Prevalence of Hypervirulence-Associated Pathogenicity Loci Among *Klebsiella pneumoniae* Bloodstream Isolates at a United States Hospital

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Abstract

*Klebsiella pneumoniae* is a major threat to human health worldwide. “Classical” *K. pneumoniae* (cKp) strains commonly cause multidrug-resistant (MDR) infections in debilitated patients residing in hospitals and long-term care facilities. In contrast, hypervirulent *K. pneumoniae* (hvKp) strains may cause invasive, community-acquired infections in otherwise healthy individuals. These highly virulent strains were first identified in Taiwan in 1986 and have since disseminated across the globe. The prevalence of hvKp has been well described in Southeast Asia, but is not well understood in the United States. In this work, 141 consecutive *K. pneumoniae* blood stream isolates were collected at Northwestern Memorial Hospital from 2015-2017. To screen this collection for hvKp, isolates were tested for the hypermucoviscous phenotype and the presence of aerobactin and salmochelin biosynthesis genes, biomarkers that have been used to distinguish hvKp from cKp. These biomarkers were identified in 12 (8.5%), 6 (4.3%), and 5 (3.5%) isolates, respectively. Whole genome
sequencing was performed on the six isolates that contained aerobactin biosynthesis genes and demonstrated that these genes were contained on large plasmids. Five isolates contained plasmids nearly identical to previously described hvKp virulence plasmids while one was similar to a plasmid from a swine isolate of *K. pneumoniae*. These findings indicate that several non-clonal hvKp-like strains are present in the U.S.

**Introduction**

*Klebsiella pneumoniae* (Kp) is a leading cause of nosocomial infections worldwide including pneumonia, bloodstream, and urinary tract infections (1). Kp can cause severe, secondary pneumonia in patients with respiratory viral infections and was recently identified as a leading cause of secondary pneumonia in patients with the novel coronavirus SARS-CoV-2 (2). Kp pathogenicity is due to a variety of virulence factors, including siderophores, fimbriae, and a polysaccharide capsule of various types. The polysaccharide capsule is the major virulence factor for Kp as it allows this bacterium to evade host immune defenses, such as complement-mediated phagocytosis (3, 4). The most common type of Kp are referred to as classical strains (cKp), which infect debilitated patients residing in hospitals and long-term care facilities, (5). cKp strains are increasingly resistant to antibiotics, including carbapenems (6-11). Genes conferring antimicrobial resistance are often carried by conjugative plasmids allowing for extensive dissemination among cKp strains. Thus, it is not surprising that the IDSA, WHO, and the CDC have each deemed Kp as a serious public health priority in need of new therapeutic development (12-14). As such, multidrug-resistant (MDR) cKp strains have been the focus of research within the United States.

A second type of Kp, referred to as hypervirulent Kp (hvKp), was first identified in Taiwan in 1986 as a common cause of pyogenic liver abscesses (PLA) in young, otherwise healthy individuals living in the community (15-22). These strains are now
common in Asia, but little is known about their prevalence in the U.S. Case reports
document the presence of hvKp infections and PLA in North America, but only a few
surveillance studies have been performed (21, 24-26). hvKp strains frequently express a
highly mucoid capsule, leading to a “hypermucoviscous” colony phenotype, and are
considerably more virulent in mouse models than cKp strains (23). Their increased
virulence is usually attributed to the presence of one of a number of large, non-
conjugative, “hypervirulence” plasmids (27-29). The limitations imposed by the hyper-
mucoid capsule on genetic exchange and the non-conjugative nature of these virulence
plasmids may restrict the hypervirulence phenotype to few sequence types (e.g. ST23, ST86, ST66) and capsule types (e.g. K1, K2). The best characterized hypervirulence
plasmids are pK2044 and KP52.145pII (Fig 1A, B) (29, 30). In addition, we previously
identified a unique plasmid with hvKp features, pTK421_2 (Fig 1C), in a bloodstream
isolate from Northwestern Memorial Hospital (NMH), suggesting considerable diversity
among the hypervirulence plasmids of hvKp (31). Each hypervirulence plasmid contains
two distinct pathogenicity loci (PAL-1 and PAL-2) (Fig 1D). PAL-1 usually consists of a
mucoid regulator gene (rmpA2), the aerobactin receptor gene (iutA), and aerobactin
biosynthesis genes (iucABCD). PAL-2 usually consists of a distinct mucoid regulator
operon (rmpADC), the salmochelin receptor gene (iroN), and salmochelin biosynthesis
genes (iroBD) (Fig 1D). Plasmids KP52.145pII and pTK421_2 contain an incomplete
PAL-1 that lacks rmpA2 (Fig 1D). The mucoid regulators rmpA and rmpA2 play a major
role in virulence by increasing capsule production and hypermucoviscosity (28), but the
roles of the different siderophores (aerobactin, salmochelin, and yersiniabactin) in hvKp
virulence remain controversial (30, 32). A recent report suggested that aerobactin was
the only siderophore required for the hypervirulent phenotype of the ST86-K2 capsule
type strain hvKp1 (32). In contrast, another report showed that only a triple siderophore
mutant (yersiniabactin, aerobactin, and salmochelin) of the ST23-K1 strain NTUH-K2044
was attenuated in the mouse intraperitoneal model (30). In addition, strain ATCC43816
(also called KPPR1) is hypervirulent in mice (4, 33) but is otherwise atypical in that it
lacks a hypervirulence plasmid and PAL-1 but contains a version of PAL-2 integrated
into a chromosomal integrative and conjugative element (ICEKp1, Fig 1D). In KPPR1,
yersiniabactin promoted disease in the lung and salmochelin promoted dissemination to
tissues (34). Thus, it is clear that the mucoid regulators and siderophores play roles in
conferring the hypervirulent phenotype, but significant strain-to-strain variability of
virulence factors exists.

A microbiological definition of hvKp has remained elusive. Initially,
hypermucoviscosity was used as a proxy for the hvKp phenotype. However, several
reports have described hypermucoviscous Kp strains that are not hypervirulent in mice
and lack pathogenicity loci usually associated with hvKp (35-37). As a result, genetic
biomarkers have been used to define hvKp, but these definitions have varied from study-
to-study. Recently, Russo and colleagues systematically examined the accuracy of using
rmpA, rmpA2, peg-344, iucA, and iroB, genes found on virulence plasmids, as diagnostic
biomarkers for hvKp (23, 38). In their cohort of 175 isolates, the presence of iucA or iroB
or a hypermucoviscosity phenotype distinguished hvKp from cKp with an accuracy of
96%, 97%, and 90%, respectively. Here, we use these biomarkers to describe the
prevalence of hvKp at NMH in Chicago, Illinois. From 2015 – 2017, we collected 141
consecutive Kp bloodstream isolates and tested them for these biomarkers by
polymerase chain reaction and the string test. We identified twelve hypermucoviscous
isolates and six isolates that contain hypervirulence pathogenicity loci located on large
hvKp-like plasmids.

Results

Identification of hvKp using diagnostic biomarkers
To assess the prevalence of hvKp-like strains at NMH, we collected 141 consecutive Kp isolates from patients with positive blood cultures from 2015–2017. Given that hvKp strains are thought to disseminate through the bloodstream, we hypothesized that bloodstream isolates would be enriched for hvKp. We screened all 141 isolates for the following previously published diagnostic biomarkers of hvKp: iucA, iroB, and the hypermucoviscous phenotype. The presence of iucA and iroB was evaluated by PCR. Among NMH bloodstream isolates, six (4.3%) were positive for iucA and five (3.5%) were positive for iroB (Table 1). Five isolates were positive for both, and a single isolate was iucA+ and iroB- (Table 1). The collection was screened for hypermucoviscosity by both string test and centrifugation. Twelve (6.7%) and 24 (13.5%) isolates, respectively, were positive for hypermucoviscosity by these two tests (Table 1).

In addition, isolates were tested for tellurite resistance, a phenotype commonly encoded by pK2044 plasmids (Fig 1). Six isolates (4.3%) were able to grow on tellurite, including three isolates that were positive for iroB and iucA (Table 1). This suggested that these three isolates may contain pK2044-like plasmids while the other double-positive (iroB+, iucA+) isolates may contain KP52.145pII-like plasmids or unique hypervirulence plasmids. Collectively, these findings indicate that Kp strains with features of hvKp are circulating at NMH and suggest that these strains may contain multiples types of hvKp-associated pathogenicity loci.

**Analysis of hvKp Plasmid Content**

Since hvKp pathogenicity loci are commonly encoded on a large virulence plasmid, we next focused on the six isolates that contained either salmochelin or aerobactin biosynthesis genes. To determine whether hvKp-associated genes of these six isolates were carried on large plasmids similar to those found in characterized hvKp isolates, complete genome sequences were obtained using both Nanopore and Illumina platforms (Table 2). Whole-genome sequencing revealed that each of these isolates
harbored large plasmids with some hvKp pathogenicity loci. Three isolates (KpN8, KpN89, and KpN115) contained plasmids with near complete alignment with pK2044, including PAL-1, PAL-2, and tellurite resistance genes (Fig 2A). KpN49 and KpN165 harbored plasmids with poor overall alignment with plasmid pK2044, (Fig 2B) but with nearly complete alignment with KP52.145pII (Fig 3). Similar to KP52.145pII, these two plasmids contained a version of PAL-1 with iucABCD and iutA but lacking rmpA2 (Fig 1). The remaining isolate, KpN23, harbored a conjugative virulence plasmid containing only the aerobactin biosynthesis genes and receptor (Fig 3, 4A and 4B). pKpN23_1 was highly homologous to a plasmid harbored by a swine isolate collected in Thailand in 2016, KpCTRSRTH01_p2, but had minimal alignment with pK2044 or KP52.145pII. We had previously identified pTK421_2, a Kp plasmid with PAL-1, PAL-2, and conjugation genes (Fig 1). This plasmid was also distinct from pK2044 or KP52.145pII (Fig 2B & 3).

We therefore aligned pKpN23_1 to pTK421_2 but found that these plasmids shared relatively little alignment beyond PAL-1 (Fig 4B). Overall, among the 141 bloodstream isolates, we identified six isolates that cumulatively contained three distinct plasmids with pathogenicity loci associated with hypervirulent Kp.

**Discussion**

Case reports indicate that hvKp strains exist in the U.S., but their prevalence is unclear and has only been investigated in two surveillance studies (24, 25). In this work, we determined the prevalence of hvKp at NMH by identifying isolates using diagnostic criteria of hvKp. The three criteria we tested were iroB, iucA, and hypermucoviscosity. We identified five isolates (3.5%) that met all three of these examined diagnostic criteria for hvKp; each contained a large plasmid nearly identical to previously described hvKp plasmids. We identified one additional isolate that contained iucA on a large plasmid but was not hypermucoviscous and lacked iroB. Currently, it is unclear if isolates positive for
only a single biomarker are highly virulent and cause the types of infections
characteristic of hvKp. Further studies are warranted to understand the level of virulence
in strains such as this one with incomplete pathogenicity loci. In addition, we identified
12 isolates that were hypermucoviscous by string test and 24 that were
hypermucoviscous by centrifugation. It is unclear why some isolates were
hypermucoviscous by centrifugation but not the string test. These data agree with
previous reports suggesting that the string test is less sensitive than centrifugation for
measuring hypermucoviscosity and that hypermucoviscosity does not accurately predict
hypervirulence (37, 39). However, all strains that contained \( rmpA \) were positive by both
string and centrifugation tests (Table 2).

Since hvKp pathogenicity loci are normally encoded on a large plasmid, we
performed long-read sequencing of the six isolates that contained \( iucA \). Whole genome
sequencing confirmed that each of these isolates harbored a large plasmid. Five of
these isolates harbored a plasmid highly similar to one of the two previously described
hvKp virulence plasmids, pK2044 or KP52.145pII, and the other, KpN23, harbored a
unique plasmid containing only aerobactin. Taken together, these data suggest that
multiple clones of hvKp isolates are circulating at NMH.

Our study agrees with the two published U.S. surveillance studies, which
describe the hvKp prevalence in the U.S. as 3.5%-6.25% of Kp infections. In 2010, 64
Kp strains were analyzed from two hospitals in the greater Houston area (25). They
identified four strains (6.25%) containing hvKp pathogenicity-associated genes, based
on the presence of \( rmpA \) and K1 capsule type by PCR amplification. In 2018, a larger
study in New York City characterized Kp isolates from 462 patients (24). This study
identified 17 (3.7%) isolates by PCR amplification that contained the hvKp biomarkers
\( rmpA \) and \( iucA \). This is much lower than the prevalence of hvKp isolates in China in
2013 (37.8%, defined as iuc+), where hvKp is endemic (40). A recent genomic surveillance study of seven countries in South and Southeast Asia analyzed the sequences of 365 bloodstream isolates collected from 2010-2017 (41). Sequencing revealed that 26% (95/365) were iuc+, 17% (63/365) were iuc+ and either rmpA+ or rmpA2+, and an additional 0.8% (3/365) were iro+ and rmpA+. Of the iuc+ isolates, 13.7% (13/95) contained carbapenemase genes, but only 1 was positive for iuc, rmpA/2 and a carbapenemase gene. Our results together with these reports indicate that while hvKp-like isolates are circulating within the United States, they remain relatively rare compared to Southeastern Asia.

The presence of hvKp in U.S. hospitals has important clinical implications. In patients, hvKp infections are associated with invasive infections most commonly characterized by the development of PLA. Extrahepatic complications such as endogenous endophthalmitis, necrotizing fasciitis, and meningitis have also been reported (42). These invasive infections are associated with significant mortality and morbidity; patients with PLA take longer to recover and 9-18% have recurrent PLA (43, 44). In addition, one study showed that 10/14 patients with endogenous endophthalmitis progressed to blindness (45). Interestingly, while hvKp isolates cause severe invasive infections, several studies reported no difference in mortality following bloodstream infection with hvKp or cKp (46, 47). This is likely related to the fact that community acquired hvKp infections tend to occur in young healthy patients, and outcome of bloodstream infection is largely dependent on the time it takes to initiate appropriate antimicrobial therapy. Delay in effective antimicrobial therapy, which is more likely to occur in infections cause by multidrug-resistant bacteria, leads to poor outcomes in bloodstream infection (48, 49). None of the six hvKp-like isolates identified at NMH contained antimicrobial resistance genes. Thus far, antimicrobial resistant hvKp
infections are exceedingly rare in the United States. However, antimicrobial resistant hvKp infections are on the rise in Asia, likely due to the increase in hospital acquired hvKp infections (46, 50, 51). While this study is limited to a single institution, the findings presented here describe the prevalence and diversity of hvKp plasmids at a major U.S. academic medical center and call attention to the need for further studies evaluating the risk and clinical outcomes of these diverse hvKp isolates.

Materials and Methods

Bacterial isolates and growth conditions

The isolates used in this study were collected from patients with a positive blood culture from 2015-2017 and are described in Table S1. These isolates were identified as Kp on a VITEK-2 (52). Kp was grown at 37°C in Lysogeny broth (LB) or on LB agar plates. For screening of tellurite resistance, Kp was grown at 37°C on LB agar plates supplemented with 3 µg/mL potassium tellurite (Sigma-Aldrich, USA).

Screening for iroB and iucA

Aerobactin and salmochelin biosynthesis genes were detected by polymerase chain reaction using custom designed primers:

`iroB Fwd- ATTTCCGCGCTACCTCTTCAG; iroB Rev-
TGGGCTATTGTATCCTGTGCTATCTCTG; iucA Fwd-
GTTGTGGTCGTTTCAGATAACAGAAAAAGTT; iucA Rev-
AATTCTCATCATGAGCAGGTGAGATCTGT`

Hypermucoviscosity testing

A string test was used to assess the hypermucoviscosity of each Kp isolate as described previously (53). Isolates were grown overnight at 37°C on LB agar. A single colony was lifted with a loop to evaluate the formation of a viscous string between the loop and the
colony. A positive string test was defined as a string length ≥ 5 mm. Hypermucoviscosity was also assessed by centrifugation of overnight cultures (5 mL LB) at 3,220 rcf for 10 minutes (39). Hypermucoviscous isolates were identified by the persistence of turbidity.

**Whole-Genome Sequencing**

Genomic DNA (gDNA) was prepared from a single colony cultured overnight at 37°C in LB using the Maxwell 16 system (Promega Corp., Madison, WI). Nanopore sequencing was performed as described previously (54, 55). Briefly, libraries were prepared from gDNA using the ligation sequencing kit (SQK-LSK109, Oxford Nanopore, UK). Libraries were sequenced on the MinION using a FLO-MIN106 flow cell and base calling and demultiplexing of sequence reads was performed using Guppy v3.4.5. Libraries for Illumina sequencing were prepared using Nextera XT library kits (Illumina, Inc., San Diego, CA) and sequenced on an Illumina NextSeq instrument to generate paired-end 150-bp reads. Hybrid assembly and circularization of Nanopore and Illumina reads were performed using Flye v2.6. Nanopore sequencing errors were corrected by aligning Illumina reads to the assembly using BWA v0.7.17 and correcting the errors with serial rounds of Pilon v1.23. Annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline.

**Molecular typing and identification of virulence genes**

Assembled whole genome sequences were analyzed with the bioinformatics tools Kleborate and Kaptive to evaluate multi locus sequence type, capsule locus typing, and virulence gene content (56, 57). Plasmids identified by whole genome sequencing were aligned using BLAST Ring Image generator (BRIG) with the alignment threshold set at 85% identity (58).


and magA in two tertiary hospitals in Houston, TX, USA. J Med Microbiol 65:1047-8.


**Figure Legends**

**Figure 1. Virulence plasmids associated with hypervirulence in *Klebsiella pneumoniae***. Genomic elements and plasmid architecture are depicted for pK2044 (**A**), KP52.145pII (**B**) and pTK421 (**C**). PAL-1 and PAL-2 contents and organization are depicted for pK2044, KP52.145pII, pTK421_2, and ICEKp1 (**D**). (Drawings not to scale.)
Figure 2. The bloodstream isolates KpN115, KpN8, and KpN89 harbor pK2044-like plasmids. DNA sequences for plasmids identified by nanopore sequencing were aligned with either pK2044 (A), or KP52.145pII (B) using blast ring image generator (BRIG). A sequence identity threshold of 85% was used. Aerobactin biosynthesis genes are indicated with green arrows, salmochelin with orange arrows, rmpA with blue arrows, and rmpA2 with red arrows. Plasmid and PALoc alignments are shown in the indicated colors.

Figure 3. The bloodstream isolates KpN49_2 and KpN165_1 harbor KP52.145pII-like plasmids. DNA sequences for plasmids identified by Nanopore sequencing were aligned with KP52.145pII using blast ring image generator (BRIG). A sequence identity threshold of 85% was used. Aerobactin biosynthesis genes are indicated with green arrows, salmochelin with orange arrows, and rmpA with blue arrows. Plasmid and PALoc alignments are shown in the indicated colors.

Figure 4. A unique plasmid containing aerobactin biosynthesis genes was identified in strain KpN23. DNA sequences for plasmid KpCTRSRTH01_p2 and plasmids from NMH bloodstream isolates (A) or pK2044, KP52.145pII, and pTK421_2 (B) were aligned with pKpN23_1 using blast ring image generator (BRIG). A sequence identity threshold of 85% was used. Aerobactin biosynthesis genes are indicated with green arrows. Plasmid and PALoc alignments are shown in the indicated colors.
### Table 1. Prevalence of hypervirulence features in blood stream isolates

<table>
<thead>
<tr>
<th>Hypervirulence Marker</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n(%)</td>
</tr>
<tr>
<td>iroB</td>
<td>5 (3.5)</td>
</tr>
<tr>
<td>iucA</td>
<td>6 (4.3)</td>
</tr>
<tr>
<td>iroB/iucA</td>
<td>5 (3.5)</td>
</tr>
<tr>
<td>Centrifugation Test</td>
<td>12 (8.5)</td>
</tr>
<tr>
<td>Tellurite Resistance</td>
<td>6 (4.3)</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of bloodstream isolates containing hvKp pathogenicity loci

<table>
<thead>
<tr>
<th>Strain</th>
<th>ST</th>
<th>K-Locus</th>
<th>iuc</th>
<th>iro</th>
<th>hmv*</th>
<th>rmpA/2</th>
<th>Plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPN8</td>
<td>ST23</td>
<td>KL1</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA, rmpA2</td>
<td>pK2044</td>
</tr>
<tr>
<td>KPN23</td>
<td>ST881</td>
<td>KL2</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA</td>
<td>pKPN23_1</td>
</tr>
<tr>
<td>KPN49</td>
<td>ST66</td>
<td>KL2</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA</td>
<td>KP52.145pIi</td>
</tr>
<tr>
<td>KPN89</td>
<td>ST23</td>
<td>KL1</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA, rmpA2</td>
<td>pK2044</td>
</tr>
<tr>
<td>KPN115</td>
<td>ST23</td>
<td>KL1</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA, rmpA2</td>
<td>pK2044</td>
</tr>
<tr>
<td>KPN165</td>
<td>ST380</td>
<td>KL2</td>
<td>iuc</td>
<td>iro</td>
<td>+</td>
<td>rmpA</td>
<td>KP52.145pIi</td>
</tr>
</tbody>
</table>

*hmv = hupermucoviscous; ST = Sequence Type
A.

- **pK2044**: 219 kb
  - **rmpA**
  - Salmochelin
  - Tellurite Res
  - Aerobactin
  - **rmpA2**

B.

- **KP52.145pII**: 121 kb
  - **rmpA**
  - Salmochelin
  - Aerobactin

C.

- **pTK421_2**: 127 kb
  - **rmpA**
  - Salmochelin
  - Aerobactin
  - Conjugation

D.

- **pK2044**
  - **rmpA2**
  - **iucABCD, iutA**

- **KP52.145pII**
  - **iucABCD, iutA**

- **pTK421_2**
  - **iucABCD, iutA**

- **ICEKp1**
  - **x**

- **PAL-1**
  - **rmpA**
  - **iucABCD, iutA**

- **PAL-2**
  - **rmpA**
  - **iroBDN**

- **ICEKp1**
  - **rmpA**
  - **iroBDN**