Research Grants Council

Areas of Excellence Scheme 2022/23 (Tenth Round)
Theme-based Research Scheme 2022/23 (Twelfth Round)
Full Proposal Application Form Template

PART I – Summary of the Research Proposal

1. Particulars of the project

(a)	Project title *(in both English and Chinese)*	Multi-disciplinary approaches to tackle the global public health threat of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* (多學科協作以解決高毒性和多耐藥肺炎克雷伯菌對全球公共衛生的威脅)
(b)	Theme	Theme 1-Promoting Good Health
(c)	Grand challenge topic	Topic 1-Infectious Diseases
(d)	Primary field code and area	Infection/Parasitology 1218
(e)	Secondary field code and area	Microbiology 1112
(f)	Third field code and area (if any)	
(g)	Project duration (months)	5 years
(h)	(i) Total direct project cost in the proposed budget ($M)	HK$ 50 million
	(ii) Total direct project cost from the RGC ($M)	HK$ 45 million
(i)	Proposed project start date	01/01/2023
(j)	Nature of application	*(* Please tick '√' as appropriate)* * ☐ New * ☑ Re-submission * ☐ Renewal Please specify the reference number of the most-recent previous application / on-going project: T11-103/21-R

3. Abstract of research comprehensible to a non-specialist

The Gram-negative bacterial pathogen *Klebsiella pneumoniae* has consistently evolved over the past two decades, generating genetic variants of mixed phenotypes of antibiotic resistance and virulence. Among them, the newly emerged multidrug resistant (MDR) and hypervirulent strains are of particular concern. To date, *K. pneumoniae* has already become the most frequently isolated bacterial pathogen in hospital settings and the most common pathogen that causes blood stream infections, with mortality rate of over 40% recorded in various countries. However, the threat imposed by new variants of this old pathogen has not been recognized. A thorough understanding of the on-going evolution trend, transmission dynamics and pathogenic mechanisms of this notorious pathogen is essential for development of effective intervention strategies to prevent occurrence of a hypervirulent and MDR *K. pneumoniae* pandemic. To this end, we propose to adopt comprehensive, multidisciplinary study approaches to depict the genetic basis of the varied phenotypic features of these organisms and identify molecular markers for design of effective methods to differentiate between high and low risk strains, and develop novel therapies to treat MDR and hypervirulent *K. pneumoniae* infections. As an internationally recognized team in *K. pneumoniae* research supported by the HMRF, GRF, CRF and RIF grants, we conducted the first national surveillance of CRKP in China, identified the first cases of ST23 type hypervirulent and

carbapenem-resistant *K. pneumoniae*, discovered the first ST11 and hypervirulent and carbapenem-resistant *K. pneumoniae* strain, and recently reported the first conjugative virulence plasmid that augmented the virulence level of *K. pneumoniae*. We have already established a collection of 7000 clinical strains and identified novel drug candidates that exhibit high efficacy, both *in vitro* and *in vivo*, in eradicating both multidrug and hypervirulent variants of *K. pneumoniae*. Support by the Theme-based Research Grant is necessary for implementation of this strategic research plan to devise feasible pre-emptive approaches to halt global dissemination of key pathogenic *K. pneumoniae* variants and protect human health.

PART II – Details of the Research Proposal

1. (a) Mission, goals and deliverables

(i) Mission

To better understand and address the rapidly aggravating global public health and clinical problems caused by hypervirulent and MDR *K. pneumoniae* strains through development of novel diagnosis, infection control and treatment strategies.

(ii) Goals

(I) To identify the risk factors of infection and clinical burden (in net mortality and co-morbidities with MDR and hypervirulent strains;

(II) To establish a strain and genome database containing retrospective and prospective *K. pneumoniae* strains recovered from healthy human populations, hospital patients, and agricultural and the environment samples collected in China and Hong Kong SAR. This database will provide essential information and materials

(III) to decipher the mechanisms of evolution and routes of transmission of hypervirulence / MDR-encoding clinical *K. pneumoniae* strains and

(IV) to decode the molecular basis of persistence and hypervirulence of clinical *K. pneumoniae* strains, so that we can utilize these basic research data

(V) to develop rapid diagnostic assays to differentiate between high and low-risk *K. pneumoniae* strains for timely and effective treatment and

(VI) to develop novel control measures and therapies to negate the clinical impact of hypervirulent and MDR *K. pneumoniae.*

(iii) Deliverables

(I) a strain and genome database covering more than 12,000 retrospective and prospective *K. pneumoniae* strains recovered from clinical patients, agricultural samples and various environmental niches in China and Hong Kong SAR;

(II) a panel of molecular targets which play key functional roles in mediating or enhancing resistance, virulence and persistence of *K. pneumoniae*; the genes concerned will be regarded as key targets for development of effective therapeutic approaches and rapid diagnostic assays;

(III) a rapid diagnostic assay to differentiate between high and low-risk *K. pneumoniae* infections to guide effective clinical treatment;

(IV) a series of novel therapies targeting the resistance and virulence determinants of *K. pneumoniae*.

1. (b) Pathways to Impact Statement *(should not exceed two A4 sides)*

Our research team performs world-leading research in the field of antibiotic resistance and virulence of various bacterial pathogens including *Klebsiella pneumoniae*, and have made a series of important discoveries in recent years regarding the evolutionary changes that resulted in the emergence of novel resistant and virulent strains of *K. pneumoniae*. We were the first research team to report that HvKP strains had evolved further to become carbapenem-resistant or CR-HvKP strains in 2015[1]. We also discovered that CRKP strains have evolved to become hypervirulent CRKP or Hv-CRKP[2]. More recently, we reported the first conjugative virulence plasmid in *K. pneumoniae* and predict that emergence such plasmid may cause rapid transmission of phenotypic high level virulence among clinical *K. pneumoniae* strains[3]. In fact, we are the first research team

which foresees and warns of an imminent threat of drug resistant and hypervirulent *K. pneumoniae* strains to mankind.

To our knowledge, we are also the only research team in the world which possesses the necessary knowledge and materials for investigating the molecular basis of rapid evolution and transmission of various resistant and virulent strains of *K. pneumoniae*. The proposed work is to pinpoint the root of the problem, which we believe involves dissemination of specific plasmids and specific *K. pneumoniae* strains which have acquired such plasmids. Our proposal is therefore organized in such a way that basic research findings are generated and utilized to devise feasible intervention approaches to solve problems related to *K. pneumoniae* infections. We will first identify key genes that encode the phenotypic features, namely high transmissibility, multidrug resistance and high-level virulence, of the strains concerned, so that we can utilize these data to devise effective diagnostic, infection control and therapeutic approaches to contain problems associated with infections by high risk *K. pneumoniae* strains.

Hong Kong has a special place in *K. pneumoniae* research. Our data indicate that various multidrug resistant and hypervirulent strains of *K. pneumoniae* actually originated from China, but comprehensive epidemiological and molecular data of these strains are currently not available. Prevalence of these newly emerged resistant and virulent strains in other countries remains relatively low but is increasing, hence the problem is increasingly being recognized and even given the term '*Klebsiella* plague' in the US. Nevertheless, the nature and the scale of the problem are still not fully understood in the scientific and medical communities in Western countries. To our knowledge, no research team in China and the Western world has the ability or plan to undergo systematic research in the area to prevent the problem from worsening. We are therefore obliged to initiate a research programme to provide new and vital information that facilitates design of effective approaches to prevent the newly emerged resistant and virulent strains of *K. pneumoniae* from further dissemination or causing fatal infections.

The proposal builds on our existing strength in performing molecular and functional analysis of *K. pneumoniae* variants. We collaborate with clinicians in mainland China and Hong Kong so that we can obtain first hand materials and clinical data for monitoring the latest evolution trend of such strains. Our laboratory has extensive experience in genomic and whole plasmid sequencing, as well as performing in depth bioinformatics analysis of the sequence data; we are therefore highly confident that we can identify genetic elements that are responsible for encoding the phenotypes of the high-risk strains. This is important for future drug development as well as for design of effective diagnostic technique to differentiate between high and low risk (or classic) *K. pneumoniae* strains. It should be emphasized that rapid identification of the high-risk strains in hospitals, especially the hypervirulent strains, is important because aggressive and timely treatment is needed to prevent invasion of such strains into the blood stream and cause fatal bacteraemia. In addition, we collaborate with chemists in University of Hong Kong to search for and design new antimicrobial compounds that are effective against the resistant and virulent *K. pneumoniae* strains. At this stage, we have already identified a number of promising drug candidates. These drug candidates represent various approaches to treat infections caused by *K. pneumoniae*. The first type is novel KPC-2 inhibitors. Caftazidime/avibactam is the current last line treatment for CRKP infections and widely used clinically worldwide. It is not only very expensive, but also become ineffective due to the increasing development of resistance in CRKP by the emergence of various KPC-2 mutations. A new class of KPC-2 inhibitor will be needed when current one become inactive. The second type are novel antibiotics targeting the new drug target, bacterial KsdA, that is involved in LPS synthesis and novel antibiotics identified through our genome mining approach. The third type are novel inhibitor of RNA polymerase, which works synergistically with rifampicin to prevent the development of resistance for this types of antibiotics. All these novel drug candidates will provide effective treatment for infection cause by CRKP/HvKP. It should also be noted that these drug candidates are actually FDA-approved drugs used for purposes other than treatment of bacterial infections, and should have the high potential to be developed into clinical antibiotics.

K. pneumoniae is a member of normal flora that resides in the human gut microbiome and may cause opportunistic infections, especially in elderly and immunocompromised patients. We should stress that emergence of multidrug resistant and hypervirulent strains of this potential pathogen poses a grave threat to human health because such strains may start to reside in the gut of even healthy individuals. This is not a local problem but a worldwide public health issue. We believe our proposal represents the most comprehensive and most feasible plan to halt transmission of multidrug resistant and hypervirulent *K. pneumoniae* strains and combat fatal infections caused by such strains. Specifically, we intend to introduce a new diagnostic test for rapid identification of high-risk strains in hospitals in 1-3 years after commencement of this research programme. This will enable clinicians to isolate infected patients and prescribe appropriate drug regimens to prevent occurrence of bacteraemia. In the medium term (4-6 years), antimicrobial drug regimens effective against a wide range resistant and hypervirulent *K. pneumoniae* strains will be available so that mortality due to *K. pneumoniae* infections can be drastically reduced. In the long term, more effective drugs targeting to specific resistance and virulence determinants, developed on the basis of our findings in the proposed work, will be available for more effective eradication of resistant and hypervirulent *K. pneumoniae* strains from clinical settings and the community. Introduction of a new diagnostic test and new therapies will tremendously reduce the workload of health care providers by significantly reducing the number of serious hospital infections, and saves lives. Benefits associated with successful prevention of drug resistant and hypervirulent *K. pneumoniae* infections from becoming an endemic disease or causing a worldwide pandemic are immeasurable.

To conclude, we truly believe that approval for funding of our proposed study, which will help prevent occurrence a *K. pneumoniae* pandemic in Hong Kong, China or other countries, would in the future be regarded as a wise and timely investment that saves countless lives of our own and our future generations. In other words, failure to conduct the proposed works, for whatever reasons, would be associated with grave consequences.

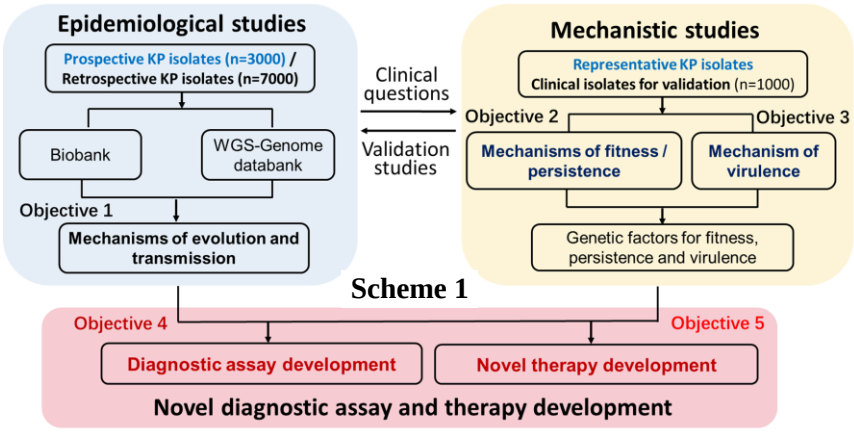
1. (c) The approach and methodology to be employed to achieve the mission, goals and deliverables

Background of study: *Klebsiella pneumoniae* has become the most important pathogen that causes hospital infections in recent years, especially in Asian countries[4-6]. Our surveillance data in China and reports from other parts of the world also show that *K. pneumoniae* is the most common clinically isolated bacterial pathogen and the most common bacterial pathogen that causes blood stream infection [7,8]. The World Health Organization describes MDR *K. pneumoniae* as a 'critical priority 1 organism" and the leading global pathogen that causes infant sepsis (https://barnards-group.com). It has been listed as the most common bacterial pathogen that causes blood stream infection and the bacterial species that causes infections of the highest mortality rate. It should be noted that bacteraemia and death due to *K. pneumoniae* infection was extremely rare in the past. This change has been attributed to the rapid evolution of this pathogen in the past decade. In particular, certain types of classic *K. pneumoniae* (cKP) such as ST23 type have already evolved into hypervirulent *K. pneumoniae* (HvKP). First recovered from patients with liver abscess in Taiwan in late 1980s, HvKP is known to cause severe community-acquired infections in relatively young and healthy hosts. HvKP strains were also responsible for infection in a number of unusual body sites, such as meningitis, necrotizing fasciitis, endophthalmitis, epidural abscess, osteomyelitis, and a variety of non-hepatic abscesses[9,10]. These clinical presentations were in the past rarely associated with members of Enterobacteriaceae, except in hosts who were severely immunocompromised. The emergence of HvKP was due to acquisition of a hypervirulence plasmid such as the pLVPK-like plasmid[11]. The non-conjugative nature of the virulence plasmid has limited the rate of emergence and dissemination of HvKP in the past, when all HvKP strains were confined to the K1/K2 type *K. pneumoniae*[10].

On the other hand, certain types of cKP such as ST11 and ST15 in Asia and ST258 in Western countries have also evolved into hyper-resistant *K. pneumoniae* such as carbapenem-resistant *K. pneumoniae* (CRKP), which is also a clinically important pathogen which often causes untreatable

or hard-to-treat infections, and is considered an urgent threat to human health by the US CDC[12]. Recently, we and other research groups have recovered colistin, tigecycline and/or ceftazidime-avibactam-resistant *K. pneum*oniae strains in human, animal and environmental samples (**Fig 1**)[13-15]. We have formed a team composed of renowned experts on bacterial MAR studies and *K. pneumoniae* studies. Our team has made three of the most significant discoveries in AMR history namely the *bla*$_{NDM-1}$ gene, *mcr*-1 gene and hypervirulent and carbapenem-resistant *K. pneumoniae* (CR-HvKP)[2,16,17]. In the past few years, we have been engaged in *K. pneumoniae* studies and have witnessed the rapid evolution trends of this pathogen such as emergence of K1 CR-HvKP, ST11 CR-HvKP, *mcr-1*-bearing KP, *tet*A/X-bearing KP and the first conjugative plasmid that encodes both carbapenem resistance and hypervirulence[1,14,18-20]. However, our understanding of this important pathogen apparently lags far behind its evolution rate. It is therefore necessary to investigate the mechanism of rapid evolution and global transmission of major clones, and the mechanisms of resistance, persistence and virulence of *K. pneumoniae*, in order to develop rapid diagnostic assays to differentiate between high-risk infections from those caused by classic (cKP) or low-risk strains to guide timely treatment, and develop novel therapies to treat MDR and hypervirulent *K. pneumoniae* infections. The expected findings of the proposed works shall provide essential data for development of new diagnostic assays and therapeutic approaches to halt and treat potentially fatal *K. pneumoniae* infections.

Overall research plan: Problematic clinical infections caused by *K. pneumoniae* are attributed to the high-level antimicrobial resistance (AMR) and hypervirulence phenotypes of such strains, as well as efficient transmission and persistence of the resistance and virulence plasmids that they harbor. This proposal aims to construct the first and the most comprehensive Biobank and genome databank of over 10,000 strains of *K. pneumoniae* collected in clinical setting, animal and environmental niches in China and Hong Kong SAR, which will provide essential materials to investigate the evolution and transmission of HvKP and MDR *K. pneumonaie* (Objective 1), genetic basis of fitness and persistence of resistant and hypervirulent *K. pneumoniae* strains (Objective 2) and delineate the key cellular mechanisms of hypervirulence (Objective 3). These hypothesis-driven basic research projects will help identify cellular targets for development of rapid detection assays to differentiate between high and low-risk infections caused by *K. pneumoniae* (Objective 4) and novel target-specific therapies to combat *K. pneumoniae* infections (Objective 5). These intervention measures will eventually be validated using the Biobank and genome databank established in Objective 1. The proposed work, which comprises these five inter-related components, is outlined in **Scheme 1**.



Scheme 1

Objective 1: To establish a strain biobank and genome databank, and decipher the evolutional and transmission mechanisms of MDR and hypervirulent *K. pneumoniae*

Our research team has dedicated much effort in *K. pneumoniae* research in the past few years. We performed the first nationwide surveillance of CRKP in China, Hong Kong SAR and other parts of the world and have since established arguably the largest collection of clinical *K. pneumoniae* strains in the world. This collection comprises 3000 clinical *K. pneumoniae* strains (including both MDR and susceptible strains) recovered in the past 26 years (1994 to 2021) from one hospital in China, 4000 clinical *K. pneumoniae* strains (both MDR and susceptible strains) collected from 10 provinces in China during the period 2014-2021, and 500 clinical strains collected in different hospitals Hong Kong. Clinical data including treatment outcome, antibiotic prescriptions and comorbidity etc. are also available for a significant proportion of these clinical strains. This comprehensive strain collection has provided us essential materials and a preliminary

but broad overview of the problem, so that we can design and initiate appropriate experiments to depict the mechanisms of evolution and transmission of various types of clinical *K. pneumoniae* strains. In this project, we will continue to collect *K. pneumoniae* strains from various settings prospectively, characterize these strains so that we can compare to previous and existing strains and depict the latest evolution trends of MDR and hypervirulent *K. pneumoniae*. The study approaches are described below.

Isolation plan

1-I: ***Strains and clinical data collection.*** We shall collect another 3000 clinical *K. pneumoniae* strains, including both MDR and susceptible strains, from fecal, respiratory, urine and blood samples from hospitals in 10 different provinces in China and two municipal cities, Beijing and Tianjin, as well as 500 clinical isolates from Hong Kong SAR, for comparative analysis with strains collected in the past (**Scheme 2**). Clinical data including treatment outcome, antibiotic prescriptions and comorbidity etc. of the patient concerned will be recorded for each strain. In addition, one each of chicken and cattle farms in each of 10 provinces in China will be selected for collection of animal samples, and water and soil samples adjacent to the farms in order to isolate more than 2000 animal-borne and environmental *K. pneumoniae* strains. Information on farm management and antibiotic usage of each farm will also be collected. The main purpose of the prospective study is to understand the interactive evolution-promoting factors for *K. pneumoniae* that reside in human, animals and the environment, and identify the origins of virulent and multidrug resistant strains. Our goal is to expand our current strain collection to the size of over 12,000 strains from various sources. These strains will be used to create a *K. pneumoniae* Biobank with all the isolates centrally located in a barcoded storage system housed in a single location in Hong Kong, with back-up stocks placed in a second location. Such Biobank can be used to screen drugs / antibodies / phage etc. and generate a series of datasets for comparative analysis such as MIC, string test results, source of isolation, clinical information and genomic features. This Biobank shall also provide materials for testing the efficacy of the novel detection assays and therapies to be developed in the proposed works described in Objective #4 and #5, hence such materials are highly valuable resource that pharmaceutical companies worldwide may need for screening of antimicrobial agents in the future.

Scheme 2

1-II: Whole genome sequencing and bioinformatics analysis. All strains in the Biobank will be subjected to whole genome sequencing using the Illumina platform and in-depth bioinformatics analysis. Firstly, pangenome analysis will be performed using Panaroo to identify core and accessory genes [21]. Secondly, core gene alignment will be performed using MAFFT [22] , followed by construction of maximum likelihood species-wide tree using IQ-TREE [23]. Within each clonal group, mapping-based SNV calling and recombination filtering will be performed using SNVPhyl [24] to construct high-resolution phylogenies. Interactive tree of life (iTOL) v3 (http://itol.embl.de/) will be applied to modify and visualize the generated phylogenetic tree [25]. Information regarding carriage of different MDR plasmids and virulence plasmids by the test strains will be obtained through blast and alignment with known plasmids. Representative strains will be subjected to third generation sequencing, using the Nanopore platform, to obtain their complete genome and plasmid sequences using the Unicycler tool[26]. Long read only data will be assembled using Canu [27], Flye [28] and Trycycler [29] respectively, and polished by Racon [30] and Medaka [31]. All data will be deposited into the genome databank for further use and specific analysis.

1-III: Construction of genome databank of K. pneumoniae in China and Hong Kong SAR. Upon establishment of a Biobank of *Klebsiella pneumoniae* strains, an affiliated genome databank of *K. pneumoniae* strains, KPGD, will also be constructed with genomic data generated from analysis of strains deposited in the Biobank. KPGD will therefore include over 12,000 *K. pneumoniae* isolates recovered from animals, clinical and environmental settings. For each isolate, genomic information on raw illumina reads, draft genome, annotated genome, virulence genes,

AMR genes, other important genes such as plasmid replicon, ST types and other genetic traits will be obtained; phenotypic information including MICs of various antibiotics, string test results or other virulence data, as well as information on source, isolation site, isolation date; patient's clinical record including treatment and clinical outcome will also be collected and deposited into the databank. The databank will be created by a IT specialist in our team whose expertise includes website and database development. The KPGD database will initially be used internally and the database domain will be built in the server located in our laboratory but moved to the public domain after it is completed and validated. Clinical data will be aligned with MDR profiles and virulence phenotypes using regression statistical models, including OR and HR values. This databank will allow us to perform comparative analyses to depict the evolution trend of this important bacterial pathogen and identify high risk strains.

1-IV: Mechanisms governing the evolution and transmission of MDR and hypervirulent K. pneumoniae. Data generated by our team and other groups have depicted an increasing prevalence of MDR and hypervirulent *K. pneumoniae* in various samples collected from the healthy human population, animals and the environment, suggesting that active evolution and transmission of MDR and hypevirulent *K. pneumoniae* have already occurred in multiple settings. We recently discovered the first conjugative virulence plasmid in *K. pneumoniae*[3], analysis of which allowed us to identify one key evolution path of CR-HvKP strains in clinical settings. However, this conjugative virulence plasmid is not commonly present in CR-HvKP strains, suggesting that other mechanisms that mediate the evolution of CR-HvKP exist. In this proposal, we shall investigate the evolution mechanisms of CRKP, HvKP and CR-HvKP strains. The first emerging clinical isolates of CRKP, HvKP and CR-HvKP, which are housed in our strain collection, will be subjected to whole genome and plasmid sequencing to identify the plasmids associated with these early strains. *K. pneumoniae* isolates that carry the virulence plasmid based on the whole genome sequence data will be subjected to conjugation study and complete plasmid sequencing to identify potentially conjugative virulence plasmid(s). Screening of similar conjugative plasmids will be conducted in our genome databank to depict the transmission dynamics of such conjugative virulence plasmids among clinical *K. pneumoniae* strains. A phylogenetic tree will be built to decipher the transmission of specific clone in a temporal and spatial manner in China including Hong Kong SAR. In addition, we will also investigate the evolution and transmission dynamics of colistin-resistant (mediated by *mcr-1* and its variants) and tigecycline resistant (*tet*(A), *tet*(X) variants, *temxCD-top1*, as well as ceftazidime/avibactam resistant KPC-2 variants (KPC-33 and KPC-93 in China) and their encoding plasmids. These studies will help delineate the evolution and transmission paths of MDR and hypervirulent *K. pneumoniae*, and the future evolution trends of such strains, to facilitate design of intervention strategies.

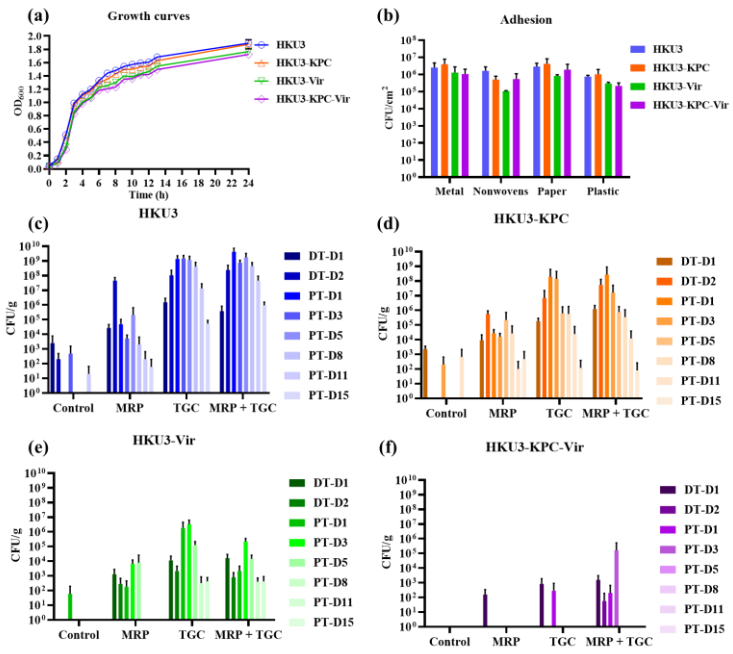


Figure 1. Adaption and fitness of virulence and resistance plasmids. *(a) growth curve; (b) competition during passages.survival of HKU3(C), HKU3-KPC(d), HKU3-Vir (e) and HKU3-KPC-Vir (f) in rat GI tracts with and without treatments of tigecycline and/or carbapenems. 3.3 is inoculation date, 3.4 is the treatment date; 3.5 is one day following treatment, and 3.19 is 14 days following treatment.*

Objective 2: To decipher mechanisms underlying the fitness and persistence of hypervirulent and/or MDR clinical *K. pneumoniae* strains

Evolution of CRKP, HvKP and CR-HvKP is known to be mediated by various plasmids, in particular the pLVPK like virulence plasmids and *bla*$_{KPC-2}$-bearing plasmids. In recent years, the prevalence of CRKP strains

increases consistently, reaching more than 50% of *K. pneumoniae* infections in certain regions of Asia including China; among the non-CRKP strains, HvKP has increasingly become more prevalent than cKP strains[32]. Most importantly, a current surveillance study conducted in different global regions showed that the prevalence of CR-HvKP increased dramatically in recent years and reaching to ~60% in China[33]. This is consistent with our data in an even larger scale surveillance in China, in which the prevalence of CR-HvKP strains was found to increase from 10~63% during 2016~2020 [34]. In addition, we have analyzed the WGS of over 1000 clinical CRKP strains isolated from 2010~2020 from our collection and found that ~95.6% of these strains carried a plasmid of IncFII replicon, which aligned well to pSWU01 (NZ_CP018455.1), a *bla*$_{KPC-2}$-bearing plasmid in CRKP strains. This suggests that pSWU01-like *bla*$_{KPC-2}$-bearing plasmid is the major plasmid that mediated the evolution of CRKP in China. These observations suggested that CRKP, HvKP and CR-HvKP adapted well to the hospital environment and the human body and have become persistent in these niches. This study aims to investigate molecular mechanisms underlying the fitness and persistence of CRKP, HvKP and CR-HvKP as follow.

2-I: Impact of bla$_{KPC-2}$-bearing plasmid and virulence plasmid on phenotypic fitness of K. pneumoniae. To investigate the impact of *bla*$_{KPC-2}$-bearing plasmids and virulence plasmids on clinical fitness and persistence of *K. pneumoniae*, isogenic strains of classic *K. pneumoniae* (cKP), CRKP, HvKP and CR-HvKP have been created including a cKP strain, HKU3, a ST11 clinical strain, a CRKP strain (HKU3-KPC, HKU3 carrying pSWU01-like, *bla*$_{KPC-2}$-bearing plasmid), a HvKP strain (HKU3-Vir, HKU3 carrying pLVPK like plasmid) and a CR-HvKP strain (HKU3-KPC-Vir, HKU3 carrying both palsmids). Growth and competition assays showed that acquisiiton of *bla*$_{KPC-2}$-bearing plasmid has no effect on fitness, whereas the virulence plasmid exerted minor fitness costs (**Fig 1a, b**). These strains will be used to test the effect of enviromental factors on the fitness of these strains, different set of isogenic strains will be subjected to the following phenotypic characterzation experiments. First, these strains will be tested in Phenotype MicroArrays for Microbial Cells (BIOLOG, PM1-PM20). The purpose of this experiment is to test the differential responses of these strains to nutrients, osmotic and ionic conditions, a range of pH and different inhibitory compounds. The data will be analysed to determine how changes in the ability to adapt to different enviromental conditions affect the ability of these organisms to survive and cause infections in the hospital environment. Factors identified by phenotype microassay will be further tested and confirmed individually. Combinations of different related factors that could mimic different environemtal condisitons will also be tested and analyzed. The data will provide us information on how the virulence plasmid affects adaptation of CRKP, HvKP and CR-HvKP strains to different environmental conditions, especially those related to hospital environment, the human GI tract and the lungs. In addition, we will also investigate if acqusition of these plasmids favors the competitiveness and survival fitness in hospital environments such as various surfaces, bedding materials, and exposure to various unfavorable conditions and chemicals such as antibacterial detergents.

Next, we will test if acquisition of *bla*$_{KPC-2}$-bearing and/or virulence plasmids may enhance the ability of CRKP strains to colonize the lungs or GI tract, which has been shown to be an important factor that contributes to the increased prevalence of such strains in clinical settings. To test this hypothesis, different sets of isogenic strains will be subjected to colonizaiton and competition assay in the lungs and GI tract of SD rat by inoculating equal amount of these strains into the lungs and GI tract of SD rat. The survival rate of different *K. pneumoniae* strains in the lungs and GI tract will be determined by plating lung homogenate and fecal suspension on LB plates containing meropenem, and the colonies obtained will be screened for the presence of virulence plasmid to determine the number of CRKP and CR-HvKP strains isolated from the lungs and GI tract, respectively. In addition, the host response to colonization by different strains of *K. pneumoniae* will be determined through measuring the physical conditions, body temperature, weight as well as immune response of the test animal such as cytokine gene expression by qRT-PCR. The microbiomes of lung and GI tract of mice infected by different strains of CRKP, HvKP and CR-HvKP will be detrmined by metagenomic sequencing, which is well developed in our team, to understand the interaction between these pathogens and the healthy gut and airway microbiome, and how these strains affect the colonization and persistence of these pathogens. If normal

micorbiome is proven to be a important factor that inhibits colonization of CRKP, HvKP and CR-HvKP strains, resonistitution of these microbiome might be a good approach to decolonize *K. pnuemoniae* in lung and GI tract. Our preliminary study showed that acquisition of virulene plasmid, but not *bla*$_{KPC}$-bearing plasmid, rendered *K. pneumonaiae* unable to survive and colonize in rat GI tract (**Fig 1c, d, e, f,** control experiments).

Another important factor that might affect colonization and persistence of *K. pneumonaiae* in the lung and GI tract are clinical practices such as antibiotic usage. To test for the effect of these factors, lung and GI tract colonization assays will be conducted as described above. Briefly mice will be subjected to treatment with differenet antibiotics that are commonly used in clincial setting to treat clinical *K. pneumoniae* infections, such as carbapenems, polymycin E, tigecycline, ceftazidime/avibactam and different combinaitons of these antibiotics. The effect of these antibiotics on the fitness and colonization of different *K. pneumoniae* strains will be determined. Importantly, the effect of these antibiotic treatments on the microbiome of lung and GI tract will also be determined as above to assess the effects of antibiotics on the clinical colonization of different *K. pneumoniae* strains. We have conducted a preliminary study on the effect of tigecycline and/or meropenem on the clinical persistence of different *K. pneumoniae* strains in rat model. Our data showed that treatment with either of tigecycline and meropenem or a combinaiton of both agents facilitate the colonization of four isogenetic strains (**Fig 1c, d, e, f,** treatment experiments). This study could explain the high prevalence of HvKP/CR-HvKP in China since tigecycline and meropenem combinaitonal therapy are commonly used to treat CRKP infections in hospitals in China.

2-II: Identification of genetic factors in K. pneumoniae that enhance physiological fitness and clinical persistence. If *bla*$_{KPC-2}$-bearing plasmids and/or virulence plasmids were proven to control to the clincial fitness and persistence of the test strains, the genetic elements located in these two types of plasmids that contribute to the clincal fitness will be idnetified and characterized. Potential genetic elements in pLVPK such as the regulatory genes *rmpA*/*rmpA2*, *iroBCDN* (encoding salmochelin), *iucABCD/iutA* (encoding aerobactin), *kfuABC* (encoding the Kfu ABC iron transport system) as well as the copper resistance and terrulite resistance genes will be tested for their role in determining the clinical adaptation potential of HvKP strains. Genetic elements in pSWU01 such as the MDR region, toxin/antitoxin systems, *tra* gene cluster for conjugation etc. will be tested for their role in determining the clinical adaptation potential of CRKP strains. In particular, we would like to see how different gene clusters interact to enhance clinical fitness and persistence in CRKP, HvKP and CR-HvKP strains. Gene knockout experiments will be conducted to test the functional role of these plasmid-borne genetic factors in encoding the phenotypes of clinical adaption and fitness. Due to the difficulty in creating genetic mutations and deletion in pLVPK-like plasmid, the following approaches will be adopted. First, plasmid gene knockout will be performed using a two-plasmid system pCasKP/pSGKP developed by our research team as previously described [35]. Briefly, the CRISPR-Cas9 system has two components: the Cas9 endonuclease from *Streptococcus pyogenes* and a single artificial chimeric guide RNA (sgRNA), will be used[36]. Second, one-step PCR product-based gene knockout system that has been shown to be successful in knocking out *rmpA* will be used as well. This system has been widely used in our laboratory to conduct gene knockout study in *E. coli*, *Salmonella* and

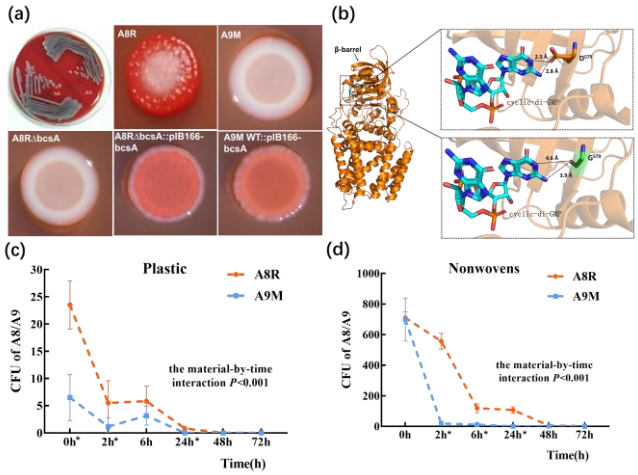
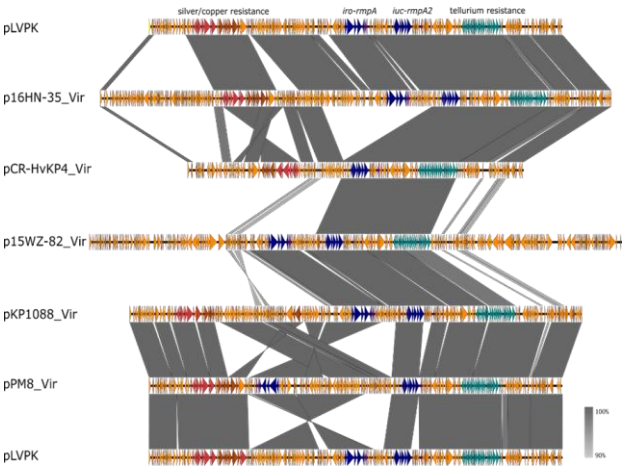


Figure 2. Mutations in UDP-forming cellulose synthase, BscA, enabled ST15 K. pneumoniae to switch to rough morphology to survive better in the hospital environment. *(a) Morphological changes of ST15 K. pneumoniae. Normal mucoid A9M acquired a mutation in bscA to change to rough morphology, A8R, which was confirmed by gene knockout and complementation. (b) BscA structure and mutation site. Better survival of A8R than A9M on various surfaces such as plastic (c) and nonwovens (d).*

K. pneumoniae [37]. Third, we will utilize the helper plasmid discovered in our laboratory to deliver different natural plasmids carrying various genetic factors to various *K. pneumoniae* strains for further testing. We possess a large number of natural virulence plasmids with various deletions in our strain collection. Upon obtaining these mutants, they will be subjected to analysis of their fitness cost, environmental adaptation potential and ability to colonize the lung and GI tract as described above.

Different ST types of *K. pneumoniae* exhibited different levels of prevalence in clinical settings, the most dominant ST type in China and Asian is currently ST11 followed by ST15, whereas the most dominant ST type in Western countries is ST258. We believe that different ST types of *K. pneumoniae* should carry different chromosomal genetic factors that facilitate clinical adaptation. To identify these factors, comparative genomic approaches will be adopted. Utilizing our large strain collection, genome sequences of each of 100 of the most dominant ST11 and ST15 *K. pneumoniae* strains, and each of 100 K1/K2 strains that exhibit low prevalence in the past several decades, will be selected to perform comparative genomics using the Genome Analysis ToolKit available in our team to identify potential genetic factors that might contribute to their clinical adaptation and persistence. These genetic factors will be further confirmed by gene knockout approached as described above. In addition, even in the same ST types such as ST11 and ST15, we observed continuous evolution of different genetic clones, some of which become dominant in recent years. To identify genetic factors that drive the evolution of different genetic clones, SNPs/InDels will be identified by mapping raw sequence reads to different reference strains using Burrows-Wheeler Aligner (BWA). Genome Analysis ToolKit will also be used for SNP identification. To narrow down the meaningful SNPs/InDels, at least 100 strains of the new clone will be used for analysis to identify the common SNPs/InDels for further confirmation. The functional role of these SNPs/InDels will be tested using gene knockout and complementation with mutated genes. It should be noted that our preliminary study has identified a chromosomal mutation in ST15 CRKP strains that allows better survival in the hospital environment (**Fig 2**).



Strains	ST/K type	Virulence plasmid	LD_{50}
KP1088	ST23/K1	+	$1*10^2$ CFU
KP1088PC	ST23/K1	-	$1*10^5$ CFU
KP1088PC/ p15WZ-82_Vir	ST23/K1	+	$1*10^3$ CFU
EH12	ST23/K1	+	$5*10^2$ CFU
EH12PC	ST23/K1	-	$1*10^7$ CFU
PM8	ST374/K2	+	$5*10^2$ CFU
PM8PC	ST374/K2	-	$1*10^5$ CFU
CR-HvKP4	ST11/K64	+	$5*10^3$ CFU
CR-HvKP4PC	ST11/K64	-	$6*10^5$ CFU
16HN-35	ST11/K47	+	$5*10^6$ CFU
16HN-35PC	ST11/K47	-	$1*10^7$ CFU
16HN-35PC/ p15WZ-82_Vir	ST11/K47	+	$2*10^6$ CFU
16HN-35PC/ pCR-HvKP4_Vir	ST11/K47	+	$5*10^6$ CFU
FJ8	ST11/K47	-	$2*10^6$ CFU
FJ8/p15WZ-82_Vir	ST11/K47	+	$2*10^5$ CFU
FJ8/pCR-HvKP4_Vir	ST11/K47	+	$1*10^6$ CFU

Figure 3. Alignment of virulence plasmids in K. pneumoniae strains with pLVPK (upper panel) and the virulence level (LD_{50}) of K. pneumoniae strains and their plasmid-free derivatives (lower panel). *Mice model, which was well developed in our group, was used to determine the LD_{50} for each strain.*

Objective 3: To decode the molecular basis of virulence of clinical *K. pneumoniae* strains

K. pneumoniae has been known to be a gut commensal bacterial species in human and animals. However, it has become the most important nosocomial bacterial pathogen that causes infections of extremely high mortality rate around the world. The mortality rate of infections caused by CRKP is around 40~50% in Asia and over 50% in Europe, and even higher for infections caused by CR-HvKP strains, reaching to 100% in some reports[8]. Little is known about the underlying mechanisms responsible for the sharp increase in mortality of patients infected by CRKP, HvKP and CR-HvKP strains. In this proposal, we aim to investigate genetic factors in CRKP, HvKP and CR-HvKP strains that govern the high virulence and mortality in human as well as the cellular pathways in human that respond to infections caused by these pathogens.

3-I: Contribution of pLVPK like virulence plasmid and chromosomal genetic factors that

contribute to phenotypic virulence of K. pneumoniae. Our preliminary data showed that acquisition of a virulence plasmid contributes to a high virulence level of different types of *K. pneumoniae* strains. Four *K. pneumoniae* strains (2 ST23/K1, 1 ST374/K2, 1 ST11/K64), carrying the pLVPK like virulence plasmid, and their virulence plasmid cured strains, were tested for their virulence potential in mouse models (**Fig 3**). Our data showed that, these four strains exhibited hypervirulence and their plasmid-free derivatives exhibited significantly lower virulence level (increase of LD_{50} by 3 logs). Another ST11/K47 strain 16HN-35, which carried a mutated virulence plasmid and exhibited low hypermucoviscosity and CPS production, was of low virulence level. Its plasmid-free derivative exhibited a LD_{50} value half of that of parental strain; such residual virulence might be contributed by other factors, such as aerobactin and enterobactin still located in the virulence plasmid. Interestingly, when we compared the virulence potential of five virulence plasmid-free derivatives, we observed that these strains exhibited signficiantly different levels of virulence in mice. These data indicated that, apart from the virulence plasmid, other genetic components, most likely chromosomal genetic elements, also contributed to the virulence of *K. pneumoniae* strains. This was further verified by introducing the same virulence plasmid p15WZ-82_Vir into strains KP1088PC, 16HN-35PC and FJ8. Upon receiving the same plasmid, these three strains exhibited different levels of virulence. We also introduced the virulence plasmid phvKP2_Vir, which only lacked the *rmpADC-iro* region when compared to pLVPK, to strains 16HN-35PC and FJ8. The results showed that these two strains exhibited lower virulence upon acquiring phvKP2_vir, when compared to p15WZ-82_Vir, indicating that the *rmpADC-iro* region contributed a certain degree virulence in *K. pneumoniae*. In this project, we intend to investigate how the virulence plasmid and chromosomal virulence factors determine the virulence level of *K. pneumoniae*. We will identify and characterize the genetic traits located in both the plasmids and the chromosome, determine specific interactions between the plasmid and chromosomal factors, and investigat how the interplay of these factors contribute to the virulence of *K. pneumoniae*.

To identify the genetic elements of virulence plasmid that contribute to phenotypic virulence, major genetic elements such as *rmpA* and *rmpA2* gene products, salmochelin (encoded by *iroBCDN*), aerobactin (encoded by *iucABCD* and *iutA*), the Kfu ABC iron transport system (encoded by *kfuABC*) as well as the copper resistance and terrulite resistance genes will be deleted sequentially to their role in expression of virulence in mouse model. In addition, these gene clusters will be cloned and introduced into *K. pneumoniae* strains to determine their contribution to virulence using the mouse infection model. The combined effect of different gene clusters will also be studied. To identify the chromosomal virulence factors, comparative genomic analysis of *K. pneumoniae* strains (without carrying virulence plasmid) exhibiting different levels of virulence will be performed by Roary; genome alignment will be performed by EasyFig to gain data of gene absence and presence. SNPs analysis will also be conducted to identify mutations that affect the virulence levels. Genes and mutations that are uniquely present in hypervirulent strains will be shortlisted for further analysis by gene knockout and functional characterization including neutrophil survival assay and cell binding and invasion assays, as well as by testing in mouse infection model. If the enhanced virulence level in some *K. pneumoniae* strains is due to regulation of specific genes or pathways, RNA-Seq approaches will be used to compare the gene expression patterns in strains with different level of virulence. We also hypothesize that the CPS loci are one of the major chromosomal virulence factors that contribute to the virulence of different K serotypes of *K. pneumoniae*. Our data and some of others have shown that CPS plays a role in virulence expression of *K. pneumoniae* [38-40]. We plan to investigate the variation in virulence level among clinically important serotypes such as K1(ST23), K2 (ST65, ST86 etc), K47 (ST11), K64 (ST11), K19 (ST15), K25 (ST494) and K36 (ST437), which are the most prevalent serotypes in mainland China and Hong Kong SAR. Classical *K. pneumoniae* strains of these serotypes will be selected and tested for their virulence in neutrophil, wax worm and mouse model to compare their virulence potential. The CPS locus of these strains will be deleted and the virulence level of these mutants will be tested as described above. The level of reduction in their virulence level when compared to the parental strains will indicate the degree of contribution of CPS to the virulence of *K. pneumoniae*. Alternatively, a K1 strain will be used as a model strain, whose CPS locus will be replaced by the CPS locus of different serotypes using the CRISPR approach modified from our

previous method[35]. The virulence level of these mutants will be compared to test the degree of contribution of CPS to virulence in strains of different serotypes. It is important to determine if CPS is the key factor that contributes to the virulence phenotype encoded chromosomally.

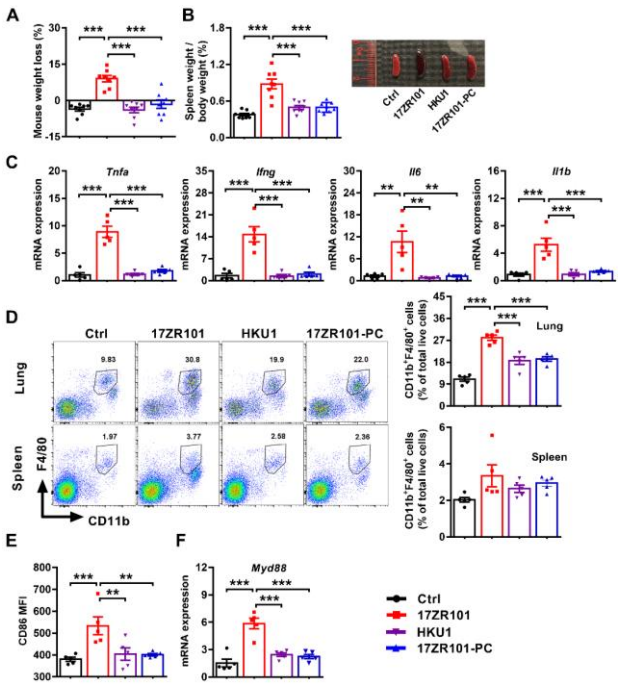


Figure 4. CR-HvKP infection induces severe inflammation in mice. (A) C57BL/6 mice were intravenously infected with 10^4 CFU of a carbapenem-resistant HvKP strain (17ZR101), 17ZR101, which lacks the virulence plasmid (17ZR101-PC), or a low virulence control cKP strain (HKU1). (A) Bodyweight loss and (B) spleen weight/body weight ratio in 17ZR101-infected mice at 12 h post-infection. (C) Expression of proinflammatory genes (*Tnfa*, *Ifng*, *Il6*, and *Il1b*) in the lungs were assessed by qPCR. (D) Macrophage infiltrations in lungs and spleens were measured by flow cytometry in 17ZR101- or mice infected by other strains. (E) Quantification of CD86 MFI in lung $CD11b^+F4/80^+$ populations. (F) Expression of *Myd88* in lungs of the test mice were assessed by qPCR. Data are means ± SEM and representative of two independent tests. texperexpeexperiments. $^{**}p < 0.01$, $^{***}p < 0.001$.

Lastly, interactions between the pLVPK like virulence plasmid and chromosomal factors will be investigated. We plan to delineate the virulence regulatory networks of 14 recently published transcription factors (TFs): KvrA, KvrB [41], IscR [42], CRP [43], SoxS, MarA, Rob, RamA [44], RmpA [45], RmpA2 [46], RmpC, RmpD, RmpD2 and OxyR [47]. To identify the genes/pathways regulated by these TFs, the ChIP-seq approach developed by our team will be used [48]. Upon acquisition of new RNA-seq and ChIP-seq data, a comprehensive network of KPVnet (*K. pneumoniae* virulence network) that illustrates the virulence-related TFs regulome and the crosstalk of multiple TFs will be created by procedures utilized in our previous study [48]. An online platform freely accessible will be developed to facilitate visualization and exploration of the virulence regulatory network, using an optimized, dynamic layout based on the visNetwork package. This will allow the user to scan through the full network by identifying one or multiple transcription factor(s) that forms a sub-network of functional importance. The KPVnet online platform will be used in master regulator analyses to identify key transcription factors that mediate activation of a biological process or pathway through inputting defaulted or customized regulatory network. These regulators and crosstalk networks can be verified by electrophoretic mobility shift assay (EMSA) and qRT-qPCR. Data used in verification of these networks will also be used to further optimize the networks generated by KPVnet. Lastly, the functional characteristics of these genes/networks will be further confirmed by gene knockout studies, cellular assays and animal studies.

3-II: Human cellular pathways govern the response to the infections by CRKP, HvKP and CR-HvKP strains. Interactions between different pathogenic *K. pneumoniae* strains and the human host are not well understood. Our team has initiated a comprehensive investigation of the host response to the infections caused by HvKP. A clinical CR-HvKp strain (17ZR101), its virulence plasmid cured strain, 17ZR101-PC, and a low virulence control cKP strain (HKU1) were used to infect mice. Both 17ZR101-PC and HKU1 exhibited low level of virulence in mice model (**Fig 4A**). After 12 h of infection, we found that animals in the 17ZR101- but not HKU1-infected group exhibited significant weight loss (**Fig 4B**) and increased spleen/weight ratio (**Fig 4C**). Besides, 17ZR101 infection was found to increase the production of inflammatory cytokines (IL-1β, IL-6, TNF-α and IFN-γ) in the lungs, but this phenomenon was not observed in HKU1- or 17ZR101-PC infected animals (**Fig 4D**). Further analysis of immune cells collected from lung and spleen showed that 17ZR101-infected animals exhibited increased infiltration of macrophages (Figure 2A), these cells were also found to express higher levels of CD86 proteins at their surface, indicating an enhanced pro-inflammatory M1 macrophage differentiation (**Fig 4E**). This M1 polarization was

not found in HKU1- or 17ZR101-PC-infected animals. Interestingly, 17ZR101 infection-induced M1 macrophage activation was associated with an increase in the level of Myd88 (**Fig 4F**). However, no significant alterations in CD206 (indicative of M2 polarization) was observed upon infection (data not shown). Taken together, our data suggested that 17ZR101 induced macrophage-mediated cytokine storms that play an essential role in host lethality.

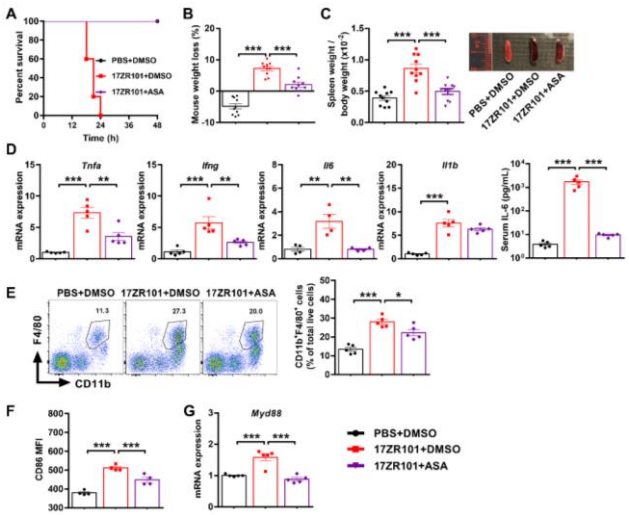


Figure 5. Acetylsalicylic Acid (ASA) treatment reduces CR-HvKP infection-induced *Myd88* expression and M1 macrophage polarization. (A) Survival curve of C57BL/6 mice intravenously infected with 10^4 CFU of 17ZR101 strain or treated with 100 mg/kg aspirin (17ZR101+ASA) 3 h after infection. (B) Bodyweight loss and (C) spleen weight/body weight ratio in 17ZR101-infected but ASA-treated mice at 12 h post-infection. (D) Expression of proinflammatory cytokines in mouse lungs (*Tnfa*, *Ifng*, *Il6*, and *Il1b*) and serum (IL-6) were assessed by qPCR or ELISA. (E) Macrophage infiltrations in lungs were measured by flow cytometry in 17ZR101-infected but ASA-treated mice. (F) Quantification of CD86 MFI in lung $CD11b^+F4/80^+$ populations. (G) Expression of *Myd88* in mouse lungs were assessed by qPCR. Data are means ± SEM and are representative of two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

To further confirm the role of cytokine storms in the pathogenesis of HvKP, we tested the therapeutic value of immune suppressant on infections caused by HvKP. Acetylsalicylic Acid (ASA) is a commonly used non-steroidal anti-inflammatory drug (NSAID) which inhibits the activity of cyclooxygenases (COX) and hence conversion of arachidonic acid to pro-inflammatory prostaglandins. ASA treatment hence inhibits M1 macrophage polarization upon LPS treatment by suppressing both the IκK/IκB/NF-κB pathway and the $COX_2/PGE_2/EP_2$/NF-κB positive feedback loop [49]. Similarly, we found that ASA treatment led to 100% survival (**Fig 5A**), retarded body weight loss (**Fig 5B**), and increased spleen/weight ratio (**Fig 5C**). This protective phenotype is associated with reduced pro-inflammatory cytokine expression (**Fig 5D**), suppressed macrophage infiltration into the lung (**Fig 5E**), inhibited CD86 expression (**Fig 5F**), and decreased *Myd88* level (**Fig 5G**). Together, these data suggest that 17ZR101 infection-promoted M1 macrophage polarization might involve a Myd88-dependent pathway. However, whether and how Myd88 regulates pulmonary macrophage polarization in 17ZR101 infection is still unclear. Based on these preliminary data, we hypothesize that HvKP infection induces Myd88-dependent production of cytokines such as IFN-γ, GM-CSF, and IL-17A, which further potentiate M1 macrophage polarization and occurrence of cytokine storm.

In this proposal, we will investigate comprehensively the entire process of HvKP infection and host response. First, the absolute and relative numbers of macrophages, neutrophils, dendritic cells, natural killer T cells, innate lymphoid cells, and Tregs from blood, intestine, spleen, tracheobronchial lymph nodes, mesenteric lymph nodes, and lungs will be measured by flow cytometry upon infections with 17ZR101, 17ZR101-PC and HKU1. Histology analysis and immunofluorescent co-staining of immune cells will be performed. We will also measure inflammatory cytokines/chemokines (IFN-α, IFN-β, IFN-γ, IL-1α/1β/1ra/6/15/17A/23, MCP-1, CCL2/3/4/11/19/21), TNF, G-CSF, MIG, IP-10 and RANTES that are associated with HvKP infection using specific ELISA kits, Cytokine Multiplex Assay Kits, and real-time PCR. Throughout, we will correlate distinct kinetics of inflammatory response in intestine, lung draining lymph nodes, lung tissues and airways with bacterial load, lung pathology, and severity of disease symptoms in the presence and absence of macrophages. Next, RNA seq of lung tissue will also be conducted to investigate the cellular pathways that mediate immune responses to HvKP infection. Comparative analysis of gene expression profile of lung tissues that are infected by 17ZR101, 17ZR101-PC and HKU1 should enable us to predict the potential pathways mediated infections by HKU1 and 17ZR101. We have conducted a preliminary RNAseq in our previous infection experiments and identify the overexpression of TLR4/ myd88/ STAT1. To further confirm this

related pathways, inhibitors targeting to these three proteins will be used to check their effect on rescue the infections caused by HvKP. In addition, TLR4-knockout and Myd88-knockout mice available in our laboratory will be used to test if removal of TLR4 or Myd88 impairs onset of cytokine storms during HvKP infections.

Second, our preliminary data have shown that HvKP strain, 17ZR101, could survive much better than cKP strain, HKU1 and 17ZR101-PC in mice after infections suggesting that HvKP strain could not be cleared by host immune system. We speculate that HvKP could escape macrophage and neutrophil cell mediated killing in mice, which is consistent with the in vitro Neutrophil cell killing data that HvKP strains are resistant to neutrophil cell killing. To investigate the mechanisms underlying the resistance to neutrophil and macrophage cell killing by HvKP strains, we plan to comprehensively examine the membrane trafficking and innate immune pathways upon infection with various HvKP and cKP stains: (1) Phagocytosis and membrane trafficking pathway. It is reported that a clinically isolated *K. pneumoniae* stain Kp43816 was able to escape phagolysosome killing by targeting the PI3K-Akt-Rab14 axis after macrophage engulfment [50]. We would like to investigate if HvKP strain utilizes similar pathway and if yes, then how HvKP manipulates the PI3K-Akt pathway. We plan to examine the vacuolar localization of the early endosomal marker Rab5, late endosomal marker Rab7, lysosomal marker LAMP2, damaged endosomal marker Galectin-3, and autophagy marker LC3, as well as the pH of *Klebsiella*-containing vacuole by confocal or time-lapse imaging. This experiment can delineate the intracellular paths of different strains after being endocytosed by macrophages in a temporal and spatial manners. Many well-studied Gram-negative pathogens utilize type III/IV secretion systems and inject effectors into host cells to manipulate membrane trafficking or vacuolar composition. However, *K. pneumoniae* does not encode any type III/IV secretion system. It would then be valuable to explore the intracellular survival mechanism used by this pathogen. (2) Pro-inflammatory cytokine signaling and programmed cell death pathways. Previous studies have shown that specific *K. pneumoniae* strain may interfere with NF-κB and NLRP3 inflammasome-meditated pyroptotic cell death [51,52]. To explore whether and how *K. pneumoniae* stimulates and modulates inflammatory responses, we plan to infect macrophages with HvKP and cKP strains and analyze the effects of activation of three pro-inflammatory (NF-κB, MAPK, and interferon) signals and onset of three types of cell death processes (apoptosis, pyroptosis, and necroptosis). We have established specific experimental assays that reflect the activation status of these pathways (e.g., IκB degradation, ERK phosphorylation, and caspase cleavage). After obtaining the results, specific activation or inhibition mechanism will be further validated and explored through genetic manipulation of the host and bacteria.

Lastly, we would like to investigate and identify the potential virulence factors that contributed to formation of cytokine storms and escape of phagocytosis during HvKP infection. Research described in **3-I** should have identified a serial of virulence factors in HvKP. Isogenic strain such as 17ZR101-PC and other cKP strains carrying different virulence factors such as *rmpA, rmpC, rmpD, ituABCD, iroNABCT*, CPS etc. will be used to infect mice to identify the major virulence factors that contribute to HvKP virulence in mice. The mechanisms underlying the contribution of these virulence plasmid and chromosomally encoded virulence factors to HvKP virulence will also be investigated. Research in this objective should help us understand the host defense mechanisms and bacterial escape strategies at the molecular level, and provide important clues for development of novel strategies for treatment of infectious diseases.

Objective 4: Development of rapid diagnostic assay for differentiation between high and low-risk *K. pneumoniae* infections to guide timely and effective treatment

Currently no effective assay is available for differentiation between high risk and low risk clinical *K. pneumoniae* infections. High risk infection is defined as those caused by clinical *K. pneumoniae* strains with high transmission ability and the ability to cause high-mortality infections. Lack of such service often results in high risk of death among patients infected by high risk strains. This study aims to identify genetic markers of high-risk *K. pneumoniae* and develop effective molecular tests to rapidly identify the high risk strains. Based on findings in objective II and III, we should be able to identify a series of genetic markers that contribute to the transmission and / or virulence of *K. pneumoniae*. We plan to validate these genetic markers using at least 1000

clinical *K. pneumoniae* strains documented in the strain and genome Biobank as described in Objective 1, for which complete genome sequence data and clinical data are available. The functional role of products of these genetic markers in contributing to high transmission potential of *K. pneumoniae* in patients and hospital environments, clinical sepsis infections, pneumonia and urinary infections will be determined. An early detection assay to differentiate between clinical infections caused by low and high-risk strains will then be developed to guide further treatment choice. Detailed study approaches are described below.

4-I: Association of virulence factors of K. pneumoniae with phenotypic hypervirulence of K. pneumoniae. Studies in Objectives #2 and #3 will identify chromosomally- and plasmid-encoded virulence factors in various categories of *K. pneumoniae* strains. To further confirm the role of these virulence factors in expression of phenotypic virulence of *K. pneumoniae*, 1000 clinical strains with complete genome sequence data will be analysed to assign these strains into different groups: 1) carriage of virulence plasmid that encodes virulence factors and chromosomal virulence-encoding genes; 2) carriage of only virulence plasmid; 3) carriage of only chromosomal virulence genes; and 4) do not carry any known virulence genes. Twenty strains from each of these groups will be selected and subjected to analysis of their virulence potential using mouse models to test the correlation between carriage of virulence genes and phenotypic virulence.

4-II: Correlation between phenotypic virulence of K. pneumoniae and clinical outcome of infected patients. The following clinical data of patients infected by the aforementioned 1000 clinical *K. pneumoniae* strains will be collected: 1) age of patient; 2) gender of patient; 3) source of sepsis including hepatobiliary, GI tract infections, respiratory, urosepsis, musculoskeletal, neurological and others or unknown sources; 4) comorbidities including diabetes mellitus, cirrhosis, congestive heart failure, CKD/ESRF (chronic kidney disease/end stage renal failure), haematological malignancy, solid tumour, steroid usage and chemotherapy; 5) supportive treatment including RRT (renal replacement therapy defined as intermittent and continuous replacement), mechanical ventilation, vasopressor, ICU care and others; 6) empirical treatment; 7) clinical outcome; 8) prior antibiotic treatment; 9) septic shock and 10) total SOFA score. These clinical data will be analyzed using the R software (R version 3.3.1). Univariate statistical tests (Fisher's exact test or Chi-square test) will be used to compare four different groups of *K. pneumoniae* strains with different virulence potential according to the analysis conducted in IV-1. Such analysis will enable us to investigate the degree of association of the virulence level of *K. pneumoniae* with clinical outcome, after eliminating effects of confounding factors such as treatment options, patients' age and underlying diseases. Multivariate logistic regression will be used to determine the level of association between clinical mortality of *K. pneumoniae* infections and different risk factors, with a particular focus on the virulence of *K. pneumoniae*. All risk factors are treated as different types of 'factor'. Holm correction will be used to adjust the P value. The Boost Regression Tree method will be used to determine the relative impact of the risk factors on the clinical mortality of *K. pneumoniae* infections. Combined analysis of the clinical risk factors and genetic traits of *K. pneumoniae* will be performed to identify both bacterial and non-bacterial risk factors responsible for bacteraemia and mortality.

4-III: Development of rapid detection assays to differentiate between high and low-risk infections caused by clinical K. pneumoniae strains. Upon identifying the relative role of specific genetic factors in contributing to occurrence of infections, an early detection assay to differentiate between clinical infections caused by low and high-risk strains will be developed to guide diagnosis and treatment of *K. pneumoniae* bacteraemia to reduce the mortality rate. Virulence factors associated with high mortality will be identified by rapid detection assays, which will be developed to screen for major virulence factors of *K. pneumoniae* in clinical specimens such as blood or sputum samples. First, Nanopore Flongle rapid detection assay, rapid lateral flow assay, and sensitive biosensors targeting these genetic biomarkers will be devised. In particular, a Nanopore

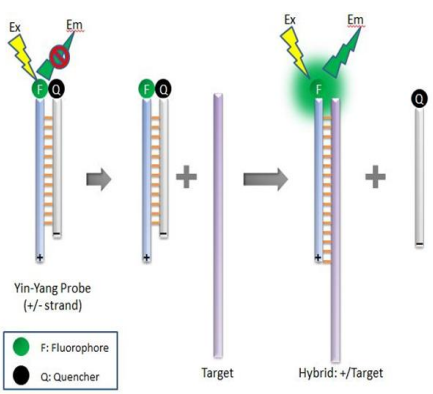


Figure 6. Schematic presentation of the Yin-Yang Probe detection method.

Flongle detection assay will be developed, in which primers targeting these genetic factors will be designed by the CLC genetic platform to amplify virulence genes from clinical *K. pneumoniae* strains. The amplification product will be subjected to sequencing by the Nanopore Flongle kit for real time detection of different genetic factors of *K. pneumoniae* that are associated with high level virulence. This assay can be completed in less than 4 hours and produces confirmative sequencing data on the potential virulence factors. It can also be directly used to detect specific virulence factors in blood, mucosa, fecal and other body fluid samples to facilitate early detection. Other genes, such as the key antimicrobial resistance genes, may also be incorporated into this assay. The advantage of this detection assay is that it is able to detect mutations that may affect the phenotypic expression of virulence factors. For example, a large number of clinical *K. pneumoniae* strains are known to carry mutations in the *rmpA/A2* genes that may affect expression of the virulence phenotypes.

Second, we will work with our collaborator, QuanDx®, an innovator in molecular diagnosis, to develop rapid detection chip for clinical uses. QuanDx utilizes the patented Yin-Yang Probe (Trademark) technology to provide fast diagnostic and prognostic for bacterial and mammalian genes (**Fig 6**). The Yin-Yang probe consists of two complementary oligonucleotides of different lengths. The longer positive strand (+) is modified with a fluorophore, while the shorter negative strand (-) is labelled with a quencher. The two probes become non-fluorescent when the fluorophore and the quench are in close proximity. Upon detecting the target oligonucleotide, the negative strand is displaced by the target and the quenched fluorophore becomes fluorescent. This simple and effective technology can discriminate single nucleotide mismatch (high specificity) and is able to detect as little as 7.5 pg target DNA (high sensitivity). It also allows simultaneous detection of multiple clinically related genes. We will use the Q-VeloxTM POCT system, which integrates nucleic extraction, real-time PCR, detection and analysis so that automated sampling to generation of diagnostic report can be achieved within 30 min to 4 h. This assay system will be capable of identifying mutations in the *rmpA/rmpA2* genes, which are common in HvKP strains and cause functional inactivation of these two genes. Other more sensitive biosensor will also be developed, depending on results of target gene screening in Objective 2 and 3.

4-IV: Assay production and clinical validation. The above developed two assays will be subjected to small scale production by WDWK Biotech (http://en.wdwkbio.com/about.html), a leading diagnostic company in mainland China founded by our Co-PI, Prof. Jianzhong Shen and QuanDx Inc. (https://www.quandx.com/products), a Molecular Diagnostic Kits, Assays and Instruments company founded by our collaborator, Dr Matt Lei. These assays will be validated in Second Affiliated Hospital of Zhejiang University hosted by our Co-PI, Prof. Rong Zhang, as well as in Prince Wales Hospital in Hong Kong hosted by our Co-PI, Prof. Margret Ip, and in Pamela Youde Nethersole Eastern Hospital in Hong Kong hosted by our Co-I, Dr Hoi-Ping Shum. The clinical validation data will be used to further optimize our assays to pave the way for clinical uses.

(a)

Antibiotic + Inhibitor	Meropenem MIC (μg/ml)		
	K. Pneumoniae KP1	CRKP2 (KPC-2)	CRKP3 (KPC-33)
MEM alone	<0.0.6	64	64
AN2718 alone	≥64	≥64	≥64
Visomitin alone	≥64	≥64	≥64
Streptozotocin alone	≥64	≥64	≥64
AN2718 2μg/ml + MRM	<0.0.6	0.5	0.5
AN2718 4μg/ml + MRM	<0.0.6	0.125	0.125
Visomitin 4μg/ml + MRM	<0.0.6	2	2
Visomitin 8μg/ml + MRM	<0.0.6	0.5	0.5

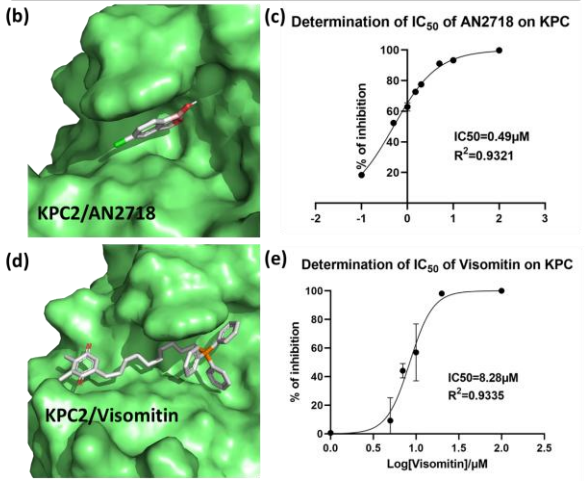


Figure 7. Potent KPC inhibitors *(a) Synergistic effect of AN2718 / visomitin and meropenem (MEM) on carbapenem-resistant K. pneumoniae (CRKP) strains and ceftazidime/avibactam-resistant K. pneumoniae; Modelled structure and IC_{50} of AN2718 (b,c) / visomitin (d, e) and KPC-2.*

Objective 5: To develop novel therapies to combat infections caused by hypervirulent and MDR *K. pneumoniae*

Current choices for treatment of CR-HvKP are extremely limited, especially in China where the incidence of fatal *K. pneumoniae* infections has increased sharply in recent years. Currently, combinations of different last resort antibiotics such as tigecycline / carbapenems and colsitin / carbapenems are used to treat *K. pneumoniae*

sepsis in China, but were only effective against CRKP strains with low level resistance to carbapenems. Ceftazidime/avibactam has been approved for clinical uses in China in 2020, however, resistance in *K. pneumoniae* has rapidly developed, resulting in emergence of various KPC variants such as KPC-33 and KPC-93. Novel antibacterial therapies are urgently required. In this project, we propose to develop inhibitors targeting the major virulence factors associated with transmission and high mortality of clinical *K. pneumoniae* infections, as well as the major resistance determinants. These include the major virulence factors identified in Objective 2 and 3. Two approaches that have been widely used in our laboratory will be used to identify inhibitors of these virulence factors, including screening of commercial compound libraries, such as those which are FDA approved or under clinical trial, natural compounds (over 6000 commercially available compounds) and our home-made libraries (over 3000 compounds and peptides from ocean sources), using specific assays. The lead compounds will be further synthesized chemically and optimized to obtain the best-fit inhibitors. Our experience indicates that these compound libraries are good resources for novel antibiotic discovery. Using these approaches, we have already identified some potential compounds that exhibit activities on various targets. These lead compounds will be developed into novel therapeutic agents for treatment of infections caused by MDR and hypervirulent *K. pneumoniae*. A series of novel therapies will be developed in this project upon identification of drug targets in Objective 2 and 3 using similar approaches.

5-I: Development of carbapenem and novel KPC-2 inhibitor combination therapy to treat clinical infections caused by K. pneumoniae. In our preliminary studies, through screening some of these libraries, we have already discovered a series of lead compounds such as AN2718 and visomitin, that specifically inhibit KPC-2, with IC_{50}=0.498 and 8.28μM. The combination of meropenem and these compounds could reduce the meropenem MIC of *K. pneumoniae* by 32~256 folds at 0.125~2μg/ml of compounds, suggesting that these compounds are good lead compounds for being developed into a potent inhibitor of KPC-2 (**Fig 7**). Avibactam is currently a potent inhibitor for KPC-2 in clinical uses, yet the rate of resistance to this inhibitor has been rising among clinical *K. pneumoniae* strains. Potent KPC-2 inhibitors are in urgent need in China and HK SAR, where KPC-2 producing *K. pneumoniae* infections is rampant. Both AN2718 and visomitin are novel types of inhibitor with different mechanisms of inhibition from existing KPC-2 inhibitor, and active on the current avibactam-resistant mutants (KPC-33) (**Fig 7**). In this project, we will perform medicinal chemistry studies for these compounds. We will chemically synthesize their analogues and test their activities to establish structure-activity relationship, from which the best compounds with high inhibition activities will be subjected to assessment of efficacy when used in combination with meropenem on CRKP strains in animal studies and further preclinical studies as described in 5-V. On the other hand, we aim to solve the complex structures of KPC-2 with these compounds and their analogues to further guide the analogue design for drug development. The high potency of these lead compounds should enable us to develop novel class of inhibitors for clinical application.

(a)

Compounds	MIC compound (μM)				
	E.coli ATCC 25922	*A. baumannii* ATCC19606	*K.pneumoniae* KP 78578	*P. aeruginosa* PAO1	*Salmonella* PY01
Aurothioglucose	>160	>160	>160	>160	>160
Gamma_Mangostin	>160	40	>160	>160	>160
Zinc_pyrithione	5	10	10	20	10
Methacycline	2.5	1.25	10	>160	2.5

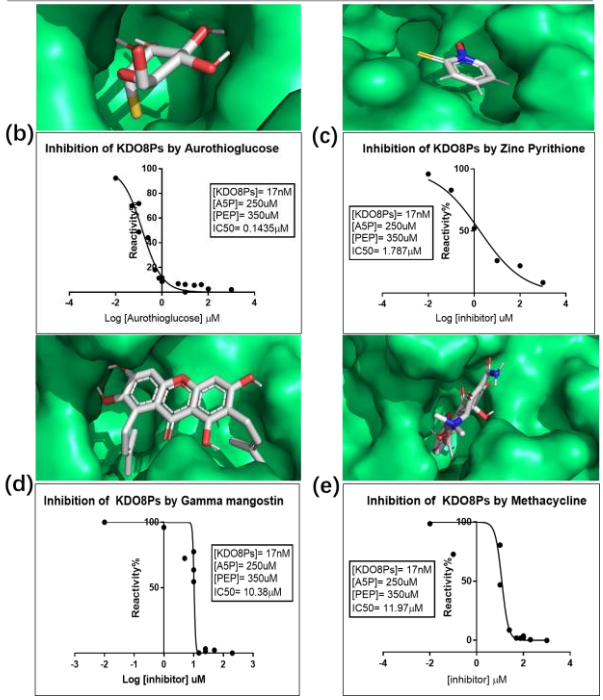


Figure 8. *(a) MIC of aurothioglucose, gamma mangostin, zinc pyrithione and methacycline on various Gram negative bacteria. The docking of compound in KPC-2 (upper panel) and the IC50 of the compound (lower panel) for (b) aurothioglucose; (c) gamma mangostin; (d) zinc pyrithione; and (e) methacycline.*

5-II: Development of novel antibiotics with a novel target, KdsA, in Gram-negative bacteria. The development of resistance in bacterial pathogens has outcompeted the rate of novel antibiotic development. We are facing the arrival

of post-antibiotic era. To win this battle, developing novel antibiotics with novel antibacterial targets is essential. Previous studies have suggested that several enzymes involved in bacterial LPS biosynthesis such as KdsA, 2-dehydro-3-deoxyphosphooctonate aldolase, which is required for lipid A maturation and cellular growth, could be potential targets for Gram negative bacteria[53-55]. We have developed KdsA high throughput screening assay and screened for several compound libraries (over 6000 compounds) available in our laboratory. We have identified over 15 compounds for KdsA and characterized four of them, with IC_{50}=0.1435, 1.787, 10.38 and 11.97Mm. Two of these compounds, zinc pyrithione and methacycline, exhibited antibacterial activity with MIC of 2.5 to 20μg/ml for various Gram negative bacteria, and another two, aurothioglucose and Gamma mangostin did not exhibit antibacterial activity (**Fig 8**). We hypothesize that these two compounds might not be able to penetrate into bacterial outer membrane. In this study, we will chemically synthesize their analogues and test their activities to establish structure-activity relationship, from which the best compounds with high antibacterial activities will be subjected to assessment of efficacy when used in combination with meropenem on CRKP strains in animal studies and further preclinical studies as described in 5-V. For aurothioglucose and gamma mangostin, we aim to improve their ability to penetrate to bacterial membrane to reach the drug target, KdsA. On the other hand, we aim to solve the complex structures of KsdA with these compounds and their analogues to further guide the analogue design for drug development. The high potency of these lead compounds should enable us to develop novel classes of inhibitors for clinical application. Upon identification of potent antibiotics, they would be subjected to further *in vivo* tests in *K. pneumoniae* sepsis model as well as preclinical studies as listed in 5-V.

5-III: Development of novel antibiotic therapy targeting to bacterial RNA polymerase. We have discovered that a FDA-approved drug, zidovudine, which is an inhibitor of reverse transcriptase of HIV and used to treat HIV infections in human, is effective in killing different *Klebsiella pneumoniae* strains including cKP, CRKP and CR-HvKP. The MIC of ZDV to these strains is as low as 2.5~5μg/ml. However, when we performed bacterial killing assay using ZDV, we found that *K. pneumoniae* could regrow after 6 hours in the killing assay when the inoculum is higher than that used in MIC assay, suggesting that ZDV-resistant mutants may emerge. This phenomenon is common for antibiotics targeting bacterial RNA polymerase, such as rifampicin (RIF) (**Fig 9a**). We then tested if the combined use of two types of RNA polymerase inhibitor could prevent generation of mutations; we performed killing assay using different combinations of these two compounds, with results showing that the combination of ZDV and RIF could effectively kill different strains of *K. pneumoniae* (**Fig 9b**). Importantly, the combined usage of these two drugs exhibited excellent *in vivo* efficacy on treating infections caused by CR-HvKP strain, but not the single drug alone (**Fig 9c**). We performed resistance development assay on these two compounds and found that when used in combination, ZDV and RIF could prevent development of resistant mutant upon 7 days' incubation, suggesting that these two compounds have high potential to be used in treatment of *K. pneumoniae* infections, in particular those caused by CR-HvKP strains (**Fig 9d**). In this project, we will further develop this cocktail therapy for clinical uses. First, we will depict the mechanisms of action of ZDV. Our preliminary data showed that ZDV could bind to RNA transcriptase of *K. pneumoniae* during ITC assay; we will further confirm the affinity of ZDV to this target. Second, we would like to determine if ZDV could inhibit the expression of virulence and antimicrobial resistance genes using RNAseq approaches. We would also investigate why combined usage of ZDV and RIF could prevent mutation development.

(a)

Bacterial strains	MIC (μg/ml)		
	ZDV	RIF	ZDV+RIF
K. pneumoniae KP1	2.5	32	<0.06
CR-HvKP-1	2.5	32	<0.06
CR-HvKP-5	5	32	<0.06
CR-HvKP-7	2.5	32	<0.06

ZDV, Zidovudine; RIF, Rifampicin

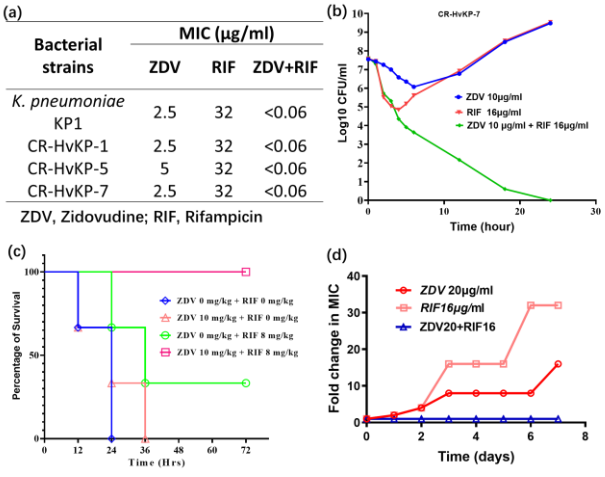


Figure 9. *(a) MIC of zidovudine (ZDV), rifampicin (RIF) and their combination on different K. pneumoniae strains (b) Time killing assay of ZDV, RIF and their combination on CR-HvKP-7 strain; (c) In vivo acvitiy assay using mouse infection model using CR-HvKP7 strain. (d) Resistance development assay for strain, CR-HvKP-7.*

Mutations inducible by ZDV and RIF alone will be identified by RNAseq; whether these mutations would impair the activity of RNA transcriptase will be determined. Lastly, this cocktail therapy will be subjected to preclinical studies as described in 5-V. These drug regimens should be ready for clinical trial upon completion of this project.

5-IV: Development of novel antibiotics against various strains of antibiotic resistant Klebsiella pneumoniae. Cationic peptides are found in all forms of life (mammals, plants, and bacteria), and are among the most widespread and structurally-diverse antibiotics in nature, with great potential for combating drug resistant Gram-negative pathogens [56,57]. In our previous studies, we conducted genome-mining based discovery for Gram-negative antibiotics through analyses of over 7000 biosynthesis gene clusters in microbes and identified two novel compounds (brevicidine and laterocidine) with strong bactericidal activities against antibiotics resistant Gram-negative pathogens, high efficacy in animal model, and a low risk of resistance including *Klebsiella pneumoniae* **(Fig 10)**[58]. In addition, we have also isolated and chemically characterized albofungins **(Fig 10)** and its novel derivatives from marine bacteria, some of which exhibited strong activities against Gram-positive bacteria (MASA, *Staphylococcus aureus*, *Bacilllus subtilis*) at a nanomolar range and notorious ESKAPE Gram-negative bacteria (*Klebsiella pneumoniae* NRRL-B-3521, *Klebsiella pneumoniae* NRRL-B-408, *Acinetobacter baumannii* B-65371, *E. cloacae* NRRL-B-425, *E. Coli* k12, and *E. Coli* MG1655) at a low micromolar range and anti-HIV activities[59]. These compounds can selectively bind with different G-quadruplexes, indicating that each of these derivatives could be a biological switch and pharmaceutical target in specific disease. In this project, we will first chemically synthesize more derivatives for structure-function relationship analyses aiming to identify optimal chemical structures. Meanwhile, we will develop more mutants of *Streptomyces coelicolor* that carry and heterologously express the albofungin gene cluster through gene editing, in order to produce other types of novel derivatives at higher yield. The compounds with high antibacterial activities will be subjected to the assessment of efficacy in CRKP/CR-HvKP infection models as well as further preclinical studies as described in 5-V. lastly, the mechanisms of action of these compounds will also be investigated using approaches as described in our previous studies[60].

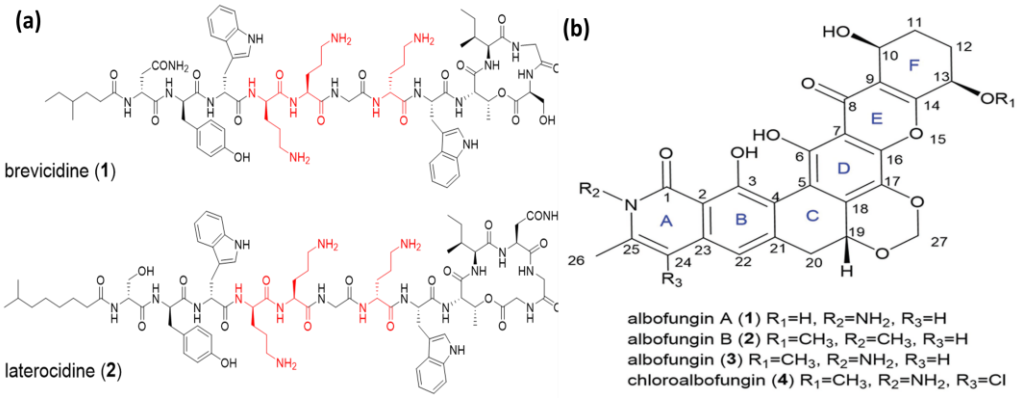


Figure 10. Three types of antibiotic compounds that show potent anti- Gram-negative bacteria, discovered through genome-mining based bioactive compound discovery[57] and bioassay-guide characterization process[58].

5-V Preclinical study for novel antibiotic cocktail therapies.

The novel antimicrobial therapies identified in this objective that exhibit high potential will be subjected to preclinical studies, which include the *in vitro* toxicity test, *in vitro* pK/pD, *in vivo* pK/pD and *in vivo* toxicity test. The procedure for preclinical studies will be described briefly due to the space limitation. First, *in vitro* toxicity tests include 1) haemolytic assays; 2) cytotoxicity assays on human cell lines (HepG2, HEK293, hPT-Kidney cells); 3) nephrotoxicity assay as previously described [61,62]; 4) Cytochrome P450 Induction Assays (Sigma) in HepaRG liver cell line, clone 5F (human); and 5) DNA binding assay and plasma protein binding assays. Second, *in vitro* PK/PD tests will be performed using an *in vitro* PK/PD model developed in a previous study [63]. Third, upon determination of PK/PD *in vitro*, *in vivo* PK/PD data will also be generated. Three major studies are involved: animal PK studies, infected animal PD studies, and PK/PD studies in infected animals. The PK study in infected model will be performed as previously described [64].

The combination analysis of *in vitro* and *in vivo* PK studies above will provide essential

information for designing the formulation of the antibiotic regimens to achieve the best efficacy and lowest toxicity. Formulations can be used in conjunction with aqueous media by adjusting pH for increasing solubility. Commonly used formulation solvents include 0.9% NaCl solution, 5% dextrose in water, propylene glycol, polyethylene glycol (PEG) 400, N-methylpyrrolidon (NMP), N,N-dimethyl acetamide (DMA) and diethylene glycol monoethyl ether (Transcutol). Surfactants including Tween 80, Cremophor EL, Cremophor RH40, Poloxamer 188, Solutol HS-15, VitE-TPGS 100 and Labrasol will be used to help reduce drug precipitation. Different formulations of regimens will be tested in *in vivo* thigh infection and sepsis model developed in the PK/PD study to identify the best formulation. In addition, antimicrobial hydrogels will also be developed for drug formulation.

Pharmacodynamics (PD) of different novel therapies will be tested in neutropenic mouse sepsis and pneumonia models. The potential for bacteria to develop resistance to combinational therapy will also be determined as described above. *In vivo* PK/PD in infected animal models will be analysed as previously described [64]. Lastly, upon identification of the best formulation of the novel therapies, the toxicity of these therapies will be tested in different *in vivo* models prior to clinical trial. The toxicity tests will involve two stages. First, *in vivo* toxicity (the maximum tolerance level) of different regimens will be determined by performing mouse LD_{50} assay through administration of female BALB/c mice by IV or IP with one single dose, or injection for seven consecutive days. Second, novel therapies exhibiting the lowest toxicity and highest antimicrobial efficacy will be selected and sent for comprehensive toxicity test in a certified toxicity laboratory, which is essential for soliciting approval of clinical trial of these new regimens.

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