Molecular evolution of *Klebsiella pneumoniae* ST278 harboring bla_{NDM-7} involved in nosocomial transmission

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Running title: Micro evolution of NDM *K. pneumoniae*

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Abstract

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 ST278 (KpN06) was obtained one 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 ST278 *K. pneumoniae* from patient 2 were likely descendants 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 resistance *K. pneumoniae* demonstrating the highly recombinant nature of this species. It also highlights the ability of NGS to clarify molecular micro-evolutionary events within antibiotic-resistant organisms.

Keywords; *K. pneumoniae*, ST278, carbapenemases, *bla*$_{NDM-7}$, plasmid, micro-evolution

Background

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, that 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 the carbapenems in *K. pneumoniae*, but the production of carbapenemases remain the most clinically relevant [1]. The Ambler class B carbapenemases or metallo-ß-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, seventeen NDM-7-producing Enterobacteriaceae were isolated from six different patients in Calgary, Canada. The resistance gene, $bla_{\text{NDM-7}}$, was harbored by an identical ~46 Kb IncX3 plasmid among these isolates [3]. The index patient (patient 1) was admitted 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 in 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 the $bla_{\text{NDM-7}}$ was absent in the KpN06; however, the mechanism underlining the resistance gene loss was unclear. In this study, next generation sequencing (NGS) was used to characterize eleven isolates (KpN01-11) collected from the two patients and their respective rooms, in order 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 media and the CDC's (Atlanta, GA) protocol was used to screen for CR Gram negative bacteria [4]. Different surfaces in patient's rooms were cultured for *K. pneumoniae* from samples collected on sterile rayon swabs that were vortexed in 5 mL of tryptic soy broth and incubated 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 MALDI-TOF MS (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO). Minimum inhibitory concentrations of drugs (Supplemental Table 1) were determined

using the Microscan NEG 38 panel (Siemens, Burlington, Ontario, Canada) and interpreted by using 2015 CLSI guidelines for broth dilution [5].

β-lactamase identification. Carbapenemases was detected using the modified Hodge test (MHT) and the MASTDISCS™ ID inhibitor combination disks [6] (Mast Group Ltd., Merseyside, United Kingdom). PCR amplification and sequencing for β-lactamases genes were undertaken 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 MEM 1µg/ml and using *E. coli* J53 (azide 100 µg/ml) as recipient.

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

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

KpN01-11 were sequenced with the MiSeq™ (Illumina, San Diego, CA) platform. Libraries were prepared with the Nextera XT kit to produce paired end reads of 250 bp for a

predicted coverage of 75X minimum. The high coverage Illumina reads were mapped to the PacBio assemblies for Kp01 and Kp06 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 (HGAP), compiled specifically for quality trimming, *de novo* assembly and polishing [14]. MiSeq reads were trimmed with TrimGalore (v.0.3.3) (http://www.bioinformatics.babraham.ac.uk/ projects/trim galore) to remove sequencing adapters and reads with Phred quality scores below 25, then 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 typed with PlasmidFinder [19] and acquired antimicrobial resistance genes were identified using SRST2 [20]. The plasmids were compared using Mauve [21] and figures 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) using SMALT (v.0.7.5) (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 (SNV) were excluded if the mapping quality or the base quality score was below 20 or the minimum alternate fraction was below 0.75. Core was defined as positions present in $\geq$ 90% of the genomes and the resultant core SNVs were used to build a phylogeny using the Core SNV Pipeline (https://github.com/apetkau/core-phylogenomics). Larger insertion and/or deletion events (>1 nucleotide) were visually identified in IGV [24] from the read mapping and were appended to

the alignment as presence/absence character states. The chromosomal integration site of IS elements were examined by ISMapper using KpN01 as reference genome [25].

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 & control (IP&C) measures

The clinical timeline for patients 1 and 2 is summarized in Figure 1. Both patients were elderly and had co-morbidities. Neither patient had travelled outside of Alberta, Canada within 1 year of the hospital admission. Positive urine and rectal swabs for CR *K. pneumoniae* (KpN01, KpN02) were obtained during June 2013 from patient 1 (he was asymptomatic during this episode) and prompted IP&C protocols (including contact, isolation and active surveillance procedures). Inner circle rectal swabs on patients in unit B were collected, however no additional positives 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, two 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 patient 1. Patient 2 previously (January 2013) received ciprofloxacin for lower UTI, had early dementia and reportedly entered other patient's rooms. 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 of revealed five additional CR *K. pneumoniae*; KpN07, KpN08, KpN09 from the room of patient 2 and KpN10,

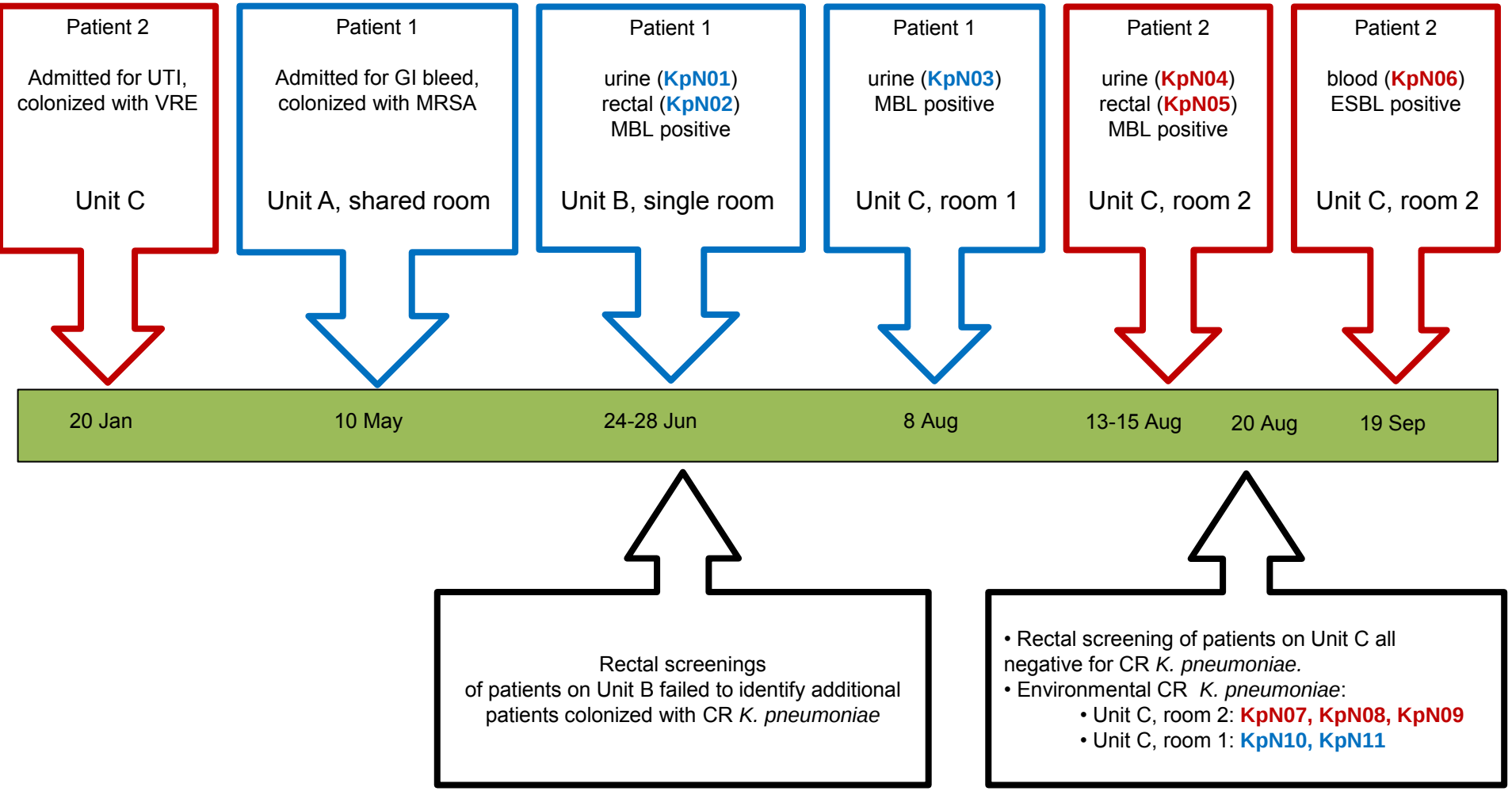

Figure 1. The timeline of events for two patients infected with MDR *K. pneumonia* within a single hospital. All events occurred within the same regional hospital in Calgary, Alberta, Canada between May and September of 2013. The clinical timeline highlights the unit transfers and positive cultures from patient 1 (blue boxes). Infection Prevention and Control (IP&C) surveillance measures on unit 3 after MDR *K. pneumoniae* was isolated our shown in the black boxes below the dateline. The timeline for patient 2 is presented in red boxes. **(**MBL) metallo-β-lactamase, (ESBL) extended-spectrum β-lactamase, (VRE) vancomycin resistant enterococcus, (CR) carbapenem resistant, (MRSA) methicillin resistant *Staphylococcus aureus,* (GI) gastro-intestinal, (UTI) urinary tract infection

KpN11 from the room of patient 1 (Figure 1). In September 2013, a CS *K. pneumoniae* (KpN06) was isolated from her blood and she was treated with intravenous colistin and meropenem but died due to sepsis.

Characterization of the CR *K. pneumoniae* showed them to be indistinguishable by traditional typing.

Susceptibilities, phenotypic and molecular tests to characterize bacterial isolates (KpN01-11) are summarized in Supplemental 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 (Supplemental Table 1). KpN01-11 belonged to ST278 and were indistinguishable by PFGE. Plasmid analysis showed the isolates obtained from patient 1 and his room contained 3 plasmids (190kb, 130kb, 50kb) while isolates obtained from patient 2 and her room also contained 3 plasmids (190kb, 100kb and 50kb). Transconjugants from KpN01-11 (except KpN06) contained the 50 kb plasmid, were positive for bla_{NDM} and typed with IncX replicon (Supplemental Table 1).

NGS revealed that the loss of carbapenem-resistance was due to a 5-kb deletion on a $bla_{\text{NDM-7}}$-harboring IncX3 plasmid.

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

Table 1. The genomic features of KpN01 and KpN06 using Pacific Biosciences RSII and Illumina MiSeq platforms

	KpN01	KpN06
Sequence type	278	278
Date of isolation	24 Jun 2013	19 Sept 2013
Source	urine	blood
β-lactamase genes	*bla*$_{\text{NDM-7}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-27}}$	*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{\text{SHV-27}}$
Additional antimicrobial resistance genes	*dfrA14, oqxA, oqxB, qnrB1, strA, strB, tetA, sul2*	*dfrA14, oqxA, oqxB, qnrB1, strA, strB, tetA, sul2*
Size (bp)	5,307,114	5,309,013
G+C content (%)	57.4	57.4
No. of gene	5,297	5,302
No. of CDS	5,153	5,157
rRNA (n)	25	25
16S	8	8
23S	8	8
5S	9	9
tRNA (n)	88	88
Plasmids (n)	4	4
Prophages (n)	7	7
ICEs (n)	3	3
IS elements (n)*	10	12
IS family (n)	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)

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 rRNA genes, 88 tRNA genes, 7 putative prophages, and 3 integrated conjugative elements (ICE). KpN01 had 10 insertion sequences (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, non-synonymous mutations. KpN06 had a 77 bp deletion within a formate hydrogen lyase activator (*fhlA*) transcription factor gene (locus no. AQD68_19305), and two transposase insertions: an IS*5* (1.2 kb) was inserted into a transcriptional regulator gene (AQD68_19830), and an IS*1* (777 bp) 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 four 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), 190,072 bp in length, with 212 putative open reading frame sequences and a 57.1% G+C content. They harbored the following antimicrobial resistance genes: $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnrB1*, *strAB*, *tetA*, *sul2* and *dfrA14*.

The IncFIB plasmids, pKpN01-SIL and pKPN06-SIL, were 134,064 and 107,110 bp 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).

Table 2. Chromosomal and plasmid variations between KpN01 and KpN06 using Pacific Biosciences RSII and Illumina MiSeq platforms

Reference	Start	End	Change	Polymorphism type	Length (with gaps)	Amino Acid Change	CDS codon #	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 alpha
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	2,437		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	two-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	**1,199**		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	5,080					AQD68_28350 - AQD68_28390	

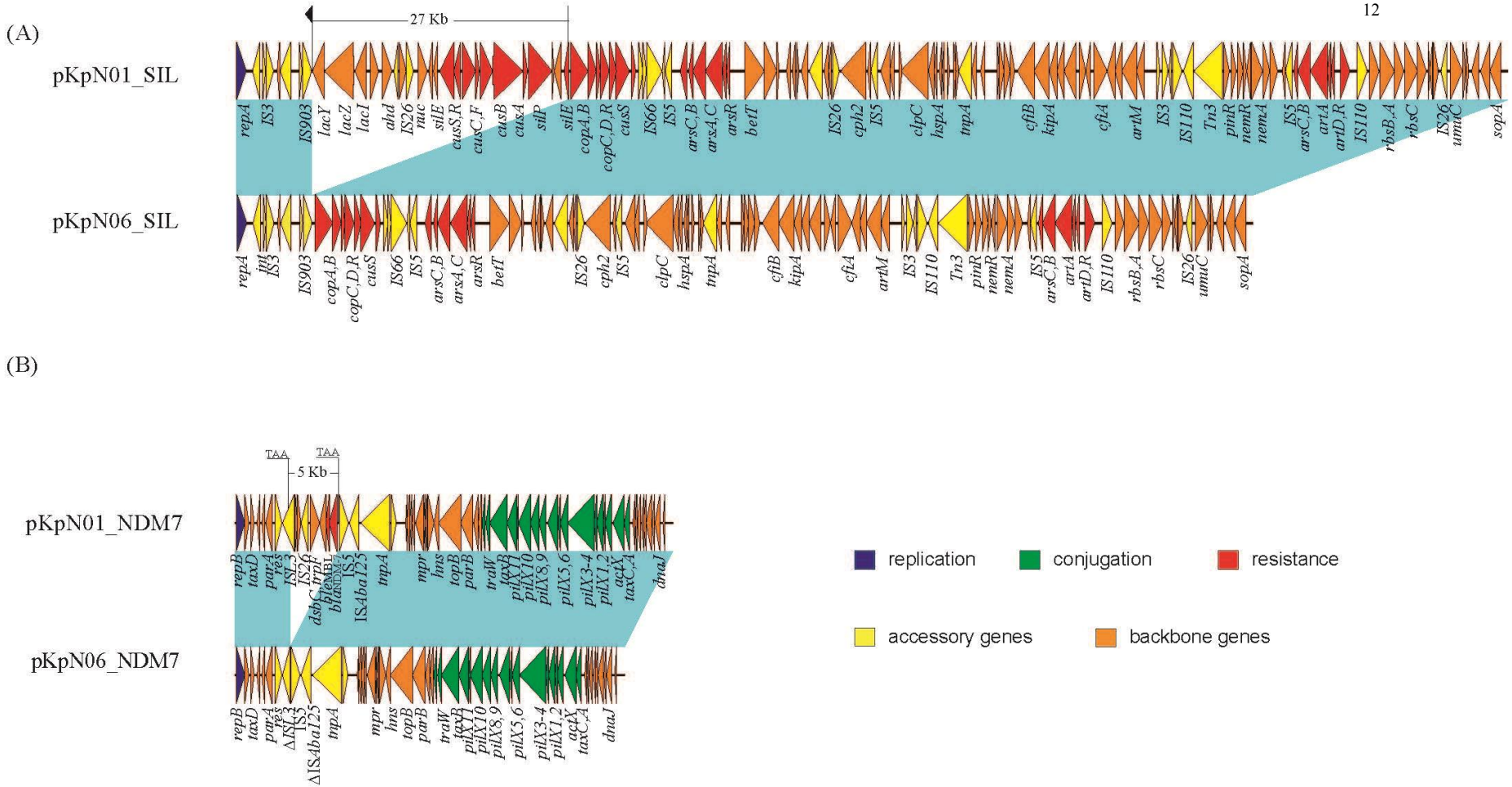

Figure 2. IncFIB and IncX plasmid structures from KpN01 and KpN06. Light blue shading denotes shared regions of homology, and open reading frames (ORFs) are portrayed by arrows and colored based on predicted gene function. The small black arrow above pKpN01_SIL denotes the downstream invert repeat of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

The IncX3 plasmid from KpN01, pKpN01-NDM7, was 46,161 bp in length and harbored *bla*$_{\text{NDM-7}}$ 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 ΔIS*L3-umuD*-IS*26-dsbC-trpF-ble*$_{\text{MBL}}$- *bla*$_{\text{NDM-7}}$-ΔIS*Aba125*, which was consistent with the loss of carbapenem resistance. Further inspection indicated that the deleted region was bracketed by two 3-bp direct repeats (TAA) (Figure 2).

The smallest plasmids from KpN01 (pKpN01-COL) and KpN06 (pKpN06-COL) were 3,223 bp in length, belonged to the ColE familyand were nearly identical (>99.9% identity) to other ColE plasmids (i.e. 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 above two completely closed ST278 genomes (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, 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 1B with the insertion/deletion events summarized in Supplementary Figure 1A. No short Indels (< 10 bp) were identified in the eleven genomes.

Table 3. Molecular characterization of *K.pneumoniae* ST278 isolates using Pacific Biosciences RSII and Illumina MiSeq platforms

Isolate	Platform	# contigs (>= 0 bp)	Total length (>= 0 bp)	N50	Plasmid (Inc)	Genome accessions
KpN01	Pacific Biosciences, Illumina	5	chromosome = 5,307,114			CP012987
			pKpN01-CTX15 = 189,853		FII	CP012988
			pKpN01-SIL = 134,064		FIB	CP012989
			pKpN01-NDM7 = 46,161		X3	CP012990
			pKpN01-COL = 3,223		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
			pKpN06-CTX15 = 190,071		FII	CP012993
			pKpN06-SIL = 107,110		FIB	CP012994
			pKpN06-NDM7 = 41,072		X3	CP012995
			pKpN06-COL = 3,121 *		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

* This contig was added from MiSeq assembly, matching the same plasmid in pKpN01-COL. May not have been in PacBio assembly due to lower coverage.

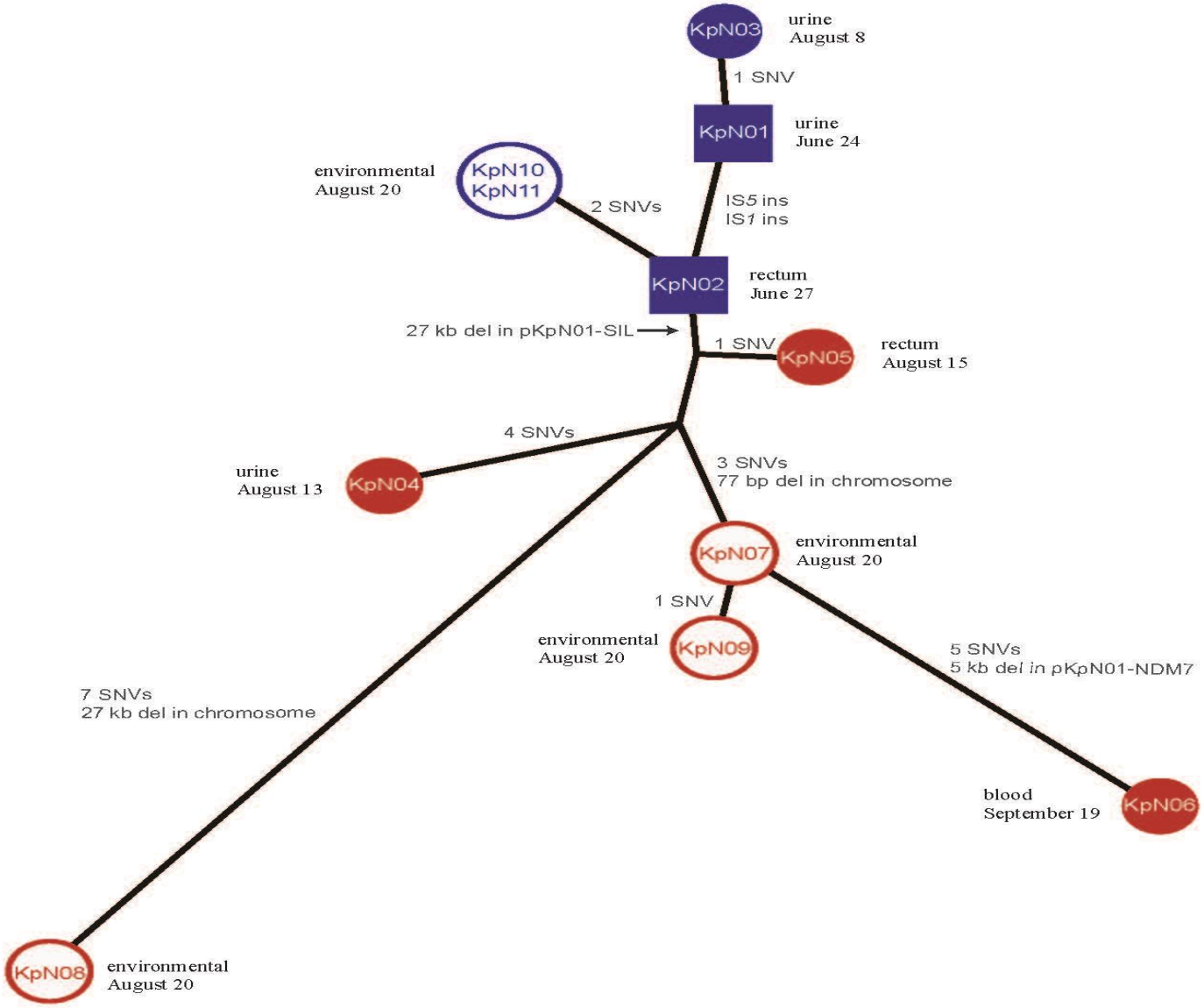

Figure 3. Maximum likelihood tree of all adaptive genetic changes from MDR *K. pneumoniae* isolates associated with patients 1 and 2 over a 3 month time period. Isolates from the biological specimens of each patient are solid colours while environmental isolates from each patients room are open (blue = 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 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.

All genomes were closely related with the core SNVs ranging from 0 – 14 (Supplementary Figure 1B). 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 three isolates from patient 1 (KpN01, KpN02, KpN03) differed by 1 SNV, while the two environmental isolates from patient's 1 room were identical. The two 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 two IS insertions (at AQD68_19830 and _25885), resulting in the gene disruption of two putative regulators. These gene deletions could be associated with selection pressure change from the urinary to gastro-intestinal tracts.

The three isolates from patient 2 [urine (KpN04), rectum (KpN05) and blood (KpN06)] differed by an average of 7 SNVs (Table 2). In addition to SNVs, KpN06 had a 77-bp 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 wasn't a direct descent from KpN04 (the first *K. pneumoniae* isolate from patient 2) but was closer to two environmental isolates (KpN07, 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, KpN06) or the environment within her room (KpN07, KpN08, KpN09).

KpN01 (collected June 24th) was the index isolate from patient 1; KpN02 (rectal from patient 1) was collected June 27th while KpN03 (urine from patient 1) was collected August 8th. This timeline would suggest that patient's 2 isolates would have evolved from KpN03. However, KpN02 acquired two IS insertions (at AQD68_19830 and _25885) that were absent in KpN01 and KpN03 but present in all of the isolates from patient 2 (i.e. KpN04, KpN05, KpN06), her

room (KpN07, KpN08, KpN09) and from patient's 1 room (KpN10, KpN11). This suggests that KpN04 -11 were likely descendants from patient's 1 rectal isolate (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 two patients admitted to adjacent rooms on the same unit despite IP&C protocols. 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 non-endemic areas including Canada [27]; however, on his initial admission to unit 1 during May 2013, patient 1 shared a semi-private room with 3 other patients. Therefore, we cannot rule out the possibility that he contracted the *K. pneumoniae* with *bla*$_{\text{NDM-7}}$ from a patient that had traveled to an endemic area.

The *K. pneumoniae* from this study belonged to ST278 that was first reported in a neonatal unit from a university hospital in Turkey [28] and more recently from Syrian patients admitted to 2 different hospitals in Northern Israel [29]. The Turkish ST278 harboured *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 were also present in rectal swabs and the most common ST associated with *bla*$_{\text{NDMs}}$ in that study [29]. However, complete genome sequencing of ST278 isolates were not conducted in these studies, and here we reported the first two complete sequenced ST278 genomes.

Genomic and phenotypic analyses demonstrated that ST278 KpN06 was carbapenem susceptible and negative for *bla*$_{\text{NDM-7}}$. The acquisition of carbapenem resistance in clinical *K. pneumoniae* had previously been reported, however, 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 number 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*$_{\text{NDM}}$ 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 3-bp 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*$_{\text{KPC}}$-harboring element reported during Tn*4401* truncation [31].

The 114kb, 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 two operons namely *cusRS* and *cusCFBA* 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 including 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 non-vertical transmissions are a major source of short term adaptive evolution in rapidly changing environmental conditions [39]. Thus the larger insertions/deletions (Supplemental Figure 1B) 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, labelled 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 timeframe (Figure 3). Our data suggests that patient's 2 isolates were likely descendants from patient's 1 rectal isolate (KpN02) and not the urine isolates (KpN01, KpN03). The 27-kb deletion in the pKpN01-SIL plasmid was the differentiating feature between patient 1-related and patient 2-related isolates. On the basis of a 77 bp 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 multidrug resistance *K. pneumoniae* demonstrating the highly recombinant nature of this species that included three deletion events, two chromosomal insertion events and seven SNVs that transpired over a three 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 micro-evolutionary event within antibiotic-resistant organisms.

Footnote

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Supplementary Data

Supplementary Table 1. Characteristics of *K. pneumoniae* from Calgary, Canada

	KpN01	KpN02	KpN03	KpN04	KpN05	KpN06	KpN07	KpN08	KpN09	KpN10	KpN11
Patient	1	1	1	2	2	2	2	2	2	1	1
Specimen	urine	rectal swab	urine	urine	rectal swab	blood	env	env	env	env	env
Date	24-Jun	27-Jun	08-Aug	13-Aug	15-Aug	19-Sep	20-Aug	20-Aug	20-Aug	20-Aug	20-Aug
Antibiotic Susceptibilities											
TZP	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4	>64/4
FOX	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
CRO	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32	>32
CAZ	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
FEP	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
ATM	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
MEM	>8	>8	>8	>8	>8	≤1	>8	>8	>8	>8	>8
ERT	>4	>4	>4	>4	>4	≤1	>4	>4	>4	>4	>4
AMK	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4	≤4
GEN	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
TOB	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
CIP	2	>2	2	>2	>2	>2	>2	2	2	>2	>2
SXT	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38	>2/38

TGC	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1	≤1
COL	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
MHT	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos
MASTDISCS™	MBL	MBL	MBL	MBL	MBL	ND	MBL	MBL	MBL	MBL	MBL
ESBL	ND	ND	ND	ND	ND	pos	ND	ND	ND	ND	ND
PCR positive β-lactamases	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 TEM-1 CTX-M-15 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27	NDM-7 CTX-M-15 TEM-1 SHV-27
PFGE pattern	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A	Pattern A
MLST	ST278	ST278	ST278	ST278	ST278	ST278	ST278	ST278	ST278	ST278	ST278
Plasmids (kb)	190, 130, 50	190, 130, 50	190, 130, 50	190, 100, 50	190, 100, 50	190, 100, 50	190, 100, 50	190, 100, 50	190, 100, 50	190, 130, 50	190, 130, 50
Transconjugant (kb)	KpN01T (50)	KpN02T (50)	KpN03T (50)	KpN04T (50)	KpN05T (50)	-	KpN07T (50)	KpN08T (50)	KpN09T (50)	KpN10T (50)	KpN11T (50)
Replicon typing	IncX	IncX	IncX	IncX	IncX	-	IncX	IncX	IncX	IncX	IncX

piperacillin-tazobactam (TZP), cefoxitin (FOX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEP), aztreonam (ATM), meropenem (MEM), ertapenem (ERT), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), ciprofloxacin (CIP), tigecycline (TGC) and trimethoprim-sulfamethoxazole (SXT), colistin (COL), modified Hodge test (MHT), Pulsed-field gel electrophoresis (PFGE), Multilocus sequencing typing (MLST), not done (ND); env, environmental

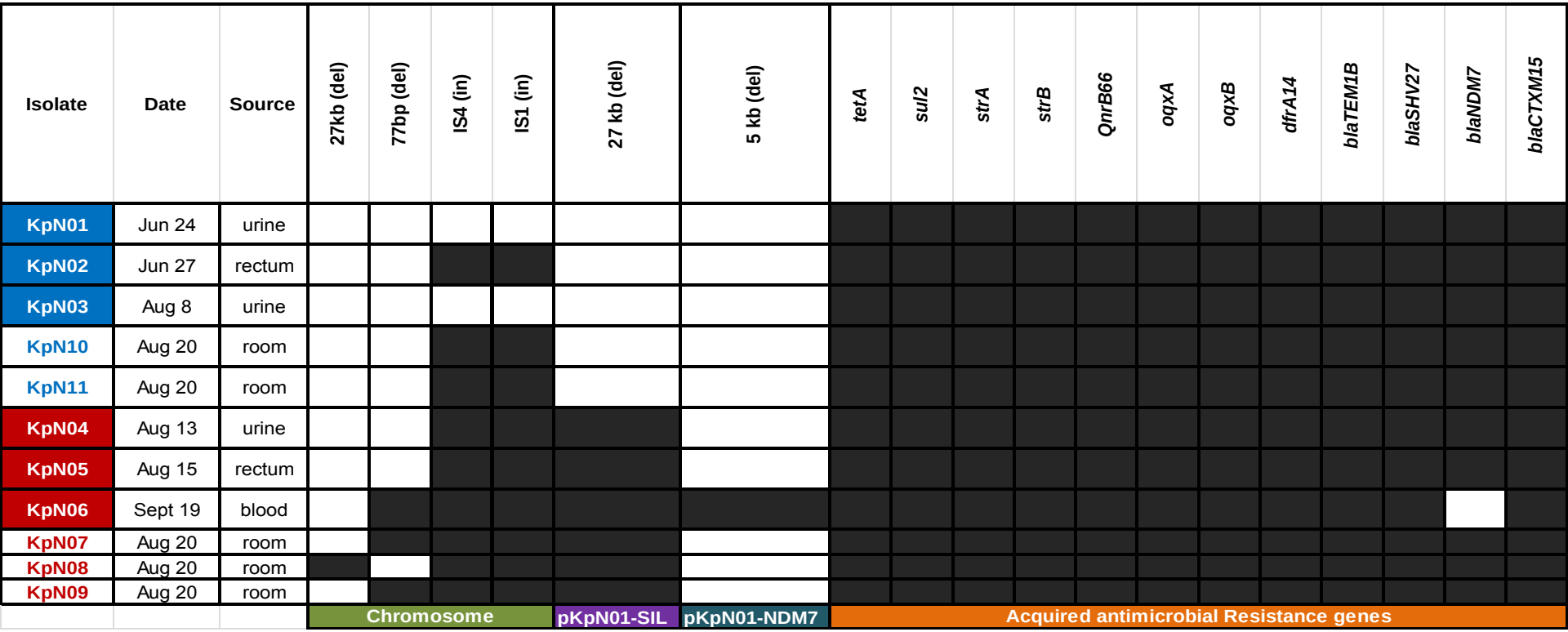
Supplementary Table 2. The core SNV positions of *K.pneumoniae* ST278 isolates

#Chromosome	Position	Kpn01	Kpn02	Kpn03	Kpn04	Kpn05	Kpn06	Kpn07	Kpn08	Kpn09	Kpn10	Kpn11
KpN01_chromosome	153886	A	A	A	A	A	A	A	T	A	A	A
KpN01_chromosome	209112	C	C	C	C	C	A	C	C	C	C	C
KpN01_chromosome	1329070	G	G	G	A	G	G	G	-	G	G	G
KpN01_chromosome	1337919	G	G	G	T	G	T	T	-	T	G	G
KpN01_chromosome	1402788	G	G	G	G	G	G	G	G	A	G	G
KpN01_chromosome	1921164	A	A	A	A	A	C	A	A	A	A	A
KpN01_chromosome	2809622	G	G	G	A	G	G	G	G	G	G	G
KpN01_chromosome	3401924	G	G	T	G	G	G	G	G	G	G	G
KpN01_chromosome	3872365	C	C	C	C	C	C	C	A	C	C	C
KpN01_chromosome	3872691	G	G	G	C	G	G	G	G	G	G	G
KpN01_chromosome	3887504	G	G	G	G	G	G	G	A	G	G	G
KpN01_chromosome	3974086	A	A	A	A	A	A	A	C	A	A	A
KpN01_chromosome	3974440	G	G	G	G	G	T	G	G	G	G	G
KpN01_chromosome	4391829	G	G	G	G	G	T	G	G	G	G	G
KpN01_chromosome	4394363	C	C	C	C	C	C	C	T	C	C	C
KpN01_chromosome	4526557	C	C	C	C	C	C	C	C	C	T	T
KpN01_chromosome	4529258	A	A	A	A	A	C	C	A	C	A	A
KpN01_chromosome	4623276	C	C	C	C	C	T	C	C	C	C	C
KpN01_chromosome	4906479	C	C	C	C	A	C	C	C	C	C	C
KpN01_chromosome	5054180	C	C	C	C	C	C	C	A	C	C	C
KpN01_chromosome	5055077	C	C	C	C	C	C	C	T	C	C	C
KpN01_chromosome	5147290	C	C	C	C	C	C	C	C	C	T	T

Supplementary Figure 1A

Isolate	KpN06	KpN08	KpN04	KpN09	KpN10	KpN11	KpN07	KpN03	KpN05	KpN01	KpN02
KpN06	0	14	9	6	9	9	5	8	8	7	7
KpN08	14	0	11	10	9	9	9	8	8	7	7
KpN04	9	11	0	5	6	6	4	5	5	4	4
KpN09	6	10	5	0	5	5	1	4	4	3	3
KpN10	9	9	6	5	0	0	4	3	3	2	2
KpN11	9	9	6	5	0	0	4	3	3	2	2
KpN07	5	9	4	1	4	4	0	3	3	2	2
KpN03	8	8	5	4	3	3	3	0	2	1	1
KpN05	8	8	5	4	3	3	3	2	0	1	1
KpN01	7	7	4	3	2	2	2	1	1	0	0
KpN02	7	7	4	3	2	2	2	1	1	0	0

Suppl Fig 1B



Supplementary Figure 1. (A) Core SNV distance matrix used to build the maximum likelihood tree along with the insertion/deletion events (B). The acquired antibiotic resistance genes were identified using SRST2.