RESEARCH ARTICLE

Molecular Dissection of an Outbreak of Carbapenem-Resistant Enterobacteriaceae Reveals Intergenus KPC Carbapenemase Transmission through a Promiscuous Plasmid

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ABSTRACT **Carbapenem-resistant *Enterobacteriaceae* (CRE) have emerged as major causes of health care-associated infections worldwide. This diverse collection of organisms with various resistance mechanisms is associated with increased lengths of hospitalization, costs of care, morbidity, and mortality. The global spread of CRE has largely been attributed to dissemination of a dominant strain of *Klebsiella pneumoniae* producing a serine β-lactamase, termed *K. pneumoniae* carbapenemase (KPC). Here we report an outbreak of KPC-producing CRE infections in which the degree of horizontal transmission between strains and species of a promiscuous plasmid is unprecedented. Sixteen isolates, comprising 11 unique strains, 6 species, and 4 genera of bacteria, were obtained from 14 patients over the first 8 months of the outbreak. Of the 11 unique strains, 9 harbored the same highly promiscuous plasmid carrying the KPC gene bla_{KPC}. The remaining strains harbored distinct bla_{KPC} plasmids, one of which was carried in a strain of *Klebsiella oxytoca* coisolated from the index patient and the other generated from transposition of the bla_{KPC} element Tn*4401*. All isolates could be genetically traced to the index patient. Molecular epidemiological investigation of the outbreak was aided by the adaptation of nested arbitrary PCR (ARB-PCR) for rapid plasmid identification. This detailed molecular genetic analysis, combined with traditional epidemiological investigation, provides insights into the highly fluid dynamics of drug resistance transmission during the outbreak.**

IMPORTANCE **The ease of horizontal transmission of carbapenemase resistance plasmids across strains, species, and genera of bacteria observed in this study has several important public health and epidemiological implications. First, it has the potential to promote dissemination of carbapenem resistance to new populations of *Enterobacteriaceae*, including organisms of low virulence, leading to the establishment of reservoirs of carbapenem resistance genes in patients and/or the environment and of high virulence, raising the specter of untreatable community-associated infections. Second, recognition of plasmid-mediated outbreaks, such as those described here, is problematic because analysis of resistance plasmids from clinical isolates is laborious and technically challenging. Adaptation of nested arbitrary PCR (ARB-PCR) to investigate the plasmid outbreak facilitated our investigation, and the method may be broadly applicable to other outbreaks due to other conserved mobile genetic elements. Whether infection control measures that focus on preventing transmission of drug-resistant clones are effective in controlling dissemination of these elements is unknown.**

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Enterobacteriaceae are responsible for a significant number of infections and death in the United States and worldwide each year, and the prevalence of antibiotic resistance in this family of bacteria continues to rise (1). One reason for this increase has been the dissemination of *Klebsiella pneumoniae* carbapenemase (KPC), a class A serine carbapenemase first isolated from *K. pneumoniae* in 1996 (2). bla_{KPC} encodes KPC and is carried within the conserved Tn*3* family transposon Tn*4401* on transferable plasmids (3). A dominant strain of KPC-producing *K. pneumoniae*, multilocus sequence type (ST) 258, has disseminated throughout the United States and other parts of the world (4, 5). Institutional outbreaks of KPC-producing *Enterobacteriaceae* due to the spread of a single strain have also been reported for other species, including *Enterobacter* spp. and *Serratia marcescens* (6, 7). While these reports highlight the clonal nature of dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE), several cases of horizontal transfer of bla_{KPC} through transferable plasmids have also been described (7–9).

Tn*4401*, a 10-kb Tn*3* family transposon, serves as the genetic support structure for bla_{KPC} and is highly conserved. Tn*4401* has been characterized in more than seven different plasmids, ranging 12 to 80 kb in size, each with a distinct in-

TABLE 1 Isolate description and carbapenem susceptibilities[a]

Patient	Isolate	Species	Date of first isolation[b]	Source(s)	MIC (μg/ml) determined by Vitek 2		Imipenem disk diffusion (mm)	Pulsed-field group	Sequence group	Plasmid	Outcome
					Imipenem	Ertapenem					
1	Kox1015	*Klebsiella oxytoca*	8/31/2007	Blood, drain, BAL fluid	4	**≥8**	16	pfg-kox1	sg2	pUVA02	Died
	Kpn1016	*Klebsiella pneumoniae*	8/31/2007	Drain, sputum	**≥16**	**≥8**	16	pfg-kpn1	sg1	pUVA01	Died
2	Kpn1017	*Klebsiella pneumoniae*	10/05/2007	Blood, sputum, urine	**8**	**4**	17	pfg-kpn1	sg1	pUVA01	Died
3	Ecl1026	*Enterobacter cloacae*	11/30/2007	Sputum, urine	4	**≥8**	20	pfg-ecl3	sg1	pUVA01	Recovered
4	Ecl1027	*Enterobacter cloacae*	12/09/2007	Urine	2	**4**	19	pfg-ecl2	sg1	pUVA01	Recovered
5	Kox1028	*Klebsiella oxytoca*	1/15/2008	Blood, sputum	4	**≥8**	20	pfg-kox1	sg2	pUVA02	Died
6	Ecl1032	*Enterobacter cloacae*	1/24/2008	Blood, ascites fluid	**≥16**	**≥8**	14	pfg-ecl4	sg1	pUVA01	Recovered
7	Eco1036	*Escherichia coli*	2/7/2008	BAL fluid	≤1	1	19	n/a[c]	sg3	pUVA03	Recovered
8	Ecl1034	*Enterobacter cloacae*	2/15/2008	Blood, sputum	2	**≥8**	18	pfg-ecl1	sg1	pUVA01	Died
9	Ecl1035	*Enterobacter cloacae*	2/20/2008	Blood, urine	4	**≥8**	18	pfg-ecl1	sg1	pUVA01	Died
10	Ecl1033	*Enterobacter cloacae*	2/20/2008	BAL fluid, sputum	2	**≥8**	17	pfg-ecl1	sg1	pUVA01	Recovered
11	Eas1043	*Enterobacter asburiae*	3/18/2008	BAL fluid, sputum, wound	4	**4**	19	n/a[c]	sg1	pUVA01	Died
	Kpn1041	*Klebsiella pneumoniae*	4/8/2008	Blood, sputum	2	**≥8**	21	pfg-kpn2	sg1	pUVA01	Died
12	Cfr1047	*Citrobacter freundii*	3/21/2008	Urine	≤1	**4**	19	n/a[c]	sg1	pUVA01	Died
13	Kox1039	*Klebsiella oxytoca*	3/31/2008	Sputum, urine	4	**≥8**	20	pfg-kox1	sg2	pUVA02	Recovered
14	Kpn1042	*Klebsiella pneumoniae*	4/6/2008	Urine	≤1	**≥8**	18	pfg-kpn3	sg1	pUVA01	Recovered

[a] Boldface values indicate intermediate or resistant susceptibilities by MIC testing. Patients 1 and 2 have been described previously (12).
[b] Month/day/year.
[c] n/a, not applicable.

sertion site (3, 10). While reassortment of the transposon has been found in an isolate from China (11), all other reports and deposited sequences in Tn*4401* to GenBank to date have a largely conserved structure and sequence.

In August 2007 we identified the first known case of CRE at our institution (12), which prompted us to screen all clinical isolates of extended-spectrum β-lactamase (ESBL)-producing enterobacteriaceae for carbapenemase production. In contrast to other reported clusters, we observed a diversity of species and genera of CRE from the outset of the outbreak. In this article, we describe the molecular epidemiological characteristics of this heterogeneous outbreak of KPC-producing CRE.

RESULTS

Detection of *KPC* and *in vitro* antimicrobial susceptibility. Approximately 280 members of the *Enterobacteriaceae* were screened for carbapenemase production by modified Hodge analysis during the study period. Sixteen isolates (5.7%) from 14 patients had positive phenotypic carbapenemase tests. Four genera and six species were represented: *Enterobacter cloacae* ($n = 6$), *K. pneumoniae* ($n = 4$), *Klebsiella oxytoca* ($n = 3$), *Escherichia coli* ($n = 1$), *Enterobacter asburiae* ($n = 1$), and *Citrobacter freundii* ($n = 1$) (Table 1). Two (14%) of 14 patients harbored clinical isolates of more than one CRE species. PCR analysis determined that all isolates carried bla_{KPC}. According to 2009 Clinical and Laboratory Standards Institute (CLSI) breakpoints, most isolates were susceptible to imipenem (81%) by automated susceptibility testing, while 1 (6%) and 4 (25%) isolates were ertapenem susceptible and intermediate, respectively (Table 1). Disk diffusion did not accurately predict *in vitro* resistance; 15 (94%) isolates were imipenem or meropenem susceptible, while one isolate (6%) was intermediate (Table 1 and data not shown).

Clinical outcomes. All patients developed clinically significant infections with CRE (Table 1). All were adult inpatients with major comorbidities who had been hospitalized for a median of 31 days prior to the first isolation of CRE. A summary of the demographic and clinical characteristics are presented in Table 2. Cases were identified throughout the hospital: eight intensive care units, five medical or surgical wards, and one oncology care unit. Investigation of the outbreak revealed possible epidemiological links between most but not all of the cases. While 93% had received broad-spectrum antibiotics prior to infection with CRE, only 36% had received a carbapenem. All-cause 28-day mortality

TABLE 2 Demographics and clinical characteristics

Characteristic	Value for case patients[a]
Mean age, yr (range)	60 (23–81)
Male	8 (57)
Comorbid conditions	
Transplant recipient	5 (36)
Diabetes mellitus	4 (29)
Renal insufficiency	4 (29)
Chronic heart disease	3 (21)
Malignancy	2 (14)
Health care-associated risk factors	
ICU stay > 48 h	9 (64)
Any ICU stay	11 (79)
Median length of stay prior to infection, days (range)	31 (3–144)
Prior anti-Gram-negative antibiotic	13 (93)
Beta-lactam/beta-lactamase inhibitor	8 (57)
Fluoroquinolone	7 (50)
Broad-spectrum cephalosporin	5 (36)
Carbapenem	5 (36)
Monobactam	2 (14)
Clinical infection	
Bacteremia	7 (50)
Urinary tract infection	7 (50)
Pneumonia	7 (50)
Intra-abdominal infection	3 (21)
Mortality	7 (50)
Bacteremia	6 (86)

[a] $n = 14$. Data are no. (%) of patients unless otherwise indicated.

was 50%, and mortality among patients with bacteremia was 86% (Table 2).

Genetic evaluation of Tn*4401*. Bidirectional sequencing of bla_{KPC} from isolates Kox1015, Kpn1016, and Eco1036 and alignment analyses revealed 100% identity to the bla_{KPC-2} allele. Using the primer sets listed in Table S1 in the supplemental material, PCR screening showed that all isolates carried bla_{KPC} and all other putative coding elements of Tn*4401* and had a genetic organization congruent with other previous reports (see Fig. S1) (3).

Several isoforms of Tn*4401* have been characterized, differing by the presence of deletions in the intergenic region between the *istB* and bla_{KPC} genes (3, 4). In this study, PCR amplification of the *istB*-bla_{KPC} intergenic region in all 16 isolates produced amplicons consistent with a 757-bp product (see Fig. S1 in the supplemental material; also data not shown), demonstrating that none contained a large deletion in this nonconserved region of Tn*4401*.

Genetic relatedness. Pulsed-field gel electrophoresis (PFGE) was performed on species that were represented by more than one isolate. A striking degree of genetic diversity was observed among the *E. cloacae* and *K. pneumoniae* isolates: four pulsed-field groups (pfg's) were identified among the six *E. cloacae* isolates, and three pfg's were identified among the four *K. pneumoniae* isolates. In contrast, all three *K. oxytoca* isolates were of a single pfg (Fig. 1; see also Table 1).

Plasmid evaluation. To identify the causative KPC-encoding plasmid during the course of the investigation, we adapted the nested arbitrary PCR (ARB-PCR) method to identify the DNA sequence flanking the Tn*4401* insertion site. A schema of this method is presented in Fig S2 in the supplemental material. This technique has previously been used in genetic studies of bacterial virulence to rapidly identify the insertion site of transposon chromosomal disruptions (13, 14).

Tn*4401*-flanking DNA sequence was obtained from 15 isolates; one isolate, Ecl1032, could not be analyzed by the ARB-PCR method despite repeated attempts. Sequence lengths were on average 561 bp (range, 249 to 703 bp). Flanking sequences of 11 isolates were determined to be of the same sequence group (sg), sg1, suggesting that the isolates carried the same KPC-encoding plasmid. The Tn*4401* insertion site for sg1 was in the transposase gene *tnpA* (99.6% identity over 509 bp; E value, 5e−202; GenBank accession no. FJ410927). The three related *K. oxytoca* isolates were of the same sequence group, sg2, with a Tn*4401* insertion site in a novel open reading frame. The solitary *E. coli* isolate has a unique sequence group, sg3, characterized by a Tn*4401* insertion site in a gene encoding a MobA-like relaxase (97.9% identity over 390 bp; GenBank accession no. AY589571). In all cases, the Tn*4401* insertion sites identified by these sequence groups were distinct from corresponding transposon insertion sites found in previously described bla_{KPC}-containing plasmids.

To verify the findings obtained by ARB-PCR, Southern blot analysis of plasmid DNA from isolates representing each pfg was performed (Fig. 2). Gel electrophoresis demonstrated that pUVA01, pUVA02, and pUVA03 were of 54, 80, and 22-kb, respectively (12; data not shown), and Southern hybridization confirmed that all isolates harbored bla_{KPC} plasmids (Fig. 2A and B). Additional Southern hybridization confirmed that all sg1 isolates (but not the sg2 or sg3 isolates) and Ecl1032 hybridized to the sg1-specific probe, while only the *K. oxytoca* and *C. freundii* isolates hybridized to the sg2-specific probe (Fig. 2C and D).

Functional carbapenemase encoded by plasmid-borne bla_{KPC} was confirmed by the generation of the pUVA01 and pUVA02 transformants AMGH01-T and AMGH07-T, respectively. The transformants had positive modified Hodge tests and were confirmed by PCR to carry bla_{KPC}, all other elements of Tn*4401*, and the original sg designation. Interestingly, AMGH01-T had significantly reduced carbapenem MICs compared to those of Kpn1016, whereas carbapenem MICs for AMGH07-T were comparable those for Kox1015 (Table 2).

Plasmid DNA of the transformants and representative CRE isolates was subjected to restriction digestion with KpnI and Southern blot analysis using bla_{KPC}, sg1, and sg2 hybridization probes (Fig. 3). For plasmid digests of the sg1 isolates Kpn1016, Ecl1027, Cfr1047, and AMGH01-T, the bla_{KPC} and sg1 probes hybridized to a 20-kb fragment. Similarly, for digests of the sg2 isolate *K. oxytoca* Kox1028 and AMGH07-T, the bla_{KPC} and sg2 probes hybridized to a 10-kb fragment. Plasmid digest of the single sg3 isolate, *E. coli* Eco1036, hybridized to the bla_{KPC} probe but neither the sg1 nor the sg2 probe. These results confirmed that sequence groups determined by ARB-PCR correctly identified the resistance plasmid carried by the CRE strain.

Southern blot hybridization of Cfr1047 with the bla_{KPC} probe also revealed a second, lower-molecular-weight fragment, suggesting the presence of two plasmids with the KPC gene (Fig 3). Furthermore, a second band in both Ecl1027 and Cfr1047 hybridized to the sg1 but not the bla_{KPC} probe. Together, these results demonstrate that the clonal *K. oxytoca* isolates harbor a single KPC resistance plasmid, pUVA02, the single *E. coli* isolate carries a unique bla_{KPC}-containing plasmid, pUVA03, and the remaining 12 isolates (representing 9 [82%] of the 11 unique outbreak strains) carry a highly pro-

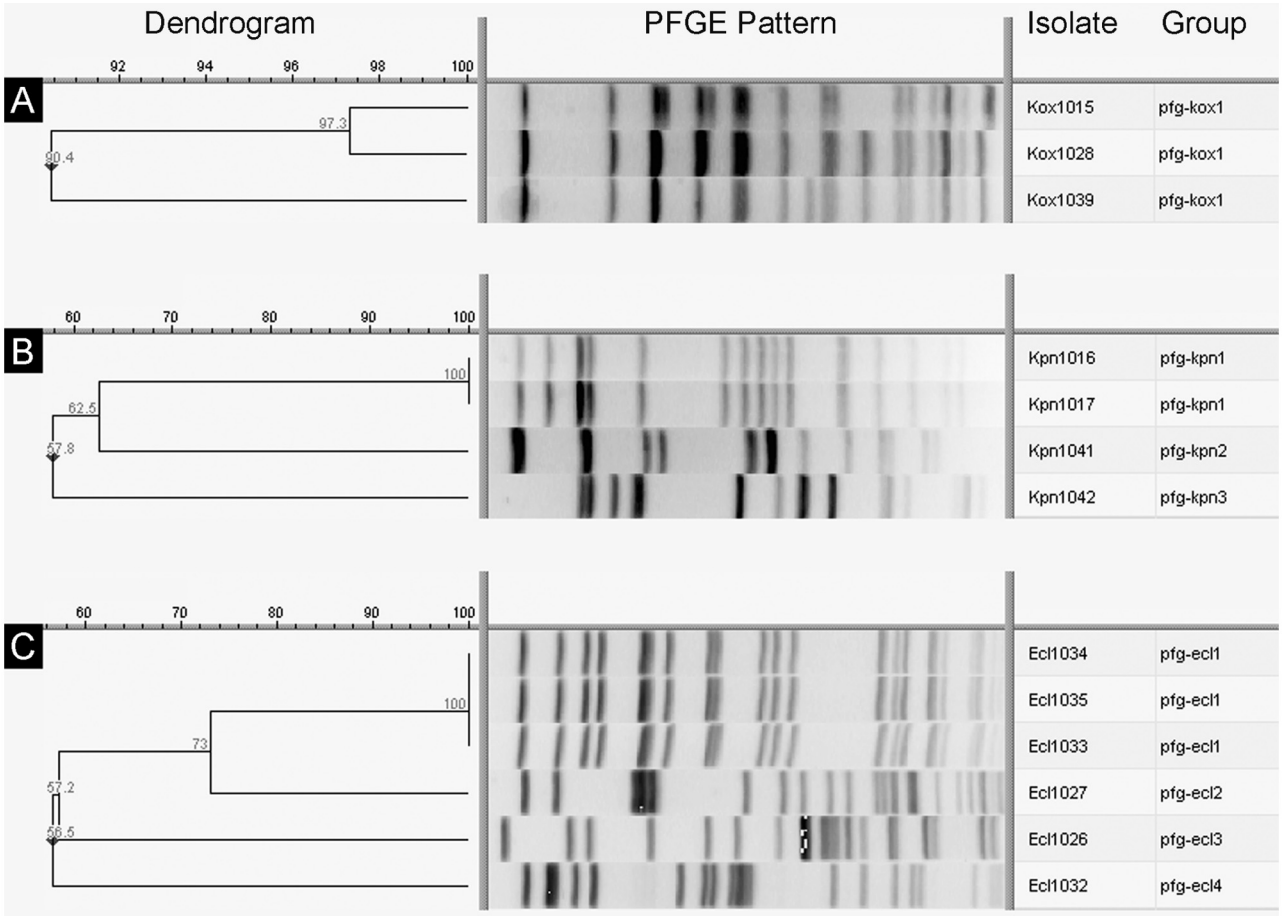

FIG 1 Pulsed-field gel electrophoresis of outbreak isolates of the same species. (A) All three *K. oxytoca* isolates are a single strain. (B) Four *K. pneumoniae* isolates are three distinct strains. (C) Six *E. cloacae* isolates are four distinct strains.

miscuous KPC resistance plasmid, pUVA01. Southern blotting also demonstrated that Cfr1047 harbors a second uncharacterized bla_{KPC} plasmid and that Ecl1027 and Cfr1047 harbor plasmids that share homology with the Tn*4401* plasmids identified by ARB-PCR. These results illustrate the diverse nature of assortments of resistance plasmids found in multidrug-resistant members of the *Enterobacteriaceae* (15).

Combining the molecular epidemiology of strain and resistance plasmid typing with classic epidemiological evaluation of CRE infection provides valuable insight into the dynamics of bla_{KPC} dissemination during this outbreak (Fig. 4). Transmission of carbapenem resistance occurred primarily through horizontal interstrain or interspecies transfer of the Tn*4401*-bearing plasmid pUVA01 and, to a lesser extent, transmission of isogenic strains between patients. The spread of bla_{KPC} correlated well with epidemiological risk factors, including the patient's geographical location, health care team, and time period of possible exposure (Table 3; see also Fig. S3 in the supplemental material). We hypothesize that pUVA03 acquired Tn*4401* via mobilization from pUVA01 or pUVA02, given the congruity of the transposon structure and the patient's epidemiological risk factors. Consequently, all CRE infections appear to be linked to the index patient.

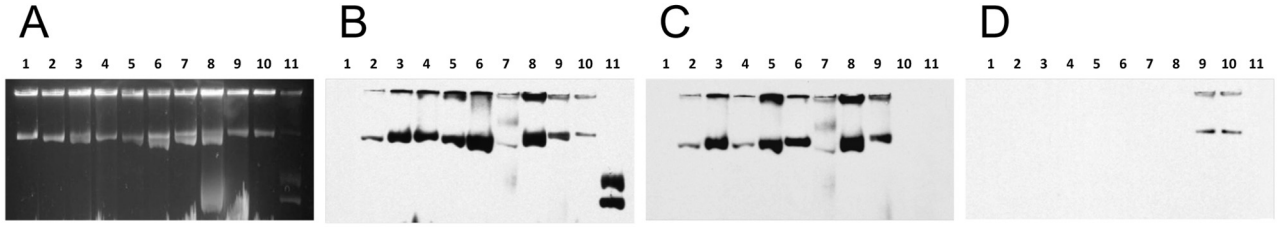

FIG 2 Plasmid electrophoresis and Southern blot analysis of all unique outbreak clones. (A) Plasmid DNA electrophoresis resolved on a 0.6% agarose gel stained with ethidium bromide. Southern blot of gel A with a bla_{KPC}-specific probe (B), sg1-specific probe (C), or sg2-specific probe (D). Lanes: 1, Kpn1014 (negative control); 2 to 4, *K. pneumoniae*; 5 to 7, *E. cloacae*; 8, *E. asburiae*; 9, *C. freundii*; 10, *K. oxytoca*; 11, *E. coli*. Supercoiled and relaxed bla_{KPC}-hybridizing plasmid DNA was obtained from *E. coli* strain Eco1036.

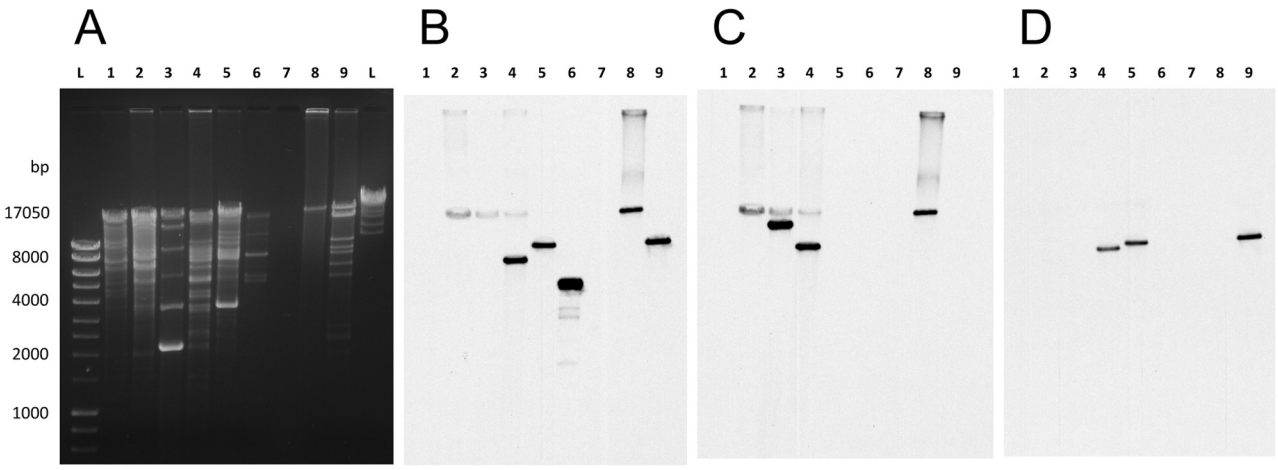

FIG 3 Southern blot analysis of KpnI-digested plasmid DNA of representative outbreak clones and resistance plasmid transformants. (A) Restriction digests of plasmid DNA electrophoresis resolved on a 0.6% agarose gel stained with ethidium bromide. (B to D) Southern blot of gel A with a bla_{KPC}-specific probe (B), sg1-specific probe (C), or sg2-specific probe (D). Lanes: L, ladder; 1, Kpn1014 (negative control); 2, Kpn1016; 3, Ecl1027; 4, Cfr1047; 5, Kox1028; 6, Eco1036; 7, blank; 8, AMGH01-T; 9, AMGH07-T.

DISCUSSION

While sometimes difficult to recognize, plasmids have previously been linked to outbreaks of drug-resistant Gram-negative bacteria. In 1985, O'Brien and colleagues reported an outbreak of plasmid-mediated gentamicin resistance, which moved to six species of the *Enterobacteriaceae* over 8 years in geographically diverse areas of the United States and Venezuela (16). Outbreaks of enterobacteriaceae resistant to sulfonamides, β-lactams hydrolyzed by ESBL, aminoglycosides, and fluoroquinolones have also been reported (17–21). Most recently, a multispecies outbreak of plasmid-mediated VIM-1 metallo-carbapenemase has been reported in Spain (22), and A/C-type plasmid carriage of the New Delhi metallo-β-lactamase gene bla_{NDM-1} has emerged in *Enterobacteriaceae* in India and the United Kingdom (22). While movement of bla_{KPC}-containing plasmids between *Enterobacteriaceae* has been described (7, 8, 23, 24), the degree of horizontal transmission between strains and species observed in this outbreak is unprecedented.

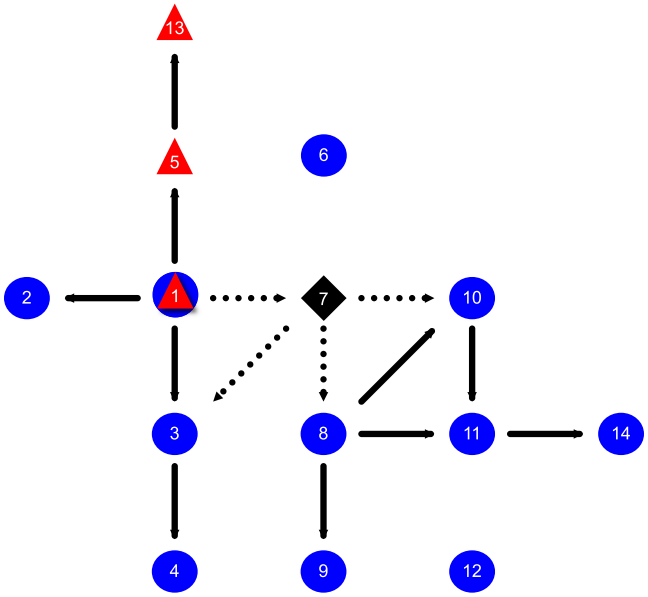

FIG 4 Hypothesized possible route(s) of spread of bla_{KPC} between patients based on plasmid profiles and epidemiological risk for CRE acquisition. Each number denotes a patient. Shape and color denote the plasmid type: blue circle, pUVA01; red triangle, pUVA02; black diamond, pUVA03. Solid arrows denote a possible route of spread based on shared plasmids and epidemiological risk factors. Dashed arrows denote a possible route of spread without a shared plasmid type. Note that arrows do not necessarily signify direct patient-to-patient transmission; the spread of CRE may have occurred to a source that shared the same epidemiological space as the infected patient.

Why did the outbreak of CRE occur in such a wide assortment of strains in our institution? One possibility is that the plasmid-mediated nature of the outbreak may indicate that pUVA01 is more mobile than other bla_{KPC} plasmids. This is supported by the observation that pUVA01 moved between a wide array of strains over a short period of time, often in the absence of carbapenem pressure. In contrast, horizontal transmission of pUVA02 or pUVA03 was not observed during the study period. Clearly, facile transfer of bla_{KPC} plasmids adds an additional layer of complexity to the epidemiology of this rapidly spreading resistance mechanism. A second possibility is that by screening all possible ESBL-producing *Enterobacteriaceae*, we may have captured non- or weakly lactose-fermenting KPC-producing organisms that may have been overlooked in a targeted surveillance and evaluation program, as well as organisms with low carbapenem MICs that would otherwise have been considered carbapenem susceptible. Regardless of the etiology, we predict that the diverse nature of this outbreak will render detection and control of CRE spread even more problematic if it becomes increasingly widespread.

TABLE 3 Transformed strains

Strain	Plasmid	MHT result[a]	MIC (μg/ml) determined by Vitek 2	
			Imipenem	Ertapenem
AMGH01-T	pUVA01	+	≤1	≤0.5
AMGH07-T	pUVA02	+	4	≥8

[a] MHT, modified Hodge test.

One important aspect of this investigation was the use of ARB-PCR to rapidly identify causative KPC resistance plasmids during the investigation. This technology allowed us to quickly recognize the high degree of genetic relatedness among the outbreak isolates at the level of plasmid carriage—relationships missed by traditional species- and strain-based analysis. Investigations of so-called "plasmid outbreaks" are difficult for all but sophisticated molecular epidemiology labs. Analysis of large resistance plasmids from clinical isolates of the *Enterobacteriaceae*, such as the ones observed in this study, can be laborious and technically challenging. ARB-PCR allows rapid determination of DNA sequences that flank a conserved element, which can be used as the molecular signature for a transmissible plasmid. Given its simplicity, ARB-PCR should be translatable to not only transposons but also resistance genes and other conserved components of mobile DNA elements. ARB-PCR does have several limitations. A product may not able to be generated from all samples, as was seen in this study. However, we were able to obtain products in all but one of our isolates without optimization of the protocol conditions or primer sets. Another limitation is that only one product will be generated from an isolate, even if it contains more than one plasmid with bla_{KPC}, as was found with isolate Cfr1047. Finally, generation of flanking sequence does not ensure that the product necessarily has a plasmid origin; transfer of drug resistance to a recipient bacterial strain and demonstration of a plasmid-encoded drug-resistant determinant are required to prove the presence of a resistance plasmid in the donor strain.

The ease of horizontal transmission of carbapenem resistance observed in this study has serious public health and epidemiological implications. Dissemination of bla_{KPC} through mobile genetic elements could allow carbapenem resistance to move to the community, as has been observed with the worldwide emergence of community-onset CTX-M-15 β-lactamase-producing *E. coli* (25–28). Additionally, transmission of bla_{KPC} to enterobacteriaceae of reduced or no virulence could lead to the establishment of a reservoir of carbapenem resistance genes in patients and/or the environment. Understanding the dynamics of bla_{KPC} horizontal gene transfer will be crucial to limiting the spread of CRE, since epidemiological and containment efforts must focus not only on the spread of multidrug-resistant strains but also on the associated resistance plasmids and transposons.

MATERIALS AND METHODS

Patients and bacterial isolates. This study was conducted at the University of Virginia (UVA) Medical Center, a 619-bed tertiary care hospital in central Virginia, from 1 September 2007 to 30 April 2008. Following the detection of the first case of CRE at UVA (12), all clinical *Enterobacteriaceae* isolates identified as possible extended-spectrum β-lactamase (ESBL) producers by automated testing (Vitek 2; bioMérieux, Durham, NC) were prospectively screened for carbapenemase production using the direct carbapenemase and modified Hodge tests (29, 30). Species identification and antibiotic susceptibility testing were performed using the Vitek 2 system with the AST-GN18 card (bioMérieux). Imipenem susceptibility was determined by the Kirby-Bauer disk-diffusion and broth microdilution methods in accordance with CLSI guidelines (31). Patient characteristics, antibiotic exposure, and outcomes were obtained from clinical records. For this study, epidemiological risk factors for acquisition of CRE were defined as hospitalization on the same floor as a patient with CRE infection or receipt of care from a health care team concurrently caring for a patient with CRE infection. Infection was defined as isolation of CRE from a clinical specimen which necessitated treatment. Mortality was defined as death from any cause within 28 days of the latest positive clinical culture with CRE. The study protocol was approved by the UVA Institutional Review Board for Health Sciences Research (no. 13558).

Bacteria, media, and reagents. Strains and oligonucleotides are listed in Table 1 (see also Table S1 in the supplemental material). Enzymes were obtained from New England Biolabs (Ipswich, MA). Strains were routinely grown at 37°C on Luria-Bertani (LB) broth or agar supplemented with freshly prepared 0.1-μg/ml meropenem (AstraZeneca, Wilmington, DE). All strains were stored at −75°C in LB broth containing 15% glycerol. DNA sequencing was performed by the UVA Biomolecular Research Facility (Charlottesville, VA).

Genetic confirmation of bla_{KPC} and Tn*4401*. PCR amplification of bla_{KPC} was performed on all isolates as previously reported (2), and bla_{KPC} allele identification was performed by bidirectional DNA sequencing for a representative strain of each strain type. Confirmation of additional elements of the Tn*4401* transposon was performed by PCR using the primer sets listed in Table S1 in the supplemental material. For each sample, whole-cell lysate was prepared as follows: an individual bacterial colony was suspended in 100 μl sterile water, boiled at 95°C for 10 min, and centrifuged at 16,100 × *g* for 1 min to sediment cell debris, and 2 μl of the supernatant was used as template DNA for PCR. PCR experiments were carried out using the following conditions: 3 min of initial denaturation at 95°C, and 30 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 57°C, a 45-s extension at 72°C, and a final extension of 5 min at 72°C with positive and negative *K. pneumoniae* controls for each run.

PFGE. Genotypic analysis was performed by PFGE of XbaI-digested total DNA with the Chef mapper system (Bio-Rad, Hercules, CA) using conditions outlined in the PulseNet protocol for the *Enterobacteriaceae* (32). Dendrogram construction was performed using the Dice coefficient and unweighted-pair group method using average linkages (UPGMA) clustering (BioNumerics 5.10; Applied Maths, Austin, TX). For this study, isolates of the same species were considered to share a common pulsed-field group (pfg) if they had a Dice similarity coefficient greater than 0.90.

Rapid determination of Tn*4401* insertion site: ARB-PCR. DNA sequences flanking the Tn*4401* plasmid insertion site were determined using the nested arbitrary PCR (ARB-PCR) method (13, 14). The method entails two sequential rounds of PCR amplification using the transposon-specific primer AM14 and the arbitrary primer Arb1.1 in the first round and the nested primers AM11 and Arb2.1 in the second round, followed by DNA sequencing using primer AM13 (see Table S1 in the supplemental material). *K. pneumoniae* Kpn1014, which lacks a KPC resistance plasmid, and sterile double-distilled water (ddH_2O) served as negative controls for both rounds of PCR amplification and DNA sequencing. Each isolate underwent ARB-PCR and DNA sequencing twice, and the resultant Tn*4401* insertion site sequences were aligned using the ClustalW software program to assess the degree of identity. Isolates carrying plasmids with DNA sequences flanking the Tn*4401* integrant that had a BLAST E value of ≤1e−50 and ≥95% identity by pairwise analysis and the same transposon insertion site, as determined by visual inspection, were considered to be of the same sequence group (sg). Unidirectional sequencing of the region flanking Tn*4401* (~1.5 to 2.4 kb) was performed on distinctive plasmids.

Plasmid characterization. To differentiate between plasmids isolated in the study, primer sets targeting the sequence groups were used for PCR analysis (see Table S1 in the supplemental material).

Southern blot hybridization using sg-specific amplicons as probes was performed to confirm sg designations. Plasmid DNA was isolated using the Qiagen Plasmid Midi kit (Qiagen) using 60°C prewarmed eluting buffer. Uncut plasmid DNA was subjected to electrophoresis in a 0.6% UltraPure DNA-grade agarose (Bio-Rad) gel at 70 V for 20 h at 4°C in 0.5× Tris-borate-EDTA buffer. Migration distances of the DNA were compared to reference plasmids of *E. coli* strain V517. For restriction site analysis, plasmid DNA was digested with KpnI at 37°C overnight and subjected to electrophoresis in a 0.7% agarose gel at 80 V for 4 h in Tris-acetate-EDTA buffer. For Southern blot hybridization, plasmid DNA was immobilized to an Amersham Hybond N^+ nylon membrane (GE Health-

care, Piscataway, NJ) via capillary transfer and cross-linked in a UV cross-linker (Agilent Technologies, Santa Clara, CA). Membranes were sequentially hybridized with PCR amplicon probes generated for bla_{KPC}, sg1, and sg2 (see Table S1 in the supplemental material) labeled using the ECL Direct nucleic acid labeling and detection system (GE Healthcare) in accordance with the manufacturer's instructions.

Transfer of plasmid and carbapenemase phenotype. In order to determine if carbapenem resistance was attributable to carriage of the Tn*4401* element, plasmid DNA extracts from Kox1015 (for pUVA02) and Kpn1016 (for pUVA01) were transferred into *E. coli* GeneHog (Invitrogen) by electroporation. Transformants were selected on LB medium supplemented with meropenem. Phenotypic carbapenemase resistance of the transformants was determined by the modified Hodge test (29). Clinical isolates and transformants were verified to carry resistance plasmids via PCR analyses and Southern blot hybridization.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00204-11/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.
Figure S3, PDF file, 0.6 MB.
Table S1, DOCX file, 0.1 MB.

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