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

Methods

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

Results

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

Conclusions

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

Background

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

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

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

**Materials And Methods**

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

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

| Strain numbers | Isolate date     | Isolate type   | AMIC  | ATM  | CRO  | CAZ  | FEP  | DMP  | CIP  | LEV  | GEN  | TOB  | SXT  | NIT  | COL  |
|----------------|------------------|----------------|-------|------|------|------|------|------|------|------|------|------|------|------|------|
| FK3106         | May 17, 2016     | drainage fluid | >8    | >6   | >4   | >8   | >2   | 2    | 2    | 2    | >0.25| >0.75| 128  | 0.5  |
| FK3108         | May 19, 2016     | pus            | 32    | 16   | >4   | >6   | 32   | 1    | 0.03 | 0.25 | 4    | >16/304| 128  | 1    |
| FK3109         | May 28, 2016     | pus            | 32    | 16   | >6   | >4   | 32   | 0.5  | 0.03 | 0.25 | 4    | >16/304| 128  | 0.25 |
| FK3129         | July 28, 2016    | drainage fluid | >6    | >6   | >8   | >8   | >2   | 2    | 0.06 | >16  | 0.125| 4    | >16/304| 256  | 16   |
| FK3141         | September 8, 2016| blood          | 32    | 2    | 4    | >2   | >5   | 4    | 0.5  | >16  | 0.5  | 4    | >16/304| 256  | 0.25 |
| FK3151         | November 9, 2016 | pus            | >6    | >6   | >6   | >6   | >2   | 1    | >8   | 1    | 0.3  | 0.5  | 1.19 | 256  | 0.25 |
| FK3152         | November 10, 2016| pus             | >6    | >6   | >4   | >4   | >2   | >8   | 4    | 0.5  | <0.25| >0.25| >0.75| 128  | 0.25 |
| FK3159         | December 11, 2016| pus            | 32    | 2    | 4    | >2   | >5   | 4    | 0.5  | <0.25| >0.25| >0.75| 128  | 0.5  |
| FK3176         | July 13, 2017    | drainage fluid | >6    | >4   | 8    | >4   | >2   | 4    | 0.5  | 2    | >16  | 0.5  | >0.25| >0.75| 128  | 0.5  |
| FK3166         | August 14, 2017  | drainage fluid | >6    | >6   | >6   | >6   | >2   | 1    | >16  | 0.25 | 2    | >16/304| 256  | 0.25 |
| FK3157         | November 13, 2016| blood          | 32    | >6   | 64   | 64   | >2   | 4    | 0.5  | 4    | >0.25| >0.75| 128  | 0.125|
| FK3113         | June 11, 2016    | pus            | 32    | 2    | >0.03| 0.125| <0.03| 0.125| 0.03 | 0.25 | 1    | >0.25| >0.75| 128  | 0.5  |
| FK3162         | August 8, 2016   | pus            | 32    | 0.5  | <0.03| 0.125| <0.03| 0.125| 0.03 | 0.06 | 0.25 | 0.5  | 0.5  | >0.25| >0.75| 128  | 0.25 |
| FK3166         | January 10, 2017 | pus             | >6    | 0.5  | <0.03| 0.125| <0.03| 0.125| 0.03 | 0.06 | 0.25 | 0.5  | 0.5  | >0.25| >0.75| 128  | 0.25 |
| FK3168         | February 9, 2017 | pus             | 32    | 0.5  | <0.03| 0.25  | <0.03| 0.25  | 0.03 | 0.06 | 0.25 | 0.5  | 0.5  | <0.25| >0.75| 128  | 0.25 |
| FK3170         | February 9, 2017 | pus             | 32    | 0.25 | <0.03| 0.125| <0.03| 0.25  | 0.03 | 0.03 | 0.25 | 0.5  | 0.5  | <0.25| >0.75| 16   | 0.25 |
| FK3181         | March 21, 2017   | pus             | 32    | 1    | <0.03| 0.25  | <0.03| 0.25  | 0.5  | 0.25 | 0.5  | 0.25 | 0.5  | <0.25| >0.75| 32   | 1    |
| FK3187         | March 24, 2017   | pus             | 32    | 1    | <0.03| 0.25  | <0.03| 0.25  | 0.5  | 0.25 | 0.5  | 0.25 | 0.5  | <0.25| >0.75| 32   | 0.25 |
| FK3194         | April 21, 2017   | pus             | 32    | 0.5  | <0.03| 0.125| <0.03| 0.25  | 0.03 | 0.06 | 0.25 | 0.5  | 0.5  | <0.25| >0.75| 32   | 0.25 |
| FK3196         | May 5, 2017      | pus             | 32    | 0.5  | <0.03| 0.125| <0.03| 0.25  | 0.03 | 0.03 | 0.25 | 0.5  | 0.5  | <0.25| >0.75| 32   | 0.5  |
| FK3199         | May 17, 2017     | drainage fluid | 32    | 0.5  | <0.03| 0.125| <0.03| 0.25  | 0.00 | 0.25 | 0.25 | 0.5  | 0.25 | <0.25| >0.75| 32   | 0.5  |
| FK3181         | June 15, 2017    | drainage fluid | >6    | >6   | >6   | >6   | >6   | >4   | >6   | >16  | 0.25 | 2    | >16/304| 256  | 0.25 |
| FK4176         | November 16, 2016| pus             | >6    | >6   | >6   | >6   | >6   | >4   | >16  | >0.25| >0.75| 16   | >0.25| >0.75| 128  | 0.5  |

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

MICs: minimum inhibitory concentrations, PLA: pyogenic liver abscess, AMIC: ampicillin, ATM: aztreonam, CRO: cefoxitin, CAZ: ceftazidime, FEP: cefepime, DMP: imipenem, CIP: ciprofloxacin, TOB: tobramycin, GEN: gentamicin, SXT: sulfamethoxazole/trimethoprim, NIT: nitrofurantoin, COL: colistin.

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

The minimum inhibitory concentrations (MICs) of ampicillin, aztreonam, ceftriaxone, ceftazidime, cefepime, imipenem, ciprofloxacin, levofloxacin, gentamicin, tobramycin, sulfamethoxazole/trimethoprim,
nitrofurantoin and colistin were confirmed by agar dilution method and microdilution broth method. The data were interpreted based on the latest guidelines published by the Clinical and Laboratory Standards Institute (CLSI; Pittsburgh, PA, USA) and the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints (http://www.eucast.org). Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 served as quality control strains. The experiment was performed in triplicate.

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

**String test and quantification of capsule**

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

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

**Biofilm formation assay**

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

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

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

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

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

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

**Table 2.** List of oligonucleotide primers used in amplification of capsular serotypes and virulence genes
| Gene   | Primer Sequence (5’-3’)                      | Tm (°C) | Amplicon size (bp) |
|--------|---------------------------------------------|---------|-------------------|
| magA   | F: GGTGCTCTTTACATCATTGC                     | 58      | 1283              |
|        | R: GCAATGGCCATTTGCGTTAG                    |         |                   |
| aerobactin | F: GCATAGGCGGATACGAACAT                  | 58      | 556               |
|        | R: CACAGGGCAATTGCTTACCT                    |         |                   |
| iroN   | F: AAGTCAAAGCAGGGGTGCCCCG                  | 58      | 665               |
|        | R: GACGCCGACATTAAGACGCAG                   |         |                   |
| kfuBC  | F: GAAGTGACGCTGT TTCTGGC                   | 58      | 979               |
|        | R: TTTCTGTGTGGCCAGTGACTC                   |         |                   |
| rmpA   | F: ACTGGGCTACCTCTGCTTCA                    | 58      | 516               |
|        | R: CTTGCATGAGCCATCTTCTCA                   |         |                   |
| wcaG   | F: GGGTGKTCA GCATCGTA                      | 58      | 169               |
|        | R: ACTATTCGCCA AACTTTTGCG                 |         |                   |
| ybtA   | F: ATGACGGAGTCCACCGCAAAAC                  | 55      | 960               |
|        | R: TTACATCAGCG TTAAAGG                     |         |                   |
| ureA   | F: GCTGACTTT AAGAGAAGCTTATG               | 58      | 337               |
|        | R: GATCATGGCGCTACCT(C/T)A                 |         |                   |
| uge    | F: TCTTCACGCCTCCTCCTCACT                   | 53      | 534               |
|        | R: GATCATCCGGTCTCCTCTGTA                  |         |                   |
| entB   | F: ATTTCCCTCAACTTCTGGGC                   | 56      | 371               |
|        | R: AGC ATCGGTGGCGGTGGTCA                  |         |                   |
| fimH   | F: TGCTGCTGGGTGGCTTGCGATG                 | 62      | 688               |
|        | R: GGGAGGGTGACGGTGACATC                   |         |                   |
| mrkD   | F: CCAACCAACTATTCCCTCGAA                  | 43      | 226               |
|        | R: ATGGAACCACATCGACATT                    |         |                   |
| K1     | F: GGTGCTCTTTACATCATTGC                   | 54      | 1283              |
|        | R: GCAATGGCCATTTGCGTTAG                   |         |                   |
| K2     | F: GGATTATGACAGCCTCTCCCT                  | 58      | 641               |
|        | R: CGACTTTGGTCCA AACTTTTT                 |         |                   |
Multilocus sequence typing (MLST)

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

### Statistical analysis

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

### Results

#### Antimicrobial susceptibility testing and growth curves

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

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

**Biofilm formation assay**

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

**Serum killing test**

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

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

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

**Polymerase chain reaction for capsular serotypes and virulence genes**

To investigate the virulence genotypes of the PLA-causing MDR strains, capsular serotypes and virulence genes were measured. Among the 12 PLA-causing MDR strains, four were K1 serotype (FK3228, FK3518, FK3599, and FK4737), one was K2 serotype (FK4276), one was K20 serotype (FK4176) and six were of non-type. Except *magA* (33.3%), *iroN* (33.3%) and *kfuBC* (50.0%), all the remaining virulence genes were present in more than half of the MDR strains (*wcaG* 83.3%, *ybtA* 83.3%, *fimH* 91.7%, *mrkD* 100%, *uge* 91.7%, *entB* 100%, and *ureA* 83.3%). The prevalence of *mpA* and *aerobactin* was 83.3% and 75.0%, respectively. The 12 typical hypervirulent strains were K1 or K2 serotype, and all the virulence genes were
present in most of the strains (**aerobactin** 100%, **rmpA** 100%, **fimH** 100%, **mrkD** 100%, **uge** 100%, **ureA** 100%, **entB** 91.7%, **wcaG** 83.3%, **magA** 83.3%, **kfuBC** 75.0%, and **ybtA** 75.0%) with the exception for **iroN** (33.3%). Additionally, the prevalence of numerous virulence genes in MDR strains was not significantly different from that of typical hypervirulent strains.

**MLST**

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

**Discussion**

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

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

The growth ability results suggested no fitness cost regarding the strains with resistant phenotype. In addition, hypermucoviscosity was considered as a surrogate marker of hvKP [5]. In this study, the percentage of hypermucoviscous MDR strains was found to be slightly lower than that of typical hypervirulent strains. However, hypermucoviscosity might not be the only indicator of hypervirulence, wherein the polysaccharide capsule can protect *K. pneumoniae* from phagocytosis by immune cells and complement-mediated bactericidal action, which acts as a major virulence characteristic for hvKP [29]. The results of capsular quantification revealed that the capsular content of PLA-causing MDR strains was higher than that of the standard strain and lower than that of the typical hypervirulent strains. The standard strain ATCC 700603 that recognized as classic *K. pneumoniae* is known for producing extended-spectrum β-lactamase (ESBL) enzymes that can hydrolyze oxyimino-β-lactams, resulting in
resistance to these drugs, and its virulence is less than the typical hypervirulent *K. pneumoniae* [30]. The data of capsular quantification was consistent with the data of the string test. Although the MDR strains and typical hypervirulent strains were sensitive to serum, the antiserum killing ability of these PLA-causing strains was significantly higher than that of the typical hypervirulent strains, which might be related to the content of capsular polysaccharide. Furthermore, the bacteria attaches to the surface of the host during the infectious process and are coated with polymers such as extracellular polysaccharides and DNA to form biofilms. The physical barrier formed by biofilms protect the bacteria from phagocytes and enzymes, improving the bacterial defenses against the host and antimicrobial resistance. This finding indicated that the biofilm formation ability of MDR strains was significantly higher than that of typical hypervirulent strains, which might be one of the reasons for the MDR strains to exhibit resistant phenotype. Moreover, *G. mellonella* larvae, acts as a model of invertebrate host infection, and has been used to explore the virulence and pathogenicity of *K. pneumoniae* strains [31]. Although the siderophore genes were different in these PLA-causing MDR strains and hypervirulent strains, the virulence was assessed in terms of lethality, thereby suggesting that the siderophore transport systems need to be investigated further to clarify the correlation between siderophore utilization and bacterial virulence. The consistency between the clinical data and the results of phenotypic assays supported the theory that the PLA-causing MDR *K. pneumoniae* strains are hypervirulent.

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

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

**Conclusions**

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

**Abbreviations**

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

**Declarations**

**Ethics approval and consent to participate**

This study has been designed in accordance with the Declaration of Helsinki (2013) (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-
involving-human-subjects/) and been approved by the Ethics Committee in Clinical Research of the First Affiliated Hospital of Wenzhou Medical University (No.2020-070).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors’ Contributions

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

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\section*{Figures}
Growth curves of PLA-causing MDR K. pneumoniae strains (red circles, n=12) were comparable to those of typical hypervirulent strains (green squares, n=12) and standard strain ATCC 700603 (grey triangles, n=1). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student's t-test. No significant difference was noted between MDR and control strains (typical hypervirulent strains and standard strain ATCC 700603), (P > 0.05).
Figure 2

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

Figure 3
Figure 4

Serum bactericidal activity of PLA-causing MDR K. pneumoniae strains (black circles, n=12) were comparable to those of typical hypervirulent strains (black squares, n=12). Data are presented as means±SD, with N=3. Statistical analysis was performed using Student’s t-test. ns, not significant.
Figure 5

Survival curves of K. pneumoniae infection model of Galleria mellonella larvae. (A) FK3068, MDR strain; (B) FK3228, MDR strain; (C) FK4603, MDR strain; (D) FK3112, typical hypervirulent strain; (E) FK3837, typical hypervirulent strain; (F) FK3914, typical hypervirulent strain; (G) 10^6 CFU/mL bacterial suspensions of MDR strains (FK3068, FK3228, FK4603), typical hypervirulent strains (FK3112, FK3837, FK3914).
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.

| Isolate number | HV type | Sequence types | capsule | siderophores | fimbriae | nitrogen |
|----------------|---------|----------------|---------|--------------|----------|-----------|
| FK3038         | NT      | 23             |         |              |          |           |
| FK3044         | NT      | 65             |         |              |          |           |
| FK3068         | NT      | 320            |         |              |          |           |
| FK3228         | K1      | 11             |         |              |          |           |
| FK3347         | NT      | 29             |         |              |          |           |
| FK3518         | K1      | 831            |         |              |          |           |
| FK3521         | NT      | 29             |         |              |          |           |
| FK3599         | K1      | 23             |         |              |          |           |
| FK4176         | +K20    | 420            |         |              |          |           |
| FK4276         | K2      | 86             |         |              |          |           |
| FK4603         | NT      | 11             |         |              |          |           |
| FK4737         | K1      | 367            |         |              |          |           |
| FK3112         | K1      | 23             |         |              |          |           |
| FK3262         | K1      | 23             |         |              |          |           |
| FK3645         | +K1     | 86             |         |              |          |           |
| FK3698         | K1      | 23             |         |              |          |           |
| FK3736         | +K1     | 65             |         |              |          |           |
| FK3818         | K1      | 23             |         |              |          |           |
| FK3837         | K1      | 23             |         |              |          |           |
| FK3914         | K1      | 23             |         |              |          |           |
| FK3953         | +K2     | 65             |         |              |          |           |
| FK3992         | +K2     | 86             |         |              |          |           |
| FK4081         | K1      | 23             |         |              |          |           |
| FK4578         | K1      | 23             |         |              |          |           |

**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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