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RESEARCH ARTICLE

Genomic Analysis of Hospital Plumbing Reveals Diverse Reservoir of Bacterial Plasmids Conferring Carbapenem Resistance

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ABSTRACT The hospital environment is a potential reservoir of bacteria with plasmids conferring carbapenem resistance. Our Hospital Epidemiology Service routinely performs extensive sampling of high-touch surfaces, sinks, and other locations in the hospital. Over a 2-year period, additional sampling was conducted at a broader range of locations, including housekeeping closets, wastewater from hospital internal pipes, and external manholes. We compared these data with previously collected information from 5 years of patient clinical and surveillance isolates. Whole-genome sequencing and analysis of 108 isolates provided comprehensive characterization of bla_{KPC}/bla_{NDM}-positive isolates, enabling an in-depth genetic comparison. Strikingly, despite a very low prevalence of patient infections with bla_{KPC}-positive organisms, all samples from the intensive care unit pipe wastewater and external manholes contained carbapenemase-producing organisms (CPOs), suggesting a vast, resilient reservoir. We observed a diverse set of species and plasmids, and we noted species and susceptibility profile differences between environmental and patient populations of CPOs. However, there were plasmid backbones common to both populations, highlighting a potential environmental reservoir of mobile elements that may contribute to the spread of resistance genes. Clear associations between patient and environmental isolates were uncommon based on sequence analysis and epidemiology, suggesting reasonable infection control compliance at our institution. Nonetheless, a probable nosocomial transmission of *Leclercia* sp. from the housekeeping environment to a patient was detected by this extensive surveillance. These data and analyses further our understanding of CPOs in the hospital environment and are broadly relevant to the design of infection control strategies in many infrastructure settings.

IMPORTANCE Carbapenemase-producing organisms (CPOs) are a global concern because of the morbidity and mortality associated with these resistant Gram-negative bacteria. Horizontal plasmid transfer spreads the resistance mechanism to new bacteria, and understanding the plasmid ecology of the hospital environment can assist in the design of control strategies to prevent nosocomial infections. A 5-year genomic and epidemiological survey was undertaken to study the CPOs in the patient-accessible environment, as well as in the plumbing system removed from the patient. This comprehensive survey revealed a vast, unappreciated reservoir of CPOs in wastewater, which was in contrast to the low positivity rate in both the patient population and the patient-accessible environment. While there were few patient-environmental isolate associations, there were plasmid backbones common to both populations. These results are relevant to all hospitals for which CPO colonization may not yet be defined through extensive surveillance.

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The Centers for Disease Control and Prevention determined that nearly 4% of the patients in acute-care hospitals in 2011 had contracted a hospital-acquired infection (HAI), with a projected estimate of 648,000 patients having had at least one HAI in United States acute-care hospitals in 2011 (1). HAIs caused by carbapenemase-producing organisms (CPOs) are of particular concern because of the substantial rates of morbidity and mortality associated with these infections (2). Carbapenemases are beta-lactamase enzymes (*bla*) that hydrolyze carbapenems, a family of antimicrobials considered to be a last line of defense against infections caused by multidrug-resistant organisms (MDROs). The most commonly detected carbapenemases in the United States include bla_{KPC}, bla_{NDM}, and bla_{OXA} (3). In 2011, the NIH Clinical Center (NIHCC) experienced an outbreak of bla_{KPC}-positive (bla_{KPC}^{+}) *Klebsiella pneumoniae* that ultimately affected 19 patients. Whole-genome sequencing (WGS) was used to track the outbreak and elucidate the chain of transmission from the index patient (4). During the 2011-2012 investigation, bla_{KPC}^{+} *K. pneumoniae* was also cultured from a handrail, a ventilator, sink drains, and surfaces within a patient's room (4, 5). Since then, extensive routine perirectal surveillance of all high-risk patients has been performed, along with environmental sampling throughout the hospital. All bla_{KPC}/bla_{NDM} PCR-positive environmental and newly identified clinical and/or patient surveillance isolates have been sequenced for both epidemiological and research purposes. The NIHCC has a very low prevalence of bla_{KPC}, bla_{NDM}, and bla_{OXA} CPOs (0.35% of patients for perirectal surveillance and 0.14% of patients for clinical cultures between 2012 and 2016), with the majority of CPO-colonized patients detected by culture-based screening at the time of admission. Notably, we have identified very few bla_{OXA}^{+} isolates; therefore, our focus for environmental screening was on CPOs that were bla_{KPC}^{+} or bla_{NDM}^{+} by PCR.

Environmental surveillance can be a useful tool to identify the source of an outbreak (6), to better understand the microbial communities within the hospital (7), and to evaluate the efficacy of environmental disinfection or other infection control measures (8). Sources of outbreaks are occasionally linked to aqueous locations such as sinks and drains (9–12), where the presence of biofilms makes remediation challenging; sink engineering modifications have been proposed to tackle these problems (13, 14). Beyond the sink, hospital sewage and wastewater are known reservoirs of CPOs around the world (15–20). One hypothesis suggests that this reservoir is, in part, due to the use of large quantities of antimicrobial agents in hospitals, which leads to the selection of MDROs and the high likelihood of horizontal gene transfer within the hospital effluent (21). A number of previous surveillance and outbreak studies have focused either on hospital effluent and wastewater treatment plants (WWTPs) (19, 22–24) or on the internal hospital environment only (25). Those studies used PCR, pulsed-field gel electrophoresis (PFGE), and culture methods to track organisms and antimicrobial resistance genes. WGS was often performed only on a small number of isolates and plasmids in these studies (18, 26, 27), limiting the genomic resolution of the analysis. Additionally, there have been limited studies comparing wastewater MDROs with patient isolates (18, 28–31).

To conduct a more in-depth analysis, we investigated the NIHCC environment to (i) determine if additional potential reservoirs of CPOs might provide information to improve our surveillance strategies, (ii) characterize similarities between chromosomes and plasmids of environmental and patient CPOs, and (iii) improve our understanding of the microbial and genetic diversity associated with carbapenemases in the health care environment. To the best of our knowledge, this is the first study to provide a combined genomic analysis of bla_{KPC}/bla_{NDM}-positive isolates from patients, from the accessible environment within the hospital, and from the external hospital effluent.

TABLE 1 Summary of environmental locations surveyed and bla_{KPC}/bla_{NDM}-positive organisms identified between January 2012 and December 2016

Location (no. of isolates)	Total no. of samples	No. (%) with organisms carrying carbapenemase genes	Species identified
Wastewater from manhole (7), pipe (8), sludge (4)	19	15 (78.9)	*Acinetobacter* spp., *Aeromonas* spp., *C. freundii* complex, *Citrobacter* sp., *Enterobacteriaceae*[a] family, *E. cloacae* complex, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Pseudomonas*[b] sp., *Serratia*[c] spp.
Housekeeping closet floor drains (79), equipment (38), surface (11)	128	16 (12.5)	*Acinetobacter* spp. (bla_{NDM}), *Aeromonas* spp., *C. freundii* complex, *E. cloacae* complex, *Leclercia* spp., *Escherichia*[d] sp., *Pantoea* spp., *K. pneumoniae*
Hospital sink drains (285), trap water (5), aerators (32), faucets (12), water (6)	340	11 (3.2)	*K. pneumoniae*, *K. oxytoca*, *E. cloacae* complex, *C. freundii* complex,
High-touch surfaces	217	3 (1.4)	*Pantoea* spp., *K. pneumoniae*
All	704	45 (6.4)	

[a]Not able to assign a genus.
[b]Not *P. aeruginosa*.
[c]Not *S. marcescens*.
[d]Not *E. coli*.

Together, these data broaden our understanding of antimicrobial resistance genes in multidrug-resistant (MDR) bacteria in the environment and hospital settings.

RESULTS

CPOs detected in the hospital environment. Extensive surveillance of the environment (Table 1), perirectal surveillance of high-risk patients upon hospital admission, and monthly whole-house surveillance of all in-house patients are performed at the NIHCC to monitor proactively and to enable an immediate response to the presence of CPOs. The Hospital Epidemiology Service focuses on surfaces accessible to patients and health care providers, particularly sink-related locations and high-touch surfaces such as countertops, handrails, furniture, patient equipment, doorknobs, carts, computers, keyboards, phones, handles, nursing stations, break rooms, ice machines, wheelchairs, elevators, and waiting areas. CPOs were recovered from only 3 (1.4%) of the 217 samples taken from high-touch surfaces over a 5-year period. One isolate was *K. pneumoniae* (KPNIH26) from a handrail that was sampled during the outbreak investigation, and the remaining two isolates were bla_{KPC}^{+} *Pantoea* spp. (PSNIH1 and PSNIH2) cultured from a shelf in an inpatient ward medication storage room and from the handrail of a public staircase, respectively (5). A large number of samples were also collected from sink components, including drains, traps, aerators, and faucets, as well as tap water. Aerators were removed, resuspended in broth prior to culture, and replaced with new parts. No potable water samples or faucet swabs grew CPOs, but 11 (3.2%) of the 340 samples from 10 drains and one aerator culture contained CPOs. Surfaces to which patients do not have direct access also contained CPOs, including 12% of the samples collected from locked housekeeping storage closets (equipment and floor drains) (Table 1).

CPOs detected in wastewater. All seven wastewater samples (100%) collected from the intensive care unit (ICU) piping system contained at least one CPO, a remarkable finding given the low prevalence of CPOs in our patient population. No CPOs were detected from two wastewater collections obtained from a different non-ICU floor of the hospital. Additionally, seven wastewater samples were collected from two external manholes associated with the NIHCC, and CPOs were recovered from every sample collected. The wastewater pipe system appears to be a reservoir for CPOs, even though our data indicate infrequent input of new CPOs on the basis of surveillance testing of all hospitalized patients and a recent point prevalence survey that did not find carriage of MDROs in the intestinal flora of NIHCC health care personnel (32).

Distinct characteristics observed in CPOs from environmental and patient samples. The 72 CPOs from the environment were compared with 36 CPOs from 30

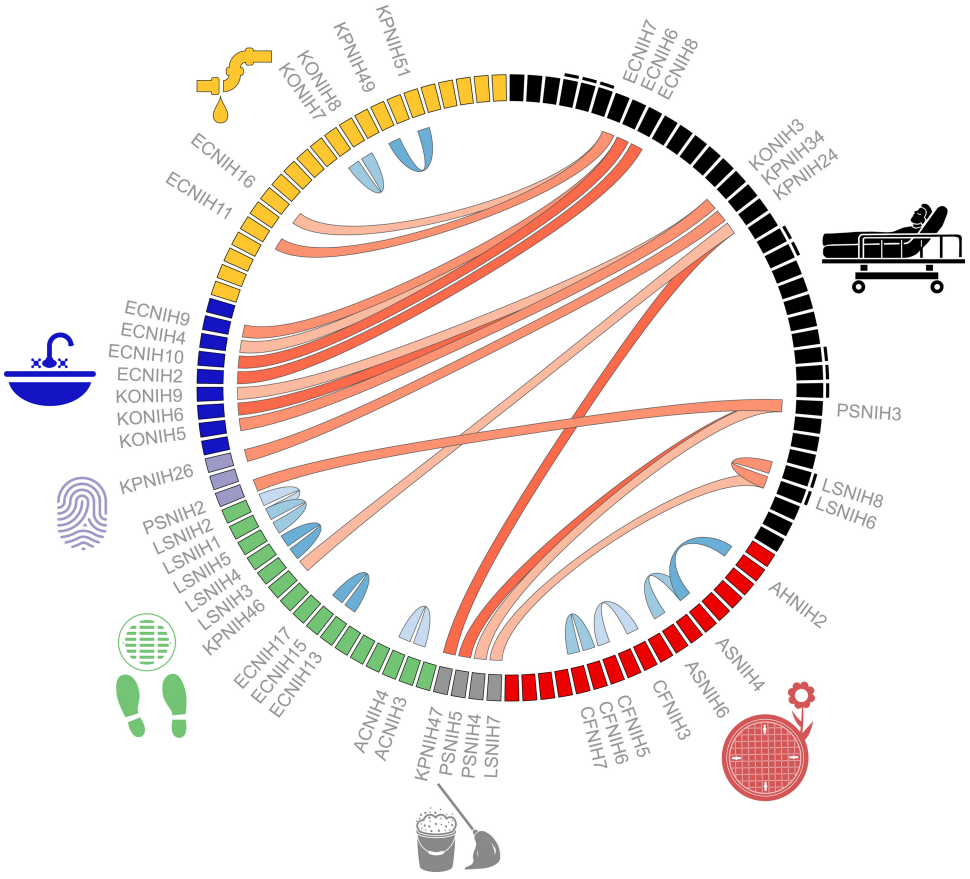

FIG 1 Connections identified on the basis of the genome sequence similarity of CPOs isolated from the environment and patients. Each rectangle in the outer ring represents a sequenced bla_{KPC}-positive isolate and is color coded on the basis of the source. Black, patient; red, wastewater manhole; light gray, housekeeping equipment; green, housekeeping closet drain; purple, high-touch surface; dark blue, sink; yellow, wastewater pipe. Four patients have multiple isolates, and these are clustered and denoted by additional black bars. Orange arcs indicate >99.90% ANI between patient and environmental isolates. Blue arcs indicate >99.90% ANI between environmental isolates. Arc color saturation does not have meaning and is used solely to aid the visual distinction of links. Redundant arcs between environmental isolates are excluded for simplicity. The isolate name is provided only if an environmental connection was found. Icon credits: Alonzo Design, Kathy Konkle, cihanterlan, pialhovik, Panpty, istrejman/Getty Images under license.

patients (Fig. 1), as some patients were colonized with more than 1 CPO. A number of environmental CPO species identified in the wastewater areas were not observed in our patient population, including *Serratia* spp. (not *Serratia marcescens*) in the wastewater and an *Escherichia* sp. (not *Escherichia coli*) found in the housekeeping closet floor drains (see Fig. S2 in the supplemental material). Diverse bla_{KPC}^{+} *Aeromonas* spp. were abundant in our environmental sampling, but only one bla_{KPC}^{+} *Aeromonas* sp. (AHNIH1), which was genetically unrelated to the environmental *Aeromonas* spp., has been detected in a single NIHCC patient to date (33). Surprisingly, no species designation could be assigned to three environmental *Enterobacteriaceae* isolates by using three clinically relevant platforms (matrix-assisted laser desorption ionization–time of flight mass spectrometry [MALDI-TOF MS], 16S rRNA sequencing, and WGS), highlighting carbapenemase genes in uniquely environmental species. The minute volume of wastewater collected for culture compared to the amount within the hospital effluent provided only a narrow representation of the vast wastewater reservoir; thus, the observed diversity of CPOs in the hospital effluent may be much greater than that shown by our data. In contrast to the environmental samples discussed above, commonality was observed between CPO species from sink drains and patients, including the *Enterobacter cloacae* complex, the *Citrobacter freundii* complex, and *Klebsiella oxytoca*.

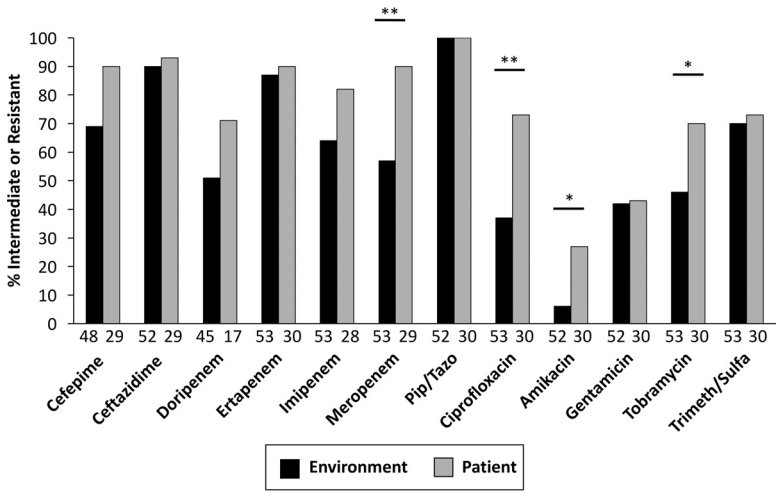

FIG 2 Antimicrobial susceptibility data on bla_{KPC}/bla_{NDM} PCR-positive *Enterobacteriaceae* from patients and the environment between January 2012 and December 2016. *Enterobacteriaceae* isolates were interpreted as intermediate or resistant to the antimicrobial agents on the basis of CLSI M100 guidelines (62). Black bars are isolates from the environment, and gray bars are isolates from patients. The values below the bars are the total numbers of isolates tested. Pip/Tazo, piperacillin-tazobactam; Trimeth/Sulfa, trimethoprim-sulfamethoxazole. (*, two-tailed $P < 0.05$; **, $P < 0.001$; Fisher's exact test.)

Susceptibility profiles of environmental and patient carbapenemase-producing members of the family *Enterobacteriaceae* were compared (Fig. 2; Table S1). Overall, *Enterobacteriaceae* environmental isolates were more susceptible to meropenem, ciprofloxacin, amikacin, and tobramycin than were patient isolates (Fig. 2). Additionally, all of the environmental *Aeromonas* spp. were susceptible to meropenem and imipenem (Table S1). Four environmental *Enterobacteriaceae* isolates were excluded from this specific comparison because we limited the study set to avoid collection bias that might inaccurately show more sensitivity of environmental isolates. Isolates that did not demonstrate growth on HardyCHROM CRE (CRE medium; Hardy Diagnostics) after original recovery from less selective HardyCHROM ESBL (ESBL medium; Hardy Diagnostics) and R2A medium were excluded because CRE medium is used for patient surveillance. Patient isolates recovered on a number of clinical culture media were included.

Potential associations between patient and environmental isolates were identified. Comparison of the genomes of 36 patient isolates and 72 environmental isolates revealed eight instances in which patient isolates showed >99.90% average nucleotide identity (ANI) with environmental isolates (Fig. 1). One *E. cloacae* complex strain (ECNIH7) from patient P, who had been hospitalized earlier in the ICU, had >99.96% ANI with two *E. cloacae* complex isolates from ICU wastewater. No other patient isolates matched any other CPOs from ICU pipe or manhole wastewater. The remaining seven patient isolates showed >99.90% ANI with isolates from sinks, housekeeping closets, and high-touch surfaces. One patient isolate (KPNIH24) showed high identity with two environmental isolates; however, this may be a widely distributed strain with little divergence since genomes with <100 single nucleotide polymorphisms (SNPs) have been identified at several institutions, based upon sequence data available in public databases. Moreover, the epidemiological link is weak, with a 3-year gap between this patient's hospitalization and detection in housekeeping areas (Fig. 1).

Pantoea spp. are known organisms in hospital environments and can be associated with outbreaks (34, 35). In 2015, two patients were found to be colonized with bla_{KPC}^{+} *Pantoea* spp., but epidemiology and genomic data indicated that they were not clonal; PSNIH3 and PSNIH6 showed <90% ANI. The positive surveillance data led to an investigation that identified two environmental *Pantoea* spp. (PSNIH4 and PSNIH5) associated with housekeeping equipment. These two environmental isolates, along with *Pantoea* PSNIH2 isolated from handrails in 2013 (5), showed >99.95% ANI with

patient isolate PSNIH3 (Fig. 1). SNP analysis identified >119 different SNPs between the patient strain and three environmental strains, which suggested closely related, but not clonal, strains. These findings emphasize the ability of *Pantoea* spp. to survive on different hospital environmental surfaces, as previously reported (5, 34).

Sink drains are a known reservoir of CPOs (9–11). We detected three examples of patient isolates showing >99.90% ANI with sink drain isolates (Fig. 1). *E. cloacae* complex isolate ECNIH6, detected in a patient in 2014, showed >99.95% ANI with three sink isolates (ECNIH4, ECNIH9, and ECNIH10) recovered from two different rooms in 2012; however, the patient was never housed in either room. Another common species identified in the sink cultures, *K. oxytoca*, has been implicated in biofilm-associated sink contamination in other hospitals (10, 11). *K. oxytoca* sink drain isolates (KONIH5 and KONIH6) were cultured from two sink drains in a room that housed patient S, who was identified as carrying KONIH3 four months prior. A third sink drain isolate (KONIH9) was detected a year later, despite pipe removal and thorough cleaning with wire brushes and bleach. (Fig. 1; Table S2). All four isolates (one from a patient, three from two sinks) show >99.99% ANI, suggesting that they are derivative isolates (Fig. 1).

Finally, a genetic connection was established between a patient isolate and a sink drain as part of a continuation of a previously published investigation (5). Sink drain isolate ECNIH2 (January 2012) carried three different bla_{KPC}-containing plasmids. One of the bla_{KPC}-containing plasmids (pKEC-39c) was traced to a plasmid in *K. pneumoniae* KPNIH27 from patient A, who stayed in that room from November 2011 to January 2012. At the time of our original study, we had no explanation for the other two bla_{KPC}-containing plasmids in ECNIH2, pKPC-272 and pKPC-f91. On further analysis, we identified a bla_{KPC}^{+} *E. cloacae* isolate from patient Y (ECNIH8), who occupied the same room (November 2010 to February 2011). This isolate is the likely recipient of the pKEC-39c plasmid. The ECNIH8 chromosome (from February 2011) differs from ECNIH2 (January 2012) by only three SNPs (99% coverage) and carries the pKPC-272 (0 SNPs; 99% coverage) and pKPC-f91 (0 SNPs; 92% coverage) plasmids. Figure 3 shows a proposed model of the persistent colonization of the sink drain and subsequent plasmid transfer. Together, these data demonstrate long-term persistence of CPOs in sink drains and the transfer of CPOs from patients to the sink drain environment. Although it is rare to capture the details of plasmid transfer as exemplified here, transfer of plasmids in sink biofilms may be a frequent occurrence and warrants further study.

Genomic analyses of bla_{KPC}-containing plasmids from environmental and patient samples illustrate a broad diversity of plasmid configurations in many species. Because transfer of carbapenemases carried on plasmids between organisms is a public health concern (36), we moved our analysis beyond a chromosomal comparison to a plasmid analysis with greater resolution. From this data set, each bla_{KPC}-containing plasmid was represented with at least one fully assembled PacBio genome. These high-quality PacBio genomes served as references and enabled us to resolve the plasmid sequences of related genomes, which may have only received short-read sequencing. Similarly, genomes with limited nucleotide identity to any previously sequenced isolate were also subjected to PacBio sequencing. In total, our analysis included 2 genomes from Roche 454 sequencing, 55 from Illumina MiSeq sequencing, and 51 from PacBio sequencing. The plasmid sequences of patient and environmental isolates from MiSeq and PacBio genomes were compared by using their k-mer composition. Sixteen-base-pair k-mers were calculated for all assemblies by using meryl (http://kmer.sourceforge.net/), and a specific bla_{KPC}-containing plasmid was defined as present if ≥95% plasmid k-mers were found in the assembly (Fig. 4; Table S2).

Some bla_{KPC}-containing plasmids were grouped together into families of plasmids for analysis purposes. For instance, newly detected pKPC-8bc0 and pKPC-79f0 were typed as incompatibility group N (IncN) (PubMLST) and exhibited a high level of genetic similarity to other IncN ST6 plasmids previously detected at NIHCC (5) (Fig. S3). We have identified this pervasive IncN family of plasmids in 7 patient and 23 environmental isolates, including high-touch surfaces, sink drains, housekeeping closets, and ICU pipe wastewater (Fig. 4). Similar to the IncN family, five newly identified bla_{KPC}-containing

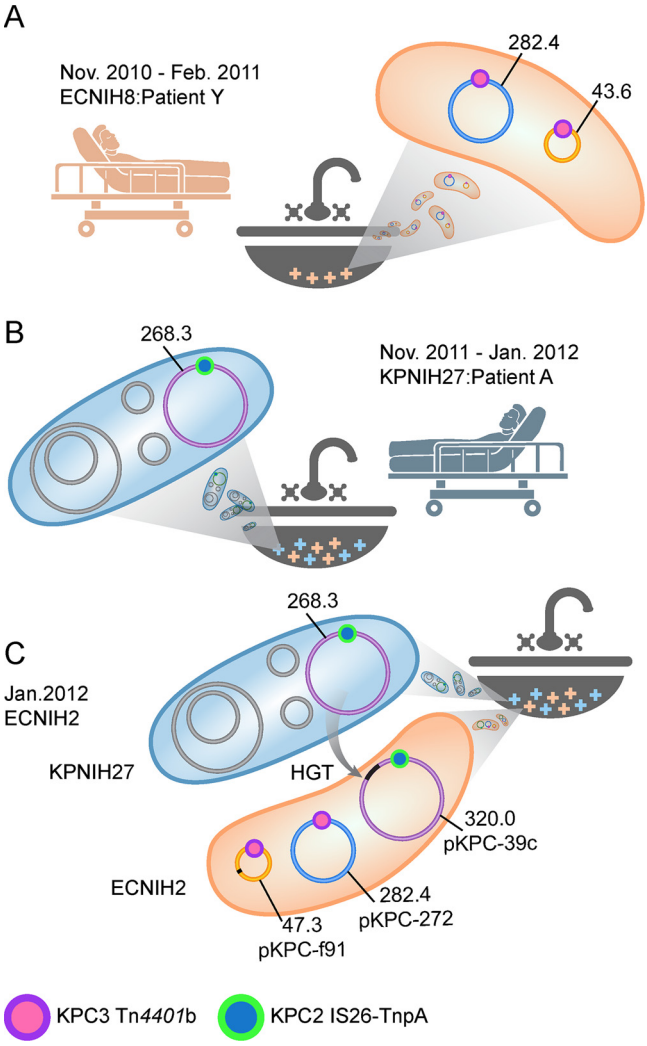

FIG 3 CPOs are able to persist and recombine in sink drains. (A) In February 2011, *E. cloacae* carrying two bla_{KPC}^{+} plasmids was cultured from patient Y. This organism may have also colonized a sink in the patient's room. (B) Eleven months later (January 2012), an isolate from patient A, who was colonized with bla_{KPC}^{+} *K. pneumoniae* upon admission, was likely introduced into the same sink (KPNIH27). (C) KPNIH27's pKPC-39c plasmid in a sink drain isolate is hypothesized to have horizontally transferred to the sink drain isolate from patient Y, generating strain ECNIH2. Plasmids carrying the bla_{KPC} gene are colored, and bla_{KPC} genes are marked by blue (KPC-2) or pink (KPC-3) circles. An insertion in the pKPC-39c plasmid is black. Non-KPC plasmids are gray. Selected plasmids are labeled with their size in kilobases. *E. cloacae* and *K. pneumoniae* isolates are orange and blue, respectively.

plasmids, which we have named the pENT-e56 family, could be grouped on the basis of sequence similarity. These plasmids were detected in 1 patient and 12 environmental isolates (Fig. 4). Interestingly, the backbone of this family of bla_{KPC}-containing plasmids was also detected in patient and sink isolates that lacked bla_{KPC} and the flanking transposon (pKPN-068, pENT-e56, and pENT-d0d) (Fig. 5), suggesting possible evolution of these plasmids through addition of the transposon.

Our study identified 27 new plasmids with carbapenemase genes based on PacBio sequencing (Table S2). To date, only a small number of these plasmids were recovered from our patient population (Fig. 4). Despite limited sampling, we detected six different bla_{KPC}-containing plasmids in *Aeromonas* spp. isolated from manhole wastewater, highlighting the high plasmid diversity within this genus. Plasmid sizes ranged from 20 to >300 kb, and large plasmids (>150 kb) were detected in both wastewater and our patient population. Interestingly, pKpQIL, a dominant bla_{KPC}-containing plasmid

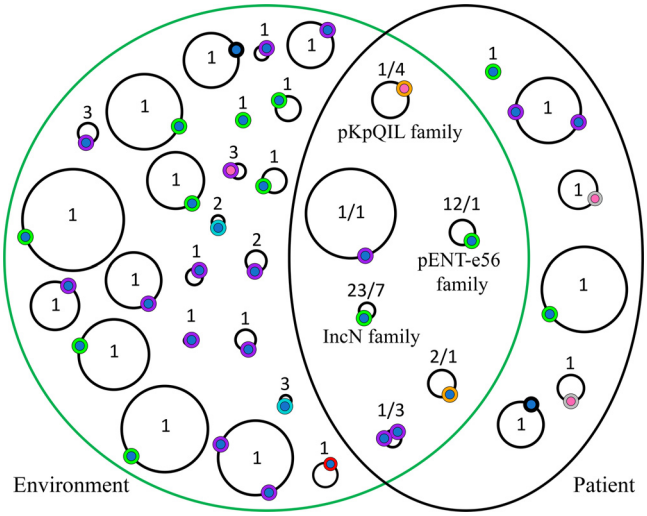

FIG 4 Venn diagram of bla_{KPC}-containing plasmids detected in the environment and patients. The presence of plasmids in isolates from patients and/or the environment was based on a k-mer inclusion approach for which a plasmid was considered present within a genome assembly if ≥95% of the plasmid k-mers were contained within the MiSeq genome assembly k-mers. Each value is the number of isolates detected in this study that carry the specified bla_{KPC}-containing plasmid. In the overlapping region, the ratio indicates the number of environmental isolates/number of patient isolates. Plasmids are drawn to scale. The inner circle represents the bla_{KPC} allele, and the outer circle represents the transposon. The bla_{KPC} alleles are blue for bla_{KPC2} and pink for bla_{KPC3}. Color was used to differentiate the flanking sequence as follows: orange, Tn4401a; purple, Tn4401b; red, Tn4401b-IS630; gray, Tn4401d; green, IS26-TnpA; light blue, Ahyd_WCHAH01; black, not determined.

among *K. pneumoniae* strains in our patient population, was not found in any of our environmental samples after the outbreak investigation in 2012.

Whereas most of the CPOs we detected carried the bla_{KPC} gene, between 2014 and 2016, bla_{NDM-1}^{+} *Acinetobacter* spp. were detected in two patients and two housekeeping closet drains. Chromosomally, no link could be demonstrated among these isolates, as they belonged to different strains or species. However, the bla_{NDM-1} gene is carried on a plasmid that has been described previously in other *Acinetobacter* spp., including *Acinetobacter lwoffii*, *Acinetobacter baumannii*, *Acinetobacter bereziniae*, and *Acinetobacter schindleri* (37–41). One of the first descriptions of this plasmid backbone was in pNDM-BJ01 (JQ001791.1) (38), and variants missing a 6-kb region encoding GroE proteins have also been described (e.g., pNDM-JN02) (42). All four isolates in this study carried a bla_{NDM-1}-containing plasmid similar to pNDM-JN02 but with a second small deletion encompassing the *trpF-nagA* genes and two SNPs. Genomic and epidemiologic data could not link plasmid transmission between the housekeeping closet drains and patient isolates, which suggests that this plasmid is widespread among *Acinetobacter* spp. beyond our institution and demonstrates a potential to persist in the environment.

Probable nosocomial transmission from environment to patient. In June 2016, a bla_{KPC}^{+} *Leclercia* sp. was isolated from the interior of a closet floor drain. *Leclercia* is a genus within the *Enterobacteriaceae* family. *Leclercia adecarboxylata*, the only species of this genus whose complete genome sequences is available, has been described as an opportunistic pathogen and occasional carrier of bla_{NDM-1} (43). A few cases of *L. adecarboxylata* infection have been documented (44–46), and at least one report describes a case of hospital-acquired pneumonia likely due to *L. adecarboxylata* (47). In August 2016, bla_{KPC}^{+} *Leclercia* sp. bacteria grew from swabs of additional housekeeping closet floor drains. In September 2016, routine perirectal surveillance revealed a new-onset bla_{KPC}^{+} *Leclercia* colonization in a hospitalized stem cell transplant recipient, patient V. In response, the Hospital Epidemiology Service collected 39 additional cultures from sink drains, housekeeping closet floor drains, nursing station surfaces, isolation carts, and patient care equipment. Given our knowledge of housekeeping

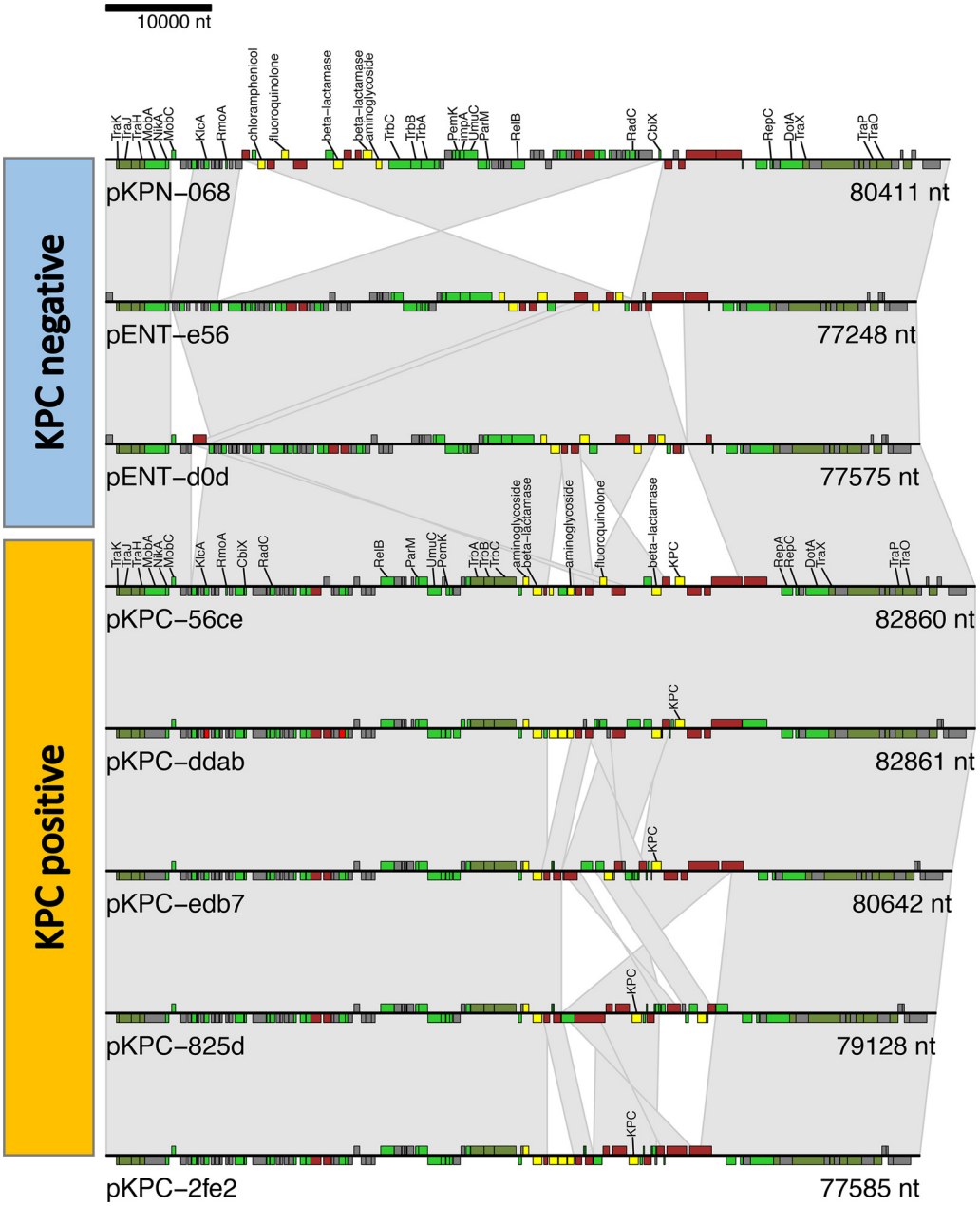

FIG 5 Genetic diversity of the pENT-e56-family of plasmids. Alignment of genetically similar bla_{KPC}-negative (top three) and bla_{KPC}-positive (bottom five) plasmids. Gray ribbons indicate regions of homology (>99.90% sequence similarity). Antimicrobial resistance genes are yellow, *tra* genes are dark green, transposase/resolvase genes are brown, and hypothetical genes are gray. Differences between the annotations of aligned regions are largely due to changes in the annotation pipeline and databases; genomes were annotated at the time of sequencing by using PGAP (versions 2.1 to 4.2).

closet floor drain isolates found in the preceding months, we reviewed housekeeping procedures and collected additional cultures from housekeeping equipment as potential transmission vectors. Review of housekeeping practices identified mop buckets as a likely point source because of their use in both patient care areas and housekeeping closets with colonized floor drains; though individual buckets are not assigned to specific floors. Seven mop buckets were sequestered and swabbed for CPO culture. Of the 39 additional samples collected, one culture from a single mop bucket grew bla_{KPC}^{+} *Leclercia* sp.

Overall, our investigation identified eight *Leclercia* isolates: two from patient V (perirectal and bronchoalveolar lavage samples), five from housekeeping closet floor

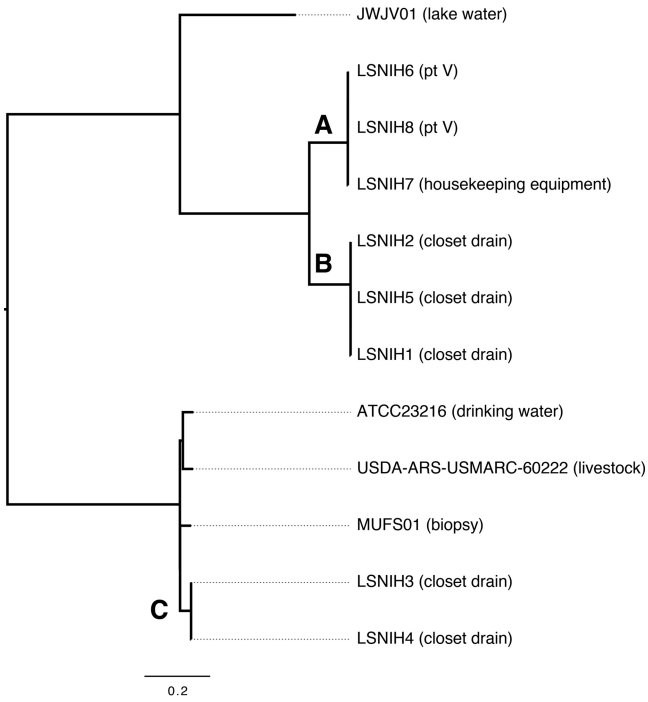

FIG 6 Phylogenetic tree of *Leclercia* spp. based on core genome alignment (ParSNP v 1.2). The tree includes eight genomes from this study (LSNIH1 to -8), two publicly available environmental isolates (NCBI accession no. JWJV01 and MUFS01), and two *L. adecarboxylata* reference strains (ATCC 23216, USDA-ARS-USMARC-60222). Isolation sources are indicated in parentheses. Core genome alignments supporting this tree covered 36% of the reference sequence.

drains, and one from a mop bucket. We compared the genome sequences by using Mash and found that the patient isolates were very similar to each other (99.99%) and to the isolate from the mop bucket (99.91%) (clade A). The remaining five isolates fell into two clusters, B and C, that differed from the patient cluster by >100,000 SNPs, corresponding to 97 and 93% identity, respectively. All three clusters carried a bla_{KPC}-containing plasmid belonging to the IncN family, a >300-kb plasmid related to plasmids (pPSP-75c and pPSP-a3c) previously identified in *Pantoea* spp., and one or two additional plasmids. We constructed a core genome phylogeny (36% of the genome) by using ParSNP (48) to compare our isolates to existing *L. adecarboxylata* genomes and found that cluster C, made up of two drain isolates, is closely related to three of four publicly available *L. adecarboxylata* genomes, including the type strain *L. adecarboxylata* ATCC 23216 (Fig. 6), suggesting identification to the species level. The A and B clades, on the other hand, may belong to a different species or subspecies. Core genome-based phylogeny was chosen over rRNA-based phylogeny because 16S rRNA sequences are unreliable for the differentiation of species of the genera *Enterobacter*, *Leclercia*, and *Citrobacter* (49). The similarity between the patient isolates and the mop bucket isolate suggests a possible common source in our hospital; however, the 166 SNPs identified (excluding a variable phage region) and their even distribution throughout the genome suggest indirect transmission. Genomic data were not sufficient to resolve the direction of transmission; however, because this organism had not been recovered from previous extensive surveillance of patients, and given our finding of six isolates belonging to at least three different clades in the hospital environment, we concluded that it was likely transmitted from an environmental source to patient V.

DISCUSSION

The presence of CPOs in the hospital environment is concerning because of the potential for spread to an immunocompromised or otherwise vulnerable patient population and because of the transmissibility of carbapenemase genes by mobile

genetic elements to other bacteria within the hospital and into the community (50). Whereas our study was not designed to investigate the water treatment systems in the community, other studies have tested the efficacy of WWTPs receiving waste from pharmaceutical drug manufacturers (51) and hospitals (24, 30, 52) or environmental water such as rivers upstream or downstream of WWTPs (53). These studies have detected MDROs throughout different steps of the treatment process, noting a reduction in numbers, but not elimination, after processing.

There have been many publications of hospital environmental investigations during outbreaks and some during nonoutbreak periods (20, 25, 27, 54, 55). Findings from the 2011 *bla*$_{KPC}^{+}$ *K. pneumoniae* outbreak investigation at NIHCC led to changes in our infection control policies, such as implementation of environmental surveillance cultures in response to newly identified CPO carriers and surveillance testing of previously positive sites after remediation. Additional testing of wastewater from internal pipes and external manholes was included in this study to identify possible new environmental CPO reservoirs that may further characterize the plasmid ecosystem. The genomic analyses of 108 identified CPOs derived from the screening of >9,000 patients and >700 environmental samples over the last 5 years have improved our understanding of CPO and plasmid relationships within our hospital and surrounding environment.

Overall, the NIHCC had a very low rate of CPO positivity on high-touch surfaces sampled within the hospital (1.4%), a higher positivity rate in sinks (3.2%) and housekeeping closets (12.5%), and an even higher positivity rate in wastewater (78.9%). For comparison, a 2.5-year multicenter prospective survey of high-touch surfaces in patient rooms found that ~40% of the rooms examined were contaminated with MDROs, including Gram-positive and Gram-negative organisms such as vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus*, MDR *K. pneumoniae*, and MDR *A. baumannii*. We did not investigate Gram-positive organisms in this study; furthermore, WGS was not used to investigate genetic relatedness in that study, so the possible comparisons of studies are limited (56).

In our study, environmental CPO isolates appeared to be more susceptible to antimicrobial agents than CPOs isolated from patients, possibly related to selective pressure of antimicrobial agents administered to patients. The diversity of *bla*$_{KPC}^{+}$ species in our hospital environment, specifically in wastewater, supports the hypothesis that environmental species can act as carriers of antimicrobial resistance genes and associated mobile elements, which could lead to transmission to clinically significant species (57). Further, we detected examples of highly similar plasmid backbones with and without the *bla*$_{KPC}$ gene. This phenomenon has been described with pKPC_UVA01-like plasmids at another hospital (58). These findings point to the utility of sequencing all identified plasmids, not just plasmids with carbapenemase genes, as these may provide the backbones that evolve into new resistance gene-containing plasmids.

One goal of this study was to characterize the plasmid population in our hospital, particularly areas proximal to the patient that are more likely to be a potential source of transmission, as well as areas more distal and less likely to be a direct source of transmission. In our hospital, the most clinically concerning CPO has been *K. pneumoniae* carrying the pKpQIL plasmid, the organism associated with the 2011 outbreak. This clonal strain was recovered repeatedly for several years from two patients who acquired the organism during the outbreak and remained persistently colonized (4, 5). However, surprisingly, pKpQIL was not detected in any environmental isolates after July 2012. This suggests that either the pKpQIL plasmid or the *K. pneumoniae* host was not well adapted to environmental persistence. Likewise, *bla*$_{NDM}$-containing plasmids have not been disseminated broadly in our hospital environment; only one common *bla*$_{NDM-1}$ plasmid backbone was detected in *Acinetobacter* spp. from two environmental samples. In contrast, there were common *bla*$_{KPC}$-containing plasmid backbones found in both environmental and patient isolates, including the IncN family of plasmids, which was detected in 7 patient and 23 environmental isolates, strongly supporting the broad host range of this plasmid family. A newly detected plasmid backbone, pENT-e56 family, was also found in 1 patient and 12 environmental isolates. However, we

detected 23 bla_{KPC}-containing plasmids exclusively in the environment. Others have described even more extensive plasmid diversity in freshwater (59) and WWTPs (53).

Environmental sources have been implicated as the cause of many hospital outbreaks (6, 60). Because wastewater is a known source of CPOs (15, 16, 19), it is striking that so few bacterial strains or plasmids were found to be shared by wastewater and patients in this study. Our study results align with findings from a few other investigations; one was a study of sewage from four different hospitals that could not correlate organisms or carbapenemase genes between patient and sewage isolates (16). Our data support the conclusion that wastewater cannot be used as a marker of patient colonization status. Another group sampled one hospital wastewater source on 16 separate occasions over a period of 2 months and analyzed 17 carbapenemase-producing *Enterobacteriaceae* isolates by PFGE. WGS of 3 of the 17 isolates was performed (18). No link could be established between wastewater and patient isolates. Important limitations of our study that could affect the interpretation of results include a very small cumulative sample volume, lack of replicate testing from the same source, and variation in culture workup.

Although few clonal connections between wastewater and patient isolates were established, we did identify several sink drain isolates that were highly similar, if not clonal, to patient isolates. A recent study by Kotay et al. experimentally showed the ease with which *E. coli* can disperse from a sink after seeding (61). We also detected other CPOs in sinks and housekeeping closets that were highly related to patient isolates, but an epidemiological link could not be established. This lack of an epidemiological connection suggests the possibility that dominant but unrelated CPOs may be in both patient and environment populations, as exemplified in a study where ESBL-producing members of the family *Enterobacteriaceae* were found in handwashing sinks in the absence of colonized patients (34). However, the lack of an identified epidemiological link could also be due to sampling error or low sensitivity of patient microbial surveillance. It is a constant challenge for infection control teams to distinguish nosocomial transmission from coincidentally recovered isolates. Detailed chromosomal and plasmid data, such as the analyses presented here, can greatly assist in the decision algorithm.

On the basis of our findings, we suggest that hospital environmental surveillance should also include housekeeping areas in addition to high-touch surfaces and sink drains. bla_{KPC}^{+} *Leclercia* spp. were not detected in NIHCC until 2016, when they were first identified in five different housekeeping closet floor drains. These five drain isolates, the isolate from a mop bucket, and two patient isolates formed three different clusters. These findings suggest that multiple strains of *Leclercia* were present in the hospital environment for some time before patient colonization occurred. *Leclercia* is not a common pathogen, and this was our first identification of bla_{KPC}^{+} *Leclercia* spp. Given that our surveillance program is more extensive than what many hospitals can support, the finding leads one to consider what might be found if more hospitals were investigated to this extent. Our data highlight the importance of consistent and proactive patient and environmental CPO surveillance. The investigation of newly identified CPO carriers, along with an immediate examination of hygiene practices, followed by remediation of the environment, are all critical actions to control environmental contamination and stop the potential spread of organisms within the patient population.

The extensive surveillance performed at the NIHCC has revealed CPOs in the environment; however, it is likely that most hospitals have some CPO colonization in wastewater and drains that remains undetected, perhaps because these sites are not sampled routinely or as part of investigative processes. The higher rates of CPO detection in water-associated environments, such as ICU pipes, sinks, housekeeping closet drains, and manhole wastewater, suggest that the hospital drainage system plays a role in providing a stable and underappreciated reservoir of MDROs within hospitals. The physical separation of wastewater from patients, combined with proper cleaning practices and active infection control responses, was largely sufficient to prevent

transmission to patients, as few clonal isolates were detected in patients and the environment. One might expect to see more transmission if there were a breakdown of good practices. The diversity of CPO-associated plasmids and transposon elements from the environment and the isolation of similar plasmid backbones with and without the bla_{KPC} gene prompt a greater appreciation of the extensive nature and mobility of plasmids in the environment.

MATERIALS AND METHODS

Setting and sample collection. Environmental samples were collected between January 2012 and December 2016 at the NIHCC (Bethesda, MD), a 200-bed clinical research hospital (Table 1). Seven wastewater samples were collected from two manholes outside the hospital, and nine wastewater samples were collected from the pipes in interstitial spaces serving the ICU and non-ICU rooms. Water samples were collected from faucets and traps of sinks in patient rooms and common areas. Surfaces were sampled with BBL culture swabs (Becton, Dickinson and Company, Franklin Lakes, NJ) moistened with saline or gauze incubated in 10 ml of tryptic soy broth (TSB; Hardy Diagnostics, Santa Maria, CA). Approximately six inches of pipe immediately below the surfaces of the sink drains was sampled with moistened BBL culture swabs (Becton, Dickinson and Company).

Sample processing, identification, and characterization of bacterial isolates. Swabs were transferred into 1 ml of TSB (Remel Inc., Lenexa, KS), vortexed, and plated directly. Gauze in 10 ml of TSB was incubated overnight at 35°C in 5% CO_2 prior to culture. Wastewater samples were plated directly. One drop of each sample suspension was cultured on CRE, ESBL, and R2A (Remel Inc.) media. A subset of environmental samples was also cultured on 5% sheep blood agar (SB; Remel Inc.). Perirectal surveillance cultures were plated directly onto CRE medium. Clinical cultures were plated onto routine clinical microbiology media, which included a combination of SB, chocolate agar (Remel Inc.), MacConkey agar (Remel Inc.), and/or Fastidious Broth (Hardy Diagnostics; Fig. S1). HardyCHROM media were incubated overnight at 35°C in ambient air, and R2A medium was incubated at 30°C in ambient air. Clinical cultures were incubated at 35°C in 5% CO_2. Colonies with different morphologies were isolated and screened by PCR for the presence of bla_{KPC} or bla_{NDM} (http://www.cdc.gov/HAI/pdfs/labSettings/KPC-NDM-protocol-2011.pdf). All isolates that tested positive for bla_{KPC} or bla_{NDM} by PCR were identified by MALDI-TOF MS (BioTyper; Bruker, Billerica, MA). Susceptibility testing was performed via broth microdilution (Trek Diagnostics, Oakwood Village, OH, or BD Phoenix, Franklin Lakes, NJ, depending on the date of isolation) or disk diffusion (Thermo Fisher Scientific, Lenexa, KS). Susceptibility interpretations were based on the most recent Clinical and Laboratory Standards Institute (CLSI) breakpoints (62) (Table S1). For this study, given our environmental screening approach, we classified as a CPO any isolate that was bla_{KPC}^{+} or bla_{NDM}^{+} by PCR. For the antimicrobial susceptibility comparison of members of the family *Enterobacteriaceae* and to eliminate bias potentially caused by the use of different culture media for clinical and environmental testing, bla_{KPC}^{+} or bla_{NDM}^{+} environmental *Enterobacteriaceae* isolates from ESBL or R2A medium that were susceptible to two or more carbapenems were tested for growth selectivity on CRE medium in accordance with CLSI M22-A3 guidelines (63). Briefly, a 0.5 McFarland suspension in saline was prepared and diluted 1:10. Ten microliters was inoculated onto CRE medium and incubated at 35°C in ambient air for 18 to 24 h. Only isolates that grew on this confirmatory CRE agar screen were included in the susceptibility comparison.

Genome sequencing and assembly. Genomic DNA extraction and library preparation for Illumina MiSeq and single-molecule real-time (SMRT) sequencing were performed as described previously (64, 65). Paired-end MiSeq reads were assembled into contigs by using SPAdes version 3.5.1 (66) and further polished by using Pilon (67). Scaffolding of refined genome assemblies to reference genomes was performed by using ABACAS version 1.3.1 (68). PacBio RSII SMRT sequencing reads (P6 polymerase binding and C4 sequencing kit, 240-min collection) were assembled by using HGAP3 (PacBio SMRT Analysis version 2.3) or CANU (version 1.3, 1.4, or 1.5) (69). The resulting contigs were polished by using Quiver (SMRT Analysis version 2.3) and manually reviewed and finished by using Gepard (version 1.3) (70). Base calling and finishing accuracy was assessed by using the Most Probable Genotype (71) software with Illumina MiSeq read alignments against the finished PacBio assembly. The Integrative Genomics Viewer (72) was used to visualize the MiSeq read alignments. Genome annotation was performed by using the National Center for Biotechnology Information (NCBI) prokaryotic Genome Annotation Pipeline (PGAP, https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Bioinformatic analyses. Genetic relatedness between genome assemblies was assessed by ANI and SNPs. ANI was estimated by using Mash version 1.1.1 with a k-mer size of 21 and a sketch size of 1,000 (73), while SNPs were called by using Snippy version 3.2 (BWA-MEM version 0.7.15, freebayes version 1.0.2) (https://github.com/tseemann/snippy). All SNPs required a read depth of ≥10 reads with ≥90% of those aligned reads supporting the variant nucleotide call. Core genome alignments were performed by using Parsnp and visualized by using Gingr (48).

Plasmid detection in shotgun assemblies was determined by using a k-mer inclusion approach. Briefly, 16-bp k-mers were generated from the fully resolved PacBio plasmid sequences along with the MiSeq genome assemblies by using meryl (13 May 2015 release, http://kmer.sourceforge.net/). A plasmid was considered present within a genome assembly if ≥95% of the plasmid k-mers were contained within the MiSeq genome assembly k-mers. A similar k-mer inclusion analysis of the PacBio-generated bla_{KPC}-positive and bla_{KPC}-negative plasmid sequences was performed. Plasmid coverage and alignments were computed by using NUCmer version 3.1 (74), laid out by using a custom Perl script, and visualized in R.

Data and material availability. This report references genomic data from previous studies (PRJNA251756). Genomic data generated for this report have been deposited under project no. PRJNA430442. Isolates can be obtained from K.M.F.; a material transfer agreement is necessary.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02011-17.

FIG S1, PDF file, 0.01 MB.

FIG S2, PDF file, 0.02 MB.

FIG S3, PDF file, 0.05 MB.

TABLE S1, XLSX file, 0.1 MB.

TABLE S2, XLSX file, 0.04 MB.

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