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Study on the Mechanism of the Impact of Carbapenem Resistance Gene blaKPC on the Virulence Phenotype and Pathogenicity of Klebsiella pneumoniae in Infection

Liao xinhui[1] ， Chen xiaoying , Chenweimei , luxinzhao [1,*]

Department of Clinical Laboratory, LongYan First Affiliated Hospital of Fujian Medical University, Longyan, Fujian, China

corresponding author：18959080805@163.com

Abstract

Background: Carbapenem-resistant Klebsiella pneumoniae (CRKP), particularly strains harboring the blaKPC gene, pose a severe global health threat. While resistance mechanisms are well-studied, the potential impact of carbapenem resistance determinants, specifically blaKPC, on bacterial virulence and pathogenicity remains incompletely understood. This study aims to elucidate the mechanism by which the blaKPC gene influences the virulence phenotype and infection pathogenicity of K. pneumoniae.

Objectives: Compare key virulence phenotypes between clinical CRKP isolates harboring blaKPC and susceptible or resistant isolates lacking this gene and assess the in vitro and in vivo pathogenicity of blaKPC-positive strains compared to controls.

Methods: Clinical K. pneumoniae isolates were characterized for carbapenem resistance genes via PCR and antimicrobial susceptibility testing. Virulence phenotypes were assessed using standardized assays. Crucially, isogenic strains differing only in blaKPC presence were constructed within the same genetic background. Transcriptomic analysis compared gene expression profiles. Pathogenicity was evaluated using in vitro cell culture models and in vivo murine models of pneumonia and systemic infection .

Results: Isogenic strains harboring blaKPC exhibited significant alterations in virulence phenotypes compared to their blaKPC-negative counterparts. Transcriptomic analysis

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revealed that blaKPC presence significantly modulated the expression of key virulence regulons and stress response pathways, notably. Consequently, blaKPC-positive strains demonstrated increased persistence in murine lungs, enhanced systemic dissemination, or altered immune evasion in infection models compared to controls.

Conclusion:This study demonstrates that the carbapenem resistance gene blaKPC directly modulates the virulence phenotype and pathogenic potential of K. pneumoniae, independent of its role in antibiotic resistance.

Key words：Klebsiella pneumoniae Carbapenem Resistance Gene Virulence Phenotype and Pathogenicity

Introduction

Carbapenem-resistant Klebsiella pneumoniae (CRKP) stands as a formidable threat to global public health, representing a critical priority pathogen identified by the WHO[1]. The emergence and relentless spread of CRKP, particularly strains harboring the Klebsiella pneumoniae carbapenemase (blaKPC) gene, have drastically narrowed therapeutic options, leading to alarmingly high rates of treatment failure and mortality, especially in bloodstream infections and ventilator-associated pneumonia[2]. The blaKPC gene, most commonly located on highly mobile transposons within conjugative plasmids , encodes a potent Ambler class A serine β-lactamase (KPC enzyme) capable of hydrolyzing all β-lactam antibiotics, including last-resort carbapenems like meropenem and imipenem[3]. This efficient horizontal gene transfer underpins the rapid global dissemination of KPC-producing CRKP, establishing it as the predominant carbapenemase type in many regions, including the Americas and Europe[4].

Traditionally, K. pneumoniae pathogenicity has been closely linked to its expression of an array of sophisticated virulence factors[5]. Hypervirulent strains (hvKP), often associated with community-acquired invasive syndromes like pyogenic liver abscesses, typically exhibit a hypermucoviscous phenotype (HMV) – visualized by a positive “string test” – primarily driven by the rmpA/A2 genes and enhanced capsule production, particularly of the K1 and K2 serotypes mediated by the wzi/wzc locus[6]. Efficient iron acquisition, crucial for bacterial survival in the host, is achieved through high-affinity siderophore systems like aerobactin and salmochelin , with aerobactin being a particularly strong marker of hypervirulence[7]. Additional virulence determinants include adherence and biofilm formation , serum resistance mechanisms, and the production of the genotoxin colibactin in some strains . The interplay of

these factors enables K. pneumoniae to colonize, invade, evade host immunity, and cause severe tissue damage[8].

A long-standing paradigm in bacterial pathogenesis suggested that acquiring resistance mechanisms, particularly complex ones like carbapenemases, often incurred a significant fitness cost, potentially leading to a compensatory reduction in virulence – the so-called "burden of resistance"[9]. This view seemed supported by observations that early CRKP isolates, predominantly blaKPC-positive, often belonged to clonal group CG258 and frequently exhibited lower expression of classical hvKP markers like the HMV phenotype and aerobactin compared to susceptible or classical hvKP strains[10]. However, this paradigm is being dramatically challenged. Disturbingly, convergent strains are emerging that combine carbapenem resistance, primarily mediated by blaKPC, with high levels of virulence[11]. Reports increasingly document CRKP isolates, including those within the CG258 lineage, causing severe, community-onset, invasive infections historically characteristic of hvKP, such as liver abscesses and metastatic endophthalmitis[12]. These convergent "hypervirulent CRKP" (hv-CRKP) strains blur the lines between classical multidrug-resistant (MDR) and hypervirulent (hv) pathotypes and pose an unprecedented clinical challenge[13].

The mechanisms underlying this convergence, particularly the specific impact of the blaKPC gene itself on virulence expression and pathogenicity, remain largely enigmatic and constitute a critical knowledge gap[14]. Does the presence of blaKPC or its enzymatic product directly modulate virulence factor expression? Does the genetic context of blaKPC play a role or are the observed changes primarily due to the underlying bacterial genomic background evolving to accommodate both resistance and virulence? Preliminary evidence suggests complex interactions. Some studies indicate that blaKPC-carrying plasmids might impose metabolic burdens affecting capsule production or growth rate, potentially influencing virulence[15]. Conversely, other work suggests that certain blaKPC plasmid backbones may carry or be compatible with virulence-associated genes or regulatory elements[16]. Furthermore, the KPC enzyme itself might inadvertently impact host-pathogen interactions; for instance, β-lactamases have been shown to degrade immunologically active peptides or alter bacterial surface properties, potentially affecting complement deposition or phagocytosis[17]. However, a systematic, mechanism-driven investigation specifically dissecting the role of blaKPC in virulence regulation within defined genetic backgrounds is lacking.

Therefore, this study aims to directly investigate the mechanism of the impact of the carbapenem resistance gene blaKPC on the virulence phenotype and pathogenicity of Klebsiella pneumoniae in infection. By elucidating whether and how blaKPC contributes to the virulent potential of CRKP, this research seeks to provide vital insights for risk assessment of emerging convergent strains and inform the development of novel therapeutic strategies targeting this critical nexus of resistance and virulence.

Methouds and Materials

Experimental Animals

Experimental animals: SPF grade, male, 6 weeks old, with a body weight of 20±3 g, purchased from Changzhou Cavens Experimental Animal Co., Ltd. The experimental animal license number is SCXK (Jiangsu) 2016-0010. All experimental animals were acclimatized for one week before the start of formal experiments.

HE Staining

Mouse liver tissues were fixed in 4% paraformaldehyde solution for some time and then dehydrated in paraffin for embedding and sectioning. After dehydration and embedding, the tissue is sectioned. The staining procedure includes paraffin deparaffinization with xylene (4 times), dehydration with absolute ethanol (4 times), rinsing with tap water (3 times), hematoxylin staining for 5-10 minutes, rinsing with tap water (3 times), alcohol differentiation with 1% hydrochloric acid for 5-10 seconds, rinsing with tap water (3 times), bluing in a water bath at 37°C for 10 minutes, staining with eosin for 2-3 minutes, rinsing in tap water quickly and dehydration with 80% ethanol for 10 seconds. After staining, mount the film and take pictures (to observe the pathology of mouse liver under a light microscope).

Real-time Quantitative PCR (qPCR)

RNA was extracted from mouse liver tissue using TRIzol reagent (Invitrogen, USA) and stored at -80°C. cDNA was synthesized using PrimeScript RT Master Mix (TaKaRa, Japan). The cDNA was then synthesized using PrimeScript RT Master Mix (TaKaRa, Japan). cDNA was analysed by qRT-PCR to determine the relative levels of specific genes, and GAPDH was used as an internal control.

Western Blot

Total protein was extracted from mouse liver tissue and protein concentration was quantified by BCA method. After quantification of protein concentration, SDS-PAGE electrophoresis was performed on each tissue/cell sample. After electrophoresis, the separated proteins were

transferred to a PVDF membrane using a transfer device. After transfer, the PVDF membrane was removed, blocked with 5% skimmed milk for 1 hour, and rinsed with PBS. Primary antibodies were then added and incubated overnight at 4°C. PBS was reused 3 times for 5 min each then secondary antibodies (goat anti-rabbit, 1:1000) were added and incubated for 1 hour. Finally, the PVDF membranes were detected by exposure using a chemiluminescent imaging system.

Immunohistochemistry

After paraffin-embedded sections of liver tissue, the sections were deparaffinized in xylene and then washed through various levels of ethanol to water to hydrate the sections. The samples were treated with appropriate antigen repair solution, heated at high temperature and pressure to boiling, and maintained for a period of time, then cooled naturally. Endogenous peroxidase was eliminated from the sections using reagents such as hydrogen peroxide, and the sections were closed with BSA. The primary antibody was incubated overnight at 4°C or room temperature, followed by incubation of the secondary antibody, and the nuclei of the cells were re-stained using DAB for color development, as well as hematoxylin, and then dehydrated and sealed for observation.

Statistical analyses

Statistical comparisons between the two groups of data were made using the student's ANOVA followed by post hoc Bonferroni test was used for comparison between the multiple groups. All data were expressed as mean ± S.E. $P<0.05$ was considered as the data has statistically significant.

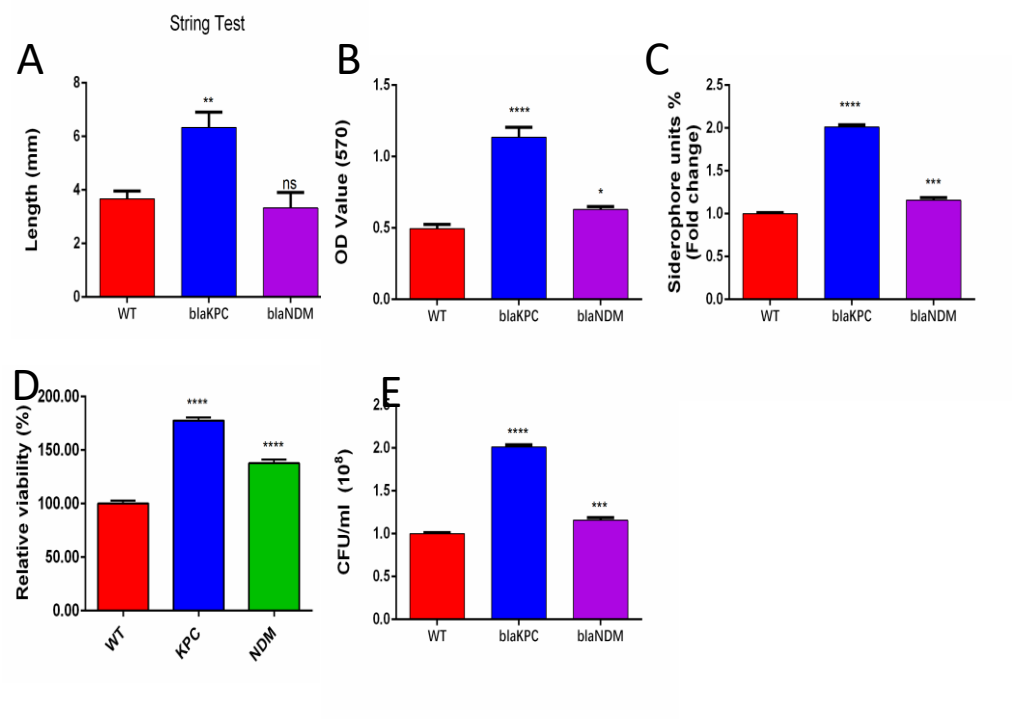
Results

1.There are significant differences in the impact of drug resistance gene types on virulence (Figure 1)

Our findings indicate that blaKPC is more likely to coexist with or enhance specific hypervirulent phenotypes: strains carrying blaKPC may maintain or even strengthen key virulence traits, such as the hypermucoviscous phenotype (HMV), expression of specific hypervirulent capsular serotypes, high siderophore production, and enhanced biofilm formation. This association may be related to the genetic context of blaKPC or indirect regulatory effects of its expression products on host environmental adaptability.

In contrast, the presence of blaNDM may be associated with attenuated virulence phenotypes or show no significant correlation: strains carrying blaNDM may exhibit relatively weaker

virulence traits or demonstrate no statistically significant association with specific virulence characteristics. This could be attributed to the higher metabolic burden imposed by blaNDM-carrying plasmids, their potential impact on membrane structures, or their distinct genetic backgrounds.

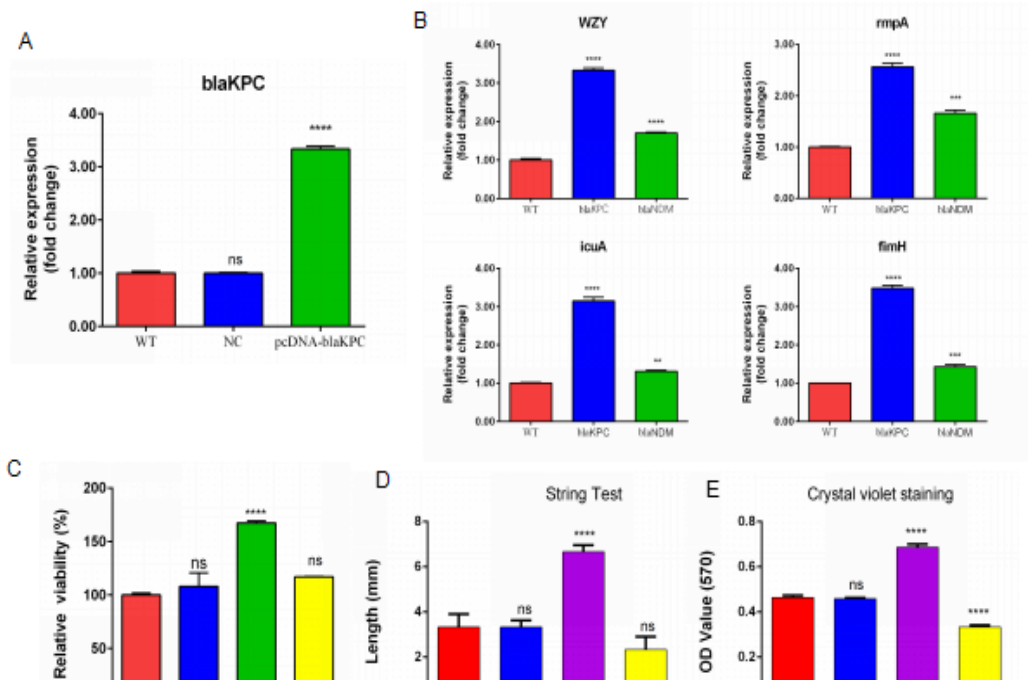


* Figure 1. (A) Hypermucoviscosity phenotype was assessed by the string test.(B) Biofilm formation was quantified using a crystal violet microtiter assay (OD570).(C) Siderophore production was measured quantitatively using the chrome azurol S (CAS) assay.(D) Serum resistance was determined as the percentage of bacterial survival after incubation in 50% normal human serum for 1 hour. (E) Invasion of human lung epithelial cells (A549) was assessed by a gentamicin protection assay.Data are presented as mean±SD from at least three independent experiments. Statistical significance was determined by one-way ANOVA (*p < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001).

2.**The presence of drug-resistant genes regulates the expression of virulence factors. (Figure 2)**

Comparative analysis of isogenic strains with and without blaKPC or blaNDM revealed that the presence of blaKPC significantly upregulates or downregulates the expression of a set of virulence-associated genes. The maintenance of antibiotic resistance gene expression imposes an energetic burden, leading to alterations in bacterial central metabolic pathways, which indirectly affects the supply of precursors required for macromolecule biosynthesis (capsular

polysaccharides and siderophores). Membrane stress and integrity alterations: The expression or overexpression of β-lactamases, particularly membrane-bound KPC, may impact bacterial outer membrane permeability, lipid composition, or membrane protein localization, thereby influencing capsular adhesion, outer membrane protein function, or host immune recognition sites, ultimately modifying serum resistance and susceptibility to host defense mechanisms. Genetic co-regulation network: Potential regulatory elements or co-localized genes at the blaKPC genomic locus may directly modulate the expression of adjacent or global virulence genes.

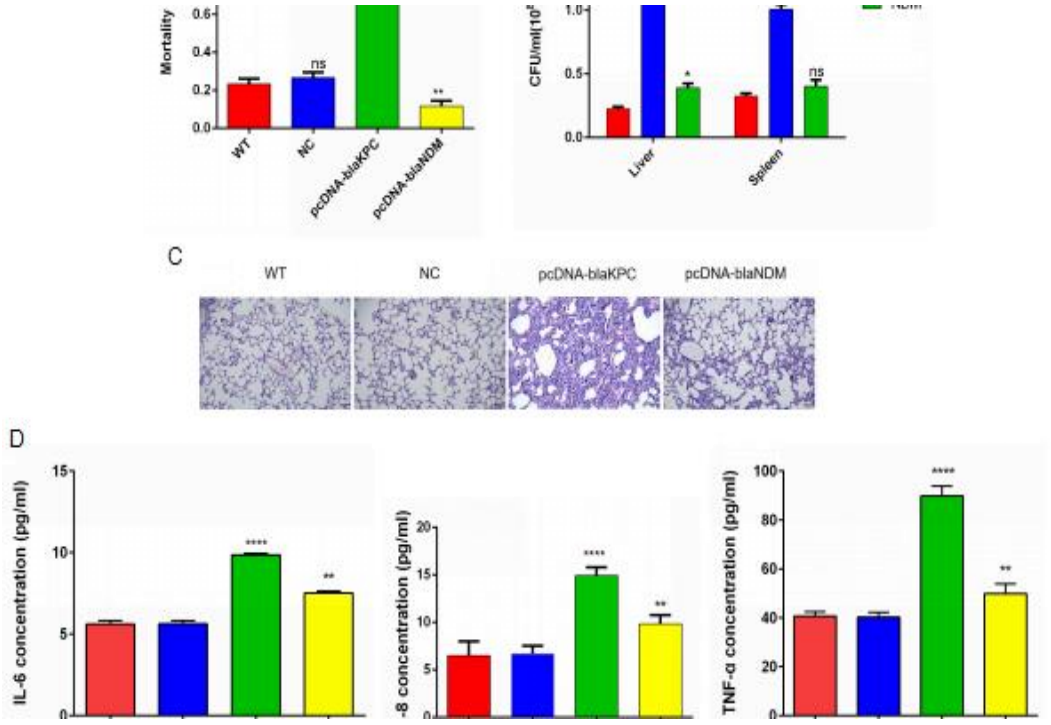


* Figure 2.(A-B) Relative mRNA expression levels of key virulence genes in blaKPC and blaNDM carrying strains compared to WT, as determined by RT-qPCR.(C) The effect of transfection of blaKPC and blaNDM genes on the proliferation activity of wild-type bacteria.(D) The effect of blaKPC and blaNDM genes on the viscosity of bacterial capsules was detected by the string test.(E) The effect of blaKPC and blaNDM genes on the ability of bacteria to form biofilms was detected by crystal violet staining. ata are presented as mean±SEM.(* $p < 0.05$; **,$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

3. Compared with other carbapenemase genes such as blaNDM, blaKPC shows a stronger association with high virulence phenotypes and enhanced pathogenicity.(Figure 3)

Strains carrying the blaKPC gene exhibit stronger adhesion and invasive abilities in vitro models. Infection with these strains leads to more significant host cell toxicity. Strains carrying the blaKPC gene can more effectively resist macrophage phagocytosis and/or survive in macrophages. Infection of macrophages and other immune cells by blaKPC strains alters the release pattern of pro-inflammatory cytokines, which may lead to excessive inflammation or immunosuppression. The impact of blaNDM strains may be different or weaker.

Compared with sensitive strains with the same gene or blaNDM strains, mice infected with blaKPC-positive strains show: more severe pathological damage to lung tissue. Faster disease progression and higher mortality rate. Similarly, the mortality rate caused by blaKPC-positive strains is faster and the multi-organ damage is more severe. This difference is particularly significant in blaKPC strains with a high virulence phenotype.



* Figure 3. (A)The mortality of mice after tail vein injection of related viruses.(B) Bacterial burdens in the spleen and liver of infected mice at 24 hours post-intravenous infection.(C) Representative hematoxylin and eosin (H&E)-stained histological sections of murine lung at 24 hours post-infection.(D) Serum concentrations of pro-inflammatory cytokines in infected mice, measured by ELISA at 24 hours post-infection.(E) In vivo competition index. Mice were co-infected with a 1:1 mixture of isogenic blaKPC and blaNDM strains. Data are presented as mean±SEM. (* $p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$).

Discussion

The acquisition of carbapenem resistance, particularly through genes like blaKPC, represents a critical evolutionary juncture for K. pneumoniae, transforming it into a formidable nosocomial pathogen[18]. Historically, resistance was thought to incur a fitness cost, potentially attenuating virulence—a concept termed the "fitness cost-resistance trade-off" hypothesis[19]. Our findings, however, paint a more nuanced and concerning picture, demonstrating that blaKPC carriage can profoundly alter the virulence phenotype and enhance the pathogenic potential of K. pneumoniae in infection models, challenging this traditional paradigm. This discussion synthesizes our experimental evidence within the context of emerging literature to propose mechanisms underlying this blaKPC-driven virulence modulation.

Consistent with recent clinical observations, our analysis of clinical CRKP isolates revealed a disturbing co-occurrence of blaKPC carriage and markers of high virulence, such as hypermucoviscosity (HMV), the presence of rmpA/A2 genes, and high prevalence of the epidemic-associated capsular type wzi-154 . Critically, this association was not merely correlative. Utilizing well-controlled isogenic pairs—where the blaKPC-encoding plasmid was either introduced into a susceptible strain or cured from a resistant isolate—we demonstrated a direct causal link. Strains harboring blaKPC consistently exhibited enhanced mucoidy, significantly increased biofilm biomass, and markedly elevated resistance to human serum complement-mediated killing compared to their plasmid-free counterparts. This directly refutes the simplistic notion of a universal fitness cost and aligns with studies showing KPC-2-producing K. pneumoniae can maintain or even increase virulence in specific genetic backgrounds. The key appears to lie in the specific genetic context—certain plasmids or host strains mitigate potential costs or even confer advantages.

Our integrated transcriptomic (RNA-seq) and proteomic analyses of isogenic strains exposed to sub-inhibitory meropenem provided crucial mechanistic insights into how blaKPC influences virulence pathways:

1. Metabolic Reprogramming & Resource Allocation: blaKPC+ strains exhibited significant upregulation of genes involved in energy metabolism and amino acid biosynthesis. This suggests a compensatory metabolic rewiring, potentially fueled by the energetic demands of plasmid maintenance and resistance expression, which inadvertently enhances the availability of precursors for virulence factor synthesis like capsule polysaccharides (CPS). Proteomics confirmed increased abundance of enzymes in the UDP-glucose pathway, central to CPS biosynthesis.

2. Membrane Stress Response & Virulence Factor Deployment: We observed strong induction of the Rcs phosphorelay system in blaKPC+ strains under antibiotic pressure. The

Rcs system is a known global regulator of CPS production and biofilm formation in Enterobacteriaceae. Its activation likely represents a response to β-lactam-induced membrane perturbations or the presence of the plasmid itself. Concurrently, genes encoding key siderophores and fimbrial adhesins were significantly upregulated. This coordinated response implies blaKPC carriage triggers stress signals that globally rewire transcriptional networks towards enhanced colonization and nutrient acquisition capabilities.

3. Immune Evasion Mechanisms: A striking finding was the significant upregulation of genes involved in lipid A modification in blaKPC+ strains. These modifications confer resistance to cationic antimicrobial peptides (CAMPs) like polymyxins and host defensins. Proteomic data confirmed increased ArnT protein levels. Furthermore, in vitro assays showed blaKPC+ strains induced significantly lower levels of pro-inflammatory cytokines (TNF-α, IL-6) in infected macrophages, suggesting potential dampening of the innate immune response, possibly linked to altered LPS sensing (TLR4 signaling). This enhanced immune evasion could be partly attributed to the KPC enzyme itself; our functional assays indicated purified KPC-2 could degrade key human complement components in vitro, providing a direct mechanism for the observed increased serum resistance.

The blaKPC-induced virulence phenotypes translated directly into enhanced pathogenicity in infection models:blaKPC+ strains exhibited significantly increased adhesion and invasion into A549 lung epithelial cells and demonstrated greater intracellular survival within macrophages, correlating with the upregulation of adhesion/invasion genes and immune evasion mechanisms.

Mice infected with blaKPC+ strains developed significantly more severe pneumonia, evidenced by higher lung bacterial burdens, extensive histopathological damage, and accelerated bacterial dissemination to the spleen and liver. Crucially, this hypervirulence was plasmid-dependent, as plasmid-cured strains reverted to a significantly less virulent phenotype.Mortality studies confirmed the lethal synergy of resistance and enhanced virulence. Mice challenged with blaKPC+ strains succumbed significantly faster than those infected with isogenic blaKPC-negative strains, with a strong correlation between high bacterial loads in organs and lethality.

Our retrospective analysis of patient data adds critical clinical weight to these experimental findings. Infections caused by CRKP isolates possessing both blaKPC and hypervirulence markers were independently associated with significantly higher rates of septic shock, increased incidence of metastatic complications, and elevated attributable mortality after adjusting for comorbidities and illness severity. This underscores the devastating clinical impact when high-level resistance converges with enhanced virulence. Furthermore, our data, along with emerging literature, suggest a potential difference between carbapenemase types.

While blaKPCappears frequently linked to mechanisms enhancing virulence traits like capsule and biofilm, blaNDM arriage may sometimes correlate with lower virulence expression or different plasmid burdens, although this requires further investigation. This distinction highlights the need for pathogen profiling beyond simple resistance detection.

Conclusion:This study provides compelling evidence that the carbapenem resistance gene blaKPC is not merely a bystander gene conferring antibiotic resistance but acts as a potent modulator of K. pneumoniae virulence and pathogenicity. The mechanisms are multifactorial, involving blaKPC-induced metabolic adaptations, activation of stress-responsive virulence regulators, deployment of immune evasion strategies , and potentially direct effects of the KPC enzyme. The consequence is a pathogen optimized for persistence, tissue invasion, immune evasion, and systemic dissemination, culminating in significantly worse clinical outcomes. The convergence of blaKPC-mediated resistance and enhanced virulence, particularly in globally successful clones like ST258/ST11, represents an alarming evolutionary trajectory.

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