ORIGINAL RESEARCH

Health Science Reports   WILEY
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Molecular detection of Class 1, 2, and 3 integrons in hypervirulent and classic *Klebsiella pneumoniae* isolates: A cross-sectional study

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Abstract

Background and Aims: The "hypervirulent" variant of *Klebsiella pneumoniae* (hvKp) is an emerging pathogen that cause life-threatening infection. The present study was conducted to identify the prevalence of hvKp and to investigate the presence class 1, 2, and 3 integrons in these isolates.

Methods: A cross-sectional study was conducted at three teaching hospitals, Ahvaz, South-west of Iran, from January 1, 2019 to December 31, 2020. Samples were collected from inpatients and included only the first samples collected from each patient. *K. pneumoniae* strains were isolated from different specimens using biochemical test and confirmed by targeting 16S–23S rDNA internal transcribed spacer. HvKp isolates were recovered using string test and were further characterized by detection virulence-associated genes (*rmpA*, *iucA*, and *magA*). Antibiotic susceptibility patterns of isolates were determined using the disc diffusion method. Isolates were screened for presence the integron genes (*intI*, *intII*, and *intIII*) and repetitive element sequence-based polymerase chain reaction (PCR) performed to determine strain relatedness. SPSS version 22 was used for the data analysis.

Results: Seventy-one (77%) of isolates showed multidrug-resistant (MDR) phenotype. HvKP accounted for 14% (13/92) of cKp isolated from blood (46%) and urinary tract infection (38%), and the great majority of them (61.5%; 8/13) exhibited MDR phenotype. Using the PCR assay, 29 of 92 isolates (31.5%) were found to have positive results for the presence of IntI. Three of the *IntI*-positive strains were hvKP. Class 2 integron was present in 8/92 cKp isolates. Integron Class 2 was found to coexist with Class 1 integron in 3/8 isolates. All integron-positive isolates (*IntI* and/or *IntII*) were resistant to at least three different classes of antibiotics and showed MDR phenotype. No Class 3 integrons were detected among the isolates.

Conclusion: The results of our study revealed that considering the role of integrons in facilitating the acquisition and dissemination of resistance genes among bacteria, monitoring the emergence of hvKp, emphasizing on the mechanism of antimicrobial resistance, can prevent from the spread of carbapenemase-producing hvKp strains.

1 | INTRODUCTION

Klebsiella pneumonia is one of the most common hospital opportunistic pathogen causing severe organ and life-threatening disease such as pneumonia, sepsis, and urinary tract infection (UTI). One of the important characteristics of *K. pneumoniae* that has led to its continuous evolution is the ability to obtain new genetic material. Therefore, a new pathotype called hypervirulent *K. pneumoniae* (hvKp) have been derived from classical *K. pneumoniae* (cKp), which differ in pathogenicity and have acquired virulence factors.[1–3]

hvKp has increased the production capacity of its capsules and aerobactin. As a result, this pathotype has changed from a hospital opportunistic pathogen to the community pathogen.[4]

Physicians are familiar with cKp and know that this bacterium is associated with infection in a single site in immunocompromised patients and people with surgical wounds, intravascular device, and endotracheal tube.[5] The majority of hvKp isolates harbors plasmids encoding virulence genes including aerobactin siderophore biosynthesis (*iuc*A), regulator of mucoid phenotype A (*rmp*A),[6] and the wzy genotype K1 (*magA*).[7] Diversity has been observed in these genetic markers, *iuc*A and *rmp*A loci.[8] These genotypic markers are used to reliable detection and confirmation of hvKp, despite the fact that a lot of diversity occurs in them.[9] *K. pneumoniae* strains harboring *mag*A were found to have a characteristic hyperviscous phenotype, detected by the positive string test. Furthermore, *mag* A-positive strains were found to have a mucopolysaccharide capsule and, compared with *mag A* mutant strain, show increased serum resistance, resistance to phagocytosis, and virulence.[10]

However, hvKp can cause infections in multiple sites in healthy people of all ages. Several studies have considered the pathogenic potential and capability of these strains to acquire mobile genetic elements including integrons and transposons. Integrons are determined as powerful mobile genetic elements that are able to transfer resistance gene cassettes among different bacteria.[11] Until now, five classes of antibiotic-resistance-encoding integrons have been identified. Class 1 integrons (*intI*) are the most frequent integron type among clinical isolates of Gram-negative bacteria such as *K. pneumonia*, class 2 integrons (*intII*) have been moderately documented, and class 3 integrons (*intIII*) have been rarely reported.[12,13]

Although several studies have reported the frequency of integrons in cKp isolated from clinical specimens in Iran,[14,15] there are very few previous studies on the prevalence of Class 1, 2, and 3 integrons in hvKp isolates from nosocomial infected patients in our region. Thus, the present study was conducted to determine the prevalence of class 1, 2, and 3 integrons in clinical hvKp isolates in Ahvaz, Iran.

2 | MATERIALS AND METHODS

2.1 | Study design and bacterial isolates and identification

A cross-sectional study was conducted after obtaining approval from the ethics committee of Ahvaz Jundishapur University of Medical Sciences (ethical code number: IR.AJUMS.MEDICINE.REC.1399.030), Ahvaz, Iran. This study was conducted at the Golestan, Imam Khomeini, and Taleqani teaching hospitals, Ahvaz, south-west of Iran, from January 1, 2019 to December 31, 2020.

2.2 | Inclusion/exclusion criteria

We included samples collected from inpatients and only included the first samples taken from each patient. Samples were collected from urine, blood, wound, tracheal tube, abscess, pleural effusion, joint aspirate, peritoneal aspirate, sputum, cerebrospinal fluid, and tonsil. We excluded any secondary cultures of the same patient so as to avoid repeated isolates. We also excluded outpatient samples. Secondary cultures were identified as a sample taken within 6 months of the first sample.

2.3 | Sample size determination and sampling methods

To obtain the sample size, according to the pilot study (including 50 samples) conducted by the researchers, the prevalence of *K. pneumoniae* was estimated to be 0.32. The following formula was used for estimating the sample size:

$$SampleSize = \frac{Z^2_{1-\alpha/2}P(1-p)}{d^2}.$$

Furthermore, the confidence level and precision were considered 95% ($Z = 1.96$) and $d = 10\%$, respectively.

2.3.1 | Specimen collection

Specimens were collected from urine, blood, wound, tracheal tube, abscess, 103 pleural effusion, joint aspirate, peritoneal aspirate, sputum, cerebrospinal fluid, and tonsils. All specimens were cultured (isolation technique) on blood agar and MacConkey agar selective medium. The colonies were then morphologically evaluated and

studied using biochemical tests and conventional method for isolation such as Methyl red-Voges Proskauer, Triple Suger İron, sulfide İndol, motility, Simmons citrate, and urea agar (Merck), and other important method for isolation. All samples were cultured on conventional media blood agar and MacConkey agar selective medium (Merck). Confirmed isolates of *K. pneumoniae* were stored at −20°C in tryptic soy broth (TSB) medium (Merck) with 30% glycerol for the next steps.

2.3.2 | DNA extraction

Total DNA was extracted using boiling method.[16] Briefly, 300 µL of bacterial suspension of 0.5 McFarland standard, which was prepared at TSB medium, was transferred to the 1.5 mL microtube. The suspension was vortexed and then incubated at 100°C in water bath for 10 min and immediately cooled on ice. This process was repeated two times. In the last step, bacterial suspension was centrifuged for 10 min at 14,000*g* and the supernatant containing total DNA was transferred to the 0.5 mL microtube, and used as a template in all molecular methods.

2.3.3 | Phonotypic and genotypic identification of cKP and hvKP

K. pneumonia subsp. *pneumonia* was confirmed based on 16S–23S rDNA internal transcribed spacer (ITS) using specific primers (described in Table 1). Polymerase chain reaction (PCR) condition was as previously described.[17] String test was used for phenotypic identification of hvKP. An inoculation loop was touched to the surface of the colony on an agar plate. Bacterial isolates that were able to form a viscous string >5 mm in length were confirmed as hvKP. For genotypic confirmation of hvKp isolates, all string-positive ones were screened for targeting *magA*, *iucA*, and *rmpA* by PCR analysis.[18–20]

The PCR reaction mixture was prepared in a final volume of 20 µL contained 12.5 µL of 2× Master mix (Amplicon), 0.5 µL of each primer (10 pM/µL), 2 µL of DNA template, and 4.5 µL of distilled water. The microtubes containing reaction mixture were transferred in the Master Cycler gradient PCR Machine (Eppendorf) and PCR was done as follows: initial denaturation at 94°C for 4 min; 35 thermal cycles of denaturation at 94°C for 45 s, annealing at 57–58°C and 61°C for ITS, *magA*, *iucA*, and *rmpA*, respectively (for 30 s), and extension at 72°C for 45 s; and a post-PCR final incubation at 72°C for 5 min. The amplified PCR products were electrophoresed on 1.5% agarose gel containing 0.5 µg/mL safety stain and visualized under a UV transilluminator.

2.4 | Antimicrobial susceptibility testing

K. pneumoniae susceptibility was examined using the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines.[21] Twelve antibiotic disks (MAST) including imipenem (IPM; 10 µg), meropenem (MEM; 30 µg), Ceftriaxone (CRO; 30 µg),

TABLE 1 Oligonucleotide primes sequences used in this study.

Primer	5′–3′ Sequence	Product size (bp)	References
16–23S rDNA ITS	F: ATTTGAAGAGGTTGCAAACGAT	130	[19]
	R: TTCACTCTGAAGTTTTCTTGTGTTC		
rmpA	F: ACTGGGCTACCTCTGCTTCA	516	[19]
	R: CTTGCATGAGCCATCTTTCA		
magA	F: GGTGCTCTTTACATCATTGC	1283	[20]
	R: GCAATGGCCATTTGCGTTAG		
iucA	F: GCATAGGCGGATACGAACAT	556	[18]
	R: CACAGGGCAATTGCTTACCT		
IntI	F: 5′-CAGTGGACATAAGCCTGTTC-3′	558	[13, 22]
	R: 5′-CCCGAGGCATAGACTGTA-3′		
IntII	F: 5′-CACGGATATGCGACAAAAAGGT-3′	789	
	R: 5′-GTAGCAAACGAGTGACGAAATG-3′		
IntIII	F: 5′-GCCTCCGGCAGCGACTTTCAG-3′	979	
	R: 5′-ACGGATCTGCCAAACCTGACT-3′		
REP 1	5′-IIIGCGCCGICATCAGGC-3′	Variable	[22]
REP 2	5′-ACGTCTTATCAGGCCTAC-3′	Variable	

Abbreviation: ITS, internal transcribed spacer.

Cefotaxime (CTX; 30 µg), Cefepime (30 µg), Amikacin (AMK; 30 µg), gentamicin (GEN; 10 µg), ceftazidime (CAZ, 30 µg), tetracycline (TET; 30 µg), AMK (30 µg), ciprofloxacin (5 µg), chloramphenicol (CL; 30 µg), and trimethoprim-sulfamethoxazole (SXT; 25 µg) were used. *K. pneumoniae* ATCC 700603 was used as the control for antibiotic resistance. MDR was outlined as acquired nonsusceptibility to a minimum of one agent in three or more antimicrobial classes.

2.5 | Integron detection

Extracted DNA by boiling method was used as the templates for integron identification. Identification of three classes of integrons was carried out for all isolates using specific primer pairs for *intI1*, *intI2*, and *intI3* genes as listed in Table 1. Cycling conditions were as follows: Initial denaturation at 94°C for 5 min; 35 thermal cycles of denaturation at 94°C for 1 min, annealing at 54°C, 58°C, and 60°C for *IntI*, *IntII*, and *IntIII*, respectively, for 1 min, and extension at 72°C for 1 min; and followed by a final elongation at 72°C for 5 min. The PCR products were electrophoresed using 1% agarose gels in 1× TBE (Tris-Boric acid-EDTA) buffer. The agarose gels were stained with 5 µg/mL safety stain and the PCR products were visualized under the UV transilluminator.[13,22]

2.6 | Repetitive element sequence-based PCR (REP-PCR)

DNA was extracted using boiling method as described previously and was diluted to 40 ng/µL. The genomic DNA of integron-positive strains and 20 integron-negative strains, including both cKp and hvKp, was amplified for fingerprinting using Rep-PCR primers, at a concentration of 50 pmol/mL. REP primer sequences are listed in Table 1. PCR was run using thermal cycles as follows: initial denaturation at 94°C for 10 min; 30 thermal cycles of denaturation at 94°C for 1 min, annealing at 48°C for for 1 min and extension at 72°C for 2 min; followed by a final extension at 72°C for 15 min. PCR products were electrophoresed on 2% agarose using a 100 bp plus DNA ladder (Thermo Fisher Scientific) as a size marker. Bionumerics software (v. 6.6) was utilized to analyze the band patterns, considering dice tolerance 2.0 and unweighted pair group method with arthmetic mean method to depict a dendrogram. Strain discrimination was done as follows: similarity > 97% is considered as indistinguishable (no differences in fingerprints), similarity > 95% as similar (one to two band difference in fingerprints), and similarity < 95% as different. In the present study, optimal cutoff for clustering was 45%.[22]

2.7 | Data quality and data management

The quality of data was evaluated using a checklist provided by Anaïs et al.[23] In this checklist, to assess the quality of the study, following items are checked: sample size, research objectives, statistical analysis, sample collection, and appropriate materials and methods. One score was assigned to each parameter and study was included if at least seven scores were achieved.[23]

2.8 | Data analysis

All statistical analyses were done using the statistical package IBM SPSS v.22.0 (IBM Corp.). The Pearson's χ^2 test or Fisher's exact test was used for categorical variables. All statistical tests were two-tailed and a $p \leq 0.05$ was considered as significant.

3 | RESULTS

3.1 | The demographic distribution of collected clinical samples

In this study, 92 nonrepetitive clinical samples including 27 urine samples (29.3%), 27 blood samples (29.3%), 11 wound samples (12%), seven tracheal lavage (7.6%), four samples of abscess (4.3%), four samples of pleural effusion (4.3%), three sputum (3.3%), three peritoneal aspirate (3.3%), three joint aspirate (3.3%), two cerebrospinal fluid (2.2%), and a sample of tonsil (1.1%) that were collected during 1 year from Imam Khomeini, Golestan, and Taleghani hospitals of Ahvaz, Iran. All *K. pneumoniae* isolates were primarily characterized by biochemical tests and finally were confirmed by targeting the 16S–23S ITS region using PCR. Among the 92 isolates, 53 samples (57.6%) were obtained from Golestan hospital, 32 samples (34.7%) were obtained from Imam Khomeini Hospital, and seven samples (7.6%) were from Taleghani Hospital. Forty-nine patients (53.3%) were male and 43 patients (46.7%) were female, and the average age for patients were 35.05 ± 14.35 years (Table 2).

3.2 | Antimicrobial resistance

The rate of resistance to convenient antibiotics in hvKp strains was lower than that in the cKp group with the exception of CL (47% for both pathotype). The results revealed that CTX (81%), CRO and MEM (79%), CAZ (77%), and SXT (71%) were the most inactive antibiotics with resistance rates above of 70%. However, the most effective antibiotics were TET, CL, and GEN with resistance rates approximately lower than 40%. There was no significant association among antimicrobial resistance, age, and gender ($p > 0.05$). The resistance rates to most tested antimicrobial agents were lower in hvKp than in cKp isolates (Figure 1), so that IPM, AMK, GEN, CL, TET, SXT, and CAZ had higher susceptibility rate (Figure 1). Carbapenem-resistant *K. pneumonia* in classic pathotype was higher than 65%, whereas in hvKp strains resistance to IPM was shown lower than 40%. Among hvKp isolates 46% (6/13) were carbapenem resistant.

TABLE 2 The demographic distribution of collected clinical samples.

| Strain number	Gender	Age	*K. pneumoniae* type	String test	Sample	Hospital	Ward
1	Female	12	Hypervirulent	Negative	Blood	Imam Khomeini	Internal medicine
2	Male	49	Classic	Negative	Urine	Imam Khomeini	Internal medicine
3	Female	35	Classic	Negative	Urine	Golestan	Internal medicine
4	Female	27	Classic	Negative	Wound	Taleghani	Burn
5	Male	5	Classic	Negative	Blood	Imam Khomeini	Pediatrics
6	Male	18	Classic	Negative	Peritoneal aspirate	Imam Khomeini	Men
7	Female	22	Hypervirulent	Negative	Urine	Golestan	Surgery
8	Female	46	Classic	Negative	Wound	Taleghani	Burn
9	Male	39	Classic	Negative	Blood	Golestan	ICU
10	Male	40	Classic	Negative	Tracheal tube	Imam Khomeini	ICU
11	Female	19	Classic	Negative	Sputum	Golestan	ICU
12	Male	26	Classic	Negative	Sputum	Golestan	ICU
13	Female	38	Classic	Negative	Blood	Golestan	Women
14	Male	34	Classic	Negative	Urine	Golestan	Men
15	Female	42	Classic	Negative	Urine	Imam Khomeini	Internal medicine
16	Female	38	Classic	Negative	Abscess	Imam Khomeini	Women
17	Male	65	Classic	Negative	Joint aspirate	Golestan	Men
18	Male	67	Classic	Negative	Cerebrospinal fluid	Taleghani	Men
19	Female	45	Classic	Negative	Urine	Imam Khomeini	Women
20	Female	16	Classic	Negative	Blood	Imam Khomeini	Women
21	Female	8	Classic	Negative	Cerebrospinal fluid	Imam Khomeini	Pediatrics
22	Male	54	Classic	Negative	Blood	Imam Khomeini	Men
23	Male	38	Classic	Negative	Blood	Golestan	Internal medicine
24	Female	45	Classic	Negative	Urine	Golestan	Internal medicine
25	Male	38	Classic	Negative	Urine	Golestan	Internal medicine
26	Male	20	Classic	Negative	Wound	Taleghani	Burn
27	Female	14	Hypervirulent	Negative	Wound	Imam Khomeini	Surgery
28	Male	78	Classic	Negative	Tonsil	Imam Khomeini	ENT
29	Male	31	Hypervirulent	Negative	Urine	Golestan	Urology
30	Male	36	Classic	Negative	Urine	Golestan	Urology
31	Male	25	Hypervirulent	Positive	Blood	Imam Khomeini	Men
32	Male	39	Classic	Negative	Blood	Golestan	Internal medicine
33	Female	36	Classic	Negative	Blood	Golestan	Internal medicine
34	Male	15	Classic	Negative	Urine	Imam Khomeini	Internal medicine
35	Male	39	Classic	Negative	Urine	Imam Khomeini	Internal medicine
36	Male	50	Classic	Negative	Urine	Golestan	ICU
37	Female	42	Classic	Negative	Blood	Golestan	ICU
38	Female	39	Classic	Negative	Blood	Golestan	ICU
39	Male	48	Classic	Negative	Blood	Golestan	Men

(Continues)

TABLE 2 (Continued)

Strain number	Gender	Age	K. pneumoniae type	String test	Sample	Hospital	Ward
40	Male	43	Classic	Negative	Abscess	Golestan	ICU
41	Male	20	Classic	Negative	Abscess	Imam Khomeini	ICU
42	Female	21	Hypervirulent	Negative	Blood	Imam Khomeini	Women
43	Female	49	Classic	Negative	Blood	Imam Khomeini	Women
44	Male	37	Classic	Negative	Urine	Golestan	Neurology
45	Female	28	Hypervirulent	Negative	Urine	Golestan	Women
46	Male	17	Classic	Negative	Sputum	Golestan	Men
47	Female	50	Classic	Negative	Joint aspirate	Golestan	Surgery
48	Male	43	Classic	Negative	Tracheal tube	Golestan	ICU
49	Male	46	Classic	Negative	Tracheal tube	Golestan	ICU
50	Male	25	Hypervirulent	Positive	Urine	Golestan	Urology
51	Male	36	Classic	Negative	Urine	Imam Khomeini	Men
52	Male	38	Classic	Negative	Blood	Golestan	Neurology
53	Female	19	Classic	Negative	Tracheal tube	Golestan	ICU
54	Female	22	Classic	Negative	Wound	Golestan	General surgery
55	Female	34	Classic	Negative	Wound	Golestan	General surgery
56	Female	45	Classic	Negative	Peritoneal aspirate	Golestan	General surgery
57	Male	31	Classic	Negative	Urine	Golestan	Men
58	Male	29	Classic	Negative	Urine	Golestan	Urology
59	Male	15	Classic	Negative	Blood	Golestan	Men
60	Female	35	Hypervirulent	Negative	Peritoneal aspirate	Golestan	Surgery
61	Female	43	Classic	Negative	Joint aspirate	Golestan	Surgery
62	Male	36	Classic	Negative	Pleural effusion	Imam Khomeini	ICU
63	Male	58	Classic	Negative	Pleural effusion	Golestan	ICU
64	Female	48	Classic	Negative	Blood	Imam Khomeini	women
65	Female	33	Hypervirulent	Negative	Blood	Imam Khomeini	Women
66	Female	35	Hypervirulent	Negative	Urine	Imam Khomeini	Women
67	Female	14	Classic	Negative	Urine	Golestan	Women
68	Male	32	Classic	Negative	Blood	Golestan	Men
69	Male	14	Classic	Negative	Blood	Golestan	Men
70	Male	26	Classic	Negative	Urine	Golestan	Urology
71	Male	74	Classic	Negative	Wound	Taleghani	Burn
72	Female	64	Classic	Negative	Wound	Taleghani	Burn
73	Female	45	Classic	Negative	Abscess	Golestan	Women
74	Female	51	Classic	Negative	Urine	Golestan	Urology
75	Male	27	Classic	Negative	Urine	Golestan	Men
76	Female	39	Classic	Negative	Urine	Imam Khomeini	Women
77	Female	45	Hypervirulent	Positive	Blood	Golestan	Women
78	Male	15	Hypervirulent	Positive	Blood	Golestan	ICU

TABLE 2   (Continued)

Strain number	Gender	Age	*K. pneumoniae* type	String test	Sample	Hospital	Ward
79	Male	33	Classic	Negative	Wound	Golestan	Surgery
80	Female	28	Classic	Negative	Wound	Imam Khomeini	Surgery
81	Male	45	Classic	Negative	Urine	Imam Khomeini	Men
82	Female	43	Classic	Negative	Blood	Imam Khomeini	Women
83	Male	32	Classic	Negative	Blood	Golestan	Men
84	Female	44	Classic	Negative	Blood	Imam Khomeini	Women
85	Male	34	Classic	Negative	Urine	Imam Khomeini	Men
86	Female	28	Classic	Negative	Tracheal tube	Golestan	ICU
87	Female	45	Classic	Negative	Tracheal tube	Golestan	ICU
88	Male	18	Classic	Negative	Tracheal tube	Imam Khomeini	ICU
89	Female	13	Classic	Negative	Pleural effusion	Imam Khomeini	ICU
90	Male	40	Classic	Negative	Pleural effusion	Golestan	ICU
91	Male	27	Classic	Negative	Blood	Golestan	ICU
92	Female	34	Classic	Negative	Wound	Taleghani	Burn
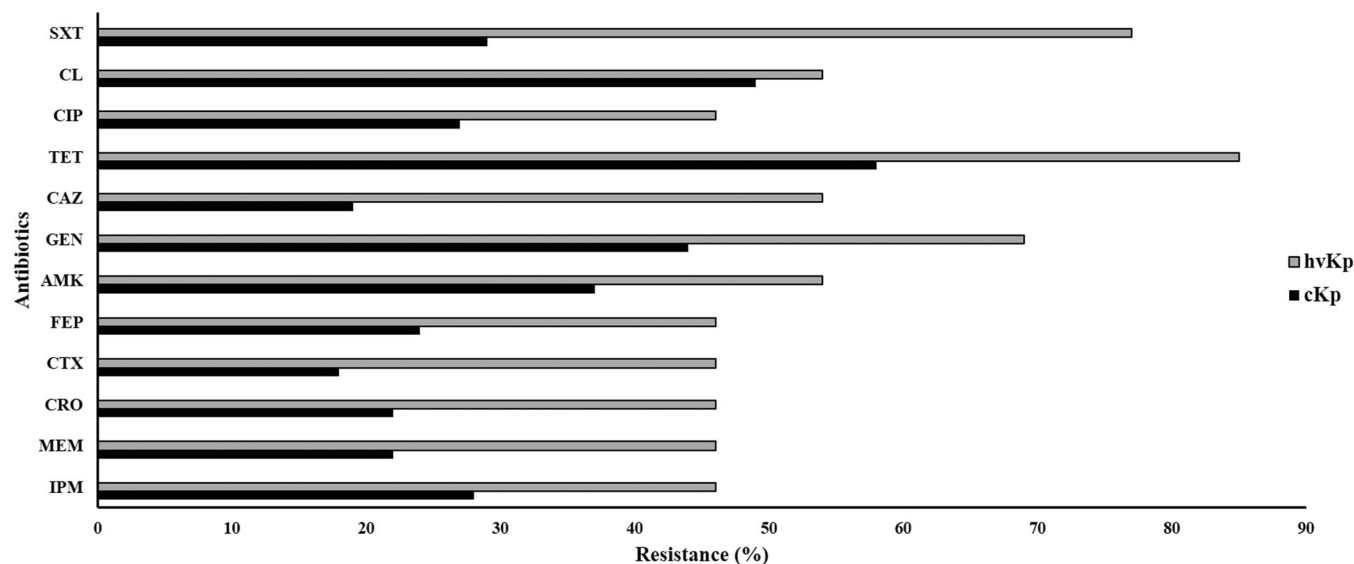
FIGURE 1   Antibiotic sensitivity graph of *K. pneumoniae* isolates. SXT: Trimethoprim/sulphamethoxazole, CL: Chloramphenicol, CIP: Ciprofloxacin, TET: tetracycline, CAZ: ceftazidime, GEN: Gentamicin, AMK: Amikacin, FEP: Cefepime, CRO: Cefteriaxone, MEM: Meropenem, IPM: Imipenem, cKp: Classic *Klebsiella pneumoniae*, hvKp: Hypervirulant *Klebsiella pneumoniae*.

3.3 | Phenotypic and genotypic characteristics of isolates

Of the 92 *K. pneumoniae* isolates, 77% (71/92) were MDR as defined by Magiorakos et al.[24] Of these 92 isolates, 85% (79/92) were considered as cKp. Therefore, the remaining isolates with string test positive (13 isolates) followed by checking for the presence virulence-associated genes (*magA*, *iucA*, and *rmpA*) confirmed as hvKp. A total of six (46%) hvKp were isolated from blood and five (38.5%) from urine, and the remaining hvKp were isolated from

wound and peritoneal fluid. Eight hvKp isolates identified as MDR (61.5%) (Table 3).

The screening of the three virulence genes (*magA*, *iucA*, and *rmp*A) and string test led to identification of 13 (14%) hvKp strains from 92 samples. Among hvKp isolates, 1/13 (7%) were *rmpA*-positive, one isolate was positive for all three genes (7%), and seven isolates (54%) were positive for *iucA*. Trigenic-positive isolates were susceptible to all tested antibiotics excepted TET. This was isolated from urine of a female patient. One uni-*magA*-positive strain isolated from blood was susceptible only to CL, TET, and SXT.

TABLE 3 Clinical and microbiological characteristics of the hvKp isolates.

ID	Sample	Ward	String	Virulence gene	Integron	Resistance phenotype
KL1	Blood	Internal ICU	+	magA	IntI	MDR
KL7	UTI	Surgery	+	magA icuA rmpA	–	–
KL27	Burn wound	Surgery	+	rmpA	intI	MDR
KL29	UTI	Urology	+	iucA	–	–
KL31	Blood	Men	+	iucA	IntI	MDR
KL42	Blood	Women	+	iucA	–	–
KL45	UTI	Women	+	iucA	–	–
KL50	UTI	Urology	+		–	–
KL60	Peritonea	Surgery	+	iucA	–	MDR
KL65	Blood	Women	+	iucA	–	MDR
KL66	UTI	Women	+	iucA	–	MDR
KL77	Blood	Women	+	–	–	MDR
KL78	Blood	ICU	+	–	–	MDR

Abbreviations: hvKp, hypervirulant *K. pneumoniae*; ICU, intensive care unit; MDR, multidrug-resistant; UTI, urinary tract infection.
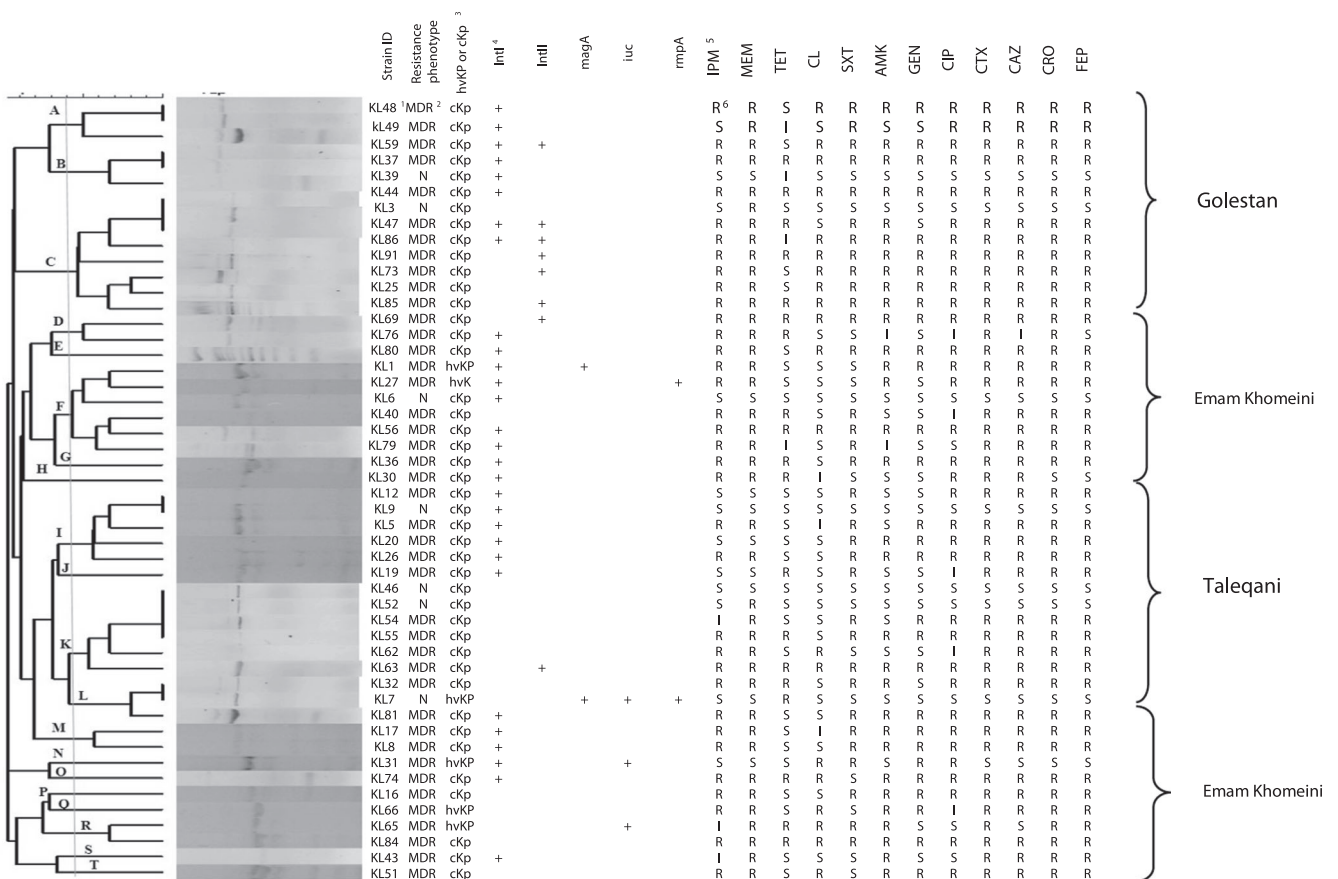
FIGURE 2 The phenotypic and genotypic characteristics of of *K. pneumoniae* isolates in this study. [1]KL: Klebsiella, [2]MDR: multidrug resistance, [3]cKp: Classic Klebsiella pneumoniae, hvKp: Hyperviruleant Klebsiella pneumoniae, [4]Int: Integron, [5]IPM: Imipenem, MEM: Meropenem, TET: Tetracycline, CL:Chloramphenicol, SXT: Trimethoprim/Sulphamethoxazole, AMK: Amikacin, GEN: Gentamicin, CIP: Ciprofloxacin, CTX: Cefotaxime, CAZ: Ceftazidime, CRO: cefteriaxone, FEP: Cefepime, [6]R: Resistance, I: Intermediate, S: Susceptible.

Using the PCR assay, 31.5% (29/92) of isolates were found to have positive results for the presence of IntI (Figure 2). Three intI-positive strains were hvKP (two strains isolated from blood [2/3, 67%] and one isolated from wound [1/3, 33%]). Other intI-positive cKp strains were isolated from blood, urine, and other samples. Class 2 integron (IntII) was present in 8/92 isolates. All IntII-positive strains were cKp pathotype. IntII was found to coexist with intI in 3/8 isolates. These strains were isolated from tracheal tube, blood, and joint aspiration, and were resistant to six different classes of antibiotics. All integron-positive isolates (IntI and/or IntII) were resistant to at least three different classes of antibotics and showed MDR phenotype. No integrons were found in the remaining isolates. No Class 3 integrons (intIII) were detected among isolates.

3.4 | Epidemiological investigations

About 10 distinctive Rep-PCR clusters (named A–D, F, I, M, K, L, and R) and 10 unique types (named E, G, H, J, N, O, P, Q, S, and T) were inferred from the band patterns (Figure 2). Cluster K consisted of integron-negative strains. Integron-positive strains distributed among other clusters. Our data showed that up to 80% of all the strains were included in 10 distinct clusters and 20% of them had unique band pattern. No significant differences was seen between Rep types of cKp and hvKp isolates (p > 0.05). However, clusters A–C belonged to Golestan Hospital, clusters D–H and M–H belonged to Emam Khomeini Hospital, whereas clusters I– L belonged to Taleqani Hospital; therefore, there was a significant difference between different Rep groups in terms of the hospitals in which the samples were taken (p < 0.001). The largest Rep type was type C including eight isolates in which all of them were classic pathotype of Golestan Hospital (Figure 2).

4 | DISCUSSION

hvKp has introduced as a new global health problem and a potential "superbug" for health settings, causing a wide range of community-acquired infections. In this research, by positive string test, 14.2% of isolates were determined as hypervirulent, which was confirmed by the detection of rmpA, iucA, and/or magA genes. Because of this, the sensitivity and specificity of string test are 66.7% and 95.2%, respectively.[25] In the present study, we amplified three virulence genes (rmpA, iucA, and magA) in all isolates for confirmation of hvKp pathotype. All string-positive isolates were positive for at least one of the mentioned biomarkers, whereas the remaining isolates in the present study, even all MDR isolates, screened negative for all three biomarkers. Despite the findings, previous studies have shown these biomarkers are superior to phenotypic-based methods in the detection of hvKp isolates.[26,27] In this study, the results of phenotypic test were consistent with genotypic test. The reason for this matching could be that the aerobactin gene was one of the confirmatory gene; according to previous studies, siderophore

production may serve as an hvKp confirmatory test in the clinical laboratory setting.[28]

We identified the hvKp in 5% (11/13) of patients with K. pneumoniae bacteremia and UTI. These isolates were mostly recovered from patients with underlying diseases such as diabetes mellitus or in those taking immunosuppressive drugs. In agreement with our findings (5/13; 38.5%), Lin et al. found only four hvKp from UTI samples (27.8%) with diabetes mellitus.[29] However, Liu et al. identified 31.4% (22/70) of patients with K. pneumoniae bacteremia, who had no underlying diseases.[30] These finding suggests that hvKp pathotype not only play an important role in community-acquired infections but also it can cause infection in people with underlying diseases.[31]

Consistent with previous reports, the rate of resistance to convenient antibiotics in hvKp strains was lower than that in the cKp group with the exception of chloramphenicol.[27] It is widely accepted that carbapenemase-producing hvKp (CRhvKp) strains cause various life-threatening infections, especially in critical patients.[5,7,9,25] In the present study, ~50% of the isolates were resistant to carbapenems. According to the results of the Li et al., only 17% of hvkp were ESBL producers.[27] However, Traghian et al.[31] in 2020 showed that all hvKps harbored ESBL genes; their finding indicated on the presence of compatibility of plasmids containing these genes with hvKp strains. Additionally, MDR-hvKp was identified in eight out of 13 (61.5%) hvKp isolates. All CRhvKp had MDR phenotype. Our findings about MDR-hvKp (61.5%) were higher than that were identified in Liu et al.[32] study at 2019 (20%). These findings indicate that hvKp strains are evolving and acquiring resistance genes, and it is better to take them seriously in controlling nosocomial infections.

In our study, 25.8% of isolates were found to have positive results for the presence of Class 1 integrons. No Class 3 integron was detected. Mirkalantari et al.[33] reported that the prevalence of Class 1 integrons was 46% in cKp isolates and Soleymanian et al.[34] reported that Class 1 integrons were found in 12.5% of the cKp isolates. Class 3 integron was not detected in Seyed-Javadi study, which is in accordance with our study.[35] In a survey in Taiwan on the prevalence of Class 1 integrons in clinical K. pneumoniae isolates collected from Taiwan, during two periods (1993 and 2004), Class 1 integrons were present in 78 isolates (34.2%) from 1993 to 129 (32.9%) from 2004.[36] The most commonly encountered integrons are those of class 1, which may be located on transmissible plasmids and transposons.[37]

The prevalence of IntII in our study was 5.5%. The results of the integron PCR analysis of Ahangarzadeh et al. (2012) study showed that 20 (13.4%) MDR K. pneumoniae isolates carried intII.[15] Furthermore, our results showed that the presence of IntI was more prevalent than that of IntII; this is similar to that observed in previous studies.[13,38,39] All integron-positive strains (both classes) had MDR phenotype. In our study, association between the presence of Class 1 and/or 2 integrons and resistance to specific antibiotic classes was not seen. However, several studies showed that the presence of IntI have been associated with resistance to aminoglycosides, cephalosporins, tetracycline and chloramphenicol as well as the presence of IntII with tetracycline resistance.[39,40]

However, hvKP strains were still susceptible to convenient antibiotics, but these strains by acquisition the mobile genetic elements has been increasingly resistant to ones.[27,41] Here we reported the appearance of six CRhvKP isolates wherein three of them harbored class 1 integron, which were recovered from bacteremia and wound infections. Although several studies have shown low prevalence of antimicrobial resistance in hvKP strains,[42] these results indicate that hvKp strains are able to acquire different types of mobile genetic elements with antibiotic resistance cassettes. The pathogenesis of these isolates and the disconcerting antimicrobial resistance pattern should be seriously considered. Therefore, we need to implement special monitoring programs to control the infection caused by them in the health system. As yet, the reason for the low prevalence of antimicrobial resistance among hvKP strains remains unclear; however, reports of these isolates may be evolutionally due to the fact that hvKp strains have thick capsules so they can adhere strongly to surfaces and form strong biofilms. Therefore, they did not need to acquire resistance genes to survive in the environment.[43]

Our Rep-PCR findings showed that the strains taken from a unique hospital grouped in the same or closely related clusters. Although the discriminatory power of Rep-PCR is less than other genotyping method such as pulse field gel electrophoresis (PFGE) or multiple locus variable number tandem repeat analysis (MLVA), but Rep-PCR still remains as a rapid, standard, and excellent reproducibility valuable tool for use in Gram-negative bacteria genotyping.[44,45] First limitation in the present study include the evaluation of relationship between the *K. pneumoniae* isolates using PCR based method, Rep-PCR. Although more advanced molecular techniques such as the whole-genome sequencing method and multi-locus sequence typing (MLST) to decipher the clonal relatedness between bacterial isolates are considered most accurate experimental methods, we recommend Rep-PCR for initial screening of isolates, followed by using a more sensitive method, such as PFGE, MLVA, or MLST for those which have the same band pattern. Another limitation in our study that must be addressed is too small number of hvKp and CRhvKp isolates to draw safe conclusions regarding the antimicrobial resistance and presence or absent integron in *K. pneumoniae* isolates, and therefore large studies are needed to validate our results.

To our knowledge, strength of this study is that the present study is the first report on the prevalence of class 1, 2, and 3 integrons among hvKp and CRhvKp isolated from different samples in Ahvaz, Iran. The results of our study revealed that hvKp strains, which were well identified by string test and tracing of *mag*A, *iuc*A, and *rmp*A genes, were more sensitive than classic pathotype to conventional antibiotics. Furthermore, the majority of hvKp strains were integron-negative, whereas the presence of three carbapenem-resistant positive integron hvKp indicates that these strains can readily acquire mobile genetic elements and virulent plasmids. Consequently considering the role of integrons in the facilitating acquisition and dissemination of resistance genes among bacteria, monitoring the emergence of hvKp with emphasizing on the mechanism of antimicrobial resistance can prevent from the spread of CRhvKp strains.

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