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RESEARCH ARTICLE

Detection of extensively drug-resistant and hypervirulent *Klebsiella pneumoniae* ST15, ST147, ST377 and ST442 in Iran

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ABSTRACT

In this study, we focused on the emergence of extensively drug-resistant (XDR), pandrug-resistant (PDR), and hypervirulent *Klebsiella pneumoniae* (hvKP) in Iran. During 2018 to 2020 a total of 52 *K. pneumoniae* isolates were collected from different clinical specimens. The hvKP isolates were identified by PCR amplification of virulence and capsular serotype-specific genes. Hypermucoviscous *K. pneumoniae* (hmKP) were identified by string test. Carbapenem-resistant hvKP (CR-hvKP), multidrug-resistant hvKP (MDR-hvKP), extensively drug-resistant hvKP (XDR-hvKP), and pandrug-resistant hvKP (PDR-hvKP) were determined by disc diffusion method, Carba-NP test and PCR method. XDR-hvKP isolates were typed by multilocus sequence typing (MLST). Among all *K. pneumoniae* isolates 14 (26.9%) were identified as hvKP and 78.6% (11/14) of them were hmKP however, none of the classic *K. pneumoniae* (cKP) isolates were hmKP. The predominant capsular serotype of hvKP was *K2* (42.85%) followed by *K1* (35.71%). The prevalence of MDR-hvKP, XDR-hvKP and PDR-hvKP isolates were 6 (42.9%), 5 (35.7%) and 1 (7.1%), respectively. ESBL production was found in 85.7% of hvKP isolates and most of them carried bla_{TEM} gene (78.6%) and 6 isolates (42.9%) were CR-hvKP. Among hvKP isolates, 1 (7.1%), 2 (14.3%), 3 (21.4%), 8 (28.6%), and 11 (78.6%) carried bla_{NDM-6}, bla_{OXA-48}, bla_{CTX-M}, bla_{SHV}, and bla_{TEM} genes, respectively. According to MLST analysis, 2, 1, 1, and 1 XDR-hvKP isolates belonged to ST15, ST377, ST442, and ST147, respectively. The occurrence of such isolates is deeply concerning due to the combination of hypervirulence and extensively drug-resistance or pandrug-resistance.

KEYWORDS

hypervirulent *Klebsiella pneumoniae*, extensively drug-resistance, multidrug-resistant hvKP, pandrug-resistance, carbapenem-resistant hypervirulent *Klebsiella pneumoniae*

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative, encapsulated, non-motile, and lactose-fermenting, facultative anaerobic bacterium [1–3]. This opportunistic pathogen remains an important public health issue, because it can lead to serious infections and life-threatening diseases. The potential of *K. pneumoniae* to acquire new genetic material is very important for its ongoing evolution. Consequently, two pathotypes called classic *K. pneumoniae* (cKP) and hypervirulent *K. pneumoniae* (hvKP) are currently in circulation, both pose specific challenges for clinicians [1, 2] and they are global pathogens. However, the incidence of hvKP infections in the Asian Pacific Rim countries is steadily growing over the past three decades [4–8]. On the

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other hand, cKP has been the most common pathotype to date in Western countries. Nonetheless, hvKP's infections are increasingly being identified outside Asia [9, 10].

The hvKP pathotype is more virulent and more threatening than the cKP. They have also an ability to cause highly invasive and fatal infections, such as liver abscesses and endophthalmitis, in both young and healthy individuals and immunocompromised individuals [11, 12].

In Taiwan, a hypervirulent *K. pneumoniae* (hvKP) variant was detected in 1986 for the first time, and these strains are increasingly being reported in Argentina, Korea, Australia, USA, and Scandinavia [1, 2, 4–8, 10]. HvKP may result in an invasive clinical syndrome, which is defined as a community-acquired liver abscess with or without extrahepatic complications like necrotizing fasciitis or endophthalmitis [9, 13, 14]. Furthermore, hvKP's infection has the ability to spread metastatically [9, 15].

There is currently no unique marker with high sensitivity for detecting hvKP strains. In previous studies, string test identifying the hypermucoviscous phenotype has been used to distinguish hvKP from cKP [10, 16, 17], but whether all hvKP strains are hypermucoviscous is not clear [11]. So, in recent studies hvKP isolates were identified based on the existence of virulence genes such as *peg-344* (putative transporter gene), *iucA* (iron-acquisition systems aerobactin biosynthetic gene), *iroB* (salmochelin biosynthetic gene), *magA* (mucoviscosity associated gene A), *rmpA* (regulator of mucoid phenotype A) and *rmpA2* (regulator of mucoid phenotype A2) [18–24].

Similar to other members of *Enterobacteriaceae* family, *K. pneumoniae* has an extracytoplasmic outer membrane that is made up of a lipid bilayer with related proteins, lipopolysaccharides (LPS), and lipoproteins. Outside the outer membrane is where the capsular polysaccharide is located and K1, K5, K2, K20, K54, and K57 are the most common hvKP capsular types [11, 19, 24, 25].

The combination of virulence factors exhibited by hvKP and antimicrobial resistance determinants possessed by cKP on the same or coexisting plasmids has recently encountered clinicians with an even greater threat. The result is the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) hvKp [26–29]. Furthermore, the rise of carbapenem-resistant hypervirulent *K. pneumoniae* (cr-hvKP) in Brazil and America, and also the growing antimicrobial resistance of hvKP in China are the global challenging issues [17, 30, 31].

In Iran, data on the prevalence of XDR and PDR hypervirulent *K. pneumoniae* strains are rare. The current research was performed to assess evolution of extensively drug-resistant hypervirulent *K. pneumoniae* strains.

METHODS

Ethical statement

The Ethics Committee of Shahid Beheshti University of Medical Sciences authorized this report with ethical number "IR.SBMU.MSP.REC.1398.787". Participants were kept confidential in order to preserve the privacy of the patients, and no identifiable private information was obtained or included in the study.

Bacterial isolates

From September 2018 to August 2020, *K. pneumoniae* isolates (*n*: 52) were recovered from patients hospitalized in Taleghani, Labbafinejad, Shohada Tajrish, Imam Hossein and Modarres hospitals, Tehran, Iran. The isolates were cultured from different clinical specimens, including sputum, urine, blood, and pus.

All clinical samples were transported to the diagnostic microbiological laboratory of the Department of Microbiology of the School of Medicine, SBMU, Tehran, Iran. Bacterial isolates were identified by conventional microbiological and biochemical tests, such as citrate utilization test, urease test, ornithine decarboxylase (OD) test, methyl red/Vogues-Proskauer (MR/VP) test and reactions on SH2/indole/motility (SIM) and triple sugar iron (TSI) agar media [32] and stored at −70 degrees centigrade in trypticase soy broth (Merck, Germany) supplemented with 20% glycerol until used.

PCR detection of the virulence-associated and capsular genes

According to the manufacturer's instructions, genomic DNA from single bacterial colonies grown on primary culture plates was extracted using a DNA extraction package (High Pure PCR Template Preparation Kit-Roche, Germany, Lot. No. 10362400). PCR analysis was performed to detect capsular serotype-specific genes (*K1*, *K2*, *K5*, *K20*, *K54*, and *K57*) and virulence genes (*peg-344*, *iucA*, *iroB*, *rmpA*, *rmpA2*, and *magA*) using specific primers which were used in the previous studies (Table 1) [25, 28, 33–41] and the results were validated by sequencing. PCR was performed in a total volume of 25 µl reaction mixture which was contained 12.5 µl of 2 × Master Mix (SinaClon-Iran, CAT. No., PR901638) containing 1 × PCR buffer, 0.4 mmol L^{-1} dNTPs, 3 mM $MgCl_2$, and 0.08 U/µl Taq DNA polymerase, 1 µl of 10 pmol of each primer, 1 µl (20 ng) of DNA template, and 9.5 µl of sterile distilled water. A thermal cycler (Eppendorf, Master Cycler Gradient, Germany) was used to perform the amplification reactions under the following thermal cycling conditions: initial denaturation at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 45s, annealing at 50–60°C, according to the melting temperature (Tm) of the each primer for 45s, extension at 72°C for 45s, and a final extension at 72°C for 5 min [42]. PCR products were electrophoresed by 1–1.5% agarose gel, visualized by DNA Safe stain (SinaClon, Tehran, Iran), and photographed under UV light. Positive control strains were kindly provided by Medical Microbiology Department of SBMU. Sequencing of the amplicons was performed with an ABI PRISM 3700 sequencer (Macrogen Co., Korea) to confirm PCR results.

Table 1. PCR primers used in this study

Target virulence genes	Primer Sequence (5′-3′)	Reference
k1 (magA)	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	[34]
k2	F: GACCCGATATTCATACTTGACAGAG R: CCTGAAGTAAAATCGTAAATAGATGGC	[35]
k5	F: TGGTAGTGATGCTCGCGA R: CCTGAACCCACCCCAATC	[35]
k20	F: CGGTGCTACAGTGCATCATT R: GTTATACGATGCTCAGTCGC	[23]
k54	F: CATTAGCTCAGTGGTTGGCT R: GCTTGACAAACACCATAGCAG	[36]
k57	F: CTCAGGGCTAGAAGTGTCAT R: CACTAACCCAGAAAGTCGAG	[23]
rmpA	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	[37]
rmpA2	F: CTTTATGTGCAATAAGGATGTT R: CCTCCTGGAGAGTAAGCATT	[38]
iucA	F: ATAAGGCAGGCAATCCAG R: TAACGGCGATAAACCTCG	[38]
iroB	F: GGGATGCTTGATTACGCAGG R: GGACTGTATTAAGCTGCCGC	[39]
peg-344	F: CTTGAAACTATCCCTCCAGTC R: CCAGCGAAAGAATAACCCC	[40]
bla_{TEM}	F: TCGGGGAAATGTGCGCG R: TGCTTAATCAGTGAGGCACC	[48]
bla_{SHV}	F: TTAGCGTTGCCAGTGCTC R: GGTTATGCGTTATATTCGCC	[49]
$bla_{\text{CTX-M}}$	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	[49]
bla_{PER}	F: CCTGACGATCTGGAACCTTT R: GCAACCTGCGCAATGATAGC	[50]
bla_{VEB}	F: CGACTTCCATTTCCCGATGC R: GGACTCTGCAACAAATACGC	[51]
bla_{GES}	F: TTGCAATGTGCTCAACGTTC R: TAGTTGTATCTCTGAGGTCG	[52]
bla_{KPC}	F: CGTCTAGTTCTGTCTTG R: CTTGTCATCCTTGTTAGGCG	[53]
bla_{VIM}	F: GATGGTGTTTGGTCGCATA R: CGAAATGCGCAGCACCAG	[54]
bla_{IMP}	F: GGAATAGAGTGGCTTAATTCTC R: CCAAACYACTAAGTTATCT	[54]
bla_{NDM}	F: GGTTTGGCGATCTGGTTTTC R: CGGAATGGCTCATCACGATC	[55]
$bla_{\text{OXA-48}}$	F: AAGGAATGGCAAGAAAACAAAA R: CCATAATCGAAAGCATGTAGCA	[56]

String test

The string test was carried out as mentioned previously [43] to evaluate mucoid phenotype. Briefly, all isolates were cultured overnight at 37 °C on blood agar plates (Merck, Germany). The colonies were gently touched and lifted using an inoculating loop. The visual observation of mucoid string >5 mm in length, representing the hypermucoviscosity characteristic, was defined as a positive result.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of hypervirulent *K. pneumoniae* isolates was assessed on cation-adjusted Mueller Hinton agar (Merck, Germany) by the Kirby-Bauer disk diffusion method based on the recommendations provided by the Clinical and Laboratory Standards Institute (CLSI) [44]. The following antimicrobial disks (Mast Group, Merseyside, UK) were used: β-lactam/β-lactamase inhibitor combinations

[piperacillin/tazobactam (PTZ, 100/10 μg)], penicillins [piperacillin (PIP, 100 μg)], cefalosporins [cefpodoxime (CPD, 30 μg), cefotaxime (CTX, 30 μg), cefepime (FEP, 30 μg), ceftazidime (CAZ, 30 μg)], monobactams [aztreonam (ATM, 30 μg)], carbapenems [imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), ertapenem (ETP, 10 μg), doripenem (DOR, 10 μg)], aminoglycosides [gentamicin(GEN, 10 μg), amikacin (AK, 30 μg)], fosfomycin/trometamol (FOT, 200 μg), folic acid synthesis inhibitors [trimethoprim-sulfamethoxazole (TS, 2.5 μg)], fluoroquinolones [ciprofloxacin (CIP, 5 μg)], tigecycline (TGC, 15 μg), and nalidixic acid. The minimum inhibitory concentrations (MICs) of seven antibiotics, including meropenem, imipenem, ciprofloxacin, ceftazidime, cefepime, cefotaxime, and colistin were determined by broth microdilution method, and the results were interpreted according to the CLSI guidelines 2019 [44]. *Escherichia coli* ATCC 25922 was used for controlling the quality of the test.

Phenotypic detection of extended-spectrum β-lactamase (ESBL)

ESBL production was examined using the combination disk diffusion test (CDDT). Antimicrobial disks containing ceftazidime (CAZ) and cefotaxime (CTX) alone and combined disks, including ceftazidime 30 μg + clavulanic acid 10 μg and cefotaxime 30 μg + clavulanic acid 10 μg (Mast Group, Merseyside, UK) were applied. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC700603 were used as negative and positive controls for ESBL production, respectively [45].

Screening for carbapenemase production

Carbapenemase activity in hvKP isolates was investigated by CarbaNP-test [46, 47]. All isolates were cultured overnight at 37 °C on blood agar plates. Cell lysis was done by the use of Commercial protein extract reagent (B-PER-II, Thermo Scientific Pierce, IL, USA). *K. pneumoniae* ATCC BAA-1706 and ATCC BAA-1705 were included as negative and positive controls, respectively.

PCR detection of carbapenemases and extended-spectrum β-lactamase genes

ESBL-encoding genes (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{PER}, bla_{VEB}) as well as class A (bla_{GES} and bla_{KPC}), class B (bla_{VIM}, bla_{IMP}, and bla_{NDM}), and class D (bla_{OXA-48}) carbapenemase genes were examined by PCR assay using specific primers (Table 1) [45, 48–58] and the PCR results were confirmed by sequencing of corresponding amplicons. Positive control strains were kindly provided by Medical Microbiology Department of SBMU. Sequencing of the amplicons was performed with an ABI 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA) to confirm PCR results.

Multilocus sequence typing (MLST)

According to the protocol mentioned on the Pasteur Institute MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html), multilocus sequence typing (MLST) was performed for extensively drug-resistant hypervirulent *K. pneumoniae* isolates (XDR-hvKP) by PCR amplification and subsequent sequencing of seven housekeeping genes, including *infB*, *phoE*, *mdh*, *tonB*, *gapA*, *rpoB*, and *pgi* [59].

Statistical analysis

Statistical Package for Social Sciences (SPSS) software version 19 for Windows (SPSS Inc, Chicago, IL, USA) was used to interpret the results. Numbers and percentages are used to represent qualitative data. For categorical data comparison, the Pearson's Chi-square test or Fisher's exact test is used. Differences with a *P*-value of less than 0.05 were considered statistically significant.

RESULTS

Patients and bacterial isolates

A total of 52 non-duplicated *K. pneumoniae* isolates were collected from 31 males (58.7%) and 21 females (41.3%). The patients ranged in age from 1 to 86 years. The clinical sources of isolates were sputum (61.5%, 32/52), blood (19.2%, 10/52), urine (15.3%, 8/52), and pus (3.8%, 2/52).

PCR results of capsular- and virulence-associated genes

Among all isolates, capsular serotype *K1* (11.5%, 6/52), *K2* (15.4%, 8/52), *K5* (13.5%, 7/52), *K20* (34.6%, 18/52), *K54* (5.8%, 3/52), *K57* (11.5%, 6/52), and other capsular serotypes (7.7%, 4/52) were identified. Distribution of capsular serotypes among all 52 isolates, classic *K. pneumoniae* isolates, and hypervirulent *K. pneumoniae* isolates are compared in Fig. 1.

The PCR analysis revealed that 12 isolates (23.1%, 12/52) harbored the *rmpA* gene which is in charge of upregulating the synthesis of capsular polysaccharide [60] and *rmpA2*, a trans-acting activator for capsular polysaccharide biosynthesis, was detected in five isolates (9.6%, 5/52) [60]. The gene *magA*, a mediator in polysaccharide synthesis in *K1* capsulated strains [61], was found in five isolates (9.6%, 5/52) (Fig. 2). Altogether 11 isolates were positive for *iucA* (21.2%, 11/52), suggesting the presence of aerobactin uptake as the most important component of the siderophore system that is involved in hypervirulence feature [62] (Fig. 2).

Besides, 38 isolates harbored *iroB* gene (73.1%, 38/52) which indicates the existence of salmochelin (Fig. 2).

Above all, 12 isolates (23.1%, 12/52) were positive for *peg-344* which is responsible for the formation of putative inner membrane transporter (Fig. 2).

Collectively, isolates harbored either *peg-344* or *iucA* genes were designated hvKP [22]. Accordingly, 14 (26.9%, 14/52) isolates were considered as hvKP and the remaining 38 (73.1% 38/52) isolates were categorized as cKP (Fig. 2).

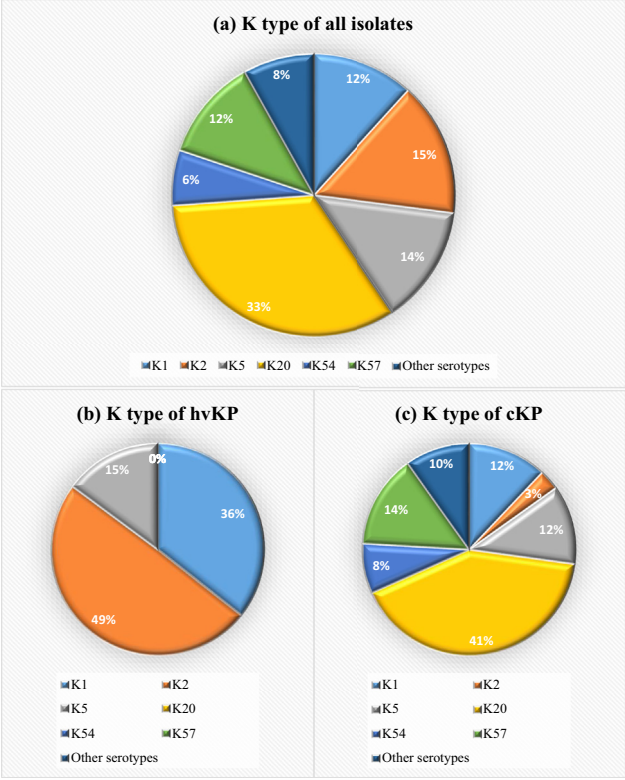

Fig. 1. Distribution of capsular genes (K types). (a) Distribution of K types in total 52 isolates. (b) The distribution of K types in 14 isolates of hvKP (c) The distribution of K types in 38 isolates of cKP

Identification of the hypermucoviscous *K. pneumoniae* isolates using string test

Based on the results of the string test, capsular PCR screening, and virulence-associated genes, 14 (26.9%, 14/52) and 38 (73%, 38/52) isolates were identified as hypervirulent and classic phenotypes, respectively. String test revealed that 11 out of the 14 hvKP isolates (78.6%, 11/14) were hypermucoviscous (hmKP) phenotype, while no cKP isolates were found to be hmKP (0%, 0/38) (Fig. 2).

Antimicrobial susceptibility

The patterns of antibiotic resistance of hypervirulent *K. pneumoniae* isolates ($n = 4$) are presented in Table 2. Resistance to minocycline (7.1%, 1/14) and colistin (7.1%, 1/14) was found to be the lowest. Among all hvKP isolates, 1 (7.1%), 5 (35.7%) and 6 (42.9%) isolates were pandrug-resistant (PDR), extensively drug-resistant (XDR) and multidrug-resistant (MDR), respectively.

Table 2. Antimicrobial susceptibility test results for hypervirulent *K. pneumoniae* isolates

Antibiotics	Sensitive No. (%)	Intermediate resistant No. (%)	Resistant No. (%)
Amikacin	9 (64.3%)	1 (7.1%)	4 (28.6%)
Aztreonam	0 (0%)	6 (42.9%)	8 (57.1%)
Cefepime	0 (0%)	0 (0%)	14 (100%)
Ceftazidime	2 (14.3%)	0 (0%)	12 (85.7%)
Cefotaxime	0 (0%)	0 (0%)	14 (100%)
Ciprofloxacin	3 (21.4%)	1 (7.1%)	10 (71.4%)
Colistin	13 (92.9%)	0 (0%)	1 (7.1%)
Doripenem	6 (42.9%)	3 (%)	5 (35.7%)
Ertapenem	8 (57.1%)	0 (0%)	6 (42.5%)
Fosfomycin	11 (78.6%)	1 (7.1%)	2 (14.2%)
Gentamicin	6 (42.5%)	4 (28.6%)	4 (28.6%)
Imipenem	8 (57.1%)	0 (0%)	6 (42.5%)
Meropenem	9 (64.3%)	0 (0%)	5 (35.7%)
Minocycline	11 (78.6%)	2 (14.3%)	1 (7.1%)
Nalidixic acid	2 (14.3%)	3 (21.4%)	9 (64.3%)
Piperacillin	0 (0%)	0 (0%)	14 (100%)
Piperacillin_tazobactam	6 (42.9%)	3 (21.4%)	5 (35.7%)
Trimethoprim sulfamethoxazole	5 (35.7%)	0 (0%)	9 (64.3%)

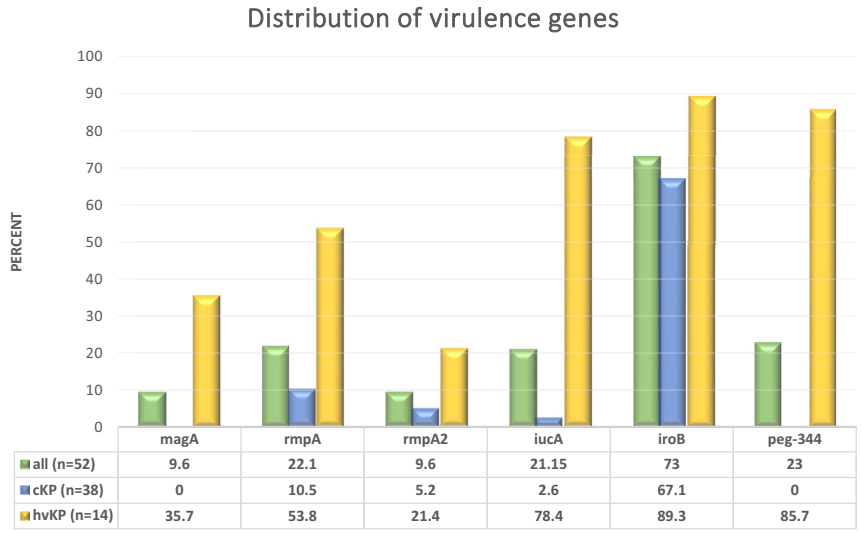

Fig. 2. The distribution of virulence genes among all *K. pneumoniae* isolates, classic *K. pneumoniae* (cKP) isolates, and hypervirulent *K. pneumoniae* (hvKP) isolates

Carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) isolates

Among 14 hypervirulent *K. pneumoniae* isolates, 6 isolates (42.9%, 6/14) were resistant to both ertapenem and imipenem and were referred as carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) and 1, 2, 3, and 1 CR-hvKP had imipenem MICs of ≥32 µL/mL, 16 µL/mL, 8 µL/mL, and 4 µL/mL, respectively.

ESBL phenotype

Of the 14 hvKP isolates, 12 (85.7%, 12/14) were ESBL-positive with the CDDT.

Carbapenemase phenotype

6 hvKP isolates (42.9%, 6/14) produced carbapenemase enzymes, according to the results of the CarbaNP test. Besides, all isolates which produce carbapenemase were resistant to carbapenem antimicrobials ($P < 0.05$).

PCR screening results of antimicrobial resistance genes

Totally, 12 out of the hvKP isolates (85.7%, 12/14) carried ESBL-encoding determinants and according to PCR screening, 3 (21.4%, 3/14), 8 (28.6%, 4/14), and 11 (78.6%, 11/14) of hvKP isolates carried $bla_{\text{CTX-M}}$, bla_{SHV}, and bla_{TEM} genes, respectively; while no isolates carried bla_{PER}, bla_{VEB}, and bla_{GES} genes.

The class D $bla_{\text{OXA-48}}$ carbapenemase gene was detected in 2 isolates (14.3% 2/14), while no isolates were positive for the presence of class B bla_{IMP} and bla_{VIM}, and class A bla_{KPC} carbapenemase genes. In addition, 1 isolate (7.1%, 1/14) carried class B $bla_{\text{NDM-6}}$ gene.

Multilocus sequence typing (MLST) analysis

As shown in Table 3, MLST identified four STs among 5 extensively drug-resistant hypervirulent *K. pneumoniae* isolates, including ST15 (40%, 2/5), ST377 (20%, 1/5), ST442 (20%, 1/5), and ST147 (20%, 1/5).

Table 3. Summary of XDR-hypervirulent *K. pneumoniae* isolates showing MLST, antimicrobial resistance (AMR) genes and virulence genes

S. no	Isolate identity	Sequence type (ST)	AMR genes	virulence genes
1	hvKP1	ST442	bla_{TEM}, bla_{SHV}	*iroB, iucA, peg-344*
2	hvKP3	ST15	bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-48}}$	*iroB, peg-344, rmpA2*
3	hvKP9	ST147	bla_{TEM}, bla_{SHV}	*iroB, iucA, rmpA2, rmpA*
4	hvKP10	ST377	bla_{TEM}, bla_{SHV}, bla_{NDM}	*iroB, iucA, rmpA2, rmpA*
5	hvKP12	ST15	bla_{TEM}	*iroB, iucA, peg-344, rmpA2, rmpA*

DISCUSSION

The prevalence of hvKP as clinically significant pathogens, can cause infections such as liver abscess in both healthy and immunocompromised individuals [63] and importantly, antimicrobial-resistant hvKP isolates are becoming more common, as is the global spread of mobile genetic elements [17, 63]. In this study, we reported the emergence of extensively drug-resistant and pandrug-resistant hypervirulent *K. pneumoniae* isolates in Iran for the first time.

The lack of a test to distinguish cKP from hvKp strains has been one of the problems in researches on hvKP. This study attempts to classify *K. pneumoniae* isolates into two pathotypes cKP or hvKP depending on the existence of *peg-344, rmpA, rmpA2, iroB*, and *iucA* virulence genes. These biomarkers have recently been shown to be extremely accurate in distinguishing hvKP strains from cKP strains, considering that the performance of the string test has been reported to be sub-optimal in areas with low prevalence [24].

In this study, among all *K. pneumoniae* isolates examined ($n = 52$), 14 isolates (29.9%, 14/52) were hypervirulent *K. pneumoniae* (hvKP). Although three hvKP isolates did not have a positive string test, carried three important biomarkers indicating the hvKP phenotype (*peg-344*, *iucA* and *iroB*) in PCR analysis, as previously described as more accurate predictors of hypervirulence characteristic among *K. pneumoniae* isolates than the string test [24].

The prevalence of hvKP which was reported by Rastegar et al. (15.1%) in Iran [64], Cubero et al. (5.4%) in Spain [65] and Peirano et al. (8.2%) in Alberta, Canada [66], and Pajand et al. (2.5%) in Iran [32] was lower than the results of current study. On the contrary, the prevalence of hvKP reported by Jung et al. (42.4%) in Korea [67], Yu et al. (38%) in Taiwan [18], Li et al. (33%) in China [17], Hyun et al. (37.4%) in Korea [68], and Liu (45.7%) in China [69] was higher than those of our study. Several previous studies have reported that East Asian countries show a higher prevalence of the hvKP than European countries [16, 65, 67, 70, 71], indicating that differences in geographical locations may be partly responsible for such discrepancies.

Because *K1*, *K2*, *K5*, *K20*, *K54*, and *K57* capsular serotypes are thought to be highly virulent phenotypes linked to serious human infections [72], they were evaluated in the present study. Most of the hvKP isolates belonged to *K2* (42.85%) and *K1* (35.71%) serotypes. Four isolates (14.3%) were classified as *K5*, while no *K20*, *K54*, and *K57* capsular serotypes were identified (Fig. 1).

In this study we focused on antimicrobial resistance of hvKP isolates because it was reported that hvKP isolates remained susceptible to antimicrobials [73, 74], but recently,

there has been a notable increase in reports of MDR-hvKP, especially from China, India, Brazil, and, Iran [32, 75–78]. Also, the convergence of hypervirulence and carbapenem-resistance provides a significant urgent problem for clinical care, infection prevention, and public health [79].

Historically, In Taiwan from 1997 to 2005, antimicrobial resistance was uncommon, with just 2% of isolates showing resistance to screened first-generation cephalosporins [25] but due to recent studies multidrug-resistant hvKP is becoming more common, making this already difficult-to-treat infection much more challenging to treat [17, 28, 68, 69, 73, 76, 80, 81].

Liu et al. reported 50% (79/158) hvKP clinical isolates from two hospitals in Beijing, China, of which 31 (39.2%, 31/79) were multidrug-resistant (MDR), 30 (37.9%, 30/79) produced ESBLs and 16 (20.3%, 16/79) were carbapenem-resistant [82].

In this study, MDR-hvKP isolates accounted for 42.9% (6/14). In addition, 85.7% of hvKP isolates (12/14) were found to be ESBL-producer and a significant percentage carried bla_{TEM} gene (78.6%, 11/14). The 42.9% (6/14) were carbapenem-resistant hypervirulent *K. pneumoniae* (CR-hvKP) which is higher than the study conducted by Liu et al. in China [82].

Above all, one of the most threatening hvKP isolates was found in 2014 which was a new issue for clinicians. In 2014, Huang et al. identified an XDR ST11 KPC-2-producing hvKP strain in Taiwan [83] which poses significant challenges for control. Also, Zhu et al. isolated *K. pneumoniae* strain XJ-K1 in Shanghai, China, that was identified as XDR-hvKP [84]. In our study, XDR-hvKP and PDR-hvKP isolates accounted for 35.7% (5/14) and 7.1% (1/14), respectively which are showing a great concern.

Although ST23 has been suggested as the most common clone of hvKP in Asia [73], in this study 2, 1, 1, and 1 XDR-hvKP isolates belonged to ST15, ST377, ST442, and ST147, respectively (Table 3).

Combination of multidrug-resistance along with virulence traits on a single plasmid vector in MDR- *K. pneumoniae* ST15 was reported by Lam et al., 2019 [85]. In our study, two ST15 isolates were identified as XDR-hvKP.

In a study performed by Pajand et al., ST147 has been shown to be associated with $bla_{\mathrm{SHV,}}$ CTX-M-G1, *armA* and *aac(6′)-Ib-cr* [32]. Also, ST147 has been identified as a pandemic clone which was regarded as a global threat to public health [86].

In this study, ST147 was determined as XDR-hvKP. Bean et al., have reported ST147 as an MDR and hypermucoviscous *K. pneumoniae* isolate which was considered as a probable emerging high-risk clone responsible for serious infections in India [87].

In a study conducted by Shelenkov et al. in 2020, three ST377 isolates were found and all of them were MDR [88]. Besides, based on the data available in the Institut Pasteur database, this sequence type is relatively uncommon and is commonly associated with MDR or extensively drug-resistant (XDR) phenotypes [89]. In current study, ST377 is reported as XDR-hvKP.

CONCLUSION

This is the first report of XDR hypervirulent *K. pneumoniae* and PDR hypervirulent *K. pneumoniae* isolates in Iran. The prevalence of such isolates is especially concerning due to the combination of hypervirulence and extensively drug-resistance or pandrug-resistance. This is a new challenge for clinicians and microbiologists to tackle, as it poses a significant public health danger. Despite the fact that there have been many studies on hvKp, it is important to establish new, reliable diagnostic methods for detecting XDR-hvKP and PDR-hvKP so that, more future studies are necessary.

Ethics approval and consent to participate: The Ethics Committee of Shahid Beheshti University of Medical Sciences authorized this report with ethical number "IR.SBMU.M-SP.REC.1398.787". Participants were kept confidential in order to preserve the privacy of the patients, and no identifiable private information was obtained or included in the study.

Competing interests: There are no conflicts of interest.

Authors' contributions: SD, AH, HG, MG, JYS, and EF conceived, designed and performed the experiments and analyzed the data. SD, AR and AH wrote the paper.

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