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Major Article

A cluster of carbapenemase-producing *Enterobacter cloacae* complex ST171 at a tertiary care center demonstrating an ongoing regional threat

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Background: In Minnesota and North Dakota, a clonal strain of bla_{KPC-3}-producing *Enterobacter cloacae* complex has been reported with increasing frequency.
Methods: Between July 2015 and February 2016, 13 carbapenem-resistant *E. cloacae* complex isolates were identified at our institution. Five bla_{KPC}-positive isolates were identified by polymerase chain reaction and underwent pulsed-field gel electrophoresis and whole genome sequencing. Medical records of these patients were reviewed.
Results: All 5 case-isolates belonged to sequence type 171 and were bla_{KPC-3}-positive. Three pulsed-field gel electrophoresis patterns with >90% similarity were identified in the 5 case-isolates. We identified overlaps in time and location between case patients. Plasmid types and resistance genes were nearly identical between the isolates. Whole genome sequencing showed isolates A, B, and D to be closely related with <10 core single-nucleotide polymorphisms differences. Isolates C and E were also closely related to each other, but more distantly to A, B, and D; all belonged to the clonal lineage of the major circulating *E. cloacae* complex strain in Minnesota and North Dakota. Despite having overlapping hospital stays, isolates for patients C and D were not identical.
Conclusions: Isolates A and D were nearly identical, indicating possible transmission during hospitalization. Transmission of the other isolates may have occurred elsewhere. This report highlights the importance of using both epidemiologic and molecular data to track the spread of carbapenemase-producers.
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Carbapenemase-producing (CP) bacteria represent a public health threat because of their resistance to antibiotics and high associated mortality. In addition, carbapenemases are readily transmitted between gram-negative bacteria because of their location on conjugative plasmids. In the United States, *Klebsiella pneumoniae* carbapenemase (KPC) is the most commonly reported carbapenemase, first identified in 2001.[1,2] Clonal spread nationwide has been demonstrated by multilocus sequence typing (MLST), establishing *K. pneumoniae* sequence type 258 as the most commonly identified clone.[3] According to laboratory-based surveillance conducted by the Centers for Disease Control and Prevention (CDC), *K. pneumoniae* is the most common carrier of KPC, however KPC is also commonly reported in *Escherichia coli* and *Enterobacter* spp.[4]

The Minnesota Department of Health (MDH) conducts statewide surveillance of carbapenem-resistant *Enterobacteriaceae* (CRE) via the Multisite Gram-negative Surveillance Initiative in partnership with the CDC. In 2013, the crude annual incidence of CRE in Minnesota per 100,000 population was 2.32, compared to 2.93 among the 7 sites

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`reporting.[4] Unique to certain parts of the country, *Enterobacter cloacae* complex represent a significant proportion of the reported CP bacteria in Minnesota.[5] Species of the *E. cloacae* complex are common nosocomial pathogens, colonizing the gut flora of hospitalized patients, and can easily spread from patient to patient. The *E. cloacae* complex comprises several species and genetic clusters, including *E. cloacae* and *E. hormaechei*. Over the past 2 decades, there has been an increasing number of reports documenting the spread of carbapenem-resistant and CP-*E. cloacae* in health care settings seemingly driven by acquisition of bla_{KPC}-bearing plasmids followed by clonal spread of strains adapted for nosocomial environments.[6,7] Previous reports from Minnesota and North Dakota have identified a circulating lineage of clonal *E. cloacae* complex producing KPC-3 with MLST type 171 (ST171), which has recently been re-classified as *E. hormaechei*.[8,9]

The University of Minnesota Medical Center (UMMC) is an 847-bed tertiary-care hospital that receives many referrals from North Dakota. It is common for patients to have received medical care in North Dakota prior to hospitalization at the UMMC. We report on 5 patients with CP-*E. cloacae* complex belonging to the circulating lineage identified over 7 months at our institution and describe their clinical and laboratory characteristics. This investigation was initiated after new CRE infections were reported in 2 patients from the same intensive care unit over a short period of time, raising concern for nosocomial spread.

METHODS

CRE are routinely submitted to the MDH-Public Health Laboratory (MDH-PHL) by clinical laboratories as part of a statewide surveillance program. Isolates meeting a specific case definition for carbapenem resistance or characterized as producing a carbapenemase by phenotypic test (eg, a modified Hodge test, modified carbapenem inactivation method) are submitted to the MDH-PHL. During 2015-2016, isolates were tested by multiplex real-time polymerase chain reaction for bla_{KPC} and bla_{NDM} carbapenemase genes based on a modified CDC protocol.[10] The case definition in 2015 was an isolate that is resistant to all third generation cephalosporins tested and non-susceptible (≥ 2 μg/mL) to imipenem, doripenem, or meropenem. Isolates were prepared for molecular analysis by boiling lysis[11] followed by template amplification using a Bio-Rad CFX96 real-time polymerase chain reaction detection system instrument (Bio-Rad Laboratories Inc, Hercules, CA). Results were evaluated with Bio-Rad CFX Manager 3.1 software (Bio-Rad Laboratories).

Between July 2015 and February 2016, 13 carbapenem-resistant *E. cloacae* complex isolates were identified at the UMMC and sent to the MDH-PHL for further testing, 5 of which were KPC-positive. The Vitek 2 system (bioMerieux, Lyon, France) was used for the initial organism identification and antimicrobial susceptibility testing (GN-ID and GN-73 cards). If the imipenem or meropenem minimum inhibitory concentration was >1, then an ertapenem screening E-test or disk diffusion test was performed. If the ertapenem result was resistant or intermediate, then a modified Hodge test was performed to screen for carbapenemase production. Isolates that were resistant to carbapenem drugs were sent to the MDH-PHL for carbapenemase testing.

The medical records of the source patients for the 5 KPC-positive isolates were reviewed. Owing to a possible link between 2 patients admitted concurrently in November 2015, screening for CP bacteria was performed on patients located on 2 adjacent intensive care units (ICU). Both of these patients were mechanically ventilated, so common respiratory therapy links were investigated. Infection preventionists gathered lists of common nurses, providers, respiratory therapists, and procedures that these patients may have shared. All intubated patients on these 2 units were screened via an established rectal swab protocol[12] and tracheal aspirate culture.

The 5 KPC-positive isolates were further examined for similarities and typed by pulsed-field gel electrophoresis (PFGE) and whole genome sequencing, both performed by the MDH-PHL molecular epidemiology unit. *E. cloacae* complex isolates were typed by PFGE according to the previously published protocol by Ribot et al,[13] with modifications. Isolate DNA was digested with XbaI enzyme and electrophoresis conditions were altered to an initial switch time of 4.0 seconds and a final switch time of 40.0 seconds; gels were run for 18 hours. PFGE patterns were analyzed using BioNumerics software (Applied Maths, Austin, TX). Resulting dendrograms were established using the Dice coefficient with a band position tolerance of 1%. Patterns with no discernible differences were assigned the same PFGE pattern designation and considered indistinguishable.

E. cloacae complex isolates characterized in this study were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA). DNA extraction was performed on a Qiagen QIAcube with the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD) following the manufacturer's protocol. Quantification of DNA was carried out using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, Waltham, MA). The Nextera XT DNA sample prep kit (Illumina) was used to complete dual-indexed sequencing libraries following manufacturer's guidelines. Paired-end V2 chemistries following standard FASTQ-only generation protocols were used to produce 250-base pair, paired-end nucleotide reads.

Genome assembly of MiSeq reads was carried out in CLC Genomics Workbench, version 9.0 (CLC Bio, Boston, MA). Assembly parameters followed default settings. Antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance database[14] and compared with results detected using ResFinder 2.1.[15] PlasmidFinder was used to define plasmid types.[16] Raw data for each isolate were mapped to an ST171 *E. cloacae* complex reference genome using CLC Genomics Workbench and single-nucleotide polymorphisms (SNP) extracted for analysis with Molecular Evolutionary Genetics Analysis software version 6 (MEGA6, Pennsylvania State University, Pennsylvania, PA).[17] Phylogenetic analysis was performed as described previously.[8]

RESULTS

Clinical results

All 5 patients (corresponding to isolates A-E) with CP-*E. cloacae* complex were hospitalized at the UMMC between July 2015 and February 2016 (Fig. 1 and 2). Isolates A-E were collected from endotracheal, urine, blood, bronchoalveolar lavage, and ascites cultures, respectively. Isolates were collected during hospitalization except isolate B, which was collected during an outpatient visit almost 2 months after hospitalization. Patients A and E were known CRE carriers prior to hospitalizations during the reference period, and were placed in contact precautions on admission.

The CP-CRE isolates from all 5 patients had similar antimicrobial susceptibility profiles (Table 1). All were resistant to cephalosporins, fluoroquinolones, and β-lactam antibiotics, including carbapenems. All showed susceptibility to amikacin, tigecycline, and colistin. Newer drug therapies, such as ceftazidime-avibactam, were not available for testing at the time of this study.

Patients A and B were hospitalized on the same unit (non-intensive care) 6 days apart. Patient A was hospitalized a second time in November 2015, being discharged a few days prior to hospitalizations for patients C and D. Patients A and D occupied the same ICU room 13 days apart. Patients C and D were concurrently hospitalized in the same ICU, overlapping for 9 days; both patients died during this hospitalization. The epidemiologic investigation into links between these 2 patients did not reveal any common nurses or procedures between the patients, but there were common physicians, residents, and respiratory therapists. Patients C and E were both from North Dakota.

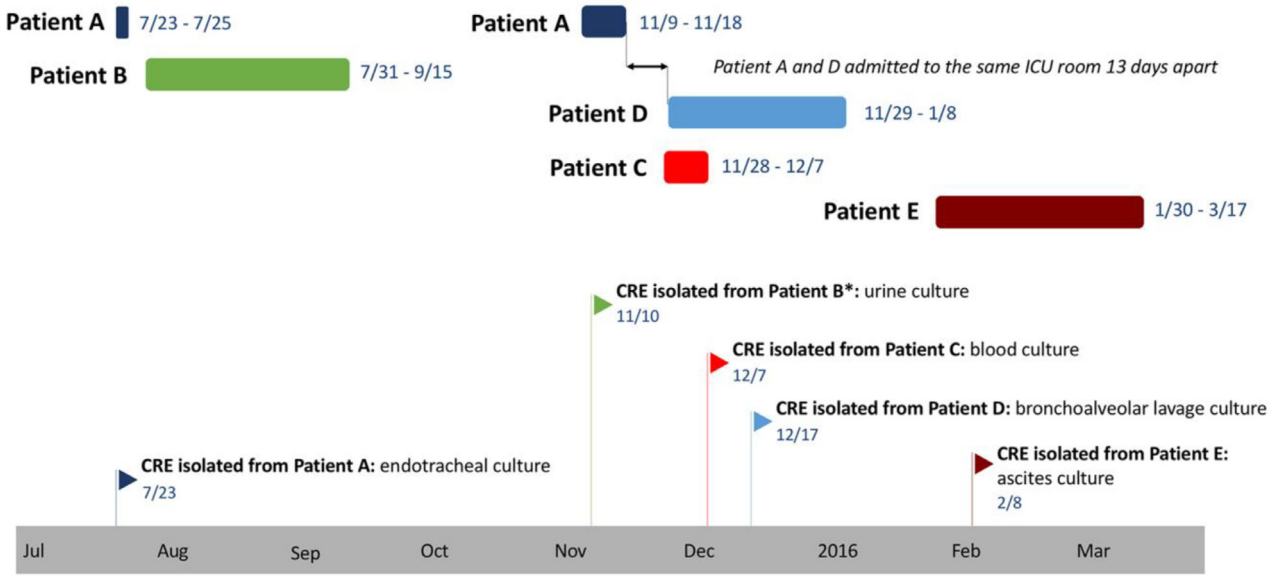

Fig 1. Hospital admission timeline for 5 patients with carbapenemase-producing *Enterobacter cloacae* complex. *CRE*, carbapenem-resistant *Enterobacteriaceae*; *ICU*, intensive care unit. *Culture not collected during University of Minnesota Medical Center hospitalization.

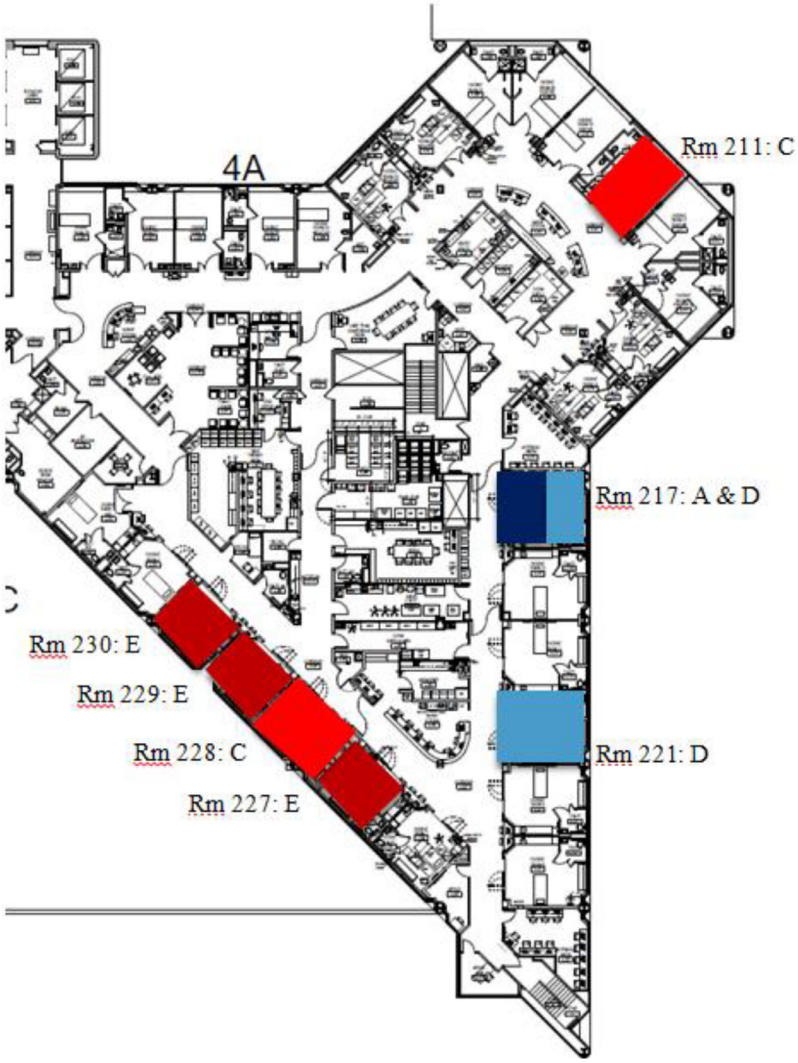

Fig 2. Room (Rm) locations of 4 patients admitted to the same intensive care unit during the study period. Patient B was never admitted to this unit.

Table 1
Minimum inhibitory concentration (μg/mL) and Clinical Laboratory Standards Institute interpretations of 5 *Enterobacter cloacae* complex isolates

	Isolates					
Drug	A	B	C	D	E	Interpretation
Amikacin	≤4.0	≤2.0	4	≤2	≤2	Susceptible
Cefepime	>16.0	≥64.0	≥64.0	16	≥64	Resistant
Ciprofloxacin	>2.0	>4.0	≥4	≥4	≥4	Resistant
Ertapenem	32.0	32.0	>32.0	16.0	32.0	Resistant
Meropenem	16.0	≥16.0	>32.0	≥16	>16.0	Resistant
Piperacillin/Tazobactam	>64.0	≥128.0	≥128	≥128	≥128	Resistant
Tigecycline	≤1.0	≤1.0	2.0	2.0	2.0	Susceptible
Colistin	0.38	0.19	0.125	0.125	0.125	No interpretation available

Patient C was transferred directly from a North Dakota hospital to the UMMC. Patient E was hospitalized at a separate North Dakota hospital 2 months prior to the UMMC hospitalization.

Laboratory results

All 5 *E. cloacae* complex isolates examined in this study belonged to the recently described clonal lineage of the major circulating strain in Minnesota and North Dakota; a single PFGE pattern (ECL18) identified in 2 study isolates (C, E) matched a pattern observed in the previous study.[8] The other 2 PFGE patterns detected during this analysis were ECL64 (isolates A, D) and ECL74 (isolate B), patterns not previously observed by the MDH-PHL. All 3 identified PFGE patterns were >90% similar to each other (Fig 3), placing the 5 isolates into the circulating lineage; the PFGE similarity cut-off for circulating lineage isolates was defined in a previous study as ≥85%.[8] In contrast, an unrelated UMMC isolate from early 2015 was only 80% similar by PFGE (data not shown).

Whole genome sequencing *in silico* MLST supported PFGE results and demonstrated that all 5 *E. cloacae* complex isolates belonged to ST171, the circulating lineage sequence type. An SNP comparison of the core *E. cloacae* complex genome using the MEGA software Maximum Parsimony method with 1,000 bootstrap replicates revealed isolates A, B, and D to be closely related to each other with <10 SNP differences between them. Isolates C and E were also closely related to each other but more distantly related to A, B, and D. Strains MNCRE07, MNCRE09, MNCRE56, and NDA were included as reference strains for previously characterized Minnesota and North Dakota *E. cloacae* complex from different phylogenetic branches of the circulating lineage (Fig 4).

Two plasmid types were identified among the 5 UMMC isolates [IncFIA(HI1) and IncX3 plasmid] by whole genome sequencing; both appear to be similar to previously characterized circulating lineage plasmids.[8] The resistance gene profile, which included gene encoding elements for ß-lactam, aminoglycoside, fluoroquinolone, sulfonamide and trimethoprim resistance, was nearly identical for all isolates, and included bla_{KPC-3} inserted in transposon *Tn4401*.[8] Notably, isolates B and C also appeared to harbor an IncX3 plasmid present in some circulating lineage clones that encodes a bla_{SHV-12} ßeta-lactam resistance gene (Table 2).

After patients C and D were identified as being infected with KPC-positive *E. cloacae* complex, screening for CP-CRE was performed on 15 ICU patients currently admitted on 2 adjacent ICUs. Two rectal swabs and 1 endotracheal aspirate were collected from each patient and tested for the presence of CP-CRE. All samples were negative for CP bacteria, suggesting no additional transmission of *E. cloacae* complex or other CRE within the ICU during this time period.

DISCUSSION

This epidemiologic investigation was prompted by 2 patients diagnosed with serious KPC-positive *E. cloacae* complex infections on the same ICU, 2 weeks apart. Infection preventionists reviewed culture results from other patients on the unit to determine whether any other *E. cloacae* complex isolates had been identified from other patients. No additional isolates were found during the investigation. Because of the temporal, locational, and staff epidemiologic links between the patients, we engaged the MDH-PHL with their assistance in strain typing.

The PFGE testing and whole genome sequencing were undertaken because of concern for local hospital unit transmission of CRE. In particular, local acquisition was of concern for patients B, C, and D, who were not known to be previously colonized with CRE. Patient B was hospitalized on the same unit as patient A, separated in time by 6 days, but the isolate for patient B was recovered >3 months later

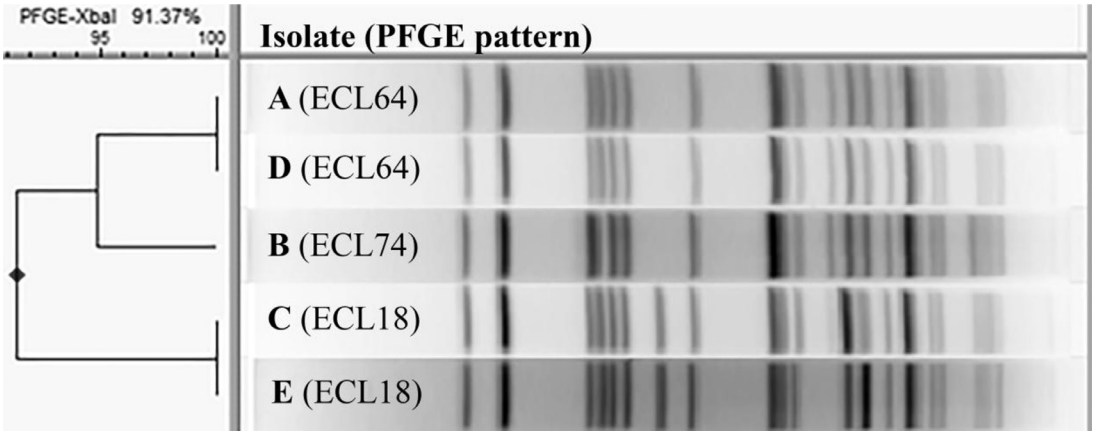

Fig 3. Dendrogram and pulsed-field gel electrophoresis patterns of 5 *Enterobacter cloacae* complex multilocus sequence type 171 isolates.

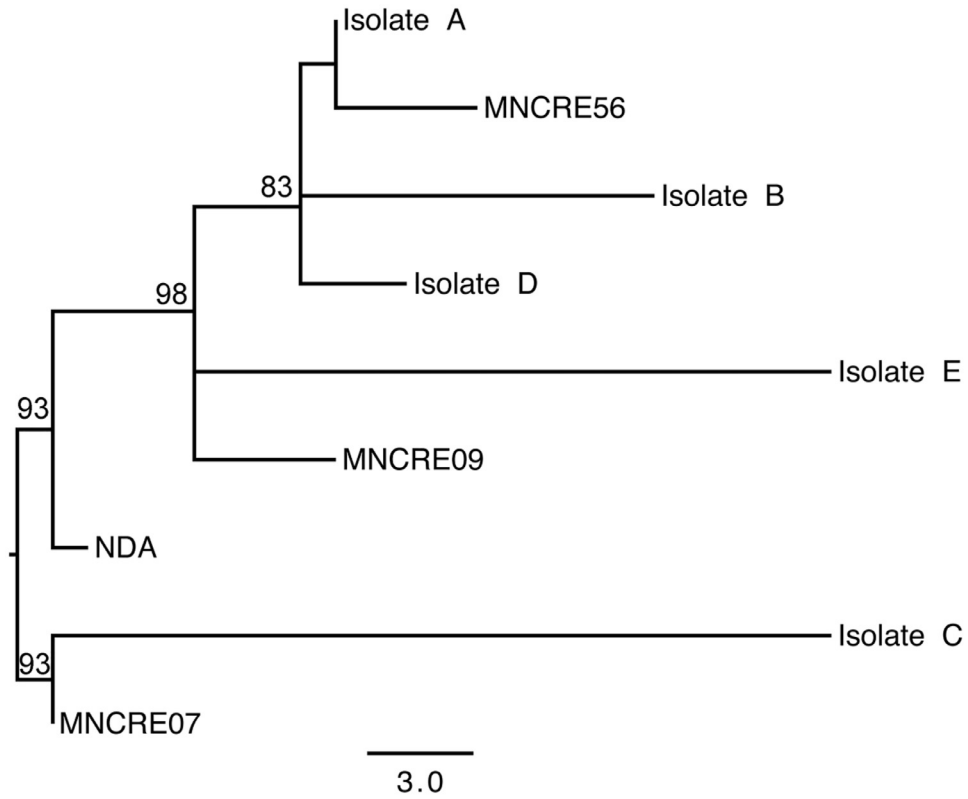

Fig 4. Dendrogram of the genetic relationship between the 5 *Enterobacter cloacae* complex isolates. Phylogenetic analysis was conducted using MEGA software Maximum Parsimony method with 1,000 bootstrap replicates. There were a total of 70 positions in the dataset. Scale represents single-nucleotide polymorphism differences across the core genome. Strains MNCRE56, MNCRE09, MNCRE07, and NDA were included as reference strains.

Table 2
Characteristics of 5 *Enterobacter cloacae* complex isolates

		Plasmid types		Resistance genes		
Isolate	PFGE pattern	IncFIA(HI1)	IncX3	*aadA1*, *strA*, *aacA4*, *strB*, *sul2*, *dfrA14*, *aac(6′)Ib-cr*, bla_{OXA-9}, bla_{TEM-1A}, *aph(3′)-Ia*	bla_{KPC-3}	bla_{SHV-12}
A	ECL64	X		X	X	
B	ECL74	X	X	X	X	X
C	ECL18	X	X	X	X	X
D	ECL64	X		X	X	
E	ECL18	X		X	X	

PFGE, pulsed-field gel electrophoresis.

during an outpatient encounter. Patient D was hospitalized in the same ICU room as patient A, separated in time by 13 days. Despite contact precautions being in place for patient A, proximity in time and location raised concerns for a break in infection control practices or failure of environmental cleaning.

Previous reports have identified a circulating strain of CP-*E. cloacae* complex in North Dakota and Minnesota.[8,9] Using various typing methods, 5 isolates were identified as belonging to the circulating strain at our institution. Combining the data collected from different typing methods with traditional source investigations, potential connections were identified between the patients and their isolates.

PFGE patterns identified for isolates A and D were identical, whereas isolate B differed by 3 bands. Subsequent whole genome sequencing SNP analysis showed that isolates A, B, and D were closely related. After further sequence analysis, isolate B was shown to be distinct from isolates A and D, harboring an IncX3 plasmid. The presence of the IncX3 plasmid in isolate B might account for the variance in PFGE pattern observed between this isolate and isolates A and D.

Patients C and D overlapped during their hospitalizations by time and location. PFGE patterns differed by 2 bands. However, SNP analysis showed isolate D to be more closely related to isolates A and B, rather than isolate C. Isolate C also differed from the former strains as it possessed an IncX3 plasmid and the resistance gene bla_{SHV-12}. Patients C and E were linked only by residence in North Dakota, and their hospitalizations were separated by almost 2 months. Isolates C and E possessed the same PFGE pattern. It is likely that a single precursor ST171 strain containing the IncX3 plasmid served as the ancestor for all strains described in this study. However, the lineage is distorted by differences in time when the isolates were collected. Bacterial strains have a propensity to gain or lose resistance genes or plasmids, or acquire point mutations over time. These changes can be driven by differences in environmental and host pressures such as patient microbiome composition and exposure to distinct antibiotics.

CONCLUSIONS

As more precise molecular techniques, such as whole genome sequencing, become available to the hospital epidemiologist, it will be important to combine traditional methods with new

technologies to better understand hospital outbreaks. In the current report, the possible local transmission of a CP-*E. cloacae* complex must be interpreted in the setting of a circulating strain encompassing 2 states. Therefore, efforts to improve local infection prevention practices may help prevent local outbreaks, but do little to curb the resistant reservoir in the surrounding community. The clinical impact of carbapenem-resistant bacteria is clearly a public health threat. As such, the documented rise and spread of CP-*E. cloacae* complex ST171 in the Northeast and Midwestern United States, in addition to the dominant *K. pneumoniae* sequence type 258, deserve closer attention.[8,9,18] Awareness of regional differences is important in understanding the spread of CP-CRE. In addition, this report serves to highlight that organisms appearing to be epidemiologically linked and genomically related may not be genomically identical. However, until sequencing results are more readily-available, hospital epidemiologists and infection preventionists must still rely on traditional methods of investigation to drive rapid interventions in the event of a possible outbreak.

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