The rise of multidrug-resistant (MDR) Enterobacte-
riaceae prompted the World Health Organization
to classify carbapenem-resistant Enterobacteriaceae, of
which Klebsiella is the most common genus, on the
global priority list of antibiotic-resistant bacteria in
2017 (1). Carbapenem-resistant Klebsiella pneumoniae
(CRKP,
also including K. quasipneumoniae) infections are gen-
erally hospital acquired, particularly among elderly and
immunocompromised patients (2,3). The major
carbapenemases include K. pneumoniae carbapen-
emase (KPC), New Delhi metallo-β-lactamase, and
carbapenem-hydrolyzing class D β-lactamase (OXA),
all of which have spread globally (4–7).

The Carbapenemase-Producing Enterobacteri-
ceae in Singapore (CaPES) study initiated in 2013 re-
vealed that the rate of incident carbapenem-resistant
Enterobacteriaceae clinical cultures in government hos-
pitals in Singapore increased during 2011–2013 and
plateaued thereafter (8). The number of cases of hy-
pervirulent K. pneumoniae has increased in the past
3 decades in parts of Asia, and likewise, the number
of cases of monomicrobial Klebsiella-induced liver ab-
cesses has also increased (9,10).

The prevalence of antimicrobial resistance among
hypervirulent K. pneumoniae isolates is rare compared
with that of standard isolates (11,12); hypervirulent
K. pneumoniae and CRKP seem to have their own par-
ticular reservoirs and remain mostly segregated from
each other. However, hypervirulent K. pneumoniae
and CRKP isolates can converge in the same organ-
ism, leading to the emergence of superbugs resistant
to antimicrobial drugs of even the last line of treat-
ment that are capable of infecting healthy persons.
This emergence has already been reported in China,
Brazil, and the United Kingdom (13–15). The fatal
outbreak that occurred in a hospital in China in 2016
was caused by a carbapenem-resistant hypervirulent
K. pneumoniae strain that had acquired a virulence
plasmid by a classic sequence type (ST) 11 strain (16).
In a study of a collection of >2,200 K. pneumoniae ge-
nomes, distinct evolutionary patterns of horizontal
genome transfer were observed in MDR isolates versus
hypervirulent isolates (17). The authors of that study postulated that hypervirulent clones might be subject to some sort of constraint against horizontal gene transfer and show more conserved pangenomic diversity than MDR clones. If that hypothesis is correct, MDR clones acquiring virulence genes or K. pneumoniae virulence plasmids would be more likely than hypervirulent clones acquiring MDR genes. To investigate this hypothesis, we searched for hypervirulent isolates among 556 CRKP isolates collected at public hospitals of Singapore.

Materials and Methods

Bacterial Isolates and Microbiologic Methods
During 2010–2015, all microbiology laboratories in Singapore had been mandated to submit their carbapenem-resistant Enterobacteriaceae isolates to the National Public Health Laboratory of Singapore. Using this library, we collected isolates from the CaPES study. We performed species identification, assessed carbapenem resistance, and determined carbapenemase genes as previously described (8).

Whole-Genome Sequencing and Data Analysis
We performed whole-genome sequencing using the MiSeq platform (Illumina, https://www.illumina.com) as previously described (18). In addition, we sequenced the complete genomes of 5 isolates from 3 patients, obtaining long reads using the GridION X5 system (Oxford Nanopore Technologies, https://nanoporetech.com) to close the gaps. We de novo assembled the Illumina sequence reads using SPAdes 3.11.1 (19) and completed genome assembly using a combination of Illumina and Oxford Nanopore Technologies data with the hybrid assembler Unicycler version 0.4.7 (Appendix Table, https://wwwnc.cdc.gov/EID/article/26/3/19-1230-App1.pdf) (20). We deposited whole-genome sequencing data in GenBank (BioProject numbers PRJNA342893, PRJNA557813, and PRJNA591409). We screened genome assemblies for virulence loci and K. pneumoniae virulence plasmid-associated loci using kleborate (21-23). We resolved missing loci and ambiguous alleles by mapping short reads to reference sequences using breaq (24) and screened assemblies for antimicrobial resistance genes using ResFinder 3.1 (25) and CARD (26). We resolved any discrepancies between these 2 gene identifiers by searching blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using translated gene sequences. We identified plasmid replicons in all completely sequenced genomes using PlasmidFinder with default settings (27). For all isolates, we performed core-genome single-nucleotide polymorphism (SNP) analysis against reference genome SGH10 chromosome (GenBank accession no. CP025080) using Parsnp 1.2 (28). In plasmid analyses, we generated alignments by mapping assemblies to reference plasmids using bowtie2 (29) on the REALPHY server (30). We inferred approximate maximum-likelihood phylogenetic trees using FastTree 2 (31) and screened completed assemblies for origin of transfer (oriT) sites and other transfer-related modules using oriTfinder (32).

Determining Hypermucoviscosity
We assessed hypermucoviscosity of all isolates using the string test (33) and a quantitative centrifugation assay (34). We used SGH10 as the positive control and SGH10 with rmpA deleted as the negative control.

Mouse Infection
We infected female 7–8-week-old C57BL/6j mice (InVivos, http://www.invivos.com.sg) with 1 × 10⁶ CFU of bacteria diluted in 100 µL phosphate-buffered saline through the intraperitoneal route and assessed for death every 8–16 h. Animal experiments were approved under protocol R18–0252 by the National University of Singapore Institutional Animal Care and Use Committee in accordance with the National Advisory Committee for Laboratory Animal Research guidelines.

Conjugation Experiments
We measured the transmissibility of the \(\text{bla}_{\text{KPC-2}}\)-carrying plasmid using a previously described method (35). In this experiment, carbapenem-resistant hypervirulent K. pneumoniae isolates were the donors and a kanamycin-resistant Escherichia coli MG1655 mutant SLC568 strain (36) was the recipient. We carried out conjugation on 0.22-µm nitrocellulose filters with donors and recipients incubated at a 1:1 ratio on lysogeny broth (LB) agar plates for 4 h at 37°C. We enumerated transconjugants on LB agar plates containing carbenicillin (100 µg/mL) and kanamycin (50 µg/mL) and recipients on LB agar plates containing kanamycin only. We confirmed transfer of the \(\text{bla}_{\text{KPC-2}}\) gene by PCR.

Antimicrobial Susceptibility Testing
We performed antimicrobial susceptibility testing following the Clinical and Laboratory Standards Institute guidelines. We determined MICs (37) and interpreted breakpoints (38) of antimicrobial drugs as described.
Hypervirulent K. pneumoniae, Singapore

Statistical Methods
We performed statistical analyses using GraphPad Prism version 8 (https://www.graphpad.com). We compared samples using the unpaired t-test with Welch correction.

Results

Discovery of Hypervirulent Features of CRKP
We retrieved 1,312 carbapenem-resistant Enterobacteriaceae collected from 6 public hospitals in Singapore during 2010–2015 through the CaPES program and National Public Health Laboratory of Singapore; 1,251 isolates were whole-genome sequenced with Illumina technology, and 556 isolates were K. pneumoniae. We searched K. pneumoniae isolate genomes for the presence of K. pneumoniae virulence plasmid–associated virulence determinants, rmpA, rmpA2, ird (the salmochelin locus), and iuc (the aerobactin locus) by using kleborate. We identified 18 isolates (originating from just 7 patients) harboring all of these loci, and 14 of these isolates came from the same 3 patients. We screened the genome assemblies of these 18 isolates for virulence features and compared the characteristics of these isolates with those of 2 known hypervirulent strains, SGH10 (serotype K1 liver abscess–associated isolate from Singapore) (6,39) and CG43 (serotype K2 clinical isolate from Taiwan) (40). We then performed a phylogenetic analysis of the core genomes of all these isolates.

The differences found among isolates from the same patient were small (0–15 SNPs) (Figure 1, panel A), suggesting that patients with multiple isolates were infected with a single strain. All isolates from patients A2, A4, and A6 were ST23 and serotype K1 (same as SGH10) and, except for ENT1256, carried the same virulence loci as SGH10 (Table 1); ENT1256 had a different allele for rmpA2. The core genomes of

Figure 1. Maximum-likelihood trees of genes from carbapenem-resistant Klebsiella pneumoniae isolates, Singapore, 2013–2015. A) Analysis generated using 63,297 single-nucleotide polymorphism sites in the core genome. The chromosomal sequence of SGH10 (GenBank accession no. CP025080) was used as reference. Isolates are closely related to hypervirulent strains SGH10 and CG43. Scale bar indicates number of single-nucleotide polymorphisms. B) Analysis generated from the alignment of K. pneumoniae virulence plasmids from the first isolates collected from different patients. The sequences of K. pneumoniae virulence plasmid pSGH10 (GenBank accession no. CP025081) was used as reference. Scale bar indicates nucleotide changes per base pair. Trees were drawn using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree) and rooted at the SGH10 branch. Labels indicate isolate no._patient no._K serotype_sequence type. Ref, reference; ST, sequence type.
isolates are generally
clonally related, as indicated by pulsed-field gel
analysis. The virulence plasmids were present in
all isolates, and the pKVP-1 element was
identical to that carried by the hypervirulent
K. pneumoniae K2 reference strain CG43.

However, the differences between isolates from
patients A2, A4, and A6 were much greater (200–300
SNPs) than the differences between isolates from patient
A2 (15 SNPs), indicating that the bacteria from
these patients were unlikely to have originated from
the same strain. The isolates from patients A14 and
A15 were the same serotype (K2) but different
sequence types (Table 1), and their core genomes con-
tained many differences (>20,000 SNPs). The isolates
from patient A14 were phylogenetically close to the
other hypervirulent strain, CG43 (Figure 1, panel A); however, unlike CG43, which carried no yersinia-
abactin (ybt) and colibactin (clb) loci, 2 A14 isolates
(ENT1192 and ENT1988) had a ybt9 locus on integrative
conjugal element K. pneumoniae 3 (Table 1). The
isolates from the remaining 2 patients (A8 and
A12) were the same serotype (K20) but had different
sequence types and many core genome differences
(>20,000 SNPs). ENT1332 had an unknown ybt locus,
and ENT1381 and a ybt9 locus on integrative conjuga-
tive element K. pneumoniae 3. Both of these isolates
did not have the clb locus. All isolates had hyperviru-
lence backgrounds.

We also performed a whole-genome phylo-
genet analysis on the K. pneumoniae virulence plasmid
carried in the first isolates obtained from all patients,
using the K. pneumoniae virulence plasmid sequence
from SGH10 (pSGH10) as reference. The K. pneumoniae
virulence plasmids appeared to form 2 separate
clades, 1 for all K1 isolates and the other for K2 and
K20 isolates (Figure 1, panel B).

The virulence potentials of all isolates were high
(Table 1); the virulence scores predicted by kleborate
for all isolates (≥4) were higher than the score (3) pre-
dicted for the hypervirulent K2 reference strain CG43.
Hypervirulent K. pneumoniae isolates are generally
defined as carrying K. pneumoniae virulence plas-
mid–associated loci and having a hypermucoviscous
phenotype (including a positive string test result),
which is dependent on regulator RmpA (40,41). We
measured the hypermucoviscosity of the first
isolates from all patients using both the string test and
a centrifugation assay; 6 of 7 isolates formed strings,
and 5 of 7 isolates were hypermucoviscous according
to the centrifugation assay (Figure 2, panel A). Only
ENT596 was negative by both tests.

The virulence potential of the carbapenem-
resistant hypervirulent K. pneumoniae isolates was
further determined in an intraperitoneal mouse in-
fec tion model. The first isolates from 6 of 7 patients
killed >50% of the infected mice within 96 hours; only
ENT596 (like control isolate ENT495, a CKRP strain
carrying pKPC2 but not hypervirulent) did not kill any
mice (Figure 2, panel B). Serotype K1 isolates were the
most virulent, and serotype K20 isolates took longer
to kill. The virulence of ENT596 did not correlate with
its predicted score; this isolate demonstrated concur-
rent loss of hypermucoviscosity and virulence in mice.
Hypervirulent *K. pneumoniae*, Singapore (Table 1; Figure 2), which might have been caused by the loss of expression of the virulence genes. The average length of hospitalization of the 7 patients harboring these isolates was 97 days, which was much longer than the average length of stay for 249 patients with carbapenem-resistant *Enterobacteriaceae* infection (38 days) (8). Taking into account all our evidence, we conclude that the isolates from 6 of 7 patients (A2, A4, A6, A8, A12, and A14) are phenotypically hypervirulent, and the isolates from patient A15 are phenotypically nonhypervirulent (although these isolates have a hypervirulent genetic background).

**Highly Conserved Plasmid Harboring bla*KPC-2***

**on All Carbapenem-Resistant Hypervirulent *K. pneumoniae***

We screened the assemblies of all 18 carbapenem-resistant hypervirulent *K. pneumoniae* isolates for acquired antimicrobial resistance genes using ResFinder (25) and CARD (26). Except for endogenous penicillin resistance, hypervirulent *K. pneumoniae* isolates are generally considered to be susceptible to antimicrobial drugs. Our search results showed that SGH10 harbored the β-lactam resistance gene bla*SHV-11* and resistance genes against fluoroquinolones (*qpxA* and *qpxB*) and fosfomycins (*fosA6*) (Table 2). In total, 17 of 18 isolates carried these 3 resistance genes; 1 isolate from patient A15 (ENT686) did not have the *qpxA* and *qpxB* genes, and these genes were also not detectable by PCR. All isolates carried an identical set of the following 4 genes: *bla*KPC-2, *bla*TEM-1A*, *bla*TEM-1B*, and *mhp*(A). In the 5 completely sequenced genomes (ENT494, ENT646, and ENT1734 [patient A2]; ENT1192 [patient A14]; and ENT607 [patient A15]; Appendix Table), we located these 4 genes on a 71,861-bp plasmid, which we named pKPC2. The sequences of the pKPC2s in the 4 isolates from patients A2 and A14 were identical. The pKPC2 in ENT607 from patient A15 had only 1-bp difference.

We performed a phylogenetic analysis of the pKPC2 sequences carried in the first isolates and other select isolates from all 7 patients, including the pKPC2 from ENT494 (i.e., pKPC2_494) as reference (Figure 4). This analysis revealed that all isolates carried sequences almost identical to pKPC2_494 (coverage and identity >99%). Because all isolates carried the 4-gene set, they probably had a plasmid closely related to pKPC2_494. Three patients had multiple isolates with pKPC2. The clinical records show long time-interval gaps of antimicrobial drug nonexposure between some isolates. The comparison also shows that the pKPC2-related plasmids were remarkably stable; they were maintained in bacteria with few changes for 90–281 days in the patients not undergoing antimicrobial drug treatment (Figure 4).
Besides resistance genes, pKPC2 had a complete set of conjugative machinery with all 4 essential modules (oriT, relaxase, type IV coupling protein, and a type IV secretion system cluster), suggesting the plasmid is self-transmissible. The Salmonella plasmid pSA20021456.2 has a relaxase, type IV coupling protein, and type IV secretion system cluster similar to pKPC2 but no oriT site. We selected 3 isolates that had only 1 antimicrobial drug resistance plasmid to assess the transmissibility of pKPC2. We performed filter mating on LB agar using the kana-mycin-resistant E. coli strain SLC568 as the recipient. After 4 hours of incubation, 80% of recipients (≈0.8 x 100 transconjugate/recipient) had received pKPC2 from ENT596 and ENT1061 (Figure 5). The conjugation frequency was 0.2% (≈2.0 x 10^{-4} transconjugate/recipient) when ENT494 was the donor. We confirmed the transconjugants acquired the bla_{KPC-2} gene by PCR (using 10 colonies for each donor). K serotype, sequence type, and hypermucoviscosity of

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Table 2. Isolation date, sampling site, and resistance genes of carbapenem-resistant hypervirulent *Klebsiella pneumoniae* isolates, Singapore, 2013–2015

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Patient no.</th>
<th>Date of isolation</th>
<th>Sampling site</th>
<th>β-lactam resistance genes</th>
<th>Other resistance genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENT494</td>
<td>A2</td>
<td>2013 Jun 7</td>
<td>Sputum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT646</td>
<td>A2</td>
<td>2013 Sep 18</td>
<td>Blood</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), qnrB1, aac(6')-lb-cr, catB3, dfrA14</td>
</tr>
<tr>
<td>ENT1734</td>
<td>A2</td>
<td>2014 Dec 19</td>
<td>Rectum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), catB3, aac(6')-lb-cr</td>
</tr>
<tr>
<td>ENT1061</td>
<td>A4</td>
<td>2014 Mar 13</td>
<td>Blood</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), aac(6')-lb-cr, adaA16, qnrB6, arr-3, sul1, tet(A), dfrA27</td>
</tr>
<tr>
<td>ENT1256</td>
<td>A6</td>
<td>2014 Jun 20</td>
<td>Rectum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT1332</td>
<td>A6</td>
<td>2014 Jul 13</td>
<td>Rectum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT1381</td>
<td>A12</td>
<td>2014 Aug 10</td>
<td>Midstream urine</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT1998</td>
<td>A14</td>
<td>2014 May 24</td>
<td>Rectum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA5, mph(A), aac(6')-lb-cr, qnrB1, catB3, tet(A), dfrA14</td>
</tr>
<tr>
<td>ENT596</td>
<td>A15</td>
<td>2013 Aug 22</td>
<td>Urine</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT607</td>
<td>A15</td>
<td>2013 Aug 22</td>
<td>Sputum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), adaA1, cmiA1, arr-2, sul1</td>
</tr>
<tr>
<td>ENT686</td>
<td>A15</td>
<td>2013 Oct 4</td>
<td>Tracheostomy aspirate</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT715</td>
<td>A15</td>
<td>2013 Oct 17</td>
<td>Trachea aspirate</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT750</td>
<td>A15</td>
<td>2013 Oct 31</td>
<td>Blood</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT758</td>
<td>A15</td>
<td>2013 Oct 31</td>
<td>Tracheal aspirate</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT883</td>
<td>A15</td>
<td>2013 Dec 14</td>
<td>Sputum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT1072</td>
<td>A15</td>
<td>2014 Mar 26</td>
<td>Sputum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A)</td>
</tr>
<tr>
<td>ENT1077</td>
<td>A15</td>
<td>2014 Mar 28</td>
<td>Rectum</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), tet(A), dfrA14</td>
</tr>
<tr>
<td>ENT495*</td>
<td>A15</td>
<td>2013 Jun 8</td>
<td>Not known</td>
<td>bla_{SHV-11}, bla_{KPC-2}, bla_{TEM-1A}, bla_{TEM-1B}, bla_{OX-1}</td>
<td>oqxA, oqxB, fosA6, mph(A), tet(A), dfrA14, aac(6')-lb-cr, adaA1, arr-2, cmiA5, ereA, qnrB1, sul1</td>
</tr>
<tr>
<td>SGH10</td>
<td>Not applicable</td>
<td>Blood</td>
<td>bla_{SHV-11}</td>
<td>oqxA, oqxB, fosA6</td>
<td></td>
</tr>
</tbody>
</table>

*Control carbapenem-resistant *K. pneumoniae* isolate (classified as *K. quasipneumoniae* by GenBank).

Figure 3. Main features of pKPC2 from *Klebsiella pneumoniae* isolate ENT494, Singapore, 2013, and comparison with pSA20021456.2. Image was generated by using SnapGene Viewer (https://www.snapgene.com) and Easyfig (https://github.com/mjsull/Easyfig). KPC, *Klebsiella pneumoniae* carbapenemase; oriT, origin of transfer; T4SS, type IV secretion system.

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donors could not explain the observed differences in conjugation efficiency.

To assess the role of pKPC2 in conferring antimicrobial drug resistance to host strains, we measured the MICs of antimicrobial drugs against the 3 isolates from patient A2 and compared with SGH10 (Table 3). SGH10 was resistant to penicillins and fosfomycin but susceptible to the 2 fluoroquinolones tested, even though the strain carried the fluoroquinolone efflux pump genes \( qpxA \) and \( qpxB \) (Table 2). This finding is consistent with the low \( qpxB \) expression seen for most \( K. pneumoniae \) strains (42). SGH10 was susceptible to cephems and carbapenems. In contrast, all 3 isolates from patient A2 were resistant to ceftriaxone, imipenem, and meropenem (Table 3), most likely because of the presence of pKPC2. These data show that pKPC2 is a highly transmissible plasmid that confers resistance to all 3 types of \( \beta \)-lactams.

**Within-Patient Microevolution of Carbapenem-Resistant Hypervirulent \( K. pneumoniae \) Isolates**

Using multiple isolates from the same patient, we set out to determine the changes that occurred in the genome of 1 carbapenem-resistant hypervirulent \( K. pneumoniae \) population over the course of an infection. This analysis enabled us to track the carriage of genes conferring carbapenem resistance and MDR in the bacteria versus antimicrobial drug exposure over time. With the available clinical data from patient A2, we reconstructed a timeline of the evolution of the 3 isolates from this patient, showing their plasmid content and antimicrobial drug exposure (Figure 6).

In addition to the chromosome, \( K. pneumoniae \) virulence plasmid, and pKPC2, all 3 isolates from patient A2 carried 2 or 3 additional plasmids (Appendix Table). Isolates were of the same infecting strain but lost or gained MDR plasmids over time. The \( K. pneumoniae \) virulence plasmid and pKPC2 were stable, showing few changes over a year. Treatment with gentamicin and ciprofloxacin correlated with the appearance of an MDR plasmid in ENT646 that encoded resistance genes to both classes of antimicrobial drugs. This 165-kb MDR plasmid (named pMDR646) carried \( aac(6')-lb-cr \), \( bla_{OXA-1'} \).
virulence plasmids (\(\ast\), and a virulence plasmid or a plasmid carrying the \(katB3\) gene) in 16 isolates from 5 of 7 patients were resistant to ciprofloxacin (Table 3). Both ENT646 and ENT1734 carried broad-range resistance genes. The last isolate, ENT1077, had acquired tetracycline and trimethoprim resistance genes not seen in other isolates.

### Discussion

We report the coexistence of hypervirulence and carbapenem resistance within the same \(K. pneumoniae\) isolates in Singapore. These isolates dated back to 2013; however, their existence could have occurred even earlier because collection started in 2010. All carbapenem-resistant hypervirulent \(K. pneumoniae\) isolates in this study harbored a \(bla_{KPC-2}\) gene. In studies conducted in China, most patients infected with carbapenem-resistant hypervirulent \(K. pneumoniae\) isolates also carried the \(bla_{KPC-2}\) gene (16,43–45). We and others have described hypervirulent \(K. pneumoniae\) in community-acquired liver abscesses in Singapore, where most isolates are capsular serotypes K1 and K2 (6,46). Most (>80%) liver abscess isolates are estimated to belong to sublineage CG23-1 (includes reference strain SGH10) (39). The carbapenem-resistant hypervirulent \(K. pneumoniae\) isolates from 5 of 7 patients in this study were K1 or K2 serotype and 6 of 7 isolates were highly virulent, as predicted. Our results suggest that \(pKPC2\) can be stably maintained in a hypervirulent \(K. pneumoniae\) bacterial host. Thus, the possible dissemination of the \(bla_{KPC-2}\) gene to hypervirulent strains present in a carriage state within communities is a concern.

In a study in China, 1,838 isolates were analyzed, and 21 carbapenem-resistant hypervirulent \(K. pneumoniae\) isolates were found and classified as ST11, ST65, ST268, ST595, and ST692 (44). In another report, 5 ST11 CRKP isolates were documented as having acquired \(K. pneumoniae\) virulence plasmids (16). In these reports, a \(K. pneumoniae\) virulence plasmid or parts of one was probably co-opted into the prevalent CRKP strain in that region, which was an ST11 strain carrying the \(bla_{KPC-2}\) gene. According to our previous study, only 5% of CRKP isolates in Singapore were ST11 (8). However, in another study, 7 carbapenem-resistant hypervirulent \(K. pneumoniae\) isolates were identified, 4 of which were ST23 and serotype K1 (47). In 2 case studies, ST23 (hypervirulent) isolates were

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**Table 3. MICs of antimicrobial drugs against 3 carbapenem-resistant hypervirulent Klebsiella pneumoniae isolates from patient A2, Singapore, 2013–2014, compared with reference strain SGH10**

<table>
<thead>
<tr>
<th>Antimicrobial drug group and drug</th>
<th>SGH10</th>
<th>ENT494</th>
<th>ENT646</th>
<th>ENT1734</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>&gt;64</td>
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<td>Polymyxin B</td>
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*Bold numbers indicate resistance as interpreted by Clinical and Laboratory Standards Institute interpretative criteria for MICs (38).*
Hypervirulent \textit{K. pneumoniae}, Singapore reported to have acquired carbapenem-resistance genes (14,48). All our isolates in this investigation had the hypervirulence background and acquired carbapenem-resistance and MDR genes; the MDR genes appeared to move in and out of the parental strain over time within the patient. Our observations are consistent with earlier reports in China involving ST65 isolates, which were likely hypervirulent, indicating that hypervirulent isolates can acquire antimicrobial drug resistance. Therefore, whether a superbug is more likely to arise from a carbapenem-resistant isolate acquiring a \textit{K. pneumoniae} virulence plasmid or some of its genes versus a hypervirulent isolate acquiring MDR genes is unclear. Both ways of exchange appear possible, perhaps depending on the prevalence of the circulating sequence type in a particular setting and in vivo selection pressures.

We also show that, although isolates from different patients were similar in terms of the virulence and carbapenem-resistance plasmids, these isolates do not arise from transmission events. For patients with multiple isolates, the core genomes were highly conserved, suggesting a single infecting isolate gained or lost various antimicrobial drug resistance genes over time. For patient A2, our longitudinal data suggest that the use of distinct antimicrobial drugs drove the acquisition of resistance, though we have no direct proof of cause and effect. Of note, in patient A15, the rectal isolate had additional antimicrobial drug resistance, perhaps reflecting that the colon might serve as a reservoir where genetic exchange can take place. Six of the 18 isolates, including the last acquired isolates from the 3 patients with multiple isolates, were collected from feces or rectal swab specimens, suggesting that the intestines are a likely reservoir for persistent carriage. Last, we demonstrated as a proof-of-principle that pKPC2 can be transferred from the \textit{K. pneumoniae} isolates to \textit{E. coli} in vitro with high efficiency. Determining the particular characteristics of pKPC2 that make it so transmissible and stable, particularly during interactions with hypervirulent isolates, and how pKPC2 is acquired by the recipient is essential.

Therefore, this report serves to alert infectious disease clinicians to the possible presence of hypervirulence in MDR or carbapenem-resistant colonizing isolates; patients harboring these isolates are at risk of developing invasive infections. With timely (rather than retrospective) whole-genome sequencing of bacteria, identifying patients harboring hypervirulent and multidrug- or carbapenem-resistant isolates at high risk for death should be possible. These patients can be selected for appropriate infection control measures, treatment, and close monitoring. Developing strategies to decolonize the gastrointestinal tracts of patients with such isolates would help minimize the release of these potential superbugs into the community.
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We thank the CaPES study group for their support. This group included Benjamin Cherng, Deepak Rama Narayana, Douglas Chan Su Gin, De Partha Pratim, Hsu Li Yang, Indumathi Venkathachalam, Jeanette Teo, Kalisvar Marimuthu, Koh Tse Hsien, Nancy Tee, Nares Smitasin, Ng Oon Tek, Ooi Say Tat, Raymond Fong, Raymond Lin Tzer Pin, Su Rinder Kaur Pada, Tan Thean Yen, and Thoon Koh Cheng. We thank Ewan M. Harrison for his critical reading of the manuscript.

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About the Author
Mr. Chen is a research associate at the Yong Loo Lin School of Medicine at National University of Singapore, Singapore. His main research interests are regulation of virulence and antimicrobial drug resistance in bacterial pathogens, including K. pneumoniae.

References


Address for correspondence: Yunn-Hwen Gan, National University of Singapore, Yong Loo Lin School of Medicine, Department of Biochemistry, 8 Medical Dr, 117596, Singapore; email: bchgangyh@sus.edu.sg
Acquisition of Carbapenem-Resistance Gene $\textit{bla}_{\text{KPC-2}}$ in Hypervirulent, \textit{Klebsiella pneumoniae}, Singapore

**Appendix**

**Appendix Table.** Features of complete genomes*

<table>
<thead>
<tr>
<th>Isolate/ subject, Replicon, Accession number</th>
<th>Size (bp)</th>
<th>Incompatibility group</th>
<th>oriT</th>
<th>Self-transmissible modules</th>
<th>Features</th>
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</thead>
<tbody>
<tr>
<td>ENT494 / A2</td>
<td>5443212</td>
<td>-</td>
<td>N</td>
<td>3/4</td>
<td>$\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes</td>
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<td>Chromosome_494, VONF01000001.1</td>
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<td>IncHI1B</td>
<td>N</td>
<td>1/4</td>
<td>$\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes</td>
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<td>pKpVp_494, VONF01000002.1</td>
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<td>IncFIA</td>
<td>Y</td>
<td>4/4</td>
<td>$\textit{bla}<em>{\text{KPC-2}}, \textit{bla}</em>{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic</td>
</tr>
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<td>pENT494_7kb, VONF01000005.1</td>
<td>71861</td>
<td>Not identified</td>
<td>Y</td>
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</tr>
</tbody>
</table>

| ENT646 / A2                                 | 5444482   | -                      | N    | 3/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes; $\textit{pKpVp}_{494} + 1 \text{IS110 aerobactin and salmochelin loci, rmpA genes; pKpVp}_{494} + 1 \text{IS110}$ |
| Chromosome_646, VONE01000001.1              | 223140    | IncHI1B                | N    | 1/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes; pKpVp + two IS110 |
| pKpVp_646, VONE01000002.1                   | 165414    | IncFIB, IncFII         | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |
| pMDR_646, VONE01000003.1                    | 95210     | IncFIA                 | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |

| ENT1734 / A2                                | 5448145   | -                      | N    | 3/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes; pKpVp + two IS110, multiple deletions of pMDR_646 |
| Chromosome_1734, VOND01000001.1             | 224835    | IncHI1B                | N    | 1/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes; pKpVp + two IS110, multiple deletions of pMDR_646 |
| pKpVp_1734, VOND01000002.1                  | 99794     | IncFIB                 | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |
| pMDR_1734, VOND01000003.1                   | 95210     | IncFIA                 | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |

| ENT1192 / A14                               | 5246080   | -                      | Y    | 2/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA5}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes |
| Chromosome_1192, VOMY01000001.1             | 215341    | IncHI1B                | N    | 1/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA5}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes |
| pKpVp_1192, VOMY01000002.1                  | 109672    | IncFIB, IncFII         | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |

| ENT607 / A15                                 | 5416548   | -                      | N    | 3/4                        | $\textit{bla}_{\text{SHV-11}}, \textit{oxqA}, \textit{oxqB}, \textit{fosA6}$, aerobactin and salmochelin loci, $\textit{mpA}$ genes |
| Chromosome_607, VOMV01000001.1              | 235891    | IncHI1B                | N    | 1/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |
| pKPC2_607, VOMV01000002.1                   | 104712    | IncFIB, IncFII         | Y    | 4/4                        | $\textit{bla}_{\text{KPC-2}}, \textit{bla}_{\text{TEM-1A}}, \textit{bla}_{\text{TEM-1B}}, \textit{mph(A)}$, cryptic; identical to pENT494_95kb |

*KPC, \textit{Klebsiella pneumoniae} carbapenemase; KpVP, \textit{K. pneumoniae} virulence plasmid; MDR, multidrug resistance; N, no; Y, yes.
Appendix Figure. A) Alignment of pMDR_646 and pMDR_1734. The alignment was performed using Mauve. B) Feature-comparison of pMDR_646 and pMDR_1734. The map was generated using BRIG-0.95. MDR, multidrug resistance.