

# Virulence Factors 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

**Jianming Cao**

Wenzhou Medical University

**Tieli Zhou** (✉ [wyztli@163.com](mailto:wyztli@163.com))

Wenzhou Medical University First Affiliated Hospital <https://orcid.org/0000-0002-2171-4710>

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## Research article

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# Abstract

**Background:** To date, little is known about virulence characteristics of pyogenic liver abscess (PLA) causing multidrug resistant (MDR) *Klebsiella pneumoniae* (*K. pneumoniae*). It may be that these strains are rare. The aim of this study was to analyze the virulence characteristics and molecular epidemiology of 12 MDR strains from 163 PLA cases in a tertiary teaching hospital from the perspective of clinical characteristics, virulence phenotypes and genotypes.

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

**Conclusions:** This study is the first analysis of the virulence factors in PLA-causing MDR strains. Our data exhibited the coexistence of hypervirulence and multidrug resistance in PLA-causing MDR *K. pneumoniae* strains, and the clones of those PLA-causing MDR strains were diverse and scattered. The study was firstly found one ST11 carbapenem-resistant hypervirulent strain in PLA.

## Background

*Klebsiella pneumoniae* has emerged as a major Gram-negative bacterium for urinary tract infections, pneumonia, bacteremia, and intra-abdominal infections worldwide [1-2]. During the past three decades, *K. pneumoniae* has been divided into two largely non-overlapping populations. One is termed classic *K. pneumoniae* (cKP), which is considered as the pathogen causing hospital-acquired infections in immunocompromised patients and is notorious for acquiring antimicrobial resistance, and the other is hypervirulent *K. pneumoniae* (hvKP) [1-3]. In contrast to cKP, the emerging variant, which was first reported in Taiwan in 1986, is hypervirulent for causing severe invasive community-acquired infections and disseminates infections among immunocompetent individuals. In addition, hvKP exhibits hypervirulent phenotypes and genotypes, and is susceptible to conventional antimicrobial agents except for being intrinsically resistant to ampicillin [1-3].

Pyogenic liver abscess (PLA) is a potentially life-threatening suppurating infection of hepatic parenchyma disease which occurs worldwide [2,4,5]. *Klebsiella pneumoniae* has emerged as a predominant pathogen of PLA across Asian and European countries, as well as the United States, and there is no denying that *K. pneumoniae*-induced pyogenic liver abscess (KP-PLA) remains a mortality-associated serious clinical challenge [5-6]. Hypervirulent *K. pneumoniae*-induced PLA usually occurs in young and healthy community individuals with cryptogenic abscess in which no source of infection can be identified, and then, migrates to distant sites, leading to extrahepatic complications, such as endophthalmitis, meningitis, and necrotizing fasciitis [2,5,7]. Most of isolates from KP-PLA are

susceptible to the majority of antibiotics, and the antibiotic resistance rates are less than 10% [8]. Antibiotic resistance is a phenomenon associated primarily with cKP. Alarming, recent reports revealed the convergence of virulence and resistance in *K. pneumoniae*, 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 tended to be 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 in virulence characteristics and molecular epidemiology [2,7,12]. Whether these MDR *K. pneumoniae* isolates were indeed traditional cKP or combined with hypervirulence is unknown. Since the virulence of *K. pneumoniae* can assist pathogen to resist host innate immunity and infect the host invasively with high pathogenicity [1,13], the convergence of virulence and resistance in *K. pneumoniae* will pose more challenges in therapy of KP-PLA, gaining knowledge of virulence and molecular epidemiological characteristics in PLA-causing multidrug resistant *K. pneumoniae* has become more urgent. In addition, hypervirulent strains usually possess thick capsular polysaccharide, anti-serum capacity and multiple virulence factors (hypermucoviscosity, capsular serotype, virulence genes, related clones and so on) [14,15].

The purpose of the present study was to investigate the virulence factors and molecular epidemiology of PLA-causing multidrug resistant *K. pneumoniae*, by collecting strains over a 2-year period from KP-PLA patients in a tertiary teaching hospital, in order to provide significant insights for the development of further effective therapeutic strategies for clinical trials of KP-PLA.

## Results

### Antimicrobial susceptibility testing

Among 12 PLA-causing MDR strains, the resistance rates to cephalosporins (ceftriaxone, ceftazidime, cefepime), quinolones (ciprofloxacin), sulfamethoxazole/trimethoprim and nitrofurantoin were high (50% -100%). Three of the strains were resistant to carbapenem, one of which was resistant to colistin, but these strains were more sensitive to aminoglycosides. However, the typical hypervirulent strains were sensitive to all tested antibacterials with the exception for being intrinsically resistant to ampicillin (Table 1).

Table 1

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

Strain numbers	MICs ( $\mu\text{g/mL}$ , $\text{mg/L}$ )													
	AMP	ATM	CRO	CAZ	FEP	IPM	CIP	LEV	GEN	TOB	SXT	NIT	COL	
FK3038	> 64	2	> 64	16	4	> 64	> 8	1	2	1	< 0.25/4.75	128	0.5	
FK3044	32	16	> 64	> 64	32	1	0.03	2	0.125	4	> 16/304	128	1	
FK3068	32	16	> 64	16	32	0.5	0.03	2	0.5	4	> 16/304	128	0.25	
FK3228	32	> 64	> 64	> 64	> 32	4	0.06	> 16	0.125	4	> 16/304	256	16	
FK3347	32	2	4	8	> 32	1	0.5	> 16	0.5	4	> 16/304	256	0.25	
FK3518	> 64	2	> 64	16	2	1	> 8	1	0.5	0.5	1/19	256	0.25	
FK3521	> 64	2	> 64	16	16	1	> 8	4	0.5	< 0.25	< 0.25/4.75	128	0.25	
FK3599	32	2	4	2	0.25	4	8	1	0.5	< 0.25	< 0.25/4.75	256	0.25	
FK4176	> 64	2	64	8	16	0.5	2	4	16	0.5	< 0.25/4.75	128	0.5	
FK4276	> 64	2	> 64	32	16	1	4	1	16	< 0.25	< 0.25/4.75	32	1	
FK4603	32	> 64	64	64	> 32	1	4	> 16	0.25	2	> 16/304	256	0.25	
FK4737	32	32	> 64	32	8	2	4	4	0.5	4	> 16/304	128	0.125	
<b>FK3112</b>	32	1	< 0.03	0.125	< 0.03	0.125	0.03	2	0.25	1	0.5/9.5	32	0.5	
<b>FK3262</b>	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	
<b>FK3645</b>	> 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	
<b>FK3698</b>	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	
<b>FK3736</b>	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	

Table 1

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

Strain numbers	MICs ( $\mu\text{g/mL}$ , $\text{mg/L}$ )												
	AMP	ATM	CRO	CAZ	FEP	IPM	CIP	LEV	GEN	TOB	SXT	NIT	COL
<b>FK3818</b>	32	1	< 0.03	0.25	< 0.03	0.25	0.5	0.25	0.25	0.5	< 0.25/4.75	32	1
<b>FK3837</b>	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
<b>FK3914</b>	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
<b>FK3953</b>	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
<b>FK3992</b>	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
<b>FK4081</b>	> 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
<b>FK4578</b>	> 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

### String test and quantification of capsule

String test disclosed that two (2/12, 16.6%) MDR strains had hypermucoviscosity, while five (5/12, 41.7%) strains had hypermucoviscosity among the hypervirulent control strains. Quantification of capsule further revealed the capsular polysaccharide content of MDR strains was significantly lower than that of hypervirulent control strains, but significantly higher than ATCC 700603, and the differences were statistically significant ( $P < 0.05$ ) (Figure 2).

### Serum killing test

All MDR strains and hypervirulent control strains isolated from KP-PLA were susceptible to serum. The anti-serum ability was no significant difference between the two groups ( $P > 0.05$ ) (Figure 3).

## Biofilm formation assay

As shown in Figure 4, the OD values of biofilms formed by MDR strains ranged from 0.31 to 0.80, with an average value of  $0.58 \pm 0.19$ ; while the OD values of biofilms formed by hypervirulent control strains ranged from 0.06 to 0.39, with an average value of  $0.27 \pm 0.10$ . The biofilm formation ability of MDR strains was significantly higher than that of hypervirulent control strains ( $P < 0.05$ ).

## Infection model of *Galleria mellonella* larvae

As shown in Figure 5, mortality of larvae depended on inoculation concentration and action time of the three MDR strains and three hypervirulent control strains ( $P < 0.05$ ) (Figure 5A, B, C, D, E, F). In addition, the lethality of MDR strains and hypervirulent control strains was similar when using  $10^6$  CFU/mL bacterial suspensions to infect 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

As shown in Figure 6, among 12 PLA-causing MDR strains, there were four of K1 serotype, one of K2 serotype, one of K20 serotype and six of non-type. Except for *magA*, *iroN* and *kfuBC* (ranging from 33.3% to 50.0%), all remaining virulence genes were presented in more than half of MDR strains (ranging from 75.0% to 100%). The prevalence of *rmpA* and *aerobactin* was 83.3% and 85.3%, respectively. Among 12 hypervirulent control strains, all were K1 or K2 serotype, and all virulence genes were presented in most strains (ranging from 75.0% to 100%) with the exception for *iroN* (33.3%). Additionally, the prevalence of numerous virulence genes in MDR strains was not significantly different with hypervirulent control strains.

## Multilocus sequence typing

The clones of the 12 MDR strains were diverse and scattered. They were categorized into nine sequence types (ST23, ST11, ST29, ST65, ST86, ST320, ST367, ST420, ST831). Among the hypervirulent control 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 well documented, MDR *K. pneumoniae* usually causes infections of patients with basic diseases and was considered as cKP with a high resistance rate but hypovirulence [2,12,14]. However, *K. pneumoniae* isolates from KP-PLA converged hypervirulence and high antibiotic resistance limited the clinical treatment options largely [16]. To date, little is known concerning virulence characteristics of PLA-causing MDR strains. Therefore, 12 MDR *K. pneumoniae* strains were collected from 163 KP-PLA cases and virulence and molecular epidemiology were further analysed. To the best of our knowledge, this study is the first analysis of the virulence factors in PLA-causing MDR strains.

Numerous studies have reported that antibiotic resistance rates were low in KP-PLA [8,11,12]. Moreover, MDR strains were rare, and patients infected with them were more likely to accompany with hepatobiliary diseases compared to non-MDR ones (Table S1). Importantly, the uncontrollable infections and ineffective prognosis in patients with hepatobiliary diseases may be associated with recurrent bacteremia due to MDR bacteria, suggesting that these MDR isolates may not be the traditional cKP and the acquisition of MDR may not compromise the overall virulence which needs further verification. However, the actual virulence of these MDR strains has not been well evaluated so far.

The results of growth ability suggested there was no fitness cost of the strains with resistant phenotype. In addition, the hypermucoviscosity was considered as a surrogate marker for hvKP [5]. Here, we found the percentage of hypermucoviscous MDR strains was slightly lower than that of hypervirulent control strains. However, it may not be suitable to consider hypermucoviscosity as the only indicator of hypervirulence, the polysaccharide capsule can protect *K. pneumoniae* from phagocytosis of immune cells and bactericidal action of complement or antimicrobial peptides and could act as a major virulence factor for hvKP [17]. Based on the data of capsular quantification, results showed the capsular content of PLA-causing MDR strains was higher than that of the standard strain and lower than that of the hypervirulent control strains, which was consistent with the results of string test. Although the MDR strains and hypervirulent control strains were all sensitive to serum, the antiserum killing ability of these PLA-causing strains was significantly higher than that of the hypovirulent standard strains, which may be related to the content of capsular polysaccharide. Furthermore, bacteria attach to the surface of 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 bacteria from attacking by phagocytes and enzymes, which improves the bacterial defenses against host and resistance to antimicrobials [18]. It also appears that biofilm formation ability of MDR strains was significantly higher than that of hypervirulent control strains, which may be one of the reasons for the MDR strains to exhibit resistant phenotype. Moreover, *G. mellonella* larvae, as a good model of invertebrate host infection, has been applied to explore the virulence and pathogenicity of PLA-causing MDR *K. pneumoniae* strains [19]. The consistency between the clinical data and the results of the 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, as well as being pathogenic to humans strongly [17,20]. In the present study, K1 or K2 serotypes accounted for less than half of the PLA-causing MDR strains, while the hypervirulent control strains were all 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 to enhance virulence [22-23]. *aerobactin* is essential for the growth and virulence of *K. pneumoniae* via regulation of iron supply [1]. In the present study, the prevalence of *rmpA* and *aerobactin* in the MDR strains was slightly lower than that of hypervirulent control strains, reflecting that PLA-causing MDR strains may be combined with hypervirulence from the perspective of virulence genes. Importantly, *wcaG*, *magA*, and *uge* genes related

to capsule synthesis were also prevalent in PLA-causing MDR strains [24-25]. Moreover, the high prevalence of siderophores genes *ybtA*, *entB*, and *kfuBC* in PLA-causing MDR strains suggested that the ability to uptake iron may be equivalent to that of hypervirulent control strains. Furthermore, almost all PLA-causing MDR strains carried *fimH* (related to type 1 fimbriae), *mrkD* (related to type 3 fimbriae), and *ureA* (an  $\alpha$ -subunit of the urease, associated with invasion) [24,26], genetically corroborated virulence phenotype results. Therefore, clinicians should be advised to pay more attention to the MDR strains, and also carefully select appropriate managements 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 hypervirulent control strains were all belong to hypervirulent clones, and ST23 was the predominated type consistenting with previous reports [27]. It has previously been common for ST11-type *K pneumoniae* to be 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, and this kind of real superbug could pose a serious threat to public health [9,15]. Upon previous literatures, ST11 carbapenem-resistant hypervirulent strains have not been found in KP-PLA. To the best of our knowledge, this is the first time that one ST11 carbapenem-resistant strain which may be MDR-hypervirulent *K. pneumoniae* has been described in KP-PLA. Importantly, further surveillance and implementation are needed to implement to control the dissemination in hospital settings and the 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 lead to further emergence of a “post-antibiotic” scenario. Importantly, it reminded that clinicians should be highly prudent in prescribing antibiotics on such KP-PLA patients due to severe antibiotic resistance and take inspection measures timely in view of hypervirulence-induced invasive infections, and supervisors should implement stricter control measures to prevent such real superbug from further disseminating in patients and hospitals. Moreover, further research is needed to elucidate the mechanisms between host, pathogen, and host-pathogen interactions, which will lay a foundation to raise the awareness of MDR-hvKP and provide effective treatments for KP-PLA patients.

## Methods

### Bacterial isolates and antimicrobial susceptibility testing

During 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) with 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 fluid (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 bioMérieux VITEK-2 (BioMérieux, Marcy-l'Étoile, France) initially. Multidrug resistant 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 hypervirulent strains were selected as the 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 standard strain [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 the agar dilution method and microdilution broth method. The results were interpreted by 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* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were served as the quality control strains.

### **Growth curves**

The growth curves of 12 PLA-causing MDR *K. pneumoniae* isolates were measured by following previous methods [31]. In brief, overnight cultures of selected *K. pneumoniae* clinical isolates from KP-PLA and *K. pneumoniae* ATCC 700603 were diluted 1:100 by Luria-Bertani (LB) broth. The cultures were incubated at 37 °C with constant shaking at 200 rpm. Bacteria suspensions were collected at 0, 2, 4, 6, 8, 10, 12, 18, 24 h and the absorbance at 600 nm was determined. Each suspension was measured in triplicates and averages of absorbance values were used for analysis. The growth of PLA-causing MDR *K. pneumoniae* was evaluated by plotting the values of OD<sub>600</sub> against time.

### **String test and quantification of capsule**

The bacterial colonies of *K. pneumoniae* strain on an agar plate were stretched by an inoculation loop. The string test was considered positive when the strain generates a viscous string with a length of >5 mm, and this strain was also considered hypermucoviscous [30].

Capsule was quantified as described previously with some modifications [10,32]. Briefly, 500 µL of cultured bacteria suspensions were resuspended and adjusted to 10<sup>8</sup> CFU/mL, and 1.2 mL sodium tetraborate in sulfuric acid were added in the resuspensions that placed in ice bath and incubated for 5 min at 100°C, and then left on ice for 10 min. A 20 µL volume of 1.5 mg/mL m-hydroxyphenyl was then added and mixed. After a 5 min incubation at room temperature, the absorbance at 590 nm was measured. The glucuronic acid content was determined from a standard curve of glucuronic acid and expressed as µg/10<sup>8</sup> CFU. Results were presented as the mean of the data of three independent experiments.

## **Serum killing test**

Serum bactericidal activity was measured using the method as described previously [6]. Bacteria suspensions in nutrient broth were collected during logarithmic phase and adjusted to  $10^6$  CFU/mL of concentration. 25  $\mu$ L of bacteria suspension was added to 75  $\mu$ L of pooled human sera in the tube. 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 and diluted corresponding fold by adding Mueller-Hinton broth, and then cultured to determine the number of viable bacteria after exposure to serum. Results were expressed as a percentage of the inoculum and graded, then a strain was considered serum resistant or serum sensitive according to the standards, and each strain was tested at least three times.

## **Biofilm formation assay**

The biofilm formation assay was measured using the method of Wilksch et al. [33]. Briefly, clinical isolates were grown to logarithmic phase in LB broth and diluted 1:100 with fresh LB broth. A total of 200  $\mu$ L of each dilution were added to a 96-well polystyrene microtiter plate and blank controls were set at the same time, and per strain was set three duplicate wells. Then, the plate was incubated at 37°C for 24 h. Planktonic cells were removed, and the wells were washed three times with sterile water, and then stained with 250  $\mu$ L 0.1% crystal violet for 10 min and rinsed three times with sterile water. Stained biofilms were solubilized with 95% ethanol and quantified by measuring the OD<sub>600</sub>. Each sample was measured in triplicates, and the averages of absorbance values were used for analysis.

## **Infection model of *Galleria mellonella* larvae**

The model of *G. mellonella* larvae was carried out on the three PLA-causing MDR isolates (FK3068, FK3228, FK4603) and three control isolates (FK3112, FK3837, FK3914) that were randomly selected and standard strain ATCC 700603 to investigate the virulence and pathogenicity of the strains [34-35]. A serial concentration gradient bacterium suspension of each strain ( $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$  CFU/mL) was prepared in advance. Eight larvae weighing of 200 mg - 250 mg were randomly selected for each strain and each concentration. A 10  $\mu$ L of bacterial suspension was injected into the last left proleg by using a 25  $\mu$ L Hamilton precision syringe. Larvae injected with 10  $\mu$ L phosphate-buffered saline were used as control. And then, the insects were incubated at 37°C in the dark and observed after 24 h, 48 h, 72 h and 96 h. Larvae were considered dead when they repeatedly failed to respond to physical stimuli.

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

Crude genomic DNA was extracted from PLA-causing *K. pneumoniae* isolates. Subsequently, 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*, *entB*, and *ureA*) were amplified by PCR using specific primers as previously described [24,36-38]. In addition, strains with these genes determined by PCR and DNA sequencing were selected as positive control for the subsequent PCR experiments.

## Multilocus sequence typing (MLST)

In this study, 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://bigsd.b.pasteur.fr/klebsiella/klebsiella.html/>). The alleles and STs 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 mean values  $\pm$  SD or median (25th - 75th percentile), whereas categorical variables were described as the number and percentage of subjects. Comparisons for continuous variables were made using either the Student's *t* test or the Mann–Whitney *U* test, and comparisons for categorical variables using either the Chi-square test or Fisher's exact test. The mortality of *G. mellonella* were assessed by Kaplan-Meier analysis and log-rank test.

## 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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