Effects of iron on the growth, biofilm formation and virulence of *Klebsiella pneumoniae* causing liver abscess

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Abstract

Background: *Klebsiella pneumoniae* is considered as the most clinically relevant species of *Enterobacteriaceae*, known to cause severe infections including liver abscesses. To the best of our knowledge, iron in human body primarily gathers in liver. Further understanding of effects of iron on *K. pneumoniae* causing liver abscess is a promising way to elucidate the mechanism of poor prognosis of liver abscess.

Results: All tested *K. pneumoniae* including those collected from liver abscess and other sterile sites and *K. pneumoniae* ATCC 700603 cultured in LB broth containing 50 μ M iron grew optimally and were in possession of the strongest biofilm formation ability, while worst growth and biofilm formation ability of the strains cultured in iron-deplete environment were observed. The infection model of *G. mellonella* larvae showed the virulence of *K. pneumoniae* cultured with additional iron was significantly higher than cultured with iron chelator ($P < 0.05$). The relative expression level of the four siderophores genes (*iucB*, *iroB*, *irp1*, *entB*) in *K. pneumoniae* strains isolated from liver abscess cultured with additional iron was lower than that with iron chelator.

Conclusions: It is suggested by our research that iron in the environment can promote the growth and enhance the biofilm formation ability of *K. pneumoniae* and the lower expression of siderophores genes may be one of the mechanisms of iron affecting the virulence of liver abscess-causing *K. pneumoniae*. Further deeper evaluation of these phenomena is warranted.

Background

Klebsiella pneumoniae is considered as the most clinically relevant species of *Enterobacteriaceae*, known to cause both community-acquired and nosocomial infections, including liver abscesses, pneumonias, urinary tract infections and bacteremias worldwide [1]. In the past two decades, a distinct hypervirulent variant of *K. pneumoniae*, characterized by its hypermucoviscous phenotype, was firstly isolated from liver abscess in Asia and has emerged as a clinically significant pathogen responsible for highly invasive infections [2]. Unlike classical *K. pneumoniae*, hypervirulent *K. pneumoniae* (hvKp) can spread from the original site of infection to other organs. Once invasive dissemination occurs, patients are often accompanied by severe and irreversible refractory sequelae, such as blindness and central nervous system damage [2,3]. The conditions of patients with hypervirulent *K. pneumoniae* causing liver abscess are serious due to the limitation of therapy, posing a great threat to patients' health and has attracted the attention of clinicians.

The pathogenicity of *K. pneumoniae* mainly arise from various virulence factors which allow it to overcome innate host immunity and to maintain infection in a mammalian host. The main virulence factors that play an important role in pathogenicity are capsular polysaccharide, lipopolysaccharide, pili and siderophore [4]. Thereinto, K1 and K2 capsular types are considered to be highly pathogenic to human [5]. *ompA* is an important regulator of activated capsular production, resulting in the formation of

hypermucoviscous phenotype and enhancement of virulence, while Aerobactin (*iucB*), yersiniabactin (*irp1*), salmochelin (*iroB*) and enterobacterin (*entB*) are the four siderophore of *K. pneumoniae* [6,7]. Therefore, understanding the virulence characteristics of *K. pneumoniae* and taking appropriate measures are critical for clinical assessment, controlling the prognosis and reducing mortality risk of patients with *K. pneumoniae* infection.

It's recognized that most organisms require iron as an essential element in a variety of metabolic and informational cellular pathways [8]. However, ferric ion (Fe^{3+}) and its derivatives are less soluble, and most organisms are difficult to directly use. It is essential to synthesize and secrete siderophore to meet the demand for iron and participate in bacterial growth and metabolism. At present, patients with liver abscess caused by *K. pneumoniae* have a serious prognosis, for the reason that liver is an important organ for storing iron. Therefore, our study was designed to evaluate the effects of iron on the growth, biofilm formation and virulence of liver abscess-causing *K. pneumoniae*.

Methods

Bacterial strains and antimicrobial susceptibility profiling

Six *K. pneumoniae* clinical isolates (FK3065, FK3087, FK3170, FK3226, FK3992, FK4003) were collected from patients in the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, China in 2016 and 2017. Among them, FK3226, FK3992, FK4003 were isolated from patients with liver abscess, and FK3065, FK3087, FK3170 were isolated from the sterile sites of non-liver abscess patients (ascites, biliary drainage fluid). Identification and antimicrobial susceptibility testing were conducted on all isolates using a VITEK2 system (bioMérieux, Marcy L'Etoile, France). *K. pneumoniae* ATCC 700603 was served as the control strain.

Growth curves and biofilm formation

Six *K. pneumoniae* clinical isolates (FK3065, FK3087, FK3170, FK3226, FK3992, FK4003) and *K. pneumoniae* ATCC 700603 were grown in Luria-Bertani (LB) broth containing 50 μM iron, 30 μM iron, 10 μM iron, no additional iron, and 50 μM iron with 200 μM iron chelating agent (2,2'-Dipyridyl), respectively [9,10]. Samples were collected at 0 h, 2 h, 4 h, 6 h, 8 h, 16 h, 24 h and the absorbance was read at 600nm.

The biofilm assay was performed as described previously. Briefly, six *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 700603 were grown overnight in LB broth with 50 μM iron, 30 μM iron, 10 μM iron, no additional iron, and 50 μM iron with 200 μM iron chelating agent. The overnight culture of cells was then diluted 1:100 in fresh LB broth with or without iron. A total of 100 μL of each dilution were added to a 96-well polystyrene microtiter plate and incubated at 37 °C for 24 h. Wells containing media alone were used as negative control. Planktonic cells were removed and the wells were washed twice with sterile water, the wells were stained with 150 μL 0.1% crystal violet for 10 min and wells were rinsed twice with sterile water [11]. Stained biofilms were solubilized with 95% ethanol and quantified by measuring the OD_{600} using a microplate reader.

Detection of virulence genes of isolates

Virulence genes (*magA*, *iucB*, *iroB*, *entB*, *irp1*, *iroN*, *kfuBC*, *mpaA*, *wcaG*, *allS*, *ybtA*, *ureA*, *uge*, *wabG*, *fimH* and *mrkD*) and capsular serotypes (K1, K2, K5, K20, K54 and K57) of the six *K. pneumoniae* clinical isolates and *K. pneumoniae* ATCC 700603 were amplified by Polymerase Chain Reaction (PCR). Primers for the aforementioned genes were listed in Table 1 [12,13]. Positive PCR products were sequenced by Beijing Genomics Institute Technology Co. Ltd (Shanghai, China). Nucleotide sequences were compared using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Infection model of Galleria mellonella (G. mellonella) *G. mellonella* killing assays were carried out on five clinical isolates and *K. pneumoniae* ATCC 700603 as described previously, with minor modifications [14]. Eight *G. mellonella* weighing between 200mg–250mg were randomly selected for each strain. Larvae were injected with 10 µL of bacterial suspension (108 CFU/mL) in phosphate-buffered saline (PBS), into the last left proleg using a 25 µL Hamilton precision syringes. The bacterial suspension was prepared by culturing the strains in LB broth containing 50 µM iron, and 50 µM iron with 200 µM iron chelating agent for 24 h. Larvae injected with 10 µL PBS were used as control. The insects were incubated at 37°C in dark and observed after 24 h, 48 h and 72 h. Larvae were considered dead when they repeatedly failed to respond to physical stimuli. The primary outcome for the insect model was rapidity and extent of mortality of *G. mellonella* assessed with Kaplan-Meier analysis and log-rank test. $P < 0.05$ was considered as statistically significant.

Quantitative reverse transcription PCR (qRT-PCR)

The effects of iron on the expression levels of *K. pneumoniae* siderophore genes (*iucB*, *iroB*, *entB*, and *irp1*) were evaluated using quantitative reverse transcription PCR (qRT-PCR). For RNA isolation, *K. pneumoniae* isolates were grown in fresh LB medium with 50 µM iron, and 50 µM iron with 200 µM iron chelating agent at 37 °C for 24h. Total RNA was extracted from culture using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA samples were stored at -80 °C. Purified RNA was reverse transcribed into cDNA for qRT-PCR analysis using a cDNA synthesis kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. Gene expression levels were measured with qRT-PCR using a 7500 RT-PGE system (TOYOBIO, Osaka, Japan) and SYBR Green qRT-PCR Kit (TOYOBIO) with the specific primers listed in Table 2 [15]. We selected several isolates to analyze the expression of *iucB*, *iroB*, *entB*, and *irp1* genes according to the gene carrying conditions (Table 3). The *rpoB* gene was used as an internal control to normalize the data. Each sample was measured in triplicates and mean of Ct values were used for analysis. Gene expression levels were calculated using $2^{-\Delta\Delta C_t}$ method.

Results

Antimicrobial Susceptibility testing

Five *K. pneumoniae* isolates (FK30650, FK3170, FK3226, FK3992, FK4003) were sensitive to conventional antimicrobials (levofloxacin, ciprofloxacin, cefepime, ceftazidime, imipenem, gentamicin, amikacin, tobramycin, ampicillin/sulbactam, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, aztreonam, cefotetan, ertapenem, ceftriaxone). However, FK3087 was resistant to ampicillin/sulbactam, levofloxacin, ciprofloxacin, gentamicin and trimethoprim/sulfamethoxazole.

Effect of iron on growth and biofilm formation of the K. pneumoniae isolates

The *K. pneumoniae* isolates and *K. pneumoniae* ATCC700603 grew optimally in LB broth containing 50 μ M iron, superior to the growth in LB broth containing 30 μ M iron, 10 μ M iron, and no additional iron, separately. The growth of these strains in the iron-depleted environment caused by the iron chelating agent was the worst (Figure 1).

We found that appropriate concentration of iron is present, it would promote the biofilm formation of *K. pneumoniae*. The strains presented the strongest biofilm formation ability in LB broth containing 50 μ M iron, which was superior to the biofilm formation ability of the strains in LB broth containing 30 μ M iron, 10 μ M iron and no additional iron. The strains owned the weakest biofilm formation ability in iron-poor environment (Figure 2).

The virulence factors of K. pneumoniae isolates

Three liver abscess-causing *K. pneumoniae* isolates (FK3226, FK3992 and FK4003) carried at least nine virulence genes, including four kinds of siderophores genes (*entB*, *iucB*, *iroB*, *irp1*).. And the serotypes of these three isolates were K1, K2, and K1, respectively. Three non-liver abscess-causing *K. pneumoniae* isolates (FK3065, FK3087 and FK3170) carried four virulence genes, including one of the siderophores gene *entB*, and the serotypes of them are not a common capsule classification. The carrying status of virulence genes was shown in Table 3.

Infection model of G. mellonella larvae

The mortality rates of *G. mellonella* after infecting the liver abscess-causing *K. pneumoniae* isolates (FK3226, FK3992) and *K. pneumoniae* ATCC700603 cultured with additional iron were significantly higher than that cultured with iron chelator ($P < 0.05$). While the mortality rates of *G. mellonella* after infecting the non-liver abscess-causing *K. pneumoniae* isolates (FK3065, FK3087 and FK3170) were not significantly different under these two conditions (Figure 3).

Quantification of siderophores genes expression with qRT-PCR

The relative expression levels of the four siderophores genes (iucB, iroB, irp1, entB) of the liver abscess-causing K. pneumoniae strains cultured after additional iron were lower than that cultured after adding iron chelator ($P<0.05$). On the contrary, the relative expression level of the siderophore gene entB of the non-liver abscess-causing K. pneumoniae isolates cultured after additional iron was higher than that cultured after adding iron chelator ($P<0.05$) (Figure 4).

Discussion

The Gram-negative bacillus K. pneumoniae is a leading cause of healthcare-associated infections, including urinary tract infections, surgical sites infections, soft tissues infections, bacteremia and pneumonia [16]. In the 1980s, case reports from Taiwan described community acquired liver abscesses caused by hvKp in patients with serious concomitant endorgan manifestations, such as meningitis and endophthalmitis [17]. Since the first report in Taiwan, the sporadic spread of hvKP has been observed in many countries in Asia, Europe and America [17]. In this study, three liver abscess-causing K. pneumoniae isolates and three non-liver abscess-causing K. pneumoniae isolates were selected to growth, biofilm and virulence investigations under different iron ion conditions.

We found that the presence of moderate amounts of iron promoted the growth of K. pneumoniae. A slower growth of K. pneumoniae isolates was found in iron-deficient environments. At the same time, it was found that iron promoted the biofilm formation of K. pneumoniae, and the ability of bacterial biofilm formation was significantly reduced after the addition of iron chelator. Furthermore, previous studies have shown that: iron can promote the initial formation of lactic acid bacteria biofilm and enhance the biofilm stability by stimulating the secretion of EPS and bridging; in the iron-deficient environment, the biofilm formation ability of Pseudomonas aeruginosa was significantly reduced, and the biofilm formation increased significantly after the iron complement of iron deficiency environment, indicating that iron plays an important role in the formation of P. aeruginosa biofilm [18]. The presence of an appropriate amount of iron is also conducive to the biofilm's formation of Escherichia coli and Staphylococcus aureus [19,20]. These findings were consistent with our finding that iron is one of the trace elements essential for microbial growth and plays a crucial role in the process of bacterial biofilm formation.

In this study, the virulence genes and capsular serotypes of seven experimental strains were detected by PCR. These three liver abscess-causing K. pneumoniae isolates carried more virulence genes, including four kinds of siderophores genes iucB, iroB, irp1, entB, while the strains isolated from the sterile site only carried four virulence factors. Previous studies have shown that different concentrations of iron could affect the surface characteristics of the pili and porin and influence the virulence of Acinetobacter baumannii [21]. At the same time, considering the different iron content among the liver and other tissue, we speculate that iron concentration may be an important factor to affect the virulence of K. pneumoniae.

Siderophores are the important substance for bacterial transport iron to meet the growth requirements. In this study, the effect of iron concentration on the relative expression of siderophores genes was detected

by qRT-PCR. After the addition of iron, the relative expression level of the four siderophores genes (*iucB*, *iroB*, *irp1*, *entB*) of the *K. pneumoniae* strains causing liver abscess was lower than that of the strains which added iron chelator. The reason may be the liver abscess-causing *K. pneumoniae* isolates used to exist in liver, where is rich in iron content. We speculated the strain itself carried four kinds of siderophore genes, and could not need to express the corresponding siderophore genes to secrete excessive iron carrier to transport iron. It can use the abundant iron in the environment to meet the needs of the bacteria itself; when it is in the iron-deficient environment, it needs to express the siderophore gene to transport the iron in the environment to meet its own needs. In addition, we found that the siderophore gene *entB* of non-liver abscess-causing *K. pneumoniae* isolates was expressed higher after adding iron than in the environment of iron deficiency and iron chelating agent deficiency. We speculated that these strains may only carry one siderophore gene, and the previously environment was not adequate about iron. When the iron in the surrounding environment increases, it needs to express the iron carrier gene to secrete more iron carriers. Transporting iron in the surrounding environment to make it grow better and exert its corresponding virulence; but when it is in an iron-depleted environment, the strain itself may have adapted to the environment because of the iron content in the environment. The siderophore genes were not overexpressed. Furthermore, to investigate the effects of iron on the virulence of *K. pneumoniae*, we determined the virulence of *K. pneumoniae* through the wax larvae infection models. To the best of our knowledge, the wax larvae are a confirmed invertebrate host model that can be used to detect the virulence of *K. pneumoniae* [14]. According to the results of the *G. mellonella* infection model, three strains caused higher mortality rate after adding iron than iron-deficient growth environment, indicating the protentional virulence in vivo. The results of qRT-PCR showed that the relative expression of siderophores genes could be considered as one of the regulators of virulence changes. Due to the various needs of different strains for iron, the expression of siderophores genes was not exactly same. Other mechanisms by which iron affect the virulence of *K. pneumoniae* need to be further explored.

Conclusions

In summary, iron can promote the growth, biofilm formation and affect virulence of *K. pneumoniae* causing liver abscess. Moreover, the diverse expression of siderophores genes may be one of the factors that regulate the variety of virulence. To Further understanding of these phenomenons, more deeper evaluation of the phenomenons is warranted.

Declarations

List of abbreviations

PCR: Polymerase Chain Reaction; qRT-PCR: Quantitative Reverse Transcription Polymerase Chain Reaction.

Ethics approval and consent to participate

The need for ethics approval and consent is deemed unnecessary in this research according to the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

Authors' contributions

TC and GFD did the same contribution to this study. TC and GFD wrote this manuscript under supervision of JMC, TLZ and QW. TC, GFD, SQZ, XCZ and YJZ performed the research. TC and GFD performed the data analysis. The research plan for this project was conceived based on several rounds of discussions among all co-authors. All authors read and approved the final version of the manuscript.

- *Competing interests*
- *The authors declare that they have no competing interests.*
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References

1. Paczosa MK, Mecsas J. *Klebsiella pneumoniae*: going on the offense with a strong defense. *Microbiol Mol Biol Rev.* 2016; 80(3): 629–661.
2. Prokesch BC, TeKippe M, Kim J, Raj P, TeKippe EM, Greenberg DE. *Primary osteomyelitis caused by hypervirulent Klebsiella pneumoniae.* *Lancet Infect Dis.* 2016; 16(9):

e190–e195. 3. Shon AS, Bajwa RP, and Russo TA. Hypervirulent (hypermucoviscous) *Klebsiella pneumoniae*: a new and dangerous breed. *Virulence*. 2013; 4(2): 107–118. 4. Podschun R, Ullmann U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clin Microbiol Rev*. 1998; 11(4): 589–603. 5. Catalán-Nájera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermucoviscosity: Two different but complementary *Klebsiella* spp. phenotypes? *Virulence*. 2017; 8(7): 1111-1123. 6. Fang CT, Chuang YP, Shun C, Chang SC, Wang JT. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. *J Exp Med*. 2004; 199(5): 697–705. 7. Hsieh PF, Lin TL, Lee CZ, Tsai SF, Wang JT. Serum-induced iron acquisition systems and TonB contribute to virulence in *Klebsiella pneumoniae* causing primary pyogenic liver abscess. *J Infect Dis*. 2008; 197(12): 1717-1727. 8. Miethke M, Marahiel MA. Siderophore-based iron acquisition and pathogen control. *Microbiol Mol Biol Rev*. 2007; 71(3): 413-451. 9. Palacios M, Broberg CA, Walker KA, Miller VL. A Serendipitous Mutation Reveals the Severe Virulence Defect of a *Klebsiella pneumoniae* *fepB* Mutant. *MSphere*. 2017; 2(4). pii: e00341-17. 10. Pasqua M, Visaggio D, Lo Sciuto A, et al. Ferric Uptake Regulator Fur Is Conditionally Essential in *Pseudomonas aeruginosa*. *J Bacteriol*. 2017; 199(22). pii: e00472-17. 11. Wilksch JJ, Yang J, Clements A, et al. MrkH, a novel c-di-GMP-dependent transcriptional activator, controls *Klebsiella pneumoniae* biofilm formation by regulating type 3 fimbriae expression. *PLoS Pathog*. 2011; 7(8): e1002204. 12. Candan ED, Aksoz N. *Klebsiella pneumoniae*: characteristics of carbapenem resistance and virulence factors. *Acta Biochim Pol*. 2015; 62(4): 867-874. 13. Wasfi R, Elkhatab WF, Ashour HM. Molecular typing and virulence analysis of multidrug resistant *Klebsiella pneumoniae* clinical isolates recovered from Egyptian hospitals. *Sci Rep*. 2016; 6: 38929. 14. Insua JL, Llobet E, Moranta D, et al. Modeling *Klebsiella pneumoniae* pathogenesis by infection of the wax moth *Galleria mellonella*. *Infect Immun*. 2013; 81(10): 3552-65. 15. Nadasy KA, Domiati-Saad R., Tribble MA. Invasive *Klebsiella pneumoniae* syndrome in North America. *Clin Infect Dis*. 2007; 45(3): e25–e28. 16. Corbella M, Caltagirone M, Gaiarsa S, et al. Characterization of an Outbreak of Extended-Spectrum beta-Lactamase-Producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit in Italy. *Microb Drug Resist*. 2018; 24(8): 1128-1136. 17. Corbella M, Caltagirone M, Gaiarsa S, et al. Characterization of an Outbreak of Extended-Spectrum beta-Lactamase-Producing *Klebsiella pneumoniae* in a Neonatal Intensive Care Unit in Italy. *Microb Drug Resist*. 2018; 24(8): 1128-1136. 18. Wiens JR, Vasil AI, Schurr MJ, Vasil ML. Iron-regulated expression of alginate production, mucoid phenotype, and biofilm formation by *Pseudomonas aeruginosa*. *MBio*. 2014; 5(1): e01010-13. 19. Alves JR, Pereira AC, Souza MC, et al. Iron-limited condition modulates biofilm formation and interaction with human epithelial cells of enteroaggregative *Escherichia coli* (EAEC). *J Appl Microbiol*. 2010; 108(1):246-255. 20. Lin MH, Shu JC, Huang HY, Cheng YC. Involvement of iron in biofilm formation by *Staphylococcus aureus*. *PLoS One*. 2012; 7(3):e34388. 21. Eijkelkamp BA, Hassan KA, Paulsen IT, Brown MH. Investigation of the human pathogen *Acinetobacter baumannii* under iron limiting Conditions. *BMC Genomics*. 2011; 12(1): 126.

Tables

Table 1. Primers used for PCR amplification of virulence genes

<i>Genes</i>	<i>Sequence</i>	<i>T_m[°C]</i>
<i>magA</i>	<i>F: 5'- GGTGCTCTTTACATCATTGC -3'</i> <i>R: 5'- GCAATGGCCATTTGCGTTAG -3'</i>	58
<i>iroN</i>	<i>F: 5'- GGCTACTGATACTTGACTATTTC -3'</i> <i>R: 5'- CAGGATACAATAGCCCATAG -3'</i>	58
<i>kfuBC</i>	<i>F: 5'- GAAGTGACGCTGTTTCTGGC -3'</i> <i>R: 5'- TTTCGTGTGGCCAGTGA CTC -3'</i>	58
<i>rmpA</i>	<i>F: 5'- ACTGGGCTACCTCTGCTTCA -3'</i> <i>R: 5'- CTTGCATGAGCCATCTTTCA -3'</i>	58
<i>wcaG</i>	<i>F: 5'- GGTTGGKTCAGCAATCGTA -3'</i> <i>R: 5'- ACTATTCCGCCAACTTTTGC -3'</i>	58
<i>alls</i>	<i>F: 5'- CCGAAACATTACGCACCTTT -3'</i> <i>R: 5'- ATCACGAAGAGCCAGGTCAC -3'</i>	58
<i>ybtA</i>	<i>F: 5'- ATGACGGAGTCACCGCAAAC -3'</i> <i>R: 5'- TTACATCACGCGTTTAAAGG -3'</i>	55
<i>ureA</i>	<i>F: 5'- GACAAGCTGTTGCTGTTTACC -3'</i> <i>R: 5'- CGGGTTGTGAACGGTGAC -3'</i>	58
<i>uge</i>	<i>F: 5'- GATCATCCGGTCTCCCTGTA -3'</i> <i>R: 5'- TCTTCACGCCTTCCTTCACT -3'</i>	53
<i>wabG</i>	<i>F: 5'- CGGACTGGCAGATCCATATC -3'</i> <i>R: 5'- ACCATCGGCCATTTGATAGA -3'</i>	58
<i>fimH</i>	<i>F: 5'- TGCTGCTGGGCTGGTCGATG -3'</i> <i>R: 5'- GGGAGGGTGACGGTGACATC -3'</i>	62
<i>mrkD</i>	<i>F: 5'- CCACCAACTATTCCCTCGAA -3'</i> <i>R: 5'- ATGGAACCCACATCGACATT -3'</i>	43
<i>iucB</i>	<i>F: 5'- ATGTCTAAGGCAAACATCGT -3'</i>	58

	<i>R: 5'- TTACAGACCGACCTCCGTGA -3'</i>	
<i>entB</i>	<i>F: 5'- ATTCCTCAACTTCTGGGGC -3'</i>	56
	<i>R: 5'- AGCATCGGTGGCGGTGGTCA -3'</i>	
<i>iroB</i>	<i>F: 5'- GCATAGGCGGATACGAACAT -3'</i>	58
	<i>R: 5'- CACAGGGCAATTGCTTACCT -3'</i>	
<i>irp1</i>	<i>F: 5'- ATGGATAACTTGCGCTTCTC -3'</i>	58
	<i>R: 5'- CGGTATAGCCGACCTTTCTG -3'</i>	
<i>K1</i>	<i>F: 5'- GGTGCTCTTTACATCATTGC -3'</i>	54
	<i>R: 5'- GCAATGGCCATTTGCGTTAG -3'</i>	
<i>K2</i>	<i>F: 5'- GACCCGATATTCATACTTGACAGAG-3'</i>	58
	<i>R: 5'- CCTGAAGTAAAATCGTAAATAGATGGC -3'</i>	
<i>K5</i>	<i>F: 5'- TGGTAGTGATGCTCGCGA -3'</i>	58
	<i>R: 5'- CCTGAACCCACCCCAATC -3'</i>	
<i>K20</i>	<i>F: 5'- CGGTGCTACAGTGCATCATT -3'</i>	58
	<i>R: 5'- GTTATACGATGCTCAGTCGC -3'</i>	
<i>K54</i>	<i>F: 5'- CATTAGCTCAGTGGTTGGCT -3'</i>	58
	<i>R: 5'- GCTTGACAAACACCATAGCAG -3'</i>	
<i>K57</i>	<i>F: 5'- CTCAGGGCTAGAAGTGTCAT -3'</i>	58
	<i>R: 5'- CACTAACCCAGAAAGTCGAG -3'</i>	

Table 2. Primers used for qRT-PCR of siderophore genes

Gene	Sequence	Reference
<i>iucB</i>	F: 5'-ATGTCTAAGGCAAACATCGT-3' R: 5'-TTACAGACCGACCTCCGTGA-3'	This study
<i>iroB</i>	F: 5'-ACGACGGCGAACCCATTATT-3' R: 5'-GACTTCACTGGCGGAATCCA-3'	This study
<i>entB</i>	F: 5'-ATATCCCGGCGAACAAGGTC-3' R: 5'-CGGCGATATTAGCCACCACT-3'	This study
<i>irp1</i>	F: 5'-CTCAGTGGCAACAACAGTGC-3' R: 5'-GATGGCGCGGTGAATGTTAC-3'	This study
<i>rpoB</i>	F: 5'-AAGGCGAATCCAGCTTGTTTCAGC-3' R: 5'-TGACGTTGCATGTTTCGCACCCATCA-3'	[15]

Table 3. The virulence genes of *K. pneumoniae*

Isolates	Virulence genes
FK3065	<i>entB</i> ∥ <i>uge</i> ∥ <i>mrkD</i> ∥ <i>fimH</i>
FK3087	<i>entB</i> ∥ <i>uge</i> ∥ <i>mrkD</i> ∥ <i>fimH</i>
FK3170	<i>entB</i> ∥ <i>uge</i> ∥ <i>mrkD</i> ∥ <i>fimH</i>
FK3226	<i>iucB</i> ∥ <i>iroB</i> ∥ <i>entB</i> ∥ <i>irp1</i> ∥ <i>rmpA</i> ∥ <i>wcaG</i> ∥ <i>ybtA</i> ∥ <i>magA</i> <i>ureA</i> ∥ <i>uge</i> ∥ <i>wabG</i> ∥ <i>mrkD</i>
FK3992	<i>iucB</i> ∥ <i>iroB</i> ∥ <i>entB</i> ∥ <i>irp1</i> ∥ <i>rmpA</i> ∥ <i>ybtA</i> ∥ <i>ureA</i> ∥ <i>uge</i> ∥ <i>wabG</i> ∥ <i>mrkD</i>
FK4003	<i>iucB</i> ∥ <i>iroB</i> ∥ <i>entB</i> ∥ <i>irp1</i> ∥ <i>rmpA</i> ∥ <i>ureA</i> ∥ <i>uge</i> ∥ <i>wabG</i> ∥ <i>mrkD</i>
ATCC700603	<i>iucB</i> ∥ <i>iroB</i> ∥ <i>entB</i> ∥ <i>irp1</i> ∥ <i>rmpA</i> ∥ <i>ybtA</i> ∥ <i>ureA</i> ∥ <i>wabG</i> ∥ <i>mrkD</i>

Figure 1

Figure 1

The growth curves of *K. pneumoniae*. Effects of different concentrations of iron on the growth of *K. pneumoniae*. DP : 2,2'-Dipyridyl, added as iron chelating agent.

Figure 2

Figure 2

. Biofilm-forming capabilities of *K. pneumoniae* in the presence and absence of iron. The biofilm-forming capabilities of *K. pneumoniae* isolates incubated with 50 µM iron were strongest, which is superior to biofilm formation ability in LB broth containing 30 µM iron, 10 µM iron and no additional iron. DP : 2,2'-Dipyridyl, added as iron chelating agent.

Figure 3

Figure 3

K. pneumoniae infection of *Galleria mellonella*. A. FK3065, B. FK3087, C. FK3170, D. FK3226, E. FK3992, F. FK4003, G. ATCC 700603. The virulence of FK3226, FK3992 and ATCC 700603 with additional iron was significantly higher than that of the strain after adding iron chelator ($P < 0.05$). DP : 2,2'-Dipyridyl, added as iron chelating agent. The PBS was control group.

Figure 4

Figure 4

Expression ratio of *K. pneumoniae* virulence genes in the presence or absence of iron. A. *iucB* gene. B. *iroB* gene. C. *irp1* gene. D. *entB* gene. DP : 2,2'-Dipyridyl, added as iron chelating agent.