Virulence Characteristics and Molecular Epidemiology of Pyogenic Liver Abscess Causing Multidrug Resistant *Klebsiella pneumoniae* in Wenzhou, China

Zhongyong Wang
The First Affiliated Hospital of Wenzhou Medical University
Siqin Zhang
Hangzhou Hospital of Traditional Chinese Medicine
Na Huang
The First Affiliated Hospital of Wenzhou Medical University
Shixing Liu
The First Affiliated Hospital of Wenzhou Medical University
Ye Xu
The First Affiliated Hospital of Wenzhou Medical University
Yajie Zhao
Wenzhou Medical University School of Laboratory Medicine and Life Sciences
Jianming Cao
Wenzhou Medical University School of Laboratory Medicine and Life Sciences
Tieli Zhou (wyztli@163.com)
The First Affiliated Hospital of Wenzhou Medical University https://orcid.org/0000-0002-2171-4710

Research Article

Keywords: pyogenic liver abscess, multidrug resistance, Klebsiella pneumoniae, virulence characteristics, molecular epidemiology

Posted Date: April 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-42660/v4

Abstract

Background

To date, little is known about the virulence characteristics of pyogenic liver abscess (PLA) that cause multidrug resistant (MDR) *Klebsiella pneumoniae* (*K. pneumoniae*), which might be due to the rarity of these strains. This study aimed to analyze the virulence characteristics and molecular epidemiology of 12 MDR strains obtained from 163 PLA cases in a tertiary teaching hospital from the perspective of clinical characteristics, virulence phenotypes, and genotypes.

Methods

The MDR strains were obtained from sterile fluid samples collected from patients with PLA. The antimicrobial susceptibility testing was confirmed by the agar dilution method and microdilution broth method. The virulence phenotypes were analyzed by the growth curves, string test, capsular quantification, serum killing test, biofilm formation assay and infection model. Polymerase chain reaction (PCR) was used to investigate the virulence genotypes. The molecular epidemiology was identified by multilocus sequence typing (MLST).

Results

The results of growth curves, string test, capsular quantification, serum killing test, biofilm formation assay, and infection model revealed that the virulence phenotypes of the 12 PLA-causing MDR *K. pneumoniae* were similar to or more obvious than those of the typical hypervirulent *K. pneumoniae* strains. These MDR strains were mainly non-K1/K2 serotypes and carried multiple virulence genes. The results of MLST illustrated that the MDR strains were categorized into 9 sequence types.

Conclusions

This is the first study to analyze the virulence characteristics in PLA-causing MDR strains. The data revealed the coexistence of hypervirulence and MDR in PLA-causing MDR *K. pneumoniae* strains, and the clones of these strains were diverse and scattered. Also, one ST11 carbapenem-resistant hypervirulent strain was identified in PLA.

Background

Klebsiella pneumoniae (*K. pneumoniae*) is a major Gram-negative bacterium responsible for urinary tract infections, pneumonia, bacteremia, and intra-abdominal infections worldwide [1–2]. In the past three decades, *K. pneumoniae* has been divided into two non-overlapping populations. One is termed as classic *K. pneumoniae* (cKP), a pathogen that causes hospital-acquired infections in immunocompromised

patients and is notorious in acquiring antimicrobial resistance, while the other is hypervirulent *K. pneumoniae* (hvKP) [1–3]. In contrast to cKP, an emerging variant that was first reported in Taiwan in 1986 is hypervirulent in causing severe invasive community-acquired infections and disseminates infections among immunocompetent individuals. According to recent studies, hvKP exhibits hypervirulent phenotypes and genotypes, and is susceptible to conventional antimicrobial agents except for the intrinsic resistance to ampicillin [1–3].

Pyogenic liver abscess (PLA) is a potentially life-threatening suppurative infection of hepatic parenchyma worldwide [2, 4, 5]. *K. pneumoniae* has emerged as a predominant pathogen of PLA across Asian and European countries, as well as the USA. Indubitably, *K. pneumoniae*-induced pyogenic liver abscess (KP-PLA) is a severe clinical challenge due to its association with mortality [5–6]. Hypervirulent *K. pneumoniae*-induced PLA usually occurs in young and healthy community individuals without an identified source of infection, and the cryptogenic abscess migrates to distant sites, leading to extrahepatic complications, such as endophthalmitis, meningitis, and necrotizing fasciitis [2, 5, 7]. A large number of the isolates from KP-PLA are susceptible to most of the antibiotics, and the antibiotic resistance rates are < 10% [8]. Antibiotic resistance is primarily associated with cKP. Interestingly, most of the recent reports revealed the convergence of virulence and resistance in *K. pneumoniae*, and most of these phenomena were commonly caused by plasmid-mediated resistance traits and virulence genes transfer [9–10]. In our previous study [11], 12 multidrug resistant (MDR) *K. pneumoniae* were isolated from non-cryptogenic PLA, which are considered as cKP. Although KP-PLA caused by antibiotic-susceptible hypervirulent strains has been well reported, MDR *K. pneumoniae* isolates from KP-PLA are rare and have not yet been well-identified, especially with respect to virulence characteristics and molecular epidemiology [2, 7, 12]. Whether these MDR *K. pneumoniae* isolates were indeed traditional cKP or combined with hypervirulence is yet to be elucidated. Since the virulence of *K. pneumoniae* can assist the pathogen to resist host innate immunity and infect the host invasively with high pathogenicity [1, 13], the convergence of virulence and resistance of *K. pneumoniae* pose challenges in treating KP-PLA. In addition, hypervirulent strains possess thick capsular polysaccharide, anti-serum capacity, and multiple virulence factors (hypermucoviscosity, capsular serotype, virulence genes, and related clones) [14, 15]. Therefore, acquiring knowledge about virulence characteristics and molecular epidemiology in the PLA-causing MDR *K. pneumoniae* is an urgent requisite.

Hence, in this study, the virulence characteristics and molecular epidemiology of PLA-causing MDR *K. pneumoniae* were investigated by collecting the strains over a 2-year period from KP-PLA patients in a tertiary teaching hospital to provide an in-depth insight in the development of effective therapeutic strategies for KP-PLA.

Materials And Methods

Bacterial isolates, antimicrobial susceptibility testing, and growth curves

From June 1, 2016 to December 31, 2017, a total of 163 KP-PLA cases were collected from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China), which has an annual admission of more than 160,000 inpatients. KP-PLA was diagnosed based on the clinical criteria [7, 16]. Initial strains were isolated from sterile fluids (including pus, blood, and drainage fluid) of KP-PLA patients and identified as *K. pneumoniae* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS; bioMérieux, Lyons, France). Antimicrobial susceptibility testing of *K. pneumoniae* isolates was conducted by bioMerieux VITEK-2 (BioMérieux, Marcy-l'Étoile, France). MDR strains were defined as non-susceptible to three or more different antimicrobial categories [17]. A total of 12 MDR *K. pneumoniae* were detected in 163 KP-PLA cases. An equal number of antimicrobial-susceptible typical hypervirulent strains were selected as experimental hypervirulent control strains (isolated from healthy, ambulatory patients with KP-PLA and carried both *aerobactin* and *rmpA* genes) and the standard strain ATCC 700603 as the hypovirulent strain (Table 1) [1, 18].

Table 1. The MICs of PLA-causing multidrug resistant strains and control strains against antimicrobial agents

Strain numbers	Isolate date	Isolate type	MICs (μg/mL, mg/L)												
			AMP	ATM	CRO	CAZ	FEP	IPM	CIP	LEV	GEN	TOB	SXT	NIT	COL
FK3038	May 17, 2016	drainage fluid	>64	2	>64	16	4	>64	>8	1	2	1	<0.25/4.75	128	0.5
FK3044	May 19, 2016	pus	32	16	>64	>64	32	1	0.03	2	0.125	4	>16/304	128	1
FK3068	May 26, 2016	pus	32	16	>64	16	32	0.5	0.03	2	0.5	4	>16/304	128	0.25
FK3228	July 26, 2016	drainage fluid	32	>64	>64	>64	>32	4	0.06	>16	0.125	4	>16/304	256	16
FK3347	September 08, 2016	blood	32	2	4	8	>32	1	0.5	>16	0.5	4	>16/304	256	0.25
FK3518	November 09, 2016	pus	>64	2	>64	16	2	1	>8	1	0.5	0.5	1/19	256	0.25
FK3521	November 10, 2016	pus	>64	2	>64	16	16	1	>8	4	0.5	<0.25	<0.25/4.75	128	0.25
FK3599	December 13, 2016	pus	32	2	4	2	0.25	4	8	1	0.5	<0.25	<0.25/4.75	256	0.25
FK4176	July 13, 2017	drainage fluid	>64	2	64	8	16	0.5	2	4	16	0.5	<0.25/4.75	128	0.5
FK4276	August 14,2017	drainage fluid	>64	2	>64	32	16	1	4	1	16	<0.25	<0.25/4.75	32	1
FK4603	November 13, 2017	blood	32	>64	64	64	>32	1	4	>16	0.25	2	>16/304	256	0.25
FK4737	December 26, 2017	pus	32	32	>64	32	8	2	4	4	0.5	4	>16/304	128	0.125
FK3112	June 13.2016	pus	32	1	<0.03	0.125	<0.03	0.125	0.03	2	0.25	1	0.5/9.5	32	0.5
FK3262	August 08,2016	pus	32	0.5	<0.03	0.125	<0.03	0.125	0.03	0.06	0.25	0.5	0.5/9.5	32	0.25
FK3645	January 10,2017	pus	>64	0.5	<0.03	0.125	<0.03	0.125	0.03	0.06	0.5	0.5	<0.25/4.75	32	1
FK3698	February 08,2017	pus	32	0.5	<0.03	0.25	<0.03	0.25	0.03	0.06	1	<0.25	<0.25/4.75	16	0.25
FK3736	February 20,2017	pus	32	0.25	<0.03	0.125	<0.03	0.25	0.03	1	0.5	0.5	<0.25/4.75	16	0.5
FK3818	March 21.2017	pus	32	1	<0.03	0.25	<0.03	0.25	0.5	0.25	0.25	0.5	<0.25/4.75	32	1
FK3837	March 24.2017	pus	32	1	<0.03	0.25	<0.03	0.25	0.03	0.06	0.25	0.5	<0.25/4.75	32	0.25
FK3914	April 21,2017	pus	32	0.5	<0.03	0.125	<0.03	0.25	0.03	0.06	0.25	0.5	<0.25/4.75	32	0.25
FK3953	May 05, 2017	pus	32	0.5	<0.03	0.125	<0.03	0.25	0.03	0.5	0.5	0.5	<0.25/4.75	32	0.5
FK3992	May 17, 2017	drainage fluid	32	0.5	<0.03	0.125	<0.03	0.25	0.008	0.25	0.25	0.5	<0.25/4.75	32	0.5
FK4081	June 15.2017	drainage fluid	>64	0.5	<0.03	0.125	<0.03	0.125	0.03	0.06	0.25	0.5	<0.25/4.75	32	1
FK4578	November 06, 2017	pus	>64	0.5	<0.03	0.125	<0.03	0.25	0.03	0.06	0.25	<0.25	<0.25/4.75	16	1

The MICs of PLA-causing multidrug resistant strains and control strains against antimicrobial agents

MICs minimum inhibitory concentrations, PLA pyogenic liver abscess, AMP ampicillin, ATM aztreonam, CRO ceftriaxone, CAZ ceftazidime, FEP cefepime, IPM imipenem, CIP ciprofloxacin, LVX levofloxacin, GEN gentamicin, TOB tobramycin, SXT sulfamethoxazole/trimethoprim, NIT nitrofurantoin, COL colistin.

Strain numbers were underlined: multidrug resistant strains; strain numbers were bolded: hypervirulent control strains. gray shading: resistance, white shading: intermediate or susceptible

The minimum inhibitory concentrations (MICs) of ampicillin, aztreonam, ceftriaxone, ceftazidime, cefepime, imipenem, ciprofloxacin, levofloxacin, gentamicin, tobramycin, sulfamethoxazole/trimethoprim,

nitrofurantoin and colistin were confirmed by agar dilution method and microdilution broth method. The data were interpreted based on the latest guidelines published by the Clinical and Laboratory Standards Institute (CLSI; Pittsburgh, PA, USA) and the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (http://www.eucast.org). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 served as quality control strains. The experiment was performed in triplicate.

The growth curves of 12 PLA-causing MDR *K. pneumoniae* isolates were measured as described previously [19]. Briefly, overnight cultures of selected *K. pneumoniae* clinical isolates from KP-PLA and *K. pneumoniae* ATCC 700603 were diluted in 1:100 by Luria-Bertani (LB) broth. The cultures were incubated at 37°C by constantly shaking at 200 rpm. The bacterial suspensions were collected at 0, 2, 4, 6, 8, 10, 12, 18, and 24 h, and the absorbance was measured at 600 nm. Each suspension was measured in triplicate, and the average of absorbance values was used for analysis. The growth of PLA-causing MDR *K. pneumoniae* was evaluated by plotting optical density 600 nm (OD_{600}) values against time. The experiment was carried in triplicate.

String test and quantification of capsule

The bacterial colonies of *K. pneumoniae* strain on an agar plate were scratched by an inoculation loop. The string test was considered positive when a viscous string of > 5 mm length was generated by the strain, which was also considered hypermucoviscous [18].

The capsule was quantified as described previously with some modifications [10, 20]. Briefly, 500 μL of cultured bacterial suspensions were resuspended and adjusted to 10^8 CFU/mL, and 1.2 mL sodium tetraborate was added to sulfuric acid in the resuspensions that were placed in ice bath and incubated for 5min at 100°C, and then kept on ice for 10 min. Then, a 20 μL volume of 1.5 mg/mL m-hydroxyphenyl was mixed. After incubating for 5min at room temperature, the absorbance was measured at 590 nm. The glucuronic acid content was determined by a standard curve of glucuronic acid and expressed as μg/10^8 CFU. The results were presented as the mean data from three independent experiments.

Biofilm formation assay

The biofilm formation assay was measured using the method described by Wilksch et al. [21]. Briefly, the clinical isolates were grown to logarithmic phase in LB broth and diluted at 1:100 ratio with fresh LB broth. Each dilution (200 μL) was added to 96-well plates, with three duplicate wells per strain; also, blank controls were set. The plates were then incubated at 37 ℃ for 24 h. The planktonic cells were removed, and the wells were washed thrice with sterile water, stained with 250 μL 0.1% crystal violet for 10 min and then rinsed three times with sterile water. The stained biofilms were solubilized with 95% ethanol and quantified by measuring the OD_{600}. Each sample was measured in triplicate, and the average of absorbance value was used for analysis.

Serum killing test

The serum bactericidal activity was measured as described by previous method [6]. The bacterial suspensions in the nutrient broth were collected during the logarithmic phase and adjusted to 10^6 CFU/mL. A volume of 25 μL bacterial suspension was added to 75 μL of pooled human sera in the tube and incubated for 0, 1, 2, or 3 h. An aliquot of each bacterial suspension was analyzed at the designated time point, diluted to the corresponding fold by adding Mueller-Hinton broth, and then cultured to determine the number of viable bacteria after exposure to serum. The strain was considered serum-resistant or serum-sensitive according to the data expressed as the viable counts and graded, and each strain was tested at least three times.

Infection model of *Galleria mellonella* larvae

The model of *G. mellonella* larvae was established based on three PLA-causing MDR isolates (FK3068, FK3228, and FK4603) and three typical hypervirulent strains (FK3112, FK3837, and FK3914) that were randomly selected and standard strain ATCC 700603 to investigate the virulence and pathogenicity of the strains [22–23]. Serially diluted bacterial suspension of each strain (10^7, 10^6, 10^5, and 10^4 CFU/mL) was prepared in advance. Eight larvae weighing 200-250mg were randomly selected for each strain and concentration. A 10 μL of bacterial suspension was injected into the last left proleg using a 25 μL Hamilton precision syringe. The larvae injected with 10 μL phosphate-buffered saline (PBS) were used as controls. Subsequently, the insects were incubated at 37 °C in the dark and observed after 24, 48, 72 and 96 h. The larvae were considered dead when they repeatedly failed to respond to physical stimuli. All experiments were conducted in triplicate.

Polymerase chain reaction (PCR) for capsular serotypes and virulence genes

Crude genomic DNA was extracted from PLA-causing *K. pneumoniae* isolates. Subsequently, the capsular serotype-specific genes (for serotypes of K1, K2, K5, K20, K54, and K57) and virulence genes (*aerobactin*, *rmpA*, *iroN*, *kfuBC*, *wcaG*, *ybtA*, *magA*, *fimH*, *mrkD*, *uge*, e*ntB*, and *ureA*) were amplified by PCR using specific primers, as described previously (Table 2) [24, 25–27]. In addition, the strains with these genes determined by PCR and DNA sequencing were selected as positive controls for subsequent PCR experiments.

Table 2. List of oligonucleotide primers used in amplification of capsular serotypes and virulence genes

Gene	Primer Sequence (5'-3')	Tm (°C)	Amplicon size (bp)
magA	F: GGTGCTCTTTACATCATTGC	58	1283
	R: GCAATGGCCATTTGCGTTAG		
aerobactin	F: GCATAGGCGGATACGAACAT	58	556
	R: CACAGGGCAATTGCTTACCT		
iroN	F: AAGTCAAAGCAGGGGTTGCCCG	58	665
	R: GACGCCGACATTAAGACGCAG		
kfuBC	F: GAAGTGACGCTGTTTCTGGC	58	979
	R: TTTCGTGTGGCCAGTGACTC		
rmpA	F: ACTGGGCTACCTCTGCTTCA	58	516
	R: CTTGCATGAGCCATCTTTCA		
wcaG	F: GGTTGGKTCAGCAATCGTA	58	169
	R: ACTATTCCGCCAACTTTTGC		
ybtA	F: ATGACGGAGTCACCGCAAAC	55	960
	R: TTACATCACGCGTTTAAAGG		
ureA	F: GCTGACTTAAGAGAACGTTATG	58	337
	R: GATCATGGCGCTACCT(C/T)A		
uge	F: TCTTCACGCCTTCCTTCACT	53	534
	R: GATCATCCGGTCTCCCTGTA		
entB	F: ATTTCCTCAACTTCTGGGGC	56	371
	R: AGCATCGGTGGCGGTGGTCA		
fimH	F: TGCTGCTGGGCTGGTCGATG	62	688
	R: GGGAGGGTGACGGTGACATC		
mrkD	F: CCACCAACTATTCCCTCGAA	43	226
	R: ATGGAACCCACATCGACATT		
K1	F: GGTGCTCTTTACATCATTGC	54	1283
	R: GCAATGGCCATTTGCGTTAG		
K2	F: GGATTATGACAGCCTCTCCT	58	641
	R: CGACTTGGTCCCAACAGTTT		

K5	F: TGGTAGTGATGCTCGCGA	58	280
	R: CCTGAACCCACCCCAATC		
K20	F: CGGTGCTACAGTGCATCATT	58	741
	R: GTTATACGATGCTCAGTCGC		
K54	F: CATTAGCTCAGTGGTTGGCT	58	881
	R: GCTTGACAAACACCATAGCAG		
K57	F: CTCAGGGCTAGAAGTGTCAT	58	1037
	R: CTCAGGGCTAGAAGTGTCAT		

Multilocus sequence typing (MLST)

Seven housekeeping genes of *K. pneumoniae* (*gapA, mdh, phoE, tonB, infB, pgi*, and *rpoB*) were amplified and sequenced to characterize the genotypes of PLA-causing isolates, according to the protocols (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html/). The alleles and sequence types were assigned according to the online database of the Pasteur Institute MLST for *K. pneumoniae*.

Statistical analysis

All statistical analyses were performed using SPSS 22.0 software (IBM, Armonk, NY, USA). Continuous variables were expressed as means ± SD or median (25th – 75th percentile), whereas categorical variables were described as the number and percentage of subjects. Student's *t*-test or Mann–Whitney *U* test was performed to evaluate the differences in continuous variables. Chi-square test or Fisher's exact test was applied to evaluate the differences in categorical variables. The mortality of *G. mellonella* was assessed by Kaplan–Meier analysis and log-rank test. Statistical tests were performed with a two-tailed significance level of 0.05.

Results

Antimicrobial susceptibility testing and growth curves

Among 12 PLA-causing MDR strains, the resistance rates to cephalosporins (ceftriaxone, ceftazidime, cefepime), quinolones (ciprofloxacin), sulfamethoxazole/trimethoprim, and nitrofurantoin remained high (50–100%). 3/12 strains (FK3038, FK3228, and FK3599) were resistant to carbapenem, and one of it (FK3228) was resistant to colistin. However, these strains were sensitive to aminoglycosides. The typical hypervirulent strains were sensitive to all the tested antibacterial agents with the exception of intrinsic resistance to ampicillin (Table 1). According to the growth curves, there was no significant difference between MDR strains and typical hypervirulent strains and standard strain ($P > 0.05$) (Fig. 1).

String test and quantification of capsule

The string test and quantification of capsule were performed to evaluate the hypermucoviscosity and the capsule of PLA-causing MDR strains. The results of string test revealed that 2/12 (16.6%) MDR strains (FK4176 and FK4737) had hypermucoviscosity, while 5/12 (41.7%) strains (FK3645, FK3736, FK3914, FK3953, and FK3992) had hypermucoviscosity among the typical hypervirulent strains. The quantification of the capsule further revealed that the capsular polysaccharide content of MDR strains was significantly lower than that of typical hypervirulent strains, but was significantly higher than that of ATCC 700603 ($P < 0.05$) (Fig. 2).

Biofilm formation assay

In addition to measuring the capsule, biofilm formation assay was also conducted. The OD values of the biofilms formed by MDR strains ranged from 0.31 to 0.80, with an average value of 0.58 ± 0.19, and the OD values of the biofilms formed by typical hypervirulent strains ranged from 0.06 to 0.39, with an average value of 0.27 ± 0.10. The biofilm formation ability of MDR strains was significantly higher than that of typical hypervirulent strains ($P < 0.05$) (Fig. 3).

Serum killing test

To evaluate the sensitivity of PLA-causing MDR strains to serum, the serum killing test was performed. All MDR strains and typical hypervirulent strains isolated from KP-PLA were found to be susceptible to serum, and no significant differences were detected between the two groups ($P > 0.05$) (Fig. 4).

Infection model of *Galleria mellonella* larvae

To further verify the pathogenicity of the PLA-causing MDR strains, an in vitro model of *G. mellonella* larvae was constructed. The mortality of the larvae depended on the inoculum concentration and action time of the three MDR strains and three typical hypervirulent strains ($P < 0.05$) (Fig. 5A, B, C, D, E, F). In addition, the lethality of MDR strains and typical hypervirulent strains was similar when using 10^6 CFU/mL bacterial suspensions to infect the larvae; however, both were significantly higher than that of the standard strains ATCC 700603 and PBS controls ($P < 0.05$) (Fig. 5G).

Polymerase chain reaction for capsular serotypes and virulence genes

To investigate the virulence genotypes of the PLA-causing MDR strains, capsular serotypes and virulence genes were measured. Among the 12 PLA-causing MDR strains, four were K1 serotype (FK3228, FK3518, FK3599, and FK4737), one was K2 serotype (FK4276), one was K20 serotype (FK4176) and six were of non-type. Except *magA* (33.3%), *iroN* (33.3%) and *kfuBC* (50.0%), all the remaining virulence genes were present in more than half of the MDR strains (*wcaG* 83.3%, *ybtA* 83.3%, *fimH* 91.7%, *mrkD* 100%, *uge* 91.7%, e*ntB* 100%, and *ureA* 83.3%). The prevalence of *rmpA* and *aerobactin* was 83.3% and 75.0%, respectively. The 12 typical hypervirulent strains were K1 or K2 serotype, and all the virulence genes were

present in most of the strains (*aerobactin* 100%, *rmpA* 100%, *fimH* 100%, *mrkD* 100%, *uge* 100%, *ureA* 100%, e*ntB* 91.7%, *wcaG* 83.3%, *magA* 83.3%, *kfuBC* 75.0%, and *ybtA* 75.0%) with the exception for *iroN* (33.3%). Additionally, the prevalence of numerous virulence genes in MDR strains was not significantly different from that of typical hypervirulent strains.

MLST

To assess the molecular epidemiology of the PLA-causing MDR strains, multilocus sequence typing was performed. The clones of the 12 MDR strains were diverse and scattered and categorized into 9 sequence types (ST23, ST11, ST29, ST65, ST86, ST320, ST367, ST420, and ST831). Among the typical hypervirulent strains, the predominant type was ST23 (8/12, 66.7%), followed by ST65 (2/12, 16.7%) and ST86 (2/12, 16.7%) (Fig. 6).

Discussion

As reported previously, the MDR *K. pneumoniae* causes infections in patients with underlying diseases and is considered as cKP with high resistance rate but hypovirulence [2, 12, 14]. However, *K. pneumoniae* isolates from KP-PLA has converged hypervirulence and high antibiotic resistance, which limit the clinical treatment options [28]. To date, little is known about the virulence characteristics of PLA-causing MDR strains. Therefore, 12 MDR *K. pneumoniae* strains were collected from 163 KP-PLA cases, and the virulence characteristics and molecular epidemiology were analyzed. To the best of our knowledge, this is the first study to analyze the virulence characteristics of the PLA-causing MDR strains.

Numerous studies have reported that antibiotic resistance rates are low in KP-PLA [8, 11, 12]. Moreover, the MDR strains were rare, and the patients infected were more likely accompanied by hepatobiliary diseases compared to patients infected with non-MDR strains (Table S1). Importantly, the uncontrollable infections and ineffective prognosis in patients with hepatobiliary diseases might be associated with recurrent bacteremia due to MDR bacteria. This phenomenon suggested that these MDR isolates might not be related to traditional cKP, and acquisition of MDR might not compromise the overall virulence, requiring further verification. However, the actual virulence of these MDR strains has not yet been well-evaluated.

The growth ability results suggestesd no fitness cost regarding the strains with resistant phenotype. In addition, hypermucoviscosity was considered as a surrogate marker of hvKP [5]. In this study, the percentage of hypermucoviscous MDR strains was found to be slightly lower than that of typical hypervirulent strains. However, hypermucoviscosity might not be the only indicator of hypervirulence, wherein the polysaccharide capsule can protect *K. pneumoniae* from phagocytosis by immune cells and complement-mediated bactericidal action, which acts as a major virulence characteristic for hvKP [29]. The results of capsular quantification revealed that the capsular content of PLA-causing MDR strains was higher than that of the standard strain and lower than that of the typical hypervirulent strains. The standard strain ATCC 700603 that recognized as classic *K. pneumoniae* is known for producing extended-spectrum β-lactamase (ESBL) enzymes that can hydrolyze oxyimino-β-lactams, resulting in

resistance to these drugs, and its virulence is less than the typical hypervirulent *K. pneumoniae* [30]. The data of capsular quantification was consistent with the data of the string test. Although the MDR strains and typical hypervirulent strains were sensitive to serum, the antiserum killing ability of these PLA-causing strains was significantly higher than that of the typical hypervirulent strains, which might be related to the content of capsular polysaccharide. Furthermore, the bacteria attaches to the surface of the host during the infectious process and are coated with polymers such as extracellular polysaccharides and DNA to form biofilms. The physical barrier formed by biofilms protect the bacteria from phagocytes and enzymes, improving the bacterial defenses against the host and antimicrobial resistance. This finding indicated that the biofilm formation ability of MDR strains was significantly higher than that of typical hypervirulent strains, which might be one of the reasons for the MDR strains to exhibit resistant phenotype. Moreover, *G. mellonella* larvae, acts as a model of invertebrate host infection, and has been used to explore the virulence and pathogenicity of *K. pneumoniae* strains [31]. Although the siderophore genes were different in these PLA-causing MDR strains and hypervirulent strains, the virulence was assessed in terms of lethality, thereby suggesting that the siderophore transport systems need to be investigated further to clarify the correlation between siderophore utilization and bacterial virulence. The consistency between the clinical data and the results of phenotypic assays supported the theory that the PLA-causing MDR *K. pneumoniae* strains are hypervirulent.

The analysis of virulence genotypes also validated our hypothesis. *K. pneumoniae* strains are presented as 78 capsular serotypes, among which K1 and K2 are related to hvKP, and are strongly pathogenic to humans [29, 32]. In the present study, K1 or K2 serotypes accounted for half of the PLA-causing MDR strains, while all the typical hypervirulent strains belonged to K1 or K2 serotypes. Although K1 or K2 serotypes can regulate the virulence of *K. pneumoniae*, hypervirulence is not unique to these capsular serotypes [33]. In addition, *rmpA* and *aerobactin* are vital genes for hypervirulence [1]. *rmpA* regulates the synthesis of extracellular polysaccharide capsule to enhance the virulence [34–35], and *aerobactin* is essential for the growth and virulence of *K. pneumoniae* by regulating iron supply [1]. In the present study, the prevalence of *rmpA* and *aerobactin* in the MDR strains was slightly lower than that of typical hypervirulent strains, indicating that the PLA-causing MDR strains are combined with hypervirulence. Importantly, *wcaG*, *magA*, and *uge* genes related to capsule synthesis are also prevalent in PLA-causing MDR strains [24, 36]. The inconsistency between the results of the capsule-related genes and hypermucoviscosity suggested that hypermucoviscosity is not the optimal factor for assessing hypervirulence, which should be assessed in conjunction with genotypes, other phenotypes, and clinical characteristics. Moreover, the high prevalence of siderophore genes, such as *ybtA*, *entB*, and *kfuBC* in the PLA-causing MDR strains suggested that the ability of iron uptake might be equivalent to that of typical hypervirulent strains. Furthermore, almost all the PLA-causing MDR strains carried *fimH* (related to type 1 fimbriae), *mrkD* (related to type 3 fimbriae), and *ureA* (an α-subunit of the urease, associated with invasion) [24, 37] and genetically corroborated with the virulence phenotype results. The results of these genes and adherence or invasion in biofilm formation in MDR strains might explain the enhanced resistance of PLA-causing MDR strains to antibiotics compared to the PLA-causing typical hypervirulent

strains. Therefore, clinicians should focus on the MDR strains and select appropriate management strategies to treat KP-PLA to reduce bacterial adhesion and colonization.

MLST analysis uncovered the molecular epidemiology of PLA-causing MDR strains. The clones of these MDR strains were diverse and scattered, while the clones of typical hypervirulent strains almost belonged to ST23, as described previously [38]. ST11-type *K. pneumoniae* is resistant to carbapenems, but not hypervirulent. However, the new ST11-type strain that has emerged in recent years is simultaneously hypervirulent, multidrug resistant, and transmissible, which could pose a serious threat to public health [9, 15]. Previous studies demonstrated that ST11 carbapenem-resistant hypervirulent strains are not found in KP-PLA. To the best of our knowledge, this is the first study to describe that one ST11 carbapenem-resistant strain might be MDR-hypervirulent *K. pneumoniae* and has been described in KP-PLA. Nonetheless, further surveillance and implementation are needed to control the dissemination of infection in hospital settings and community.

Conclusions

Combining the virulence phenotypes and genotypes, the convergence of hypervirulence and multidrug resistance in PLA-causing MDR *K. pneumoniae* strains was observed, which in turn lead to a “post-antibiotic” scenario. It also reminded the clinicians to be prudent in prescribing antibiotics to KP-PLA patients due to severe antibiotic resistance and providing timely inspection measures for hypervirulence-induced invasive infections; supervisors should implement meticulous control measures to prevent such real superbug from further disseminating to patients and hospitals. Nevertheless, further research is needed to elucidate the mechanisms among the host, pathogen, and host-pathogen interactions. This will in turn lay a foundation to raise the awareness regarding MDR-hvKP and provide effective treatments for KP-PLA patients.

Abbreviations

PLA
pyogenic liver abscess; MDR:multidrug resistant; MLST:multilocus sequence typing; cKP:classic *K. pneumoniae*; hvKP:hypervirulent *K. pneumoniae*; KP-PLA:*K. pneumoniae*-induced pyogenic liver abscess; MICs:minimum inhibitory concentrations; PCR:polymerase chain reaction; STs:sequence types; PBS:phosphate-buffered saline

Declarations

Ethics approval and consent to participate

This study has been designed in accordance with the Declaration of Helsinki (2013) (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-

involving-human-subjects/) and been approved by the Ethics Committee in Clinical Research of the First Affiliated Hospital of Wenzhou Medical University (No.2020-070).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by research grants from the National Natural Science Foundation of China (No.81741059 and 81971986) and the Health Department of Zhejiang Province of the People's Republic of China (No. 2019KY098)

Authors' Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published,and agree to be accountable for all aspects of the work.

Acknowledgements

We thank the National Natural Science Foundation of China (No.81741059 and 81971986) and the Health Department of Zhejiang Province of the People's Republic of China (No. 2019KY098) for providing financial funding.

References

1. Russo TA, Marr CM. Hypervirulent *Klebsiella pneumoniae*. *Clin Microbiol Rev*. 2019, 32(3).
2. Siu LK, Yeh KM, Lin JC, Fung CP, Chang FY. *Klebsiella pneumoniae* liver abscess: a new invasive syndrome. Lancet Infect Dis. 2012;12(11):881–7.
3. Liu YM, Li BB, Zhang YY, Zhang W, Shen H, Li H, Cao B. Clinical and molecular characteristics of emerging hypervirulent *Klebsiella pneumoniae* bloodstream infections in mainland China. Antimicrob Agents Chemother. 2014;58(9):5379–85.
4. Tsai FC, Huang YT, Chang LY, Wang JT. Pyogenic liver abscess as endemic disease, Taiwan. Emerg Infect Dis. 2008;14(10):1592–600.

5. Shon AS, Bajwa RP, Russo TA. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: a new and dangerous breed. Virulence. 2013;4(2):107–18.
6. Siu LK, Fung CP, Chang FY, Lee N, Yeh KM, Koh TH, Ip M. Molecular typing and virulence analysis of serotype K1 *Klebsiella pneumoniae* strains isolated from liver abscess patients and stool samples from noninfectious subjects in Hong Kong, Singapore, and Taiwan. J Clin Microbiol. 2011;49(11):3761–5.
7. Chen J, Zhang M, Chen J, Ning Y, Cai X, Zhang L, Xu H, Guo J. Cryptogenic and non-cryptogenic liver abscess: A retrospective analysis of 178 cases revealed distinct characteristics. J Int Med Res. 2018;46(9):3824–36.
8. Kong H, Yu F, Zhang W, Li X. Clinical and microbiological characteristics of pyogenic liver abscess in a tertiary hospital in East China. Med (Baltim). 2017;96(37):e8050.
9. Gu D, Dong N, Zheng Z, Lin D, Huang M, Wang L, Chan EW, Shu L, Yu J, Zhang R, et al. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. Lancet Infect Dis. 2018;18(1):37–46.
10. Yang X, Wai-Chi Chan E, Zhang R, Chen S. A conjugative plasmid that augments virulence in *Klebsiella pneumoniae. Nat Microbiol*. 2019, 4(12):2039–2043.
11. Zhang S, Zhang X, Wu Q, Zheng X, Dong G, Fang R, Zhang Y, Cao J, Zhou T. Clinical, microbiological, and molecular epidemiological characteristics of *Klebsiella pneumoniae*-induced pyogenic liver abscess in southeastern China. Antimicrob Resist Infect Control. 2019;8:166.
12. Jun JB. *Klebsiella pneumoniae* Liver Abscess. Infect Chemother. 2018;50(3):210–8.
13. Liu Y, Liu PP, Wang LH, Wei DD, Wan LG, Zhang W. Capsular Polysaccharide Types and Virulence-Related Traits of Epidemic KPC-Producing *Klebsiella pneumoniae* Isolates in a Chinese University Hospital. Microb Drug Resist. 2017;23(7):901–7.
14. Ye M, Tu J, Jiang J, Bi Y, You W, Zhang Y, Ren J, Zhu T, Cao Z, Yu Z, et al. Clinical and Genomic Analysis of Liver Abscess-Causing *Klebsiella pneumoniae* Identifies New Liver Abscess-Associated Virulence Genes. Front Cell Infect Microbiol. 2016;6:165.
15. Lee CR, Lee JH, Park KS, Jeon JH, Kim YB, Cha CJ, Jeong BC, Lee SH. Antimicrobial Resistance of Hypervirulent *Klebsiella pneumoniae*: Epidemiology, Hypervirulence-Associated Determinants, and Resistance Mechanisms. Front Cell Infect Microbiol. 2017;7:483.
16. Foo NP, Chen KT, Lin HJ, Guo HR. Characteristics of pyogenic liver abscess patients with and without diabetes mellitus. Am J Gastroenterol. 2010;105(2):328–35.
17. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18(3):268–81.
18. Russo TA, Olson R, Fang CT, Stoesser N, Miller M, MacDonald U, Hutson A, Barker JH, La Hoz RM, Johnson JR. Identification of Biomarkers for Differentiation of Hypervirulent *Klebsiella pneumoniae* from Classical K. pneumoniae. *J Clin Microbiol.* 2018, 56(9).

19. Palacios M, Broberg CA, Walker KA, Miller VL. A Serendipitous Mutation Reveals the Severe Virulence Defect of a *Klebsiella pneumoniae* fepB Mutant. *mSphere*. 2017, 2(4).
20. Filisetti-Cozzi TM, Carpita NC. Measurement of uronic acids without interference from neutral sugars. Anal Biochem. 1991;197(1):157–62.
21. Wilksch JJ, Yang J, Clements A *et al*. MrkH, a novel c-di-GMP-dependent transcriptional activator, controls *Klebsiella pneumoniae* biofilm formation by regulating type 3 fimbriae expression. *PLoS Pathog*. 2011, 7(8): e1002204.
22. Zhang X, Zhao Y, Wu Q, Lin J, Fang R, Bi W, Dong G, Li J, Zhang Y, Cao J, et al. Zebrafish and *Galleria mellonella*: Models to Identify the Subsequent Infection and Evaluate the Immunological Differences in Different *Klebsiella pneumoniae* Intestinal Colonization Strains. Front Microbiol. 2019;10:2750.
23. Insua JL, Llobet E, Moranta D, et al. Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella. Infect Immun*. 2013; 81(10): 3552–65.
24. Zhang S, Yang G, Ye Q, Wu Q, Zhang J, Huang Y. Phenotypic and Genotypic Characterization of *Klebsiella pneumoniae* Isolated From Retail Foods in China. Front Microbiol. 2018;9:289.
25. Candan ED, Aksoz N. *Klebsiella pneumoniae*: characteristics of carbapenem resistance and virulence factors. Acta Biochim Pol. 2015;62(4):867–74.
26. Luo Y, Wang Y, Ye L, Yang J. Molecular epidemiology and virulence factors of pyogenic liver abscess causing *Klebsiella pneumoniae* in China. Clin Microbiol Infect. 2014;20(11):O818–24.
27. Wasfi R, Elkhatib WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from Egyptian hospitals. Sci Rep. 2016;6:38929.
28. Su SC, Siu LK, Ma L, Yeh KM, Fung CP, Lin JC, Chang FY. Community-acquired liver abscess caused by serotype K1 *Klebsiella pneumoniae* with CTX-M-15-type extended-spectrum beta-lactamase. Antimicrob Agents Chemother. 2008;52(2):804–5.
29. Fung CP, Chang FY, Lin JC, Ho DM, Chen CT, Chen JH, Yeh KM, Chen TL, Lin YT, Siu LK. Immune response and pathophysiological features of *Klebsiella pneumoniae* liver abscesses in an animal model. Lab Invest. 2011;91(7):1029–39.
30. Elliott AG, Ganesamoorthy D, Coin L, et al. Complete Genome Sequence of Klebsiella quasipneumoniae subsp.similipneumoniae Strain ATCC 700603. Genome Announc. 2016;26(3):e00438-16. 4(.
31. McLaughlin MM, Advincula MR, Malczynski M, et al. Quantifying the clinical virulence of *Klebsiella pneumoniae* producing carbapenemase *Klebsiella pneumoniae* with a *Galleria mellonella* model and a pilot study to translate to patient outcomes. BMC Infect Dis. 2014;14:31.
32. Podschun R, Ullmann U. *Klebsiella spp.* as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev. 1998;11(4):589–603.
33. Yu WL, Ko WC, Cheng KC, Lee CC, Lai CC, Chuang YC. Comparison of prevalence of virulence factors for *Klebsiella pneumoniae* liver abscesses between isolates with capsular K1/K2 and non-K1/K2 serotypes. Diagn Microbiol Infect Dis. 2008;62(1):1–6.

34. Yu WL, Ko WC, Cheng KC, Lee HC, Ke DS, Lee CC, Fung CP, Chuang YC. Association between *rmpA* and *magA* genes and clinical syndromes caused by *Klebsiella pneumoniae* in Taiwan. Clin Infect Dis. 2006;42(10):1351–8.
35. Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: Going on the Offense with a Strong Defense. Microbiol Mol Biol Rev. 2016;80(3):629–61.
36. Yeh KM, Lin JC, Yin FY, Fung CP, Hung HC, Siu LK, Chang FY. Revisiting the importance of virulence determinant *magA* and its surrounding genes in *Klebsiella pneumoniae* causing pyogenic liver abscesses: exact role in serotype K1 capsule formation. J Infect Dis. 2010;201(8):1259–67.
37. Struve C, Bojer M, Krogfelt KA. Identification of a conserved chromosomal region encoding *Klebsiella pneumoniae* type 1 and type 3 fimbriae and assessment of the role of fimbriae in pathogenicity. Infect Immun. 2009;77(11):5016–24.
38. Lam MMC, Wyres KL, Duchene S, Wick RR, Judd LM, Gan YH, Hoh CH, Archuleta S, Molton JS, Kalimuddin S, et al. Population genomics of hypervirulent *Klebsiella pneumoniae* clonal-group 23 reveals early emergence and rapid global dissemination. Nat Commun. 2018;9(1):2703.

Figures

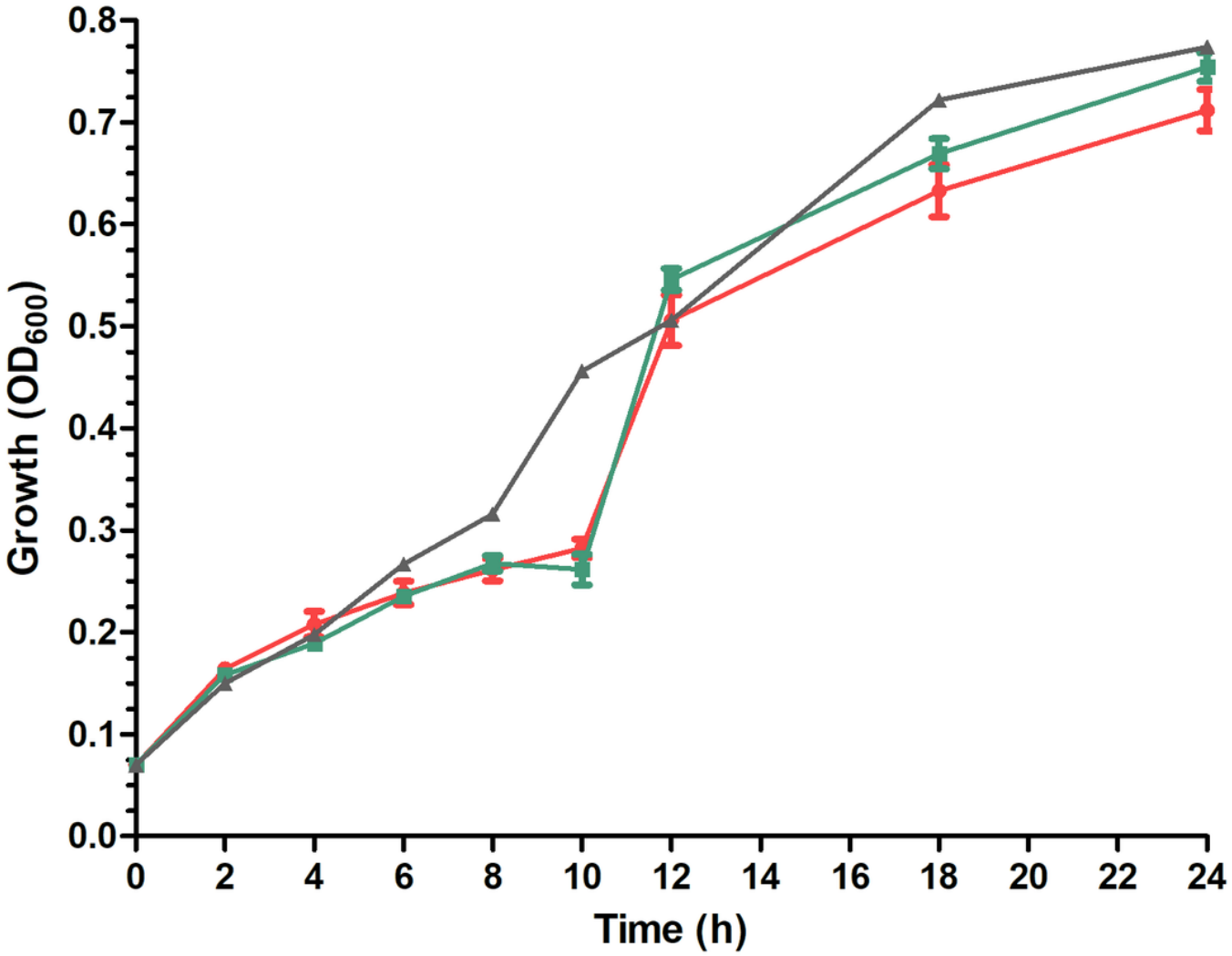

Figure 1

Growth curves of PLA-causing MDR K. pneumoniae strains (red circles, n=12) were comparable to those of typical hypervirulent strains (green squares, n=12) and standard strain ATCC 700603 (grey triangles, n=1). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student's t-test, No significant difference was noted between MDR and control strains (typical hypervirulent strains and standard strain ATCC 700603), ($P > 0.05$).

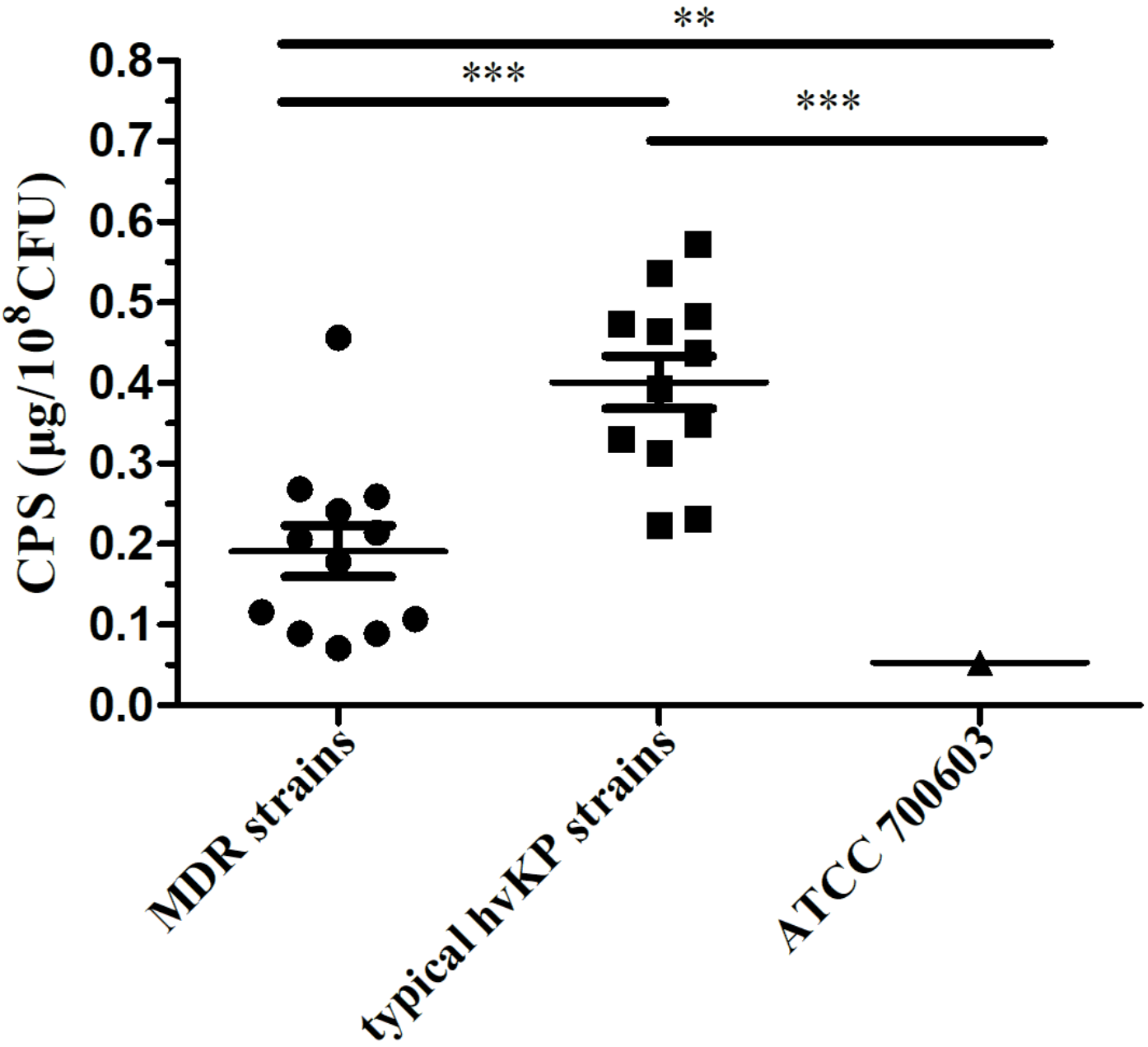

Figure 2

Capsular quantification of PLA-causing MDR K. pneumoniae strains (black circles, n=12) were comparable to those of typical hypervirulent strains (black squares, n=12) and standard strain ATCC 700603 (black triangles, n=1). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

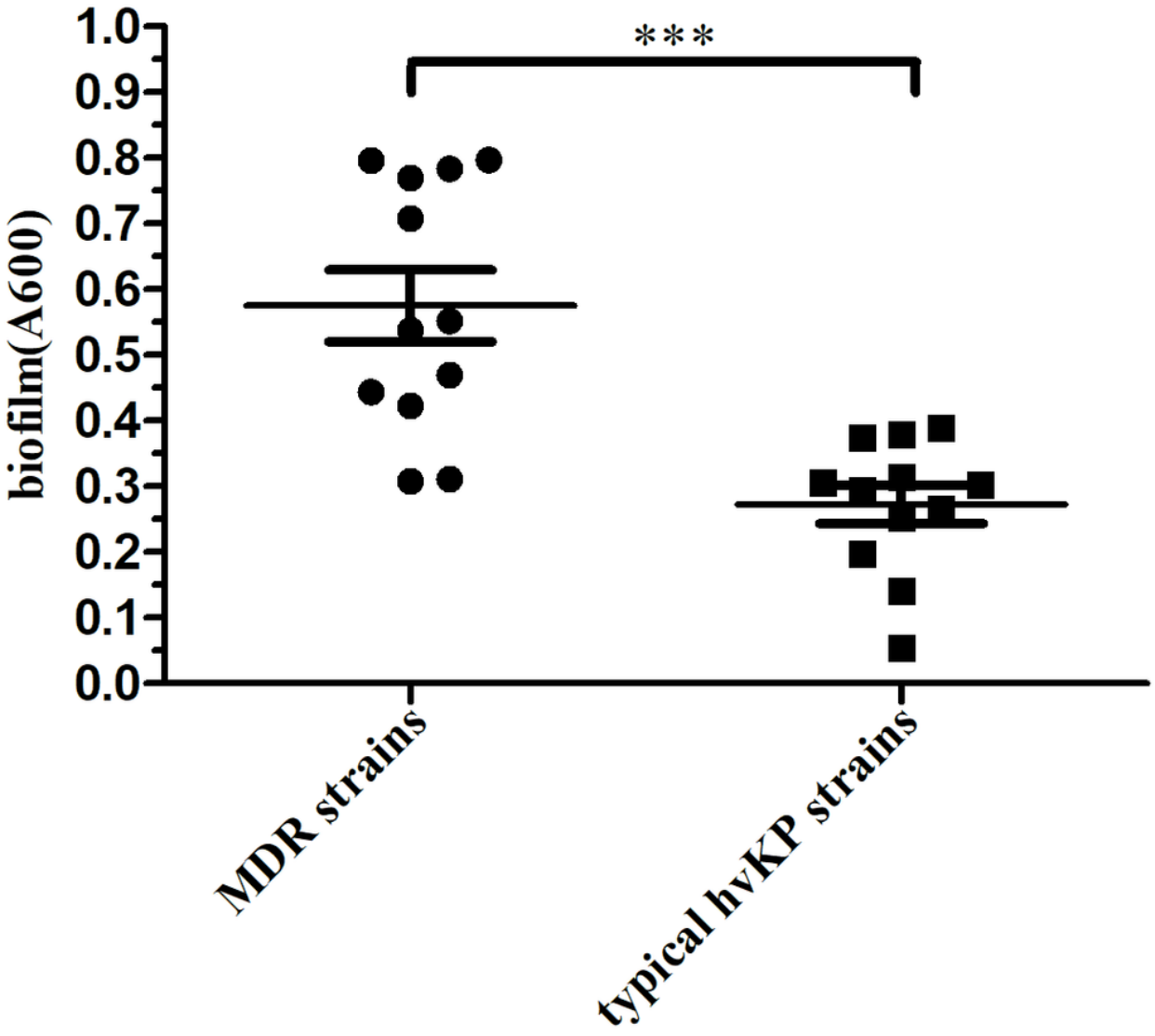

Figure 3

Biofilm formation ability of PLA-causing MDR K. pneumoniae strains (black circles, n=12) were comparable to those of typical hypervirulent strains (black squares, n=12). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student's t-test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

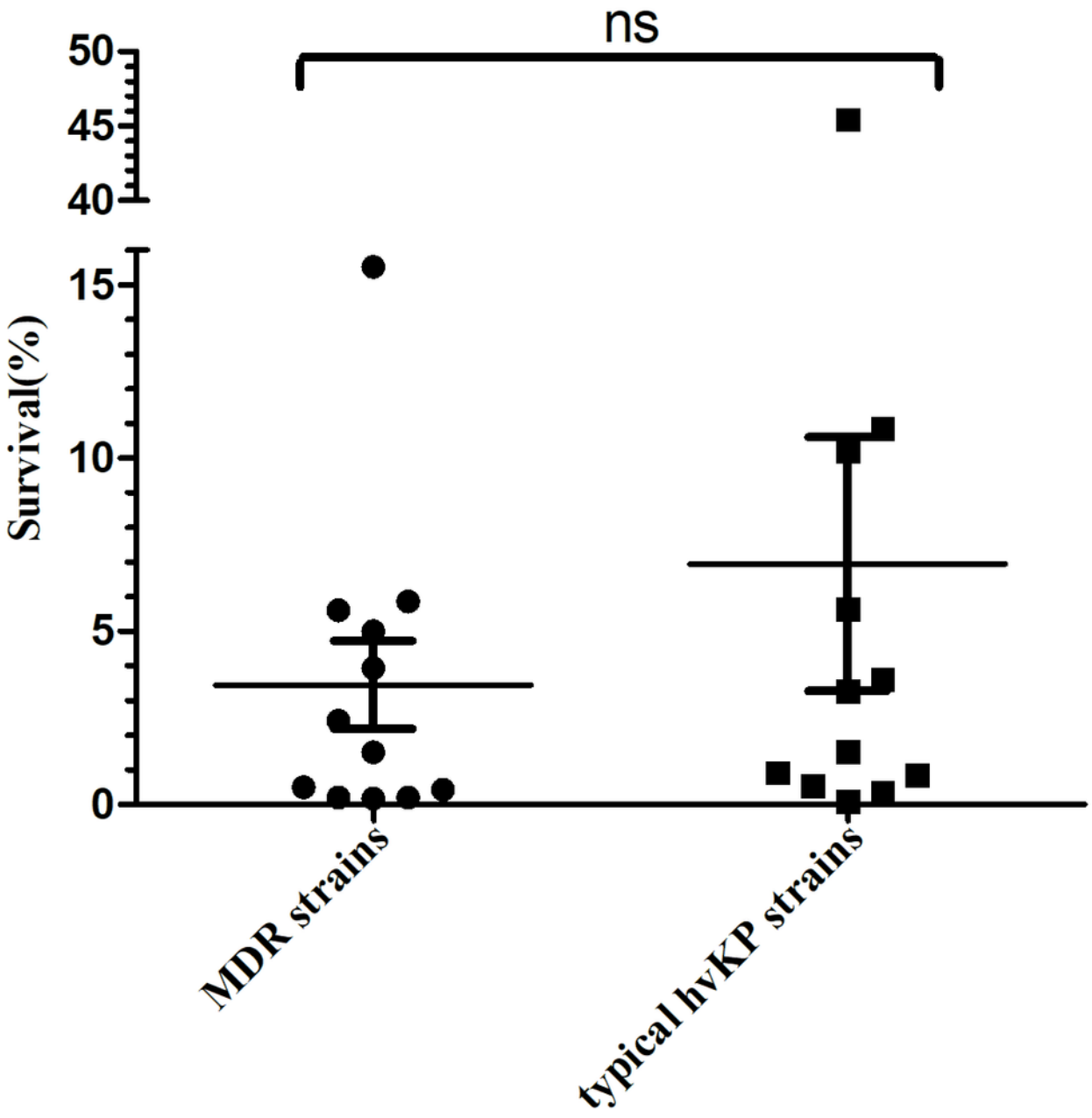

Figure 4

Serum bactericidal activity of PLA-causing MDR K. pneumoniae strains (black circles, n=12) were comparable to those of typical hypervirulent strains (black squares, n=12). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student's t-test. ns, not significant.

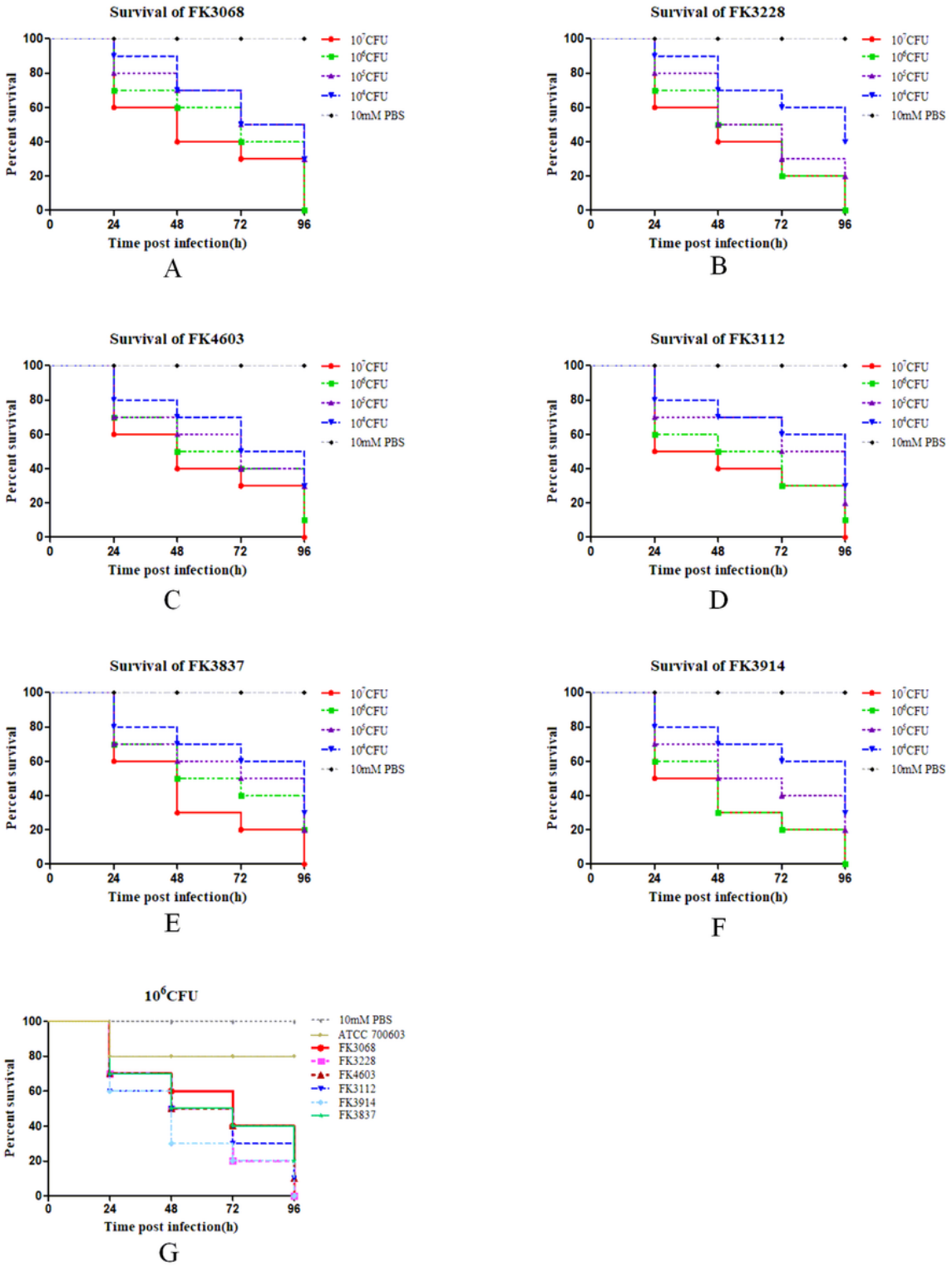

Figure 5

Survival curves of K. pneumoniae infection model of Galleria mellonella larvae. (A) FK3068, MDR strain; (B) FK3228, MDR strain; (C) FK4603, MDR strain; (D) FK3112, typical hypervirulent strain; (E) FK3837, typical hypervirulent strain; (F) FK3914, typical hypervirulent strain; (G) 106 CFU/mL bacterial suspensions of MDR strains (FK3068, FK3228, FK4603), typical hypervirulent strains (FK3112, FK3837,

FK3914), a standard strain ATCC 700603, and a blank control PBS. Data is presented as percentage, with N=3. Statistical analysis was performed using Kaplan-Meier analysis and log-rank test.

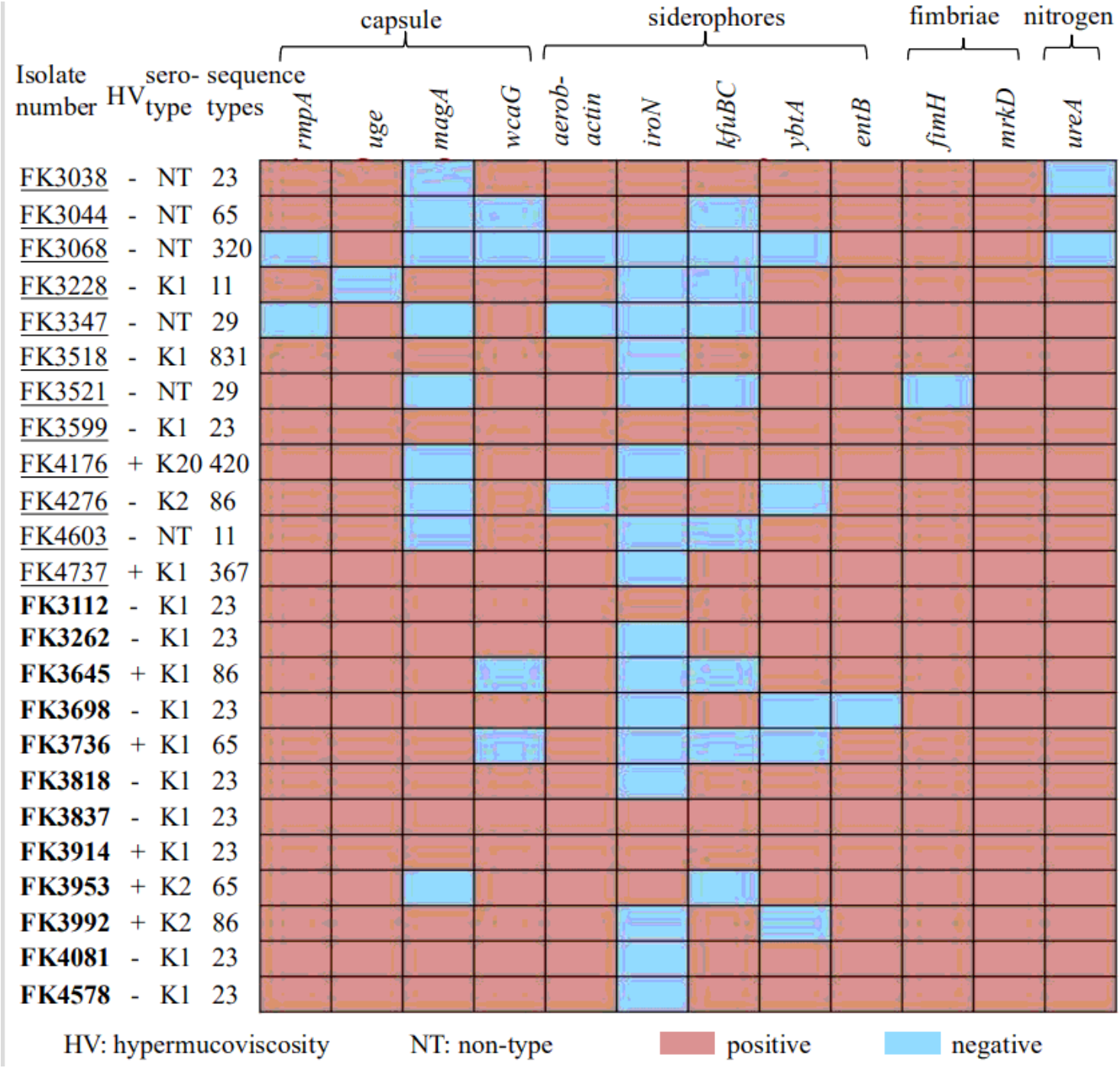

Figure 6

Genotype map of PLA-causing MDR and typical hypervirulent strains. Strain numbers were underlined: multidrug resistant strains; strain numbers were bolded: typical hypervirulent strains.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.docx