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EPIDEMIOLOGY AND SURVEILLANCE

Dynamics of bla_{KPC-2} Dissemination from Non-CG258 *Klebsiella pneumoniae* to Other *Enterobacterales* via IncN Plasmids in an Area of High Endemicity

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ABSTRACT Carbapenem-resistant *Enterobacterales* (CRE) pose a significant threat to global public health. The most important mechanism for carbapenem resistance is the production of carbapenemases. *Klebsiella pneumoniae* carbapenemase (KPC) represents one of the main carbapenemases worldwide. Complex mechanisms of bla_{KPC} dissemination have been reported in Colombia, a country with a high endemicity of carbapenem resistance. Here, we characterized the dynamics of dissemination of bla_{KPC} gene among CRE infecting and colonizing patients in three hospitals localized in a highly endemic area of Colombia (2013 and 2015). We identified the genomic characteristics of KPC-producing *Enterobacterales* recovered from patients infected/colonized and reconstructed the dynamics of dissemination of bla_{KPC-2} using both short and long read sequencing. We found that spread of bla_{KPC-2} among *Enterobacterales* in the participating hospitals was due to intra- and interspecies horizontal gene transfer (HGT) mediated by promiscuous plasmids associated with transposable elements that was originated from a multispecies outbreak of KPC-producing *Enterobacterales* in a neonatal intensive care unit. The plasmids were detected in isolates recovered in other units within the same hospital and nearby hospitals. The gene "epidemic" was driven by IncN-pST15-type plasmids carrying a novel Tn*4401*b structure and non-Tn*4401* elements (NTE_{KPC}) in *Klebsiella* spp., *Escherichia coli*, *Enterobacter* spp., and *Citrobacter* spp. Of note, *mcr-9* was found to coexist with bla_{KPC-2} in species of the *Enterobacter cloacae* complex. Our findings suggest that the main mechanism for dissemination of bla_{KPC-2} is HGT mediated by highly transferable plasmids among species of *Enterobacterales* in infected/colonized patients, presenting a major challenge for public health interventions in developing countries such as Colombia.

KEYWORDS *Klebsiella pneumoniae* non-CG258, *Enterobacterales*, bla_{KPC-2}, Colombia, whole-genome sequencing, IncN plasmid, outbreak

Carbapenem-resistant *Enterobacterales* (CRE) currently pose a significant threat to global public health because the resulting infections are associated with high morbidity and mortality and very limited treatment options (1). In addition, the

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widespread transmission of carbapenem resistance via mobile genetic elements remains a main reason for concern (2). The most important mechanism for carbapenem resistance is the production of carbapenemases. Indeed, the *Klebsiella pneumoniae* carbapenemase (KPC) constitutes the most common class A beta-lactamase enzyme. KPC circulates worldwide and, along with New Delhi metallo-β-lactamase (NDM class B), Verona integron-encoded metallo-β-lactamase (VIM class B), imipenemase metallo-β-lactamases (IMP class B), and oxacilinase-48 (OXA-48 class D), is the most common carbapenemase identified worldwide (3, 4).

Since the first case of KPC identified in a *K. pneumoniae* clinical isolate from North Carolina in 1996 (5), international spread has occurred, and multiple outbreaks have been reported worldwide. In particular, a single clonal group (CG), designated CG258, has been responsible for the majority of infections, with two principal sequence types (STs) ST258 and ST512 (a single locus variant of ST258) representing the majority of bla_{KPC}-containing *K. pneumoniae*. CG258 is responsible for >80% of outbreak isolates in the United States and about 90% of infections in Israel (6–9). The allelic variants bla_{KPC-2} and bla_{KPC-3} are the predominant genes associated with *K. pneumoniae* in Europe (Greece and Italy), Middle East (Israel), South America (Colombia and Argentina), and North America (especially the United States) (6–13). The bla_{KPC} gene is often located on the mobile transposon Tn*4401* and also on diverse plasmids, including broad-host-range plasmids of the incompatibility groups IncA/C, IncL/M, and IncN (14). Outbreaks of KPC-producing *Enterobacterales* caused by transferable plasmids have also been reported, specifically, by "promiscuous" plasmids of incompatibility group N (IncN) that harbor the bla_{KPC-2} gene (15).

In South America, the first detection of bla_{KPC} on a conjugative plasmid was reported in Colombia (16), and since then, the country has become a region where strains producing carbapenemases are endemic (17). In Colombia, outbreaks of KPC-producing *K. pneumoniae* were initially attributed to strains belonging to clonal group CG258 (12, 13). Previous published data also suggested cocirculation of *K. pneumoniae* CG258 strains carrying bla_{KPC-3} and non-CG258 (from different STs) harboring bla_{KPC-2} (18, 19). However, a recent comprehensive genomic study indicated that the likely emergence of carbapenem resistance in Colombian hospitals was driven by the horizontal transfer of promiscuous plasmids harboring bla_{KPC-2} among Gram-negative bacteria instead of clonal dissemination of CG258 strains (19).

Here, we characterized the dynamics of transmission of bla_{KPC} genes among CRE infecting and colonizing patients in an area of endemicity in Colombia around the city of Medellín, encompassing more than 2 million inhabitants. We provide evidence that the major mechanism for the spread of carbapenemase resistance (bla_{KPC-2}) is the horizontal gene transfer (HGT) mediated by highly transferable plasmids, leading to a high endemicity of carbapenem resistance among *Enterobacterales*.

RESULTS

Infection/colonization by KPC-producing *Enterobacterales* in neonates and adults. Between July 2013 and August 2015, 185 CRE clinical isolates of infected and colonized patients were collected from three hospitals serving the Medellin metropolitan area and adjacent communities (Central area of Colombia). Among the recovered organisms, 131 (70.8%) were positive for bla_{KPC} (based on PCR), with 125 (95,4%) and 6 (4.6%) isolates carrying bla_{KPC-2}, and bla_{KPC-3}, respectively. These isolates were obtained from a group of 110 patients, encompassing 58 (53%) adults, 51 (46%) newborns, and one infant patient (1%) (see Data Set S1 in the supplemental material).

Among adult patients, 27 (47%) were colonized and 31 (43%) were considered infected. A total of 6 infected patients were previously colonized by KPC-producing *Enterobacterales*. More than half of the patients were males (56.9%, $n = 33$) and the majority were older (median age of 61 years; interquartile range [IQR] = 48 to 79 and 70 years; IQR = 56 to 81 years in colonized and infected patients, respectively). In the infected patients, KPC-producing *Enterobacterales* were most frequently isolated from urine and respiratory samples (32.3 and 25.8%, respectively). Overall, patients exhibited

several underlying conditions, among which diabetes (34.5%), chronic obstructive pulmonary disease (20.7%), and chronic kidney disease (20.7%) were the most common. A total of 14 (24.1%) patients had previous antibiotic exposure. The most frequent antibiotics were cephalosporins (13.8%), beta-lactam/beta-lactamase inhibitor combination and carbapenems (10.3% for each) (see Table S1 in the supplemental material).

Among newborns, 46 (90.2%) were colonized, and 5 (9.8%) were infected by KPC-producing *Enterobacterales*. In colonized newborns, the mean length of hospital stay before sampling was 12 days (IQR = 5 to 30 days). A total of 26.1% ($n = 12$) were premature, and 36.9% ($n = 17$) had been previously exposed to antibiotics, mainly to aminoglycosides (26.1%), aminopenicillins (26.1%), and cephalosporins (23.9%). Several patients were colonized by up to 2 ($n = 7$) or 3 ($n = 1$) different species of *Enterobacterales* (see Table S2).

A total of 48 neonates (45 colonized and 3 infected) were involved in an outbreak that occurred during the study period in the neonatal intensive care unit (NICU) in hospital 1. Indeed, on 7 March 2014, 11 cases of CRE colonization in newborns were detected in the weekly surveillance cultures. Overall, 82 and 7 newborns, respectively, were colonized and infected by CRE and linked to the outbreak, which was declared over on 30 December 2014. Subsequently, five sporadic cases of colonization by carbapenem-resistant *Enterobacter cloacae* were detected between January and August 2015.

Non-CG258 *K. pneumoniae* in the outbreak setting shared an IncN plasmid. Of the 57 isolates previously identified as carbapenem-resistant *K. pneumoniae*, 51 (89.5%) and 6 (10.5%) were positive for bla_{KPC-2} and bla_{KPC-3}, respectively (see Data Set S1). Of these, 29 isolates were selected for WGS ($n = 23/bla_{KPC-2}$, $n = 6/bla_{KPC-3}$) based on initial characterization by rep-PCR/DiversiLab. The majority ($n = 25$) were identified as *K. pneumoniae*, 3 were identified as *Klebsiella quasipneumoniae*, and 1 was identified as *Klebsiella variicola*.

The KPC-2-producing *K. pneumoniae* isolates involved in the neonatal outbreak in hospital 1 (in 2014), exhibited a variety of genetic backgrounds that were not related to CG258. One main lineage was ST502 ($n = 10$) harboring capsular type *wzi* 50. Other isolates belonged to ST140 ($n = 2$), ST36 ($n = 1$), and ST101 ($n = 1$) exhibiting different capsular types (*wzi* 306, 394, and 29, respectively) and virulence factors. Despite the major differences in genetic backgrounds the isolates harbored a similar IncN plasmid replicon type and carried bla_{SHV}, *sul1*, *fosA*, and *aac(6′)-Ib-cr* conferring, respectively, β-lactam, sulfonamide, fosfomycin, and aminoglycoside/quinolone resistance (Fig. 1 and Table 1). In addition, the bla_{KPC-2} gene was located on a novel Tn*4401*b transposon, which was flanked by a GATCT target site duplication and modified by the insertion of the Tn*5403* element which was flanked by 34-bp inverted repeats into the IS*Kpn6* element (see Fig. S1 in the supplemental material).

We also detected bla_{KPC-2} in non-CG258 *K. pneumoniae* (ST15, -17, -25, -433, and -964; Fig. 1) from colonized and infected adults in the participating hospitals (2013 to 2015). Some isolates that belonged to ST15 and ST17 (C1-205 and C1-01) carried bla_{KPC-2} on the novel Tn*4401*b. Of note, we found similar resistance genes and plasmid replicons (IncN) to that found in isolates that had been involved in the NICU outbreak (Fig. 1 and Table 1). In particular, a *K. pneumoniae* ST433 (C1-201-2) isolate carried bla_{KPC-2} on a non-Tn*4401* genetic element (NTE_{KPC}) of Tn*3* elements-Δbla_{TEM}-bla_{KPC-2}-ΔTn*1721*, previously described (20). This isolate carried five plasmid replicons, including IncN and genes conferring resistance to several antibiotics, including sulfonamides, rifampin, trimethoprim, aminoglycosides, and quinolones (Table 1).

Furthermore, *K. variicola* (C1-76) and *K. quasipneumoniae* (C2-116) isolates recovered from colonized adults at hospital 1 and hospital 2, also carried the bla_{KPC-2} on the novel Tn*4401*b. We also identified the IncN plasmid replicon and resistance determinants similar to those isolates from the neonatal outbreak (Fig. 1 and Table 1). Overall, our results suggest that the spread of bla_{KPC-2} across different clones of *K. pneumoniae* non-CG258, *K. variicola*, and *K. quasipneumoniae* strains was due to the same IncN type

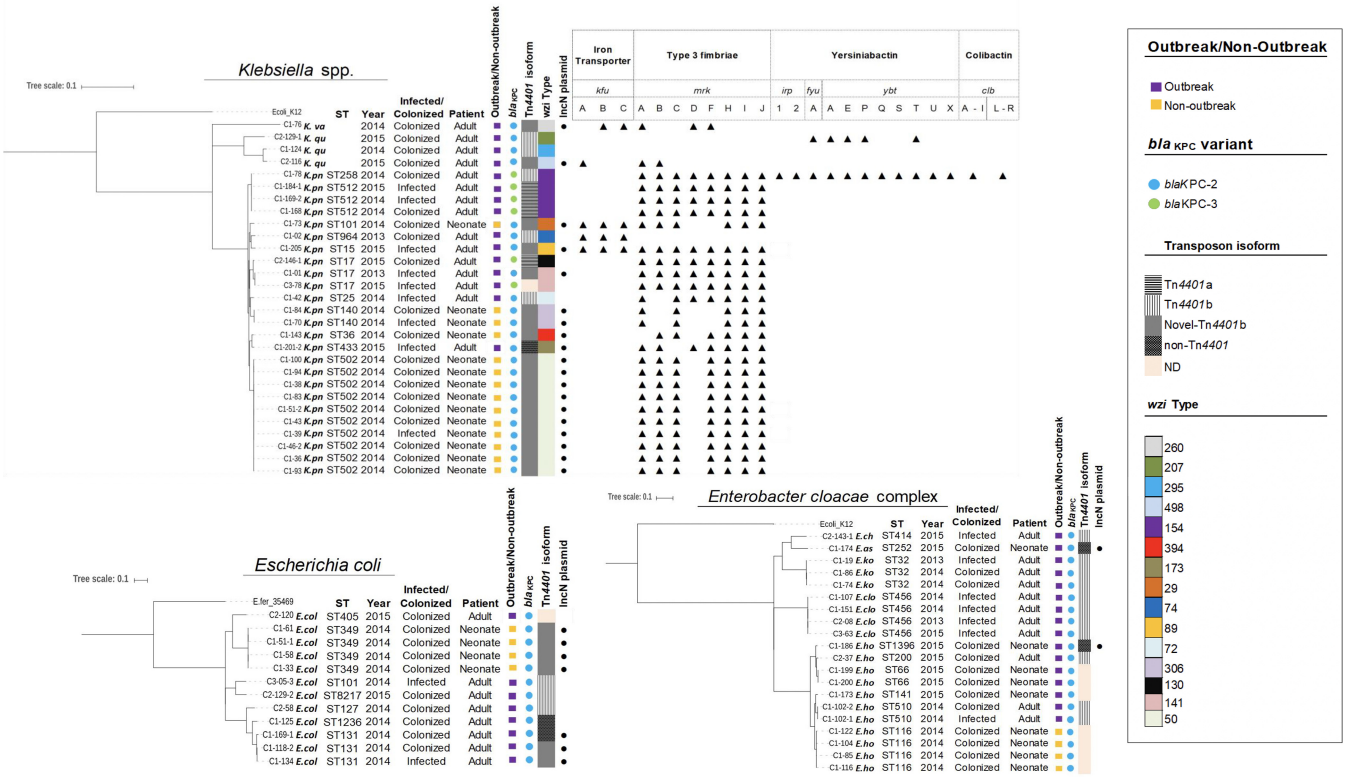

FIG 1 Phylogenetic tree showing the genetic relationships among isolates of *Klebsiella* spp. ($n = 29$), *E. coli* ($n = 12$), and *Enterobacter cloacae* complex ($n = 20$) harboring bla_{KPC}. The results were generated by RAxML and visualized by the iTOL program. For species assignation, "*K.va*" indicates *Klebsiella variicola*, "*K.qu*" indicates *Klebsiella quasipneumoniae*, "*K.pn*" indicates *Klebsiella pneumoniae*, "*E.col*" indicates *Escherichia coli*, "*E.ch*" indicates *Enterobacter chengduensis*, "*E.as*" indicates *Enterobacter asburiae*, "*E.ko*" indicates *Enterobacter kobei*, "*E.clo*" indicates *Enterobacter cloacae*, and "*E.ho*" indicates *Enterobacter hormaechei*. Isolates were characterized by ST and year of isolation. We show whether the isolate was recovered from an infected/colonized patient, a neonate or an adult patient, and from a outbreak/nonoutbreak. We indicate the bla_{KPC} variant, transposable element carrying bla_{KPC}, capsular type (*wzi* typing), IncN replicon plasmid identified in the different species of *Enterobacterales* harboring bla_{KPC} and virulence determinants identified in *Klebsiella* spp.

conjugative plasmid that carried the gene bla_{KPC-2} into the modified Tn*4401*b via insertion of a Tn*5403* element. This plasmid seems to have the ability to broadly disseminate between hospitals.

Simultaneous circulation of *K. pneumoniae* CG258 harboring bla_{KPC-3} favors dissemination of carbapenem resistance. Phylogenetic analyses showed four CG258 *K. pneumoniae* isolates that harbored bla_{KPC-3}. They belonged to ST512 (C1-184-1, C1-169-2, and C1-168) and ST258 (C1-78). All of them contained the *wzi* 154 capsular type and were recovered from infected and colonized adult patients from a single hospital (Fig. 1). In the ST258 isolate, bla_{KPC-3} was located on a Tn*4401*b transposon, and we were able to identify virulence factors, such as type 3 fimbriae, yersiniabactin, and colibactin, as well as five plasmid replicons, including IncI2 (Fig. 1 and Table 1). A BLAST analysis indicated high similarity to an IncI2 plasmid previously described in US isolates and to a *K. pneumoniae* isolate from Colombia (COL-Kpn48) (19, 21) (see Fig. S2). In contrast, the bla_{KPC-3} of ST512 isolates was located on a Tn*4401*a transposon and contained only the type 3 fimbriae (Fig. 1). We also identified a set of four plasmid replicons that were shared, including the IncFIB(pQIL) plasmid replicon (two isolates, C1-168 and C1-169-2) (Table 1). Of note, the IncFIB(pQIL) plasmid is similar to a ST512 strain previously associated with an outbreak in the city of Medellin (19). Thus, our findings suggest that carbapenem-resistant *K. pneumoniae* CG258 isolates are also circulating concomitantly with non-CG258 *K. pneumoniae*, favoring the dissemination of carbapenem resistance.

Interspecies transmission of IncN plasmids carrying bla_{KPC-2} carbapenemase. During the sampling period, we collected 74 isolates from species other than *K.*

TABLE 1 Molecular characteristics of 72 KPC-producing *Enterobacterales* isolates sequenced by Illumina platform

Species	ST[a]	Hospital	No. of isolates	Patient type	Variant of bla_{KPC-2}	Transposon isoform[b]	Additional resistance genes	Plasmid replicon type[c]
Klebsiella spp.								
K. pneumoniae	ST502	1	10	Neonate	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-62}, *sul1*, *aac(6′)-Ib-cr*, *fosA*	**IncN**, IncR
	ST140	1	2	Neonate	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-1}, *sul1*, *aac(6′)-Ib-cr*, *str*(A,B), *oqx*(A,B), *fosA*	**IncN**, IncFIA, IncFII
	ST101	1	1	Neonate	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-1}, *sul1*, *aac(6′)-Ib-cr*, *aph(3″)-Ib*, *aph(6′)-Id*, *str*(A,B), *oqx*(A,B), *catA1*, *tet*(D), *fosA*	**IncN**, IncFIB, IncFIB(K), IncFII, IncFII(K)
	ST36	1	1	Neonate	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-11}, *sul1*, *aac(6′)-Ib-cr*, *str*(A,B), *oqx*(A,B)	**IncN**, IncR, IncFIB, IncFII
	ST17	1	1	Adult	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-11}, *sul1*, *aac(6′)-Ib-cr*, *oqx*(A,B), *tet*(D), *fosA*	**IncN**, IncFIB(K), IncFII
		2	1	Adult	bla_{KPC-3}	Tn*4401*a	bla_{SHV-11}, *sul1*, *sul2*, *aadA2*, *oqx*(A,B), *erm*(B), *cmlA1*, *tet*(B,D), *fosA*, *dfrA15*	IncFIB(K), IncFIB(pQIL), IncFII, IncFII(K)
		3	1	Adult	bla_{KPC-3}	ND	bla_{SHV-11}, *sul1*, *oqx*(A,B), *catA1*, *tetD*, *fosA*	IncHI1B, IncFIB
	ST15	1	1	Adult	bla_{KPC-2}	Novel Tn*4401*b	*sul1*, *aac(6′)-Ib-cr*, *aadA1*, *oqx*(A,B), *qnrB19*, *mph*(A), *tet*(A), *fos*(A)	**IncN**, ColpVC, IncFIB(K), IncFII(K), IncI1
	ST433	1	1	Adult	bla_{KPC-2}	Non-Tn*4401*	bla_{SHV-11}, *sul*(*1*,*2*), *aac(6′)-Ib-cr*, *aadA16*, *oqx*(A,B), *qnrB6*, *fosA5*, *arr3*, *dfrA27*	**IncN**, ColRNAI, IncFIB(K), IncFII(K), IncX3
	ST964	1	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{SHV-1}, *aac(3″)-IIa*, *aph(3″)-Ib*, *aph(6′)-Id*, *oqx*(A,B), *qnrB19*, *fosA*	IncFIA, IncFIB(K), IncFII(K)
	ST25	1	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{SHV-1}, *oqx*(A,B), *fosA*	ND
	ST258	1	1	Adult	bla_{KPC-3}	Tn*4401*b	bla_{SHV-28}, *sul*(*1*,*3*), *aac(3)-IVa*, *aac(6′)-Ib*, *aph(4)-Ia*, *aad*(*A2*,*A24*), *oqx*(A,B), *catA1*, *cmlA1*, *fosA*, *dfrA12*	ColRNAI, IncR, IncFIB(K), IncFII(K), IncI2
	ST512	1	3	Adult	bla_{KPC-3}	Tn*4401*a	bla_{SHV-11}, *sul1*, *aac(6′)-Ib*, *aph(3)-Ia*, *aadA2*, *oqx*(A,B), *catA1*, *fosA*, *dfrA12*	ColRNAI, IncFIB(K), IncFIB(pQIL), IncFII(K), IncX3
K. variicola		1	1	Adult	bla_{KPC-2}	Novel Tn*4401*b	bla_{LEN25}, *sul1*, *aac(6′)-Ib-cr*, *oqx*(A,B).	**IncN**, IncFIB(K), IncFII(K)
K. quasipneumoniae		1	1	Adult	bla_{KPC-2}	Tn*4401*b	$bla_{OKP-A-5}$, *sul1*, *oqx*(A,B), *fosA*	IncR, IncFII(K)
		2	1	Adult	bla_{KPC-2}	Novel Tn*4401*b	$bla_{OKP-A-5}$, *sul1*, *aac(6′)-Ib-cr*, *oqx*(A,B), *fosA*	**IncN**, IncR, IncFII(K)
		2	1	Adult	bla_{KPC-2}	Tn*4401*b	$bla_{OKP-B-3}$, *sul2*, *rmtG*, *oqx*(A,B), *qnrB19*, *fosA*	IncL/M(pMU407), IncHI1B, IncFIB, IncFII
E. coli	ST349	1	4	Neonate	bla_{KPC-2}	Novel Tn*4401*b	bla_{OXA-1}, *sul1*, *aac(6′)-Ib-cr*, *aadA1*, *catA1*, *tet*(B)	**IncN**, IncFIB
	ST131	1	2	Adult	bla_{KPC-2}	Novel Tn*4401*b	bla_{SHV-5}, *sul1*, *sul3*, *aac(3)-IV*, *aac(6′)-Ib-cr*, *aph(4)-Ia*, *aad*(A1,A2), *cmlA1*	**IncN**, IncHI2, IncHI2A, IncFIB, IncFII, IncI1
		1	1	Adult	bla_{KPC-2}	Non-Tn*4401*	*sul*(*1*,*2*), *aac(3)-IId*, *aph(3″)-Ib*, *aph(3)-IId*, *aadA16*, *qnrB6*, *tet*(A), *arr3*, *dfrA12*, *dfrA27*	**IncN**, IncFIA, IncFIB, IncFII
	ST1236	1	1	Adult	bla_{KPC-2}	Non-Tn*4401*	bla_{TEM-1A}	ColRNAI, IncY
	ST405	2	1	Adult	bla_{KPC-2}	ND	bla_{CMY-2}, *sul*(*1*,*2*), *aac*(*3*)-*IId*, *aadA5*, *aph(3″)-Ib*, *aph(6′)-Id*, *str*(A,B), *mph*(A), *tet*(A), *dfrA17*	IncFIB, IncFII, IncI1
	ST8217	2	1	Adult	bla_{KPC-2}	Tn*4401* b	$bla_{CTX-M-12}$, *sul*(*1*,*2*), *aadA1*, *rmtG*, *qnrB19*, *mdf*(A)	IncL/M(pMU407), IncHI1B, IncFIB, IncFII, IncI1
	ST127	2	1	Adult	bla_{KPC-2}	Tn*4401*b	*aac(3″)-IIa*, *str*(A,B), *qnrB19*	IncN2, IncFII
	ST101	3	1	Adult	bla_{KPC-2}	Tn*4401*b	*sul*(*2*,*3*), *aad*(A1,A2), *str*(A,B), *qnrB19*, *cmlA1*, *floR*, *tet*(A), *tet*(M), *dfrA12*	IncL/M(pMU407), IncN2, IncFIB, IncX1
Enterobacter cloacae complex								
E. hormaechei	ST1396*	1	1	Neonate	bla_{KPC-2}	Non-Tn*4401*	bla_{ACT-7}, *sul1*, *aac(6′)-Ib-cr*, *aph(3″)-Ib*, *aadA16*, *qnrB6*, *fosA*, *arr-3*, *dfrA27*	**IncN**, IncFIB(K)
	ST116	1	4	Neonate	bla_{KPC-2}	ND	bla_{ACT-7}, *sul2*, *aph(3″)-Ib*, *aph(6)-Id*, *str*(A,B), *tet*(D), *fos*(A), *dfrA14*	IncFIB, IncFII
	ST66	1	2	Neonate	bla_{KPC-2}	ND	bla_{ACT-7}, bla_{ACT-16}, *fosA*, *aph(6′)-Id*	IncFIB, IncFII
	ST141	1	1	Neonate	bla_{KPC-2}	ND	bla_{ACT-7}	IncFIB(K), IncFII
	ST510	1	2	Neonate	bla_{KPC-2}	Tn*4401*b	bla_{ACT-7}, *sul*(*1*,*2*), *aac(3)-IId*, *aadA2*, *mph*(A), *tet*(D), *fosA*, *dfrA12*	IncFII, IncL/M, IncX5
	ST200	2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{ACT-16}, $bla_{CTX-M12}$, *sul*(*1*,*2*), *aac(3)-IId*, *aac(6′)-Ian*, *aph(3″)-Ib*, *aph(6′)-Id*, *aadA2*, *qnrE1*, *mph*(A), *catA2*, *tet*(D), *fosA*, *dfrA12*, *mcr-9*	IncN2, IncHI2, IncHI2A, IncFIB, IncFII, IncX5

(Continued on next page)

TABLE 1 (Continued)

Species	ST[a]	Hospital	No. of isolates	Patient type	Variant of bla_{KPC-2}	Transposon isoform[b]	Additional resistance genes	Plasmid replicon type[c]
E. asburiae	ST252	1	1	Neonate	bla_{KPC-2}	Non-Tn*4401*	bla_{ACT-3}, bla_{ACT-7}, *sul1*, *aac(6′)-Ib-cr*, *aadA16*, *qnrE1*, *qnrB6*, *fosA*, *arr3*, *dfrA27*	**IncN**, IncFIB(K)
E. cloacae	ST456	1	2	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMH-3}, *sul1*, *aac(6′)-Ib-cr*, *aac(6′)-Ib3 aac(6′)-II*, *aadA1 aadA2*, *mphA*, *fosA*, *dfrA1*, *dfrA12*	ND
		2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMH-3}, $bla_{CTX-M-12}$, *sul*(*1*,*2*), *aac(6′)-Ib-cr*, *aac*(*3*)-*IId*, *aac(6′)-Ib3*, *aac(6′)-II*, *aadA1*, *aadA2*, *rmtG*, *qnrB19*, *mph*(A), *tet*(D), *fosA*, *dfrA*(*1*,*12*)	IncL/M
		3	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CHM-3}, $bla_{CTX-M-12}$, *sul1*, *aac*(*3*)-*IId*, *aac(6′)-Ib3*, *aac(6′)-II*, *aad*(A1,A2), *aac(6′)-Ib-cr*, *mph*(A), *tet*(*D*), *fosA*, *dfr*(A1,A12)	ND
E. kobei	ST32	1	2	Adult	bla_{KPC-2}	Tn*4401*b	bla_{ACT-9}, *aph(3″)-Ib aph(6)*-Id, *aacA4*, *fosA*	IncN2
		1	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{ACT-9}, *sul1*, *aac(3″)-IIa*, *aac*(*3*)-*VIa*, *aph(3″)-Ib aph(6)-Id*, *aadA1*, *qnrB19*, *tet*(A), *fosA*, *mcr-9*	IncN2
E. chengduensis	ST414	2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{ACT-6}, *aac(3″)-IIa*, *aph(3″)-Ib*, *aph(6′)-Id*, *qnrB19*, *fosA*	IncN2
Citrobacter spp.								
C. portucalensis		1	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMY-77}, *sul*(*1*,*2*), *aac(6′)-Ib-cr*, *aad*(A2,A16), *qnrB19*, *qnrB6*, *mph*(A), *tet*(D), *arr-3*, *dfrA12 dfrA27*	**IncN**, IncHI1A(CIT), IncHL1B(CIT), IncX5
C. freundii	ST215	1	1	Adult	bla_{KPC-2}	Tn*4401*b	$bla_{CMY-110}$, *sul1*, *aac(6′)-Ib-cr*, *aadA16*, *qnrB6*, *arr-3*, *dfrA27*	**IncN**, RepA
	ST169	2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMY-65}, *qnrB38*	Col440I, IncFIB(pHCM2), IncX5
	ST504*	2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMY-65}, $bla_{CTX-M15}$, *sul1*, *aac(3″)-IIa*, *aac(6′)-Ib-cr*, *ant(2″)-Ia*, *aph(3″)-Ib*, *aph(6′)-Id*, *aadA1*, *qnrB60*, *qnrB38*, *mph*(A), *erm*(B), *catB3*, *cmlA1*	IncN2, IncFII(K), RepA
	ST22	2	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{CMY-48}, *sul*(*1*,*2*), *aac(3″)-IIa*, *aac(6′)-Ian*, *aph(3″)-Ib*, *aph(3′)-Ia aph(6′)-Id*, *aad*(A10,A1), *qnrB19*, *tet*(B), *tet*(D), *dfrA1*	IncN2, RepA
Serratia marcescens		1	4	Adult	bla_{KPC-2}	Tn*4401* b	bla_{SRT-2}, $bla_{CTX-M-12}$, *sul*(*1*,*2*), *aac(3″)-IIa*, *aac(6′)-Ic*, *qnrB19 aac(6′)-Ib-cr*, *tet*(41)	IncA/C, IncA/C2, IncN2, IncR, IncHI1A(CIT), IncHL1B(CIT), IncP6
		3	1	Neonate	bla_{KPC-2}	Tn*4401*b	*sul*(*1*,*2*), *aac(3″)-IIa*, *aac(6′)-Ic*, *aac(6′)-Ian*, *aph(3″)-Ib*, *aph(3′)-Ia*, *aph(6′)-Id*, *aadA10*, *catA1*, *dfrA1*	IncA/C2, IncN2
		3	1	Adult	bla_{KPC-2}	Tn*4401*b	bla_{SRT-2}, $bla_{CTX-M-12}$, *sul1*, *aac(6′)-II*, *aac(6′)-Ic*, *ant(2″)-Ia*, *aadA5*	IncL/M

[a]*, new sequence type (ST).
[b]ND, not detected.
[c]The IncN plasmid replicon type is indicated in boldface.

pneumoniae carrying bla_{KPC-2} in colonized/infected neonates and adults (see Data Set S1). In total, 43 of these isolates were selected for WGS based on initial characterization by rep-PCR/DiversiLab. The organisms identified were *Escherichia coli* ($n = 12$, 7 STs), *Enterobacter cloacae* complex (including *Enterobacter hormaechei* [$n = 11$, 5 STs, 1 new ST], *Enterobacter cloacae* [$n = 4$, 1 ST], *Enterobacter kobei* [$n = 3$, 1ST], *Enterobacter asburiae* [$n = 1$, 1 ST], and a recently described species of *Enterobacter chengduensis* [$n = 1$, 1 ST] [22]) (Fig. 1), *Citrobacter freundii* ($n = 4$, 3 STs, 1 new ST), *Citrobacter portucalensis* ($n = 1$), and *Serratia marcescens* ($n = 6$) (see Fig. S3).

Most isolates carried genes conferring resistance to several antibiotics, including amynoglycosides, sulfonamides, tetracyclines, trimethroprim, quinolones, phenicols, fosfomycin, and β-lactams (Table 1). Notably, two isolates of *Enterobacter cloacae* complex (*E. kobei* ST32 and *E. hormaechei* ST200) harbored the *mcr-9* gene, related to resistance to polymyxins (23) (Table 1). Of note, the genetic environment of *mcr-9* was similar to the one found in the chromosome of *E. hormaechei* strain S5 (accession number CP031571.1) recovered in the United States in 2015 (24) (see Fig. S4).

The bla_{KPC-2} gene was located on the Tn*4401*b isoform in 25/43 isolates sequenced. The remaining isolates carried the novel Tn*4401*b, including six *E. coli* isolates (ST349 [$n = 4$] and ST131 [$n = 2$]) (Fig. 1). The non-Tn*4401* element (NTE_{KPC}) found in *K. pneumoniae* ST433 (see above) was also detected in two *E. coli* isolates (ST131 and ST1236). In *E. hormaechei* ST1396 and *E. asburiae* ST252, NTE_{KPC} was identified with a insertion of Tn*5403* into the *tnpA*(Tn*3*). Of note, these isolates shared the IncN plasmid replicon (Table 1). In *E. coli* ST405 ($n = 1$) and *E. hormaechei* ST116 ($n = 4$), ST66 ($n = 2$), and ST141 ($n = 1$), bla_{KPC-2} was not detected in the genome, suggesting the loss of the plasmid or bla_{KPC} gene during storage or subculture processing prior to sequencing.

Taken together, our results support interspecies dissemination of bla_{KPC-2} among *Enterobacterales* driven by promiscuous plasmids, predominantly of the incompatibility N group (IncN) carrying a novel Tn*4401*b structure and non-Tn*4401* elements (NTE_{KPC}).

Characterization of IncN plasmids involved in dissemination of bla_{KPC-2}. We evaluated the transferability of bla_{KPC-2} by mating assays, selecting one *K. pneumoniae* ST15 (C1-205) isolate carrying the bla_{KPC-2} on the novel Tn*4401*b and IncN plasmid replicon type as donor. We readily transferred bla_{KPC-2} into *E. coli* J53 with a conjugation efficiency of 1×10^{-2} per donor cells. We selected the transconjugant (*E. coli* Tc_C1-205) and other *Enterobacterales* (C1-70, C1-83, C1-93, C1-94, C1-143, C1-134, C1-205, and C2-116) to characterize the genetic location of bla_{KPC-2}. Our results indicated that all isolates had bla_{KPC2} located on an ~56-Kb plasmid (see Fig. S5).

To reconstruct the complete plasmid sequence, we selected one *K. pneumoniae* ST502 isolate (C1-94) and sequenced the genome using the MinION platform (long-read sequencing). The 56,321-bp plasmid was designated pIncN_C1-94_KPC and determined to be ST15 at the pMLST (Fig. 2a). We confirmed that the plasmid carried the novel Tn*4401*b associated with a class 1 integron and other resistance genes [*sul1*, *qacEDelta*1, *aac(6′)Ib-cr*]. Subsequently, we used this plasmid sequence to determine its presence in each isolate using the Illumina data (short read). We identified the plasmid in the transconjugant strain (*E. coli* Tc_C1-205) and 24 *Enterobacterales* isolates (Fig. 2a). Our findings support the notion that pIncN_C1-94-KPC mediated the initial neonatal outbreak, spreading to other units within the same hospital, and was circulating simultaneously in other regional hospitals into different *Enterobacterales*.

We also detected a second IncN plasmid in some isolates that carried the bla_{KPC-2} on non-Tn*4401* elements (NTE_{KPC}) (C1-169-1, C1-201-2, C1-186, and C1-174). This plasmid was similar to a completely sequenced pEC881_KPC plasmid (accession number CP019026.1) from an *E. coli* strain recovered in Cali, Colombia, in 2013 (20) (Fig. 2b). Likewise, we identified the backbone of this plasmid in *Citrobacter portucalensis* (C1-154) and *C. freundii* ST215 (C1-185) isolates recovered from adults at hospital 1 (Fig. 2b).

DISCUSSION

In this study, we characterized the dynamics of dissemination of bla_{KPC-2} in different genera and species of *Enterobacterales* in hospitals serving an area of endemicity in Colombia with more than 2 million inhabitants. We show that broad dissemination of bla_{KPC} is facilitated by intra- and interspecies HGT mediated by promiscuous plasmids associated with transposable elements. This mechanism of spread led initially to a multispecies outbreak of KPC-2-producing *Enterobacterales* in a neonatal care unit. Subsequently, the bla_{KPC-2}-carrying plasmids were detected in *Enterobacterales* recovered from adult units in the same hospital and nearby hospitals over 3 years. The gene "epidemic" was driven by IncN-pST15-type plasmids carrying a novel Tn*4401*b structure and non-Tn*4401* elements (NTE_{KPC}).

Previous studies (18, 19) had suggested that dissemination of bla_{KPC-2} among *K. pneumoniae* non-CG258 in Colombia was mainly due to HGT, as well as its dissemination in other *Enterobacterales* (25, 26). Our study, using and extended and comprehensive evaluation of *Enterobacterales* recovered from colonized and infected patients in a high-endemicity area of Colombia, supports that concept and shows the efficient and expanding spread of carbapenem resistance, occurring mainly from hospitalized patients with the possibility of "spillover" to the community maintaining high circulation

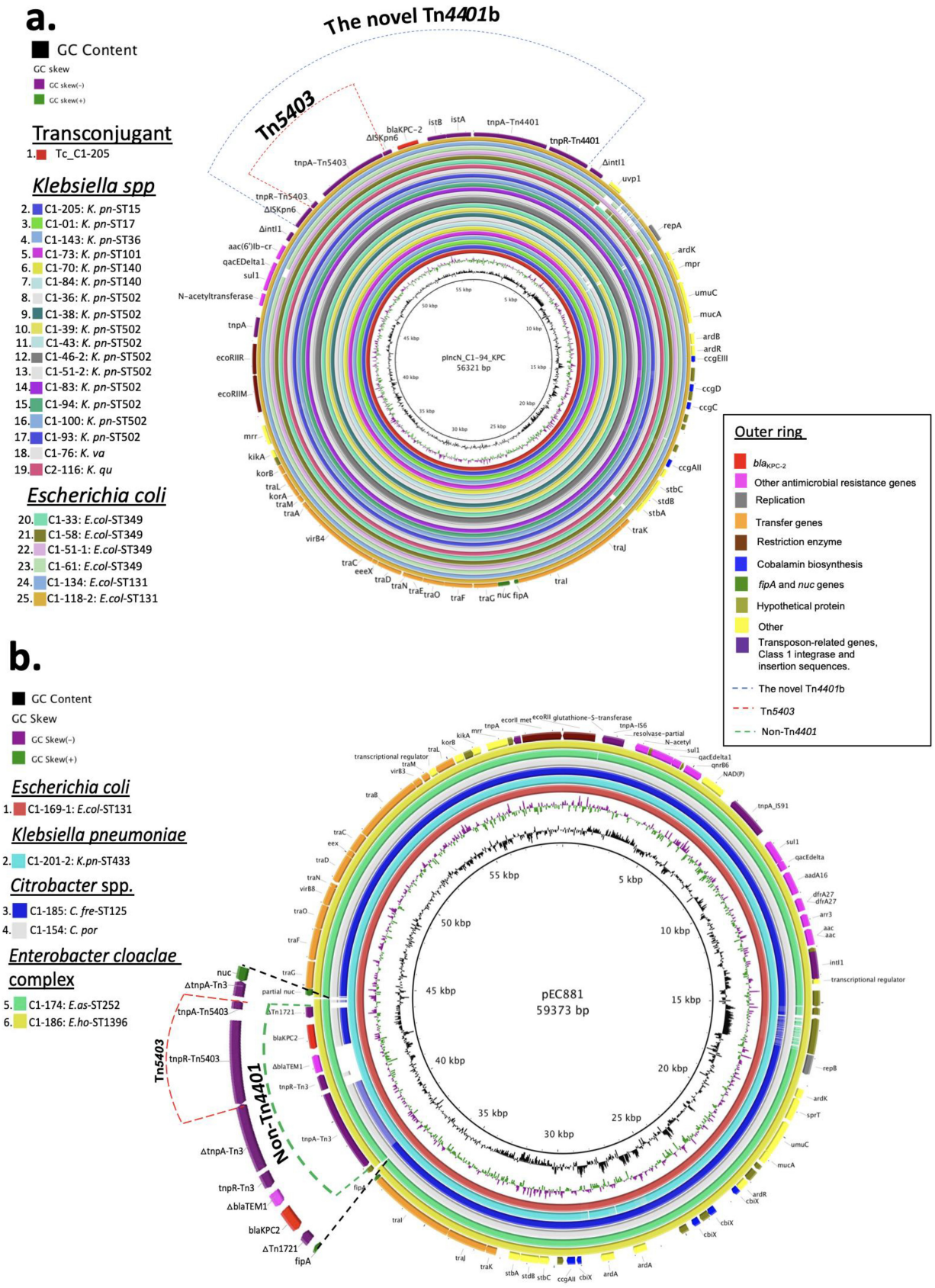

FIG 2 (a) BLAST Ring Image Generator (BRIG 0.95 and BLASTN v2.2.29) alignment of 24 sequenced bla_{KPC-2}-carrying *Enterobacterales* and the Tc_C1-205 transconjugant harboring the annotated pIncN_C1-94_KPC (accession number "contig_241_1" within WGS genome SAMN07291374;

(Continued on next page)

of these genes. Consistent with previous observations, *K. pneumoniae* isolates belonging to CG258 and carrying bla_{KPC-3} also circulated simultaneously with other genetic backgrounds (mostly harboring bla_{KPC-2}). This phenomenon has created a "perfect storm" for dissemination of carbapenem resistance in Colombia (19).

Interestingly, multiple species of KPC-2-producing *Enterobacterales* ($n = 24$) were recovered in the NICU outbreak and adult units from hospital 1 and, subsequently, in nearby hospitals. The isolates exhibited heterogeneous genetic backgrounds but had in common the location of bla_{KPC-2} gene within a novel Tn*4401*b element which harbors an insertion of Tn*5403* transposon into the IS*Kpn*6 (Fig. 1 and see Fig. S1). The Tn*5403* element was previously identified as a "helper" element that participated in the transfer of nonconjugative plasmids, found in isolates of *K. pneumoniae* recovered from polluted aquatic environments (27). It was also found in isolates of *Raoultella ornithinolytica*, a microorganism widely distributed in aquatic environments, insects, and fish (28). Of note, Tn*5403* only contains genes related to transpositions function and is known to transpose by replicative transposition. The element seems to play as "disruptive" or "reorganizing" force via transposition within plasmids from clinical isolates (29). Thus, it is tempting to speculate that this novel Tn*4401*b structure could have originated through the introduction of Tn*5403* element mediated by homologous recombination between environmental and human-adapted hospital isolates.

Our analysis of short- and long-read whole-genome sequencing revealed that the novel Tn*4401*b element was harbored within the pIncN_C1-94_KPC plasmid, which was identified in the 24 *Enterobacterales* isolates described above (Fig. 2a), showing a high conjugation efficiency, as has been previously described in IncN-type plasmids (30–32). An additional non-Tn*4401* element "carrier" of bla_{KPC-2} (NTE_{KPC}) was also identified in different species of *Enterobacterales* from neonates and adults (Table 1), harbored in a plasmid similar to the IncN pEC881_KPC reported previously (20) (Fig. 2b). Thus, these two IncN plasmids played a key role in the transmission interspecies of bla_{KPC-2} in some of the participating hospitals. Presumably, transposition of Tn*5403* occurred and facilitated plasmid rearrangements (29).

Our findings are in agreement with previous studies (15, 32–37) by showing that the interspecies spread of bla_{KPC-2} through plasmids adds an additional layer of complexity to the molecular investigation of multispecies outbreaks and strongly suggest conjugation capacity and high plasticity of IncN ST15 plasmids in the gastrointestinal environment within a patient. Thus, the data in the study indicated that dissemination of the bla_{KPC-2} gene started in July 2013 in a *K. pneumoniae* ST17 isolate from an infected adult patient localized in non-ICU in hospital 1 that carried the bla_{KPC2} within the novel Tn*4401*b into pIncN_C1-94_KPC plasmid. This plasmid seems to be the source of the subsequent outbreaks in the neonatal unit.

Figure 3 attempts to reconstruct the events of dissemination of bla_{KPC2} of the outbreak. Our data suggest that, in the beginning of the outbreak (March 2014), the clonal dissemination of bla_{KPC2} by non-CG258 lineage of *K. pneumoniae* ST502 occurred, followed by emergence of KPC-2-producing *E. coli* ST349 by acquisition of the pIncN_C1-94_KPC plasmid (Fig. 2a) and subsequent spread to other species of *Enterobacterales* (March to December 2014). In parallel, an *E. coli* ST131 recovered from an adult patient in non-ICU setting carried the bla_{KPC-2} on NTE_{KPC} within a plasmid highly similar to pECC881_KPC (Fig. 2b). After the outbreak was controlled (2015), we detected sporadic cases in neonates colonized by *E. asburiae* ST252 (C1-174) and *E. hormaechei* ST1396 (C1-186) carrying bla_{KPC-2} in the same plasmid pEC881_KPC, with some varia-

FIG 2 Legend (Continued)
inside ring). Each isolate and the Tc_C1-205 transconjugant are indicated by a ring of different color (left panel). The outer ring shows resistance genes, as well as the structural genes of pIncN_C1-94_KPC indicated by different colors (right panel). The novel transposon Tn*4401* (blue dotted line) harboring an insertion of Tn*5403* (red dotted line) into IS*kpn6* is also shown. (b) BLAST Ring Image Generator (BRIG 0.95 and BLASTN v2.2.29) of six sequenced bla_{KPC-2}-carrying *Enterobacterales* isolates using plasmid pEC881 as reference (accession number CP019026.1) from an *E. coli* recovered in Cali, Colombia, 2013 (inside ring). Each isolate is indicated by a ring of different color (left panel). The outer ring shows the resistance and structural genes indicated by different colors (right panel). The NTE_{KPC} element (green dotted line) harboring an insertion of Tn*5403* (red dotted line) into *tnpA*(Tn*3*) in C1-174 and C1-186 isolates is also shown.

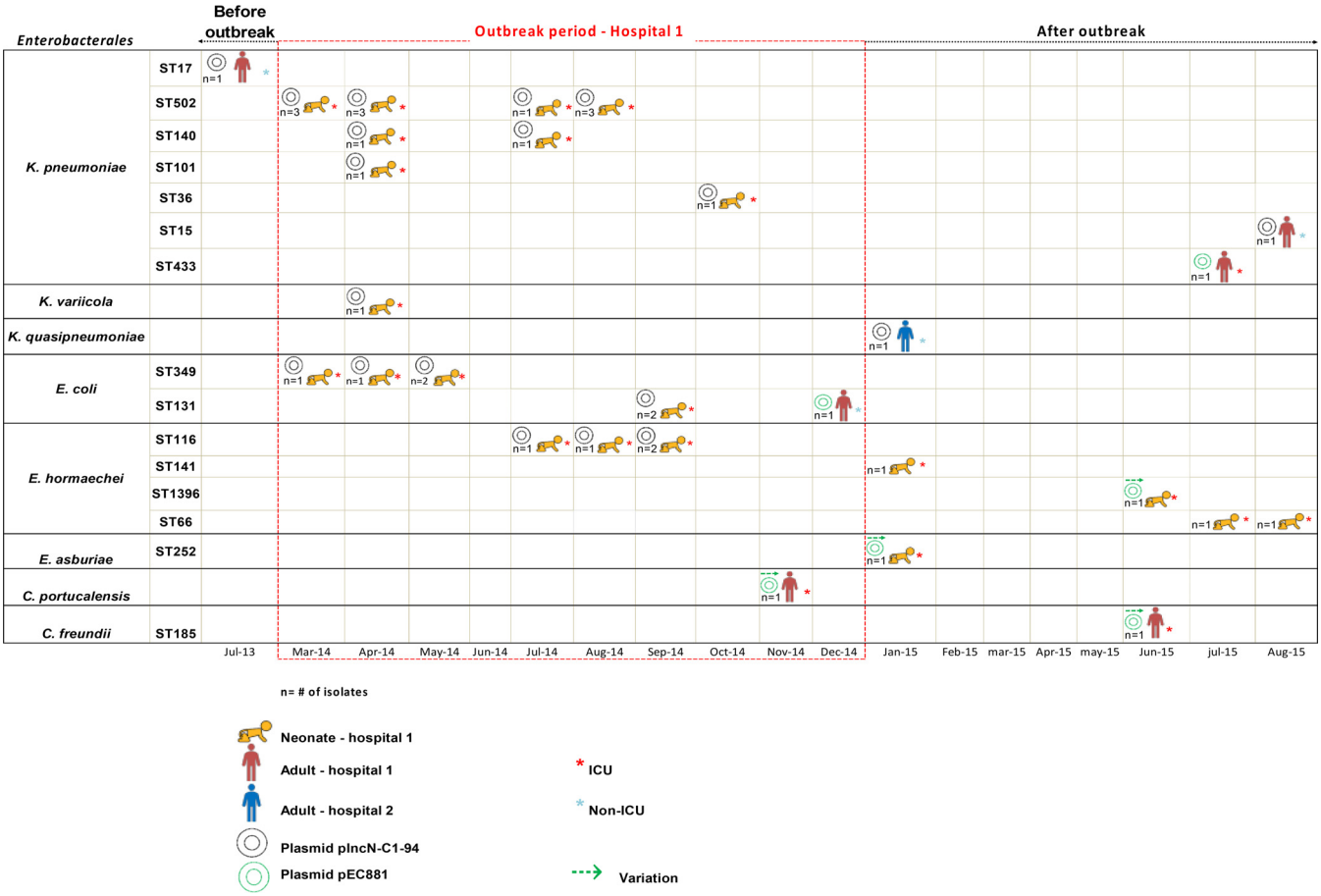

FIG 3 Timeline of the outbreak and dissemination of bla_{KPC2} among different *Enterobacterales* in hospitals located in Medellin and surrounding areas (2013 to 2015). The neonates are in orange, adult from hospital 1 in brown and adult from hospital 2 in dark blue. The asterisk indicates the place of stay in ICU (red) and non-ICU (light blue) at the time of sampling. The type of plasmid identified in the isolates are indicated with black and green ring. Those that exhibited variations within mobile elements are indicated with a green arrow pointing to the right.

tions in the region containing the bla_{KPC2}. Also, in the adult unit *C. freundii* ST215 (C1-185) and *C. portucalensis* (C1-154) were identified carrying the backbone of pEC881_KPC, albeit carrying a Tn*4401*b (Fig. 2b), suggesting gene rearrangement likely through transposition and homologous recombination. Likewise, one isolate of *K. quasipneumoniae* (C2-116) recovered from an adult patient in hospital 2 (January 2015), harbored the pIncN_C1-94_KPC plasmid.

Another important finding in our study is the identification of a group of closely related *E. cloacae* ST456 isolates ($n = 4$) carrying bla_{KPC-2} on Tn*4401*b (Fig. 1), a finding only previously reported in Norway (38). Of note, *E. kobei* ST32 and *E. hormaechei* ST200 isolates recovered from infected and colonized adult patients carried the *mcr-9* gene within a genetic environment similar to a previous isolate described in USA (Fig. S2). The *mcr-9* was described initially in *Salmonella enterica* and *E. coli* (23, 39), and later in *Enterobacter* spp. and *Klebsiella pneumoniae* and has been associated with carbapenemase-encoding genes (bla_{NDM-1}, bla_{VIM-1}, bla_{VIM-4}, and bla_{OXA-48}) in the United States, China, and European countries (24, 40, 41). In South America, it has recently been described in *K. quasipneumoniae* associated with bla_{NDM-1} in Argentina (42). To our knowledge, this is the first report of *Enterobacter* spp. harboring *mcr-9* and bla_{KPC-2}. However, at the time of isolation, the organisms were susceptible to colistin therefore it is likely that the *mcr-9* gene was not expressed, a finding consistent with previous reports (43). Of note, *mcr-9* had not been previously reported in Colombia.

Some limitations of the study include the limited number of hospitals in the region and that we were not able to resolve all plasmid sequences by long-read sequencing.

Seven isolates of *E. hormaechei* involved in the NICU outbreak could have lost the bla_{KPC} gene or the plasmid that contained it during storage or subculture processing prior to sequencing. Therefore, it was not possible to define the genetic environment of bla_{KPC} gene in those isolates. However, we used the analysis of those genomes in the study to describe aspects related to the outbreak in NICU from hospital 1.

In conclusion, the KPC carbapenemase epidemic in an area of high endemicity in Colombia is driven by the horizontal transfer of promiscuous plasmids harboring bla_{KPC-2} among members of the *Enterobacterales*. This phenomenon has resulted in the occurrence of multispecies outbreak and high-level of genetic diversity of KPC-producing *Enterobacterales*. Our findings provided evidence of an epidemic of plasmid carrying bla_{KPC} rather than clonal expansion of successful genetic lineage. Interrupting plasmid transmission is a challenge for public health interventions in developing countries.

MATERIALS AND METHODS

Strain selection and clinical and epidemiological characterization. We conducted a prospective surveillance study of CRE in three tertiary-care hospitals in Colombia between 2013 and 2015. Hospital 1 is located in Medellín and, hospitals 2 and 3 are located in surrounding areas (202, 143, and 146 beds, respectively). All hospitals have functional intensive care units (ICU) for adults and two of them have neonatal intensive care units (NICU).

We isolated *Enterobacterales* from infected or colonized patients. Colonization was defined as a CRE recovered from a surveillance rectal culture (only in two hospitals) or a clinical sample without associated symptoms or disease. Infection was defined by a compatible clinical syndrome with confirmation by the infectious disease services and/or infection control committee, retrospectively. Rectal swabs were cultured on a selective chromogenic medium (chromID CARBA; bioMérieux). The collected *Enterobacterales* exhibited nonsusceptibility to at least one carbapenem (imipenem, meropenem or ertapenem). Multiple CRE isolates from an individual patient, in the same or different specimens, were also included.

Surveillance rectal swabs were collected in the following patients: (i) individuals with previous stay in the ICU, (ii) patients who received broad-spectrum antibiotic treatment (piperacillin-tazobactam, cefepime, quinolones, or carbapenems) within 90 days before admission, (iii) immunosuppressed patients due to hematologic disease or cancer, (iv) patients who underwent kidney transplantation, (v) use of a long-term urinary catheter or surgical wound drainage, (vi) suspected infection at a different institution, and (vii) hemodialysis and peritoneal dialysis. Clinical and demographic data from colonized and infected patients (i.e., age, sex, colonization or infection by more than one CRE, ICU admission, antibiotic use, use of invasive medical devices, underlying diseases, and comorbidities) were collected from electronically medical records and recorded in a Microsoft Access Database. This study was approved by the Institutional Review Board (IRB) of each participating hospital.

Bacterial identification, antibiotic susceptibility testing, and detection of the bla_{KPC} gene. Identification and antimicrobial susceptibility of *Enterobacterales* were performed by using the automated Vitek-2 system (bioMérieux Marcy-l'Étoile, France). The antimicrobial agents tested included ertapenem, imipenem, meropenem, cefotaxime, ceftazidime, ceftriaxone, cefepime, gentamicin, amikacin, and ciprofloxacin. MICs results were interpreted following the Clinical and Laboratory Standards Institute (CLSI) breakpoints (44).

All isolates classified as carbapenem-resistant were tested by PCR for the presence of the bla_{KPC} gene using primers and assays previously described (5). *Klebsiella pneumoniae* ATCC BAA-1705 and *Klebsiella pneumoniae* ATCC BAA-1706 were used as positive and negative controls for the PCR assay, respectively. DNA sequencing was performed on PCR amplification products and the results were compared and aligned with reference sequence using the online BLAST database for detection of variants of the bla_{KPC} gene.

Whole-genome sequencing. A total of 72 isolates of CRE carrying bla_{KPC} were selected for whole-genome sequencing (WGS) based on initial characterization by rep-PCR/DiversiLab (bioMérieux Marcy-l'Étoile, France) and choosing representatives strains of major clonotypes. Genomic DNA was extracted with the GeneJET Genomic DNA purification kit (Thermo Fisher Scientific, Waltham, MA). DNA libraries were prepared using a NexteraXT DNA sample preparation kit and multiplexed with a NexteraXT index primer kit on the Illumina platform (Illumina, San Diego, CA). Genomic libraries were sequenced on a MiSeq sequencer to obtain 250-bp paired-end reads using kit v2 and v3 (Illumina). The readings were processed to eliminate low quality bases and contamination with sequences of adapters and later assembled by *de novo* assembly. Cleaning and assembly were carried out using CLC Genomics Workbench assembler, version 8.5. The genomes were annotated using the RAST server (http://rast.nmpdr.org).

In order to reconstruct a fully closed plasmid sequence from a *Klebsiella pneumoniae* isolate carrying the bla_{KPC-2} and isolated from hospital 1, we used the MinION (Oxford Nanopore Technologies, Oxford, UK) platform to obtain long-read sequences. Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen), and MinION libraries were prepared using the SQK-LSK208 kit according to the manufacturer´s protocol. We used both Illumina and MinION reads to fully generate the plasmid sequence. The BLAST Ring Image Generator (BRIG) (45) was used to align the assembled reads of the sequenced clinical isolates and the transconjugant Tc_C1-205 strain to the bla_{KPC-2} carrying *K. pneu-*

moniae C1-94 plasmid (pIncN_C1-94_KPC) (accession number "contig_241_1" within WGS genome SAMN07291374). In addition, we aligned the assembled reads of some isolates sequenced to the reference plasmids carrying bla_{KPC} included pEC881_KPC (accession number CP019026.1) and pBK15692 (accession number NC_022520).

***In silico* MLST, genome annotation, virulence factors, and capsular typing.** The assemblies were typed on the web server of the Center for Genomic Epidemiology using the multilocus sequence typing (MLST; MLST 2.0 server; https://cge.cbs.dtu.dk/services/MLST/) (46). The determination of resistance elements, the plasmid replicon, and plasmid multilocus sequence type (pMLST) were identified using Resfinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder/), Plasmidfinder 2.1 (https://cge.cbs.dtu.dk/services/PlasmidFinder/), and pMLST 2.0, (https://cge.cbs.dtu.dk/services/pMLST/), respectively (47, 48), using an identity percentage higher than 95% and a coverage cutoff greater than 90%. The Tn*4401* isoforms were determined by BLASTn comparing the region surrounding each bla_{KPC} gene to the sequences of the Tn*4401* isoforms as described previously (49).

In addition, we identified virulence genes and capsular typing (*wzi* sequencing) of isolates of *K. pneumoniae* using the Institut Pasteur's Klebsiella BigsDB site (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

Phylogenetic analyses. The phylogenetic reconstruction of the isolates was carried out by detecting single nucleotide polymorphisms (SNP) against reference genomes specific to each family of *Enterobacterales*: *Klebsiella spp* (accession number NZ_LAKK01000012.1), *Escherichia coli* (accession number CP010876.1), *Citrobacter freundii* (accession number NZ_CP011612.1), *Enterobacter complex* (accession number NRIS01000001.1), and *Serratia marcescens* (accession number NZ_HG326223.1). We also included the following reference genomes as an external group: *E. coli* K-12 MG1655 (accession number NC_000913), *Escherichia fergusonii* ATCC 35469 (accession number NC_011740.1), and *Salmonella enterica* CT18 (accession number NC_003198.1). An SNP matrix was constructed and used to reconstruct the phylogeny of the strains with RAxML (50). We used the general time reversal model with a GAMMA distribution and the Lewis correction for the parameters to determine the best phylogenetic reconstruction by maximum likelihood because the SNP matrix constitutes a matrix without invariant characters. We performed 20 runs and chose the one with the best score. In addition, 100 bootstraps were made to support the reconstructions. The trees obtained were visualized by iTOL (51).

Transferability of the bla_{KPC} gene and plasmid analysis. We selected one isolate of *K. pneumoniae* (C1-205) from hospital 1 carrying the bla_{KPC} gene (according to short-read WGS-base analysis) and used it as donor in a conjugation experiment, with sodium azide-resistant *E. coli* strain J53 as the recipient. The experiments were performed as previously described (37). Briefly, donor and recipient were grown overnight with agitation at 37°C in tryptic soy broth (TSB). Cultures were transferred into fresh TSB (ratio 1:100) and incubated with agitation for approximately 5 h at 37°C, until the culture reached exponential growth (i.e., an optical density at 600 nm of 0.5 to 0.6). Donor and recipient cultures were mixed at a 1:10 ratio in fresh TSB and incubated at 37°C without agitation for 20 h. The conjugation mixture was diluted in phosphate-buffered saline, and dilutions were plated on tryptic soy agar (TSA) containing imipenem (4 μg/ml) and azide (150 μg/ml) for selection of transconjugants. The conjugation efficiency was calculated by dividing the number of transconjugant by donor cells. Antimicrobial susceptibility testing and PCR amplification of bla_{KPC} gene were subsequently performed to confirm the transconjugant Tc_C1-205.

To confirm the localization of bla_{KPC-2} gene on a plasmid, we selected eight isolates of different *Enterobacterales* of hospitals 1 and 2 carrying bla_{KPC} genes (C1-70, C1-83, C1-93, C1-94, C1-143, C1-134, C1-205, and C2-116) based on short-read WGS analysis and the *E. coli* transconjugant (Tc_C1-205). S1 digestion and pulsed-field gel electrophoresis was performed using the previously described protocol (52, 53), followed by Southern hybridization using a digoxigenin (DIG) DNA-labeled bla_{KPC} probe (DIG-High Prime DNA labeling and detection starter kit II; Roche, Germany), following the manufacturer's instructions. A transconjugant (Tc_C1-205) was sequenced on the Illumina MiSeq platform (Illumina), and the reads were assembled *de novo* using CLC Genomics Workbench assembler (v8.5) with default parameters, and the contigs were mapped against *Escherichia coli* J53 reference genome (accession number AICK01). Identification of plasmids associated with transfer of the bla_{KPC} gene was performed with BLASTn, using the unmapped contig harboring bla_{KPC} in *E. coli* Tc_C1-205 (accession number SAMN12815781).

Data availability. The sequence data for the isolates included in this study were submitted to the NCBI GenBank database under BioProject number PRJNA391501.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 7.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

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