polymerase chain reaction (PCR) was performed to detect the *int1* gene of the class I integrons. We also investigated the suitability of the two pairs of primers for the detection of the *intl* gene. **Results**: Antibiotic susceptibility testing revealed 90% resistance to ceftizoxime, cefotaxime, and cefepime, 88.6% to cefazolin, gentamicin, ticarcillin, and ceftriaxone, 83.3% to imipenem, 60% to ciprofloxacin, 56.6% to ofloxacin, and 36.6% to amikacin. The PCRs with two pairs of primers, one designed previously and the other in this study, detected *int1* in 36.6% and 60% of samples, respectively. **Conclusion:** The *int1* gene was of high prevalence (60%) in *K. pneumoniae* isolates. This factor could play a significant role in the spread of MDR strains. Also, failure to adhere to essential points in the design of the primer can lead to the production of primers with low specificity and efficiency, which reduces the proper identification of antibiotic resistance genes.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic gram-negative bacteria that cause broad spectrum of diseases. Besides pneumonia, it causes meningitis, urinary tract infection, bacteremia, and wound infection, particularly in patients admitted to the ICU [1-4]. Various antibiotics are prescribed for the treatment of *K. pneumoniae*-associated infections, and this bacterium confronts them with different strategies [5]. Increased multidrug-resistant (MDR) *K. pneumonia* strains has become a global concern and has limited options for the treatment of this infection. Multidrug-resistant (MDR) is defined as the resistance of a microorganism to at least one antimicrobial drug in three or more antimicrobial categories [6,7].

Mobile genetic elements (MGEs) like integrons play a crucial role in the acquisition, evolution, and distribution of antibiotic resistance in *K. pneumonia* strains [8, 9]. Integrons can carry and spread the antibiotic resistance genes among bacteria and are among the most significant routes of distribution of resistance genes and the emergence of MDR strains via horizontal transfer [10, 11]. The most common types of integrons are the transportable class I (Tn402 derivatives) integron in *K. pneumonia* strains; hence, the study of the molecular characteristics of class I integrons gene in *K. pneumonia* is essential [12]. Integrons constitute three parts based on conserved sequence (CS): the 5'-CS, the variable segment, and the 3'-CS [13-15]. The integrons are recognized by the presence of an *intI* gene (encoding integrase), a recombination site (*attI*), and a promoter (P_c) [15-17].

The spreading of drug-resistant pathogenic bacteria is one of the most crucial concerns in societies [18]. This study aimed to determine class I integron and antibiotic resistance profile in *K. pneumonia* isolates, and investigate the accuracy of two different pairs of primers in the diagnosis of class I integron.

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MATERIAL AND METHODS

Clinical samples and laboratory identification. In this cross-sectional study, from January 2010 to June 2010, we collected 67 clinical samples from patients admitted to the ICU at Shahid Beheshti Hospital of Babol in the north of Iran. The written consent was obtained from all the participants, and the study was performed under the declaration of Helsinki's statement for medical research involving human subjects. The Ethics Committee of Babol University of medical sciences, Mazandaran, Iran approved the study (code No. 8213110).

The isolates were confirmed as *K. pneumoniae* by conventional biochemical and microbiological tests as described elsewhere [19]. All strains were stored in Luria-Bertani broth (Merck, Germany) containing 20% glycerol at -80˚C until used.

Antibiotic susceptibility test. In accordance with Clinical and Laboratory Standards Institute guideline [20], antimicrobial susceptibility test was performed on the Mueller-Hinton agar plates (Merck, Germany) using the standard disk-diffusion agar method for the following antibiotics: ciprofloxacin (CIP, 5 μg), ceftriaxone (CRO, 30 μg), cefotaxime (CTX, 30 μg), amikacin (AK, 30 μg), ofloxacin (OFX, 5μg) imipenem (IMI, 10 μg), cefepime (CPM, 30 μg), ticarcillin (TC, 75 μg), gentamicin (GM, 10 μg) (MAST Diagnostics, Merseyside, UK) and cefazolin (CZ, 30 μg), ceftizoxime (CZX, 30 μg) (Rosco Diagnostics, Denmark). *K. pneumoniae* ATCC 13883 was used as a positive control.

DNA Extraction. Pure colonies were obtained from previously cultured isolates preserved in glycerol at -80˚C. DNA extraction was performed using a high pure template PCR preparation kit (Roche, Germany) according to the manufacturer's instruction. The extractions were examined by NanoDrop (quantitative) and gel electrophoresis (qualitative) to ensure the purity of the product.

Primer design and PCR. In this study, besides the primers used by others [21], we designed another primer pairs to investigate the presence of the *int1*gene in *K. pneumoniae* isolates (Table 1). The primers were designed using Gene Runner and Primer3plus software and examined by the oligo analyzer software. The PCR reaction mix with a final volume of 50 µL contained 10 µL of DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5U *Taq* DNA polymerase, 50 pM of each primer (Amplicon Co., Denmark), and sterilized water to the final volume. DNA of *K. pneumoniae* ATCC1209 and *K. pneumoniae* ATCC1053 were used as the positive and negative controls, respectively. Amplification was performed in a thermocycler (Corrbet, Australia), and programmed as reflected in table 1. PCR products were analyzed by electrophoresis on 1.5% agarose gels containing SYBR™ Safe DNA gel stain and then sequenced in both directions by the Sanger method (Copenhagen, Denmark). The sequence obtained in this study were compared with similar sequences available at the GenBank database using BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/).

Nucleotide sequence accession number. The nucleotide sequence generated in the current study was deposited in the GenBank database under the accession number KC543110.

Table 1. Primers used for PCR amplification of *intl* gene

Gene	Primer sequence (5'→ 3')	PCR product (bp)	Amplificationprogram	Reference
intI	F- TCTCGGGTAACATCAAGG R- AGGAGATCCGAAGACCTC	243	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 53°C, and 30 s at 72°C); 5 min at 72°C	[21]
intI	F-GAAATCCAGATCCTTGACCCG R- GGTTCGTGCCTTCATCCGTT	361	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 59°C, and 30 s at 72°C); 5 min at 72°C	This study

RESULTS

Bacterial isolation. Over the six months, 30 *K. pneumonia* clinical isolates were identified from 67 clinical samples of patients admitted to the ICU of Shahid Beheshti hospital, Babol, north of Iran.

Antibiotic resistance profile. Antibiotic susceptibility testing (AST) for various antibiotics revealed 90% resistance to ceftizoxime, cefotaxime, and cefepime, 88.6% to cefazolin, gentamicin, ticarcillin, and ceftriaxone, 83.3% to imipenem, 60% to ciprofloxacin, 56.6% to ofloxacin, and 36.6% to amikacin (Table 2). The highest resistance rates belonged to CZX, CTX, and CPM. About 96.6% of isolates showed resistance to at least one antimicrobial drug in three or more antimicrobial categories and were defined as MDR.

PCR and BLAST analysis. Both PCR assays detected the *intI* gene (Fig. 1); the detection rate by the primers designed by Rao *et al.* (2006) (Table 1) was 36.6%, while the primers designed in the present study detected the gene in 60% of the isolates. In the BLAST analysis, our generated sequence showed 100% identity with many similar sequences in the GenBank database, including *Pseudomonas aeruginosa* strain PSE6684 (aac. No. CP053917), *Providencia rettgeri* strain YPR31 (acc. No. CP053897), *Proteus mirabilis* strain YPM35 (acc. No. CP053899), *Escherichia coli* isolate J31 (acc. No. CP053788), and *Klebsiella pneumoniae* strain BA2275 (acc. No. CP053365).

Table 2. Antibiotic resistance profile of *K. pneumoniae* isolates

	CZ n (%)	IMI n (%)	CRO n (%)	CZX n (%)	CTX n (%)	AK n (%)	OFX n (%)	CIP n (%)	CPM n (%)	TC n (%)	GM n (%)
S	3 (9.9)	4 (13.2)	2 (6.6)	2 (6.6)	1 (3.3)	13 (43.2)	10 (33.3)	10 (33.3)	2 (6.6)	1 (3.3)	1 (3.3)
I	1 (3.3)	8 (26.6)	3 (9.9)	1 (3.3)	2 (6.6)	6 (20)	3 (9.9)	2 (6.6)	1 (3.3)	3 (9.9)	3 (9.9)
R	26 (88.6)	18 (60)	25 (83.3)	27 (90)	27 (90)	11 (36.6)	17 (56.6)	18 (60)	27 (90)	26 (88.6)	26 (88.6)

CZ, cefazolin; IMI, imipenem; CRO, ceftriaxone; CZX, ceftizoxim; CTX, cefotaxime; AK, amikacin; OFX, ofloxacin; CIP, ciprofloxacin; CPM, cefepime; TC, ticarcillin; GM, gentamicin; S, susceptible; I, intermediate; R, resistant

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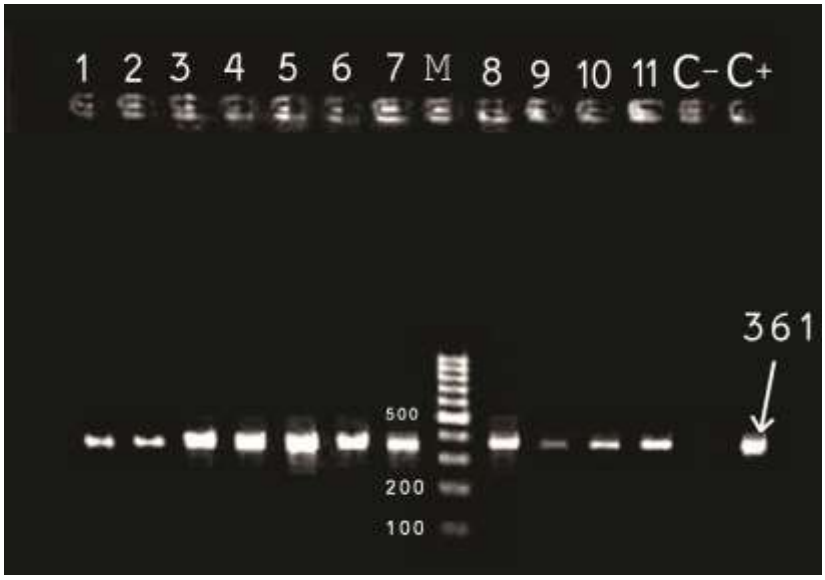

Fig. 1. Amplification of the *int1* gene in *K. pneumoniae* isolates. Lane M, DNA marker (100 bp); lane 1-11, positive samples; C-, negative control (ATCC1053); C+, positive control (ATCC1209).

Table 3. Multidrug resistance (MDR) profiles of *K. pneumoniae* isolates

Type of resistant	Pattern of antibiotic-resistant	No. of isolates	Total
Resistant to 1 agent	GM	1	1
Resistant to 4 agent	GM, CZX, CZ, TC	1	1
Resistant to 6 agent	CZX, TC, CTX, CPM, IMI, CIP	1	2
	GM, CZX, TC, CTX, CPM, CRO,	1	
Resistant to 7 agent	GM, CZX,CZ, CTX, CPM, CRO, AK,	1	1
Resistant to 8 agent	CZX, CZ, TC, CTX, CPM, CRO, AK, IMI	1	7
	GM, CZX, CZ, TC, CTX, CPM, CRO, AK,	4	
	CZX, TC, IMI CTX, CPM, CRO, AK, OFX,	1	
	GM, CZX, CZ, TC, CTX, CPM, OFX, CIP	1	
Resistant to 9 agent	CZX, CZ, CTX, CPM, CRO, AK, IMI, OFX, CIP	1	2
	CZX, CZ, CTX, CPM, CRO, AK, TC, GM, CIP	1	
Resistant to 10 agent	GM, CZX, CZ, TC CTX, CPM, CRO, IMI, OFX, CIP	13	16
	GM, CZX, CZ, TC, CTX, CPM, CRO, AK, IMI, CIP	2	
	GM, CZX, CZ,TC CTX, CPM, CRO, AK, OFX, CIP	1	
Total	14	30	30

CZ, cefazolin; IMI, imipenem; CRO, ceftriaxone; CZX, ceftizoxim; CTX, cefotaxime; AK, amikacin; OFX, ofloxacin; CIP, ciprofloxacin; CPM, cefepime; TC, ticarcillin; GM, gentamicin

DISCUSSION

The results of this study showed that 96.6% of the identified *K. pneumonia* isolates were MDR (Table 3). These results correspond with those of a previous study in Iran that reported 99.3% of *K. pneumonia* isolates to be MDR [22]. Several factors contribute to the development of MDR strains, *e.g.*, misusing and overusing different antibacterial agents in the health care setting and the transfer of resistance genes by MGEs, such as the plasmids, transposons (TEs), integrons *(int),* and bacteriophages [23, 24]. In our study, the *K. pneumonia* isolates exhibited the highest resistance rate (90%) to cefepime, cefotaxime, and ceftizoxime. In similar studies in Iran and Brazil, the highest antibiotic resistance rates showed to be to cefepime and cefotaxime [12, 25, 26]. Antibiotic resistance in this bacterium poses a severe threat [27], especially in hospital settings, and claim many lives worldwide and results in healthcare costs as well. Hence, the world health organization (WHO) declared 2011 as the year of antibiotic resistance [28,29].

The detection of the *int1* by two different pairs of primers yielded different results. The primers designed by Rao *et al.* (2006) detected *int1* in 36.6% of the isolates, while the primers we designed in the present study detected the gene in 60% of the isolates. Two similar studies in China have reported the frequency of *int1* in *K. pneumonia* isolates 60.1% and 51.1% [21,30], which are almost in agreement with our results.

In PCR assays, primer design is one of the most crucial factors affecting the amplification of a particular sequence. Two features, specificity, and efficiency are

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essential in primer design; the frequency of mispriming events defines specificity. Primers with poor specificity tend to produce undesired amplicons. Efficiency is defined as the ability of primers to amplify a product with a two-fold increase per cycle. The main factors in primer design include primer length, GC content, 3' end sequence, Tm temperature, and specific sequences. A change in any of these characteristics affects the specificity and efficiency of primers.

The production of new antibiotics and their improper use in treating bacterial diseases have resulted in antibiotic resistance, which involves different mechanisms. Determination of resistance pattern to antibiotics in common pathogens is crucial to adopt a specific therapy against a particular pathogen. So, the detection of drug resistance in *K. pneumoniae* isolates via the *int1* gene is vital for planning the control measures to prevent the spread of MDR in hospitals. Also, designing alternative primers in different geographical areas might result in the higher detection rate of resistance genes.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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