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

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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 these strains are rare. The aim of this study was to analyze the virulence 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 twelve PLA-causing MDR *K. pneumoniae* were similar or even more obvious than those of sensitive hypervirulent *Klebsiella pneumoniae* control strains. These MDR strains were mainly non-K1/K2 serotypes and carried multiple virulence genes. Multilocus sequence typing (MLST) revealed the PLA-causing MDR strains had low clone correlation.

Conclusions: This study was the first analysis on virulence factors of PLA-causing MDR strains. The virulent factors exhibited the coexistence of hypervirulence and multidrug resistance in PLA-causing MDR *K. pneumoniae* strains, and these MDR strains were mainly polyclonal spread and there 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, notorious for acquiring antimicrobial resistance [1–3]. The other is hypervirulent *K. pneumoniae* (hvKP). 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, and it exhibits hypervirulent phenotypes and genotypes, which 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 worldwide [2, 4, 5]. *K. 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 could be identified, and then, migrates to distant sites, thereby 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 antimicrobials, and the antimicrobial 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], twelve multidrug resistant (MDR) *K. pneumoniae* were isolated from non-cryptogenic PLA and tended to be considered as cKP. Although, KP-PLA caused by antimicrobial-susceptible hypervirulent strains has been well reported, MDR *K. pneumoniae* isolates from KP-PLA are rare and have not been well identified, particularly in the virulence and molecular epidemiology in the literature [2, 7, 12]. Whether these MDR *K. pneumoniae* isolates were indeed typical cKP or combined with hypervirulence was unknown. Since the convergence of virulence and resistance in *K. pneumoniae*, as well as the challenge of KP-PLA itself, gaining knowledge of virulence and molecular epidemiological characteristics in PLA-causing multidrug resistant *K. pneumoniae* has become more urgent. The virulence of *K. pneumoniae* is closely related to the ability of aggression and defense, and hypervirulence can assist pathogen to resist host innate immunity and infect the host invasively with high pathogenicity [1, 13]. In addition, hypervirulent strains usually possess thick capsular polysaccharide, anti-serum capacity and multiple virulence factors, including hypermucoviscosity, capsular serotype, virulence genes, and related clones [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%). Thereinto, three strains were resistant to carbapenem, one of which was resistant to colistin, but these strains were more sensitive to aminoglycosides. However, the 12 hypervirulent control 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 (µg/mL, 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
FK3112	32	1	< 0.03	0.125	< 0.03	0.125	0.03	2	0.25	1	0.5/9.5	32	0.5
FK3262	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	> 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	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	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	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	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	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	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

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

Strain numbers	MICs (µg/mL, mg/L)												
	AMP	ATM	CRO	CAZ	FEP	IPM	CIP	LEV	GEN	TOB	SXT	NIT	COL
FK3992	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	> 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	> 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													

Growth curves

The growth ability of MDR strains was comparable with that of hypervirulent control strains and the standard strain ATCC 700603 (Fig. 1).

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 ($P < 0.05$) (Fig. 2).

Susceptibility to serum killing

All MDR strains and hypervirulent control strains isolated from KP-PLA were susceptible to serum killing. The anti-serum ability of MDR strains was more sensitive than that of hypervirulent control strains, but there was no significant difference between the two groups ($P > 0.05$) (Fig. 3).

Biofilm formation assay

As shown in Fig. 4, the OD values of biofilms formed by MDR strains were ranged from 0.31 to 0.80, with an average value of 0.58 ± 0.19 ; while the OD values of biofilms formed by hypervirulent control strains were 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 hypervirulent control strains ($P < 0.05$).

Infection model of *Galleria mellonella* larvae

As shown in Fig. 5, three MDR strains and three hypervirulent control strains were all time-dependent and concentration-dependent on the lethality of larvae ($P < 0.05$), while the corresponding concentration gradients of MDR strains and hypervirulent control strains had no significant difference in the lethality of larvae (Fig. 5A, B, C, D, E, F). In addition, the lethality of MDR strains and hypervirulent control strains was similar by 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 ($P < 0.05$) (Fig. 5G).

Polymerase chain reaction for capsular serotypes and virulence genes

As shown in Fig. 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 33.3–50.0%), all remaining virulence genes were presented in more than half of MDR strains (ranging from 75.0–100%). The prevalence of *ompA* and *aerobactin* was 83.3% and 85.3%, respectively. Among 12 hypervirulent control strains, all of them were K1 or K2 serotype, and all virulence genes

were presented in most strains (ranging from 75.0–100%) 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

MLST revealed nine sequence types (ST23, ST11, ST29, ST65, ST86, ST320, ST367, ST420, ST831) in the MDR strains. 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%) (Fig. 6).

Discussion

MDR *K. pneumoniae* often infect inpatients with basic diseases and were considered as cKP with a high resistance rate but hypovirulence [2, 12, 14]. It is reported that *K. pneumoniae* isolates from KP-PLA converged hypervirulence and highly resistance that greatly limited the treatment options of KP-PLA, and the condition posed a threat to patients by repeated infections and poor prognosis, but those isolates has not been explored further [16]. To date, little is known about virulence characteristics of these PLA-causing MDR strains. Hence, in the present study, we analyzed the virulence and molecular epidemiology of 12 MDR strains from 163 KP-PLA cases in a tertiary teaching hospital from the perspective of clinical characteristics, virulence phenotypes and genotypes. To the best of our knowledge, this study was firstly analyzed the virulence factors of PLA-causing MDR strains.

The general resistant rates were lower in KP-PLA, MDR strains were rare and usually were treated as traditional cKP, and patients infected with MDR strains were more likely to accompany with hepatobiliary diseases than non-MDR ones (Table S1). The general understanding is that these patients are related to hepatobiliary diseases per se and abnormal bilioenteric anatomy, as well as a history of more antibiotic exposure, the hypovirulent cKP with severe resistance rate can easily cause nosocomial infections in these patients, while hypervirulent strains usually infect healthy community residents and are generally fully sensitive to antibacterial drugs. It is worth noting that the high infection-related death rate in patients with PLA of hepatobiliary diseases may be associated with recurrent bacteremia due to MDR bacteria, which can cause uncontrollable infections and more deaths, and clinical symptoms, laboratory examination, and clinical outcomes comparisons between MDR and non-MDR strains were similar (Table S1), 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 for the decline in growth ability due to the resistant phenotype. The hypermucoviscosity was considered as a surrogate marker for hvKP [5]. The percentage of hypermucoviscous MDR strains in our study was slightly lower than that of hypervirulent control strains. However, recent studies consider that hypermucoviscosity is unreliable [18]. A polysaccharide capsule can protect *K. pneumoniae* from phagocytosis of immune cells and bactericidal action of complement or antimicrobial peptides and acts as a major virulence factor for hvKP [17]. The results of capsular quantification suggested that the capsular content of PLA-causing MDR strains was between the hypervirulent control strains and the standard strain, which was consistent with the results of string test. When the patients are infected by bacteria, antibodies in human serum specifically will bind to the bacterial epitopes and combine with the complement system to recruit and then kill bacteria for an immune response, and capsular polysaccharide can hinder this process [6]. 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 hinder the attack of bacteria by phagocytes and enzymes, which improves the bacterial defenses against host and resistance to antimicrobials [18]. The 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 virulence *in vivo* and pathogenicity of MDR strains was comparable with

hypervirulent control strains. In brief, the PLA-causing MDR *K. pneumoniae* strains may be hypervirulent with analysis of virulence phenotypes.

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]. The high 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. Next, *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 α -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. These MDR strains were mainly polyclonal spread, while the clones of hypervirulent control strains were more concentrated, all of which were hypervirulent clones, and ST23 was the predominated type consistent with previous reports [27]. ST11 is a common carbapenem-resistant sequence type in China and even converges hypervirulence, highlighting the great threat of such real superbugs [9, 15]. To the best of knowledge, ST11 carbapenem-resistant hypervirulent strains have not been found in KP-PLA. In this study, there was firstly found one ST11 carbapenem-resistant strain which may be MDR-hypervirulent *K. pneumoniae*.

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 and cautious in prescribing antibiotics on such KP-PLA patients because of severe drug resistance and take inspection measures timely in view of hypervirulence-induced invasive infections. Moreover, additional studies need to be conducted to elucidate the mechanisms between host, pathogen, and host-pathogen interactions. Further studies can be helpful 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 OD₆₀₀ 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 10⁸ 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-hydroxyldiphenyl 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/10⁸CFU. Results were presented as the mean of the data of three independent experiments.

Susceptibility to serum killing

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 10⁶ CFU/mL of concentration. 25 µL of bacteria suspension was added to 75 µL 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 µL 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 µL 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 OD₆₀₀. 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 μ L of bacterial suspension was injected into the last left proleg by using a 25 μ L Hamilton precision syringe. Larvae injected with 10 μ 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 primes 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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Figures

Figure 1

The growth curves of PLA-causing multidrug resistant *K. pneumoniae* strains

Figure 2

The capsular quantification of PLA-causing multidrug resistant *K. pneumoniae* strains

Figure 3

The anti-serum ability of PLA-causing multidrug resistant *K. pneumoniae* strains

Figure 5

Survival curves of *K. pneumoniae* infection model of *Galleria mellonella* larvae

Figure 6

Genotype map of PLA-causing multidrug resistant and hypervirulent control strains

Supplementary Files

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