Original Article

Virulence Factors, Capsular Serotypes and Antimicrobial Resistance of Hypervirulent *Klebsiella pneumoniae* and Classical *Klebsiella pneumoniae* in Southeast Iran

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Conflict of Interest

No conflicts of interest.

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ABSTRACT

Background: The present study was conducted to investigate the distribution of virulence factors, capsular serotypes and antibiotic resistance properties of classical *Klebsiella pneumoniae* (cKP) and hypermucoviscous/hypervirulent *Klebsiella pneumoniae* (hvKP) isolated from different clinical specimens in Kerman, south-east of Iran.

Materials and Methods: A total of 146 *K. pneumoniae* isolates were obtained from different clinical specimens. HvKP isolates were identified using the string test. Genes of capsular serotypes K1, K2, K5, K20, K54 and K57 and virulence-associated genes, *rmpA*, *kfu*, *fimH*, *mrkD*, *allS*, *iutA*, *magA*, *entB* and *ybtS* were evaluated by PCR. Antimicrobial susceptibility was also determined using the disc diffusion method.

Results: Out of 146 *K. pneumoniae* isolates, 22 (15.1%) were hvKP. More than half of the hvKP isolates, 13 (59.1%), belonged to non-K1, K2, K5, K20, K54, K57 serotypes. Out of 22 hvKP isolates, 3 and 3 had K1 and K2 serotypes respectively. Among all isolates, *entB* 140 (95.9%) and *mrkD* 138 (94.5%) were the most common virulence genes. *RmpA*, *iutA* and *kfu* were associated with hvKP isolates (P -value <0.05). However, no significant difference was found in *fimH*, *allS*, *mrkD*, *entB* and *ybtS* genes between hvKP and cKP strains. HvKP exhibited significantly lower resistance rates to all antimicrobial agents than cKP, except to trimethoprim-sulphamethoxazole and ampicillin (P -value <0.05).

Conclusion: The frequency of hvKP was low, but overall, the prevalence of virulence-related genes was higher in hvKP than cKP. HvKP was not related to specific serotypes. Furthermore, hvKP isolates were more susceptible to antimicrobial agents compared to cKP isolates.

Keywords: *Klebsiella pneumoniae*; Virulence factors; Capsular serotypes; Antibiotic resistance

INTRODUCTION

Klebsiella pneumoniae (Kp) is a Gram-negative, encapsulated, non-motile bacterium, that is one of the most common causes of both health care and community-associated infections,

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including urinary tract infections, bacteremia, and pneumonia [1]. Two different groups of *K. pneumoniae* have been described: classical (cKP) and hypervirulent (hvKP). Currently, most *K. pneumoniae* infections, particularly in immunocompromised patients are due to cKP strains [2]. However, between the mid-1980s and 1990s, reports from Taiwan described a new and hypervirulent clinical variant of hvKP [3]. Although hvKP infection appears to occur often in diabetic patients, the importance of this variant is linked to its ability to cause community-acquired, life-threatening infection among young and healthy individuals [4]. HvKP strains often produce colonies with a hypermucoviscous phenotype that can be defined semi-quantitatively by a positive “string test,” a method that has been extensively used for identification of hvKP [4]. This hypermucoviscous phenotype can be related to overexpression of capsule polysaccharides. Most of hvKp isolates belong to capsular serotype K1 and K2 [5]. A capsule with mucoid character protects *Klebsiella* from phagocytosis and bactericidal effect of serum [6, 7]. A number of potential virulence factors, principally including mucoviscosity-associated gene A (*magA*) and regulator of mucoid phenotype A (*rmpA*), have been reported to be associated with hypermucoviscous phenotype [3]. Initially, *magA* was considered to mediate the hypermucoviscous phenotype. Further studies showed that *magA* is responsible for capsular serotype K1 of *K. pneumoniae* [8]. The *rmpA* gene is localized on a 180-kilobase plasmid and is a positive regulator of extracapsular polysaccharide synthesis [9].

Other virulence genes that are more prevalent in hypervirulent *K. pneumoniae* than the classical *K. pneumoniae* include siderophores such as enterobactin (Ent), the prototypical catecholate siderophore; aerobactin, a hydroxamate siderophore whose receptor is encoded by *iutA*; yersiniabactin (YbtS), a phenolate-type siderophore that is structurally distinct from enterobactin and Kfu, which mediates uptake of ferric [10]. Likewise, *allS* (a gene associated with allantoin metabolism) have been shown to be present more often in hvKP strains with a K1 serotype [10]. The *fimH* and *mrkD* genes which encode type 1 and type 3 fimbriae respectively are responsible for attachment to host cells [11]. These factors are known to contribute to virulence and are responsible for colonization, invasion, and pathogenicity. Alarming, some studies reported that multi-drug-resistant, even carbapenem-resistant hvKP isolates have emerged, which is becoming a major public health concern [3, 12]. In the current study, we investigated the distribution of various virulence genes, capsular serotypes and antibiotic resistance of cKP and hypermucoviscous/hvKP isolated from different clinical specimens in Kerman, south-east of Iran.

MATERIALS AND METHODS

1. Collection and identification of *K. pneumoniae* clinical isolates

From January 2017 to October 2018, a total of 146 non-duplicate *K. pneumoniae* isolates were collected from clinical specimens of different patients referred to two teaching hospitals of Kerman, Iran. The samples were cultured on EMB and blood agar media and then were incubated at 37°C for 24 hours. *K. pneumoniae* was identified by standard biochemical tests including morphology of colony, Gram staining, oxidase, lactose, triple sugar iron medium (TSI), SIM medium (Sulfide hydrogen, Iodole, Motility), Methyl Red and Voges-Proskauer (MR-VP), urease, lysine decarboxylase, arginine dihydrolase and Simmons citrate agar [13].

2. String test

The hypermucoviscosity phenotype of the isolates was assessed by string test as described previously [14]. The string test was performed to distinguish hvKP from cKP strains. The result

Table 1. List of primers used in this study.

Primer name	DNA sequence (5'→3')	Target gene	Amplicon size (bp)	Reference
<i>MagAF1</i>	GGTGCTCTTTACATCATTGC	Capsular type K1	1,283	[16]
<i>MagAR1</i>	GCAATGGCCATTTCGTTAG			
<i>K2wzy-F1</i>	GACCCGATATTACACTTGACAGAG	Capsular type K2	641	[16]
<i>K2wzy-R1</i>	CCTGAAGTAAATCGTAAATAGATGGC			
<i>K5wzxF360</i>	TGGTAGTGATGCTCGCGA	Capsular type K5	280	[16]
<i>K5wzxR639</i>	CCTGAACCCACCCCAATC			
<i>wzyK20F</i>	CGGTGCTACAGTGCAATCATT	Capsular type K20	741	[16]
<i>wzyK20R</i>	GTTATACGATGCTCAGTCGC			
<i>wzxK54F</i>	CATTAGCTCAGTGGTTGGCT	Capsular type K54	881	[16]
<i>wzxK54R</i>	GCTTGACAAACACCATAGCAG			
<i>wzyK57F</i>	CTCAGGGCTAGAAGTGCAT	Capsular type K57	1,037	[16]
<i>wzyK57R</i>	CACTAACCCAGAAAGTCGAG			
<i>ybtS_for ybtS_rev</i>	GACGGAAACAGCACGGTAAA GAGCATAATAAGGCGAAAGA	Siderophore	242	[10]
<i>entB_for entB_rev</i>	GTCAACTGGGCCTTTGAGCCGTC TATGGGCGTAAACGCCGGTGAT			
<i>iutA_for iutA_rev</i>	GGGAAAGGCTTCTCTGCCAT TTATTGCCACACGCTCTT	Siderophore	920	[10]
<i>rmpA_for rmpA_rev</i>	CATAAGAGTATTGGTTGACAG CTTGCAATGAGCATCTTTCA			
<i>mrkD_for mrkD_rev</i>	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	Adhesin type 3 fimbriae	340	[10]
<i>FimHF</i>	ATGAACGCCTGGTCTTTGC			
<i>FimHR</i>	GCT GAACGCCTATCCCTGC	Adhesin type 1 fimbriae	688	[11]

was regarded as positive when an inoculation loop was able to generate a viscous string of ≥ 5 mm in length by stretching bacterial colonies on a blood agar plate. A negative string test was considered when the length of the string was less than 5 mm or no string was present [14].

3. PCR for amplification of virulence associated genes.

Bacterial genomic DNA was extracted by boiling at 100°C for 15 minutes and centrifugation at 12,000 rpm for 7 minutes [15]. Detection of capsular serotype-specific genes including K1, K2, K5, K20, K54, K57 and virulence genes (*rmpA*, *kfu*, *fimH*, *mrkD*, *allS*, *iutA*, *magA*, *entB* and *ybtS*) was carried out by polymerase chain reaction [10, 11, 16]. The sequences of the primers utilized in this study are shown in Table 1.

4. Antimicrobial susceptibility testing

Antibiotic susceptibility testing was carried out for all *K. pneumoniae* isolates by the disk diffusion method according to Clinical and Laboratory Standards Institute guidelines [17]. The antibiotics included amikacin, imipenem, nalidixic acid, ofloxacin, ciprofloxacin, trimethoprim-sulfamethoxazole, aztreonam, ceftriaxone, ceftazidime, cefotaxime, ampicillin, tetracycline, and gentamicin. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used in this study as controls for antimicrobial susceptibility testing.

5. Statistical analysis

The resulting data were finally analyzed using the Statistical Package for Social Sciences (SPSS) version 19 (SPSS Inc, Chicago, IL, USA). Qualitative data are presented as numbers and percentages. The Chi-square test or Fisher's exact test were applied for comparison of categorical data. Differences with $P < 0.05$ were considered statistically significant.

RESULT

1. Clinical Characteristics of hvKP and cKP Isolates

A total of 146 *K. pneumoniae* isolates were obtained from clinical specimens derived from hospitalized patients. Of these, only 22 (15.1%) isolates were positive for the string test and identified as hvKP, the remaining 124 (84.9%) isolates were categorized as cKP. The hvKP isolates were obtained from clinical specimens as follows: 9 from urine, 5 from respiratory secretions, 4 from blood and 4 from wounds. The cKP isolates were collected from urine (51), blood (40), respiratory secretions (21) wounds (11) and CSF (1). No statistically significant difference was found between hvKP and cKP isolates in terms of the type of clinical specimens.

2. Genetic comparison between hvKP and cKP isolates

All *K. pneumoniae* isolates were investigated for the presence of genes encoding capsule K antigens. The majority of isolates (82.2%, 120/146) were associated with non K1, K2, K5, K20, K54, K57 serotypes. Moreover, more than half of the hvKP isolates (59.1%, 13/22) belonged to non K1, K2, K5, K20, K54, K57 serotypes. Three of the isolates belonged to K1 capsular type since they carried *magA* gene. All of these three isolates were hvKP. K2 was also positively associated with hvKP (3 out of the 6 isolates that belonged to capsular serotype K2 were hvKP). One isolate belonged to K5 and one isolate belonged to K54. The string test showed that both of these isolates were hvKP. K20 serotype which accounted for 15 (10.3%) of all isolates was the most frequent capsular type. However, 14 out of these 15 isolates were cKP. Table 3 shows the frequency of capsular serotypes and virulence-associated genes among hvKP and cKP isolates.

Among all isolates, *entB* 140 (95.9%) and *mrkD* 138 (94.5%) were the most common virulence genes. *RmpA* was only detected in four hvKP isolates. In addition, *iutA* and *kfu* were also associated with hvKP isolates ($P = 0.000$ and $P = 0.031$ respectively) than cKP isolates. However, no significant difference was found in *fimH*, *allS*, *mrkD*, *entB* and *ybtS* genes between hvKP and cKP isolates (Table 2).

Table 2. Comparison of serotypes and virulence genes of hypervirulent *Klebsiella pneumoniae* (hvKP) and classical *K. pneumoniae* (cKP)

Characteristic	hvKP (n = 22)	cKP (n = 124)	P value
Serotypes			
K1	3 (13.6%)	0 (0.0%)	0.003 ^a
K2	3 (13.6%)	3 (2.4%)	0.044 ^a
K5	1 (4.5%)	0 (0.0%)	0.151
K20	1 (4.5%)	14 (11.3%)	0.470
K54	1 (4.5%)	0 (0.0%)	0.151
K57	0 (0.0%)	0 (0.0%)	n
Virulence gene			
<i>rmpA</i>	4 (18.2%)	0 (0.0%)	0.000 ^a
<i>kfu</i>	10 (45.5%)	29 (23.4%)	0.031 ^a
<i>fimH</i>	5 (22.7%)	32 (25.8%)	0.760
<i>mrkD</i>	21 (95.5%)	117 (94.4%)	1.000
<i>allS</i>	2 (9.1%)	4 (3.2%)	0.223
<i>iutA</i>	5 (22.7%)	3 (2.4%)	0.002 ^a
<i>magA</i>	3 (13.6%)	0 (0.0%)	0.003 ^a
<i>entB</i>	20 (90.9%)	120 (96.8%)	0.223
<i>ybtS</i>	15 (68.2%)	71 (57.3%)	0.337

^aP-value of <0.05 was considered to be statistically significant.

n, No statistics are computed because variable is a constant.

Table 3. Antimicrobial resistance pattern of hypervirulent *Klebsiella pneumoniae* (hvKP) and classical *Klebsiella pneumoniae* (cKP)

Antimicrobial agent	hvKP (n = 22)	cKP (n = 124)	P value
Ampicillin	20 (90.9%)	121 (97.6%)	0.163
Cefotaxime	7 (31.8%)	82 (66.1%)	0.002 ^a
Ceftriaxone	6 (27.3%)	79 (63.7%)	0.001 ^a
Ceftazidime	5 (22.7%)	79 (63.7%)	0.000 ^a
Aztreonam	6 (27.3%)	77 (62.1%)	0.002 ^a
Trimethoprim/Sulfamethoxazole	9 (40.9%)	68 (54.8%)	0.228
Gentamicin	3 (13.6%)	65 (52.4%)	0.001 ^a
Nalidixic acid	3 (13.6%)	62 (50.0%)	0.002 ^a
Tetracycline	4 (18.8%)	56 (45.2%)	0.018 ^a
Amikacin	0 (0.0%)	48 (38.7%)	0.000 ^a
Imipenem	2 (9.1%)	47 (37.9%)	0.008 ^a
Ofloxacin	1 (4.5%)	40 (32.3%)	0.008 ^a
Ciprofloxacin	1 (4.5%)	31 (25.0%)	0.047 ^a

^aP-value of <0.05 was considered to be statistically significant.

3. Antimicrobial Resistance Profile of hvKP and cKP Isolates

A larger proportion of the cKP isolates were found to be resistant to antimicrobial agents including ampicillin (97.6%), cefotaxime (66.1%), ceftriaxone (63.7%), ceftazidime (63.7%), aztreonam (62.1%) trimethoprim/sulfamethoxazole (54.8%), gentamicin (52.4%), nalidixic acid (50%), tetracycline (45.2%), amikacin (38.7%), imipenem (37.9%) ofloxacin (32.3%). Ciprofloxacin was found to be the most effective antibiotic against cKP isolates (75% of isolates were sensitive). HvKP exhibited significantly lower resistance rates to all antimicrobial agents than cKP, except to trimethoprim-sulphamethoxazole and ampicillin (Table 3).

DISCUSSION

The present study was conducted on 146 *K. pneumoniae* isolates collected from patients hospitalized in Kerman hospitals from January 2017 to October 2018. HvKP isolates were defined on the basis of a positive string test. Of all *K. pneumoniae* isolates tested in this study, 22 isolates (15.1%) were hvKP. The hvKP prevalence of our study was lower than those reported by Li et al. (33%) in china [18], Yu et al. (38%) in Taiwan [19] and a study conducted by Jung et al. (42.4%) in Korea [20], but higher than the reports from a hospital in Spain (5.4%) [21] and a study in Alberta, Canada (8.2%) [22]. These variations in prevalence can be partly attributed to the geographical location. For example, previous studies showed that the prevalence of the hvKP in European countries is lower than East Asian countries [2, 20, 21, 23, 24].

In the current study, we evaluated six serotypes (K1, K2, K5, K20 K54 and K57), which are considered as highly virulent phenotypes associated with severe infections in humans [25]. The majority of hvKP isolates were associated with non-K1/K2/K5/K20/K54/K57 serotypes, and only 27.3% of hvKP isolates had K1/K2 serotypes. Although the K1/K2 capsular serotypes are common among hvKP isolates, some studies have shown that a considerable proportion of the hvKP strains may have a non-K1/K2 serotype [1, 25]. Our results revealed that serotypes other than K1/K2/K5/K20/K54/K57 may also play key roles in the hvKP infections. In this study K20 was the most frequent capsular type, but it was detected almost exclusively in the cKP isolates. This finding is consistent with the results of previous studies in which the K20 has been detected more commonly in non-hvKP strains [2, 18, 20]. Therefore, it seems that the role of K20 capsule in the pathogenesis of hypervirulent *K. pneumoniae* may not be as important as previously thought.

Besides hypermucoviscosity, a number of virulence factors have been suggested to contribute to the pathogenesis of hvKP strains. To date, *rmpA* and *magA* are the most frequently reported factors that have direct correlation with hvKP virulence [3, 26]. Although *rmpA* and *magA* were associated with positive string test, we found only 5 *rmpA* and/or *magA* positive among the 22 string test positive isolates. These results imply the presence of mucoid factors other than *magA* and *rmpA*; for example, some variations in the composition of lipopolysaccharide [21].

Iron acquisition factors including enterobactin (Ent), aerobactin, aerobactin receptor (IutA), and yersiniabactin (YbtS) and Kfu have been described as virulence factors in *K. pneumoniae* strains [27]. In our study, a majority of both hvKP and cKP isolates were demonstrated to carry *ent* gene, a finding in line with most previous reports [3, 10, 27]. More than half of the isolates also possessed *ybtS* and there was no significant difference in the prevalence of this gene between hvKP and cKP isolates. These results are almost in accordance with the findings of Yan et al. and Gao et al. [3, 27]. In agreement with other studies, the prevalence of *iutA* and *kfu* in hvKP isolates were significantly higher than those in cKP isolates [11, 27]. Moreover, an association between *iutA* and *rmpA* genes was found. This association is not surprising since both genes are located on the same 180-kb plasmid [20].

Fimbrial related genes, such as *mrkD* and *fimH* are also involved in the virulence of *K. pneumoniae* but, no significant differences in the prevalence of these genes were observed between hvKP and cKP strains. Guo et al. reported that only hypermucoviscous/hypervirulent isolates were positive for *mrkD* [3] but in many other studies, this gene was observed in both cKP and hvKP isolates [14, 27]. Moreover, consistent with most prior studies, we found that almost all of the *K. pneumoniae* strains carried *mrkD* [10, 14, 27].

In the present investigation, cKP isolates exhibited significantly higher antimicrobial resistant rate for all antimicrobial agents than hvKP isolates with the exception of ampicillin and trimethoprim/sulfamethoxazole. The same studies reported a high rate of resistance to ampicillin among hvKP isolates which is in agreement with our findings [3, 27, 28]. Although previous studies also indicated that hvKP strains are generally susceptible to antimicrobial agents, the reason for this difference is unclear. However, it can be hypothesized that hvKP strains cannot acquire resistance-related plasmids, or that some drug-resistant genes are lost when they become hypervirulent [18].

In the current study, cKP isolates were resistant to most of the antibiotics tested, including imipenem. Similarly, a high rate of imipenem resistance in *K. pneumoniae* was reported from Iran [29, 30]. Our previous studies demonstrated that New Delhi Metallo-beta lactamase (NDM) is responsible for carbapenem resistance in *K. pneumoniae* in Kerman, Iran [31, 32]. Although the prevalence of imipenem-resistant hvKP strains in our study was low, the enhanced virulence of these strains and the possibility of acquiring antimicrobial resistance are a cause for concern.

In conclusion, the prevalence of hvKP was low, but overall, the prevalence of virulence-related genes was higher in hvKP than cKP. HvKP was not related to specific serotypes. Furthermore, although hvKP isolates were more susceptible to clinically common used antimicrobial agents relative to cKP isolates, the emergence of antimicrobial resistance among these strains may become a future threat.

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