

Higher Virulence Renders K2 *Klebsiella pneumoniae* a Stable Share Among Those from Pyogenic Liver Abscess

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Objective: To explore why serotype K2 accounts for a stable share in *Klebsiella pneumoniae* from pyogenic liver abscess (PLA).

Methods: Totally 15 K2 *K. pneumoniae* strains from PLA, 21 K2 from non-PLA, and 31 K1 from PLA were collected from China. Sequence typing, molecular serotyping, regular PCR, and *Galleria mellonella* lethality were performed. A total of 12 virulence genes were detected: *peg-344*, *allS*, *p-rmpA*, *p-rmpA2*, *c-rmpA*, *fimH*, *mrkD*, *iucA*, *iroN*, *irp2*, *entB*, and *wzi*. The differences between K2 *K. pneumoniae* strains from PLA and non-PLA were investigated along with K1 ones.

Results: Significant differences were found between K2 strains from PLA and non-PLA for the rates of virulence genes *peg-344* and *iucA*. The latter group also showed more diverse sequence types than the former. Significant differences were only found for virulence genes *allS* and *irp2* between K1 and K2 strains from PLA. Based on the equal virulence factors backgrounds other than serotypes, K2 strain is more virulent than K1 as *G. mellonella* lethality confirmed. Gene *p-rmpA* only brings equal virulence to *p-rmpA* plus *p-rmpA2* in K2 strain.

Conclusion: Based on the same virulence factors backgrounds except serotypes, K2 *K. pneumoniae* is more virulent than K1 from PLA, which provides a survival advantage to maintain a stable share.

Keywords: *Klebsiella pneumoniae*, pyogenic liver abscess, serotype, virulence, gene

Introduction

Pyogenic liver abscess (PLA) is a relatively common but fatal infectious disease, accounting for 13% of intra-abdominal infections and 48% of visceral abscesses.¹⁻³ In China, the incidence of PLA was 17.59 per 100,000 hospital admissions, while it was 1.07 to 3.59 in the west,⁴ which is still on the rise.⁵ With the use of antibiotics and percutaneous catheter drainage, the mortality rate of PLA decreased from over 31.0% 60 years ago⁶ to around 10.0% in recent years.^{7,8} The agents of PLA include various bacteria, of which *Klebsiella pneumoniae* and *Escherichia coli* are the most documented, particularly in Asia.^{9,10} In the past 3 to 4 decades, *K. pneumoniae* has replaced *E. coli* as the foremost agent of PLA,¹¹ the share of which exceeded 80.0%.¹² The mean length of hospital stay for PLA was 10.3 days in New Zealand¹³ while it was 15.6 in China mainland.¹⁴

K. pneumoniae is now a notorious Gram-negative bacillus, causing series of infections, such as pneumonia, urinary tract infection, bacteremia, and PLA.¹⁵ As a notable member of ESKAPE, *K. pneumoniae* is also well

known for its rapidly evolved drug resistance.¹⁶ Furthermore, *K. pneumoniae* is also amazing for its hypervirulence and the convergence of hypervirulence and extreme drug resistance.¹⁷ Based on the capsular polysaccharide, *K. pneumoniae* strains are denoted as 78 serotypes.¹⁸ For *K. pneumoniae* strains causing PLA, although a couple of serotypes are involved, e.g. K1, K2, K5, K16, K20, K54, K57, and K64, K1 and K2 are usually the predominant 2 types, which accounted for nearly 70.0% in sum;^{8,19} the ratio of K1 versus K2 is about 2:1. Therefore, K2 *K. pneumoniae* also plays an indispensable role in PLA. It is noted that the proportion of K1 *K. pneumoniae* declined, while that of K2, accounting for around 20.0%, was retained, as indicated by the 3 reports,^{8,12,20} which included *K. pneumoniae* strains from PLA during different years and regions. It is intriguing as to why K2 maintained the share over the past several years.

Typically, K1 and K2 *K. pneumoniae* strains are both hypervirulent.¹⁵ However, the same serotype strains from different specimen sources may differ in various backgrounds, such as virulence genes and sequence types (STs), which could also be found between different serotype strains.²¹ The steady-going share of K2 indicates that the traits of K2 ones from PLA need to be unveiled, in particular its virulence versus K1 ones and those of K2 from non-PLA. As Qiucheng Shi reported,²² K1 ST23 *K. pneumoniae* was the dominant clone in the moderate virulence-level group, although it was the most prevalent clone of the hypervirulent *K. pneumoniae* as *Galleria mellonella* lethality test was conducted; 24/36 K1 strains showed moderate virulence level, while 8/11 K2 presented high virulence level. K2 strains are generally hypervirulent with median lethal dose (LD₅₀) <10³ colony forming units (CFUs) while mouse lethality test was performed.²³ However, the comparison of K1 versus K2 was not noted, although K1 strain typically shows an LD₅₀ of 10² CFU.²⁴ Different batches and conductors may yield different results, although mouse lethality test is done for the same strain. To unveil the traits of K2 strains from PLA, we collected 15 K2 from PLA, 21 K2 from non-PLA, and 31 K1 from PLA. Their virulence genes, factors, STs, and virulence were compared to elucidate the underlying reason for their stable share.

Materials and Methods

K. pneumoniae Strains

Totally 21 K2 *K. pneumoniae* strains from non-PLA were included in this study, which were collected from 5 hospitals in 4 provinces of China between January 2017 and February 2018: Huashan Hospital, 10 strains; Jinshan Hospital, 3 strains; Taizhou Municipal Hospital, 2 strains; The First Affiliated Hospital of Guangxi Medical University, 1 strain; Sixth Hospital of Shanxi Medical University, 5 strains. The other 15 K2 *K. pneumoniae* strains from PLA were isolated from such 3 hospitals: The First Affiliated Hospital, College of Medicine, Zhejiang University, 10 strains; The First Affiliated Hospital of Xiamen University, 3 strains; Taizhou Municipal Hospital, 2 strains. The non-PLA specimen sources included: sputum, 8; urine, 5; blood, 4; pus, 4.

The 30 K1 *K. pneumoniae* strains from PLA were included in this study, which were collected from 4 hospitals in 4 provinces of China between January 2017 and February 2018: Huashan Hospital, 2 strains; The First Affiliated Hospital, College of Medicine, Zhejiang University, 20 strains; The First Affiliated Hospital of Xiamen University, 8 strains. Another one was NTUH-K2044 from National Taiwan University Hospital.

Strains were identified using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry system (Bruker Daltonics Inc., Fremont, CA, USA), with the standard strains ATCC 27853, ATCC 700603, and ATCC 25922 as the controls.

Strain NTUH-K2044 (GenBank accession number: AP006725.1) is a typical hypervirulent K1 *K. pneumoniae*, which was isolated from a patient with PLA and meningitis.²⁵ *K. pneumoniae* strain HS11286 (GenBank accession number: CP003200.1), serotyped as K47, harbours *bla*_{KPC-2} and shows hypovirulence, which was isolated from a patient with pneumonia at Huashan Hospital, Fudan University, Shanghai, China.²⁶ Strains NTUH-K2044 and HS11286 were used as controls for *G. mellonella* lethality tests. All the strains were kept at -80 °C prior to use.

Multilocus Sequence Typing (MLST)

Genomic DNA of *K. pneumoniae* strains were extracted using QIAamp DNA mini kit (QIAGEN Co.; Catalog number: 51,304). Seven housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) were amplified using an Applied Biosystems Veriti PCR system (ABI, San Ramon, CA, USA). The yielded products were then sequenced by an ABI 3730XL DNA Analyzer (ABI, San Ramon, CA, USA). In comparison with the *K. pneumoniae* MLST database website http://bigsd.b.pasteur.fr/cgi-bin/bigsd/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=sequenceQuery, ST of each strain was obtained. The primers were shown in [Table S1](#).

Determination of Serotypes and Virulence Genes

Serotypes K1 and K2 were identified using PCR methods.²⁷ Virulence genes (*peg-344*, *allS*, *entB*, *irp2*, *iroN*, *iucA*, *fimH*, *mrkD*, *p-rmpA2*, *c-rmpA*, *p-rmpA*, and *wzi*)^{28–30} were determined using the former Veriti PCR system and agarose electrophoresis. The primers were shown in [Table S1](#). Virulence genes *p-rmpA/p-rmpA2* were used to predict the presence of virulence plasmids.

Lethality Test

Wax moth (*G. mellonella*) larvae lethality test was done as a reference.³¹ Ten larvae, weighting about 300 mg each, were injected per group with 10 µL of *K. pneumoniae* at the logarithmic growth phase. Surveillance time points were set at 6, 12, 24, 48, 72, and 96 h, respectively, after infection. The tested strains H2, H4, and H8 were from different lineages as pulse-field confirmed by gel electrophoresis.⁸

Statistical Analysis

Statistical analyses were performed using the GraphPad Prism 8 software (GraphPad Software Inc., USA). Chi-square and survival tests were used to analyze comparisons between groups; $p < 0.05$ was considered statistically significant.

Results

Traits of *K. pneumoniae* Strains Involved in *G. mellonella* Lethality Tests

[Table 1](#) shows the backgrounds of the 5 *K. pneumoniae* strains involved in *G. mellonella* lethality tests, including STs, virulence genes, and predicted virulence factors. Two K2 and one K1 clinical strains were tested with NTUH-K2044 (K1) being the positive control and HS11286 (K47) being the negative control. All the 5 strains except HS11286 were from PLA. Strains H4 and H8 had same backgrounds except for serotypes.

Traits of K2 *K. pneumoniae* Strains from PLA and Non-PLA

[Figure 1](#) shows that significant differences were only found for virulence genes *peg-344* and *iucA* between K2 *K. pneumoniae* strains from PLA and non-PLA; higher rates of *peg-344* and *iucA* were both found in K2 *K. pneumoniae* strains from PLA

Table 1 Traits of *K. pneumoniae* Strains Involved in *G. mellonella* Lethality Tests

Strain	ST	Serotype	<i>c-rmpA</i>	<i>p-rmpA</i>	<i>p-rmpA2</i>	Enterobactin	Yersiniabactin	Salmochelin	Aerobactin	Fimbriae I	Fimbriae 3
NTUH-K2044	23	K1	+	+	+	+	+	+	+	+	+
H8	2159	K1	-	+	+	+	-	+	+	+	+
H4	375	K2	-	+	+	+	-	+	+	+	+
H2	380	K2	-	+	-	+	+	+	+	+	+
HS11286	11	K47	-	-	-	+	+	-	-	+	+

Note: Virulence factors enterobactin, yersiniabactin, salmochelin, aerobactin, Fimbriae I, and Fimbriae 3 were in line with the results of *entB*, *irp2*, *iroN*, *iucA*, *fimH*, and *mrkD*, respectively.

Abbreviations: ST, sequence type; +, positive; -, negative.

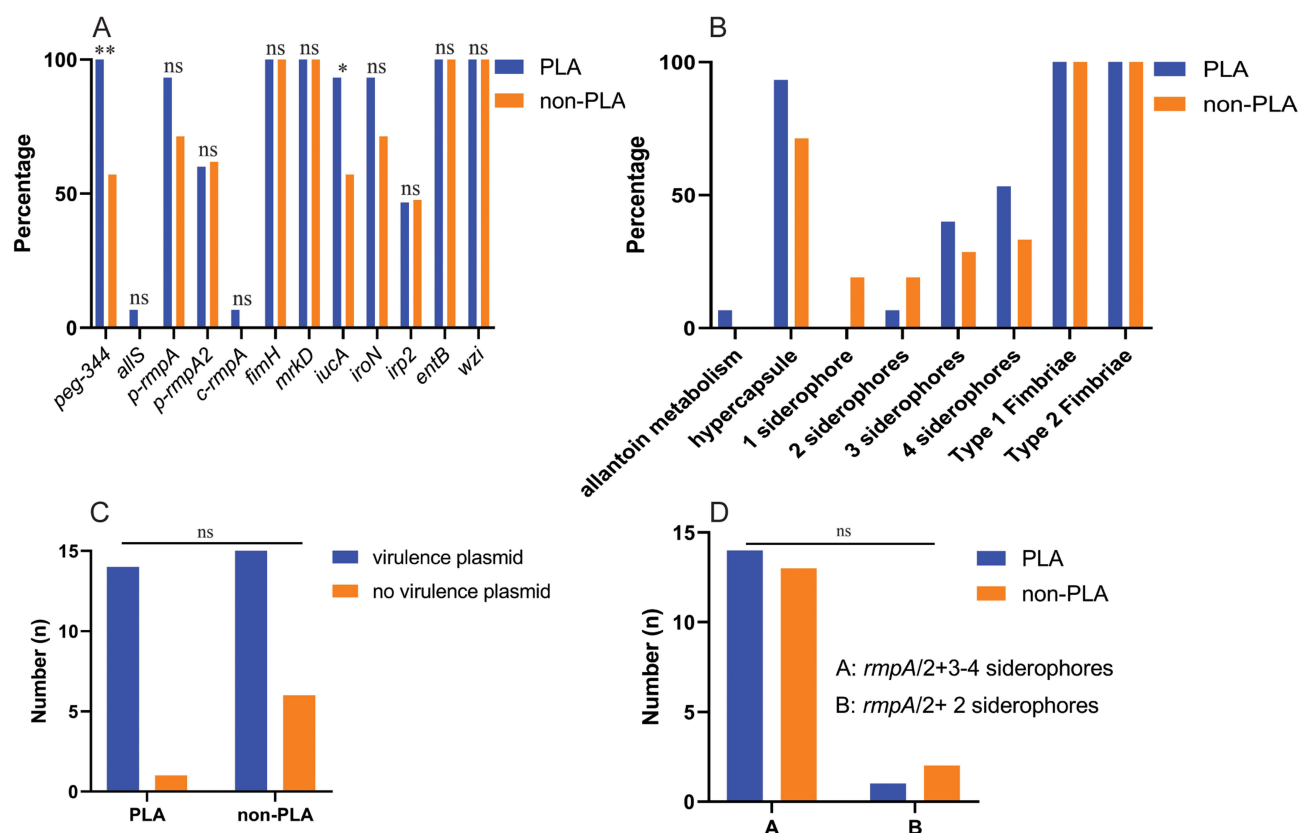


Figure 1 The traits of K2 *K. pneumoniae* strains from PLA and non-PLA. **(A)** Prevalence of twelve virulence genes; **(B)** Prevalence of virulence factors; **(C)** Prevalence of virulence plasmid; **(D)** Prevalence of *rmpA/2+3-4* siderophores and *rmpA/2+2* siderophores. **: $p < 0.01$; *: $p < 0.05$; ns: not significant; PLA: pyogenic liver abscess; non-PLA: non-pyogenic liver abscess. The corresponding virulence genes were *allS*, *rmpA/2*, *iucA/iroN/irp2/entB*, *fimH*, and *mrkD*, respectively, for predicted virulence factors allantoin metabolism, hypercapsule, siderophore, Types 1 and 3 fimbriae. Genes *p-rmpA* and *p-rmpA2* were used to predict a virulence plasmid.

(Figure 1A). No significant differences were found in Figure 1B-D. Except for *peg-344*- and *iucA*-related virulence genes, the others and virulence plasmids were without significant differences.

STs of K2 *K. pneumoniae* Strains from PLA and Non-PLA

Figure 2 shows more diverse STs in *K. pneumoniae* strains from non-PLA (12 STs) than those from PLA (7 STs). However, ST65 both accounted for about 30% in the two groups.

Traits of K1 and K2 *K. pneumoniae* Strains from PLA

Figure 3 shows that significant differences were only found for virulence genes *allS* and *irp2* between K1 and K2 *K. pneumoniae* strains from PLA, which means the different rates of allantoin metabolism and yersiniabactin. The rates of *allS* and *irp2* were both higher in K1 than in K2 strains (Figure 3A). Except for allantoin metabolism and yersiniabactin, the others and virulence plasmid were without significant differences (Figure 3B-D).

Survival Curves of *G. mellonella* Infected by *K. pneumoniae* Strains

To elucidate the virulence traits of K2 strains from PLA, K1 and K2 with *rmpAs* and siderophores were investigated along with the positive control NTUH-K2044 and negative control HS11286. Figure 4A suggests equal hypervirulence of NTUH-K2044 and H4, which was higher than that of H8. The lack of *c-rmpA* and *irp2* led to less virulence of H8 than NTUH-K2044 although they were both K1. With the same backgrounds except serotypes (Table 1), more hypervirulence of H4 than that of H8 indicated K2 strains are more virulent than K1 although they are both hypervirulent.

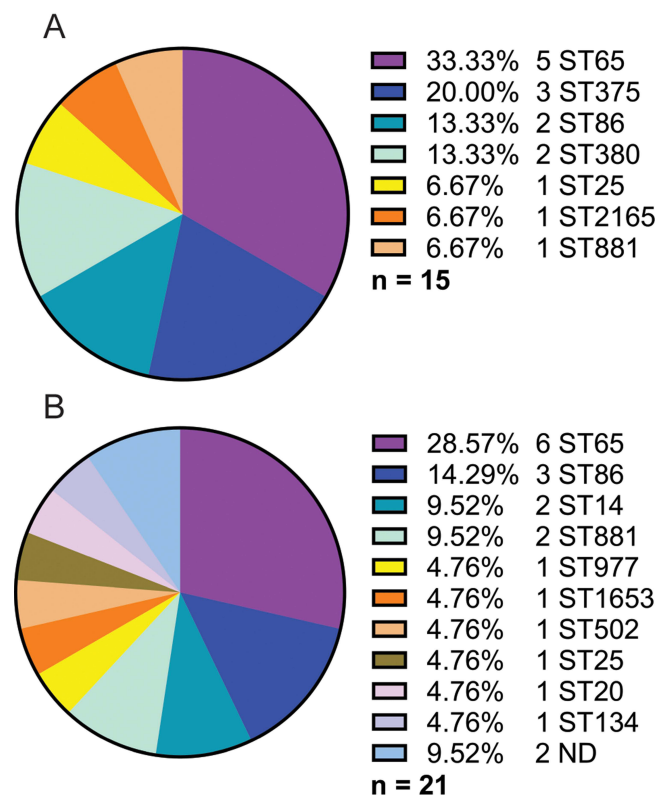


Figure 2 STs of K2 *K. pneumoniae* strains from PLA and non-PLA. **(A)** STs of 15 K2 *K. pneumoniae* strains from PLA; **(B)** STs of 21 K2 *K. pneumoniae* strains from non-PLA. ST: sequence type; ND: not defined.

Figure 4B confirms the equal hypervirulence of NTUH-K2044, H4, and H2. As shown in Table 1, H4 and H2 were both typed as K2 but harboured different *rmpA*s and siderophores. Therefore, with *rmpA* or *rmpA2*, the other *rmpA/2* or excessive siderophores are redundant for the hypervirulence of K2 strains.

Discussion

The reason is still unknown as to why K2 *K. pneumoniae* strains maintain a stable proportion among *K. pneumoniae* from PLA. As revealed in this study, based on the same virulence factors backgrounds other than serotype, K2 strains are more virulent than K1 ones.

As Figure 1 shows, higher rates of *peg-344* and *iucA* were both found in K2 *K. pneumoniae* strains from PLA than those from non-PLA. Gene *iucA* means siderophore aerobactin while the function of *peg-344* remains to be elucidated.³² The less diversity of STs in K2 strains from PLA than those from non-PLA indicated the less divergent origins of the former while ST65 presented an equal share ($p > 0.9999$). Different from another report,²³ ST66, ST373, ST374, and ST384 were absent while ST25, ST2165, and ST1881 were present in our study. As Figure 2 shows, higher rates of *allS* and *irp2* were both found in K1 than K2 *K. pneumoniae* strains from PLA. Gene *allS* means allantoin metabolism, which facilitates the colonization of K1 strains in the gut.³³ Unlike the absence of *allS* from another 2 reports,^{23,34} one K2 strain harboured it in this study. Virulence of K2 strains from PLA was rarely compared with K2 from non-PLA and K1 from PLA. According to Table 1 and Figure 4A, K2 strain H4 was more virulent than K1 strain H8. Without *c-rmpA* and *irp2*, H4 still presented the same hypervirulence with NTUH-K2044, which is likely to be the most virulent K1 strain. Figure 4B indicates that with one *rmpA/2*, the other *rmpA/2* or excessive siderophores are redundant for the hypervirulence of K2 strains. Figure 4 suggests that based on the same virulence factors backgrounds except serotypes, K2 is more virulent than K1, which is consistent with another report.²² It should also be noted that simultaneous 3 *rmpA*s in K1 strains are rare.⁸

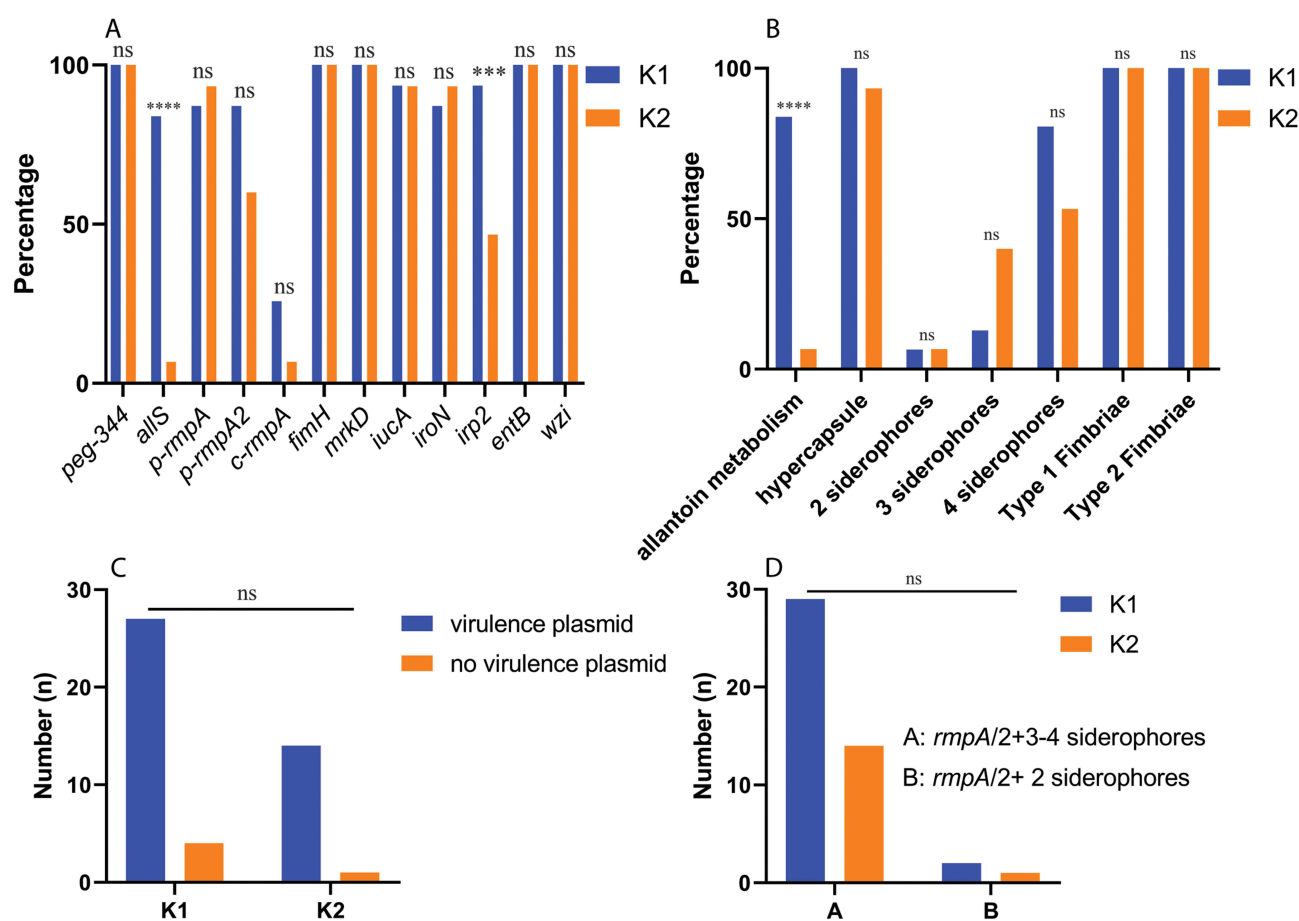


Figure 3 The traits of K1 and K2 *K. pneumoniae* strains from PLA. **(A)** Prevalence of twelve virulence genes; **(B)** Prevalence of virulence factors; **(C)** Prevalence of virulence plasmid; **(D)** Prevalence of *rmpA/2+3-4* siderophores and *rmpA/2+2* siderophores. ***: $p < 0.001$; ****: $p < 0.0001$; ns: not significant; PLA: pyogenic liver abscess; non-PLA: non-pyogenic liver abscess. The corresponding virulence genes were *allS*, *rmpA/2*, *iucA/iroN/irp2/entB*, *fimH*, and *mrkD*, respectively, for predicted virulence factors allantoin metabolism, hypercapsule, siderophore, Types 1 and 3 fimbriae. Genes *p-rmpA* and *p-rmpA2* were used to predict a virulence plasmid.

Since K2 *K. pneumoniae* strains are more virulent than K1 ones, why is that the share of K2 is less than that of K1? Although not rigorous, other related investigations may provide reasonable clues. First, the carriage of K1 may be higher. According to a report from South Korea,³⁵ 3/11 patients with PLA carried K1 *K. pneumoniae* strains in stool while 1/11 did K2. In healthy Korean adults, another report included 1174 Koreans and found 248 *K. pneumoniae* strains from stools, of which 57 were K1 (4.9%) and 54 belonged to ST23.³⁶ Second, K1 and K2 strains always coexist with O antigen 1.³⁷ However, different serotypes mean different capsular polysaccharides. Without the same O1 antigen, O-antigen mutant of serotype K1 became susceptible to liver clearance and caused mild abscess formation, but its serotype K2 counterpart maintained their wild-type virulence.³⁸ These findings, together with those in this study, indicate the most important role of capsular polysaccharides in determining the higher hypervirulence of K2 strains.

This study has some limitations. First, the sample size is somewhat small due to the difficulty of collecting K2 *K. pneumoniae* strains from PLA. Second, virulence factors are often regulated by series of virulence genes and therefore their relations are not inevitable. Third, the same serotype strains may present diverse virulence. However, only 3 clinical strains (2 K2 and 1 K1) were included in *G. mellonella* lethality tests.

Taken together, based on the same virulence factors backgrounds except serotypes, K2 *K. pneumoniae* is more virulent than K1 one from PLA, which provides a survival advantage to maintain a stable share.

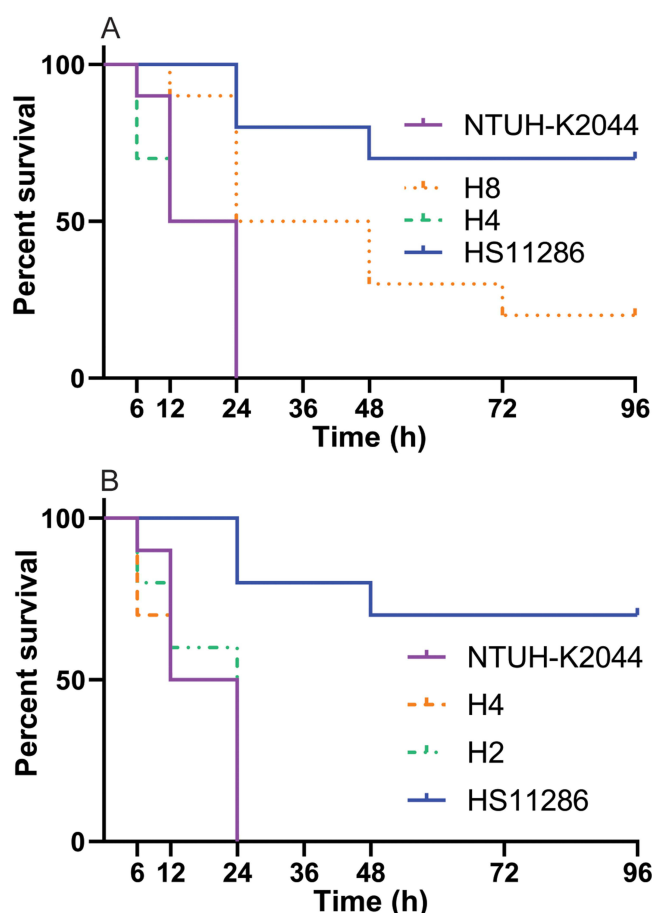


Figure 4 Survival curves of *G. mellonella* injected by *K. pneumoniae* strains. For (A), Log rank (Mantel–Cox) test yielded values of $\chi^2 = 25.0246$, $p < 0.0001$ for the four groups; $\chi^2 = 10.6681$, $p = 0.0048$ for the three groups (NTUH-K2044, H8, and H4); $\chi^2 = 7.6182$, $p = 0.0058$ for the two groups (NTUH-K2044 and H8); $\chi^2 = 0.0770$, $p = 0.7814$ for the two groups (NTUH-K2044 and H4); $\chi^2 = 7.8064$, $p = 0.0052$ for the two groups (H8 and H4). For (B), Log rank (Mantel–Cox) test yielded values of $\chi^2 = 21.2928$, $p < 0.0001$ for the four groups; $\chi^2 = 0.2624$, $p = 0.8771$ for the three groups (NTUH-K2044, H4, and H2).

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Ethics Approval and Consent to Participate

The studies involving human participants were reviewed and approved by the Ethics Committee of Huashan Hospital (Shanghai, China) (ethical approval No. 2021-484). The consent for participation was waived for this study in accordance with the national legislation and the institutional requirements. As per Item No. 34 of Measures for Ethical Review of Biomedical Research Involving Human Beings (National Health and Family Planning Commission of China, 2014), the strains involved in this study belong to anonymous recorded materials and therefore the ethical approval was needed while consent for participation was waived. All the methods were carried out in accordance with relevant guidelines and regulations for the present study.

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Shanxi Medical University, Taiyuan, Shanxi, China), and Meng Li (Department of Clinical Laboratory, The First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi, China).

Author Contributions

All authors made a significant contribution to the work reported, whether that was 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 there are no competing interests to declare.

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