



A Nationwide Screen of Carbapenem-Resistant *Klebsiella pneumoniae* Reveals an Isolate with Enhanced Virulence and Clinically Undetected Colistin Heteroresistance

Jessie E. Wozniak,^{a,b,c,d} Victor I. Band,^{a,b,c,d} Andrew B. Conley,^f Lavanya Rishishwar,^f Eileen M. Burd,^{c,d} Sarah W. Satola,^{c,d} Dwight J. Hardy,^h Rebecca Tsay,^h Monica M. Farley,^{c,d,e}  Jesse T. Jacob,^{c,d} Ghinwa Dumyati,^h I. King Jordan,^{f,g}  David S. Weiss^{b,c,d,e}

^aEmory University, Atlanta, Georgia, USA

^bEmory Vaccine Center, Atlanta, Georgia, USA

^cEmory University School of Medicine, Atlanta, Georgia, USA

^dEmory Antibiotic Resistance Center, Atlanta, Georgia, USA

^eAtlanta VA Medical Center, Decatur, Georgia, USA

^fHRC Applied Bioinformatics Laboratory, Georgia Institute of Technology, Atlanta, Georgia, USA

^gSchool of Biological Sciences, Georgia Institute of Technology, Atlanta, Georgia, USA

^hUniversity of Rochester Medical Center, Rochester, New York, USA

ABSTRACT The convergence of hypervirulence and multidrug resistance in *Klebsiella pneumoniae* is a significant concern. Here, we report the first screen for hypermucoviscosity, a trait associated with increased virulence, using a U.S. surveillance collection of carbapenem-resistant (CR) *K. pneumoniae* isolates. We identified one hypermucoviscous isolate, which carried a gene encoding the KPC-3 carbapenemase, among numerous resistance genes. The strain further exhibited colistin heteroresistance undetected by diagnostics. This convergence of diverse resistance mechanisms and increased virulence underscores the need for enhanced *K. pneumoniae* surveillance.

KEYWORDS KPC, *Klebsiella*, colistin, heteroresistance, hypermucoviscous

Several recent reports have described carbapenem-resistant (CR), hypervirulent *Klebsiella pneumoniae* strains in China (1–4). Alarming, strains described by Gu et al. harbored plasmids containing virulence genes, including those controlling capsule-mediated hypermucoviscosity, and antibiotic resistance genes, including that encoding the KPC-2 carbapenemase (1). Previously, hypervirulent/hypermucoviscous *Klebsiella pneumoniae* (hvKP) strains were largely antibiotic susceptible. While the majority of infections with hvKP occur in Asia, we were interested in systematically assessing the prevalence of carbapenem-resistant hvKP (CR-hvKP) strains in the United States.

We tested 265 carbapenem-resistant *K. pneumoniae* clinical isolates collected between 2012 and 2015 from the Emerging Infections Program (EIP) Multi-site Gram-negative Surveillance Initiative (MuGSI) which has a surveillance population of >15 million people in 8 states across the United States. We phenotypically screened isolates using the string test to detect hypermucoviscosity, which has been strongly correlated with hypervirulence. *K. pneumoniae* isolates collected through MuGSI between 2012 and 2015 were streaked out on blood agar plates (tryptic soy agar [TSA] with 5% sheep blood; Remel, Thermo Fisher Scientific). The string test was performed to detect hypermucoviscosity, using a 1- μ l inoculation loop on a single colony. A positive string test is >5mm in length. One isolate collected in 2014 from New York, termed CDC98, was positive by the string test (Fig. S1 and Movie S1). Subsequent testing by six independent scientists who were blind to the strains being tested confirmed CDC98 as being string test positive compared to three nonhypermucoviscous negative-control strains (Fig. S2).

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Address correspondence to David S. Weiss, david.weiss@emory.edu.

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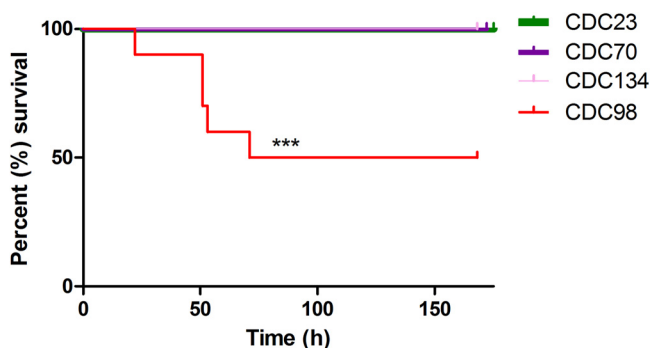


FIG 1 CDC98 displays increased virulence compared to that of nonhypermucoviscous *K. pneumoniae* isolates. Mice were infected intraperitoneally with CDC98 or the indicated nonhypermucoviscous *K. pneumoniae* isolates. Results shown are the combination of two independent experiments ($n = 10$ total). Comparison of survival curves was performed with the Mantel-Cox test ($P = 0.0004$).

Whole-genome Illumina sequencing revealed that CDC98 is sequence type 258 (ST258), capsule type KL107, and contains numerous resistance genes, including *kpc3*. ST258 is the current global pandemic strain of *K. pneumoniae*, and although the reasons for its success as a global pathogenic strain are uncertain, it is likely to have a fitness and selective advantage, as well as success, due to its antibiotic-resistance elements (5, 6). Additionally, we discovered by sequencing that the isolate harbors a previously unreported hybrid of plasmids pKPN-498 and pUHKPC45-117, encoding antibiotic resistance [*SHV-12*, *sull*, *catA1*, *mph(A)*, *LEN-4*, *dfrA12*, and *aadA2*] and virulence (*sil* and *pco*) genes. Interestingly, the isolate does not harbor the virulence plasmid pLVPK (or its close derivatives, collectively referred to here as pLVPKs) found in most Asian hvKP strains or the previously identified regulators of hypermucoviscosity and hypervirulence, *rmpA* and *rmpA2* (7). The genes *rmpA* and *rmpA2* have been thought to be responsible for the hypermucoviscous phenotype by direct regulation of the *cps* locus, although examples have been published where hypermucoviscosity was observed in the absence of these genes (8–13). In addition to the putative virulence genes encoded on the hybrid plasmid, CDC98 possesses chromosomal copies of the siderophore receptor genes *iutA* and *iroN*, which are found on pLVPKs in many Asian hvKP isolates.

The level of virulence of CDC98 was difficult to predict, since it is hypermucoviscous yet lacks pLVPK. We directly assessed this using a murine *in vivo* infection model; C57BL/6 mice were infected intraperitoneally with 10^6 CFU of the indicated *K. pneumoniae* strains. Mice were provided with food and water *ad libitum* and monitored for signs of illness or weight loss. Mice exhibiting weight loss below 80% of starting weight were euthanized. CDC98 was indeed more virulent than three nonhypermucoviscous ST258 *K. pneumoniae* isolates from the same study site (New York; Fig. 1). However, while CDC98 displays significantly increased virulence compared to the other *K. pneumoniae* isolates tested, this level of virulence is far lower than that observed in canonical hypervirulent *K. pneumoniae* strains (14).

As the sequencing of CDC98 indicated the presence of multiple antibiotic-resistance genes, antibiotic susceptibility testing was performed using the MicroScan WalkAway 96 plus instrument (Beckman Coulter, Inc., Brea, CA) and Neg Breakpoint Combo 44 panel, and the resultant antibiogram of this strain revealed extensive drug resistance to multiple classes of antibiotics, including carbapenems (Table S1). In the case of multidrug-resistant strains, such as CDC98, last-line antibiotics are often required for treatment. One such drug is the cationic polymyxin antibiotic colistin, which disrupts the membranes of Gram-negative bacteria.

Additional susceptibility testing by population analysis profile (PAP), whereby serial dilutions of an overnight bacterial culture are plated on increasing concentrations of an antibiotic on agar plates, revealed CDC98 to be heteroresistant to colistin (Fig. 2A). Heteroresistance describes a phenomenon wherein a subpopulation of bacteria in an

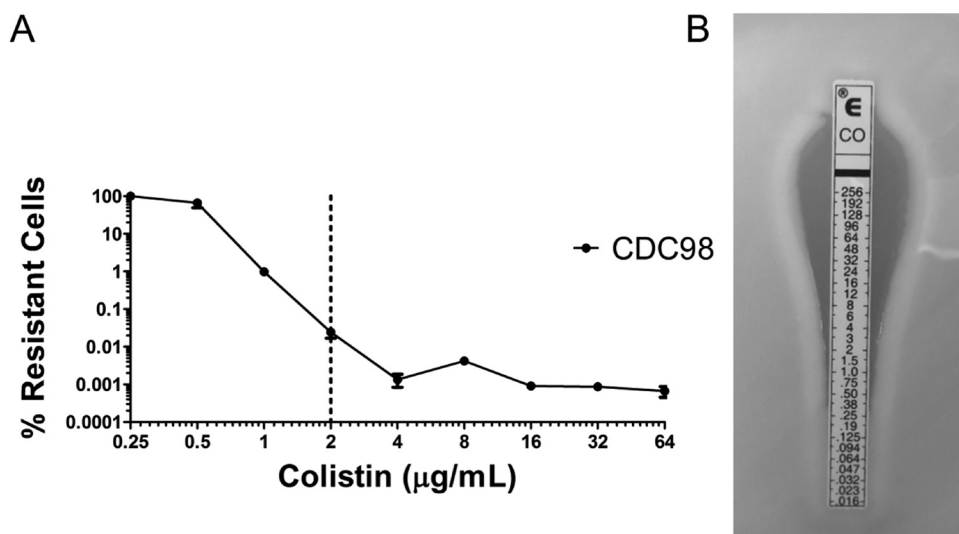


FIG 2 CDC98 displays heteroresistance to the last-line antibiotic colistin. (A) Population analysis profile (PAP) of CDC98 reveals a subpopulation of colistin-resistant cells. The dotted line represents the CLSI epidemiological cutoff value (ECV) for determining *K. pneumoniae* susceptibility or resistance to colistin (16). (B) CDC98 was designated by Etest as susceptible to colistin.

otherwise homogenous population displays antibiotic resistance. Importantly, previous work has shown that colistin heteroresistance mediates antibiotic treatment failure in mice, and it has been postulated that this may in part contribute to unexplained antibiotic treatment failure in the clinic (15). Diagnostic testing for colistin susceptibility by Etest (Fig. 2B) or broth microdilution (BMD; MIC < 0.5 µg/ml) classified CDC98 as susceptible, indicating that the heteroresistance was not detected.

This is the first report of a systematic survey of carbapenem-resistant *Klebsiella pneumoniae* isolates for hypermucoviscosity in the United States. Discrepancies between Asian CR-hvKP strains (such as strain K1; [1]) and CDC98 suggest that a distinct lineage of carbapenem-resistant *K. pneumoniae* may be emerging in the United States, as CDC98 exhibits phenotypic similarities to contemporary hypervirulent *K. pneumoniae* strains while containing significant genetic differences (Table S2). These findings are further supported by a recent U.S. isolate exhibiting *rmpA* and/or *rmpA2*-independent increased capsular polysaccharide production and enhanced virulence, which was not as virulent as classical hvKP strains (8). Together, these results are indicative of the convergent evolution of distinct lineages of *K. pneumoniae* in Asia and the United States, leading to acquisition of enhanced virulence and antibiotic resistance. This convergence, taken together with emerging antibiotic resistance mechanisms, like heteroresistance, that can often go undetected by current diagnostics, pose a new and worrisome threat to public health.

Recent discussion in the field has suggested that using hypermucoviscosity as the singular criterion for identifying hypervirulence is insufficient. Hypermucoviscous isolates with enhanced virulence, such as CDC98, can lack the common genomic biomarkers for hypervirulence (*rmpA*, *rmpA2*, K1 capsular serotype, etc.). Therefore, while our findings reveal that CR-hvKP isolates have been relatively rare, both phenotypic and genotypic screening will be critical to gain a comprehensive understanding of CR-hvKP in the United States.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00107-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

SUPPLEMENTAL FILE 2, MOV file, 10.8 MB.

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We declare no competing financial interests.

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