Comparative analysis of Clinial Carbapenem-Resistant (CR), Hypermucoviscous (HM) and CR-HM *Klebsiella pneumoniae* isolates in China

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Research article

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Abstract

Background: Although carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and hypermucoviscous *K. pneumoniae* (HMKP) were largely non-overlapping, the recent emergence of CR-HMKP has raised great alarm in the world. We compared the molecular characteristics of CRKP, HMKP and CR-HMKP isolates.

Results: 220 cases of *K. pneumoniae* isolates was collected and identified between Jan 2015 and Dec 2016 from Renji Hospital. Carbapenem resistance test and string test were performed to screen CRKP, HMKP and CR-HMKP isolates. All the CRKP, HMKP and CR-HMKP isolates were investigated for capsular genotyping, virulence genes and resistance genes by PCR and DNA sequencing. Multilocus sequence typing (MLST) was used to characterize isolates sequence types (STs). Serum killing assay and mouse lethality assay were respectively performed to confirm the virulence of the isolates *in vitro* and *in vivo*. Of 220 *K. pneumoniae*，71 HMKP, 84 CRKP and 8 CR-HMKP were identified. Resistance rate to carbapenems was significantly higher in CRKP than HMKP and CR-HMKP. For MLST and serotyping, ST23 (26.8%)，K1 (33.8%) and K2 (23.9%) serotypes were the most common in HMKP isolates while ST11 (84.5%, 100%) and K-nontypable (91.6%, 100%) were the predominant types in CRKP and CR-HMKP isolates. The existence of virulence genes *rmpA*, *magA* and *iutA* was significantly higher in HMKP while the prevalence of resistance gene bla_{KPC-2} was higher in CRKP and CR-HMKP. Virulence test *in vivo* and *in vitro* both showed the lower virulence of CRKP and CR-HMKP compared to HMKP.

Conclusions: In spite of low virulence, the emergence of CR-HMKP indicates a confluence of hypermucoviscous phenotype and carbapenem resistance. Furthermore, the similar molecular characteristics between CRKP and CR-HMKP suggested that CR-HMKP might evolve from CRKP.

Background

Klebsiella pneumoniae familiar by clinicians is capable of causing pneumonia, bacteremia, pyogenic liver abscess, and urinary tract infections [1]. The ability of acquiring new gene elements contributes to its ongoing evolution. As a result, two pathotypes termed carbapenem-resistant K. pneumoniae (CRKP) and hypermucoviscous K. pneumoniae(HMKP)were the dominant circulating pathogens in the past few decades [1, 2].

In 1996, the first K. pneumoniae producing Klebsiella pneumoniae carbapenemase (KPC) type carbapenemase was identified in North Carolina [3]. Since then, New Delhi metallo-β-lactamase-1 (NDM-1), Imipenemase(IMP) and Verona Integron-Mediated Metallo-β-lactamase (VIM) types carbapenemase have been found worldwide [4]. Infections caused by CRKP mainly occurred in health care setting and have high rates of morbidity and mortality due to the scarcity of effective treatments [5]. According to a work report by CDC in 2013, CRKP infections caused 11% of nosocomial K. pneumoniae infections, equaling 7,900 infections and resulted in 520 deaths based on a 2011 survey of 183 hospitals in the United States [6].

In 1986, the first hypervirulent K. pneumoniae (hvKP) causing liver abscess (LA) was identified in Taiwan, and it has been increasingly reported globally [7]. HMKP/hvKP isolates exhibit a striking capacity to cause life-threatening community acquired infection among young and relatively healthy people, with diabetes being the major risk factor[8]. Furthermore, hvKP strains possess a propensity of causing metastatic infections, such as necrotizing fasciitis, endophthalmitis, and meningitis [9]. With the increasing incidence of hvKP infection, hvKP infection has been recognized not only in Asia, but also in Europe [10], South America [11], Australia [12] and North America [13–15].

Most recently, the confluence of carbapenemase resistance determinants possessed by CRKP and virulence factors possessed by hvKP in the same strain has resulted in the emergence and spread of carbapenem-resistant HMKP/hvKP (CR-HMKP/CR-hvKP), which is a even greater challenge faced by clinician in the near future [16].

However, significant knowledge gaps exist on clinical and molecular characteristics comparison of CRKP, HMKP and CR-HMKP. In this study, we conducted a retrospective comparative analysis of CRKP, HMKP and CR-HMKP strains collected from Renji Hospital from January 2015 to December 2016.

Results

Isolates Characteristics

A total of 220 K. pneumoniae strains were collected in Renji hospital from January 2015 to December 2016. 32.3% (71/220) isolates were HMKP, 38.2% (84/220) were CRKP and 3.6% (8/220) were CR-HMKP. The strains were mainly isolated from sputum (CRKP 53.6% vs HMKP 38% vs CR-HMKP 75%), blood (CRKP 13.1% vs HMKP 21.1%, vs CR-HMKP 0%), liver abscesses (CRKP 0% vs HMKP 5.6% vs CR-HMKP12.5%). Community-associated infections were mainly involved in HMKP (35.2%) than CRKP (21.4%) and CR-HMKP (12.5%), while CRKP and CR-HMKP were mostly detected in health-care setting ((87.5%, 78.6%) compared with HMKP (64.8%).

Antimicrobial Susceptibility Testing

Notably, the prevalence of CRKP and CR-HMKP strains exhibiting resistance to carbapenem antimicrobial was higher than that of HMKP (Fig. 1). All of the HMKP were sensitive to the three agents while all the CRKP and CR-HMKP were resistant to them. For HMKP, CRKP and CR-HMKP, MIC50 /90 of imipenem was 0.25/0.5, 128/128 and 128/256,MIC50/90 of meropenem was 0.125/0.125, 128/256 and 128/256, MIC50 /90 of ertapenem was 0.125/0.125, 256/256, 256/256.

Serotyping, MLST profiles and virulence-associated genes

To identify the serotype of 71 HMKP isolates, PCR was performed using primers for K1, K2, K5, K20, K54 and K57 serotypes. A total of 53 (74.6%) were positive for K1, K2, K20 and K57. Twenty-four (33.8%) isolates displayed the K1 serotype, 17 (23.9%) isolates showed K2 serotype, while 6 (8.4%) isolates were K20 and 6 (8.4%) isolates were K57. However, no K5 or K54 serotype was found. MLST analysis identified 22 STs among the 71 HMKP. The most prevalent ST in HMKP was ST23 (26.8%, 19/71), followed by ST65 (12.7%，9/71), ST11(5.6%，4/71) and ST86 (4.2%，3/71). There was strong association between STs and K serotypes. Sixteen of 24 (66.7%) K1 isolates belonged to ST23. Among the 17 K2 isolates, 58.8% (10/17) were ST65 and 23.5% (4/17) were ST375. Virulence genes *rmpA*, *magA* and *iutA* were found in 88.7% (63/71), 35.2% (25/71) and 84.5% (60/71) HMKP isolates respectively. Notably, all the *magA* positive HMKP were K1 serotype.

For 84 CRKP, 4.8% (4/84) isolates showed serotype K2 and 3.6% (3/84) were serotype K20 and the others (91.6%) were K-nontypable. MLST analysis showed 84.5% (71/84) were ST11, followed by ST15 (4.76%, 4/84). Furthermore, another 9 isolates displayed 6 different STs, including ST15, ST1869, ST323, ST45, ST709, ST722 and ST332. 1.2% (1/84) *rmpA*, 0% *magA* and 2.4% (2/84) *iutA* were positive among CRKP isolates. 85.7% (72/84) were blaKPC-2 positive.

All the 8 CR-HMKP isolates produced KPC-2 carbapenemase and belonged to ST11 by MLST while they were all K nontypable. Virulence genes *rmpA*, *magA* and *iutA* were found in 37.5% (3/8), 0 and 25% (2/8) CR-HMKP isolates.

Serum Killing

Seven respective HMKP, CRKP and CR-HMKP isolates were randomly selected to perform serum killing to compare the ability of anti-phagocytosis. Survival rates of HMKP, CRKP and CR-HMKP isolates were evaluated in the presence of normal human serum over a 2-h period (Fig. 2). HMKP isolates exhibited markedly higher survival rates in the presence of normal human serum compared with CRKP and CR-HMKP.

Mouse Lethality Assay

Mouse lethality assay showed that the LD_{50} of all the 7 CR-HMKP and CRKP strains was $> 10^6$ CFU, which indicated very low virulence while LD_{50} of HMKP was less than 5×10^4 CFU. Furthermore, survival test indicated that mice treated with CRKP and CR-HMKP significantly enhanced survival compared to that for HMKP treated mice (Fig. 3a). Finally, mice sacrifice their life using 100% CO_2 for CFU in blood and liver. As results, CRKP and CR-HMKP treated mice exhibited reduced bacterial burden (CFU) in blood and liver compared to HMKP-treated mice (Fig. 3b and c).

Discussion

The retrospective study was conducted in 220 K. pneumoniae culture-positive patients hospitalized during the period of January 2015 to December 2016 in the Renji Hospital. Analysis of the molecular characteristics indicated that the proporation of CRKP and HMKP were both increasing. In our study, 71 HMKP among 220 K. pneumoniae isolates (32.3%) were identified. Since the emergence of CR-HMKP, several CR-hvKP infections have been reported in China[16, 17]. In this study, we found 8 (3.5%) K. pneumoniae exhibiting both hypermucoviscosity and carbapenem resistance, termed as CR-HMKP. This finding, together with several reports about of CR-hvKP or CR-HMKP [16–18], suggests CR-HMKP has become an important pathogen in cases of bacteria infection in China.

hvKP were often susceptible to antibiotics while CRKP carrying ESBLs or carbapenemase always showed highly resistant to antibiotics. In our study, all of the HMKP were sensitive to the three agents while all the CRKP and CR-HMKP were resistant to them. For CR-HMKP, MIC50 /90 of the three carbapenem antibiotics was up to128/128 which indicated high resistance of CR-HMKP. hvKP were often involved in ST23, ST65 and ST86 while CRKP mainly associated with CC258 (mainly ST11 and ST258). In our study, ST23 (26.8%) were the most common in HMKP isolates while ST11 (84.5%, 100%) were the predominant types in CRKP and CR-HMKP isolates which agrees with previous study [19, 20].

The emergence of CR-HMKP was the results of plasmid spread and bi-directional evolution between CRKP and HMKP. Whether carbapenem resistant clonal population evolved to be hypermucoviscous or the hypermucoviscous population evolved to be carbapenem resistant remains unknown. Giving the 8 isolates belonged to ST11 clones, KPC-producing, and lower virulence, it can be speculated that CRKP acquire virulence-harboring plasmid and became hypermucoviscous. Furthermore, Hypermucoviscosity was always been considered as a mark of hypervirulence. However, in our study, in vitro serum killing and mouse lethality assay both showed that the virulence of 7 CR-HMKP was low, which indicated that the use of the term hypermucoviscous to define the virulence of isolates has proven to be problematic. Our findings agree with the following views: not all hvKP strains are hypermucoviscous and some cKP has this phenotype [21, 22]. Lin YC et al. found that hypermucoviscosity-negative strains are more prone to cause severe infections than hypermucoviscous K. pneumonia [23]. Therefore, precise definition of hvKP is more important. Russo TA et al. proposed iuc and/or either rmpA or rmpA2 would be predicted to be the best markers to define hvKP [24]. However, we have 2 CR-HMKP both harboring iuc and rmpA which showed low virulence. In my opinion, the most accurate markers for defining hvKP should meet the criteria that the isolate will no longer hypervirulent if the factors are lost. With the discovery of more and more new virulence factors, defining the hvKP will be more difficult. Serotypes, sequence type and genomic background should be considered when defined hvKP.

Conclusion

In conclusion, our study revealed that the lower virulence of CR-HMKP compared to HMKP and high resistance compared to CRKP. Furthermore, CR-HMKP isolates belonged to ST11 clones, were KPC-producing and lower virulence, these properties indicated that CR-HMKP might evolve from CRKP. Hypermucoviscous was just a character of klebsiella pneumoniae but not a indicator of hypervirulence.

Accurate definition hvKP has a long way to go. A limitation of our study was that the small size of isolates and single center was investigated. Multiple-center and large sample are needed to illuminate the clinical characterization and pathogenicity of CR-HMKP strains.

Method

Bacterial strains

A retrospective study was conducted on 220 K. pneumoniae strains collected from Renji Hospital from January 2015 to December 2016. CRKP was defined as isolates non-susceptibility to any of imipenem, meropenem and ertapenem in accordance with the breakpoints of Clinical and Laboratory Standards Institute (CLSI) guidelines. HMKP was defined as positive “string test” isolates [25]. A inoculation loop is able to generate a viscous string of > 5 mm in length by stretching bacterial colonies on an agar plate was defined as positive string test[26]. CR-HMKP was defined as isolates harboring both carbapenem-resistance and positive “string test”. All the strains were confirmed using the VITEK 2 Compact system (bioMérieux, France).

Antimicrobial Susceptibility Testing And β-lactamase Characterization

Antimicrobial susceptibility testing was performed by the agar dilution method. Three frequently-used carbapenem agents including imipenem, meropenem, ertapenem were tested. The results were interpreted following the criteria of CLSI.

Serotyping and detection of virulence-associated and carbapenem resistance genes by PCR

Capsular polysaccharide synthesis (CPS) genotyping of K serotype-specific alleles for K1, K2, K5, K20, K54 and K57 were amplified by PCR as previously described [27]. The following virulence genes: rmpA, mucoviscosity-associated gene (magA), ferric iron uptake system genes (kfu, entB, ybtS), aerobactin (iucA), and fimbrial adhesions such as fimH and mrkD and carbapenem resistance genes such as bla_{KPC}, bla_{OXA-48} and bla_{NDM-1} were detected by PCR as previously described[28, 29]. The PCR products were sequenced and the DNA sequences obtained were compared with those available in the NCBI GenBank database using BLAST searches.

Multilocus Sequence Typing

Multilocus sequence typing (MLST) was performed as described previously [30]. Sequence of seven housekeeping genes was compared with that on the Pasteur Institute MLST website

(http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html).

Susceptibility To Serum Killing

Serum bactericidal activity was measured as previously described with minor modifications [31]. 0.1 ml early-log-phase cells, washed and suspended in 0.9% NaCl (ca.1 × 10^6 CFU/ml) bacteria were added to 0.1 ml pooled human serum from healthy volunteers for 30 min. Viable counts were obtained at 0 and 2 h of incubation at 37 °C on Mueller-Hinton agar. Each strain was tested at least three times.

Mouse Lethality Study

To determine the 50% lethal dose (LD_{50}) in a murine model, 6- to 8-week-old, male, pathogen-free BALB/c mice (body weight 20–25 g) were obtained from the Shanghai Jiaotong University School of Medicine Animal Center. Six mice were used as a sample population for each strain. Ten-fold serial dilution of cell-forming unit (CFU) of K. pneumoniae was made from 10^6CFU/ml to 10 CFU/ml. The BALB/c mice received eye intravenous injections with 0.1 ml suspension of each concentration. Inoculated mice were observed for 14 days for symptoms of infection. Survival of the inoculated mice was recorded daily. Finally, mice were euthanized using 100% CO_2 at a flow rate of 22 per minute.

Statistical analysis

Data was conducted with SPSS16.0 software (SPSS Inc., USA) and compared using 2-way ANOVA with Tukey's multiple-comparison test and the log-rank test. A value of $P < 0.05$ was considered to be statistically significant.

Abbreviations

CRKP: Carbapenem-resistant Klebsiella pneumoniae; HMKP: Hypermucoviscous Klebsiella pneumoniae; CR-HMKP: Carbapenem-resistant hypermucoviscous Klebsiella pneumoniae; MLST: Multilocus sequence typing; STs: sequence types; PCR: Polymerase Chain Reaction; KPC: Klebsiella pneumoniae carbapenemase; NDM-1: New Delhi metallo-β-lactamase-1; IMP: Imipenemase; VIM: Verona Integron-Mediated Metallo-β-lactamase; CDC: Centers for Disease Control; LA: liver abscess

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Shanghai Jiaotong University School of medicine (Shanghai, China). All procedures performed in studies involving human participants were in accordance

with the ethics standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethics standards.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Study design: JYZ and GYW Study conduct: GYW and QW. Data collection: ZS. Data analysis: QL and RPZ. Data interpretation: ML. Drafting manuscript: JYZ and GYW. Revising manuscript content: ML. Approving final version of manuscript: ML. JYZ take responsibility for the integrity of the data analysis. All authors read and approved the final manuscript.

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Figures

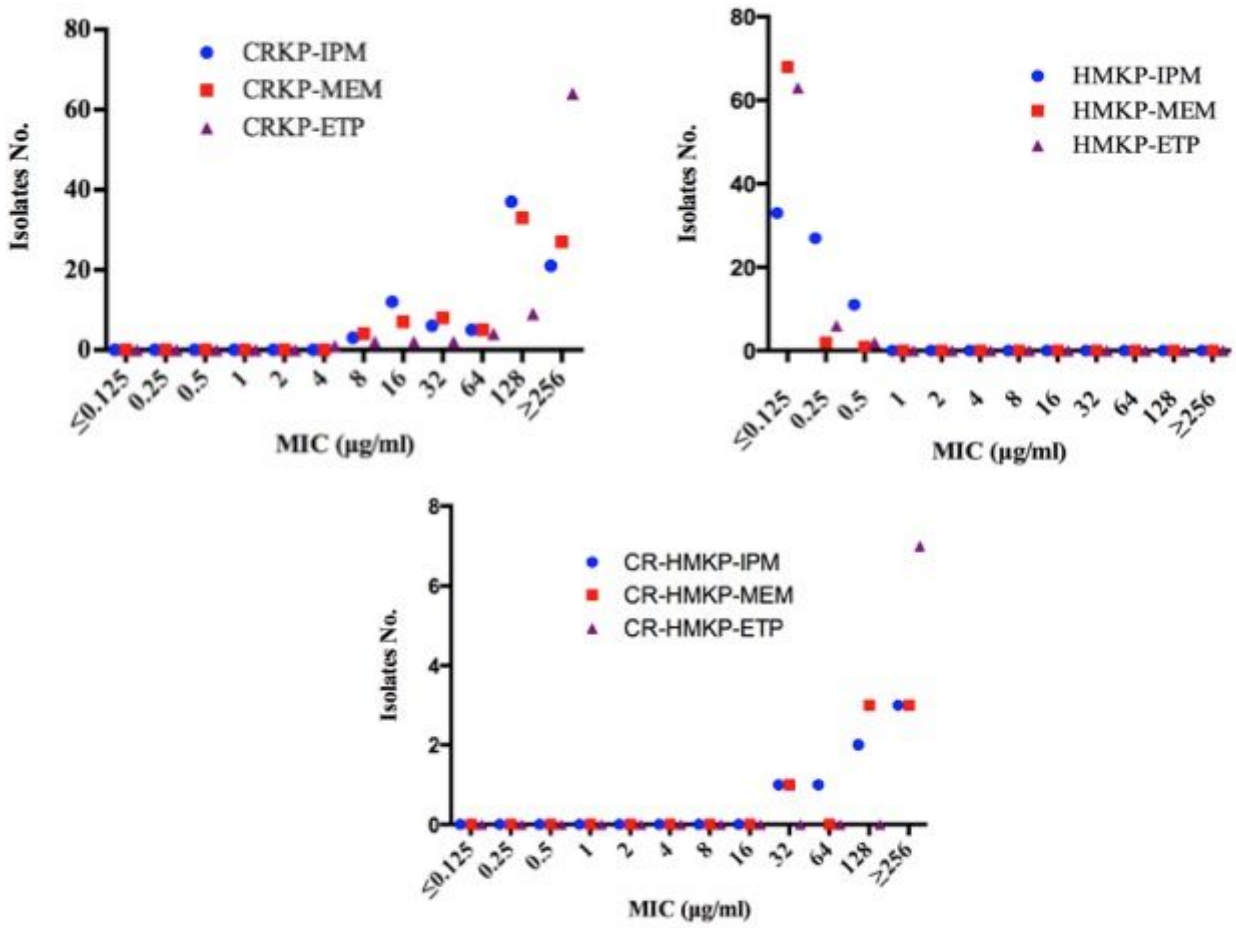

Figure 1

All the HMKP, CRKP and CR-HMKP isolates was performed susceptibility testing to imipenem, meropenem and ertapenem using agar dilution method. Isolates No. : Isolates number

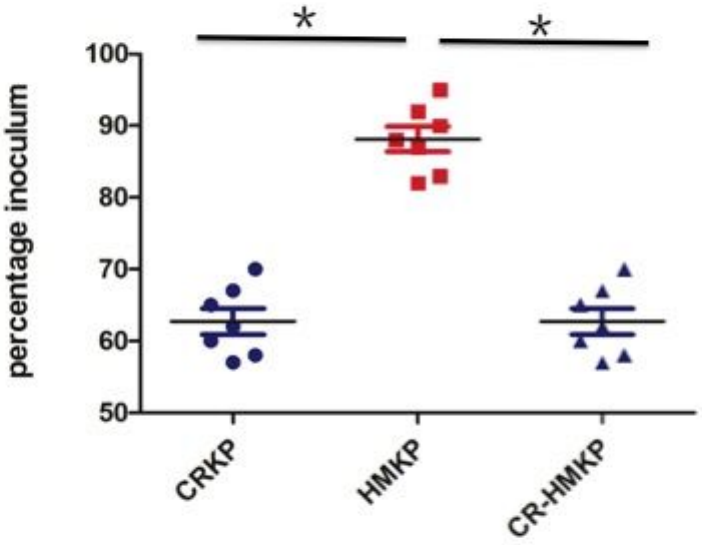

Figure 2

Serum resistance is represented as percent viability (no. of colonies at 2h of incubation/no. of colonies for the initial mixture)

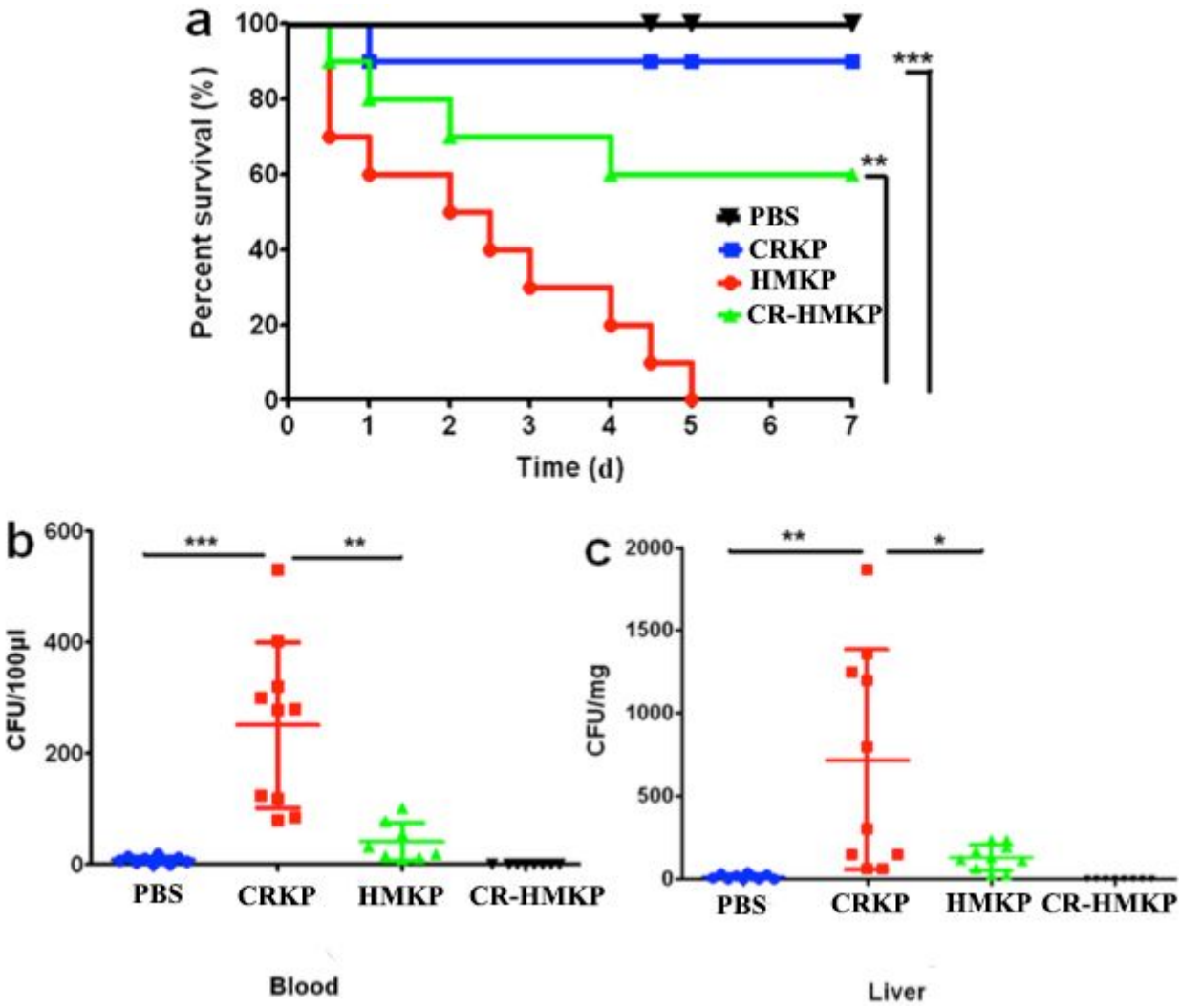

Figure 3

(a) Survival analysis of mice infected with CRKP, HMKP and CR-HMKP. (b and c) shown are CFU number in blood and liver at 24h postinfection. ***: p<0.001; **: p<0.05; *:p<0.01.

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