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Short Communication

First Report of Class 1 and Class 2 Integrons in Multidrug-Resistant *Klebsiella pneumoniae* Isolates from Northwest Iran

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SUMMARY: We investigated the prevalence of multidrug resistance, production of extended-spectrum β-lactamases (ESBLs), and presence of class 1 and 2 integrons in 150 clinical isolates of *Klebsiella pneumoniae* from northwest Iran by performing phenotypic confirmatory tests and polymerase chain reaction (PCR) analysis. Of the 150 isolates, 149 (99.3%) were multidrug resistant (MDR). Of the MDR isolates, 124 (83.2%) were ESBL positive. The results of the PCR analysis showed that 117 (78.5%) and 20 (13.4%) MDR *K. pneumoniae* isolates carried *intI1* and *intI2*, respectively, and 16 (10.7%) MDR *K. pneumoniae* isolates contained the integrase genes of both class 1 and 2. Resistance of the isolates to gentamicin, tetracycline, ceftazidime, cephalothin, chloramphenicol, and nalidixic acid was observed to be significantly associated with the presence of class 1 integrons; however, the resistance to tetracycline was observed to be associated with the presence of class 2 integrons alone. This study showed that integrons are widely prevalent in the clinical isolates of *K. pneumoniae* from northwest Iran, and that they may be playing an important role in attributing multidrug resistance to the clinical *K. pneumoniae* isolates. To the best of our knowledge, this is the first report showing the presence of class 1 and class 2 integrons in MDR *K. pneumoniae* isolates from clinical settings in northwest Iran.

Klebsiella pneumoniae is an important opportunistic pathogen that frequently causes urinary tract infections and pneumonia in immunocompromised individuals. After *Escherichia coli*, it is the most common cause of Gram-negative septicemia and nosocomial infections. The worldwide development of multidrug resistant (MDR) strains of *K. pneumoniae* is a matter of great concern (1). Transfer of antibiotic resistance genes between different species of bacteria is facilitated by mobile DNA elements such as transposons and plasmids. In the recent years, a substantial portion of the resistance genes present on the plasmids and transposons of Gram-negative bacilli have been observed to be integrated into DNA elements called integrons (2,3). Integrons are important systems involved in the spread and maintenance of multidrug resistance in microorganisms (4). They are specialized genetic elements capable of capturing, integrating, and mobilizing gene cassettes, which mainly encode the antibiotic resistance genes (5), through site-specific recombination. Four classes of integron have been identified on the basis of the homology of the integrase proteins (6). The 5′-conserved sequences (5′-CS) of all integrons encode a DNA integrase (*IntI*) that mobilizes and inserts gene cassettes (1,4). This region also contains a promoter sequence, P_{ant}, required for the expression of most of the genes carried by the gene cassettes (7,8). Class 1 integrons are the most frequently detected class of integrons in clinical microbial isolates; however, class 1 integrons share their cassette pool with integrons of other classes (9–11). The integrase gene found in class 2 integrons is primarily present in the Tn7 transposons and their derivatives that express a defective integrase protein (5). The class 3 integron-associated integrase genes are similar to the corresponding genes in class 2 integrons. The class 3 integrons are rarely detected in clinical isolates. Only a single class 3 integron, isolated from a carbapenem-resistant *Serratia marcescens* strain, has been described (12). A fourth class of integrons harboring hundreds of cassettes and associated with the *intI4* integrase gene has recently been detected in the small chromosome of *Vibrio cholerae* (6). However, class 1 integrons are the most frequently detected integrons in clinical isolates and are observed to be strongly associated with multiple-antibiotic resistance observed in the hospital environments (13). Reports from different countries have described a high prevalence of class 1 and class 2 integrons in Gram-negative clinical isolates (14–17). These data suggest that integrons are relatively common, especially in Enterobacteriaceae, and that they contribute to the spread of antimicrobial drug resistance in healthcare settings. However, only a few studies have analyzed the frequency of occurrence of integrons, and the association between integron carriage and antimicrobial susceptibility in Iran. To the best of our knowledge, this is the first report on the association between presence of class 1 and class 2 integrons and resistance to selected

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antimicrobial agents, and the frequency of occurrence of integrons in the clinical isolates of *K. pneumoniae* in northwest Iran.

A total of 10,948 clinical specimens, including urine, wound exudate, blood, cerebrospinal fluid, tracheal aspirates, bronchial secretions, sputum, peritoneal fluid, and throat swabs were collected from different teaching hospitals in northwest Iran and analyzed between January 2009 and March 2010. Among these 150 non-duplicate *K. pneumoniae* isolates were identified by performing standard bacteriological analysis. Once identified, the isolates were preserved at −70°C in Trypticase soy broth (Difco Laboratories, Detroit, Mich., USA) containing 15% v/v glycerol (18). The isolates were tested for their susceptibility to 12 antimicrobial agents using the Kirby-Bauer disc diffusion method, which was as per the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI) (19). The following antimicrobials agents were employed in the susceptibility tests: amikacin (30 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), chloramphenicol (10 μg), gentamicin (10 μg), cephalothin (30 μg), imipenem (10 μg), nitrofurantoin (300 μg), cotrimoxazole (25 μg), tetracycline (30 μg), norfloxacin (10 μg), and nalidixic acid (30 μg) (Mast, Co., Merseyside, UK). *E. coli* ATCC 25922 was used as the control strain in antibiotic susceptibility tests. Multidrug resistance was considered if the isolate showed resistance to more than two unrelated drugs (19). Screening for extended-spectrum β-lactamase (ESBL) production was carried out by performing the phenotypic confirmatory test according to the CLSI guidelines (19). The DNA of the isolates were extracted by the boiling method, as described by Yu et al. (15), after which the DNA samples were used as a source of templates for the polymerase chain reaction (PCR) amplification. Names of the primers used in the detection of *intI1* and *intI2* genes and the sizes of the expected PCR products are listed in Table 1. The primers were obtained from Alpha DNA (Montreal, Canada). PCR amplifications were performed using 25 μl of a reaction mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 50 pmol of each primer, 2.5 U of *Taq* DNA polymerase (Takara, Otsu, Japan), and 2 μl of the template DNA. We performed the amplification in an Eppendorf Thermal Cycler in the following sequence: 5 min at 94°C, followed by 35 cycles of 1 min each at 94°C (1 min at 55°C for integron class 1 and 1 min at 50°C for integron class 2), 30 s at 72°C, and a final extension step at 72°C for 10 min. The PCR products were separated by performing agarose gel electrophoresis (1.5% w/v agarose). The products were stained using ethidium bromide (0.5 mg/ml) for 30 min and visualized under ultraviolet light (2). All the data were analyzed using SPSS software for Windows, version 13.0. The significance of differences between the resistance patterns of the isolates was determined using the chi-square test. A P value of ≤ 0.05 was considered to be statistically significant.

Of the 150 isolates tested, 53.3% (n = 80) had been isolated from female patients and 46.7% (n = 70) had been isolated from male patients; 109 (72.7%) isolates were obtained from hospitalized patients, and 41 (27.3%) isolates were from outpatients. Most of the isolates were resistant to nitrofurantoin (96.7%) and cotrimoxazole (95.3%). The rate of resistance to each antibiotic were as follows: ceftazidime, 84.7%; cephalothin, 84%; gentamicin, 76%; tetracycline, 64.7%; nalidixic acid, 60.7%; chloramphenicol, 57.3%; amikacin, 50.7%; and ciprofloxacin, 43.3%. The most effective drugs against *K. pneumoniae* were imipenem (86.7%) and norfloxacin (59.7%). Moreover, 149 isolates (99.3%) were detected to be MDR, out of which 124 (83.2%) were identified to be ESBL producers. These isolates had high resistance against cephalothin (98.9%) and ceftazidime (92.9%) as well as other tested antimicrobial agents. In the 149 MDR isolates, the class 1 integrase gene (*intI1*) showed a dominant presence; it was present in 117 isolates (78.5%), and only 20 isolates (13.4%) had the class 2 integrase gene (*intI2*). Moreover, 16 (10.7%) isolates had both *intI1* and *intI2* genes. One isolate that was sensitive to the anibiotics had no integron. Our results showed that the presence of class 1 integron, and not class 2 integron, was significantly ($P < 0.05$) associated with multidrug resistance and production of ESBLs in *K. pneumoniae* isolates (details not showed). We also observed a significant relationship between the presence of an integron and the phenotypic resistance to some antimicrobial agents tested. A positive association existed between the presence of the *intI1* gene and resistance to gentamicin, tetracycline, ceftazidime, cephalothin, chloramphenicol, and nalidixic acid. A positive association was also observed between the presence of the *intI2* gene and resistance to tetracycline (Table 2).

Since knowledge about antibiotic susceptibility, prevalence of MDR, and presence of integrons in clinical isolates can facilitate selection of the appropriate treatment agents and also help control nosocomial infections, in the present study, we investigated the antimicrobial susceptibility patterns and the presence of class 1 and class 2 integrons in the clinical isolates of *K. pneumoniae* from northwest Iran. The results of the present study showed a high level of antimicrobial resistance among *K. pneumoniae* isolates. Of the 150 *K. pneumoniae* isolates analyzed in this study, up to 50%

Table 1. Oligonucleotide primers used in the PCR assay

Primer	Nucleotide sequence (5′–3′)	PCR target	Expected size (bp)	Reference
IntI1 F	TCT CGG GTA ACA TCA AGG	Class 1 integrase gene	250	2, 14
IntI1 R	AGG AGA TCC GAA GAC CTC			
IntI2 F	TTA TTG CTG GGA TTA GGC	Class 2 integrase gene	233	20
IntI2 R	ACG GCT ACC CTC TGT TAT C			

F, forward primer; R, reverse primer.

Table 2. Association of resistance to various antimicrobial agents and presence of integron genes in *K. pneumoniae* isolates

Antibiotic	No. (%) of resistant isolates with genes:		No. (%) of intermediate isolates with genes:		No. (%) of sensitive isolates with genes:		Association of resistance with[1]:	
	intI1	*intI2*	*intI1*	*intI2*	*intI1*	*intI2*	*intI1*	*intI2*
Gentamicin	99 (86.8)	14 (12.3)	2 (40)	1 (20)	16 (51.6)	5 (16.1)	***P* = 0.01**	*P* = 0.77
Tetracycline	83 (85.6)	7 (7.2)	10 (66.7)	2 (13.3)	24 (63.2)	11 (28.9)	***P* = 0.01**	***P* = 0.04**
Ceftazidime	108 (85)	18 (14.2)	—	—	9 (40.9)	2 (9.1)	***P* = 0.01**	*P* = 0.75
Co-trimoxazole	113 (79)	19 (13.3)	1 (100)	—	3 (50)	1 (16.7)	*P* = 0.21	*P* = 0.90
Imipenem	15 (75)	—	1 (100)	—	101 (78.3)	20 (15.5)	*P* = 0.82	*P* = 0.15
Ciprofloxacin	53 (84.1)	9 (14.3)	17 (81)	1 (4.8)	47 (71.2)	10 (15.2)	*P* = 0.19	*P* = 0.45
Norfloxacin	49 (83.1)	8 (13.6)	8 (72.7)	3 (27.3)	60 (75)	9 (11.3)	*P* = 0.47	*P* = 0.34
Cephalothin	106 (84.8)	18 (14.4)	1 (20)	—	10 (50)	2 (10)	***P* = 0.01**	*P* = 0.58
Amikacin	64 (84.2)	9 (11.8)	22 (88)	4 (16)	31 (63.3)	7 (14.3)	*P* = 0.09	*P* = 0.84
Nitrofurantoin	115 (79.3)	19 (13.1)	—	—	2 (50)	1 (25)	*P* = 0.06	*P* = 0.72
Chloramphenicol	75 (86.2)	12 (13.8)	20 (71.4)	5 (17.9)	22 (62.9)	3 (8.6)	***P* = 0.01**	*P* = 0.54
Nalidixic acid	74 (81.3)	11 (12.1)	25 (92.6)	4 (14.8)	18 (56.3)	5 (15.6)	***P* = 0.02**	*P* = 0.85

[1]: Significant values are in bold.

of resistance was observed against different antimicrobial agents, including nitrofurantoin, co-trimoxazole, ceftazidime, cephalothin, gentamicin, tetracycline, nalidixic acid, chloramphenicol, and amikacin. Our results are in accordance with those from many previous studies, indicating a major prevalence of resistant *K. pneumoniae* isolates in clinical settings. *K. pneumoniae* isolates showing a high degree of resistance to cephalothin (67.5%), gentamicin (53%), ceftazidime (51.3%), and nalidixic acid (47.5%) have been reported in Iran (21). In other parts of the world, several studies have identified *K. pneumoniae* isolates showing high rates of resistance (up to 50%) to different antimicrobial agents, including ceftazidime, amikacin, gentamicin, co-trimoxazole (22,23), and ciprofloxacin (23,24). Multidrug resistance was observed to be common in our isolates. In our study, 149 (99.3%) isolates showed MDR. The rate of occurrence of MDR *K. pneumoniae* isolates observed in our study is similar to that reported by Khadri et al. (22) and Taslima et al. (25). We also observed the production of ESBLs in about 83% of our MDR isolates, which is consistnet with the results of other studies and indicates a worrisome prevalence rate of MDR strains (21,23). Moreover, our results indicate that integrons are widespread in *K. pneumoniae* isolates. Among 149 MDR isolates, 137 (91.9%) were observed to have integrons, which is an uncommonly high rate of presence of integrons. We also observed the presence of multiple integrons in some isolates. We also found a significant ($P < 0.001$) relationship between the MDR phenotype and integrons, whereas Martinez-Freijo et al. had described only a tendency of development of multidrug resistance in strains with integrons (13). This difference in the observations can be explained by the fact that we studied the resistance of the isolates against nitrofurantoin, co-trimoxazole, and cephalothin as well; moreover, the study duration, integron detection method, and epidemiological conditions in our study differed from those in the previous study. We used an integrase PCR assay, whereas Martinez-Freijo et al. had performed PCR using CSs.

The extent of presence of class 1 integrons in our isolates was 78.5%; this is similar to that observed in hospital strains in the U.S. (14). As previously noted (26), the presence of class 1 integrons was more than that of class 2 integrons. Our study found an association between the presence of class 1 integrons and lack of susceptibility to gentamicin, tetracycline, ceftazidime, cephalothin, chloramphenicol, and nalidixic acid. Previous studies have also reported an association between the *intI1* gene and resistance to many antibiotics, including aminoglycosides, cephalothin, and nalidixic acid (11,14,27). However, we did not observe a statistically significant association between presence of integrons and resistance to nearly half of the antibiotics that we tested, including co-trimoxazole, imipenem, ciprofloxacin, norfloxacin, amikacin, and nitrofurantoin. Acquisition of antimicrobial resistance genes by elements other than integrons and production of metallo-β-lactamase (MBL) enzymes would be an alternative approach for acquiring drug resistance in MDR isolates.

Our results show that a significant association exists between class 2 integrons and resistance to only tetracycline, which indicates that resistance determinants against other tested drugs are not frequently carried by class 2 integrons. The alarming resistance against cephalosporins observed in the ESBL-producing isolates in this study underlines the need for accurate identification of such *K. pneumoniae* isolates before starting empirical therapy with drugs such as extended-spectrum cephalosporins. Imipenem, norfloxacin, ciprofloxacin, and amikacin are supposed to be the most effective in vitro antibiotics. However, the clinical efficacy of these antibiotics in *K. pneumoniae* infections remains to be assessed.

In conclusion, the differential association of class 1 and class 2 integrons with resistance in our isolates suggests that integrons may be facilitating the spread of antimicrobial resistance in our region. Further studies are required to determine the types of gene cassettes in these integrons and the production of MBLs in the clinical isolates of *K. pneumoniae*. To the best of our knowledge, this is the first report on the association of multidrug resistance with the presence of class 1 and class 2 integrons in *K. pneumoniae* isolates from clinical settings in northwest Iran.

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Conflict of interest None to declare.

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