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Investigation of a suspected nosocomial transmission of bla_{KPC3}-mediated carbapenem-resistant *Klebsiella pneumoniae* by whole genome sequencing

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ABSTRACT

Whole genome sequencing (WGS) was compared to pulse-field gel electrophoresis (PFGE) of XbaI-digested genomic DNA, as methods by which to evaluate a potential transmission of carbapenem-resistant *Klebsiella pneumoniae* between 2 hospital inpatients. PFGE result demonstrated only 1-band difference between the isolates, suggesting probable relatedness. In contrast, while WGS data demonstrated the same sequence type and very similar chromosomal sequences, over 20 single nucleotide variants were identified between the isolates, bringing into question whether there was a transmission event. WGS also identified an additional plasmid, with an XbaI restriction site in the isolates of the second patient that was not identified by PFGE. While WGS provided additional information that was not available by PFGE, in this study, neither method could definitively conclude the relatedness between the isolates.

1. Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) are an evolving threat to public health. At the present time in the United States, carbapenem resistance among the Enterobacteriaceae is predominantly mediated by the class A β-lactamase, KPC (Gupta et al., 2011; Munoz-Price et al., 2013; Temkin et al., 2014; Tzouvelekis et al., 2012). bla_{KPC} genes are typically encoded on plasmids that harbor resistance determinants for several other antimicrobial classes (Nordmann et al., 2011; Schultsz and Geerlings, 2012). As a result, such CRE are resistant to many, if not all, currently available antimicrobials and are extremely difficult to treat (Temkin et al., 2014; Tzouvelekis et al., 2012). The bla_{KPC}-harboring plasmids are efficiently mobilized both between isolates of the same species and across genera within the family Enterobacteriaceae, providing an opportunity for rapid dissemination of carbapenem resistance in healthcare settings (Cuzon et al., 2010; Mathers et al., 2011; Nordmann et al., 2009; Sidjabat et al., 2009). Evaluation of hospital-based outbreaks of KPC-producing *Klebsiella pneumoniae* can be challenging, as the majority of KPC-producing isolates in the United States are of a common lineage, sequence type (ST) 258 (Kitchel et al., 2009). Furthermore, KPC-producing *K. pneumoniae* can colonize the gastrointestinal tract of exposed patients for prolonged periods of time, silently introducing these isolates into healthcare facilities, and potentially only causing clinical infections weeks to months after hospital admission. Pulse-field gel electrophoresis (PFGE) remains the gold standard method used to evaluate isolates of CRE in cases of suspected intrafacility transmission (Goering, 2010). However, in regions where KPC is endemic, PFGE data may not yield the level of discrimination required to differentiate intrafacility transmission from introduction of a closely related strain from another healthcare facility. In contrast, sequencing of

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whole bacterial genomes has been shown to be a powerful tool by which to track transmission of KPC-producing isolates within healthcare facilities (Snitkin et al., 2012). Whole genome sequence (WGS) data allow not only high-level resolution of genomic differences between bacteria but investigation of antimicrobial resistance genes and their associated mobile genetic elements such as transposons and plasmids (Mathers et al., 2015c).

At our institution, the proportion of Enterobacteriaceae resistant to carbapenems is <0.5%, and KPC accounts for the carbapenem-resistant phenotype in 90% of these isolates (Pollett et al., 2014). Nearly, all patients with CRE to date have had recent history of treatment in long-term acute care hospitals and/or nursing homes, and for the vast majority of cases, isolates were recovered in the first 48 hours of hospitalization. As such, when carbapenem-resistant *K. pneumoniae* (CRKP) were isolated from 2 patients, both after prolonged hospitalization in our surgical intensive care unit (ICU), we launched an investigation to determine if these cases might represent hospital-based transmission. We compared WGS to PFGE as tools to aid in this investigation, at our facility with a low baseline incidence of CRE.

2. Materials and methods

2.1. Clinical isolates and antimicrobial susceptibility testing

Three CRKP isolates recovered from bile fluid from patient A (CRKP-A) obtained hospital day 100 and from respiratory secretions (CRKP-B1) and blood (CRKP-B2) from patient B collected hospital day 134 in 2014 were evaluated. Two additional CRKP (CRKP-X and CRKP-Y), isolated from 2 different patients hospitalized in our facility around the same time, were evaluated as controls. Antimicrobial susceptibility was performed by the Clinical and Laboratory Standards Institute reference broth microdilution method, on panels prepared in-house, as described elsewhere (Pollett et al., 2014).

2.2. PFGE analysis

PFGE analysis of the CRKP isolates was performed using an XbaI digestion of DNA from the 2 CRE isolates (CRKP-A and CRKP-B1), as previously described for *Escherichia coli* (http://www.cdc.gov/pulsenet/protocols.htm). PFGE data analysis was performed using BioNumerics V. 6.6 11 (Applied Maths, Inc., Austin, TX).

2.3. WGS

DNA was extracted from the isolates using a tissue DNA extraction kit on a BioRobot EZ1 (Qiagen, Valencia, CA, USA). Genomic shotgun libraries were generated using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's instruction. WGS was performed using an Illumina MiSeq with a 2X250bp v2 sequencing protocol.

2.4. WGS data analysis

Between 1.1 and 1.8 million reads per sample were acquired, and de novo assembly was performed using SPAdes 3.1.1 (http://bioinf.spbau.ru/spades). For each isolate, the number of contigs ranged from 111 to 132; the largest contigs were 368 kb to 430 kb; and the total genome lengths ranged from 5.55 Mb to 5.71 Mb. Raw paired reads from Illumina MiSeq for each isolate were processed using Galaxy tools (Goecks et al., 2010) and submitted for single nucleotide variant (SNV) analysis and generation of SNV phylogenetic tree (maximum likelihood method) using Center of Genomic Epidemiology (CGE) CSI Phylogeny (Kaas et al., 2014) (https://cge.cbs.dtu.dk/services/CSIPhylogeny). The SNV calling was based on similar quality filter criteria described elsewhere (Salipante et al., 2015a): 1) a minimum of 15 reads, 2) relative depth at SNV positions of 50%, 3) minimum distance between SNVs of 10, 4) minimum SNV quality of 30, and 5) minimum read mapping quality of 25 and minimum Z-score of 1.96. Multilocus sequencing type (MLST) was identified through use of CGE MLST 1.7 (https://cge.cbs.dtu.dk/services/MLST). Antimicrobial resistance genes were identified by both RAST (http://rast.nmpdr.org) and CGE ResFinder (https://cge.cbs.dtu.dk/services/ResFinder). The contigs containing antimicrobial resistance genes were analyzed by BLAST to identify closely matched plasmids; the entire contigs were then mapped to these plasmids using CONTIGuator (Galardini et al., 2011) (http://contiguator.sourceforge.net). The closest plasmid was then used as reference sequence to perform mapping from the raw paired-end reads by using Geneious (Biomatters, Auckland, New Zealand), generating a hypothetical plasmid map based on the consensus sequence. The WGS data have been deposited in GenBank with accession nos. SAMN03997506 (CRKP-A), SAMN03997511 (CRKP-B1), and SAMN03997513 (CRKP-B2).

2.5. PFGE band DNA gel purification and Sanger sequencing analysis

The regions of the gel corresponding to the 121.6–198.2 kb size bands were cut out and DNA was recovered and purified using Zymoclean Large Fragment DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Several sets of PCR primers were designed (Table 1) with 1 set amplifying a 648-bp region immediately upstream of XbaI restriction site on the pRMH760-like plasmid, 1 set amplifying a 757-bp region immediately downstream of the XbaI site, and 1 set amplifying a 735-bp region across the XbaI site. Two more primer sets were designed to amplify the region containing *aadB* gene and *aph(3′)-lb* gene present on this plasmid, respectively. PCR was performed using AmpliTaq Gold Fast PCR Master Mix (Thermo Fisher, Carlsbad, CA, USA) and visualized by conventional agarose gel electrophoresis. The same PCR primers were diluted 10-fold and used as sequencing primers. Sanger sequencing analysis was performed using BigDye Terminator v3.1 cycle Sequencing Kit and ABI Prism 3130xl Genetic Analyzer (Thermo Fisher).

3. Results

3.1. Case summaries and epidemiologic investigation

Patient A had end-stage liver disease due to alcoholic cirrhosis that was complicated by ascites and renal failure. The patient was transferred to our surgical ICU following a short stay at an outside hospital for management of sepsis secondary to peritonitis and evaluation for liver transplantation. On hospital day 43, a CRKP was isolated from patient A's respiratory secretions and 57 days later from the patient's bile fluid (CRKP-A, obtained hospital day 100). Patient A was placed on contact precautions per hospital policy. Patient B had hepatitis C and alcoholic liver cirrhosis and was transferred from a long-term acute care facility after a prolonged hospitalization for complicated cholecystitis, sepsis, respiratory failure, and acute renal failure. Patient B was admitted to the same surgical ICU as patient A, 42 days after patient A's admission and the day prior to the first isolation of CRKP from patient A. After 93 days hospitalization, the first 40 of which were in a room adjacent to patient A, CRKP was isolated from patient B's abdominal drainage. Forty-one days later, CRKP-B1 (collected hospital day 134) was isolated from respiratory secretions; and CRKP-B2 (collected hospital day 134), from blood. Review of medical records and unit logs revealed several shared care personnel between the patients, including physicians, nurses, care partners, allied health workers (e.g., dialysis), and housekeeping. Additionally, both patients underwent endoscopic procedures, liver transplantation, intubation, and central line placement prior to isolation of CRKP. Due to the multitude of commonalities, we determined that there was a high likelihood of unit-based transmission of CRKP from patient A to patient B. No surveillance cultures were performed on these patients, either at admission or during their hospitalization, to evaluate for CRKP colonization.

Table 1
Primers for PCR and Sanger sequencing.

Target	Amplicon size	Forward primer	Reverse primer
Xba1Up	648 bp	5′-TGTCCATGATTCAGCAGAGAGCT-3′	5′-TTG GCT GAT GCT ATC GAC CCT-3′
Xba1Down	757 bp	5′-CACACTGAGTGTCATGGTTGGT-3′	5′-CGC ACC TTA TGG CAA TAC CGA-3′
Xba1Cross	735 bp	5′-TGTCACGACAGCACCATCACT-3′	5′-CCT CAG CTA AGT CAG CAG TTC GAT-3′
aadB	701 bp	5′-GGTAAGCTGTAATGCAAGTAGCGT-3′	5′-AGC CTG TAG GAC TCT ATG TGC T-3′
aph(3′)Ia	689 bp	5′-GGTAGCGTTGCCAATGATGTTACA-3′	5′-GTC AGC GTA ATG CTC TGC CA-3′

3.2. Evaluation of isolates relatedness by PFGE and WGS

PFGE analysis demonstrated a difference of 1 band (indicated by arrow) between CRKP-A (collected hospital day 100) and CRKP-B1 (collected hospital day 134), indicating that the isolates were closely related (Fig. 1A). SNV phylogenetic tree generated from the WGS data, and 30 other *K. pneumoniae* genomes available on the NCBI database showed both patients clustered closely to a reference genome KPNIH33 (Conlan et al., 2014) (Fig. 1B). MLST from WGS data identified ST258 for all 3 CRKP. bla_{KPC3}, flanked by a 10.0-kb Tn4401 isoform d transposon, was the sole carbapenemase gene in all 3 isolates (Table 1). The contigs that harbored the Tn4401 isoform d transposons in these 3 isolates were of the same size (25.3 kb) and were 100% identical. However, due to the limitation of short sequencing reads, we were unable to generate a complete plasmid sequence for this 25.3-kb sequence element. Using KPNIH33 as the reference genome, 24 and 22 high-confidence SNVs (defined as >20 reads at the variant site with >95% relative depth) were identified between CRKP-A and CRKP-B1 or CRKP-B2, respectively (Table 2). Only 2 SNVs were found between CRKP-B1 and CRKP-B2, suggesting low level of intrinsic variability in the WGS assay and that these isolates (with ≤3 SNVs) were indistinguishable at the genomic level (Tables 2 and 3). Most of these SNVs were synonymous, with the exception of 5 which affected various genes (Table 3). In contrast, 145 pairwise variants were observed between CRKP-A and KPNIH33; 133, between CRKP-B1 and KPNIH33; and 131, between CRKP-B2 and KPNIH33 (Data not shown). Similarly, when we evaluated 2 CRKP isolated from 2 other patients hospitalized at our facility, we found a very different ST919 strain (CRKP-Y) that harbored bla_{KPC3} on a Tn4401b transposon and a ST258 strain (CRKP-X) that harbored bla_{KPC3} on a Tn4401d transposon (Fig. 1B). Although CRKP-X also clustered closely to KPNIH33, there were 55–67 SNVs between this isolate and CRKP-A, CRKP-B1, and CRKP-B2 (data not shown), suggesting a significant difference. Interestingly, none of the SNVs identified between CRKP-A and CRKP-B1 were predicted to affect an XbaI restriction site and, therefore, could not account for the altered PFGE band pattern noted in Fig. 1A. It is possible this XbaI site was present in a region of suboptimal sequence coverage and excluded from our SNV analysis by our quality filters.

3.3. Identification of an additional plasmid in the isolates from patient B

Several contigs identified in CRKP-B1 and CRKP-B2 were absent in CRKP-A—these mapped to the plasmid pRMH760 (Harmer and Hall, 2014). Raw read mapping of these contigs, using pRMH760 as reference,

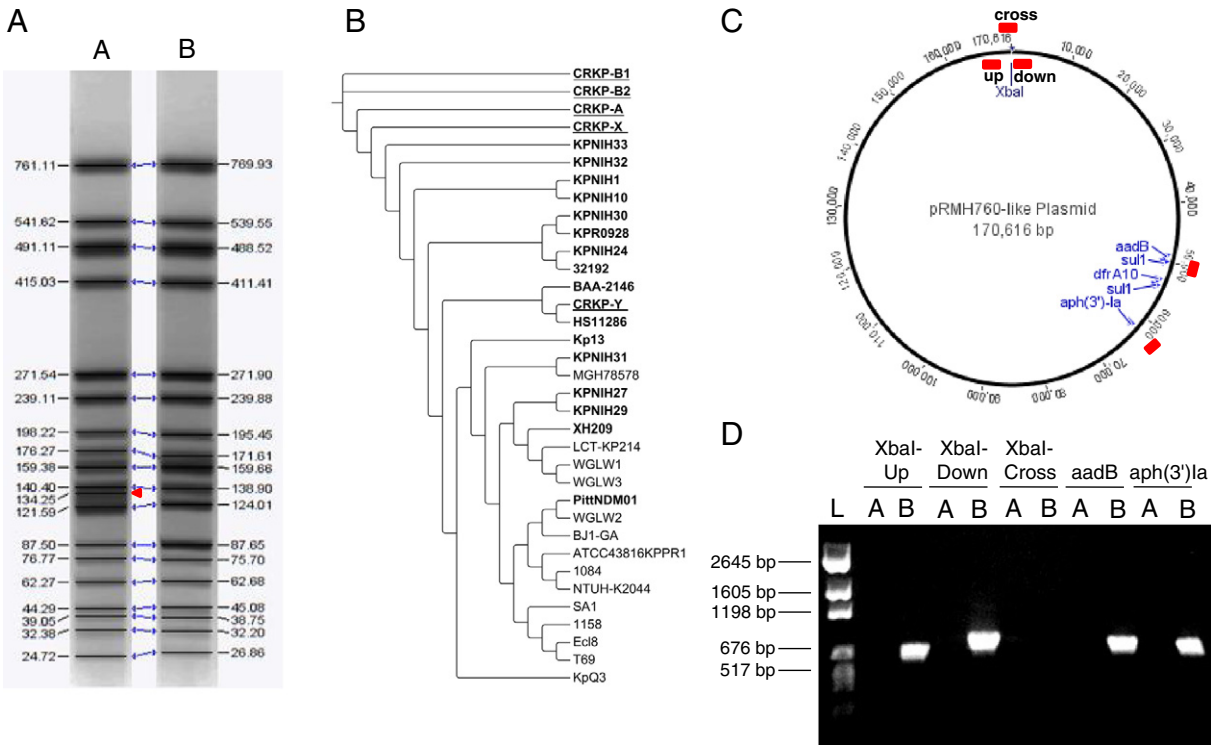

Fig. 1. A, PFGE gel of CRKP from patient A (isolate A) and patient B (isolate B1). Blue lines indicate matching bands, and red arrow indicates the 1-band difference. PFGE agarose gel between 121.59 kb and 198.22 kb in lanes A and B were cut out, and DNA was recovered for PCR and Sanger sequencing. B, Maximum likelihood phylogenetic tree based on SNV for CRKP A, B1, and B2 and CRKP X and Y (underlined), compared to 30 *K. pneumoniae* reference genomes (carbapenem-resistant isolates are in boldface). C, Five sets of primers were designed to amplify regions (red bars) on the pRMH760-like plasmid. D, Gel electrophoresis demonstrating robust amplification of 4 regions of the pRMH760-like plasmid in the DNA recovered from the B lane of the PFGE gel, but absent in the DNA recovered from A lane of the PFGE gel. Primers flanking the XbaI site did not amplify in either lane, indicating a complete XbaI enzyme digestion.

Table 2
Antimicrobial susceptibility and molecular characterization of the carbapenem-resistant *K. pneumoniae* isolates.

Isolate	Patient	Specimen Type	MIC (μg/mL)						Sequence type	No. of SNVs	Resistance genes		
			Imipenem	Meropenem	Ertapenem	Amikacin	Gentamicin	Tobramycin	Trim/Sulfa		β-Lactams	Aminoglycoside	Folate pathway inhibitors
CRKP-A	A	Bile Fluid	R (>8)	R (>16)	R (>2)	S (16)	I (6)	R (10)	R (>4/80)	ST-258	24 (A vs B1), 22 (A vs B2)	*aac(6′)Ib*, *strA*, *strB*	*sul2*, *dfrA14*
											[a]*blaKPC3*, *blaOXA9*, *blaSHV11*, *blaTEM1A*		
CRKP-B1	B	Respiratory	R (>8)	R (>16)	R (>2)	**I (32)**	**R (>10)**	R (10)	R (>4/80)	ST-258	24 (B1 vs A), 2 (B1 vs B2)	*aac(6′)Ib*, *strA*, *strB*, **aadB**, **aph(3″)Ia**	*sul2*, *dfrA14*, **sul1**, **dfrA10**
											[a]*blaKPC3*, *blaOXA9*, *blaSHV11*, *blaTEM1A*		
CRKP-B2	B	Blood	R (>8)	R (>16)	R (>2)	**I (32)**	**R (>10)**	R (10)	R (>4/80)	ST-258	22 (B2 vs A), 2 (B2 vs B1)	*aac(6′)Ib*, *strA*, *strB*, **aadB**, **aph(3″)Ia**	*sul2*, *dfrA14*, **sul1**, **dfrA10**
											[a]*blaKPC3*, *blaOXA9*, *blaSHV11*, *blaTEM1A*		

R = resistant; I = intermediate; S = susceptible.
Boldface results highlighted the difference between *K. pneumoniae* from patients A and B.
[a] Carbapenemase gene flanked by Tn4401 isoform d.

was used to generate a 170.6-kb consensus sequence with 98.5% homology to pRMH760. This pRMH760-like putative plasmid harbored the aminoglycoside resistance genes *aadB* and *aph(3′)Ia*, the trimethoprim resistance gene *dfrA10*, and 2 copies of sulfonamide resistance gene *sul1* (Table 1). Interestingly, both isolates from patient B demonstrated slightly higher MICs to amikacin and gentamicin, which were determined by reference broth microdilution (CLSI, 2014), when compared to CRKP-A (Table 1). In addition, the pRMH760-like plasmid harbored an XbaI restriction site; the predicted XbaI-digested linear DNA of this plasmid was predicted to comigrate with the 171.6-kb genomic DNA fragment observed on the PFGE gel. Sequences corresponding the upstream region of the pRMH760-like plasmid XbaI site, downstream of the XbaI site, *aadB* and *aph(3′)Ia* (Fig. 1C), were successfully amplified in the PFGE gel–recovered DNA from CRKP-B1, but not CRKP-A (Fig. 1D). Sanger sequencing of the amplicons yielded sequences that matched the WGS data. As a control, primers designed to span across the XbaI digestion site did not yield amplification, indicating complete XbaI enzyme digestion of the DNA. Notably, this difference was not observed by the BioNumerics software on the PFGE gel (Fig. 1A).

4. Discussion

In recent years, next-generation sequencing (NGS) technologies have evolved to the point where sequencing of whole bacterial genomes is possible in clinical settings, including the availability of benchtop sequencers such as the 454 GS Junior, Ion Torrent PGM, and Illumina MiSeq (Dunne et al., 2012; Fournier et al., 2014; Sherry et al., 2013). We sought to determine if the use of WGS data for our investigation could provide a more refined analysis on strain relatedness than was available by PFGE, as has been shown in other studies (Mathers et al., 2015a; Salipante et al., 2015b). We found 22–24 high-quality SNVs between the CRKP isolated from patients A and B. Recently, Salipante et al. (2015a) proposed a cutoff of ≤3 genomic variants between isolates to define them as indistinguishable, ≤12 genomic variants for isolates to be considered closely related, and ≥13 variants to be considered unrelated, based on a study using WGS for bacterial strain typing of a collection of vancomycin-resistant *Enterococcus faecium*, methicillin-resistant *Staphylococcus aureus*, and *Acinetobacter baumannii*. For CRKP isolates specifically, up to 17 SNV differences were observed in an outbreak at the National Institutes for Health hospital (Snitkin et al., 2012), and up to 10 (Weterings et al., 2015) or 15 (Mathers et al., 2015b) SNV differences were observed in 2 other ST258 *K. pneumoniae*–related outbreaks. In our study, the number of SNVs (22–24) between the CRKP isolated from patients A and B seemed to be slightly greater than what other outbreak investigations have observed, but the lack of a consensus definition for relatedness makes it very challenging to reach a definitive conclusion regarding transmission, based on the present data. It should be mentioned that our analysis was based on short-read MiSeq data, which do not allow construction of complete genome or plasmid maps, potentially yielding an incomplete SNV profiling. Furthermore, we employed a high-stringency filter for our SNV calls, as has been done elsewhere (Salipante et al., 2015a), to help mitigate overcalling genomic diversity due to SNVs introduced to the data, as sequencing errors. Such evaluation of only regions with the highest quality sequence coverage likely also results in undercalling of the total number of sequence variations between isolates. In contrast, long-read sequencing platforms, such as the PacBio RS, are associated with a higher inherent error rate, which may overcall SNVs and require correction by another NGS platform such as the Illumina system.

It should be noted that the SNV cutoffs proposed by Salipante et al. (2015a) may be overly restrictive. Bacterial isolates are known to diversify within a single infected/colonized host, and each individual patient who acquires a CRKP will develop, over time, a genomically diverse population of the organism (Worby et al., 2014). CRKP-A and CRKP-B1 were isolated 76 days apart—which may have provided ample time for the accumulation of SNVs across the CRKP genome, within the

Table 3
Single nucleotide variants among *K. pneumoniae* isolates.

	Reference (KPNIH33) location	CRKP-A	CRKP-B1	CRKP-B2	Variant type	Affected gene
1	1104614	A	G	G	Synonymous	
2	1120295	C	T	T	Nonsynonymous	AIW70704.1 = LysR family transcriptional regulator
3	1434384	A	G	G	Synonymous	
4	1583898	T	C	C	Synonymous	
5	1712388	G	T	T	Synonymous	
6	1713375	C	A	C	Synonymous	
7	1982119	A	T	T	Synonymous	
8	2142962	C	T	C	Synonymous	
9	2238744	G	A	A	Synonymous	
10	2492974	A	C	C	Nonsynonymous	AIW70970.1 = ATP-dependent helicase HrpA
11	2578845	T	G	G	Noncoding	
12	2639561	A	G	G	Nonsynonymous	AIW71106.1 = urea ABC transporter ATP-binding protein
13	2840365	A	T	T	Synonymous	
14	3095316	A	G	G	Synonymous	
15	3415321	T	C	C	Synonymous	
16	3614084	T	C	C	Synonymous	
17	4239941	C	T	T	Synonymous	
18	4239950	T	A	A	Nonsynonymous	AIW72527.1 = hypothetical protein
19	4568820	A	G	G	Synonymous	
20	4592807	C	A	A	Nonsynonymous	AIW72874.1 = oligogalacturonate lyase
21	4603942	A	G	G	Synonymous	
22	4604101	G	A	A	Synonymous	
23	4828372	T	C	C	Synonymous	
24	5283801	A	T	T	Noncoding	

host(s). To this point, Golubchik et al. (2013) found as many as 40 SNVs across the genomes of different colonies of *S. aureus* isolated from a single nares swab collected from a colonized individual. Both the present study and other outbreak investigations have sequenced a limited, representative number of isolates from each patient, which may grossly underestimate the diversity of the bacterial population present in a given patient and result in correspondingly incorrect inferences of transmission events (Worby et al., 2014). In our particular evaluation, we do not know how long patients A (or B) may have been colonized with the CRKP prior to development of infection, but it is clear that long-term colonization with CRE—up to years—is common, and both patients had extensive exposure to the healthcare system in which CRE are prevalent (Marquez et al., 2013). Little data are available that document the rate at which bacteria and, in particular, members of the Enterobacteriaceae undergo genomic diversification within a single patient. The estimated rate at which *E. coli* accumulates point mutations is 1.6×10^{-10} per base pair per generation (Barrick et al., 2009), and the rate of change for *K. pneumoniae* is thought to be in the range of 5–10 SNVs/genome/year (Bowers et al., 2015; Mathers et al., 2015c). However, this is in the absence of selective pressures such as host response, antimicrobials, and other medications (Zdziarski et al., 2010). SNVs may accumulate significantly more rapidly under the selective pressure of an infected or colonized host. For example, a study of *E. coli* used for therapeutic bladder colonization found the rate at which SNVs accumulated was 1.3–2.5 times that of the same *E. coli* when propagated *in vitro*. The rate of mutation in this study was dependent on the individual host, indicating diversification rates are host specific (Zdziarski et al., 2010). Thus, while the presence of 24 SNVs between CRKP-A and CRKP-B1 indicates a certain degree of divergence between these isolates, a transmission between the 2 patients is still a possibility, considering both patients had extensive exposure to antimicrobial therapy, which could theoretically increase the rate of mutation of the CRKP *in vivo*. Furthermore, the 25.3-kb regions flanking Tn4401d were 100% identical between the isolates in the present study, providing another supporting evidence for relatedness. Of note, while the Tn4401d isoform is relatively uncommon (Mathers et al., 2015c), we identified it in isolate CRKP-Y, which was isolated from a third patient 9 months after discharge of these 2 patients from our hospital. We cannot rule out the possibility that both patients acquired CRKP from the hospital environment, or other colonized patients, via 2 independent acquisition events. As such, while WGS data may aid in the investigation of transmission events, absent very low (i.e. ≤3 SNVs, which would indicate recent, direct transmission) or high (i.e. for example >100 SNVs, which would indicate significant genomic distance and no recent transmission event) diversity, conclusive definitions of isolates as part of an outbreak remain unresolved. Nonetheless, standardization of WGS data analysis and development of consensus guidelines by which to define relatedness of CRE and other important hospital-associated pathogens will significantly improve our ability to evaluate such events.

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