

## REVIEW ARTICLE

# The making of hypervirulent *Klebsiella pneumoniae*

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**Abstract**

*Klebsiella pneumoniae* is a notorious bacterium in clinical practice. Virulence, carbapenem-resistance and their convergence among *K. pneumoniae* are extensively discussed in this article. Hypervirulent *K. pneumoniae* (HvKP) has spread from the Asian Pacific Rim to the world, inducing various invasive infections, such as pyogenic liver abscess, endophthalmitis, and meningitis. Furthermore, HvKP has acquired more and more drug resistance. Among multidrug-resistant HvKP, hypervirulent carbapenem-resistant *K. pneumoniae* (Hv-CRKP), and carbapenem-resistant hypervirulent *K. pneumoniae* (CR-HvKP) are both devastating for their extreme drug resistance and virulence. The hypervirulence of HvKP is primarily attributed to hypercapsule, macromolecular exopolysaccharides, or excessive siderophores, although it has many other factors, for example, lipopolysaccharides, fimbriae, and porins. In contrast with classical determination of HvKP, that is, animal lethality test, molecular determination could be an optional and practical method after improvement. HvKP, including Hv-CRKP and CR-HvKP, has been progressing. R-M and CRISPR-Cas systems may play pivotal roles in such evolutions. Hv-CRKP and CR-HvKP, in particular the former, should be of severe concern due to their being more and more prevalent.

## 1 | INTRODUCTION

*Klebsiella pneumoniae* (*K. pneumoniae*) is a gram-negative and non-motile bacterium and belongs to *Enterobacteriaceae*, which was first described by Carl Friedlander in 1882 as a bacterium and isolated from the lungs of patients who had died of pneumonia. *K. pneumoniae* is ubiquitously found in water, soil, humans, and animals and can colonize healthcare-related sites.<sup>1,2</sup> *K. pneumoniae* could colonize a variety of sites in human body, for example, axilla, intestinal tract, and nasopharynx.<sup>3–5</sup> In the past decades, *K. pneumoniae* was of greater and greater concern worldwide, mainly due to its enhanced resistance and recently focused hypervirulence.<sup>6,7</sup> Despite SHV-1  $\beta$ -lactamase, encoded on the chromosome and being intrinsic resistance, *K. pneumoniae* could antagonize antimicrobials through various pathways, for example, hydrolyzing enzymes, missing porins,

efflux overexpression, topoisomerase, and lipopolysaccharide (LPS) modification.<sup>8</sup> Alarming, carbapenem-resistant *K. pneumoniae* (CR-KP) has reached a rate of over 30.0% among *K. pneumoniae* strains and brought formidable challenges in clinical practice.<sup>9</sup> In 2016, The World Health Organization (WHO) made a list of the most “critical” bacteria at a global level with an urgent need for new treatments, among which carbapenem-resistant *Enterobacteriaceae* (CRE) was classified as a critical priority organism.

CR-KP could come into existence via multiple mechanisms, that is, (i) production of carbapenemase; (ii) decreased expression or loss of outer membrane proteins with overexpression of extended-spectrum  $\beta$ -lactamases and AmpC cephalosporinases; and (iii) activation of efflux pumps.<sup>10</sup> Carbapenemases include *K. pneumoniae* carbapenemases, New Delhi metallo- $\beta$ -lactamase, Verona integron-encoded metallo- $\beta$ -lactamase, imipenemase, and oxacillinase.<sup>11</sup>

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Disruption or deficiency of OmpK35 and OmpK36 could result in CR-KP.<sup>12</sup> Overexpression of efflux pumps also confers carbapenem resistance.<sup>13</sup> While the reasons of CR-KP differ remarkably worldwide, it is dominantly conferred by the mobile genetic elements harboring a variety of antibiotic-resistance genes, for example, beta lactamase *K. pneumoniae* carbapenemases gene (*bla<sub>KPC</sub>*), New Delhi metallo- $\beta$ -lactamase gene (*bla<sub>NDM</sub>*), and oxacillinase-48 gene (*bla<sub>OXA-48</sub>*).<sup>14,15</sup>

Hypervirulent *K. pneumoniae* (HvKP), first recognized as a unique clinical pathogen in the 1980s in Taiwan,<sup>16</sup> is an entity of being more virulent than classical *K. pneumoniae* (cKP), which is determined by animal (mice, wax moth larvae) lethality tests, neutrophil assay, and so on.<sup>17-19</sup> cKP is often associated with nosocomial infections, for example, pneumonia, urinary tract infection (UTI), and bacteremia, among those at extremes of age or with underlying immunodeficiencies while HvKP often induces infections, such as pyogenic liver abscess (PLA), lung abscess, endophthalmitis, in otherwise healthy individuals (Table 1). The prevalence of HvKP varies in different regions, ranging from 12% to 45% in HvKP-endemic areas.<sup>20-24</sup> Recent studies unveiled a convergent trend of CR-KP and HvKP,<sup>25</sup> which needs our deeper insights. Here, we try to focus on the determinants of HvKP, detection, and its evolution.

## 2 | CONCEPTION AND CLASSIFICATION

cKP is a group of *K. pneumoniae* that lacks hypercapsule, macromolecular exopolysaccharide, or excessive siderophores, which incurs a high median lethality dose (LD<sub>50</sub>) and rarely induces diseases in otherwise healthy individuals (except UTI) regardless of its being multidrug-resistant (MDR). cKP often causes infections, for example, pneumonia, UTI, bacteremia, or meningitis in immunocompromised individuals.<sup>26-28</sup> cKP brings a LD<sub>50</sub> of >10<sup>7</sup> colony forming unit (CFU) in a BALB/c mouse pneumonia model.<sup>29</sup> By contrast, HvKP is another type of *K. pneumoniae* that harbors hypercapsule, macromolecular exopolysaccharide, or excessive siderophores, which incurs a lower LD<sub>50</sub> and induces infections in both immunocompromised and otherwise healthy individuals. HvKP yields a LD<sub>50</sub> of <10<sup>2</sup> CFU in a BALB/c mouse pneumonia model.<sup>29,30</sup> HvKP could be divided into two categories: drug-sensitive and MDR. Among MDR-HvKP, hypervirulent carbapenem-resistant *K. pneumoniae* (Hv-CRKP) and carbapenem-resistant hypervirulent *K. pneumoniae* (CR-HvKP) are both notorious for their super drug-resistance and hypervirulence, the former being much more frequent than the latter. The characteristics of cKP and HvKP are listed in Table 1.<sup>31</sup>

The basis of Hv-CRKP is classical CR-KP while CR-HvKP evolves from HvKP (serotypes K1, K2, K5, K10, K20, K25, K27, and K57) acquiring carbapenem-resistant plasmids.<sup>69,83</sup> As reported in the document,<sup>62</sup> 521 *K. pneumoniae* strains were collected from GenBank as of May 13, 2020; Molecular combinations predicted 29 strains to be *bla<sub>KPC</sub>*-positive and hypervirulent, of which 7 were CR-HvKP and 22 were Hv-CRKP; 94 and 165 strains were HvKP and *bla<sub>KPC</sub>*-positive *K. pneumoniae*, respectively. As of May 31, 2021, 890 *K. pneumoniae*

genomes from GenBank were analyzed, and 53 Hv-CRKP and 17 CR-HvKP strains were designated<sup>84</sup>; 478 and 168 strains were CR-KP and HvKP, respectively. Among another 530 clinical *K. pneumoniae* strains collected in Mainland China from January 2017 to February 2018, 28 and 6 were Hv-CRKP and CR-HvKP respectively<sup>84</sup>; 227 and 171 were CR-KP and HvKP, respectively, showing constituent ratios of 12.3% for Hv-CRKP among CRKP and 3.5% for CR-HvKP among HvKP. Such surveillance results suggest Hv-CRKP is far more prevalent than CR-HvKP.

## 3 | DETERMINANTS

### 3.1 | Hypercapsule

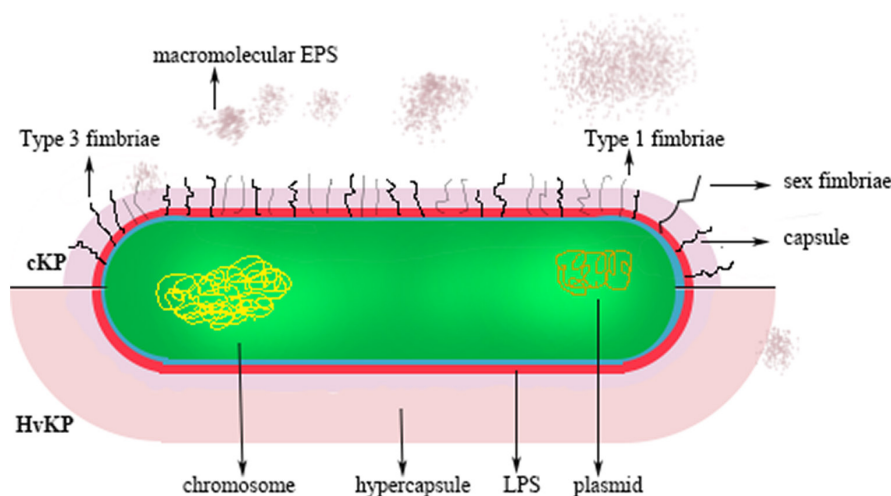
Capsule (Figure 1) is an essential layer of polysaccharide bound on the surface protein Wzi of *K. pneumoniae*,<sup>1,53,85,86</sup> the loss of which would render *K. pneumoniae* remarkably less virulent or nonvirulent.<sup>30,85,87,88</sup> To date, there are in total 79 serotypes for *K. pneumoniae*.<sup>52</sup> In contrast with cKP, HvKP could produce a hypercapsule, which contributes to hypervirulence.<sup>52,65</sup> A basic production and the serotype of capsule is controlled by a chromosomal operon, *cps*, which harbors a couple of genes, that is, *wzi*, *wza*, *wzb*, *wzc*, *gnd*, *wca*, *cpsB*, *cpsG*, and *galF*.<sup>89,90</sup> (Table 2). Sequencing of *wzi* and *wzc* could be a shortcut to determine serotypes of *K. pneumoniae* instead of a traditional serological method.<sup>18,55,91</sup> More importantly, hypercapsule could be regulated by several specific virulence genes, that is, *c-rmpA*, *c-rmpA2*, *p-rmpA*, *p-rmpA2*, and *wzy-K1*. Genes *c-rmpA*, *c-rmpA2*, and *wzy-K1* are both in chromosome while *p-rmpA* and *p-rmpA2* are plasmid-borne.<sup>92-94</sup> Each of the 5 virulence genes could result in hypercapsule. However, their different combinations may yield different production of capsule. *c-rmpA* and *c-rmpA2* in the ICEKp genomic island exist only in serotype K1 *K. pneumoniae* with a positive rate of <50.0%.<sup>95</sup> HvKP shows positive rates of 55–100% for *rmpA* or *rmpA2* while cKP rarely harbors.<sup>21,95</sup> *RmpA*, described in 1989, is a regulator of mucoid phenotype in pK100,<sup>96</sup> along with *RmpB*, another virulence plasmid-encoded regulator.<sup>97</sup>

The expression of *rmpA* depends on RcsB, KvrA, and KvrB.<sup>98</sup> KvrA, KvrB, and RcsB contribute to capsule regulation through the control of the *rmpA* promoter and through additional mechanisms. *K. pneumoniae* strains with deletions of *kvrA* and *kvrB* are less virulent than wild type.<sup>99</sup> Genes *c-rmpA*, *c-rmpA2*, *p-rmpA*, and *p-rmpA2* positively regulate the *cps* locus at the transcriptional level.<sup>94,97,100,101</sup> Gene *wzy-K1* was firstly termed as *mgaA*, which was discovered in 2004<sup>102</sup> and specific to serotype K1 *K. pneumoniae*.<sup>103-105</sup> *Wzy* is a polymerase present in the inner membrane,<sup>106</sup> which combines Wzx into “Wzx/Wzy pathway”<sup>107</sup> or “Wzx/Wzy secretion system”<sup>108</sup> and is imperative for polymerization of undecaprenyl diphosphate-linked K-antigen sugar moiety in the periplasmic region. *Wzy* polymerizes the repeating oligosaccharides following their being flipped by Wzx in a blockwise manner.<sup>109</sup> *Wzy* then releases the oligosaccharide moiety from its lipid carrier to the nascent polymer.<sup>110</sup> In addition, the regulation of capsule A (*cpsA*) and B (*cpsB*) genes can also result

TABLE 1 Characteristics of cKP and HvKP

Parameters	cKP	HvKP	Hv-CRKP or CR-HvKP	References
Typical infections	Pneumonia, UTI, bacteremia	PLA; lung, neck and kidney abscesses; endophthalmitis; necrotizing fasciitis; meningitis; pneumonia; cellulitis; myositis; septic arthritis; osteomyelitis	Combined infections by cKP and HvKP	19,26–33
Susceptible populations	Immunocompromised (diabetics, patients with malignancies or transplant, bedridden individuals)	Diabetics, otherwise healthy individuals	Combined populations	19,20,28,29,34–37
Serotypes	K1-K79	Mostly K1 and K2, seldom K5 and K57	K64, K47, K20, K2 and K20	1,28,29,38–50
Siderophores (positive rates, %)	Enterobactin (100), yersiniabactin (17–46), salmochelin (2–4), aerobactin (6)	Enterobactin (100), yersiniabactin (90), salmochelin (>90), aerobactin (>93)	Enterobactin (100), yersiniabactin (90), salmochelin (40), aerobactin (>93)	40,51–56
Geographical prevalence	Worldwide	Mostly the Asian Pacific Rim, the trend to the world	Mainly Asia, the trend to the world	29,30,32,38,57–76
Commonly acquired infection type	Primarily nosocomial	Community acquired	Often nosocomial and seldom community-acquired	29,59,77–80
Drug-resistance	Frequent (for example ESBLs and carbapenemase-producing)	Rare except penicillin-resistance	Carbapenemase-producing	9,19,28,29,81,82

Abbreviations: cKP, classical *Klebsiella pneumoniae*; CR-HvKP, carbapenem-resistant hypervirulent *Klebsiella pneumoniae*; ESBLs, extended-spectrum  $\beta$ -lactamases; HvKP, hypervirulent *Klebsiella pneumoniae*; Hv-CRKP, hypervirulent carbapenem-resistant *Klebsiella pneumoniae*; PLA, pyogenic liver abscess; UTI, urinary tract infection ; PLA, pyogenic liver abscess.



**FIGURE 1** The schematic diagram of *Klebsiella pneumoniae* and the difference between cKP and HvKP

Genes	Related factor	Plasmid	Chromosome
<i>wzy-K1, wzx, wzc, wza, wzb, wzi, gnd, wca, cpsA, cpsB, cpsG, galF</i>	Capsule/ exopolysaccharide		✓
<i>allS</i>	Allantoin		✓
<i>rmpA, rmpB, rmpA2</i>	Capsule	✓	✓
<i>kvrA, kvrB; rcsA, rcsB</i>	Capsule		✓
<i>p-rmpA, p-rmpA2</i>	Capsule	✓	
<i>c-rmpA, c-rmpA2</i>	Capsule		✓
<i>entABCDEF</i>	Enterobactin	<i>entC/E/F</i>	✓
<i>fepABCDG</i>	Enterobactin	✓	✓
<i>irp, ybt, fyu</i>	Yersiniabactin	✓	✓
<i>iroABCDE</i>	Salmocheilin	✓	✓
<i>iucABCD, iutC</i>	Aerobactin	✓	✓
<i>fim</i>	Type 1 fimbria	<i>fimC/D</i>	✓
<i>mrkABCD</i>	Type 3 fimbria		✓
<i>wb, waa, lpx, uge, wabG</i>	LPS		✓
<i>pks, clbA-S</i>	Colibactin	✓	✓
<i>terZA-Z, terWXY</i>	Tellurite resistance	✓	
<i>silS</i>	Silver resistance	✓	
<i>peg-344</i>	Metabolite transporter	✓	

**TABLE 2** Locations of virulence-related genes in this study

Abbreviations: LPS, lipopolysaccharide.

in hypercapsule. Glucose could also be a signal to increase capsule production.<sup>94,111</sup>

Capsule could protect *K. pneumoniae* through the following strategies: inhibiting phagocytosis by hosts' immune cells, preventing activation of the early immune response, and hampering lysis by complement and antimicrobial peptides.<sup>31</sup> Capsule confers resistance to opsonophagocytosis in *K. pneumoniae* regardless of opsonins.<sup>112</sup> Capsule could also contribute to biofilm formation,<sup>113,114</sup> which is contrary to another report.<sup>115</sup> Capsule induces a defective immunological host response, for example, maturation of dendritic cells (DCs) and pro-Th1 cytokine production by hampering bacterial binding and internalization.<sup>116</sup> The rate of phagocytosis by immune

cells is inversely proportional to the amount of capsule on the bacterial cell surface. Capsule induces DCs maturation with upregulation of CD83, CD86, and toll-like receptor (TL) and downregulation of CD14 and DC-SIGN. Capsule also suppresses the host immunological responses by inducing lower cytokines TNF- $\alpha$ , IFN- $\gamma$ , and IL-6 in the early stages of lung infection, reverse in the late stage.<sup>88,117</sup> In contrast, capsule also induces persistently higher IL-10 level, which down-regulates the expression of pro-inflammatory cytokines.<sup>117</sup> *Klebsiella* inhibits Rac1 activation; and inhibition of Rac1 activity triggers a NOD1-mediated CYLD and MKP-1 expression, which in turn attenuates IL-1 $\beta$ -induced IL-8 secretion. Purified capsular polysaccharide (CPS) neither reduces IL-1 $\beta$ -induced IL-8 secretion nor

induces the expression of CYLD and MKP-1, thereby indicating that CPS is necessary but not sufficient to attenuate inflammation.<sup>118</sup> More immune cells were recruited to lungs infected with acapsular than capsular *K. pneumoniae* strains.<sup>88</sup> Capsule could protect *K. pneumoniae* against human defensin-mediated bactericidal activity,<sup>119</sup> attenuate the production of human defensins in vitro,<sup>119</sup> and enhance pneumonia in mouse models.<sup>120–122</sup> The hypercapsule would render HvKP more prominent defense in comparison with cKP.

CPS, but not LPS O side chain is a major complement resistance factor in *K. pneumoniae* isolates due to its modulating the deposition of C3 and protecting the microorganisms against human alveolar macrophage phagocytosis.<sup>87,123</sup> Anionic capsule, but not cationic or uncharged, blocked the bactericidal activity of antimicrobial peptides or proteins, for example, human neutrophil defensin 1, beta-defensin 1, lactoferrin, and protamine sulfate, by binding them, thereby reducing the amount of peptides reaching *K. pneumoniae* surface.<sup>124,125</sup> Capsule both protects *K. pneumoniae* from the bactericidal action of defensins and impedes their expression via the expression of CYLD and MKP-1.<sup>119</sup>

### 3.2 | Macromolecular exopolysaccharide

Despite of polysaccharides bound on Wzi, namely CPS, *Enterobacteriaceae* including *K. pneumoniae* could also synthesize and secrete a variety of extracellular saccharides with low or high molecular weight, included in which are alginate, cellulose, colonic acid, curdlan, dextran, diutan, gellan, hyaluronic acid, levan, succinoglycan, welan, and xanthan.<sup>126</sup> Among such exopolysaccharides, colonic acid is closely related to HvKP for its macromolecular weight and ability to form biofilm, which is produced via "Wzx/Wzy pathway".<sup>127–129</sup>

Exopolysaccharides could be produced via 4 pathways: (1) the so-called Wzx/Wzy-dependent pathway; (2) the ATP-binding cassette (ABC) transporter-dependent pathway; (3) the synthase-dependent pathway and (4) the extracellular synthesis by use of a single sucrose protein.<sup>126</sup> "Wzx/Wzy pathway" is closely related with HvKP, which per se includes a couple of virulence proteins, that is, Wzx, Wzy, Wzc, Wza and Wzb. Hypervirulence via "Wzx/Wzy pathway" is limited to serotype K1 *K. pneumoniae* strains. Wzy-K1, wxz, and wzc but not wza and wzb are always positive simultaneously, suggesting they are one integrity to form hypervirulence; The following knockout of each of them resulted in non-mucoviscous colonies by "string test"<sup>44</sup> and disappeared macromolecular exopolysaccharides by Periodic Acid Schiff stain.<sup>102</sup>

Wzx is a flippase<sup>109</sup> and Wzc is an inner membrane tyrosine autokinase. Following the action of Wzx and Wzy, nascent K antigen is translocated onto the bacterial surface by synergetic action of Wza, Wzc, and Wzb.<sup>130</sup> Wzc and Wzb control the length and amount of K antigen.<sup>131,132</sup> Wzc and Wzb are cognate low molecular weight phosphotyrosine phosphatase.<sup>133</sup> Wzc belongs to polysaccharide copolymerase 2a subfamily, which is essential for the assembly of Group 1 capsule 1.<sup>134</sup> Wza is an outer membrane translocon that translocates

the nascent capsular polysaccharides on the bacterial surface.<sup>135,136</sup> Wza octamerizes across the periplasmic and outer membrane regions.<sup>137,138</sup> Wza is widely distributed in various *K. pneumoniae* including HvKP and cKP, loss of which induces ineffective exporting CPS.<sup>139,140</sup> To date, the structures and exact functions of Wzx, Wzy, Wzc, Wza, and Wzb were primarily elucidated in *Escherichia coli*, which is a close member with *K. pneumoniae* in *Enterobacteriaceae*.

### 3.3 | Excessive siderophores

Iron is an essential element for bacteria to strive during infection, which is restricted by the host,<sup>141</sup> a process called nutritional immune.<sup>142</sup> Iron supply could affect the proliferation of *K. pneumoniae* and consequent bacterial count, which is also a vital factor for virulence. The majority of iron is deposited in a bound manner in the host, for example, transferrin and ferritin, little being free. Therefore, to capture iron is a prerequisite for *K. pneumoniae* to survive and propagate.<sup>143</sup> *K. pneumoniae* could harbor 4 kinds of siderophores, that is, enterobactin, yersiniabactin, salmochelin, and aerobactin, which possess higher affinity than host transport proteins and can thus steal iron successfully from hosts' iron-chelating proteins.<sup>143</sup> At least one siderophore is harbored by *K. pneumoniae* with the positive rate of enterobactin being 100.0%. The affinity of the 4 siderophores is not equal, ranging from aerobactin with the lowest to enterobactin with the highest.<sup>144,145</sup>

Enterobactin is the basic iron uptake system in *K. pneumoniae*,<sup>57,146–148</sup> with its biosynthesis and transport being encoded by the chromosomal gene cluster *entABCDEF* and *fepABCDG*.<sup>58,149</sup> Enterobactin could be neutralized by the host-secreted lipocalin-2,<sup>59,150,151</sup> which has several antimicrobial capabilities and is secreted by many cell lineages, for example, neutrophils. Lipocalin-2 is upregulated by the host in the respiratory tract infection of *K. pneumoniae*.<sup>152–154</sup> In addition, lipocalin-2 also has proinflammatory effects, leading to neutrophil recruitment to the site of infection via IL-8.<sup>155,156</sup> The presence of lipocalin-2 aids in the clearance of *K. pneumoniae* with only one siderophore: enterobactin.<sup>60</sup>

Yersiniabactin is another "basic" siderophore in *K. pneumoniae* with a positive rate of over 75.0%,<sup>62</sup> which originated from *Yersinia* and is also encoded by chromosome. Yersiniabactin is positive in 18% of cKP strains and 90% in HvKP.<sup>58,60</sup> *irp* gene cluster encodes proteins for yersiniabactin synthesis, *ybt* and *fyu* encode transporters for the secretion of enterobactin and *ybtO* encodes the uptake receptor of enterobactin.<sup>58,59,157</sup> During lung infection, yersiniabactin together with enterobactin is highly expressed and it is not inhibited by lipocalin-2 in vivo.<sup>59,60,143</sup> Yersiniabactin alone is not capable of acquiring the iron for *K. pneumoniae* and lack of the other 3 siderophores would render *K. pneumoniae* not capable of disseminating from the lungs,<sup>60</sup> which may be the reason why the positive rate of enterobactin is 100.0%. In addition to lung infection, yersiniabactin may be important for *K. pneumoniae* to induce PLA.<sup>58,158</sup>

Salmochelin is an additional siderophore in *K. pneumoniae*, which is per se a c-glucosylated form of enterobactin.<sup>159,160</sup> The

c-glucosylation is carried out by *iro* gene cluster, that is, *iroABCDE* on either the chromosome or a plasmid.<sup>58</sup> IroN contributes to the transport of salmochelin carrying iron.<sup>149,161</sup> Salmochelin is not neutralized by lipocalin-2<sup>159</sup> and induces *K. pneumoniae* colonization of the nasopharynx<sup>155</sup> and consequent pneumonia. Salmochelin is seldom present in cKP with a rate of 2–4% but usual in HvKP with a rate of > 90%.<sup>57,58,60</sup>

Aerobactin is a citrate-hydroxamate siderophore, which is rarely present in cKP with a rate of ~ 6% but common in HvKP rating over 90.0%.<sup>46,57,61,146</sup> Aerobactin is usually associated with a hypercapsule while *K. pneumoniae* with hypercapsule does not inevitably harbor aerobactin.<sup>46,61,143</sup> Aerobactin is controlled by the gene cluster *iucABCD* and its transport is determined by *iutA*, both of which are often present on the same pLVPK-like plasmids carrying *p-rmpA*.<sup>58,93,97,162,163</sup> Aerobactin is not neutralized by lipocalin-2. Aerobactin is crucial for some HvKP causing lung infection<sup>158</sup> and it accounts for the majority of the total siderophores in HvKP.<sup>164</sup> Aerobactin, but not enterobactin, yersiniabactin, or salmochelin, is essential for successful infection by HvKP in pneumonic and subcutaneous mouse infection models.<sup>158,164</sup> However, in a mouse *i.p.* HvKP infection model, aerobactin along with yersiniabactin and salmochelin is seemingly redundant unless the 3 are all deleted.<sup>58</sup>

In addition to the aforementioned 4 siderophores, other iron-stealing systems may also support the pathogenesis of HvKP. *K. pneumoniae* NTUH-K2044 also encodes ferrichrome, ferric, ferrous, and haem-iron uptake systems.<sup>58</sup> Intriguingly, another study reported a CR-HvKP strain with a positive string test and K2 ST14 types,<sup>165</sup> which lacked *rmpA*, *rmpA2*, and yersiniabactin, but harbored receptors (not biosynthetic genes) for aerobactin and salmochelin. It suggests curious capability of “stealing” siderophores from other species with their coexistence and the following transformation into being hypervirulent.

### 3.4 | Other virulence factors

Apart from hypercapsule, exopolysaccharide, and excessive siderophores, which are all vital for HvKP, HvKP could also possess a couple of other virulence factors, such as fimbriae, LPS, colibactin, tellurite and silver resistance and allantoin metabolism.

Fimbriae in KP are classified as type 1 and 3 with type 1 being thin, thread-like protrusions and type 3 being helix-like filaments.<sup>31</sup> Type 1 fimbriae, encoded by *fim* gene cluster,<sup>31</sup> are widely distributed in *K. pneumoniae* with a positive rate of over 90.0%,<sup>166,167</sup> which was over 95.0% in our study.<sup>62</sup> Type 3 fimbriae, encoded by *mrk-ABCD* gene cluster,<sup>168</sup> are “mannose insensitive” and therefore do not bind mannose. Type 1 fimbriae contribute to UTI<sup>169</sup> and biofilm formation, including on urinary catheters.<sup>170</sup> Type 3 fimbriae have been shown to bind extracellular matrix proteins such as type IV and V collagens<sup>171</sup> and contribute to biofilm formation.<sup>170,172</sup> Type 3 fimbriae are also over 95.0% positive in *K. pneumoniae* strains.<sup>62</sup>

LPS, also termed as endotoxin, typically consists of an O antigen, a core oligosaccharide and lipid A, which are ubiquitous and

encoded by *wb*, *waa*, and *lpx* gene clusters respectively.<sup>173–176</sup> LPS protects *K. pneumoniae* against humoral defenses and also serves as a strong immune activator. Lipid A is a potent ligand for TLR4, which leads to the production of cytokines and chemokines, followed by the recruiting and activating of neutrophils and macrophages. Lipid A protects *K. pneumoniae* against some cationic antimicrobial peptides.<sup>177,178</sup> *K. pneumoniae* strains with K1, K10, and K16 serotypes could mask their LPS,<sup>179</sup> which was also found in HvKP.<sup>180</sup> *K. pneumoniae* could also modify its LPS as unrecognizable by certain immune receptors.<sup>177</sup> For *K. pneumoniae*, LPS is the primary means of protection against complement, even in the presence of capsule.<sup>179</sup> The O antigen of LPS protects against C3 by binding C3b and abrogates pore formation.<sup>179,181–183</sup> *uge*, which encodes a UDP galacturonate 4-epimerase, and *wabG*, which encodes a GalA transferase, are also involved in the production of LPS.<sup>184,185</sup>

Colibactin, also termed as “genotoxin,” is a natural and genotoxic chemical compound, which is encoded by *pks* genomic island with a length of 54 Kb.<sup>186</sup> The *pks* island represents a total of 19 genes, that is, *clbA* to *clbS*.<sup>186</sup> The *pks* locus is usually present in a chromosomal integrative and conjugative element (ICE).<sup>47,187</sup> Colibactin could induce DNA double-strand breaking, chromosome aberrations, and cell cycle arrest in the G2/M phase.<sup>186,188</sup> In addition, colibactin contributes to colonization and survival of *K. pneumoniae*<sup>189</sup> and the global spread of clonal group (CG) 23.<sup>190</sup> The *pks*-related island could also encode numerous compounds<sup>191</sup> and contribute to the anti-inflammatory,<sup>192</sup> antibiotic,<sup>193,194</sup> and analgesic effects<sup>195</sup> for colibactin-producing *K. pneumoniae*. Prevalence of *pks* differs in different regions, ranging from 3.5% (5/141) in Europe to 25.6% (53/207) in Taiwan.<sup>187,196</sup> In particular, *pks* is highly prevalent in serotype K1 *K. pneumoniae* rating from 71.4% (35/41) and 78.8% (26/33).<sup>187,197</sup> Carriage of *rmpA*, *iutC*, and *ybtA* was significantly higher in the *pks*-positive isolates than the *pks*-negative isolates (95.5% vs. 13.2%,  $p < 0.001$ ), which indicates the emerging *pks* genotoxic trait is associated with increasing HvKP strains.<sup>197</sup>

Tellurite and silver resistance is encoded by *terZA* to *Z*, *terWXY* and *silS*, respectively, which may be important for systemic infections.<sup>163,198</sup> Such genes are in the virulence plasmid and not HvKP specific,<sup>92,199</sup> loss of which decreases tellurite and silver resistance in *K. pneumoniae* but did not affect virulence in a mouse pneumonia model.<sup>199</sup>

Allantoin metabolism is one pathway for *K. pneumoniae* to obtain carbon and nitrogen from allantoin,<sup>200</sup> a degradation product from nucleic acids that some microbes can use as a source of nitrogen.<sup>201</sup> Allantoin metabolism is under control of *all* gene cluster<sup>202</sup>; *all* gene cluster is enriched in strains associated with PLA versus commensal strains.<sup>203,204</sup> *allS* is present in K1 but no other serotype HvKP causing PLA with a rate of 100.0%,<sup>46</sup> which suggests an association with K1 serotype. However, if specimen types are of consideration, *allS* is not inevitably in line with K1 serotype, vice versa. The absence of *allS* does not reduce virulence.<sup>46</sup>

Peg-344 is a metabolite transporter encoded by HvKP virulence plasmid.<sup>205</sup> The exact function of *peg-344* needs to be elucidated. Peg-344 could increase RNA abundance when *K. pneumoniae* is



grown in human ascites, which suggests Peg-344 may transport an unidentified growth factor present in ascites.<sup>205</sup> It may be per se a coincidence that *peg-344* could be a rather good indicator of HvKP because of its being in virulence plasmid.

## 4 | ROLE OF RACE FACTOR

PLA is a typical infection caused by HvKP. PLA is endemic in East Asia,<sup>69,75,83,206</sup> which indicates a close relationship between certain races and HvKP. In North America, 78.3% of patients were of Asian origin,<sup>63</sup> who were with *K. pneumoniae*-induced PLA. A surveillance showed high carriage rate of *K. pneumoniae* (21.1%) in Korean intestinal tract<sup>207</sup>; among the strains, 23.0% were K1 serotype, predominant in PLA-derived *K. pneumoniae* strains.<sup>208</sup> Chinese ethnicity is also a major factor predisposing to intestinal colonization by serotype K1/K2 *K. pneumoniae* isolates.<sup>3</sup> The colonization of HvKP in intestinal tract is a vital step for the formation of PLA. As carbapenem resistance is taken into consideration, CR-HvKP and Hv-CRKP are mainly distributed in healthcare settings<sup>209</sup> and presents no significant correlation with race.

On the contrary, the role of race factor is some limited. For instance, immigration to western countries decreased the carriage of *K. pneumoniae* (5.6% vs 24.1%,  $p = 0.024$ ) in Korean intestinal tract,<sup>207</sup> which indicates the important role of environmental factors.<sup>210</sup>

## 5 | DETERMINATION

To determine a *K. pneumoniae* strain as HvKP, the golden standard should be animal tests, such as mouse lethality test,<sup>15</sup> *Galleria mellonella* lethality test.<sup>18,19,25</sup> Besides, serum killing assay is also an alternative method.<sup>211</sup> However, the aforementioned tests are cumbersome, sometimes confusing<sup>212</sup> and not ready to use for clinical laboratories. It is essential to find novel methods of determining HvKP for clinical practice.

"String test" was once used for HvKP. The formation of a viscous string >5 mm is considered positive when the colony is stretched out using a loop from a blood-agar plate.<sup>102</sup> "String test" showed a sensitivity of 89% and a specificity of 91%,<sup>92</sup> which are inconsistent with another report.<sup>213</sup> Sequence types (ST) and serotypes are both not so specific for HvKP, but the virulence gene repertoire may be one optimal clue to choose markers.<sup>54,65,81,214</sup> Virulence genes in the virulence plasmids (for example, pK2044 and pLVPK)<sup>47,93,215</sup> are more accurate for defining HvKP than those in integrative and conjugative elements (ICE),<sup>187,190,197,216</sup> among which are genes encoding yersiniabactin and colibactin, not vital for hypervirulence. Nevertheless, *iroB*, *iucA*, *peg-344*, *rmpA*, and *rmpA2* in the virulence plasmids are all optimal biomarkers for defining HvKP,<sup>92</sup> among which *iuc* and/or either *rmpA* or *rmpA2* would be the best combined markers. In the recent report of 5 HvKP strains,<sup>25</sup> *iro*, *peg-344*, and *rmpA* were all negative in the relevant plasmid. With the recognition on molecular

biomarkers progressing for defining HvKP, novel single or combined markers are likely to be designated in the near future.

On the other hand, hypervirulence of *K. pneumoniae* is restricted by series of virulence genes (Table 2), which means that the polymorphism and deletion of any of such genes may affect the eventual virulence. The lack of hypervirulent phenotype in virulence plasmid-bearing CR-KP strains was found to be due to the mutation's presence on *rmpA* and *rmpA2* genes, which rendered them non-functional, while some strains carrying wild type *rmpA* did not exhibit hypervirulent phenotype either suggesting that other factors might also contribute to the hypervirulence of CR-KP.<sup>217</sup> A large proportion (58%) of CR-KP strains in China mainland during 2014–2017 were found to harbor a virulence plasmid, while only 13% of such strains exhibited a hypervirulent phenotype by string test and neutrophil assay.<sup>217</sup> Therefore, the complexity of virulence in *K. pneumoniae* indicates the molecular combinations for hypervirulence need further investigations and optimizations.

## 6 | EVOLUTION

Clonal group (CG) 23 is associated with K1 capsule and HvKP, which accounts for over 30% of (ST) for HvKP-inducing PLA.<sup>214,218–220</sup> Another study showed over 80% of CG23 *K. pneumoniae* strains inducing PLA belong to CG23-I, which emerged in ~1928 following acquisition of ICEKp10, and then disseminated globally<sup>190</sup>; Ninety-four of the 97 strains possessed plasmid-borne *iro*, *iuc*, *rmpA*, and *rmpA2*.<sup>190</sup> The possible dates for the most recent common ancestors for the entire CG23 population, the CG23-I sublineage, and the equine strains could be 1878, 1928, and 1972, respectively.

Plasmids pK2044 (224,152 bp) and pLVPK (219,385 bp)<sup>93,215</sup> are the 2 classical ones conferring hypervirulence in *K. pneumoniae*,<sup>97,162,163</sup> which is mediated through *iuc*, *rmpA*, and *rmpA2* genes. pK2044 and pLVPK are highly similar.<sup>220,221</sup> Their descendants varied in the length, for example, 121 Kb, 90 Kb, 200 Kb, and 178 Kb.<sup>25,47,220</sup>

The first KPC-producing *K. pneumoniae* was found in a patient in a North Carolina Hospital in 1996,<sup>222</sup> while the first KPC-2-producing *K. pneumoniae* in China was found in Zhejiang Province in 2007.<sup>223</sup> Furthermore, the first Hv-CRKP, showing K2 type and ST65 emerged in China in 2013.<sup>15</sup> Another recent study unveiled an outbreak of Hv-CRKP with ST11 and a pLVPK-like virulence plasmid pVir-CR-hvKP4 (178,154 bp).<sup>25</sup> Compared with pLVPK, pVir-CR-hvKP4 had a deletion of 41,231 bp fragment, which included the virulence genes *rmpA* and *iro*. Another report from Taiwan investigated a strain TVGHCRE225 with ST11 and a pVir plasmid (297,984 bp), a hybrid HvKp virulence plasmid.<sup>224</sup> 38% of pVir shared 49% and 47% of identities with pK2044 and pLVPK, respectively, the remaining portion possessing 61% coverage with pPMK-NDM, a resistance plasmid, at 99% identity.

MDR and extremely drug-resistant (XDR) HvKP are concerning pathogens, which accounts for 7.4–15.0% among CR-KP<sup>225</sup> albeit 57% (20/35) HvKP strains inducing bacteremia were concurrently

CR-KP in a 2016 investigation from China.<sup>226</sup> Hv-CRKP and CR-HvKP, the most notorious ones among MDR and XDR HvKP, are now seemingly emerging worldwide.<sup>25,227</sup> Such evolution may occur through 2 mechanisms. The first pathway is via HvKP acquiring a plasmid carrying drug resistance determinants<sup>228,229</sup> or by the insertion of resistance genes into virulence plasmid or chromosome harbored by HvKP.<sup>23,230</sup> The second pathway is via MDR/XDR cKP acquiring a pK2044- or pLVPK-like virulence plasmid or integrated virulence genes into drug resistance plasmid.<sup>25</sup> The virulence plasmids themselves are usually non-conjugative and therefore non-self-mobilizable. However, they could be mobilized or co-transferred with the help of the self-transferable incompatibility group F plasmids.<sup>84,231</sup> Due to the hypercapsule of HvKP itself, which could mask the fimbriae and hamper the conjugation, the second pathway may be more convenient. Hv-CRKP is far more prevalent than CR-HvKP.<sup>84</sup> ST11 accounted for over 70.0% among CR-HvKP/Hv-CRKP strains.<sup>62</sup> However, such overall assessment is lacking (Table 3).<sup>7</sup> Hv-CRKP and CR-HvKP are both still progressing, obtaining other resistance, for example, to colistin.<sup>232</sup>

Integration and conjugation (Figure 2) are two primary means of acquiring foreign nucleic acids. tRNA sites are often targets for integration and 73% of *K. pneumoniae* strains had an ICE inserted into one or more of the four asparagine tRNA genes.<sup>216</sup> ICEKp1, 76 Kb long in NTUH-2044, was more prevalent in HvKP than in cKP: 38/42 vs 5/32.<sup>237</sup> Later, a more widely conserved ICEKp (KPHPI208-GM1 also designated ICEKp10) was described to possess 8 genomic modules,<sup>187</sup> which is ubiquitous in CG23 HvKP strains.<sup>47</sup> ICEKp10, the most common ICE, was found in 77% of ST23, 40% of ST258, and in 25 other STs strains.<sup>238</sup> However, ICEKp10 is poorly conserved in non-CG23 HvKP. The comparative analysis of 97 CG23 genomes

showed the 81 members of sublineage CG23-I had acquired ICEKp10 containing genes that encode yersiniabactin and colibactin.<sup>190,238</sup> ICEKp is widely distributed in both cKP and HvKP, which bore 14 variants.<sup>238</sup> ICE could also carry other virulence genes apart from those encoding yersiniabactin and colibactin. ICEKp1 encodes RmpA and salmochelin.<sup>237</sup> In addition, the integration of other ICE or genomic islands is also a commonplace.<sup>216,239</sup> The yersiniabactin carried by ICE is more beneficial in cKP than in HvKP.<sup>60,158</sup> Colibactin contributes to colonization, mucosal invasion, and/or dissemination of *K. pneumoniae*.<sup>189</sup> Furthermore, the other factors carried by ICEKp or other genomic islands may prove to play critical roles in various settings.

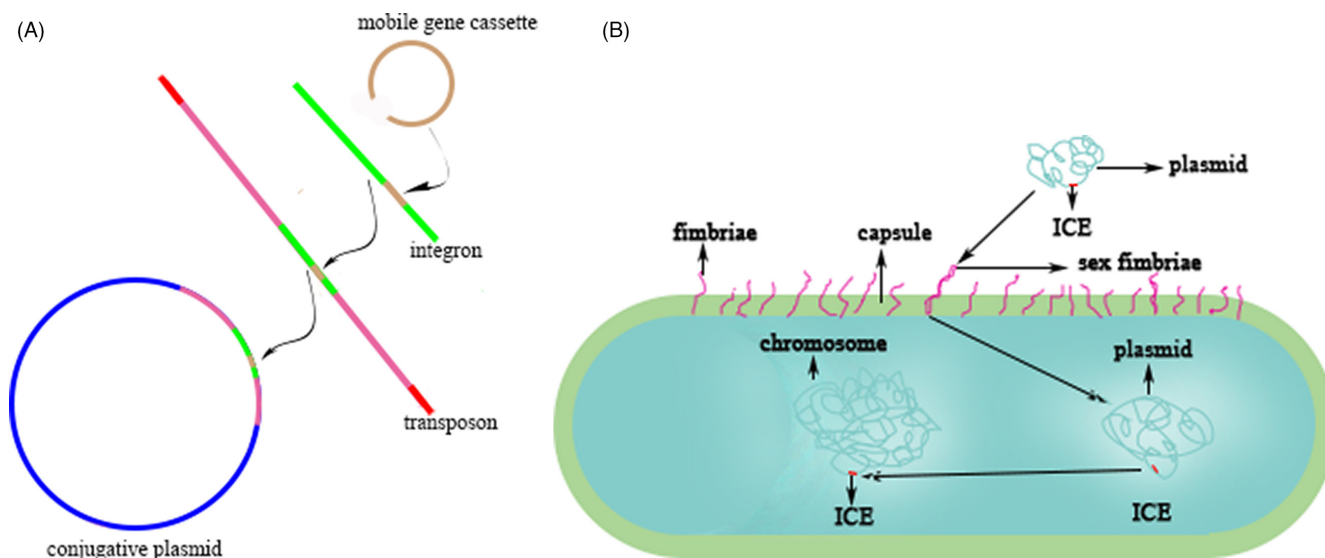
The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas), restriction and modification (R-M) are the two primary immune systems in bacteria, which are both responsible for surveillance of nucleic acids and may play salient roles in horizontal gene transfer (HGT) of drug resistance or virulence genes.<sup>240</sup> CRISPR-Cas system could cut exogenous DNA by targeting proto-spacers.<sup>241-243</sup> Recent studies<sup>244,245</sup> confirmed that CG258 *K. pneumoniae* strains lack type I-E CRISPR-Cas system and another investigation suggested such absence is associated with the dissemination of IncF epidemic drug resistance plasmids in CG258.<sup>246</sup> Furthermore, CG258 *K. pneumoniae* strains also has an impaired R-M system.<sup>247</sup> Type I R-M system consists of HsdR (slicing), HsdM (methylating) and HsdS (targeting),<sup>240,248</sup> which could hamper invasion of foreign DNA via methyltransferase and restriction endonuclease.<sup>248</sup> In addition, CRISPR-Cas and R-M systems could cooperate to combat exogenous nucleic acids.<sup>249,250</sup> Therefore, lack of CRISPR-Cas and R-M renders CG258 more prone to acquire outer plasmids carrying drug resistance or virulence

TABLE 3 Characteristics of Hv-CRKP and CR-HvKP

Classification	Location	ST	Serotype	Clinical context	Note	References
Hv-CRKP	China	ST11	K47	Ventilator-associated pneumonia	Few cases: 5 isolates; XDR; 178 Kb pLVPK-like plasmid; One clone	25
Hv-CRKP	China	ST11	K47	Retrospective study	One case; pLVPK-like plasmid and 2 drug resistance plasmid; unique feature of 5 tandem copies of <i>bla</i> <sub>KPC-2</sub>	233
CR-HvKP	USA	ST23	K1	UTI	One case; <i>bla</i> <sub>SHV-36</sub> , <i>fosA</i> , <i>oqxAB</i> on chromosome; drug resistance plasmid with <i>bla</i> <sub>KPC-2</sub> , <i>bla</i> <sub>TEM-1A</sub> and truncated <i>bla</i> <sub>OXA-9</sub>	227
CR-HvKP	China	ST23	K1	Sepsis	One case; pLVPK-like plasmid with insertion of <i>bla</i> <sub>KPC-2</sub> and <i>dfrA14</i>	23,234
CR-HvKP	China	ST36	K62	Bloodstream, burn wounds	One case; pLVPK-like plasmid and drug resistance plasmid with <i>bla</i> <sub>KPC-2</sub> , <i>fosA</i> , <i>oqxAB</i>	229
CR-HvKP	China	ST86	K2	Burn wound	One case; <i>bla</i> <sub>KPC-2</sub> and <i>bla</i> <sub>NDM-1</sub> ; 215 Kb virulence plasmid	235
CR-HvKP	China	ST65	K2	Septicemia	One case; Encodes enterobactin and aerobactin but not yersiniabactin or <i>kfu</i> ; <i>bla</i> <sub>SHV-11</sub> , <i>bla</i> <sub>TEM-53</sub> , <i>ompK35/36</i> decreased expression	15
CR-HvKP	Canada	ST86	KL2	UTI	One case; Plasmid with <i>bla</i> <sub>KPC-2</sub> as well as <i>bla</i> <sub>SHV-1</sub> and <i>fosA</i>	236

Abbreviations: CR-HvKP, carbapenem-resistant hypervirulent *Klebsiella pneumoniae*; Hv-CRKP, hypervirulent carbapenem-resistant *Klebsiella pneumoniae*; ST, sequence type; XDR, extreme drug resistance; UTI, urinary tract infection.





**FIGURE 2** The formation and transfer of drug resistance or virulence elements. (A) the formation of drug resistance or virulence elements. (B) the transfer of drug resistance or virulence elements

genes, which is called HGT. Armed with extreme drug resistance and virulence, CR-HvKP and Hv-CRKP strains can then survive better in both community and healthcare settings and cause consequent both vertical transfer and HGT, forming a vicious cycle. KPC(+) ST11 CR-KP accounted for 11 mobile genetic element clusters with type A and F sharing 20.83% and 54.76%, respectively,<sup>251</sup> showing an evident combination of HGT and vertical transfer which is likely present in Hv-CRKP.

## 7 | CONCLUSIONS

*Klebsiella pneumoniae* was first described in the late 19th century, which was more and more documented for its hypervirulence and drug resistance. Hypervirulence of *K. pneumoniae* is primarily dependent on some factors, that is, hypercapsule, macromolecular exopolysaccharide, or excessive siderophores albeit *K. pneumoniae* harbors numerous virulence genes. Molecular determination of HvKP is a practical pathway in contrast with the traditional laborious lethality tests while further improvement is needed. HvKP, including Hv-CRKP and CR-HvKP, could bring great challenges worldwide in clinical practice in the future due to their extreme virulence and drug resistance. The impaired immune systems (CRISPR-Cas and R-M) could enhance such trend.

As confirmed now and speculated in the future, Hv-CRKP is far more prevalent than CR-HvKP. They would not bring more and more novel virulence, but present more and more drug resistance, for example, polymyxin resistance. Their targeted options are rather limited. More researches are needed to be done to fight against it.

## AUTHOR CONTRIBUTIONS

Piaopiao Dai and Dakang Hu conceived of and wrote the review, which was revised by Dakang Hu.

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## CONFLICT OF INTEREST

None declared.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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