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ORIGINAL RESEARCH

The Anti-Virulence Effect of Sub-Minimal Inhibitory Concentrations of Levofloxacin on Hypervirulent *Klebsiella pneumoniae*

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Background: Hypervirulent *Klebsiella pneumoniae* (*hvKp*) is best described as a virulent pathogen and generally associated with the hypermucoviscosity phenotype. Increased capsule and aerobactin production are established important *hvKp*-specific virulence factors. Although *hvKp* strains have been relatively susceptible to antimicrobials, given the high morbidity and mortality, there is a critical need for alternative strategies for the treatment of *hvKp* infections. Thus, the anti-virulence therapy has been targeted for the *hvKp* development of therapeutics.

Materials and Methods: Four *hvKp* isolates with hypermucoviscous phenotype were used in our experiments. Mucoviscosity of the capsule can be assessed by low-speed centrifugation of cultures. CPS amount was determined by glucuronic acid content. The capsule thickness was measured under microscope after ink staining. The transcriptions of gene were measured by quantitative real-time PCR (qRT-PCR). The effect of levofloxacin on the resistance of *K. pneumoniae* to phagocytosis by macrophages and mouse lethality assay was observed.

Results: Our data revealed that sub-Minimal Inhibitory Concentrations (sub-MIC) of LVX reduce mucoviscosity and CPS production of *hvKp*. Microscopic observations demonstrated that the capsule of *hvkp* bacteria became thinned after treatment with LVX. qRT-PCR showed decreased transcript levels of *rmpA, wzi, magA, iroN and icuA* genes. Down-regulation of these virulence genes occurred leading to increased susceptibility to phagocytosis by macrophages. Mouse lethality assay revealed that the wild strain had the LD50 of 10^3 CFU, while the sub-MIC LVX-treated bacteria had the LD50 of 10^5 CFU.

Conclusion: Our data suggested that LVX may serve as a potential anti-virulence agent for refractory infection by *hvKp*.

Keywords: hypervirulent *Klebsiella pneumoniae*, hypermucoviscous, capsular polysaccharide, levofloxacin, anti-mucoviscous, anti-virulence agent

Introduction

Klebsiella pneumoniae (*kp*) is an increasingly important opportunistic pathogen capable of causing pneumonia, urinary tract infections, and bacteremia in immunocompromised or frequently healthcare-exposed individuals.[1] Recently, a new and hypervirulent clinical variant of *K. pneumoniae* has an ability to cause community-acquired and life-threatening infections among young and healthy individuals. Epidemics of hypervirulent *K. pneumoniae* (*hvKp*) have occurred mainly in Asian countries, including China, South Korea and Iran, while only sporadic spread have been reported in European and American countries. The patients infected by *hvKp* often present with multiple sites of infection and/or develop subsequent metastatic spread, which are less common in other *Enterobacteriaceae* infections. The prevalence of bacteremia caused by *hvKp* in China is high, ranging from 24.5% to 36.8%,[2,3] which is lower than 41.5% reported in

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Taiwan,[4] but higher than 6% (53/878) reported by a teaching hospital in Spain from 2007 to 2013.[5] Despite reasonable antibiotic treatment, patients with the invasive syndrome have a mortality rate ranging from 3% to 31%,[6] and patients with hypermucoviscous (HMV) *K. pneumoniae* bacteremia have a mortality rate close to 35% or higher.[3] In one case series of 12 French patients with the HMV phenotype, all 5 patients with bacteremia died.[7]

The RmpA- and/or RmpA2-mediated overproduction of capsular polysaccharide is the unique structural feature of *hvKp* strains when grown on agar plates. The capsular polysaccharides (CPS) is one of important virulence factors of *hvKp*, which can protect bacteria from phagocytosis and opsonization, provide protective shield against antimicrobial peptides, and suppresses the early inflammatory response.[8] Also, it is a key virulence factor that aids dissemination to the host bloodstream and systemic infection.[9,10] More than 70 capsular serotypes have been reported for *K. pneumoniae*, and isolates that produce CPS are generally more virulent than non-capsulated strains. Among these, most of *hvKp* isolates belong to capsular serotypes K1 and K2.[11] Most HMV *K. pneumoniae* isolates from patients with bacteremia also had the K1 or K2 serotype (78%).[12] Besides CPS, a number of virulence factors have been suggested to contribute to the pathogenesis of *hvKp* strains, including *rmp*A, *magA*, and several iron acquisition factors.

Antibiotics continue to be the only treatment option for *K. pneumoniae* infections, but it still fails to prevent the occurrence of invasive syndrome. There have been no trials assessing which antibiotics are best suited for treating *hvKp* infection and local antimicrobial resistance patterns. Many studies have shown that changes in CPS levels due to gene mutant and deletion from the *CPS* locus can lead to changes in mucus phenotypes, serum, phagocytosis, and virulence. For example, *hvKp* with mutations in *rmpA*, or other genes that affect capsule production, typically lose the HMV phenotype and show a strong reduction in virulence when tested in mice.[13,14] However, changes in CPS and other virulence-related gene expression through spontaneous and specific gene mutants or deletion may not be applied in clinical patients with *hvKp* infection. Ofloxacin has been shown to reduce mucobiscosity of *hvKp*.[15] In our study, we want to discuss levofloxacin (LVX) serves as potential anti-virulent agent for *hvKp* infection.

Materials and Methods

Bacteria

Four *hvKp* strains were isolated in blood or pyogenic fluid cultures from with liver abscess in the first affiliated hospital of Anhui Medical University. The strains were grown in Mueller–Hinton broth at 37°C with shaking at 130 rounds per minute (RPM). All the strains identified had positive string test results and carried several virulence-associated genes (*rmpA, magA, ironB*, and *iucA*). Molecular analysis revealed that the isolates produced K1 capsular polysaccharides and belong to sequence type (ST) 23. They were all susceptible to almost all commonly used antibiotics, including ceftriaxone, cefotaxime, piperacillin–tazobactam imipenem, meropenem, amikacin, and levofloxacin, except ampicillin. The minimum inhibitory concentration (MIC) values of levofloxacin for the four isolates were 0.03125–0.125μg/mL. *K. pneumoniae* ATCC 700603 is a non-hvKP control strain, which is a K6 serotype possessing neither *magA* nor *rmpA*.

Evaluation of Anti-Mucoviscous Activity of Levofloxacin

The mucoviscosity of *K. pneumoniae* strains was determined as previously described.[16] Briefly, equal numbers of phase-cultured bacteria with or without different concentrations of LVX (1/16 ×, 1/8 ×, 1/4 ×, 1/2 × of the MIC) were centrifuged at 1000 g for 5 min. Then, the supernatant was subjected to measurement of the absorbance at 600 nm. B16 isolate was used for verifying the anti-mucoviscous activity of LVX, and then confirmed the effect with other *hvKp* isolates.

Quantitative Measurement of Bacterial Capsular Polysaccharide

The bacterial extracellular polysaccharide was extracted and the uronic acid, a main component of *K. pneumoniae* K1 capsule, was quantified as previously described.[17,18] Briefly, 500 μL of overnight broth-cultured bacteria was mixed with 100 μL of 1% Zwittergent 3–14 (Sigma-Aldrich, Milwaukee, WI) in 100 mM citric acid (pH 2.0) and then incubated at 50°C for 20 min. After centrifugation, 250 μL of the supernatant was transferred and added with 1 mL of cold ethanol. The mixture was incubated at 4°C for 20 min for precipitation. After centrifugation, the pellet was dried and dissolved in 200 μL of distilled water, and then 1200 μL of 12.5 mM tetraborate in concentrated H_2SO_4 was added. After vigorous

vortex, the mixture was boiled for 5 min. After cooling, 20 μL of 0.15% 3-hydroxydiphenol (Sigma-Aldrich) was added. Then, the absorbance at 520 nm was measured.

Bacterial Growth Curves

To monitor the effects of levofloxacin on bacterial growth, growth curve was generated as previously described.[19] Bacteria cultured overnight were added to a 96-well microtiter plate at a final concentration of 10^6 CFU/mL, together with different concentrations of LVX for 18h (final volume of 200 μL). The optical density (OD) of the bacterial culture at 600 nm was measured every 2h using a spectrophotometer (U-1500, Hitachi, Tokyo, Japan). Experiments were repeated in triplicate and the average absorbance values were used for analysis.

Microscopic Observation of Capsule

Overnight cultures were mixed with equal volumes of India ink, and 2 μL samples of the mixtures placed on microscope slides and covered with a cover slip. The samples were evaluated, and images captured using an Olympus BX50 microscope (Olympus, Tokyo, Japan) with Confocal Laser Scanning Microscopy (CLSM) a 100 objective lens. To determine the change in capsule thickness over time, overnight cultures with or without 1/4MIC LVX were subcultured in fresh M-H B broth starting at 0.05 OD600 units. The cultures were incubated at 37 °C over a series of time-points. The cultures were diluted to 0.1OD600. Then, four slides were prepared according to the above ink staining method to observe and measure the capsule thickness. The shortest diameter of the clear zone around each bacterial cell was recorded as the capsule thickness. Four fields were randomly selected for each slide, all cells were measured and average capsule thickness was calculated.

Quantitative Real-Time Polymerase Chain Reaction

The expression level of *rmpA, magA, galF, wzi, manC, iucA*, and *iroN* genes in *hvKp* strains after treatment with a sub-MIC concentration of LVX were determined via quantitative real-time PCR (qRT-PCR). The primers used for qRT-PCR were synthesized according to the literature.[15] RNAs of the *K. pneumoniae* strains at the logarithmic growth stage were extracted using an RNA extraction kit (Tiangen) according to the kit's procedure. The quantity and quality of the RNAs were assessed using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Complementary DNA was synthesized using the cDNA Synthesis Kit (Takara, Japan). The qRT-PCR (three replicates) assay for each gene was performed using Applied Biosystems PowerUpTM SYBR® Green Master Mix (Thermo Fisher Scientific) in an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific). The 16S rRNA gene was used as internal control. Finally, the relative expression of the above genes was calculated by the $2^{-\Delta\Delta Ct}$ method.

Determination of the Anti-Phagocytosis

Raw264.7 cells were grown in DMEM (Gibco, Grand Island, NY, United States) containing 10% FBS at 37°C. Bacteria were grown in 5 mL of LB broth with or without LVX (1/4 MIC μg/mL) for 8–10 hours and washed once in cold phosphate-buffered saline (PBS). Raw 264.7 cells were infected with *K. pneumoniae* at a multiplicity of infection (MOI) of 100 and incubated for 2h at 37°C to permit phagocytosis. After 2h incubation, infected cells were washed three times with PBS, and incubated for another 1h with 1mL of DMEM containing 100 μg/mL of gentamycin and 10 μg/mL lysostaphin (Sigma-Aldrich) to kill the extracellular bacteria. The cells were washed three times with PBS again and lysed with 0.2 mL of sterile 0.25% Triton X-100. After being fully mixed, 10-fold serial dilutions were plated on the MHA culture plates for 16h incubation at 37°C, and the number of bacterial colonies was counted. The percentage of phagocytosis rate was expressed as the number of viable bacteria that infect RAW264.7 cells compared with the number of viable bacteria from the pretreatment and multiplied by 100.

Determination of the Virulence in Mouse Lethality Tests

ICR male mice (28–32g) were injected with graded doses of 10^2 to 10^6 CFU in 10-fold serial dilutions in 0.1 mL of normal saline via tail vein. Six mice were used to test the effect of each inoculum (10g/0.1mL). Mice receiving injections were followed for 7 days, with an in extremis state or death being used as the study end point. The 50% lethal dose

(LD50) of *K. pneumoniae* strain was calculated using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) according to the number of mice that died. Animal studies were approved by the Animal Care and Use Committee of Anhui Medical University (No. LLSC20190253). All animal care and use protocols in this study were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Results

Levofloxacin Reduce Mucoviscosity and CPS Production

HMV strains do not sediment well during centrifugation, and the supernatant remains turbid. Measurement of the turbidity after centrifugation can therefore serve as a quantitative indicator of HMV. The strains were grown as described above, with or without LVX overnight, and then subjected to low-speed centrifugation. Uronic acid (UA) is a key component of bacterial capsules and has been used as an indicator of capsule production. These results indicate that LVX can reduce the capsule production and mucoviscosity (Figure 1). Obviously, the effect was enhanced with the increase in the concentration of LVX (Figure 1).

Low Concentration of LVX Had No Obvious Effects on the Growth of *KP*

To monitor different MICs of LVX on the growth of *KP*, a 18h growth curve was generated. Based on the growth curve, we found that the growth of *hvKp* was almost unaffected when the concentration of LVX in the medium was less than or equal to 1/4MIC (Figure 2).

Microscopic Observation of Capsule

When *hvKp* was grown in the presence of sub-inhibitory concentration LVX, the capsule became thinner. When the concentration was reduced to 1/16MIC, the capsule thickness was not significantly different from that of the original strain (Figure 3A). Compared with ATCC700603, when LVX concentration was 1/2MIC, there was no obvious difference in capsule thickness between ATCC700603 and LV. However, at other concentrations of LVX, the capsule was thicker than 700603 in our study. We further evaluate the effect of LVX on bacterial capsule thickness over time following the addition or removal of LVX. After the addition of LVX, the bacterial capsule thickness gradually decreased with time and reached the minimum level after 6 hours (Figure 3B). Conversely, when LVX was removed from an overnight culture of *hvKp*, the capsule gradually regained its thickness (Figure 3C). But over 8 hours, the bacterial capsule thickness had not returned to the original strain size. Eventually, the bacterial capsule thickness could return to normal after about 12 hours. These results demonstrated that the effect of LVX on the *hvKp* capsule was reversible.

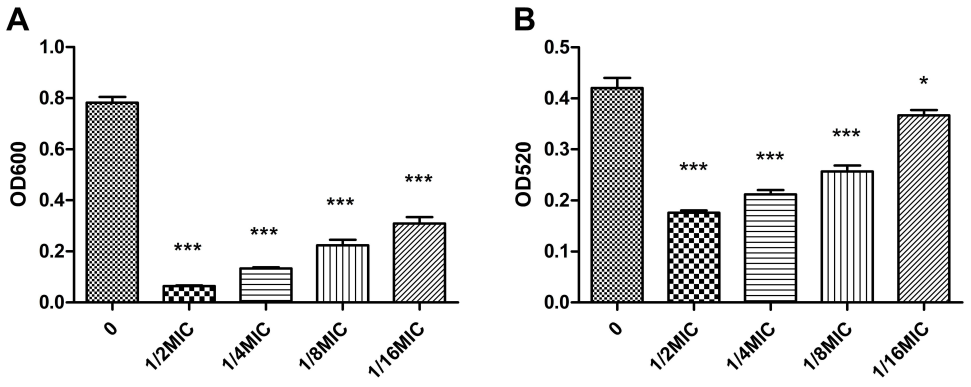

Figure 1 Mucoviscosity and CPS production are reduced in LVX-treated strains. Uronic acid mucoviscosity (**A**) and CPS production (**B**) were assessed as described in Materials and Methods. The data presented here are from a representative assay. The one-way ANOVA test was performed to determine statistically significant differences between different concentrations of LVX and without LVX, ***p < 0.001. The mucoviscosity determined by centrifugation was represented by OD600 of three independent experiments (mean±SD). (0 vs 1/2MIC, 0 vs 1/4MIC, 0 vs 1/8MIC, 0 vs 1/16MIC, ***p < 0.001). The amount of CPS was represented by OD520 of three independent experiments (mean±SD). (0 vs 1/2MIC, 0 vs 1/4MIC, 0 vs 1/8MIC, 0 vs 1/16MIC, ***p < 0.001, *p < 0.05).

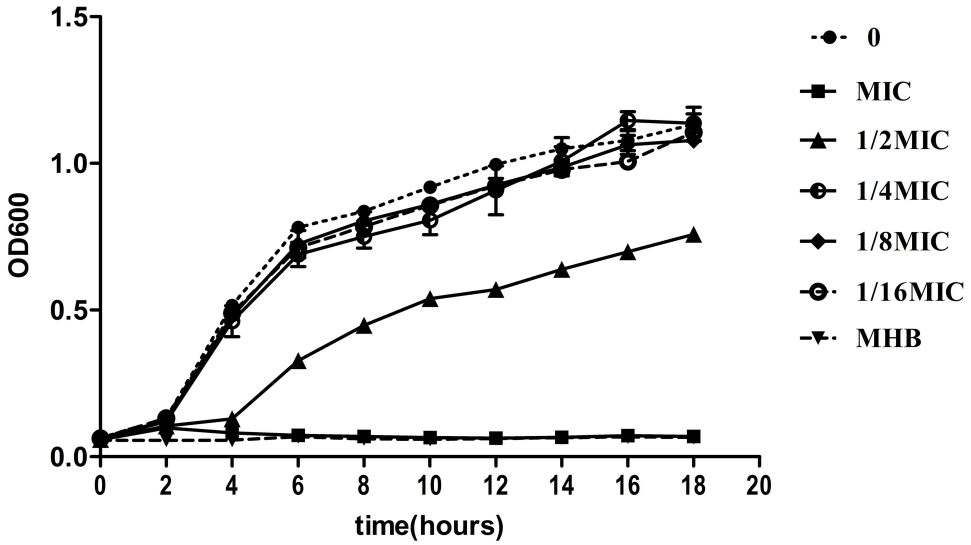

Figure 2 Bacterial growth curve at different concentrations of levofloxacin. Low concentration (≤1/4MIC) of LVX does not affect the *hvKp* growth. Bacterial cultures were grown at 37°C for 18 h.

LVX Represses Capsule Genes and Other Virulence Genes Expression

We evaluate the effect of LVX on the expression levels of the capsule-related genes (*rmpA, magA, galF, wzi*, and *manC*) and other virulence genes (*iucA* and *iroN*) in absence and presence of 1/4 MIC LVX overnight. Not all the gene transcripts were markedly lower when *hvKP* was grown in the presence of LVX compared to that in the absence of LVX (Figure 4). Transcription of *rmpA, wzi, magA, iroN* and *icuA* decreased, especially *rmpA, wzi*, and *iroN*. However, expression of *galF* and *manC* was not affected.

Phagocytosis of *hvKp*

A hallmark of the pathogenesis of *K. pneumoniae* is its resistance to macrophage-mediated phagocytosis, due to the capsule polysaccharide. Given that LVX reduced capsule expression (Figures 3 and 4), bacteria treated with 1/4MIC LVX were more likely to be phagocytosed (Figure 5).

Virulence in Mouse Lethality Tests

Mouse lethality assay revealed that the wild strain had the LD50 of 10^3 CFU, while the LVX-treated bacteria had the LD50 of 10^5 CFU. When mice were injected bacteria with doses of 10^6 CFU, All the mice in group 0 (wild strain) died within 4 days (Figure 6). On day 7, the death rate in group LVX (LVX-treated bacteria) was 50% (Figure 6).

Discussion

Antibiotics have been widely used in the treatment of bacterial infection with high efficiency since their discovery. These drug molecules can kill bacterial pathogens or inhibit their growth if they are used at the right concentration and dosage. However, if the drug concentration is below the minimum inhibitory concentration (MIC), antibiotics can regulate bacterial adaptability and gene expression.[20] For example, antibiotics acting on bacterial cell walls can inhibit the synthesis of capsular polysaccharides in Streptococcus pneumoniae.[21] In the study by Nomura et al, it was found that after the treatment of *K. pneumonia*e with different drugs in 1/4MIC, penicillins, cephalosporins and monoamides could significantly reduce the capsular thickness, quinolones had little effect on the capsular thickness, while aminoglycosides, macrolides and carbapenems showed no significant change in the capsular thickness.[22] Since the mid-1980s, *hvKp*, which is usually presented as an HMV phenotype, has become a clinically important pathogen and often causes severe disseminated infections.[23,24] The high mucinous phenotype of *hvKp* is due to the increased production of typical CPS and the presence of specific virulence genes.[8,25] Similarly, for *hvKp* strain, Namekawa H showed that some antibiotics

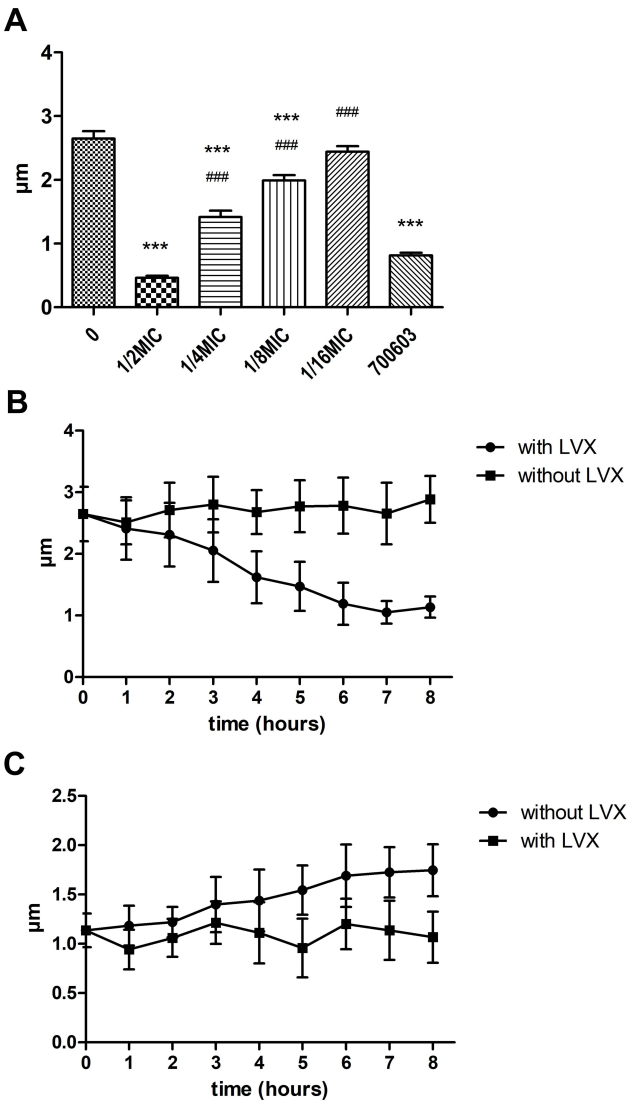

Figure 3 (**A**) Under the sub-inhibitory concentration of LVX overnight, the capsule thickness was measured after ink staining. The higher the concentration of LVX, the thinner the capsule. ***p < 0.001 (compared to 0 group), ###p < 0.001 (compared to 700603). (**B**) Bacterial capsule thickness measured with or without LVX over time. (**C**) Bacterial capsule thickness measured after removal or persistence of LVX over time.

could inhibit the high viscosity of *hvKp* and some antibiotics could promote the high viscosity of *hvKp* by testing 18 kinds of drugs at 1/4MIC concentration, among which rifampicin showed a strong inhibitory effect on capsule formation.[15] In our study, we found that LVX can inhibit the *hvKp* capsule formation. At 1/4MIC, LVX had little effect on the growth of the bacteria, but the mucoviscosity and CPS production decreased. Regardless of the effect of drugs on bacterial growth, with the increase in drug concentration, the inhibition effect on bacterial capsule became more obvious. These results can be seen from qualitative measurements of capsule content and direct measurements of capsule thickness in our study. The capsule thickness of *hvKp* began to thin after the addition of LVX for 2h. When the capsule was reduced to the minimum, the capsule thickness gradually recovered after the removal of the drug. But it did not completely recover until 12h.This indicated that the change of capsule thickness of *hvKp* caused by LVX is reversible.

Several important genes associated with the capsule production are encoded in the CPS cluster, includes three promoters, which are located upstream of *galF, wzi*, and *manC*, respectively.[26] The transcriptional regulator RmpA upregulates the activities of all these promoters to activate capsule production, resulting in the HMV phenotype and increase in virulence.[27] From the perspective of gene transcription level, LVX has different effects on the expression of these genes. We hypothesized that LVX may reduce the mucoviscosity and CPS production by inhibiting the expression of *rmpA*. Theoretically, all the CPS

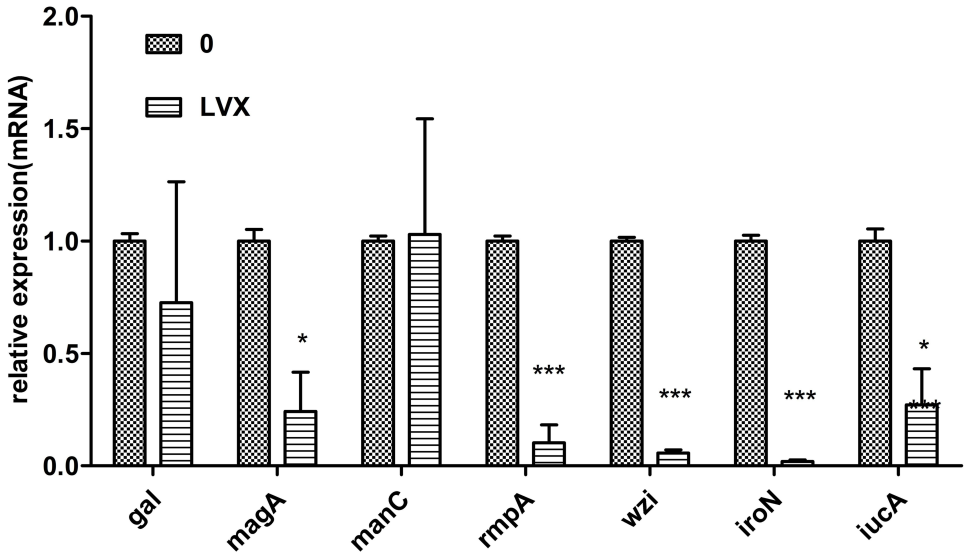

Figure 4 LVX represses the transcription of capsular and other virulence genes in B16. qRT-PCR was performed to analyze the transcriptional levels of *gal*, *magA*, *manC*, *rmpA*, *wzi*, *iroN* and *iucA* genes. Bacterial cultures with or without LVX were grown at 37°C for 8 h. 16S rRNA was used as a reference gene for normalization. Data represent the mean of three independent experiments performed in triplicates. Statistically significant with respect to the *hvKp* grown in MHB medium without LVX. *p < 0.05, ***p < 0.001.

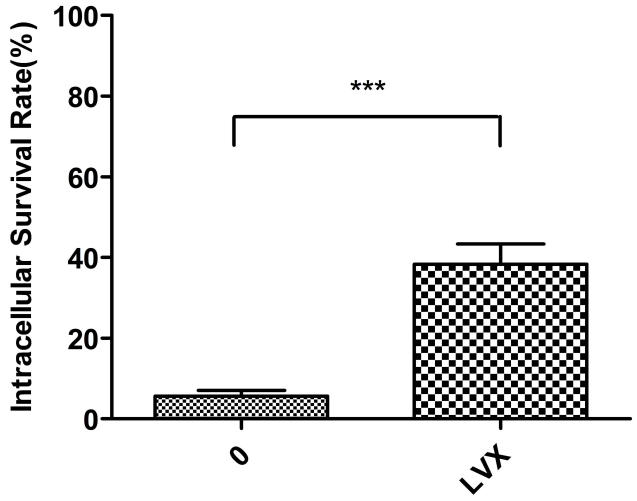

Figure 5 Phagocytosis of *K. pneumoniae*. The percentage of phagocytosis against *hvKp* increased significantly after treatment with LVX (***p < 0.001).

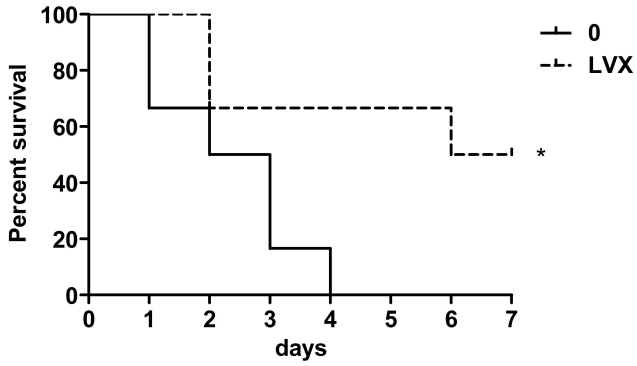

Figure 6 Survival curve of mice injected bacteria with doses of 10^6 CFU bacteria. The Log rank (Mantel–Cox) test was performed to determine statistically significant differences between the two groups. (*p<0.05).

genes are expected to be repressed simultaneously. However, in our experiment, only *wzi* was inhibited significantly, while *galF* and *manC* were not affected. The polycistronic mRNA driven by promoter P2 from *wzi* to *gnd* (in many cases, corresponds to upstream of *manC)* consisted of genes of capsule repeat unit synthesis and polymerization, as well as surface assembly.[28] It is speculated that down regulation of *wzi* has an effect on the capsule production in *hvKp* grown in the presence of LVX and may not be regulated by *rmpA*. Mucoviscosity-associated gene A (magA) of *Klebsiella pneumoniae* contributes to K1 CPS biosynthesis. MagA was predicted to be a Wzy-like polymerase based on sequence homology, conserved domain alignment, and an O-antigen synthesis assay.[28,29] MagA (K1_wzy) is essential for the synthesis of capsular polysaccharide and plays an important role in hypermucoviscous. So in our experiment, down-regulated *magA* was also involved in the reduction of the hypermucoviscosity phenotype and CPS production.

In large part, the enhanced virulence of *hvKp* is due to its ability to synthesize significantly higher levels of iron acquisition molecules.[30] These siderophores have a remarkably high affinity for iron and facilitate bacteria acquire iron in iron-depleted environments, such as the human host. *HvKp* can produce four siderophores: enterobactin, salmochelin, yersiniabactin, and aerobactin, whereas salmochelin (encoded by the *iro* operon) and aerobactin (*iuc*) are *hvKp* specific.[31] Recent data have directly demonstrated only aerobactin plays in *hvKp* growth/survival ex vivo and for the enhanced virulence of the *hvKp* in vivo.[32] As such, it has been proposed as an anti-virulence target.[33] There is a strong trend of co-occurence of *iro* and *iuc* loci in *hvKp* strains in a recent analysis of ~2500 Kp genomes, suggesting salmochelin is present in these genomes because it is important to the success of *hvKp* as well.[34] In our experiment, LVX had an inhibitory effect on both *iucA* and *iroN*, especially *iroN*. Iuc and Iro synthesis is encoded by loci (*iuc* and *iro*), that are typically co-located on the so-called 'virulence plasmids' of *K. pneumoniae*. These plasmids also carry additional virulence determinants including rmpA genes that upregulate capsule production. QRT-PCR revealed reduced transcription level of these three genes in *hvKp* grown in the presence of LVX. It can be inferred from the above observations, sub-MIC LVX may not affect bacterial growth but could modulate the overall gene-expression pattern. Specifically, genes located on virulence plasmids.

CPS is probably considered the critical factor in *K. pneumoniae* pathogenesis to protect *K. pneumoniae* from killing by phagocytosis or serum factors.[35,36] Compared with encapsulated strains, unencapsulated *K. pneumoniae* strains are more susceptible to killing by serum complement, and they are more readily phagocytosed and killed by phagocytic leukocytes.[37,38] In our study, LVX decreases the CPS production that were correlated with the enhancement of phagocytic activity of macrophages. After LVX treatment, the virulence of bacteria in mice decreased with the change of several virulence factors (CPS synthesis-related genes and siderophores expression inhibition).

Conclusion

Taken together, we here demonstrated that LVX repress CPS and siderophores production. This changed bacterial phenotype, including mucoviscosity, impairs the bacterial resistance to phagocytosis and reduce the virulence of aof bloodstream infection in a mouse model . In addition to its bactericidal properties, LVX reduces the virulence of *hvKp*, making it a perfect or even preferred antibiotic for the treatment or prevention of infections caused by *hvKp*.

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Disclosure

The authors report no conflicts of interest in this work.

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