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ORIGINAL RESEARCH

Molecular Characteristics of Virulence Genes in Carbapenem-Resistant and Carbapenem-Sensitive *Klebsiella Pneumoniae* in Relation to Different Capsule Serotypes in Ningbo, China

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Background: *Klebsiella pneumoniae* (KP) is a common nosocomial pathogen. Capsules are an important component of KP's virulence, among which the K1, K2, K5, K20, K54, and K57 serotypes are predominant and exhibit varying degrees of virulence.

Methods: The capsule and virulence genes of 150 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and 213 carbapenem-sensitive *Klebsiella pneumoniae* (CSKP) isolates were examined by polymerase chain reaction (PCR). The isolates were tested for hypermucoviscosity by string tests. Phylogenetic relationships between KP isolates were analyzed using multilocus sequence typing (MLST) and a *Galleria mellonella* infection model confirmed the differences in virulence.

Results: A total of 111 of 363 isolates of KP were detected, the highest detected serotypes were K1, K5, and K2, and CSKP was detected more frequently than CRKP. There was a greater prevalence of K1 and K2 serotypes in CSKP, while in CRKP, K5 serotypes were more prevalent. K1 isolates had the highest detection rates for hypermucoviscosity *Klebsiella pneumoniae* (hmKP) and hypervirulent *Klebsiella pneumoniae* (hvKP), and carried the most virulence genes. K54 isolates had the lowest detection rate of hmKP while K5 isolates had the lowest detection rate of hvKP and carried the fewest virulence genes. MLST results for serotypes K1, K20, and K57 showed significant homogeneity, while those for serotypes K2, K5, and K54 showed diversity. The *Galleria mellonella* infection model showed that the K1 serotype was the most virulent and the K54 serotype was the weakest.

Conclusion: CSKP isolates were detected more frequently than CRKP isolates for capsular serotype detection. K1 isolates had the most virulence gene and strongest virulence, K5 isolates carried the fewest virulence genes, and K54 isolates had the weakest virulence. Furthermore, significant homogeneity was observed among K1, K20, and K57 isolates.

Keywords: *Klebsiella pneumoniae*, capsular serotypes, virulence, MLST

Introduction

Klebsiella pneumoniae (KP) is an opportunistic Gram-negative bacillus that can lead to a variety of invasive infections including pneumonia, liver abscesses, urinary tract infections, and other severe infections.[1–3] The capsule, a polysaccharide matrix on the surface of KP, serves as a protective component that can resist the damaging effects of harmful substances such as antibiotics, complement, and antimicrobial peptides. By suppressing the host's inflammatory response, the capsule enhances the survivability and migratory capacity of KP, making it a crucial virulence factor.[1] Genetic variation in capsule polysaccharides results in different capsular serotypes known as K antigens. Currently, at least 160 serotypes[4] have been confirmed, among which serotypes K1, K2, K5, K20, K54, and K57 have been related to higher virulence.[5–11] In addition to the capsule, iron

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carriers, pili, lipopolysaccharides, and inner membrane transport proteins are important virulence factors for host colonization.[12] KP is classified into classical *Klebsiella pneumoniae* (cKP) and hypervirulent *Klebsiella pneumoniae* (hvKP) according to the virulence. Firstly, hvKP was identified by the hypermucoviscosity phenotype of the strain,[12] but with the development of research, Bulger[13] and Russo[12] suggested that genes such as " *rmpA*, *rmpA2*, *iroB*, *iucA*, and *peg-344*" could provide a diagnostic accuracy of 95% for hvKP. With the continued use of carbapenem antibiotics, the detection of carbapenem-resistant *Klebsiella pneumoniae* (CRKP) has been on the rise. High-virulence serotypes have also been increasingly detected among CRKP strains,[14,15] presenting a significant public health concern. To understand the distribution characteristics of capsule types and the epidemiological features of associated virulence genes in KP, we screened 363 clinically isolated KP strains for capsule and virulence genes. As well as analyzing the strain homogeneity using Multilocus sequence typing (MLST), and determining their virulence using *Galleria mellonella* virulence infection models. This study has contributed to a deeper understanding of the epidemiological characteristics and virulence disparities among various capsular serotypes of *Klebsiella pneumoniae* in the specified region.

Materials and Methods

Isolates Collection

A total of 363 non-repetitive CRKP (150) and CSKP (213) isolates from multiple centers in Ningbo from January 2019 to December 2021 were selected. The specimens included sputum, midstream urine, sterile blood, secretions, and throat swabs.

Identification and Drug Sensitivity of Isolates

All isolates were identified using the VITEK 2 Compact system (bioMérieux, France). A broth microdilution method was used to confirm carbapenem resistance. Quality control was ensured using *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (Ministry of Health Laboratory Center). CRKP was defined according to the 2023 CLSI MIC breakpoint standards for resistance or intermediate resistance to one or more carbapenem antibiotics.

Hypermucoviscosity Phenotype Test

The isolates were streaked on Columbia blood agar plates and incubated at 37°C in a 5% CO2 incubator for 18–24 hours. A single bacterial colony was picked using a disposable sterile inoculation loop, if the length of the mucoviscous string pulled upward exceeded 5 mm and was observed in at least two repetitions, it was considered a positive string test, indicating hypermucoviscosity *Klebsiella pneumoniae* (hmKP). Otherwise, it was considered negative, indicating non-hypermucoviscous *Klebsiella pneumoniae* (n-hmKP).

Capsule Typing Genes and Virulence Gene Detection

An extraction method involving boiling was used to obtain DNA templates. Capsule typing genes (K1, K2, K5, K20, K54, and K57), capsule polysaccharide mucoviscosity-associated genes (*rmpA, magA, rmpA2*), pili-related genes (*alls, markD, fimH*), iron carrier-related virulence genes (*aerobactin, iucA, iroB, iutA, kfu, entB, ybts*), transport protein of the inner membrane (*peg-344*), and lipopolysaccharide-related gene (*wabG*) were amplified by polymerase chain reaction (PCR). Table 1 contains primer sequences and annealing temperatures of each gene. A total of 25 μL of reaction mixture consisted of 2x Taq MasterMix (12.5μL), forward and reverse primers (1.0μL each), DNA template (2.0μL), and ddH_2O (8.5μL). The amplification conditions were as follows: initial denaturation at 94°C for 3 minutes, then 30 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds, extension at 72°C for 1 minute, and extension at 72°C for 10 minutes. The amplification products were electrophoresed on a 1.0% agarose gel at 110 V for 30 minutes and finally placed in a GELDOC XR gel imaging analysis system (Bio-Rad, USA) for imaging. The distribution of Marker bands was compared, and the negative and positive bands of the gene were determined according to the location of the target band. The positive bands were purified and then sequenced.

Table 1 PCR Primer Sequence

Gene	Primer sequences (5'-3')	Product (bp)	Annealing (°C)	Reference
K1	F: GGTGCTCTTTACATCATTGC R: GCCCAGGTTAATGAATCCGT	1283	55	[16]
K2	F:GACCCGATATTCATACTTGACAGAG R:CCTGAAGTAAAATCGTAAATAGAT	641	53	[11]
K5	F: GCCACCTCTAAGCATATAGC R: CGCACCAGTAATTCCAACAG	540	55	[16]
K20	F: CGGTGCTACAGTGCATCATT R: GTTATACGATGCTCAGTCGC	741	56	[16]
K54	F:CATTAGCTCAGTGGTTGGCT R:GCTTGACAAACACCATAGCAG	881	55	[16]
K57	F: CGACAAATCTCTCCTGACGA R:CGCGACAAACATAACACTCG	1037	50	[16]
rmpA	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	516	55	[17]
rmpA2	F: CTTTATGTGCAATAAGGATGTT R: CCTCCTGGAGAGTAAGCATT	451	52	[18]
iucA	F:AATCAATGGCTATTCCCGCTG R:CGCTTCACTTCTTTCACTGACAGG	239	59	[19]
iroB	F:ATCTCATCATCTACCCTCCGCTC R:GGTTCGCCGTCGTTTTCAA	235	58	[18]
peg-344	F:CTTGAAACTATCCCTCCAGTC R:CCAGCGAAAGAATAACCCC	508	56	[19]
aerobactin	F:GCATAGGCGGATACGAACAT R:CACAGGGCAATTGCTTACCT	556	53	[11]
ybtS	F:GACGGAAACAGCACGGTAAA R:GAGCATAATAAGGCGAAAGA	242	50	[11]
iutA	F: ACCTGGGTTATCGAAAACGC R: GATGTCATAGCCTGATTGC	1115	55	[20]
magA	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	1283	53	[17]
fimH	F:ATGAACGCCTGGTCCTTTGC R:GCTGAACGCCTATCCCCTGC	688	56	[21]
entB	F:GTCAACTGGGCCTTTGAGCCGGTC R:TATGGGCGTAAACGCCGGTGAT	400	59	[11]
alls	F:CATTACGCACCTTTGTCAGC R:GAATGTGTCGGCGATCAGCTT	764	57	[11]
mrkD	F:CCACCAACTATTCCCTCGAA R:ATGGAACCCACATCGACATT	226	54	[22]
wabG	F:ACCATCGGCCATTTGATAGA R:CGGACTGGCAGATCCATATC	683	55	[23]
kfu	F:GAAGTGACGCTGTTTCTGGC R:TTTCGTGTGGCCAGTGACTC	797	58	[24]
inf B	F: CTCGCTGCTGGACTATATTCG R: CGCTTTCAGCTCAAGAACTTC	462	56	[17]
gap A	F: TGAAATATGACTCCACTCACGG R:CTTCAGAAGCGGCTTTGATGGCTT	662	59	[17]
pgi	F: GAGAAAAACCTGCCTGTACTGCTGGC R: CGCGCCACGCTTTATAGCGGTTAAT	566	59	[17]
tonB	F: CTTTATACCTCGGTACATCAGGTT R: ATTCGCCGGCTGRGCRGAGAG	539	59	[17]

(*Continued*)

Table 1 (Continued).

Gene	Primer sequences (5'-3')	Product (bp)	Annealing (°C)	Reference
mdh	F: CCCAACTCGCTTCAGGTTCAG R: CCGTTTTTCCCCAGCAGCAG	756	59	[17]
rpoB	F: GGCGAAATGGCWGAGAACCA R: GAGTCTTCGAAGTTGTAACC	1075	56	[17]
phoE	F: ACCTACCGCAACACCGACTTCTTCGG R: TGATCAGAACTGGTAGGTGAT	602	59	[17]

Multilocus Sequence Typing (MLST) Analysis

The extraction of DNA template was the same as that of capsule typing genes. PCR was employed to amplify fragments of seven housekeeping genes of KP, which were respectively as *infB, gapA, pgi, mdh, tonB, rpoB*, and *phoE*. A total of 25 μL of reaction mixture included 2x Taq MasterMix (12.5μL), forward and reverse primers (1.0μL each), DNA template (2.0μL), and ddH_2O (8.5μL). The primer sequences and annealing temperatures were listed in Table 1. The reaction conditions outlined involve an initial denaturation at 94 °C for 5 minutes, followed by denaturation at 94 °C for 30 seconds, annealing at gene-specific temperatures for 45 seconds, extension at 72 °C for 45 seconds, and a final extension at 72 °C for 5 minutes, repeated for a total of 35 cycles. The PCR products of housekeeping genes were tested by Shanghai Shenggon Biotech. Then, the results were uploaded to the http://bigsdb.pasteur.fr/klebsiella/ website, where they were compared with the database to determine locus numbers and MLST types for each housekeeping gene.

Galleria Mellonella Virulence Infection Model Preparation

A total of 111 capsular serotype-positive isolates were selected as experimental isolates. The larvae of *Gallerella mellonella* (Huiyude, Tianjin) with a weight of 250–350 mg and light yellowish-white, and showing good motility were chosen. The larvae were considered suitable if they responded actively when touched. Ten larvae were inoculated with each isolate at the rear abdomen of the last abdominal foot. The injection process was meticulously observed for any indications of leakage. The inoculated larvae were transferred to disposable sterile culture dishes and incubated at 37°C incubator (Yiheng, Shanghai, China) in a light-free environment. Larval survival was recorded every 6 hours to plot survival curves. Twenty strains were randomly selected for the pre-experiment, and the larvae of *Galleria mellonella* were injected with 10^7CFU/mL, 10^6CFU/mL, and 10^5CFU/mL, respectively to observe the survival rate of larvae. The concentration of the bacterial solution for the formal experiment was determined to be 10^7CFU/mL. Larvae were considered dead if their bodies turned black and they showed no response to touch. To ensure the accuracy of the experiment, three control groups were established in this study. Which were respectively as saline control (injected bacterial fluid replaced with saline), hvKP negative control (injected bacterial fluid from *Klebsiella quasipneumoniae* ATCC 700603), and hvKP positive control (injected bacterial fluid from *Klebsiella quasipneumoniae* NTUH-K2044) (purchased from Ministry of Health Laboratory Center).

Statistical Analysis

WHONET 5.6 software was used for the retrospective analysis of bacterial data from multiple clinical centers in Ningbo. Data processing was performed with SPSS software (version 25.0). A Chi-square test or Fisher's exact probability test was used to compare sample rates. Analysis of the number of virulence genes carried by the different capsule serotypes was conducted using an *F*-test. The log-rank (Mantel-Cox) test was used to perform the survival analysis, with a significance level of $P<0.05$ indicating statistical significance.

Results

Detection of Capsular Genes

Among the 363 KP isolates, 35 isolates belonged to the K1 serotype (9.6%), 21 isolates belonged to the K2 serotype (5.8%), 27 isolates belonged to the K5 serotype (7.4%), 8 isolates belonged to the K20 serotype (2.2%), 8 isolates belonged to the

K54 serotype (2.2%), and 12 isolates belonged to the K5 serotype (3.3%). The prevalence of capsule serotypes K1, K5, and K2 was found to be highest in CSKP compared to CRKP, with K1 and K2 serotypes being more prevalent in CSKP and K5 serotype in CRKP. Specifically, the detection rates of K1, K2, and K54 were significantly higher in CSKP than in CRKP, while the rates of K5, K20, and K57 did not show significant differences between the two groups (Figure 1, Supplementary Table 1).

Distribution of Virulence Genes in Serotype Capsular Isolates

There were significant differences between capsular serotypes and virulence genes in the distribution of virulence genes ($P<0.05$), and the detection rates of *rmkD, fimH, entB*, and *wabG* were higher in each capsule serotype. The *rmpA, rmpA2, iroB, iucA, peg-344, iutA, magA, aerobactin, ybts, alls*, and *kfu* genes were the most prevalent in the K1 serotype, and *magA* was only present in the K1 serotype, while 85.7% of *alls* were present in the K1 serotype. *RmpA, rmpA2, iroB, iucA, peg-344, iutA, aerobactin, kfu, rmkD, fimH, entB, wabG* were the lowest-detected genes in the K5 serotype. *Ybts* were the lowest detected in the K57 serotype, and *kfu* was not detected in the K20 and K57 serotypes, as shown in Table 2 for details.

According to the heatmap of the virulence gene distribution correlation (Figure 2), *rmpA, iroB, peg-344, aerobactin*, and hmKP were usually concentrated, and *iucA* was highly positively correlated with *iutA*. *magA* and *alls* were highly positively correlated with K1 serotype, while *rmpA, iroB, aerobactin*, and hmKP were highly negatively correlated with K non-typeable. Isolates with *rmpA, iroB, aerobactin*, and hmKP usually belonged to K1, K2, K5, K20, K54, or K57 serotype.

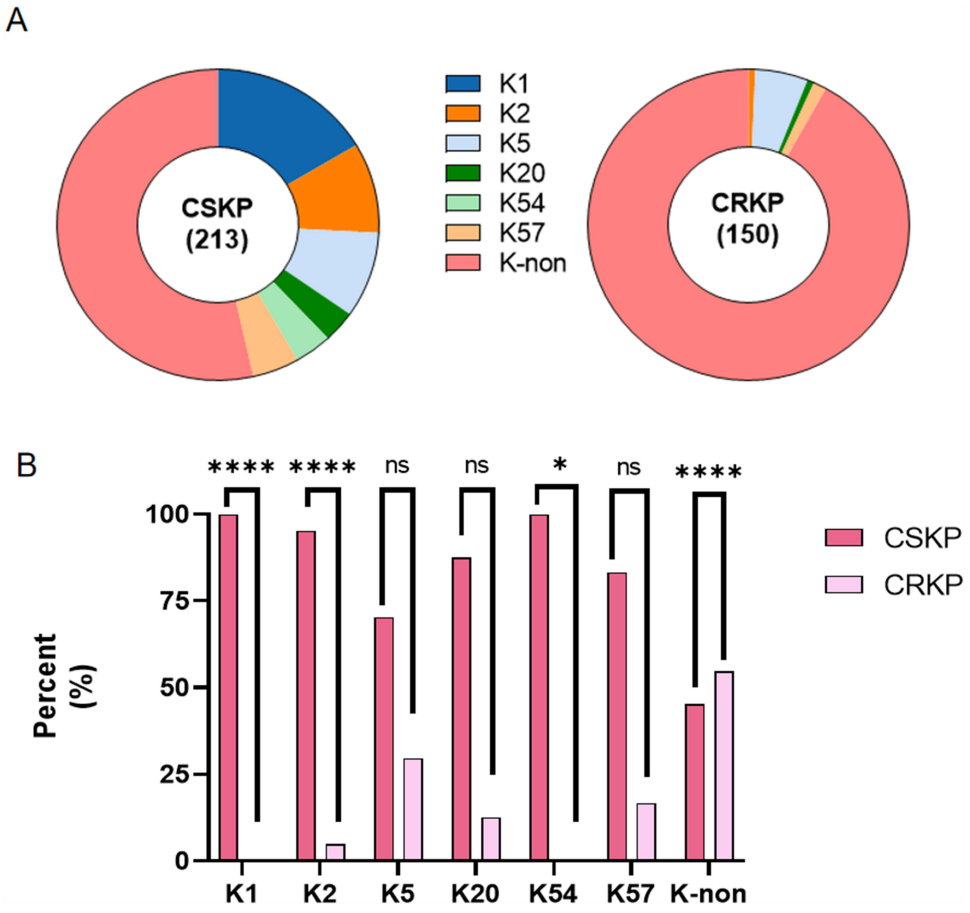

Figure 1 Proportion of capsular serotypes. Figure (**A**) shows the percentage of each capsule serotype in the CSKP and CRKP groups. The comparison of capsule serotypes between CSKP group and CRKP group was shown in Figure (**B**). "****" means $P<0.0001$, "*"means $P<0.05$, "ns" means no statistically significant difference between the two groups.

Table 2 Virulence Gene in Capsular Serotypes

Gene	K1 n=35	K2 n=21	K5 n=27	K20 n=8	K54 n=8	K57 n=12	P
rmpA	34(97.1)	20(95.2)	9(33.3)	6(75.0)	4(50.0)	10(83.3)	<0.001*
rmpA2	32(91.4)	12(57.1)	7(25.9)	7(87.5)	3(37.5)	10(83.3)	<0.001*
iucA	35(100.0)	16(76.2)	10(37.0)	6(75.0)	6(75.0)	10(83.3)	<0.001*
iroB	35(100.0)	20(95.2)	10(37.2)	5(62.2)	4(50.0)	11(91.7)	<0.001*
peg-344	34(97.1)	17(81.0)	6(22.2)	6(75.0)	4(50.0)	11(91.7)	<0.001*
iutA	34(97.1)	16(76.2)	8(29.6)	5(62.5)	6(75.0)	9(75.0)	<0.001*
magA	32(91.4)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	<0.001*
aerobactin	35(100.0)	20(95.2)	9(33.3)	6(75.0)	4(50.0)	11(91.7)	<0.001*
ybts	31(88.6)	14(66.7)	17(63.0)	5(62.5)	6(75.0)	5(41.7)	0.028*
alls	30(85.7)	0(0.0)	0(0.0)	1(12.5)	0(0.0)	0(0.0)	<0.001*
rmkD	35(100.0)	20(95.2)	16(66.7)	8(100.0)	8(100.0)	12(100.0)	<0.001*
fimH	34(97.1)	21(100.0)	18(66.7)	8(100.0)	8(100.0)	12(100.0)	0.001*
kfu	32(91.4)	4(19.0)	8(29.6)	0(0.0)	1(12.5)	0(0.0)	<0.001*
entB	35(100.0)	21(100.0)	17(63.0)	8(100.0)	8(100.0)	12(100.0)	<0.001*
wabG	35(100.0)	20(95.2)	18(66.7)	8(100.0)	8(100.0)	12(100.0)	<0.001*

Note: "*": Fisher exact probability method is used.

The Proportion of hmKP and hvKP in Each Capsule Serotype

The positivity rate of hmKP (Figure 3A) was significantly different among the different capsular serotypes. The positive rates of hmKP in the K1 (33,94.3%) and K2 (18,85.7%) serotypes were significantly higher than those in K57 (9,75.0%), K20 (5,62.5%), K5 (11,40.7%), and K54 (2,25.0%). The prevalence of hvKP was similar to that of hmKP. The prevalence of hvKP (Figure 3B) was also statistically different among capsular serotypes, with K1 (33, 94.3%) being the highest, followed by K57 (9,75%), K2 (11,52.5%), K20 (4,50%), K54 (3,37.5%) serotypes. The K5 serotype had the lowest detection rate (4,14.8%).

The Number of Virulence Genes in Each Capsule Serotype Isolates

There were 15 virulence-related genes identified. The results showed that K1 isolates carried the largest number of virulence genes, followed by K2, K57, K20, K54, and K5 carried the least virulence genes. Figure 4 shows that the number of virulence genes carried by K1 serotype isolates was the most concentrated, while the number of genes carried by K5 serotype isolates was widely distributed.

MLST of the Isolates with Positive Capsule Typing Genes

MLST was performed on 111 isolates with positive capsule typing genes. A minimum spanning tree was generated using Phyloviz, revealing differences in sequence types (STs) among various capsule types. Among the K1 serotype, 35 isolates were identified, predominantly belonging to the ST23 (27, 77.1%) sequence type, and the ST23 sequence type was exclusively found in K1 isolates. For the K2 serotype, 21 isolates were detected with diverse STs, mainly ST65 (5, 23.8%). The K5 serotype yielded 27 isolates, with the predominant ST being ST437 (7, 25.9%). In the case of the K20 serotype, 8 isolates were observed, primarily belonging to the ST268 (5, 62.5%) sequence type. The K54 serotype yielded 8 isolates, mainly associated with the ST29 (4, 50.0%) sequence type. The K57 serotype included 12 isolates, predominantly belonging to the ST412 (10, 83.3%) sequence type, as depicted in Figure 5.

The Virulence of Different Capsule Serotype Isolates

The pre-experiment was conducted to determine the appropriate injection concentration for the infection model. Results showed that larval survival decreased with increasing bacterial concentration (10^5 CFU/mL, 10^6 CFU/mL, 10^7 CFU/mL), with significant differences (P<0.001), indicating a dose-dependent response (Figure 6A). For the main experiment, an inoculum concentration of 10^7 CFU/mL was selected, and it comprised 111 isolates that were positive for capsule genes.

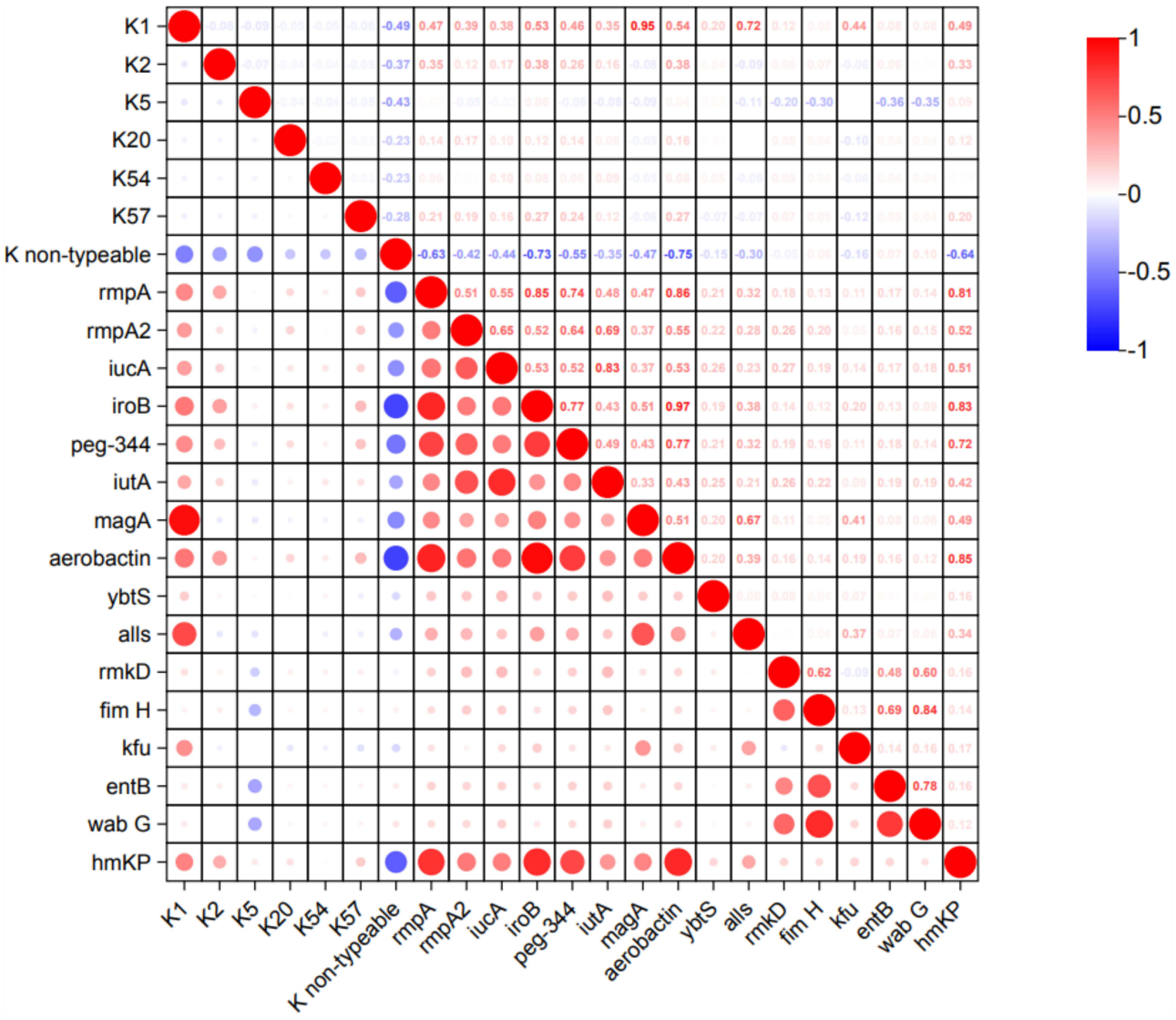

Figure 2 Heat map of virulence gene distribution and correlation. The values in each square represent the correlation between the genes on the horizontal and vertical axes. Values closer to 1 and the red circle larger indicate a stronger positive correlation, values closer to −1 and the blue circle larger indicate a stronger negative correlation, and values closer to 0 and the circle smaller indicate no correlation.

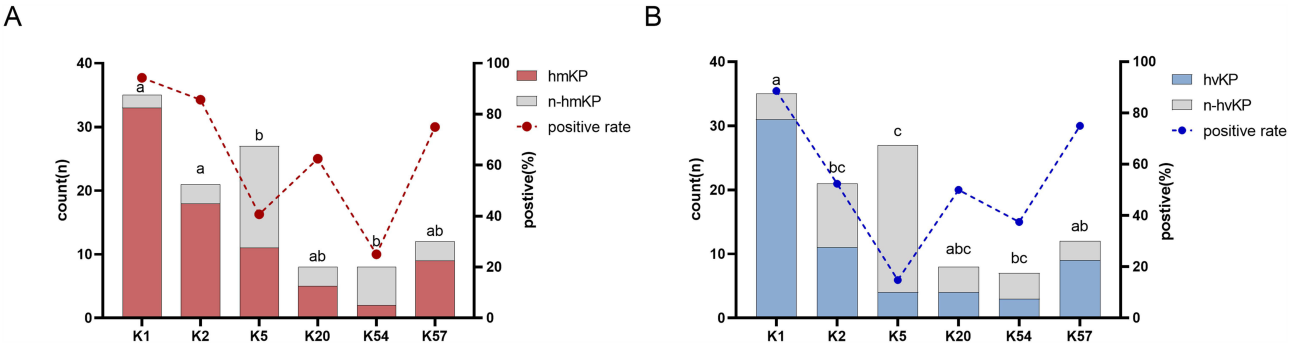

Figure 3 Detection rates of hmKP and hvKP in capsule serotypes. Figure (**A**) shows the detection rate of hmKP. The results of multiple comparisons were labeled by alphabet method, and only "(a)" and "(b)" labeled groups indicated P<0.05. Figure (**B**) shows the detection rate of hvKP in groups. The results of multiple comparisons were labeled by alphabet method, and only "(a)" and "(b,c)", "(a)" and "(c)", "(a,b)" and "(c)" labeled groups indicated *P*<0.05.

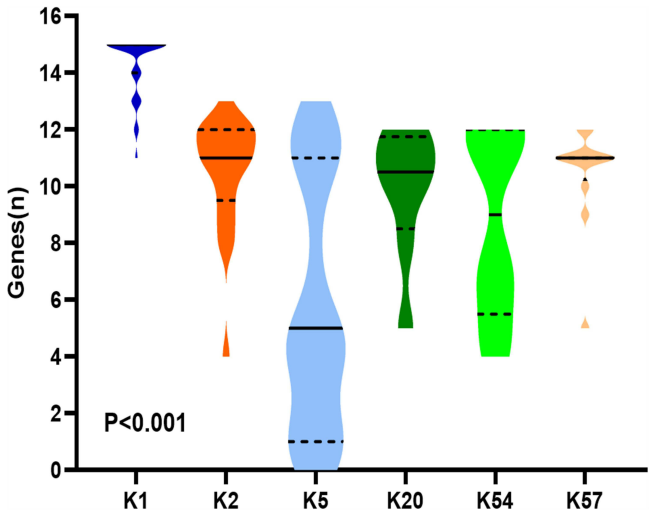

Figure 4 Number of virulence genes carried for each capsule serotype. The black line indicates the median and the dashed line indicates the quartile.

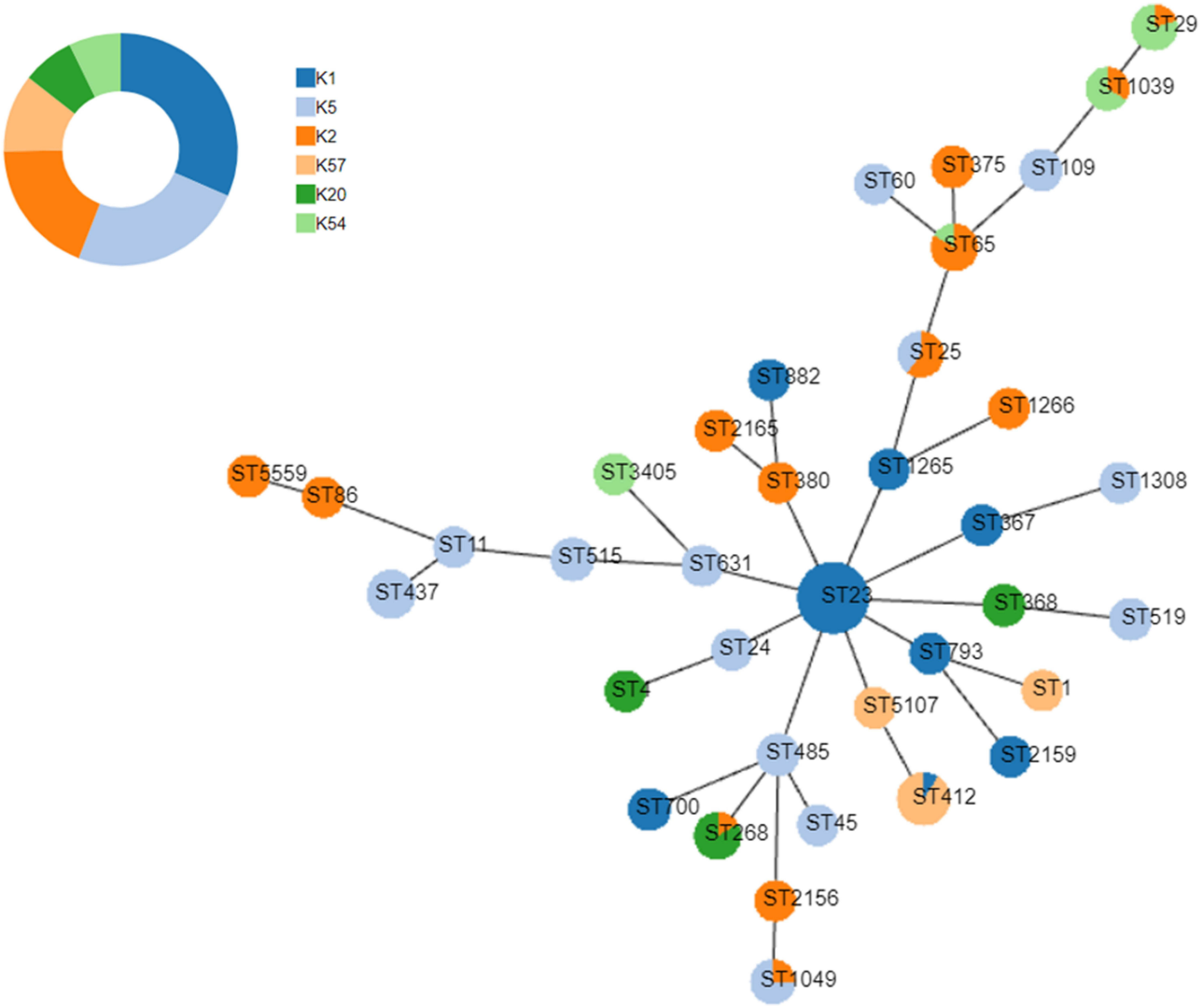

Figure 5 Minimum spanning tree of *Klebsiella pneumoniae*. The minimum spanning tree is constructed using seven allelic genes (*gapA, infB, mdh, pgi, phoE, rpoB, tonB*) of *Klebsiella pneumoniae*. The size of the nodes is proportional to the number of isolates, and the length of the lines between nodes is proportional to the number of different alleles.

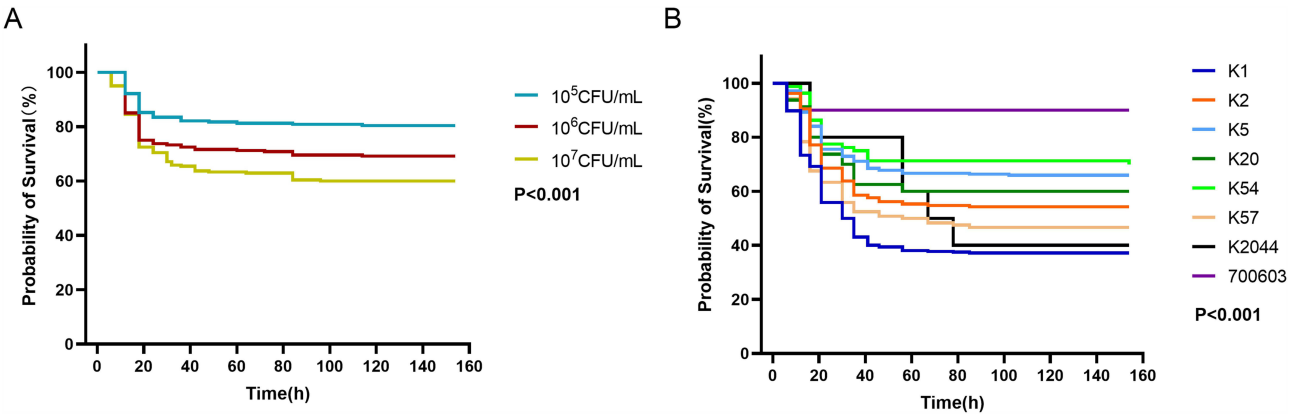

Figure 6 Survival curves of *Galleria mellonella* larvae in the infection model. Panel (**A**) shows the survival rate of *Galleria mellonella* larvae at different time points under solution concentrations of 10^7, 10^6 and 10^5CFU/mL, and *P* value is the significance among the three groups. Panel (**B**) shows the survival rate of *Galleria mellonella* larvae at different time points, ATCC 700603 (shown as 700,603), NTUH-K2044 (shown as K2044), with the *P* value indicating the significance between the six groups.

The results indicated that the average survival time of *Galleria mellonella* larvae was the shortest for the K1 serotype and the longest for the K54 serotype. Larval survival rates were as follows: K1 (38.3%) < K57 (46.7%) < K2 (54.3%) < K20 (61.5%) < K5 (66.4%) < K54 (70.0%). Refer to Figure 6B for further details.

Discussion

According to reports, *Klebsiella pneumoniae* of serotypes K1 and K2 are associated with invasive infections linked to liver abscesses and are notoriously difficult to treat.[5] Serotypes K5, K20, K54, and K57 have also been identified as highly virulent capsular types capable of causing severe intra- and extra-pulmonary infections.[6,7,25] In this study, we found that among *Klebsiella pneumoniae* strains, the K1 serotype had the highest detection rate (35, 9.6%), followed by K5 (27, 7.4%), K2 (21, 5.8%), K57 (12, 3.3%), K20 (8, 2.2%), and K54 (8, 2.2%). In Taiwan,[26] the detection rate for each capsule serotype was similar to that observed in our study. In Japan,[27] the detection rate of K1 was lower than that in our study, whereas the detection rate of K2 was higher. However, the results of the Belgian study[28] were significantly different from those of our study, which may be related to regional differences. In this study, we found that the detection rate of CSKP was higher than that of CRKP for all capsule serotypes. Our study indicate that each capsular type's detection rate in CSKP is higher than in CRKP. And there was a greater prevalence of K1 and K2 serotypes in CSKP, while in CRKP, K5 serotypes were more prevalent. A study from Guangdong, China[17] showed CRKP commonly presents as K1, K2, and K57 serotype, while K5 serotype was not detected. These results indicated that the strains prevalent in different regions were different.

Apart from capsules, siderophores, pili, and lipopolysaccharides are also important virulence factors in *Klebsiella pneumoniae*. To further study the carrying rate of virulence genes in each capsule serotype, we detected 15 virulence genes, among which, *rmkD, fimH, entB*, and *wabG* were dominant in each capsule serotype. The *rmpA, rmpA2, iroB, iucA, peg-344, iutA, magA, aerobactin, ybts, alls*, and *kfu* genes were the most prevalent in the K1 isolates. *RmpA, rmpA2, iutA, iucA, aerobactin, iroB, peg-344*, and *kfu* showed the lowest detection rate in K5 isolates. The *rmpA, iroB, aerobactin, peg-344*, and hmKP were found to be concentrated in the correlation study, and *iucA* was highly positively correlated with *iutA*. The reason may be that in KP, such virulence genes usually participate in virulence expression and pathogenesis together, and the vast majority of virulence genes are located on the common plasmid.[12] In addition, K1 serotype, *magA*, and *alls* were highly correlated, and *rmpA, iroB*, and *aerobactin* were highly negatively correlated with K non-typeable, that is, the detection of these three genes was associated with hypervirulent capsular serotypes (K1, K2, K5, K20, K54, K57). These genes provide important support for the high virulence of KP.[25] This study identified that K1 isolates carried the largest number of virulence genes and their distribution was relatively concentrated, which was also agreed by Liao[26] and Kao.[29] The number of virulence genes carried by K5 isolates was the least, and the distribution was uneven, the detection rate of most genes in K5 isolates was the lowest. In this study, K5 isolates from CRKP carried fewer virulence genes than those from CSKP, and the number of

genes carried by K5 isolates differed between the two groups, which further indicated that there was a certain competition between drug resistance genes and virulence genes.[25]

In the traditional definition, isolates with hypermucilage phenotype are considered to be hvKP.[12] With the deepening of research, genes such as " *rmpA*, *rmpA2*, *iroB*, *iucA*, and *peg-344*" have been increasingly identified as hvKP.[13] The prevalence of these genes varied among different capsular serotypes, with previous research indicating that K1 serotype exhibited the highest positive rates ranging from 81.5% to 100.0%, followed by K2 and K57 serotypes with rates between 50.0% and 100.0%. Conversely, K20 and K54 serotypes demonstrated slightly lower positive rates, potentially falling below 60.0% or even 50.0%.[26,27,30,31] Consistent with previous findings, this study observed that K1 serotype displayed the highest positive rates for the aforementioned virulence genes, followed by types K2 and K57 serotypes. In addition, this study found that hmKP was detected at the highest rate in serotype K1 and at the lowest rate in serotype K54. In terms of the detection rate of hvKP, the K1 serotype was still dominant, while the lowest detection rate of hvKP was not the K54 serotype but the K5 serotype, which was consistent with the results of the number of virulence genes carried by each capsule serotype. This indicated that hmKP was related to hvKP to some extent,[32,33] but there were still differences between them. To clarify the homology and dissemination of the different capsular serotypes in this area, a minimum spanning tree was constructed using MLST typing. The results showed significant differences in MLST types among different capsular serotypes. The ST23 sequence type was found only in the K1 serotype and was the dominant type (77.1%). This phenomenon has also been supported by previous reports.[28,34] In the K2 serotype, the dominant type was not obvious, and it was diversified, mainly the ST65 sequence type. K20 isolates are mainly ST268 sequence type, K54 isolates are mainly ST29 sequence type, and K57 isolates are mainly ST412 sequence type, showing obvious clonal dissemination, which was different from the prevalent strains in Taiwan.[26] At the same time, MLST typing of different capsular serotypes also overlapped (ST412, ST1049, ST25, etc.), suggesting that horizontal transmission also existed at the same time. Mendes[25] revealed that transposons, integrons, plasmids, insertion sequences, and ICEs are transmission vectors of KP.

To verify the virulence of each capsular serotype, a *Galleria mellonella* larval infection model was constructed. The survival rate of K1 isolates larvae was the lowest, followed by K57, K2, K20, K5, and K54 isolates. Among the six capsular serotypes, K1 isolates were the most virulent, while K54 isolates were the least virulent. A virulence study of K54 isolates in the United Kingdom[31] also found a lower prevalence of K54 isolates than of K1 isolates. According to most previous studies, K1 and K2 were the most virulent serotypes of KP. This study found that the detection rate of hvKP, the number of virulence genes carried, and the mortality rate of *Galleria mellonella* larvae in K57 isolates were higher than those in K2 isolates. This study found that in addition to K1 and K2, K57 isolates deserve more attention. Surprisingly, although the K5 isolates had the lowest prevalence of multiple virulence genes, the lowest overall number of virulence genes, and the lowest detection rate of hvKP, their virulence was not the weakest in the *Galleria mellonella* larvae model, which was stronger than that of the K54 isolates, and the detection rate of hmKP was also higher than that of the K54 isolates. This indicates that the virulence of bacteria is not determined by a single factor, but by a variety of virulence factors.[35,36]

Conclusion

The detection rate of capsule serotypes in CSKP was found to be higher than that of CRKP, with K1 being the most prevalent serotype, followed by K5 and K2. CSKP was dominated by the K1 and K2 serotypes, while CRKP was characterized mostly by the K5 serotype. Among K1 isolates, the detection rates of hvKP and hmKP were the highest, along with the number of virulence genes present. Conversely, the detection rate of hmKP in K54 isolates was the lowest, as was the detection rate of hvKP in K5 isolates, along with the number of virulence genes. Clonal relationships were observed among the K1, K20, and K57 serotypes. The K1 serotype was predominantly correlated with the ST23 sequence type, the K20 serotype was commonly linked to the ST268 sequence type, and the K57 serotype was primarily associated with the ST412 sequence type. MLST typing of the K2, K5, and K54 serotype isolates revealed a higher level of diversity. Among the six capsule serotypes, isolates of the K1 serotype demonstrated the highest level of virulence, while strains of the K54 serotype exhibited the lowest level of virulence.

Abbreviations

KP, *Klebsiella pneumoniae*; PCR, polymerase chain reaction; CRKP, carbapenem-resistant *Klebsiella pneumoniae*; CSKP, carbapenem-sensitive *Klebsiella pneumoniae*; MLST, Multilocus sequence typing; hmKP, hypermucoviscosity *Klebsiella pneumoniae*; n-hmKP, non-hypermucoviscous *Klebsiella pneumoniae*; hvKP, hypervirulent *Klebsiella pneumoniae*; n-hvKP, non-hypervirulent *Klebsiella pneumoniae*; cKP, classical *Klebsiella pneumoniae*.

Ethical Statement

All clinical data and related data in this study have been approved by the Ethics Committee of Ningbo Medical Center LiHuiLi Hospital and informed consent of the patient.The batch number is KY2023SL347-01.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

References

1. Choby JE, Howard-Anderson J, Weiss DS. Hypervirulent *Klebsiella pneumoniae* - clinical and molecular perspectives. *Journal of Internal Medicine*. 2020;287(3):283–300. doi:10.1111/joim.13007
2. Kuo G, Yen CL, Lu YA, et al. Clinical and visual outcomes following endogenous endophthalmitis: 175 consecutive cases from a tertiary referral center in Taiwan. *Journal of Microbiology, Immunology, and Infection = Wei Mian Yu Gan Ran Za Zhi*. 2022;55(1):114–122. doi:10.1016/j.jmii.2021.01.010
3. Zhang H, Zhang G, Yang Y, et al. Antimicrobial resistance comparison of *Klebsiella pneumoniae* pathogens isolated from intra-abdominal and urinary tract infections in different organs, hospital departments and regions of China between 2014 and 2017. *Journal of Microbiology, Immunology, and Infection = Wei Mian Yu Gan Ran Za Zhi*. 2021;54(4):639–648. doi:10.1016/j.jmii.2020.03.009
4. Lam MMC, Wick RR, Judd LM, Holt KE, Wyres KL. Kaptive 2.0: updated capsule and lipopolysaccharide locus typing for the *Klebsiella pneumoniae* species complex. *Microbial Genomics*. 2022;8(3). doi:10.1099/mgen.0.000800
5. Nakamura K, Nomoto H, Harada S, et al. Infection with capsular genotype K1-ST23 hypervirulent *Klebsiella pneumoniae* isolates in Japan after a stay in East *Asia*: two cases and a literature review. *Journal of Infection and Chemotherapy: Official Journal of the Japan Society of Chemotherapy*. 2021;27(10):1508–1512. doi:10.1016/j.jiac.2021.05.011
6. Sanikhani R, Moeinirad M, Shahcheraghi F, et al. Molecular epidemiology of hypervirulent *Klebsiella pneumoniae*: a systematic review and meta-analysis. *Iranian Journal of Microbiology*. 2021;13(3):257–265. doi:10.18502/ijm.v13i3.6384
7. Mukherjee S, Bhadury P, Mitra S, et al. Hypervirulent *Klebsiella pneumoniae* causing neonatal bloodstream infections: emergence of NDM-1-producing hypervirulent ST11-K2 and ST15-K54 strains possessing pLVPK-associated markers. *Microbiology Spectrum*. 2023;11(2):e0412122. doi:10.1128/spectrum.04121-22
8. Zhan L, Wang S, Guo Y, et al. Outbreak by hypermucoviscous *Klebsiella pneumoniae* ST11 isolates with carbapenem resistance in a tertiary hospital in China. *Frontiers in Cellular and Infection Microbiology*. 2017;7:182. doi:10.3389/fcimb.2017.00182
9. Yuan Y, Li Y, Wang G, et al. blaNDM-5 carried by a hypervirulent *Klebsiella pneumoniae* with sequence type 29. *Antimicrobial Resistance and Infection Control*. 2019;8(1):140. doi:10.1186/s13756-019-0596-1
10. Shao C, Xin L, Mi P, Jiang M, Wu H. Phenotypic and molecular characterization of K54-ST29 hypervirulent *Klebsiella pneumoniae* causing multi-system infection in a patient with diabetes. *Frontiers in Microbiology*. 2022;13:872140. doi:10.3389/fmicb.2022.872140
11. Zhou M, Lan Y, Wang S, et al. Epidemiology and molecular characteristics of the type VI secretion system in *Klebsiella pneumoniae* isolated from bloodstream infections. *Journal of Clinical Laboratory Analysis*. 2020;34(11):e23459. doi:10.1002/jcla.23459
12. Russo TA, Marr CM. Hypervirulent *Klebsiella pneumoniae*. *Clinical Microbiology Reviews*. 2019;32(3). doi:10.1128/CMR.00001-19
13. Bulger J, MacDonald U, Olson R, Beanan J, Russo TA. Metabolite transporter PEG344 is required for full virulence of hypervirulent *Klebsiella pneumoniae* strain hvKP1 after pulmonary but not subcutaneous challenge. *Infection and Immunity*. 2017;85(10). doi:10.1128/IAI.00093-17

14. Gu D, Dong N, Zheng Z, et al. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. *The Lancet Infectious Diseases*. 2018;18(1):37–46. doi:10.1016/S1473-3099(17)30489-9
15. Su S, Zhang J, Zhao Y, et al. Outbreak of KPC-2 Carbapenem-resistant *Klebsiella pneumoniae* ST76 and carbapenem-resistant K2 hypervirulent *Klebsiella pneumoniae* ST375 strains in Northeast China: molecular and virulent characteristics. *BMC Infectious Diseases*. 2020;20(1):472. doi:10.1186/s12879-020-05143-y
16. Liu P, Yang A, Tang B, et al. Molecular epidemiology and clinical characteristics of the type VI secretion system in *Klebsiella pneumoniae* causing abscesses. *Frontiers in Microbiology*. 2023;14:1181701. doi:10.3389/fmicb.2023.1181701
17. Lin ZW, Zheng JX, Bai B, et al. Characteristics of hypervirulent *Klebsiella pneumoniae*: does low expression of rmpa contribute to the absence of hypervirulence? *Frontiers in Microbiology*. 2020;11:436. doi:10.3389/fmicb.2020.00436
18. Zhang Y, Xu Y, Huang Y. Virulence genotype and correlation of clinical severeness with presence of the type VI secretion system in *Klebsiella pneumoniae* isolates causing bloodstream infections. *Infection and Drug Resistance*. 2022;15:1487–1497. doi:10.2147/IDR.S353858
19. Du Q, Pan F, Wang C, et al. Nosocomial dissemination of hypervirulent *Klebsiella pneumoniae* with high-risk clones among children in shanghai. *Frontiers in Cellular and Infection Microbiology*. 2022;12:984180. doi:10.3389/fcimb.2022.984180
20. Liu Y, Du FL, Xiang TX, et al. High prevalence of plasmid-mediated quinolone resistance determinants among serotype k1 hypervirulent *Klebsiella pneumoniae* isolates in China. *Microbial Drug Resistance (Larchmont, NY)*. 2019;25(5):681–689. doi:10.1089/mdr.2018.0173
21. Kot B, Piechota M, Szweda P, et al. Virulence analysis and antibiotic resistance of *Klebsiella pneumoniae* isolates from hospitalised patients in Poland. *Scientific Reports*. 2023;13(1):4448. doi:10.1038/s41598-023-31086-w
22. Dan B, Dai H, Zhou D, Tong H, Zhu M. Relationship between drug resistance characteristics and biofilm formation in *Klebsiella pneumoniae* strains. *Infection and Drug Resistance*. 2023;16:985–998. doi:10.2147/IDR.S396609
23. Candan ED, Aksöz N. *Klebsiella pneumoniae*: characteristics of carbapenem resistance and virulence factors. *Acta biochimica Polonica*. 2015;62 (4):867–874. doi:10.18388/abp.2015_1148
24. Li HF, Zhang LX, Zhang WL, Li J, Li YQ, Hu TP. Study on virulence genes, drug resistance and molecular epidemiology of *Klebsiella pneumoniae* with high virulence in inner mongolia, China. *Infection and Drug Resistance*. 2023;16:1133–1144. doi:10.2147/IDR.S391468
25. Mendes G, Santos ML, Ramalho JF, Duarte A, Caneiras C. Virulence factors in carbapenem-resistant hypervirulent *Klebsiella pneumoniae*. *Frontiers in Microbiology*. 2023;14:1325077. doi:10.3389/fmicb.2023.1325077
26. Liao CH, Huang YT, Hsueh PR. Multicenter surveillance of capsular serotypes, virulence genes, and antimicrobial susceptibilities of *Klebsiella pneumoniae* causing bacteremia in Taiwan, 2017-2019. *Frontiers in Microbiology*. 2022;13:783523. doi:10.3389/fmicb.2022.783523
27. Harada S, Ishii Y, Saga T, Aoki K, Tateda K. Molecular epidemiology of *Klebsiella pneumoniae* K1 and K2 isolates in Japan. *Diagnostic Microbiology and Infectious Disease*. 2018;91(4):354–359. doi:10.1016/j.diagmicrobio.2018.03.010
28. Anantharajah A, Deltombe M, de Barsy M, et al. Characterization of hypervirulent *Klebsiella pneumoniae* isolates in Belgium. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology*. 2022;41(5):859–865. doi:10.1007/s10096-022-04438-z
29. Kao CY, Zhang YZ, Bregente CJB, et al. A 24-year longitudinal study of *Klebsiella pneumoniae* isolated from patients with bacteraemia and urinary tract infections reveals the association between capsular serotypes, antibiotic resistance, and virulence gene distribution. *Epidemiology and Infection*. 2023;7(151):e155"2.
30. Wang TC, Lin JC, Chang JC, et al. Virulence among different types of hypervirulent *Klebsiella pneumoniae* with multi-locus sequence type (MLST)-11, Serotype K1 or K2 strains. *Gut Pathogens*. 2021;13(1):40. doi:10.1186/s13099-021-00439-z
31. Turton JF, Payne Z, Micah K, Turton JA. Capsular type K54, clonal group 29 and virulence plasmids: an analysis of K54 and non-K54 closely related isolates of *Klebsiella pneumoniae*. *Epidemiology and Infection*. 2018;146(14):1813–1823. doi:10.1017/S0950268818001826
32. Zhang Y, Zeng J, Liu W, et al. Emergence of a hypervirulent carbapenem-resistant *Klebsiella pneumoniae* isolate from clinical infections in China. *The Journal of Infection*. 2015;71(5):553–560. doi:10.1016/j.jinf.2015.07.010
33. Calfee DP. Recent advances in the understanding and management of *Klebsiella pneumoniae*. *F1000Research*. 2017;6:1760. doi:10.12688/f1000research.11532.1
34. Du P, Liu C, Fan S, Baker S, Guo J. The role of plasmid and resistance gene acquisition in the emergence of ST23 multi-drug resistant, hypervirulent *Klebsiella pneumoniae*. *Microbiology Spectrum*. 2022;10(2):e0192921. doi:10.1128/spectrum.01929-21
35. Dai P, Hu D. The making of hypervirulent *Klebsiella pneumoniae*. *Journal of Clinical Laboratory Analysis*. 2022;36(12):e24743. doi:10.1002/jcla.24743
36. Pu D, Zhao J, Chang K, Zhuo X, Cao B. "Superbugs" with hypervirulence and carbapenem resistance in *Klebsiella pneumoniae*: the rise of such emerging nosocomial pathogens in China. *Science Bulletin*. 2023;68(21):2658–2670. doi:10.1016/j.scib.2023.09.040