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MAJOR ARTICLE

Molecular Evolution of a *Klebsiella pneumoniae* ST278 Isolate Harboring bla_{NDM-7} and Involved in Nosocomial Transmission

Tarah Lynch,[1,2,5] Liang Chen,[7] Gisele Peirano,[1,2] Dan B. Gregson,[1,2,4,5] Deirdre L. Church,[1,2,4,5] John Conly,[1,2,3,4,5] Barry N. Kreiswirth,[7] and Johann D. Pitout[1,2,3,5,6]

[1]Division of Microbiology, Calgary Laboratory Services, [2]Department of Pathology and Laboratory Medicine, [3]Department of Microbiology, Immunology, and Infectious Diseases, [4]Department of Medicine, and [5]Snyder Institute for Chronic Diseases, Cummings School of Medicine, University of Calgary, Canada; [6]Department of Medical Microbiology, University of Pretoria, South Africa; and [7]Public Health Research Institute Tuberculosis Center, Rutgers University, Newark, New Jersey

During 2013, ST278 *Klebsiella pneumoniae* with bla_{NDM-7} was isolated from the urine (KpN01) and rectum (KpN02) of a patient in Calgary, Canada. The same strain (KpN04) was subsequently isolated from another patient in the same unit. Interestingly, a carbapenem-susceptible *K. pneumoniae* ST278 (KpN06) was obtained 1 month later from the blood of the second patient. Next-generation sequencing (NGS) revealed that the loss of carbapenem-resistance in KpN06 was due to a 5-kb deletion on the bla_{NDM-7}-harboring IncX3 plasmid. In addition, an IncFIB plasmid in KpN06 had a 27-kb deletion that removed genes encoding for heavy metal resistance. Phylogenetic analysis showed that the *K. pneumoniae* ST278 from patient 2 was likely a descendant of KpN02 and that KpN06 was a close progenitor of an environmental ST278. It is unclear whether KpN06 lost the bla_{NDM-7} gene in vivo. This study detailed the remarkable plasticity and speed of evolutionary changes in multidrug-resistant *K. pneumoniae*, demonstrating the highly recombinant nature of this species. It also highlights the ability of NGS to clarify molecular microevolutionary events within antibiotic-resistant organisms.

Keywords. *K. pneumoniae*; ST278; carbapenemases; bla_{NDM-7}; plasmid; microevolution.

During the 1970s, *Klebsiella pneumoniae* emerged as an important cause of nosocomial urinary tract infections, respiratory tract infections, and bloodstream-associated infections [1]. The management of infections due to *K. pneumoniae* has been recently been complicated by the emergence of resistance to the carbapenems, which are often the last line of effective therapy available for the treatment of infections caused by multidrug-resistant (MDR) isolates [2]. Several mechanisms are responsible for resistance to carbapenems in *K. pneumoniae*, but the production of carbapenemases remains the most clinically relevant [1]. The Ambler class B carbapenemases or metallo-β-lactamases (MBLs) identified in *K. pneumoniae* are most often NDMs, while VIMs and IMP types are relatively rare in this species [1].

Between May 2013 and Dec 2014, 17 NDM-7–producing Enterobacteriaceae were isolated from 6 patients in Calgary, Canada. The resistance gene bla_{NDM-7} was harbored by an identical approximately 46-kb IncX3 plasmid among these isolates [3]. The index patient (patient 1) was admitted to the hospital in May 2013, and an ST278 carbapenem-resistant (CR) *K. pneumoniae* (KpN01) was isolated from his urine during June 2013. Subsequently, in August 2013, the same CR ST278 *K. pneumoniae* (KpN04) was identified from a different patient (patient 2) from the same unit. Interestingly, a carbapenem-susceptible (CS) ST278 *K. pneumoniae* (KpN06) was then obtained from the blood of patient 2 in September 2013. Molecular analyses showed that bla_{NDM-7} was absent in KpN06; however, the mechanism underlining the resistance gene loss was unclear. In this study, next-generation sequencing (NGS) was used to characterize 11 isolates (KpN01–11) collected from the 2 patients and their respective rooms, to explore the short-term microevolution of carbapenem resistance and nosocomial transmission of *K. pneumoniae* ST278.

MATERIALS AND METHODS

Rectal and Environmental Screening for CR Enterobacteriaceae

Rectal swabs were placed into Copan M40 Transystem containing Amies gel transport medium, and the Centers for Disease Control and Prevention's (Atlanta, Georgia) protocol was used to screen for CR gram-negative bacteria [4]. Specimens collected from different surfaces in patient's rooms by means of sterile rayon swabs were cultured for *K. pneumoniae* by vortexing the swabs in 5 mL of tryptic soy broth, followed by incubation at 37°C for 24 hours. Samples exhibiting turbidity were plated on blood and MacConkey agars.

Bacterial Identification and Antimicrobial Susceptibilities

Isolates (KpN01–11) were identified using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Vitek AMS; bioMerieux Vitek Systems, Hazelwood, Missouri). Minimum inhibitory concentrations of drugs (Supplementary

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Correspondence: J. D. Pitout, Calgary Laboratory Services, #9, 3535 Research Road NW Calgary, Alberta, Canada T2L 2K8 (johann.pitout@cls.ab.ca).

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Table 1) were determined using the Microscan NEG 38 panel (Siemens, Burlington, Canada) and interpreted by using 2015 Clinical and Laboratory Standards Institute guidelines for broth dilution [5].

β-lactamase Identification

Carbapenemases was detected using the modified Hodge test and the Mastdiscs ID inhibitor combination disks [6] (Mast Group, Merseyside, United Kingdom). Polymerase chain reaction (PCR) amplification and sequencing for β-lactamase genes were undertaken by using primers and conditions as previously described [7, 8].

Plasmid Analysis

Plasmid sizes were determined as previously described and assigned to plasmid incompatibility groups by PCR-based replicon typing [9, 10]. Conjugation experiments were performed by mating-out assays with nutrient agar containing meropenem 1 μg/mL and using *Escherichia coli* J53 (azide 100 μg/mL) as recipient.

Molecular Typing

Molecular typing was performed using standardized pulsed-field gel electrophoresis (PFGE) [11] and multilocus sequencing typing [12].

NGS

DNA from KpN01 (from patient 1) and KpN06 (from patient 2) were extracted by standard methods (DNeasy Blood and Tissue Kit, Qiagen, Toronto, Canada) and sent to the Génome Québec Innovation Centre (Montreal, Canada) for long-read sequencing with Pacific Biosciences RSII platform (Pacific Biosciences, Menlo Park, California) to differentiate the chromosomal from plasmid DNA. Library preparation was optimized to include both long reads (>3 kb) and shorter, circular consensus sequencing reads. Four SMRT cells per isolate were used to ensure a minimum of 75× coverage of each genome.

KpN01–11 were sequenced with the MiSeq (Illumina, San Diego, California) platform. Libraries were prepared with the Nextera XT kit to produce paired end reads of 250 base pairs for a minimum predicted coverage of 75-fold. The high coverage Illumina reads were mapped to the PacBio assemblies for Kp01 and Kp06 by using Nesoni [13] to ensure high-quality reference genomes.

Assembly and Analysis

The KpN01 and KpN06 PacBio reads were assembled using the Hierarchical Genome Assembly Process, compiled specifically for quality trimming, de novo assembly, and polishing [14]. MiSeq reads were trimmed with TrimGalore (v.0.3.3; available at: http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) to remove sequencing adapters and reads with Phred quality scores of <25 and then were merged with FLASH (v.1.2.10) [15]. The merged and trimmed, paired-end fastq data were assembled with SPAdes (v. 3.0) [16] and assessed with QUAST [17]. All assemblies were annotated with Prokka (v.1.7) [18], the plasmids were typed with PlasmidFinder [19], and acquired antimicrobial resistance genes were identified using SRST2 [20]. The plasmids were compared using Mauve [21], and figures were produced with EasyFig [22].

Read Mapping and Variant Calling

The assembled PacBio genome of KpN01 (the index isolate) was used as the reference sequence for read mapping of the other isolates (KpN02–11) by using SMALT (v.0.7.5; available at: http://www.sanger.ac.uk/resources/software/smalt/), and the resultant mapping files were indexed and sorted with SAMtools (v. 1.0) [23] before variant calls were made with Freebayes (v. 0.9.8). Potential single-nucleotide variants (SNVs) were excluded if the mapping quality or the base quality score was <20 or if the minimum alternate fraction was <0.75. Core was defined as positions present in ≥90% of the genomes, and the resultant core SNVs were used to build a phylogeny, using the Core SNV Pipeline (available at: https://github.com/apetkau/core-phylogenomics). Larger insertion and/or deletion events (>1 nucleotide) were visually identified in Integrative Genomics Viewer [24] from the read mapping and were appended to the alignment as the presence or absence of character states. The chromosomal integration site of insertion sequence (IS) elements were examined by ISMapper, using the KpN01 genome as a reference [25].

Ethics Approval

Ethics approval was obtained from the Conjoint Health Research Ethics Board at the University of Calgary (REB13-0867_REN1).

RESULTS

CR *K. pneumoniae* Were Transferred From Patient 1 to Patient 2, Notwithstanding Infection Prevention and Control Measures

The clinical time line for patients 1 and 2 is summarized in Figure 1. Both patients were elderly and had comorbidities. Neither patient had travelled outside of Alberta, Canada, within 1 year of the hospital admission. Urine and rectal swab specimens positive for CR *K. pneumoniae* (KpN01 and KpN02) were obtained during June 2013 from patient 1 (he was asymptomatic during this episode) and prompted infection prevention and control protocols (including contact, isolation, and active surveillance procedures). Inner circle rectal swabs from patients in unit B were collected, but no additional positive swabs were found. Patient 1 was subsequently moved to unit C, where another positive urine sample was collected in early August (KpN03) before ciprofloxacin and gentamicin was administered for lower urinary tract infection (Figure 1).

In mid-August, 2 CR *K. pneumoniae* isolates were recovered from the nephrostomy tube (KpN04) and rectal swab (KpN05) of an asymptomatic elderly patient (patient 2) in a room immediately adjacent to that of patient 1. Patient 2 previously (in January 2013) received ciprofloxacin for lower urinary tract infection, had early dementia, and reportedly entered the rooms of

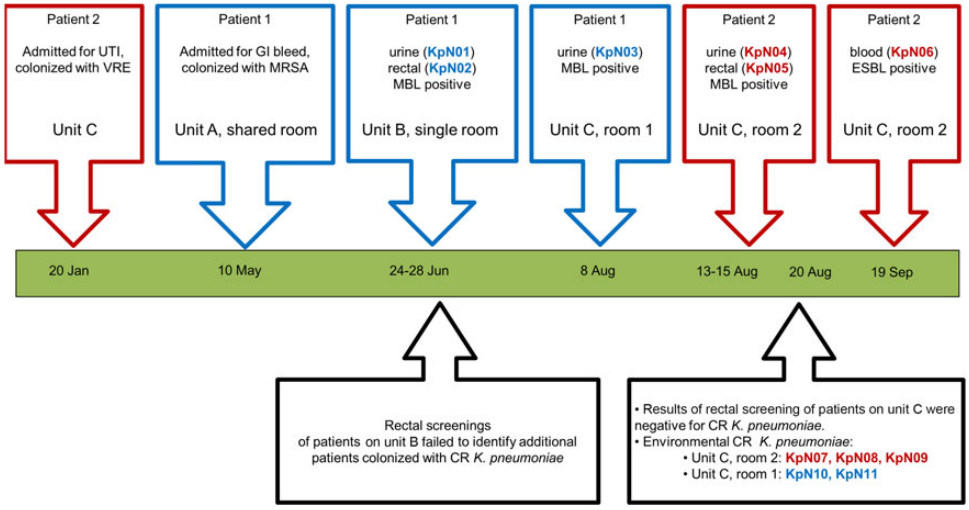

Figure 1. The time line of events for 2 patients infected with multidrug-resistant (MDR) *Klebsiella pneumonia* within a single hospital. All events occurred within the same regional hospital in Calgary, Canada, between May and September of 2013. The clinical time line highlights the unit transfers and positive culture results from patient 1 (blue boxes). Infection prevention and control surveillance measures on unit 3 after MDR *K. pneumoniae* was isolated are shown in the black boxes below the dateline. The time line for patient 2 is presented in red boxes. Isolation measures were taken with patient 2 due to vancomycin-resistant enterococcus (VRE) colonization found upon admission in May. Abbreviations: CR, carbapenem resistant; ESBL, extended-spectrum β-lactamase; GI, gastrointestinal; MBL, metallo-β-lactamase; UTI, urinary tract infection.

other patients. One patient on unit C received ertapenem during this time, and rectal screenings of all patients did not identify additional patients colonized with CR *K. pneumoniae*. Environmental screening revealed 5 additional CR *K. pneumoniae* isolates: KpN07, KpN08, and KpN09 from the room of patient 2 and KpN10 and KpN11 from the room of patient 1 (Figure 1). In September 2013, CS *K. pneumoniae* (KpN06) was isolated from a blood specimen from patient 2, and she was treated with intravenous colistin and meropenem but died due to sepsis.

Characterization of the CR *K. pneumoniae* Isolates Showed Them to Be Indistinguishable by Traditional Typing

Susceptibility, phenotypic, and molecular tests to characterize bacterial isolates (KpN01–11) are summarized in Supplementary Table 1. PCR and sequencing identified $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{SHV-27}}$ in KpN01–11. They were also positive for $bla_{\text{NDM-7}}$, with the exception of KpN06 (Supplementary Table 1). KpN01–11 belonged to ST278 and were indistinguishable by PFGE. Plasmid analysis showed that the isolates obtained from patient 1 and his room contained 3 plasmids (190 kb, 130 kb, and 50 kb), while isolates obtained from patient 2 and her room also contained 3 plasmids (190 kb, 100 kb, and 50 kb). Transconjugants from KpN01–11 (except KpN06) contained the 50-kb plasmid, were positive for bla_{NDM}, and were typed with IncX replicon (Supplementary Table 1).

NGS Revealed That the Loss of Carbapenem Resistance Was Due to a 5-kb Deletion on a blaNDM-7–Harboring IncX3 Plasmid

KpN01 (a $bla_{\text{NDM-7}}$–positive isolate from patient 1) and KpN06 (a $bla_{\text{NDM-7}}$–negative isolate from patient 2) were chosen for in-depth genomics analysis. The genomic features of assemblies are presented in Table 1, and assembly summaries are in Table 2. The average coverage for the PacBio data was >100-fold for both isolates (both the circular consensus sequences and longer reads combined). The MiSeq data coverage was similar (approximately 100-fold coverage), and as a result, no single base call corrections were made.

The chromosome lengths of KpN01 and KpN06 were 5.307 Mb and 5.309 Mb, respectively, similar in length to other *K. pneumoniae* genomes in public databases (range, 5.3–5.6 Mbp). They had an average G + C content of 57.4%, carried 25 ribosomal RNA genes, 88 transfer RNA genes, 7 putative prophages, and 3 integrated conjugative elements (ICEs). KpN01 had 10 IS elements, and KpN06 had 12 (Table 1). Table 2 details the variation in KpN06 with reference to KpN01, including 7 chromosomal SNVs that were intragenic, nonsynonymous mutations. KpN06 had a 77–base pair deletion within a formate hydrogen lyase activator (*fhlA*) transcription factor gene (locus no. AQD68_19305) and 2 transposase insertions: an IS*5* (1.2 kb) was inserted into a transcriptional regulator gene (AQD68_19830), and an IS*1* (777 base pairs) was inserted into a TetR family transcriptional regulator gene (AQD68_25885). The deletion and IS insertion mapping predicts that these genes were inactive in KpN06.

The long read sequencing led to parsing of 4 similar plasmids (that belonged to IncFII, IncFIB, IncX3, and ColE incompatibility groups) in KpN01 and KpN06.

The IncFII plasmids, pKpN01-CTX15 and pKpN06-CTX15, were highly similar (>99.9% nucleotide identities) and 190 072 base pairs in length, with 212 putative open reading frame sequences and a 57.1% G + C content. They harbored the following

Table 1. Genomic Features of KpN01 and KpN06 Determined Using Pacific Biosciences RSII and Illumina MiSeq Platforms

Feature	KpN01	KpN06
Sequence type	278	278
Date of isolation	24 June 2013	19 September 2013
Source	Urine	Blood
β-lactamase genes	bla_{NDM-7}, $bla_{CTX-M-15}$, bla_{TEM-1}, bla_{SHV-27}	$bla_{CTX-M-15}$, bla_{TEM-1}, bla_{SHV-27}
Additional antimicrobial resistance genes	*dfrA14, oqxA, oqxB, qnrB1, strA, strB, tetA, sul2*	*dfrA14, oqxA, oqxB, qnrB1, strA, strB, tetA, sul2*
Size, base pairs	5 307 114	5 309 013
G + C content, %	57.4	57.4
Genes, no.	5297	5302
CDS, no.	5153	5157
Ribosomal RNA genes, no.		
Overall	25	25
16S	8	8
23S	8	8
5S	9	9
Transfer RNA genes, no.	88	88
Plasmids, no.	4	4
Prophages, no.	7	7
ICEs, no.	3	3
IS elements, no.	10	12
IS family (no.)	IS*1* (1), IS*3* (1), IS*903* (2), IS*Kpn1* (4), IS*Kpn1400* (2)	IS*1* (2), IS*3* (1), IS*5* (1), IS*903* (2), IS*Kpn1* (4), IS*Kpn1400* (2)

Abbreviations: CDS, coding DNA sequences; ICE, integrated conjugative element; IS, insertion sequence.

antimicrobial resistance genes: $bla_{CTX-M-15}$, bla_{TEM-1}, *qnrB1*, *strAB*, *tetA*, *sul2*, and *dfrA14*.

The IncFIB plasmids, pKpN01-SIL and pKPN06-SIL, were 134 064 and 107 110 base pairs in length, respectively (Figure 2). Interestingly, pKpN06-SIL from KpN06 had a 27-kb deletion (when compared to pKpN01-SIL) that contained the *lac*, *sil*, and partial *cus* operons, encompassing *lacY-lacZ-lacI- ahd- nuc-silE-cusRS-cusCFBA -silP- silE* genes. Of note is that the 27-kb deletion region was located immediately downstream of IS*903* (Figure 2).

The IncX3 plasmid from KpN01, pKpN01-NDM7, was 46 161 base pairs in length and harbored bla_{NDM-7} with no additional antibiotic resistance determinants. The detailed plasmid structure was reported in our previous study [3]. Interestingly, the InX3 plasmid from KpN06, pKpN06-NDM7, had a 5-kb deletion (when compared to pKpN01-NDM7) that included the region of Δ*IS3-umuD*-IS*26-dsbC-trpF-ble*$_{MBL}$- bla_{NDM-7}-Δ*ISAba125*, which was consistent with the loss of carbapenem resistance. Further inspection indicated that the deleted region was bracketed by two 3-base pair direct repeats (TAA; Figure 2).

The smallest plasmids from KpN01 (pKpN01-COL) and KpN06 (pKpN06-COL) were 3223 base pairs in length, belonged to the ColE family and were nearly identical (>99.9% identity) to other ColE plasmids (ie, pKP13b [CP003994] [26], pCAV1321-3223 [CP011604], pCAV1492-3223 [CP011637], pCAV1311-3223 [CP011569], and pCAV1741-3223 [CP011652]).

NGS Unveiled That the *K. pneumoniae* ST278 Strains Belonged to Different Clusters

In addition to the 2 completely closed ST278 genomes described above (KpN01 and KpN06), 9 more isolates were sequenced using the Miseq Illumina platform (Table 3). The sequencing reads were mapped to the KpN01 genome to identify SNVs and de novo assembled to examine large insertions and deletions. Figure 3 outlines the genetic changes observed between the clinical isolates recovered from patients 1 and 2 and their rooms. A maximum likelihood tree was produced by combining core SNVs with the larger insertion and deletion events as character states (presence/absence) appended to the alignment. The core SNV positions are presented in Supplementary Table 2 and summarized in a distance matrix in Supplementary Figure 1*B*, with the insertion/deletion events summarized in Supplementary Figure 1*A*. No short Indels (<10 base pairs) were identified in the 11 genomes.

All genomes were closely related, with the core SNVs ranging from 0–14 (Supplementary Figure 1*B*). The addition of the larger insertion/deletion events to the phylogeny produced a tree that illustrates the genetic adaptations of *K. pneumoniae* within a short time scale. The 3 isolates from patient 1 (KpN01, KpN02, and KpN03) differed by 1 SNV, while the 2 environmental isolates from the room of patient 1 were identical. The 2 urinary isolates (KpN01 and KpN03), collected 45 days apart, differed by a single SNV (Table 2), while the rectal isolate (KpN02), collected 3 days after KpN01, acquired 2 IS insertions (at AQD68_19830 and _25885), resulting in the gene disruption of 2 putative regulators. These gene deletions could be associated with selection pressure change from the urinary to gastrointestinal tracts.

The 3 isolates from patient 2 (urine [KpN04], rectum [KpN05], and blood [KpN06]) differed by an average of 7 SNVs (Table 2).

Table 2. Chromosomal and Plasmid Variations Between KpN01 and KpN06 Determined Using the Pacific Biosciences RSII and Illumina MiSeq Platforms

Reference	Start	End	Change	Polymorphism Type	Length (With Gaps)	Amino Acid Change	CDS Codon No.	CDS Position	Gene	Locus_Tag	Product
KpN01_chromosome	209 112	209 112	C → A	SNP (transversion)	1	D → E	156	468	. . .	AQD68_01120	Malonate decarboxylase subunit α
KpN01_chromosome	1 337 919	1 337 919	G → T	SNP (transversion)	1	R → L	207	620	. . .	AQD68_06685	Dihydropteridine reductase
KpN01_chromosome	1 921 164	1 921 164	A → C	SNP (transversion)	1	T → P	813	2437	. . .	AQD68_09505	ATP-dependent helicase
KpN01_chromosome	3 974 440	3 974 440	G → T	SNP (transversion)	1	R → L	56	167	. . .	AQD68_19845	AraC family transcriptional regulator
KpN01_chromosome	4 391 829	4 391 829	G → T	SNP (transversion)	1	T → K	60	179	spr	AQD68_21945	Lipoprotein Spr
KpN01_chromosome	4 529 258	4 529 258	A → C	SNP (transversion)	1	E → A	106	317	. . .	AQD68_22510	Hypothetical protein
KpN01_chromosome	4 623 276	4 623 276	C → T	SNP (transition)	1	P → S	88	262	sirA	AQD68_22995	2-component system response regulator
KpN01_chromosome	3 873 994	3 874 070	. . .	Deletion	77	. . .	81	241	fhlA	AQD68_19305	Formate hydrogen lyase activator
KpN01_chromosome	3 969 188	3 969 187	. . .	Insertion	1199	. . .	49	145	. . .	AQD68_19830	Transposase
KpN01_chromosome	5 189 933	5 189 932	. . .	Insertion	777	. . .	167	500	. . .	AQD68_25885	Transposase
pKpN01-SIL	82 330	109 275	. . .	Deletion	26 945	. . .	. . .	. . .	. . .	AQD68_28045–AQD68_28145	. . .
pKpN01-NDM7	12 315	17 395	. . .	Deletion	5080	. . .	. . .	. . .	. . .	AQD68_28350–AQD68_28390	. . .

Abbreviations: ATP adenosine triphosphate; CDS, coding DNA sequence; SNP, single-nucleotide polymorphism.

In addition to SNVs, KpN06 had a 77-base pair deletion in gene *fhlA* that truncated the gene and a 5-kb deletion in the IncX3 plasmid, encompassing the carbapenem resistance gene $bla_{\text{NDM-7}}$. Interestingly, KpN06 was not a direct descent from KpN04 (the first *K. pneumoniae* isolate from patient 2) but was closer to 2 environmental isolates (KpN07 and KpN09) collected from the room of patient 2 (Figure 3). Moreover, the 27-kb deletion in pKpN01-SIL was only present in the strains obtained from patient 2 (KpN04, KpN05, and KpN06) or the environment within her room (KpN07, KpN08, and KpN09).

KpN01 (collected on 24 June) was the index isolate from patient 1; KpN02 (recovered from a rectal specimen from patient 1) was collected on 27 June, while KpN03 (recovered from a urine specimen from patient 1) was collected on 8 August. This time line would suggest that isolates from patient 2 would have evolved from KpN03. However, KpN02 acquired 2 IS insertions (at AQD68_19830 and _25885) that were absent in KpN01 and KpN03 but present in all of the isolates from patient 2 (ie, KpN04, KpN05, and KpN06), samples from her room (KpN07, KpN08, KpN09), and samples from the room of patient 1 (KpN10 and KpN11). This suggests that KpN04–11 were likely descendants from the rectal isolate from patient 1 (KpN02; Figure 3).

DISCUSSION

This report documented the transmission of highly similar $bla_{\text{NDM-7}}$–harboring *K. pneumoniae* ST278 isolates (with 0–14 core SNVs differences) between 2 patients admitted to adjacent rooms on the same unit, despite infection prevention and control protocols. The case involving patient 1 had significant public health ramifications in Alberta as it suggested that NDM *K. pneumoniae* infections may have been acquired locally, independent of international travel [3]. Autochthonous acquisition of NDM-producing *K. pneumoniae* had previously been described in areas of nonendemicity, including Canada [27]; however, on his initial admission to unit 1 during May 2013, patient 1 shared a semiprivate room with 3 other patients. Therefore, we cannot rule out the possibility that he contracted *K. pneumoniae* with $bla_{\text{NDM-7}}$ from a patient who had traveled to an area of endemicity.

The *K. pneumoniae* from this study belonged to ST278, which was first reported in a neonatal unit from a university hospital in Turkey [28] and more recently in Syrian patients admitted to 2 different hospitals in northern Israel [29]. The Turkish ST278 harbored $bla_{\text{NDM-1}}$, $bla_{\text{OXA-1}}$, and $bla_{\text{SHV-27}}$ and was isolated in 2013 from a newborn's rectal swab [28]. The ST278 from Israel was also present in rectal swabs and the most common ST associated with bla_{NDMs} in that study [29]. However, complete genome sequencing of ST278 isolates were not conducted in these studies, and here we reported the first 2 completely sequenced ST278 genomes.

Genomic and phenotypic analyses demonstrated that ST278 KpN06 was CS and negative for $bla_{\text{NDM-7}}$. The acquisition of

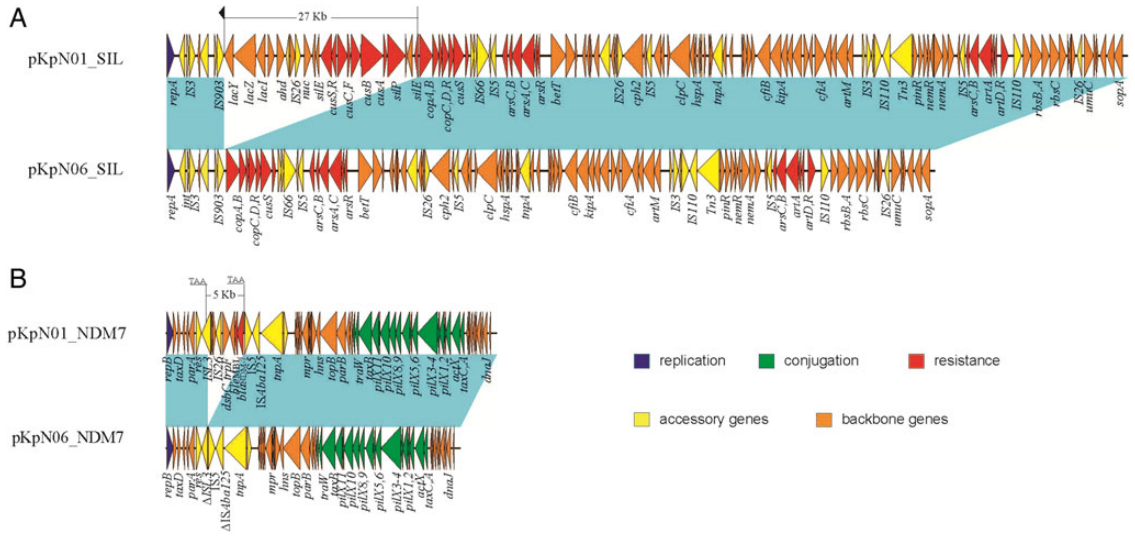

Figure 2. IncFIB and IncX plasmid structures from KpN01 and KpN06. Light blue shading denotes shared regions of homology, and open reading frames (ORFs) are portrayed by arrows and colored on the basis of predicted gene function. The small black arrow above pKpN01_SIL denotes the downstream invert repeat of IS*903*, while the 3–base pair putative direct repeats (TAA) of the 5-kb deletion on pKpN01_NDM7 are underlined.

Table 3. Molecular Characterization of *Klebsiella pneumoniae* ST278 Isolates, Using Pacific Biosciences RSII and Illumina MiSeq Platforms

Isolate and Platform(s), No. of Contigs (≥0 Base Pairs)	Total Length (≥0 Base Pairs)	N_{50}	Plasmid (Inc)	Genome Accessions
KpN01, Pacific Biosciences, Illumina				
5	Chromosome, 5 307 114	. . .	. . .	CP012987
5	pKpN01-CTX15, 189 853	. . .	FII	CP012988
5	pKpN01-SIL, 134 064	. . .	FIB	CP012989
5	pKpN01-NDM7, 46 161	. . .	X3	CP012990
5	pKpN01-COL, 3223	. . .	colE	CP012991
KpN02, Illumina				
167	5 634 950	193 867	. . .	LLWT00000000
KpN03, Illumina				
219	5 628 465	94 505	. . .	LLWU00000000
KpN04, Illumina				
279	5 576 435	52 252	. . .	LLWV00000000
KpN05, Illumina				
125	5 603 554	310 445	. . .	LLWW00000000
KpN06, Pacific Biosciences, Illumina				
5	Chromosome, 5 309 013	. . .	. . .	CP012992
5	pKpN06-CTX15, 190 071	. . .	FII	CP012993
5	pKpN06-SIL, 107 110	. . .	FIB	CP012994
5	pKpN06-NDM7, 41 072	. . .	X3	CP012995
5	pKpN06-COL, 3121[a]	. . .	colE	CP014305
KpN07, Illumina				
128	5 604 347	299 134	. . .	LLWX00000000
KpN08, Illumina				
117	5 575 333	343 007	. . .	LLWY00000000
KpN09, Illumina				
122	5 604 080	343 007	. . .	LLWZ00000000
KpN10, Illumina				
159	5 634 927	230 303	. . .	LLXA00000000
KpN11, Illumina				
154	5 643 345	276 468	. . .	LLXB00000000

Abbreviation: N_{50}, the length for which half of the bases of a draft genome are situated in contigs of that length or longer.

[a] This contig was added from the Illumina MiSeq assembly, matching the same plasmid in pKpN01-COL. It may not have been in the Pacific Biosciences assembly, owing to lower coverage.

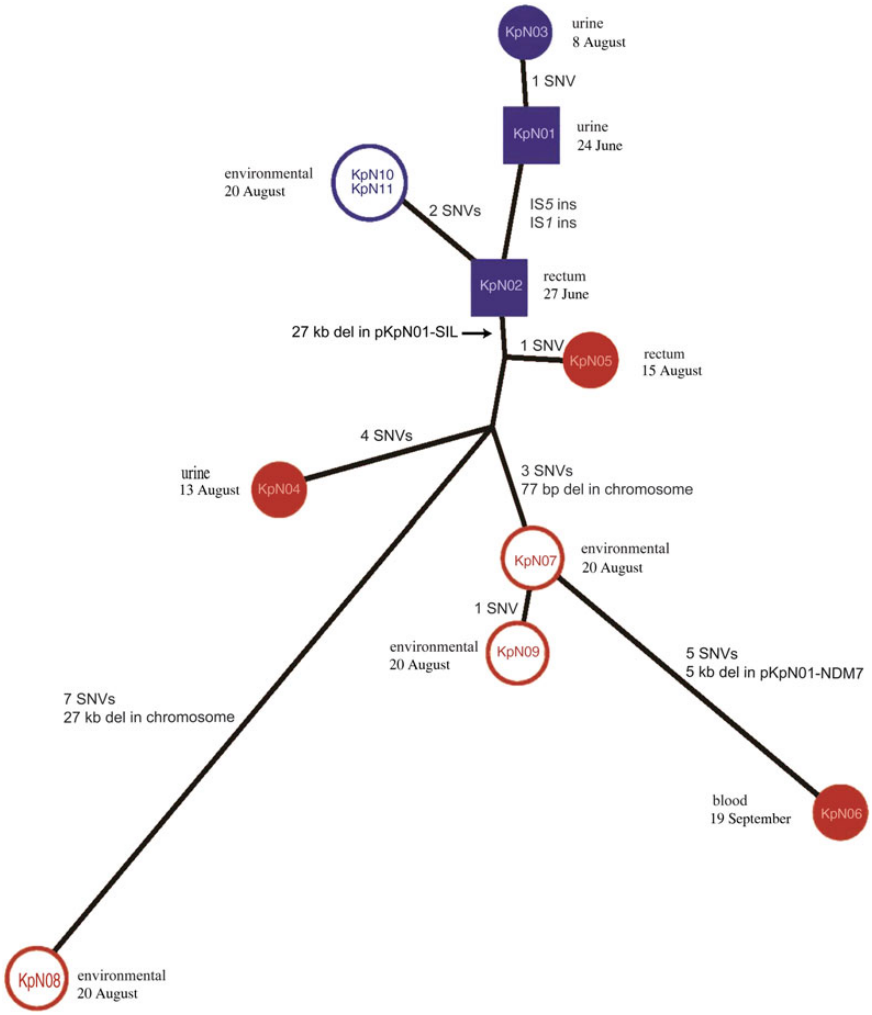

Figure 3. Maximum likelihood tree of all adaptive genetic changes from multidrug-resistant *Klebsiella pneumoniae* isolates associated with patients 1 and 2 over a 3-month period. Isolates from the biological specimens of each patient are solid colors, while isolates from environmental samples from each patient's room are open (blue, patient 1; red, patient 2). KpN01 and KpN02 are presented as squares to differentiate that these were collected while patient 1 was in unit 2 while all other isolates were collected from unit 3 (circles). All genetic events including core single-nucleotide variants (SNVs) and insertion/deletions were included in the analysis, with the latter being added as presence/absence character states to the alignment to capture all of the genetic adaptations that occurred.

carbapenem resistance in clinical *K. pneumoniae* had previously been reported, but the loss of carbapenem resistance is rare in published literature [1]. The in-vitro loss of $bla_{\text{NDM-1}}$ by *K. pneumoniae* KPX was recently described from Taiwan; $bla_{\text{NDM-1}}$ plasmid was maintained in high copy numbers when exposed to carbapenems, but carbapenem resistance was lost with the removal of selection pressure [30]. This was due to either reduced copy numbers of pKPX-1 or the loss of the $bla_{\text{NDM-1}}$ via directed repeat mediated slippage.

To the best of our knowledge, this is the first report to document the possible in vivo loss of bla_{NDM} in *K. pneumoniae*. The deletion was due to the loss of a 5-kb $bla_{\text{NDM-7}}$–harboring fragment on pKpN06-NDM7 (Figure 2). Detailed analysis of this 5-kb region identified a 3–base pair putative directed repeat sequence (TAA), and it is likely that this deletion is due to directed repeat mediated slippage, a scenario similar to the excision of 5.3-kb bla_{KPC}-harboring element reported during Tn*4401* truncation [31].

The 114-kb IncFIB plasmid pKpN06-SIL from KpN06 had 27-kb deletion that included the *lac*, *sil*, and *cus* operons (Figure 2). The *lac* operon is responsible for the transport and metabolism of lactose in Enterobacteriaceae and allows for the effective digestion of lactose when glucose is not available [32]. The proteins encoded by the *sil* operon mediate silver resistance by restricting the accumulation of silver in the cell through a combination of silver sequestration in the periplasm and active efflux [33]. The *cus* determinant consists of 2 operons, *cusRS* and *cusCFBA*, and confers copper and silver resistance [34]. Further examination the 27-kb deletion region revealed that it is located directly downstream of IS*903*, and it

is likely that the 27-kb deletion was due to IS*903*-mediated adjacent deletion as described previously [35].

The use of reference mapping and inclusion of high-quality core genome SNVs to investigate the phylogenetic relationship between genomes is common practice [36–38]. However, the phylogenetic tree using only core SNVs did not accurately describe the short-term genetic adaptations that had occurred in our study. *K. pneumoniae* strains have large accessory genomes [37] in which nonvertical transmissions are a major source of short-term adaptive evolution in rapidly changing environmental conditions [39]. Thus the larger insertions/deletions (Supplementary Figure 1*B*) were appended to the core SNV alignment for phylogenetic analysis to accurately describe the genetic changes that transpired over a 3-month period (Figure 3). The variations, labeled on the tree branches, illustrated how the clinical epidemiology aligned with the genomic adaptations of bacteria. This phylogenetic analysis revealed fascinating microevolution aspects pertaining to mobile elements in *K. pneumoniae* over a short time frame (Figure 3). Our data suggest that the isolates from patient 2 were likely descendants from the rectal isolate from patient 1 (KpN02) and not from the urine isolates (KpN01 and KpN03). The 27-kb deletion in the pKpN01-SIL plasmid was the differentiating feature between patient 1–related isolates and patient 2–related isolates. On the basis of a 77–base pair chromosomal deletion, the CS blood isolate (KpN06) was more closely related to the environmental isolate (KpN07) collected from the room of patient 2 than clinical isolates obtained earlier (KpN04 and KpN05). It is unclear whether KpN06 lost the $bla_{\text{NDM-7}}$ gene in vivo or whether patient 2 became infected with a strain found on an inanimate surface of the hospital room. The inclusion of insertion/deletion events outside core genome analysis can be useful for unveiling mechanisms underlying nosocomial spread of pathogens.

In summary, this study detailed the remarkable plasticity and speed of evolutionary changes in MDR *K. pneumoniae*, demonstrating the highly recombinant nature of this species, which included 3 deletion events, 2 chromosomal insertion events, and 7 SNVs that transpired over a 3-month period. Such rapid genetic fluctuation likely allows for the selection of strains with the ability to swiftly adapt to new environments. This study highlights the ability of NGS to clarify molecular microevolutionary event within antibiotic-resistant organisms.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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