1 Prevalence of Hypervirulence-Associated Pathogenicity Loci Among *Klebsiella* 2 *pneumoniae* Bloodstream Isolates at a United States Hospital

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10 11 **Abstract**

12 *Klebsiella pneumoniae* is a major threat to human health worldwide. “Classical” *K.*
13 *pneumoniae* (cKp) strains commonly cause multidrug-resistant (MDR) infections in
14 debilitated patients residing in hospitals and long-term care facilities. In contrast,
15 hypervirulent *K. pneumoniae* (hvKp) strains may cause invasive, community-acquired
16 infections in otherwise healthy individuals. These highly virulent strains were first
17 identified in Taiwan in 1986 and have since disseminated across the globe. The
18 prevalence of hvKp has been well described in Southeast Asia, but is not well
19 understood in the United States. In this work, 141 consecutive *K. pneumoniae* blood
20 stream isolates were collected at Northwestern Memorial Hospital from 2015-2017. To
21 screen this collection for hvKp, isolates were tested for the hypermucoviscous
22 phenotype and the presence of aerobactin and salmochelin biosynthesis genes,
23 biomarkers that have been used to distinguish hvKp from cKp. These biomarkers were
24 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 hypermucoid 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 carbapenamase genes, but only 1 was positive for *iuc*, *rmpA/2* and a carbapenamase 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- ATTTCCGCCGCTACCTCTTCAG; *iroB* Rev- TGGGCTATTGTATCCTGTGCTATCTCTG; *iucA* Fwd- GTTGTGGTCGTTTCAGATAACAGAAAAAGTT; *iucA* Rev- AATTCTCATTATCATGACGAGTGAGATCTGT

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).

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457 Figure Legends

458 **Figure 1. Virulence plasmids associated with hypervirulence in *Klebsiella***
459 ***pneumoniae*.** Genomic elements and plasmid architecture are depicted for
460 pK2044 **(A)**, KP52.145pII **(B)** and pTK421 **(C)**. PAL-1 and PAL-2 contents and
461 organization are depicted for pK2044, KP52.145pII, pTK421_2, and ICEKp1 **(D)**.
462 (Drawings not to scale.)

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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

Hypervirulence Marker	Isolates n = 141
	Positive n(%)
<i>iroB</i>	5 (3.5)
<i>iucA</i>	6 (4.3)
<i>iroB/iucA</i>	5 (3.5)
String Test	12 (8.5)
Centrifugation Test	24 (17.0)
Tellurite Resistance	6 (4.3)

Table 2. Characteristics of bloodstream isolates containing hvKp pathogenicity loci

Strain	ST	K-Locus	<i>iuc</i>	<i>iro</i>	hmv*	<i>rmpA/2</i>	Plasmid
KPN8	ST23	KL1	<i>iuc 1</i>	<i>iro 1</i>	+	<i>rmpA, rmpA2</i>	pK2044
KPN23	ST881	KL2	<i>iuc 3</i>	-	-	-	pKPN23_1
KPN49	ST66	KL2	<i>iuc 2</i>	<i>iro 2</i>	+	<i>rmpA</i>	KP52.145pII
KPN89	ST23	KL1	<i>iuc 1</i>	<i>iro 1</i>	+	<i>rmpA, rmpA2</i>	pK2044
KPN115	ST23	KL1	<i>iuc 1</i>	<i>iro 1</i>	+	<i>rmpA, rmpA2</i>	pK2044
KPN165	ST380	KL2	<i>iuc 2</i>	<i>iro 2</i>	+	<i>rmpA</i>	KP52.145pII

*hmv = hypermucoviscous; ST = Sequence Type

A **B** **C** **D**

A

B

A

B