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

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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*), and this might be due to that these strains are rare. 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.

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 or even more obvious than those of typical hypervirulent *K. pneumoniae* strains. These MDR strains were mainly non-K1/K2 serotypes and carried multiple virulence genes. The results of multilocus sequence typing (MLST) illustrated that the MDR strains were categorized into 9 sequence types.

Conclusions: This study is the first to analyze the virulence characteristics in PLA-causing MDR strains. Our data exhibited the coexistence of hypervirulence and MDR in PLA-causing MDR *K. pneumoniae* strains, and the clones of those PLA-causing MDR strains were diverse and scattered. This study first found one ST11 carbapenem-resistant hypervirulent strain in PLA.

Background

Klebsiella pneumoniae is a major Gram-negative bacterium that is responsible for urinary tract infections, pneumonia, bacteremia, and intra-abdominal infections worldwide [1-2]. In the past three decades, *K. pneumoniae* is divided into two non-overlapping populations. One is termed as classic *K. pneumoniae* (cKP), which is 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 that occurs worldwide [2,4,5]. *Klebsiella pneumoniae* has been emerged as a predominant pathogen of PLA across Asian and European countries, as well as the United States. There is no denying that *K. pneumoniae*-induced pyogenic liver abscess (KP-PLA) is a serious clinical challenge due to its association with mortality [5-6]. Hypervirulent *K. pneumoniae*-induced PLA usually occurs in young and healthy community individuals with no 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]. Most of the isolates from KP-PLA are susceptible to majority of antibiotics, and the antibiotic resistance rates are less than 10% [8]. Antibiotic resistance is a phenomenon that is primarily associated with cKP. Interestingly, most of the recent reports have 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 been well identified yet, particularly with regard to virulence characteristics and molecular epidemiology [2,7,12]. Whether these MDR *K. pneumoniae* isolates were indeed traditional cKP or combined with hypervirulence still remains to be unknown. 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* will pose more challenges in treating KP-PLA. In addition, hypervirulent strains usually possesses thick capsular polysaccharide, anti-serum capacity and multiple virulence factors (hypermucoviscosity, capsular serotype, virulence genes, related clones and so on) [14,15]. Therefore, it is necessary to gain knowledge with regard to virulence characteristics and molecular epidemiology in the PLA-causing MDR *K. pneumoniae* urgently.

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

Results

Antimicrobial susceptibility testing and growth curves

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 two (2/12, 16.6%) MDR strains (FK4176 and FK4737) had hypermucoviscosity, while five (5/12, 41.7%) strains (FK3645, FK3736, FK3914, FK3953, and FK3992) had hypermucoviscosity among the typical hypervirulent strains. 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, and the differences were statistically significant ($P < 0.05$), (Figure 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 ($P < 0.05$), (Figure 3).

Serum killing test

To evaluate the sensitivity of the 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 there were no significant differences between the two groups ($P > 0.05$), (Figure 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 ($P < 0.05$) (Figure 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, but both were significantly higher than that of the standard strains ATCC 700603 and PBS controls ($P < 0.05$), (Figure 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 with K1 serotype (FK3228, FK3518, FK3599, and FK4737), one was with K2 serotype (FK4276), one was with 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. Among the 12 typical hypervirulent strains, all were of K1 or K2 serotype, and all 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 showed no significant differences with that of typical hypervirulent strains.

Multilocus sequence typing

To conduct 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 were 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%), (Figure 6).

Discussion

As reported previously, the MDR *K. pneumoniae* usually 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 limited the clinical treatment options largely [16]. To date, little is known with regard to the virulence characteristics of PLA-causing MDR strains. Therefore, 12 MDR *K. pneumoniae* strains were collected from 163 KP-PLA cases and analyzed the virulence characteristics and molecular epidemiology. 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 remained lower in KP-PLA [8,11,12]. Moreover, the MDR strains were rare, and patients infected with these were more likely accompanied with hepatobiliary diseases when compared to patients infected with non-MDR strains (Table S1). More importantly, the uncontrollable infections and ineffective prognosis in patients with hepatobiliary diseases might be associated with recurrent bacteremia due to MDR bacteria. This 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 been well evaluated so far.

The growth ability results suggested no fitness cost regarding the strains with resistant phenotype. In addition, the 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, it might not be suitable to consider hypermucoviscosity as the only indicator of hypervirulence, wherein the polysaccharide capsule can protect *K. pneumoniae* from phagocytosis of immune cells and complement-mediated bactericidal action, which acts as a major virulence characteristic for hvKP [17]. 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* [18]. 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 can protect the bacteria from phagocytes and enzymes, improving the bacterial defenses against the host and antimicrobial resistance. This meant 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, which acts as a good model of invertebrate host infection, has been used to explore the virulence and pathogenicity of *K. pneumoniae* strains [19]. By combining with the results of virulence genes, although the siderophore genes were different in these PLA-causing MDR strains and typical hypervirulent strains, these strains still had comparable level of virulence in terms of lethality. This showed that the siderophore transport systems require further research to clarify the relationship between siderophore utilization and bacterial virulence. The

consistency between the clinical data and the results of phenotypic assays supported the notion that the PLA-causing MDR *K. pneumoniae* strains were hypervirulent.

Analysis of virulence genotypes can further validate our hypothesis. *K. pneumoniae* strains are presented in at least 78 capsular serotypes, in which K1 and K2 are related to hvKP, and are strongly pathogenic to humans [17,20]. In the present study, K1 or K2 serotypes accounted for less than 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 [21]. In addition, *rmpA* and *aerobactin* are the most important genes for hypervirulence [1]. *rmpA* regulates the synthesis of extracellular polysaccharide capsule in order to enhance the virulence [22-23], and *aerobactin* is considered 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, reflecting that the PLA-causing MDR strains might be combined with hypervirulence from the perspective of virulence genes. More importantly, *wcaG*, *magA*, and *uge* genes related to capsule synthesis were also prevalent in the PLA-causing MDR strains [24-25]. Additionally, the inconsistency between the results of the capsule-related genes and hypermucoviscosity supported the notion that hypermucoviscosity is not a good factor for assessing hypervirulence and should be assessed in conjunction with genotypes and other phenotypes, as well as 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) [24,26], and genetically corroborated with virulence phenotype results. The results of these genes and adherence or invasion in biofilm formation in MDR strains might explain as to why the PLA-causing MDR strains were more resistant to antibiotics than the PLA-causing typical hypervirulent strains. Therefore, clinicians should pay much attention to the MDR strains, and also carefully 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, and this was consistent with that reported in the previous study [27]. It is commonly known that ST11-type *K. pneumoniae* is resistant to carbapenems, but not hypervirulent. However, the new ST11-type strain that has been emerged in recent years is simultaneously hypervirulent, multidrug resistant, and transmissible, and this could pose a serious threat to the public health [9,15]. Previous studies have revealed that ST11 carbapenem-resistant hypervirulent strains were 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. Importantly, further surveillance and implementation are needed to control the dissemination of infection in hospital settings and community.

Conclusion

Combining the virulence phenotypes and genotypes, the convergence of hypervirulence and multidrug resistance in PLA-causing MDR *K. pneumoniae* strains was observed, which might in turn lead to further emergence of “post-antibiotic” scenario. Importantly, it reminded that clinicians should be highly prudent in prescribing antibiotics to KP-PLA patients due to severe antibiotic resistance and provide inspection measures timely in view of hypervirulence-induced invasive infections, and supervisors should implement meticulous control measures to prevent such real superbug from further disseminating to patients and hospitals. Moreover, 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.

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. The diagnosis of KP-PLA was conducted based on the clinical criteria [7,28]. 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) initially. MDR strains were defined as non-susceptible to three or more different antimicrobial categories [29]. A total of 12 MDR *K. pneumoniae* were found in 163 KP-PLA cases. Meanwhile, 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) [1,30].

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 results 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 done in triplicate.

The growth curves of 12 PLA-causing MDR *K. pneumoniae* isolates were measured as described by the previous methods [31]. In brief, 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 done 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 >5mm length was generated by the strain, and this was also considered hypermucoviscous [30].

The capsule was quantified as described previously with some modifications [10,32]. Briefly, 500μL of cultured bacterial suspensions were resuspended and adjusted to 10^8 CFU/mL, and 1.2 mL sodium tetraborate was added into sulfuric acid in the resuspensions that were placed in ice bath and incubated for 5min at 100°C, and then kept on ice for 10min. A 20 μL volume of 1.5 mg/mL m-hydroxyphenyl was then added and mixed. After incubating for 5min at room temperature, the absorbance was measured at 590nm. 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. [33]. 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 polystyrene microtiter plates, with three duplicate wells per strain, as well as blank controls were set at the same time. 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. 25 μL of bacterial suspension was added to 75μL of pooled human sera in the tube. Next, the tubes were shaken and incubated for 0, 1, 2, or 3 h. An aliquot of each bacterial suspension was removed at the designated time point, diluted to 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 results that were 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, FK4603) and three typical hypervirulent strains (FK3112, FK3837, FK3914) that were randomly selected and standard strain ATCC 700603 to investigate the virulence and pathogenicity of the strains [34-35]. Serially diluted bacterial suspension of each strain (10^7,10^6,10^5,10^4 CFU/mL) was prepared in advance. Eight larvae weighing 200mg-250mg were randomly selected for each strain and each concentration. A 10 μL of bacterial suspension was injected into the last left proleg by using a 25 μL Hamilton precision syringe. The larvae injected with 10 μL phosphate-buffered saline were used as controls. After that, the insects were incubated at 37°C in the dark and observed after 24 h, 48 h, 72 h and 96 h. The larvae were considered dead when they repeatedly fail to respond to physical stimuli. All experiments were done 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) [24,36-38]. In addition, the strains with these genes that were determined by PCR and DNA sequencing were selected as positive controls for subsequent PCR experiments.

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 provided 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.

List Of 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

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.

Availability of data and materials

The datasets used and analyzed during the study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Author 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.

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Tables

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

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

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		

Figures

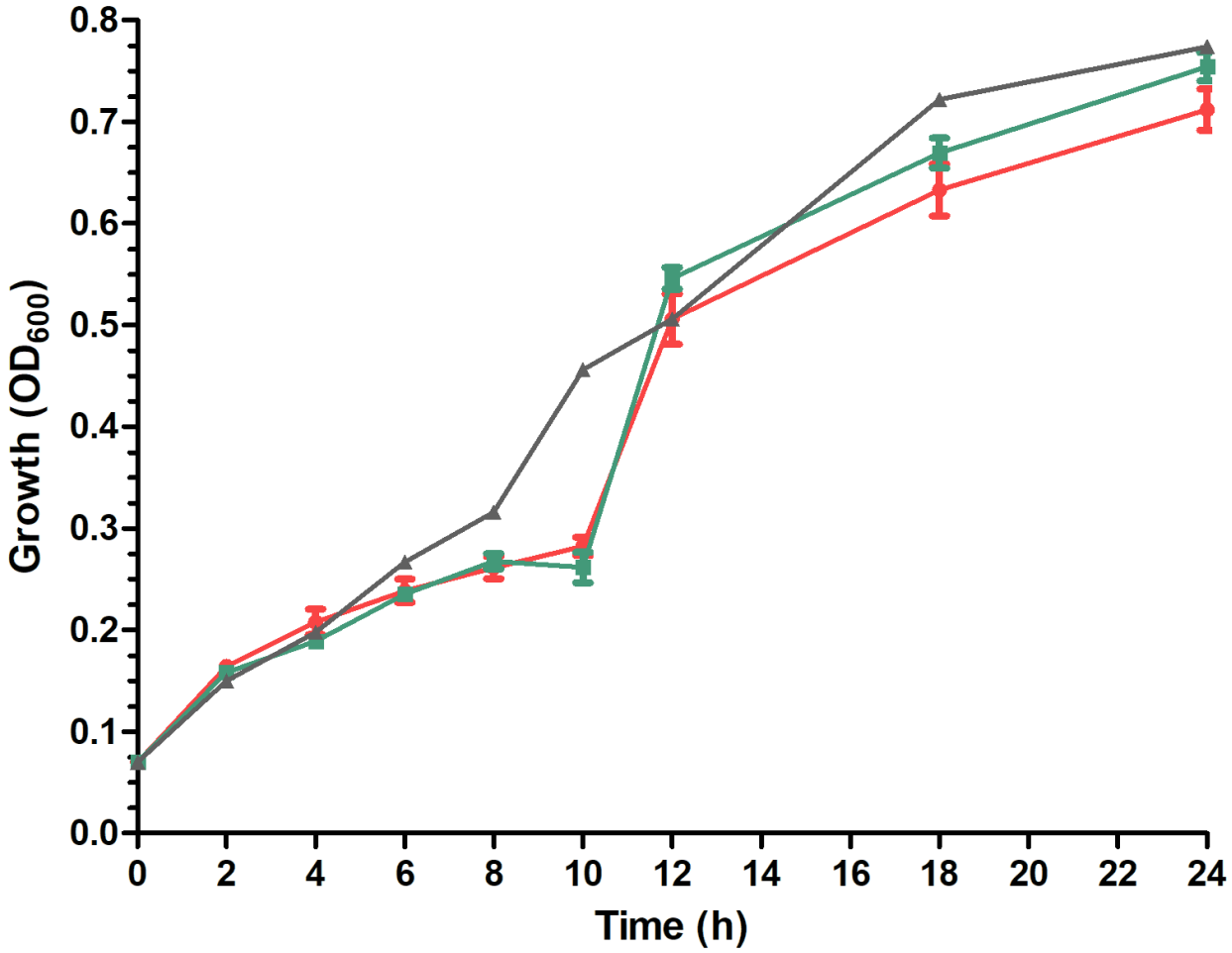

Figure 1

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

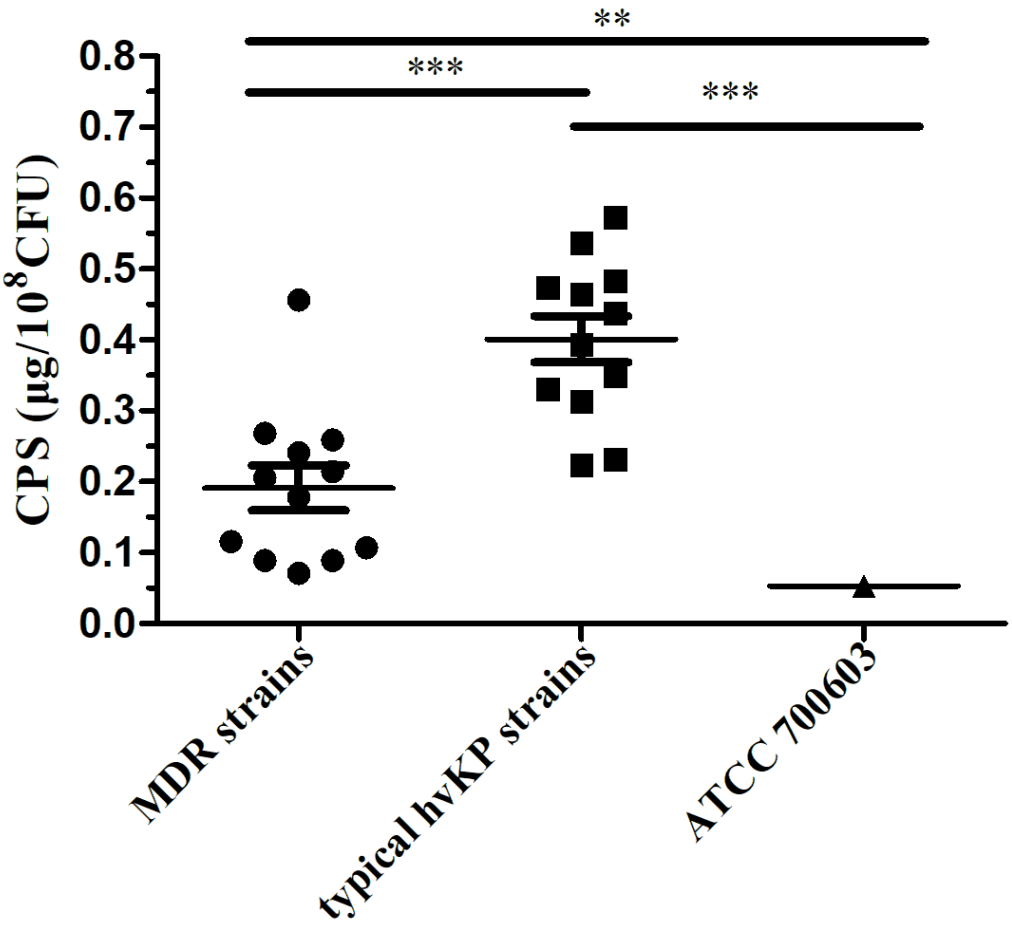

Figure 2

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

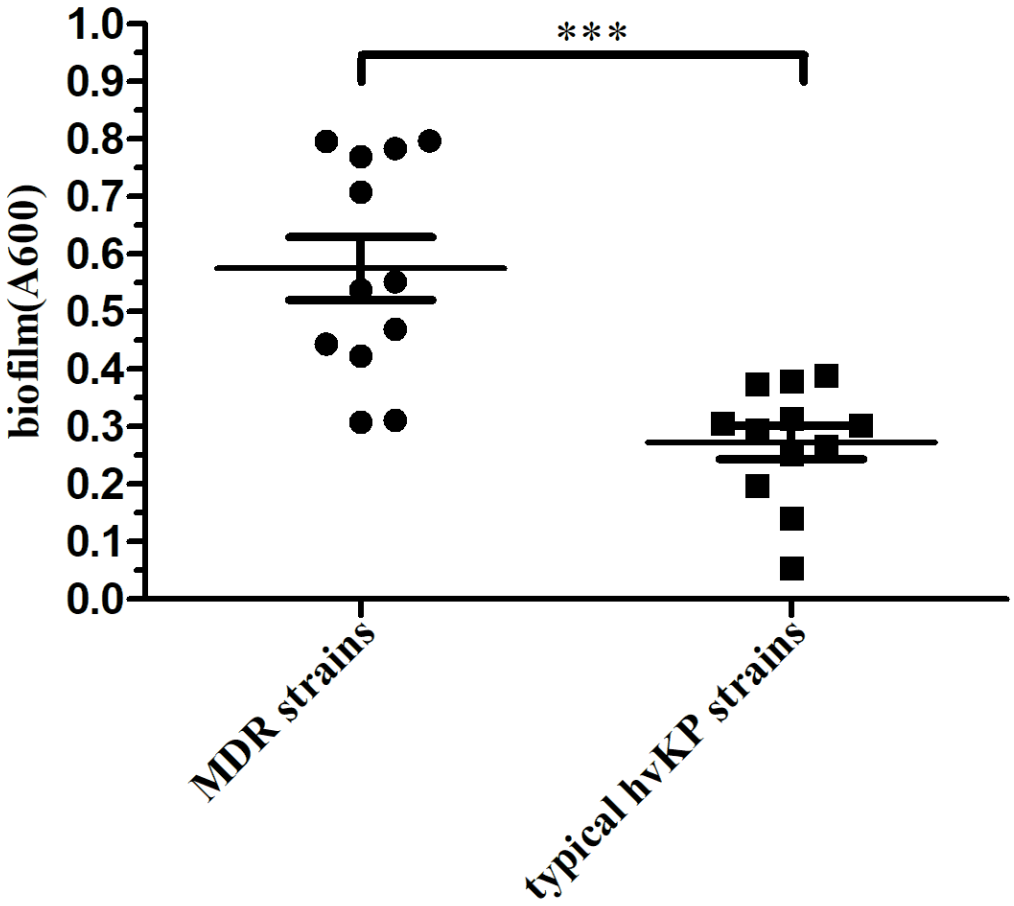

Figure 3

Biofilm formation ability of PLA-causing MDR K. pneumoniae strains (black circles, n=12) were comparable to that of typical hypervirulent strains (black squares, n=12). Data is 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.

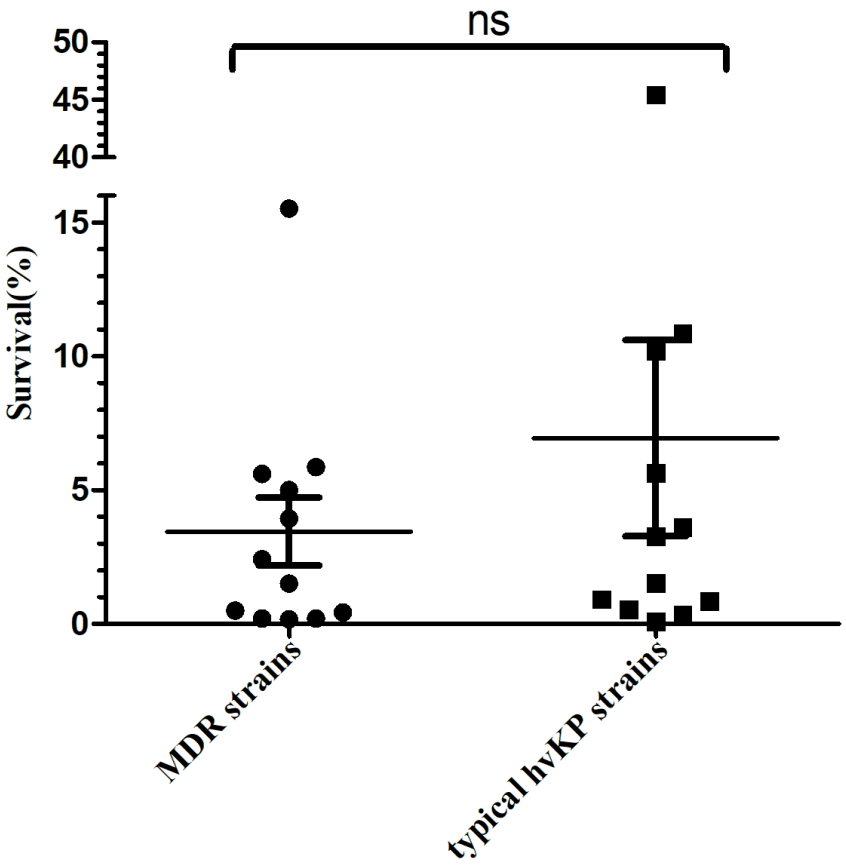

Figure 4

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

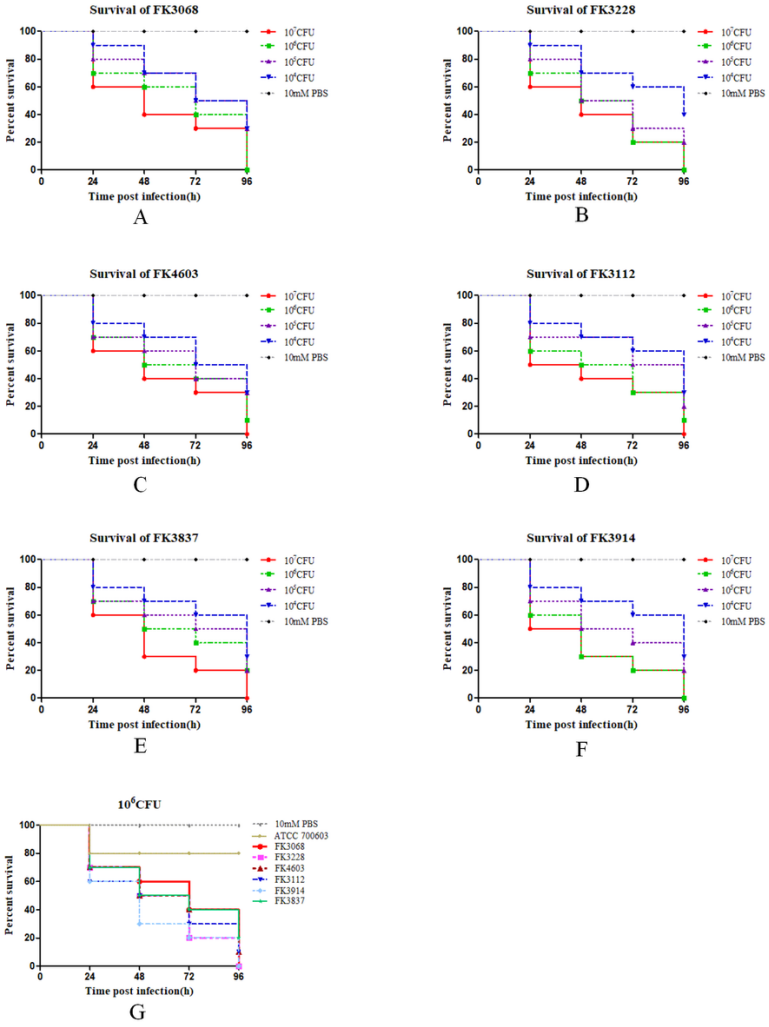

Figure 5

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.

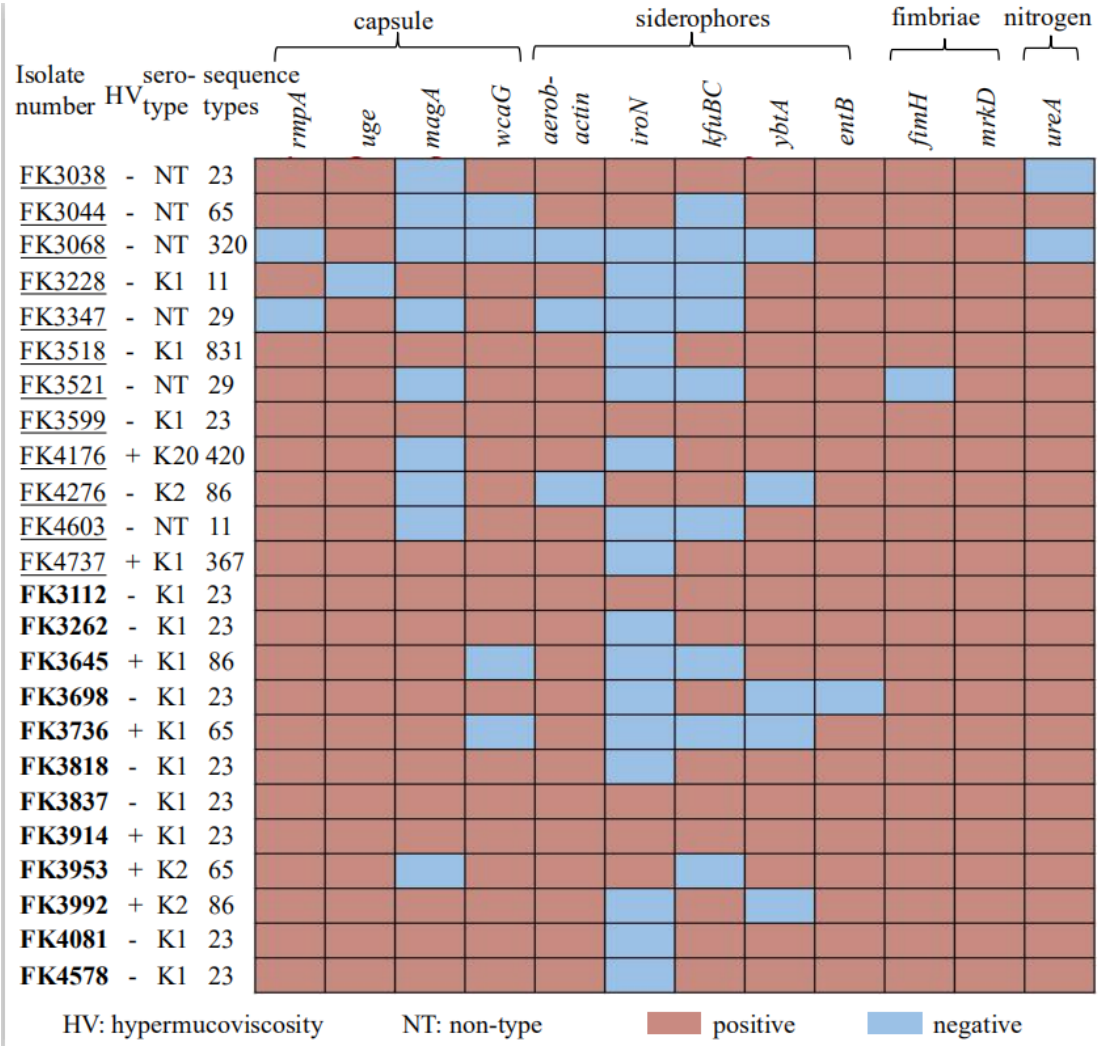

Figure 6

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

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- TableS1.docx