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MAJOR ARTICLE

Evolution and Transmission of Carbapenem-Resistant *Klebsiella pneumoniae* Expressing the $bla_{OXA-232}$ Gene During an Institutional Outbreak Associated With Endoscopic Retrograde Cholangiopancreatography

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Background. Whole-genome sequencing (WGS) is an emerging and powerful technique by which to perform epidemiological studies in outbreak situations.

Methods. WGS was used to identify and evaluate an outbreak of OXA-232–expressing carbapenem-resistant *Klebsiella pneumoniae* (CRKP) transmitted to 16 patients over the course of 40 weeks via endoscopic retrograde cholangiopancreatography procedures at a single institution. WGS was performed on 32 OXA-232 CRKP isolates (1–7 per patient) and single-nucleotide variants (SNVs) were analyzed, with reference to the index patient's isolate.

Results. Interhost genetic diversity of isolates was between 0 and 15 SNVs during the outbreak; molecular clock calculations estimated 12.31 substitutions per genome per year (95% credibility interval, 7.81–17.05). Both intra- and interpatient diversification at the plasmid and transposon level was observed, significantly impacting the antibiogram of outbreak isolates. The majority of isolates evaluated (n = 27) harbored a $bla_{CTX\text{-}M\text{-}15}$ gene, but some (n = 5) lacked the transposon carrying this gene, which resulted in susceptibility to aztreonam and third- and fourth-generation cephalosporins. Similarly, an isolate from a colonized patient lacked the transposon carrying *rmtF* and *aac(6')lb* genes, resulting in susceptibility to aminoglycosides.

Conclusions. This study broadens the understanding of how bacteria diversify at the genomic level over the course of a defined outbreak and provides reference for future outbreak investigations.

Keywords. CRE outbreak; whole-genome sequencing; ERCP; OXA-232; carbapenem-resistant *Klebsiella pneumoniae*.

Carbapenemase-producing Enterobacteriaceae (CPE) are a significant threat to patient safety [1, 2]. CPE are commonly resistant to all or nearly all antimicrobials and infection is associated with a high attributable mortality [3, 4]. Introduction of CPE to a healthcare network via infected or colonized patients can result in rapid dissemination of the organism across patients and between institutions [5, 6]. At our institution, like other areas of the United States [7], the *Klebsiella pneumoniae* carbapenemase (KPC) accounted for the carbapenem-resistance phenotype in 90% of CPE [8]. In late 2014, this distribution of carbapenemases shifted dramatically when a carbapenem-resistant *K. pneumoniae* (CRKP) harboring the OXA-232 carbapenemase was introduced by a patient with prior medical treatment in India. Over the next 3 months, every new case of CPE infection (n = 7) was due to OXA-232 CRKP, despite this carbapenemase having only been described once before in the United States at the time [9]. Epidemiological investigations ultimately identified the outbreak to be associated with endoscopic retrograde cholangiopancreatography (ERCP), affecting 17 patients, from a single source patient. In the present study, we demonstrate the utility of whole-genome sequencing (WGS) to aid with the investigation of this outbreak.

MATERIALS AND METHODS

Clinical Isolates

Thirty-two isolates from 17 patients were evaluated. These included the index patient (Pt0), 9 infected patients, and 7 colonized patients. One colonized patient (CPt3) later became infected and is referred to as CPt3_Pt9. Isolates were defined as carbapenem resistant if the meropenem minimum inhibitory concentration (MIC) was >1 μg/mL or the imipenem MIC

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was >1 μg/mL (with the exception of *Proteus*, *Providencia*, and *Morganella*, where an imipenem MIC >4 μg/mL was required).

Isolate Testing and WGS

Isolates were tested using the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution for antimicrobial susceptibilities [16]. MICs were interpreted using CLSI criteria [16], except for colistin, which was interpreted using European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria [23]. Modified carbapenem inactivation method was performed to confirm carbapenemase activity, as detailed in the Supplementary Materials. WGS was performed using both single molecule real-time sequencing conducted with a PacBio RSII instrument (Pacific Biosciences, Menlo Park, California) for isolates Pt0-1, Pt1-1, and CPt1-1, and an Illumina Miseq (Illumina, San Diego, California) for all isolates, as detailed in the Supplementary Materials. Single-nucleotide variants (SNVs) were called using the PacBio core genome assembly of Pt0-1 as the reference, as detailed in the Supplementary Materials.

Plasmid Isolation and *Escherichia coli* Transformation

The 6.1-kb $bla_{OXA-232}$ carrying plasmid pUCLAOXA232-1 was isolated from Pt1-1 using a MiniPrep Kit (Qiagen). The plasmid was introduced into a competent *Escherichia coli* HST08 strain (Clontech, Mountain View, California) by heat shock following the manufacturer's instructions. *Escherichia coli* cells were plated on a Mueller-Hinton agar with an ertapenem disk. Isolates that grew within the overall zone of growth inhibition were picked and screened for the presence of $bla_{OXA-232}$ gene by polymerase chain reaction for $bla_{OXA-232}$ [10]. Positive *E. coli* isolates was then sequenced by Illumina MiSeq to confirm the presence of the entire 6.1-kb plasmid and absence of other plasmids.

RESULTS

Summary of the Outbreak

The institution's clinical microbiology department evaluates all isolates of CPE for mechanisms of resistance, as part of an ongoing institutional review board–approved research study. Investigation of a potential CRKP outbreak was initiated following the laboratory's identification of a $bla_{OXA-232}$-positive CRKP isolated from a patient with no corresponding travel history. Seventeen patients were ultimately identified through combination of epidemiological and molecular microbiology investigations as associated with the outbreak (Figure 1). These included one source patient (Pt0) who received medical treatment in India 2 months prior to admission to our facility. Forty-four days after Pt0's admission, OXA-232 CRKP was isolated from Pt0's respiratory secretions. Twelve days prior (outbreak day [OD] 1), OXA-232 CRKP was isolated from the bile of a second patient, Pt1 (Figure 1). The mode of transmission between Pt0 and Pt1 remains unclear: they were admitted 12 days apart but housed in different hospital wards. Pt1 underwent ERCP on OD4 and OD30, using 2 different duodenoscopes (A and B, respectively, Figure 1).

Case finding was performed by evaluation of all carbapenem-resistant Enterobacteriaceae isolated in the 2014–2015 calendar years for the $bla_{OXA-232}$ gene [10]. Once the outbreak was recognized, rectal swab cultures were performed on patients with ERCP performed between OD4 and the day the outbreak was recognized and ERCP procedures were halted, on OD117. These evaluations identified 15 additional patients either infected (n = 9, designated as "Pt," Figure 1A) or colonized (n = 6, designated as "CPt," Figure 1B) with an OXA-232 CRKP. All patients, with one exception, had ERCP using one or both duodenoscopes A and B (Figure 1); the majority (12/14) had ERCP with duodenoscope A alone. Pt6 was exposed to duodenoscope A prior to developing CRKP infection and duodenoscope B after infection. Pt3 was exposed to only duodenoscope B (Figure 1). Pt10 did not have ERCP performed, but was housed in a room adjacent to Pt8. CPt3 developed a clinical infection (intra-abdominal abscess) on OD167, 13 days after recognition of colonized status—he is referred to as Cpt3_Pt9. None of the outbreak patients, with the exception of Pt0, had a travel history outside the United States. Patients were treated with a combination of antimicrobials, which were adjusted frequently given the complex nature of their infections, but included ceftazidime-avibactam; meropenem; intravenous fosfomycin that was obtained under approval from the University of California, Los Angeles (UCLA) Institutional Review Board and the US Food and Drug Administration; gentamicin; amikacin; and azithromycin. As of this writing, 5 of 11 patients with CRKP infection were alive—2 patients died as a result of complications due to their CRKP infections, whereas others died due to other complications.

Characterization of CRKP Positive for $bla_{OXA-232}$

Thirty-two isolates from 17 patients were available for study and sequenced. The chromosomes of all isolates shared 94% coverage and 99% identity to that of *K. pneumoniae* XH209, which was isolated from a patient in China in 2014 [11]. Both XH209 and the outbreak CRKP belong to sequence type (ST) 17. This ST accounts for CPE in Greece and Turkey, but is different from the epidemic ST258 that harbors KPC [12]. Four plasmids were identified in isolate Pt0-1 (Figure 2A). Two of these (pUCLAOXA232-1 and pUCLAOXA232-3) carry the 3 remarkable antimicrobial resistance (AR) genes $bla_{OXA-232}$, $bla_{CTX-M-15}$, and *rmtF* (Supplementary Figure 1). All other outbreak isolates harbored additional 2 plasmids (Figure 2A). Key plasmid-borne AR genes (Supplementary Tables 1 and 2) included the β-lactamases $bla_{OXA-232}$, $bla_{CTX-M-15a}$, and bla_{OXA-1}, and those impacting aminoglycosides (*aac(6')Ib*, *aac(6')Ib-cr, rmtF*) [13, 14], rifampin (*arr-2* and *arr-3*), chloramphenicol (*cat* and *catB3*), sulfonamides (*sul1*), trimethoprim (*dfrA1*), and fluoroquinolones (*aac(6')Ib-cr*). AR genes found on the chromosomes included bla_{SHV1} (ubiquitous in *K. pneumoniae*), efflux pump genes *oqxA* and *oqxB*, and fosfomycin resistance gene *fosA*

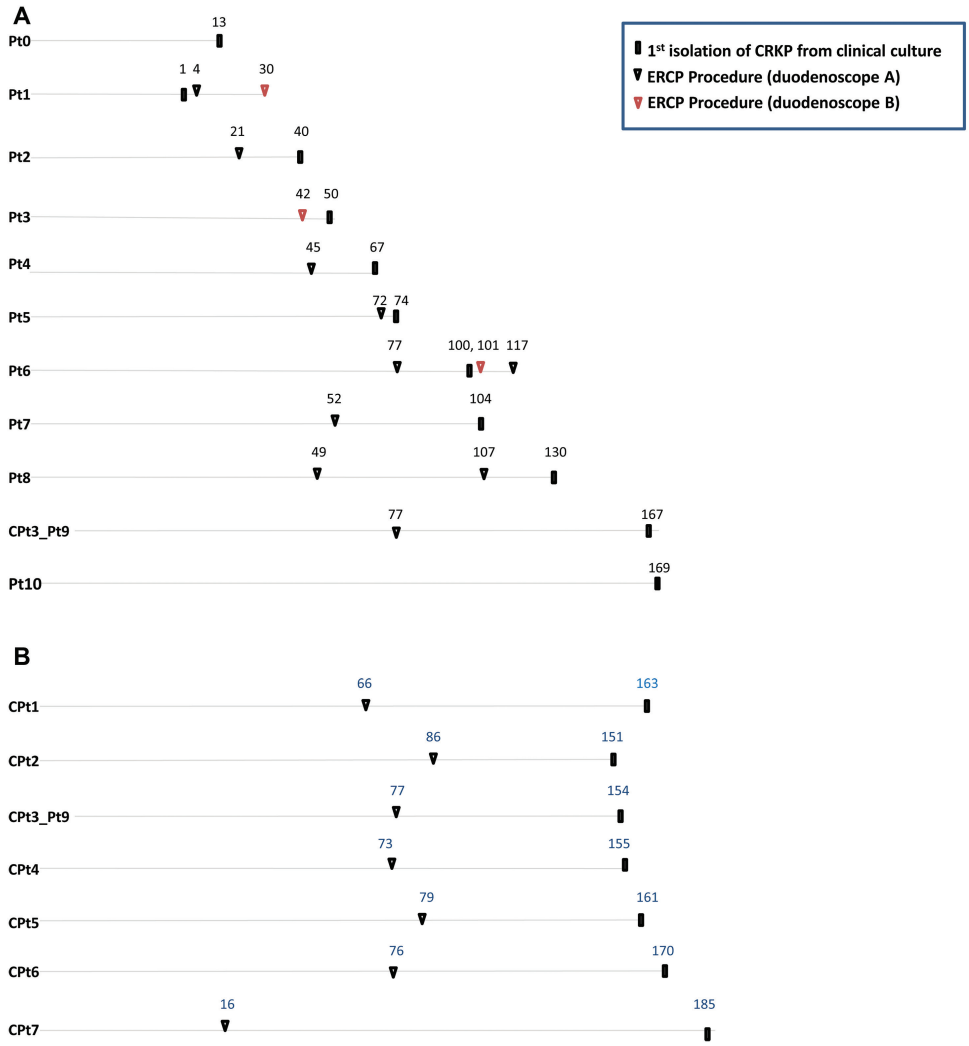

Figure 1. Outbreak timeline. Day 1 was the day the outbreak OXA-232 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) was isolated from patient (Pt) 1. Dates are presented above boxes (isolation of first CRKP) and triangles (date of endoscopic retrograde cholangiopancreatography [ERCP]). *A*, Patients identified by clinical cultures (passive surveillance). *B*, Colonized patients (CPt), identified by rectal surveillance cultures.

(Supplementary Table 1). Mutations associated with quinolone resistance were found in *gyrA* (predicted to encode Ser83Phe and Asp87Asn) and DNA topoisomerase IV *parC* gene (predicted to encode Glu84Lys) genes (Supplementary Table 1). A 10-bp deletion was found in the outer membrane porin *ompK35* gene at codon 165 and was predicted to truncate OmpK35 by 110 amino acids. Eight mutations and one 6-bp insertion were observed in the *ompK36* gene of all isolates, predicted to result in 5 amino acid substitutions.

All isolates were resistant to ampicillin-sulbactam, cefazolin, ciprofloxacin, ertapenem, levofloxacin, meropenem, piperacillin-tazobactam, and trimethoprim-sulfamethoxazole. All isolates were susceptible to ceftazidime-avibactam and most were susceptible to colistin (78.1%) and tigecycline (75.0%). No isolates harbored the *mcr-1* or *mcr-2* genes associated with transmissible colistin resistance. Several isolates (28.1%) had intermediate imipenem MICs [10]. Most isolates (84.4%) were resistant to aztreonam, ceftazidime, ceftriaxone, cefepime, and ceftolozane-tazobactam. Pt6, CPt1, and CPt3 harbored isolates susceptible to these antimicrobials, associated with loss of $bla_{CTX\text{-}M\text{-}15}$ (Figure 2B; Supplementary Table 2). Similarly, 96.9% of isolates were resistant to aminoglycosides (Supplementary Table 2), whereas the isolate CPt7-1 was susceptible, due to loss of *aac(6')Ib* and *rmtF* (Figure 2B; Supplementary Table 2) [13].

Isolate Diversification

The chromosome of isolate Pt0-1 was used as reference for genomic comparisons. Loss of large transposons occurred in isolates from 5 of 7 (71.4%) colonized patients and 1 of 10 (10.0%) infected patients. In addition, 40 kb of sequence

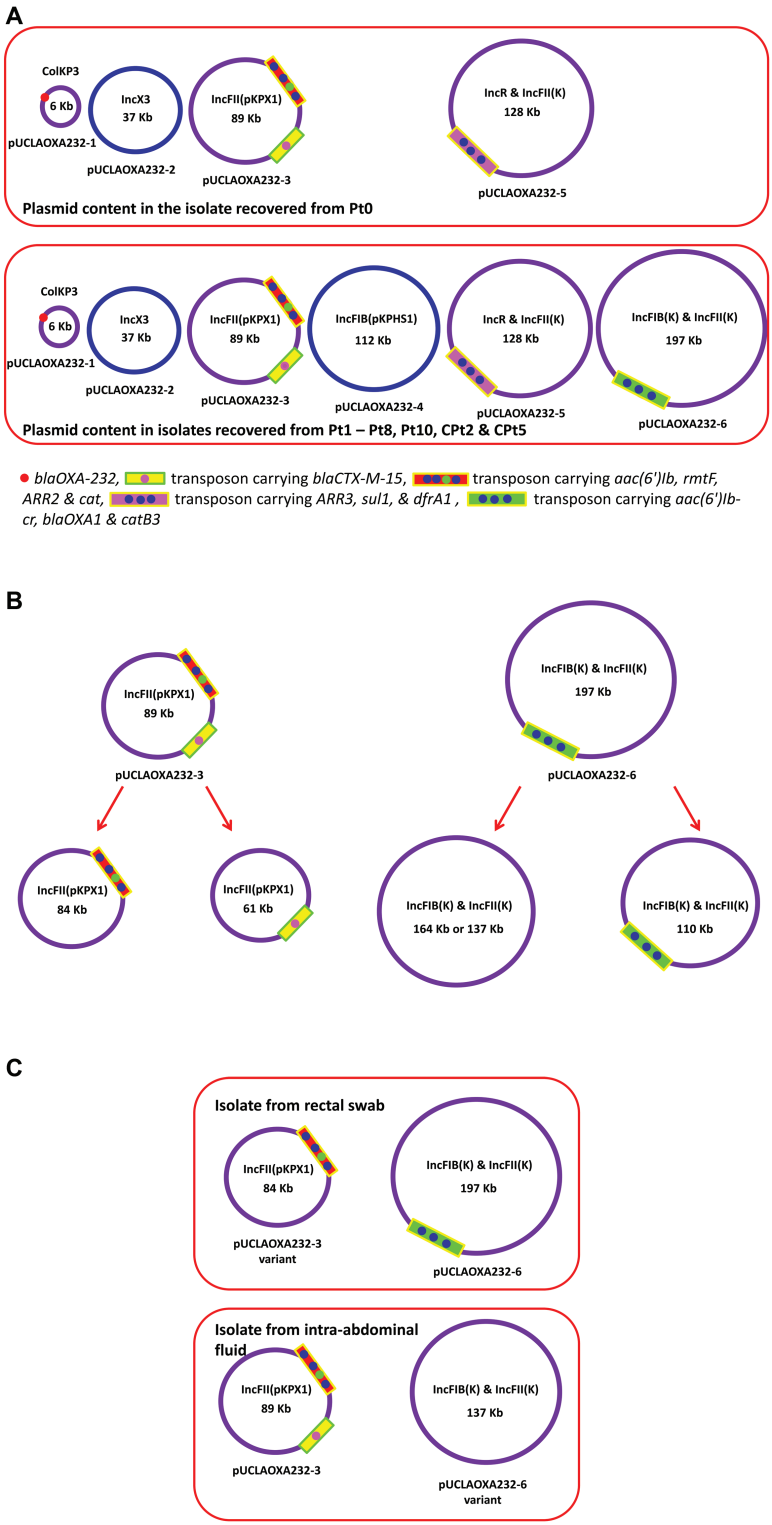

Figure 2. *A*, Plasmid content in the OXA-232 carbapenem-resistant *Klebsiella pneumoniae* isolates. *B*, Transposon loss from plasmids found in isolates recovered from CPt3_Pt9 and CPt1, CPt4, CPt6, and CPt7. *C*, Plasmid comparison between the isolates recovered from a single patient (CPt3_Pt9), demonstrating within-host plasmid diversification. Note that plasmids pUCLAOXA232-1, -2, -4, and -5 were not changed and are not shown. Abbreviations: CPt, colonized patient; Pt, patient.

comprised predominantly of phage elements was missing from the chromosomes of 8 isolates: Pt1-2, Pt6-1, Pt6-2, Pt6-6, CPt2-1, CPt3-1, CPt5-1, and CPt6-1.

Single-nucleotide variants were evaluated. SNVs at 64 unique sites across the chromosomes of the sequenced isolates were identified and detailed in Supplementary Table 3 and

Figure 3A. The number of SNVs between each isolate and the reference (Pt0-1) ranged from 3 to 11. A maximum of 15 SNVs were observed between any 2 isolates. Molecular clock calculations, performed using BEAST, revealed 12.31 substitutions per genome per year, with a 95% credibility interval between 7.81 and 17.05. The timed phylogeny reconstructed by BEAST is presented in Figure 3B.

SNV analysis both reflected and enhanced findings of epidemiological investigations performed during the outbreak. Seven isolates were sequenced from Pt1; the genomes of these isolates were diverse, with a total of 8 SNVs (Supplementary Table 4), suggesting that multiple variants were transmitted from Pt0 to Pt1 [15]. By contrast, the genomes of the 4 isolates recovered from Pt3 were almost identical, with only one SNV

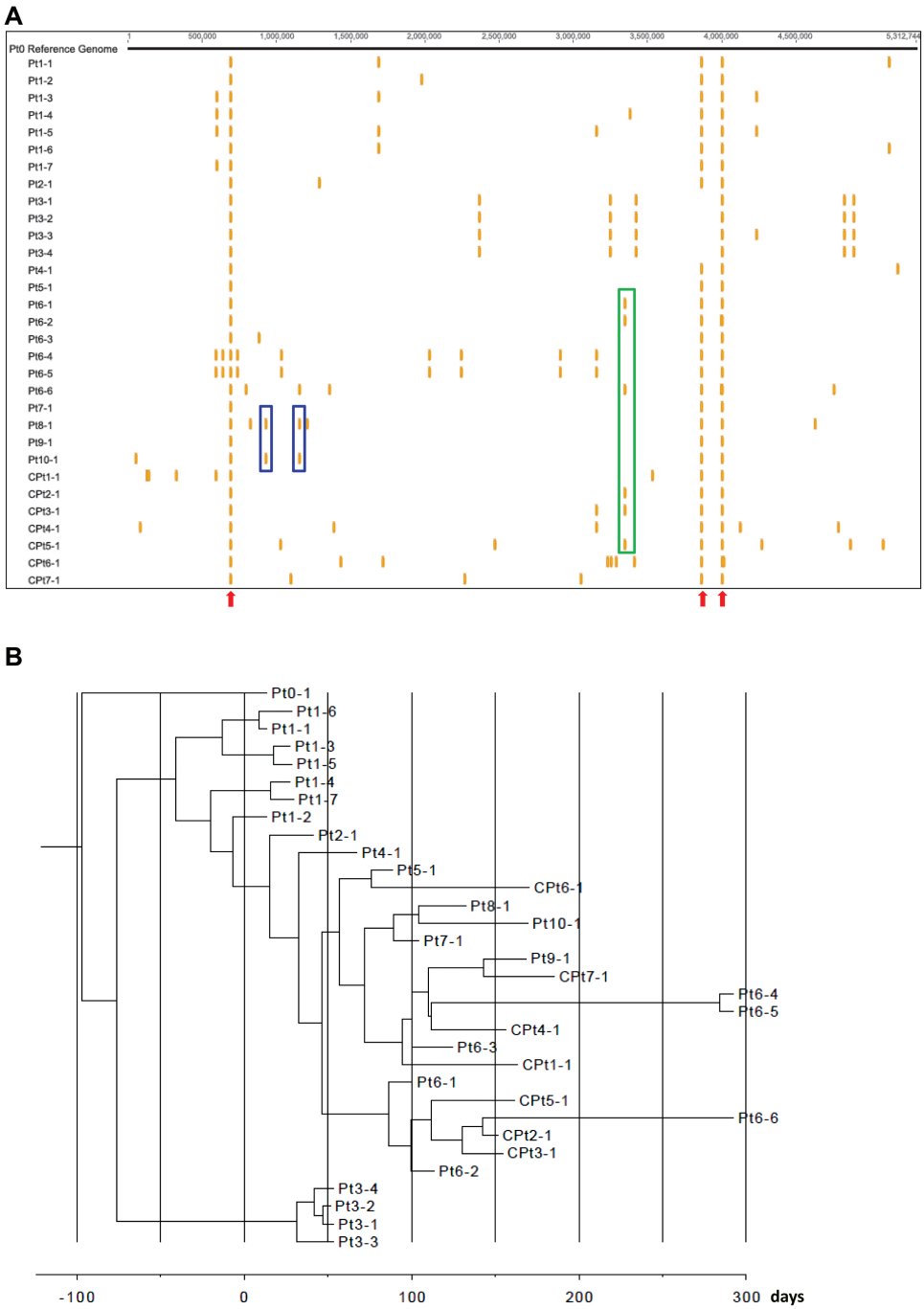

Figure 3. *A*, Overview of single-nucleotide variants (SNVs) identified among outbreak OXA-232 carbapenem-resistant *Klebsiella pneumoniae* (CRKP) isolates. *B*, Timed SNV phylogenetic tree of the outbreak CRKP isolates. Abbreviations: CPt, colonized patient; Pt, patient.

(Supplementary Table 5), suggesting transmission of a single variant to Pt3, and a single subsequent mutation occurring within Pt3. Interestingly, timed phylogeny suggested transmission occurred from Pt0 to Pt3, rather than from Pt1 to Pt3 via a contaminated duodenoscope. All outbreak isolates harbored 3 common SNVs (Figure 3A, indicated by red arrows) compared with the isolate from Pt0. However, one of these (3866613 C > T, in a noncoding region) was missing in all 4 isolates recovered from Pt3 (Figure 3A). Pt3 was the only patient in the outbreak to have ERCP performed using only duodenoscope B, consistent with an alternate route of infection. The epidemiological investigations did not identify risk factors for transmission from Pt0 to Pt3, but a clear transmission route from Pt0 to Pt1 was also not identified. Because significant genomic diversity was observed among isolates recovered from Pt1, we cannot rule out the possibility that the lineage transferred to Pt3 existed in Pt1 but was not sampled and sequenced, despite good coverage of isolates from Pt1 (n = 7 isolates sequenced).

Six isolates were sequenced from Pt6, representing 2 distinct lineages that coexisted in Pt6 for at least 150 days. The first includes isolates Pt6-1 and Pt6-2 (isolated on days 100 and 113, respectively), which were quite similar, with only one SNV, and isolate Pt6-6 (isolated on day 279) with 5 additional SNVs (Supplementary Table 6). The second lineage includes 2 identical isolates (Pt6-4 and Pt6-5, both sampled on day 267 from blood and bile, respectively) and one very different isolate (Pt6-3, isolated from a rectal swab on day 124, which harbored 9 SNVs from Pt6-4 and Pt6-5; Supplementary Table 6). Pt6 had ERCP performed on multiple occasions, including OD77 (duodenoscope A), OD101 (duodenoscope B), and OD117 (duodenoscope A), indicating that either 2 separate transmission events or a single transmission of 2 lineages occurred to Pt6. The first lineage recovered from Pt6 (isolates Pt6-1, Pt6-2, and Pt6-6) clustered with isolates CPt2-1, CPt3-1 (aka Pt9), and CPt5-1 (Figure 3B); all these patients had ERCP performed within one week, on OD77–86. The second lineage identified in Pt6 (isolates Pt6-3, Pt6-4, and Pt6-5) cluster with isolates from Cpt1-1, Cpt4-1, Cpt7-1, and Pt9-1. This latter group of patients had ERCP performed on OD17 (CPt7) through OD77 (Pt9). The fact that Pt9_CPt3 also harbored the 2 lineages found in Pt6 (one isolated from rectal surveillance culture and one isolated from clinical culture), and both patients had ERCP performed on OD77 using the same duodenoscope, strongly suggests one transmission of 2 lineages to both Pt6 and Pt9_CPt3, in particular as Pt9_CPt3 only had ERCP performed on one occasion. The other patients with one or the other lineage had only one isolate sampled, and may therefore have also harbored both lineages.

Pt10 did not have ERCP performed. The timed phylogeny strongly suggests this patient acquired the OXA-232 CRKP from Pt8 (Figure 4B). The isolates from these 2 patients shared 2 unique SNVs, 934156 G > A (*hlyD* hemolysin secretion protein D gene) and 1157344 C > T (*malZ* maltodextrin glucosidase gene, blue boxes in Figure 3A). These genomic data are supported by the fact that these 2 patients were housed in adjacent rooms for 14 days, making unit-based transmission highly likely.

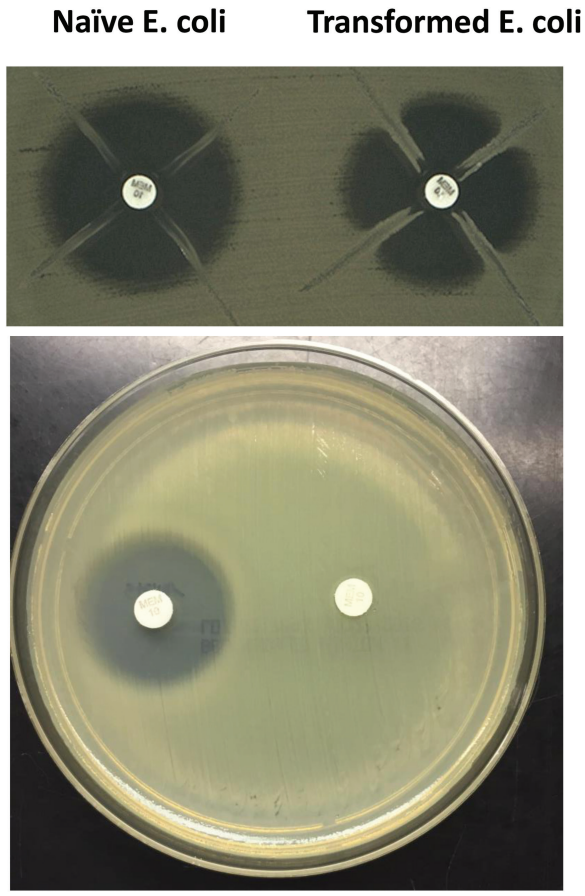

Figure 4. Modified Hodge test (top) and carbapenem inactivation method (bottom), for the naive DH5a *Escherichia coli* and an *E. coli* transformed with pUCLAOXA232-1, demonstrating carbapenem-hydrolyzing activity of the transformed isolate.

Characterization of OXA-232 Carbapenemase Activity

The 6.1-kb plasmid pUCLAOXA232-1 carrying $bla_{OXA-232}$ was isolated and introduced into a naive DH5a *E. coli* background (HST08, Takara, Otsu, Japan). The transformed isolate hydrolyzed meropenem, evidenced by a positive carbapenemase inactivation method result (Figure 4), confirming carbapenemase activity from OXA-232. The transformed *E. coli* was also resistant to ampicillin (MIC changed from 4 μg/mL to >32 μg/mL), ampicillin-sulbactam (from 4 μg/mL to 32 μg/mL), and cefazolin (from 1 μg/mL to 8 μg/mL) and intermediate to piperacillin-tazobactam (from 1 μg/mL to 32 μg/mL), as expected to occur from the presence of an active OXA-232 gene. However, the transformed *E. coli* only demonstrated no elevation in carbapenem MIC (≤0.25 μg/mL to ertapenem, meropenem, and imipenem pre-and posttransformation) [16]. We thus

retrospectively evaluated all Enterobacteriaceae isolated from blood (chosen because the laboratory saves blood isolates) from patients who had ERCP performed during the outbreak; none harbored $bla_{OXA\text{-}232}$.

DISCUSSION

WGS is emerging as a powerful tool by which to perform molecular epidemiologic studies. However, interpretation of data is challenging as methods have yet to be standardized and the anticipated rate of diversification of bacteria within a human host is poorly defined [17]. We evaluated 32 CRKP isolates recovered during an outbreak associated with ERCP procedures at a single institution. The core advantage of this investigation was clear epidemiology of transmission for the majority of patients, which allowed us to identify the putative date of acquisition and to assign a chronology to diversification in vivo. In addition, because this outbreak was caused by the highly unusual OXA-232 CRKP, we were able to identify a likely source patient and cases associated with this outbreak, through systematic testing of carbapenem-resistant Enterobacteriaceae isolated at our institution for the $bla_{OXA\text{-}232}$ gene. A diversification at a rate of 12.31 substitutions per genome per year was found in this outbreak, a number very similar to the 10.1 substitutions per genome per year identified in a recent outbreak of ST 258 KPC-producing isolates [18]. Similarly, 11 SNVs were found between 2 CRKP isolates following 18 months of carriage [19]. These data suggest that the guidelines proposed by Salipante et al to define genomic relatedness by WGS (ie, a cutoff of 4–12 SNVs as closely related and ≥13 SNVs as unrelated) [20] may need refining for CRKP. However, our findings might not necessarily extrapolate to other CPE investigations. Diversification rates may differ significantly between not only species [21], but specific lineages within a species. Furthermore, transmission of isolates between patients is generally thought to sharply reduce the genetic diversity of a given bacterial population—that is, the transmission bottleneck. Such bottlenecks were observed in our outbreak, including both narrow (as occurred to Pt3 via what was likely a non-ERCP transmission) and loose bottlenecks (as occurred for Pt6 and CPt3_Pt9, who harbored 2 distinct lineages of CRKP thought to be transmitted during a single event). These findings reinforce the limitation of sequencing one isolate per patient during outbreak investigations, and demonstrate the challenge in arbitrary cutoffs for the number of SNVs to define genetic relatedness.

Nonetheless, we were able to confirm epidemiological links identified by traditional methods by WGS data. For instance, isolates from Pt8 and Pt10 were highly related, congruent with the epidemiological finding that these patients were housed in adjacent rooms. Similarly, isolates from Pt6, CPt2, CPt3, and CPt5 formed a unique lineage (Figure 3B), consistent with the timing of their ERCP procedures. Finally, WGS data determined that isolates from Pt3 were significantly different from other outbreak isolates, strongly suggesting direct transmission from Pt0, although epidemiological investigations were unable to identify this possible route of transmission. Nonetheless, other non-ERCP-based transmission did occur on 2 other occasions during the outbreak, between Pt0 and Pt1, and between Pt8 and Pt10. Such findings served to reinforce the importance of not only reevaluating ERCP reprocessing procedures at our institution, but also of strict reinforcement of hand hygiene and contact precautions.

One interesting finding of this study was the various phenotypes identified among the outbreak isolates. In particular, the antibiogram of the outbreak isolates varied considerably, including isolates that were susceptible to the cephalosporins and aminoglycosides. While many researchers utilize antimicrobial susceptibility results to roughly estimate the likelihood of 2 isolates of a given species being related, our study clearly demonstrates the limitations of this approach. Many laboratories employ cascade reporting—that is, only reporting broad-spectrum antibiotics (eg, carbapenems), if more narrow-spectrum agents (eg, cephalosporins) test resistant. As such, cephalosporin-susceptible, carbapenem-resistant isolates may not immediately be recognized. Furthermore, as we demonstrated, OXA-48–like carbapenemases have relatively weak carbapenemase activity. High-level resistance to carbapenems typically requires concomitant permeability defects [22], such as mutations to *ompK35* and *ompK36* found in our isolates. When we transferred the OXA-232 plasmid to a naive *E. coli*, this isolate remained susceptible to the carbapenems (Figure 4). While we did not identify any carbapenem-susceptible Enterobacteriaceae that harbored this gene with limited testing, there remains a real concern that this plasmid could spread undetected, only becoming resistant as isolates developed permeability defects.

In summary, we describe the utility of WGS for investigation of an outbreak caused by CRKP. While such investigations show promise, standardization of WGS as an epidemiological tool is needed, in particular to allow comparison of data across institutions. As a result of this outbreak, our laboratory now routinely screens all carbapenem-resistant Enterobacteriaceae for carbapenemases, on the day they are isolated. These results are reported in real time to the infection prevention team for review and investigation, if necessary. Although there is the risk of OXA-232 and other carbapenemases to be present in carbapenem-susceptible isolates, there remains no practical method by which to screen all Enterobacteriaceae isolated by the laboratory for these resistance mechanisms. However, use of commercially available molecular tests to detect presence of carbapenemase genes from either blood cultures or rectal swabs collected for surveillance may allow improved detection of these isolates.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted

materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Note

Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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