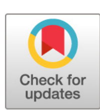

First Report of an OXA-48- and CTX-M-213-Producing *Kluyvera* Species Clone Recovered from Patients Admitted in a University Hospital in Madrid, Spain

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ABSTRACT *Enterobacterales* species other than *Klebsiella pneumoniae* also contribute to OXA-48 carbapenemase endemicity. We studied the emergence of an OXA-48-producing *Kluyvera* species clone, which expresses the novel CTX-M-213 enzyme, colonizing patients in our hospital. Rectal swabs from patients admitted in four wards (March 2014 to March 2016; R-GNOSIS project) were seeded onto Chromo ID-ESBL) and Chrom-CARB/OXA-48 chromogenic agar plates. Carbapenemases and extended-spectrum β-lactamases (ESBLs) were characterized (PCR, sequencing, cloning, and site-directed mutagenesis), and antibiotic susceptibility was determined. Clonal relatedness was established (XbaI pulsed-field gel electrophoresis [XbaI-PFGE]), and plasmid content was studied (transformation, S1 nuclease digestion-PFGE, SB-hybridization, restriction fragment length polymorphism [RFLP] analysis [DraI and HpaI], and PCR [incompatibility group and *rep*A, *tra*U, and *par*A genes]). Whole-genome sequencing (WGS) (Illumina HiSeq-2500) and further bioinformatics analysis of plasmids (PLACNET and plasmidSPAdes) were performed. Patients' charts were reviewed. Six unrelated patients (median age, 75 years [range, 59 to 81 years]; 4/6 male patients) colonized with OXA-48-producing *Kluyvera* species isolates (>95% similarity of the PFGE pattern) were identified. Nosocomial acquisition was demonstrated. In two patients, OXA-48-producing *Kluyvera* species isolates coexisted with OXA-48-producing *Raoultella ornithinolytica*, *K. pneumoniae*, and *Escherichia coli*. The bla_{OXA-48} gene was located on an ~60-kb IncL plasmid related to IncL/M-pOXA-48a and the novel $bla_{CTX-M-213}$ gene in a conserved chromosomal region of *Kluyvera* species isolates. CTX-M-213, different from CTX-M-13 (K56E) but conferring a similar β-lactam resistance profile, was identified. Genomic analysis also revealed a 177-kb IncF plasmid (class I integron harboring *sul1* and *aadA2*) and an 8-kb IncQ plasmid (IS*4*-bla_{FOX-8}). We describe the first bla_{OXA-48} plasmid in *Kluyvera* spp. and the novel chromosomal CTX-M-213 enzyme and highlight further nosocomial dissemination of bla_{OXA-48} through clonal lineages or plasmids related to IncL/M-pOXA-48a.

KEYWORDS carbapenemase-producing *Kluyvera* spp., molecular epidemiology, pOXA-48, new CTX-M variant, clonal transmission, carbapenemase-producing *Kluyvera ascorbata*

In 1981, *Kluyvera* was proposed as a new genus within the *Enterobacteriaceae* (currently named *Enterobacterales*) family, and *Kluyvera ascorbata* was designated the type species. At present, it is classified within the *Enterobacter-Escherichia* clade. The

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Kluyvera genus shares microbiological features with other *Enterobacterales* species and is considered part of the human intestinal microbiome, although it is scarcely related to clinical infections (1, 2). *K. ascorbata* is believed to contain the natural progenitor of genes encoding cefotaximases (CTX-M) of the CTX-M-1 and CTX-M-2 groups, while other species, such as *Kluyvera georgiana*, have been correlated mainly with the CTX-M-8, CTX-M-9, and CTX-M-25 groups (3–6). The mobilization of natural bla_{CTX-M} genes from the *K. ascorbata* and *K. georgiana* chromosomes to plasmids is well known. Genetic elements such as the IS*Ecp1*-like element have been demonstrated to be involved in the transfer of these genes to other *Enterobacterales* species (6–9). Additionally, plasmids carrying CTX-M genes are usually carried by multidrug-resistant and high-risk clones of common nosocomial opportunistic pathogens, which are frequently able to acquire other resistance determinants, including those coding for carbapenemases (10, 11). In the last years, the emergence and global spread of carbapenemase-producing *Enterobacterales* (CPE) have become important challenges in the treatment and prevention of infections in hospital settings.

Carbapenemases hydrolyze a wide range of β-lactam antibiotics, including carbapenems, and frequently confer coresistance to other antimicrobial agents. KPC, VIM, NDM, and OXA-48 are the most prevalent carbapenemases in Europe, and their dissemination is commonly facilitated by both horizontal transfer via mobile genetic elements and bacterial clonal dissemination (12–14). During the last decade, although *Klebsiella pneumoniae* has been considered the main reservoir of OXA-48-type carbapenemases, plasmid-mediated spread of genes encoding this enzyme among different *Enterobacterales* is being increasingly reported (15–19).

The aim of the present study was to characterize what we believe to be the first description of OXA-48-producing *Kluyvera* species isolates, recovered from six colonized patients admitted in our hospital. Epidemiological and genetic characterizations were performed in order to highlight the current emergence and dissemination of bla_{OXA-48} in *Kluyvera* spp. We also report a novel chromosome-encoded bla_{CTX-M} variant (CTX-M-213), a K56E mutant of the CTX-M-13 enzyme.

RESULTS

Bacterial isolates and clinical data. Six unrelated patients (median age, 75 years [range, 59 to 81 years]; 4/6 male patients) colonized with OXA-48-producing *Kluyvera* spp. were detected in the University Hospital Ramón y Cajal from April 2014 to July 2015. For the six isolates, the *Kluyvera* genus was determined by mass spectrometry (MS) (score values, 1.7 to 2) and 16S rRNA amplification. At the species level, higher score values were obtained for *K. ascorbata* than for *K. georgiana* by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS, whereas partial 16S rRNA gene sequencing assigned the same identity (100%) for both species (Table 1).

OXA-48-producing *Kluyvera* species isolates were detected in three different wards (gastroenterology, pneumology, and urology). The median length of stay (LOS) was 15 days (range, 9 to 40 days), and the median LOS until *K. ascorbata* detection (LOS-Ka) was 11.5 days (range, 1 to 41 days) (Table 1). It should be noted that cultures from five patients (83.3%) were negative at admission, and colonization with OXA-48-producing *Kluyvera* spp. was detected during their hospitalization. In one case (patient 5), an OXA-48-producing *Kluyvera* species isolate was found in the first sample, but according to clinical records, this acquisition could have been related to a previous admission in our hospital. All isolates exhibited a highly related pulsed-field gel electrophoresis (PFGE) pattern with a level of similarity of $>$95% (see Fig. S1 in the supplemental material). Additionally, in two patients, OXA-48-producing *Kluyvera* species isolates were recovered along with other species of OXA-48 producers, *Raoultella ornithinolytica* (patient 2) and both *K. pneumoniae* and *Escherichia coli* (patient 5).

Phenotypic and genotypic characterization of resistance. PCR amplification showed the presence of bla_{OXA-48} and also a bla_{CTX-M} gene of the CTX-M-9 group in all *Kluyvera* species isolates. Sequencing revealed that all isolates harbored a novel variant of CTX-M β-lactamase, CTX-M-213, differing from CTX-M-13 by a single amino acid

TABLE 1 Characteristics of OXA-48-producing *Kluyvera* species isolates colonizing patients detected during the R-GNOSIS project[a]

Parameter	Value for isolate					
	KA1	KA2	KA3	KA4	KA5	KA6
Score by MALDI-TOF MS[b]						
K. ascorbata	1.73	1.76	1.87	1.82	1.92	1.81
K. georgiana	1.71				1.73	1.81
% identity by 16S rRNA sequencing						
K. ascorbata	100	100	100	100	100	100
K. georgiana	100	100	100	100	100	100
Patient characteristics						
Age (yr)/sex	76/M	81/M	64/M	73/F	80/M	59/F
Ward location	G	P	U	G	P	U
Isolation date (mo and yr)	Apr 2014	Apr 2014	Aug 2014	Dec 2014	Sept 2014	July 2015
LOS (days)	40	9	14	16	29	8
LOS-Ka (days)[c]	40	9	13	16	1	8
Cocolonizing organism(s)[d]		*R. ornithinolytica*			*K. pneumoniae* and *E. coli*	
Reason for admission	Colon angiodysplasia	COPD	Orchiepididymitis	Rectal bleeding with anemia	Incipient pneumonia	Infection of renal surgical wound
Infection[e]	No	No	No	No	No	No
MIC (mg/liter)						
Amoxicillin-clavulanate	>16/8	>16/8	>16/8	>16/8	>16/8	>16/8
Piperacillin-tazobactam	>64	>64	>64	64	>64	64
Cefuroxime	>16	>16	>16	>16	>16	>16
Cefoxitin	16	≤8	16	≤8	≤8	≤8
Cefepime	≤1	≤1	≤1	≤1	≤1	≤1
Cefotaxime	>32	8	16	≤1	32	16
Ceftazidime	>16	>16	>16	≤1	>16	>16
Imipenem	≤1	≤1	≤1	≤1	2	≤1
Ertapenem	2	1	2	4	2	2
Meropenem	≤1	≤1	1	1	≤1	≤1
Other coresistance(s)		CIP, SXT	CIP, SXT	CIP, TIG	SXT	
Resistance genes						
CP gene	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}
ESBL gene	$bla_{CTX-M-213}$	$bla_{CTX-M-213}$	$bla_{CTX-M-213}$	$bla_{CTX-M-213}$	$bla_{CTX-M-213}$	$bla_{CTX-M-M213}$
Other[f]		bla_{FOX-8}				
Plasmid transformants						
Resistance profile	ERT, MER	CAZ, CTX, MER	IMP, ERT, MER	IMP, ERT, MER	MER	MER, ERT
Resistance gene(s)	bla_{OXA-48}	bla_{OXA-48}, bla_{FOX-8}	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}	bla_{OXA-48}
Size (kb)	~60	~60	~60	~60	~60	~60
Inc group	IncL	IncL	IncL	IncL	IncL	IncL
PCR result (*rep*A, *tra*U, *par*A)	+	+	+	+	+	+
RFLP pattern	A	A	A	A	A	A

[a]M, male; F, female; G, gastroenterology; P, pneumology; U, urology; LOS, length of stay; ESBL, extended-spectrum β-lactamase; CP, carbapenemase; COPD, chronic obstructive pulmonary disease; IMP, imipenem; ERT, ertapenem; MER, meropenem; CAZ, ceftazidime; CTX, cefotaxime; CIP, ciprofloxacin; SXT, trimethoprim-sulfamethoxazole; TIG, tigecycline.
[b]Score values of ≥2.0 are required for identification to the species level, and score values of ≥1.7 are required for identification to the genus level.
[c]LOS until *K. ascorbata* detection.
[d]Cocolonization with other OXA-48 producers.
[e]Infection with OXA-48-producing *K. ascorbata*.
[f]Other resistance genes against β-lactam antibiotics.

substitution (Lys-56-Glu) (Fig. 1). All isolates were resistant to ertapenem (MIC range, 1 to 4 mg/liter), and in most cases, isolates were resistant to cefotaxime (MIC range, ≤1 to >16 mg/liter) and ceftazidime (MIC range, ≤1 to >32 mg/liter). Imipenem and meropenem showed MIC values within the susceptible-to-intermediate range (MIC range, ≤1 to 4 mg/liter). Coresistance to other antimicrobials was also observed in four isolates (Table 1).

Plasmid typing. Successful transformants carrying bla_{OXA-48} were confirmed by PCR, and comparable restriction profiles (profile A) were observed among all of them by plasmid DNA digestion. In all wild-type strains and transformants, hybridization and PCRs revealed that the OXA-48 gene was harbored on a ca. 60-kb IncL plasmid. The

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                 10        20        30        40        50        60
CTX-M-13    MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTKDNTQ
KA1         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ
KA2         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ
KA3         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ
KA4         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ
KA5         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ
KA6         MFAAAACIPLLLGSAPLYAQTSAVQQKLAALEKSSGGRLGVALIDTEDNTQ

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FIG 1 Alignment of the amino acid sequences of CTX-M-213 (from isolates KA1 to KA6) and CTX-M-13 around position 56. Bold letters indicate the single amino acid substitution Lys-56-Glu.

amplification of *rep*A, *par*A, and *tra*U genes showed that the backbone of OXA-48-encoding plasmids was related to the previously reported IncL/M-pOXA-48a plasmid. Restriction fragment length polymorphism (RFLP) patterns obtained by DraI and HpaI digestion showed comparable restriction profiles (profile A) among all transformants.

All transformants exhibited high levels of nonsusceptibility to carbapenems (100% for meropenem, 66.7% for ertapenem, and 33.3% for imipenem), and only one isolate (KA2) showed resistance to extended-spectrum cephalosporins (ceftazidime and cefotaxime). Moreover, resistance to non-β-lactam antibiotics was observed (Table 1).

Resistance phenotype of *bla*$_{\mathbf{CTX\text{-}M\text{-}213}}$. *E. coli* DH5$\alpha$ cells harboring pCTX-M-213 or pCTX-M-13 were obtained by cloning and site-directed mutagenesis experiments, respectively. MIC values showed that glutamine at position 56 (CTX-M-213) was responsible for a slight decrease of β-lactam MICs compared with those obtained with CTX-M-13 (Table 2).

Genome sequencing and bioinformatics analysis. Whole-genome sequencing (WGS) analysis was performed on one representative *Kluyvera* species isolate (KA2). The KA2 strain showed an antibiotic resistance gene profile including the OXA-48 enzyme, the chromosomal novel CTX-M-213 variant, a chromosomally located *pbp2* gene, a class 1 integron harboring aminoglycoside resistance genes (*sul1* and *aadA2*), and a *bla*$_{\text{FOX-8}}$ gene on an ~8-kb IncQ plasmid.

The comparative analysis of the 10 genomes revealed a 616,933-bp core genome including 69,272 single nucleotide polymorphisms (SNPs) among them (Fig. 2). In the phylogenetic tree, we observed that the KA2 strain maintained a greater evolutionary relatedness with *K. georgiana* strains than with other *Kluyvera* species. Moreover, *K. ascorbata* WCH1410 also showed a higher identity with *K. georgiana* strains.

The *bla*$_{\text{CTX-M-213}}$ gene from the KA2 isolate was located in a chromosomal conserved region corresponding to the *bla*$_{\text{CTX-M}}$ housekeeping gene of *Kluyvera* (see Fig. S2A in

TABLE 2 MIC values for different β-lactam antibiotics in CTX-M-213- and CTX-M-13-producing *E. coli* transformants

Drug[a]	MIC range (μg/ml)		
	E. coli DH5α	*E. coli* DH5α (pCTX-M-213)	*E. coli* DH5α (pCTX-M-13)
Amoxicillin	≤8	256	256
Amoxicillin + CLA	≤8	3–4	12
Piperacillin + TZB	0.5	1–1.5	0.5–1
Cefuroxime	0.125	256	256
Cefoxitin	0.125	3–6	1.5–6
Cefepime	<0.06	1–1.5	1.5–8
Cefotaxime	<0.06	3–8	16–24
Ceftazidime	<0.06	0.38	0.75–1
Imipenem	0.125	0.19–0.25	0.125–0.19
Ertapenem	0.06	0.008–0.01	0.012–0.016
Meropenem	0.125	0.01–0.03	0.01–0.03

[a]CLA, clavulanic acid; TZB, tazobactam.

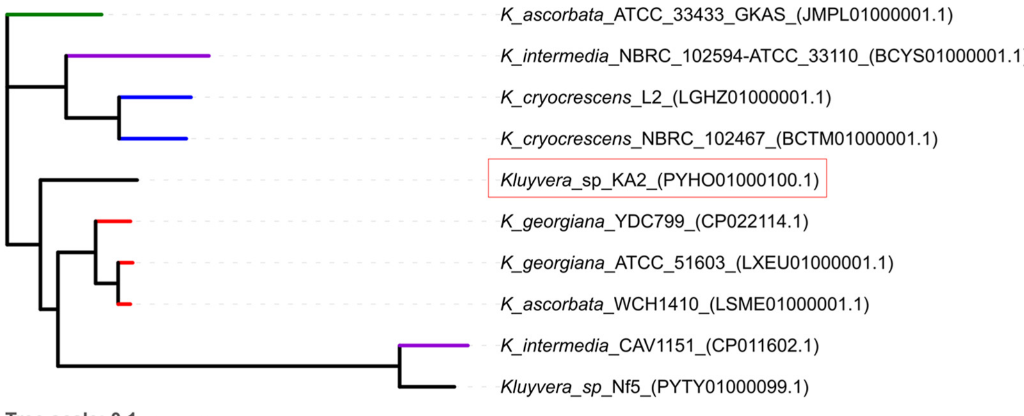

FIG 2 Phylogenetic tree of the core genomes of KA2 (boxed in red) and the nine reference strains available in the GenBank database. The units of the scale are SNPs by position.

the supplemental material), sharing a similar synteny with *K. ascorbata* WCH1410 but a different CTX-M variant (GenBank accession number NZ_LSME00000000). Moreover, an ~3-kb region containing the bla_{CTX-M} gene and four hypothetical genes was found in the *Raoultella planticola* pKnNDM1 plasmid (GenBank accession number NC023911) carrying a gene encoding a CTX-M-9 variant (Fig. S2A). This finding indicates a transfer from a *Kluyvera* chromosome to a plasmid that could facilitate the dissemination of the resistance gene. In fact, we found this ~3-kb region with 99% similarity in plasmids of other species, such as pMRVIM0813 and pQC from *Enterobacter cloacae* and pMSP071 from *E. coli* (GenBank accession numbers KP975077.1, DQ108615.1, and AF174129.3, respectively) (Fig. S2B).

The numbers and sizes of plasmids detected by S1 nuclease digestion-PFGE (S1-PFGE) and WGS analysis were congruent. The three plasmids observed were categorized by PlasmidFinder as IncL/M, IncFII, and IncQ. pOXA-48 (63,678 bp) corresponded to the IncL/M plasmid and contained 87 open reading frames (ORFs), and 43 were hypothetical proteins of unknown functions. According to protein databases, the other 44 proteins were categorized in diverse functions, such as resistance (bla_{OXA-48} [β-lactamase]) and defense mechanisms (*pemKI* and *vapI* [plasmid maintenance system]), plasmid replication or recombination (*mucAB* [UV light resistance], *xerD* [site-specific recombinase], *radC* [DNA repair protein], *ssb* [DNA binding protein], and *repA*, *repB*, and *repC* [replicases]), conjugative transfer (locus *traH* to *traW*), or the mobilome and transport (*mobA* to *mobC*) (Fig. S3).

pOXA-48 was identical (100% coverage and 99% identity) to plasmids already described for *K. pneumoniae* (pKpn-E1.Nr7 [63,581 bp], pKp_Goe_795-2 [63,593 bp], and pKPoxa-48N1 [62,592 bp]) and *E. coli* (pEC745_OXA48 [63,544 bp]), with GenBank accession numbers CP018461.1, KM406491.1, KC757416.2, and CP015075.2, respectively (Fig. S3).

The large plasmid designated pKA2_2 (~177 kb) here, and classified as IncFII according to PlasmidFinder, showed similarity with plasmids in the database (Fig. S4). The first one is a 231,294-bp plasmid of *R. ornithinolytica* strain FDAARGOS_431 (GenBank accession number CP023893.1), sharing 47% coverage with 99% identity, and the second is pOZ172 (127,005 bp) of *Citrobacter freundii* strain B38 (GenBank accession number CP016763.1) (20). The IncFII plasmid replication initiator protein RepB was 100% identical to that of pOZ172. Among the 164 ORFs contained in this plasmid, 83 were hypothetical proteins without known function. The other 81 proteins had known functions classified by the Clusters of Orthologous Groups of Proteins (COG) database as defense mechanism (*ardA* [antirestriction protein], *hsdM* [type I restriction-modification system], *hsdS* [restriction endonuclease], *mcrB* [methylcytosine-specific restriction endonuclease], *acrB-mdtA* [multidrug resistance pump protein], and *stbD*

[antitoxin component]), replication and recombination (*pinE* [site-specific DNA recombinase], *yhdJ* [DNA modification methylase], *recD* [exonuclease], *xerC* and *xerD* [site-specific recombinases], and *dinP* [DNA polymerase]), and secretion and transport (*virB4* and *traD* [type IV secretory pathway]). The plasmid also included different genes related to inorganic ion transport and heavy metal resistance: *zitB* and *czcA* (cobalt-zinc-cadmium resistance efflux pump proteins), *mgtA* (magnesium-transporting ATPase), *ftn* (ferritin), and *fepC* (Fe^{3+}-siderophore transport system). Furthermore, due to the high numbers of transposases and insertion sequences (ISs) (such as IS*5*) found in this plasmid, its sequence was not closed; however, the gene content is well characterized and described in Fig. S4 in the supplemental material. pKA2_2 corresponds to a novel plasmid containing regions of plasmids from different species, including *Citrobacter* and *Raoultella*, with evidence of recombination and genetic transfer between different bacterial genera.

The plasmid content of the KA2 isolate includes an IncQ plasmid (pKA2_3) (8,789 bp) harboring 8 ORFs, including 2 ORFs encoding hypothetical proteins lacking known function, 1 with defense function (bla_{FOX-8} [cephalosporin-hydrolyzing class C β-lactamase FOX-8]), 2 with replication and recombination functions (*repA* and *repC* [regulatory proteins]), and 2 related to transport and mobilome categories (*mobC* and *mobD* [relaxases] and IS*4*-like [insertion sequence]) (Fig. S4). The pKA2_3 IncQ plasmid shared 68% coverage and 99% identity with other ~8-kb small plasmids, like pBRST7.6 from *Aeromonas hydrophila* AO1, pKPSH212 from *K. pneumoniae* I212, or pGNB2 from an uncultured bacterium (GenBank accession numbers EU925817, KT896501, and DQ460733, respectively). The *repC*, *repA*, and *mob* genes of pKA2_3 shared 99% nucleotide identity with those of plasmids pGNB2 and pQ7 and shared 100% identity with the iterons from pGNB2 (21, 22). This IncQ small β-lactamase plasmid is an element with 60.8% GC content and harbors a novel accessory module including IS*4* and the bla_{FOX-8} gene first described here.

DISCUSSION

To the best of our knowledge, we report for the first time a *Kluyvera* species clone harboring an OXA-48-encoding plasmid and also the novel chromosomal CTX-M-213 variant. *Kluyvera* species have been described as opportunistic etiological microorganisms of adult clinical infections (23, 24). Moreover, carbapenemase production has been scarcely reported among these isolates and only in relation to KPC-2 and GES-5 genes (25, 26). For our isolates, bacterial identification to the genus level was achieved by mass spectrometry (MALDI-TOF MS) and partial 16S rRNA gene sequencing, but these methods were not conclusive at the species level. Nevertheless, data from the phylogenetic analysis and the new chromosomal CTX-M-213 β-lactamase, belonging to the CTX-M-9 group, led us to suspect that these isolates could be ascribed to *K. georgiana*. It should be noted that a recent study suggested that the *K. ascorbata* WCH1410 isolate, a control isolate also used in our study, should be reassigned as *K. georgiana* (27), and our results were consistent with that finding. These results also indicate that it would be necessary to reclassify the different species of the *Kluyvera* genus. Next-generation sequencing (NGS) tools could help to improve available databases and indirectly increase the accuracy of rapid identification techniques currently used in clinical laboratories.

Different transposable elements and plasmids have been largely involved in the mobilization of the chromosomal bla_{CTX-M} gene from *Kluyvera* spp. to other *Enterobacterales* species (10, 28). However, in the last decades, *E. coli* has been the major source of transmission and dissemination of different genes encoding CTX-M, particularly CTX-M-15 (29–31). At present, a scenario comparable to that for CTX-M enzymes is being described for the emergence and global spread of CPE, either by clonal expansion or by interspecies lateral transfer of genetic elements (32, 33).

During the study period, the incidence of CPE colonization in our institution was 2%, and it was due to mainly the success and persistence of OXA-48-producing *K. pneumoniae* clones, particularly the sequence type 11 (ST11) high-risk clone (34). Similar results have been reported in other Spanish hospitals (35–37). However, and as has

happened with bla_{CTX-M} genes, plasmid lateral transfer is taking an essential role in the emergence and global spread of the bla_{OXA-48} gene among other *Enterobacterales* species, especially *E. coli* (37–39). In fact, bla_{OXA-48} cross-species transmission has been reported in our institution, related to a dominant and very disseminated ca. 60-kb IncL plasmid described previously as IncL/M-pOXA-48a (unpublished data).

Kluyvera sp. strain KA2, beyond the bla_{OXA-48} gene, carried other relevant antibiotic resistance genes affecting aminoglycosides and sulfonamides, such as the *aadA2* and *sul1* genes, respectively. Moreover, we also found the carriage of a novel 177-kb IncFII plasmid with functions for metabolism and for the defense of the strain, like the heavy metal resistance genes or different multidrug resistance pump genes. We have few hints about its origin, but pKA2_2 shared the highest similarity with the *R. ornithinolytica* FDAARGOS_431 unnamed1 plasmid. However, pKA2_2 also shared similarity with the *Raoultella* and *Citrobacter* plasmids, with a large presence of insertion sequences (ISs), suggesting pKA2_2 dissemination and recombination throughout diverse *Enterobacterales* species. Finally, we described a third plasmid, pKA2_3, an IncQ small β-lactamase plasmid harboring a bla_{FOX-8} gene that enlarges its antibiotic resistance and increases the probability of spread to other strains and species.

In summary, the establishment of OXA-48-producing epidemic *K. pneumoniae* and *E. coli* clones along with the plasmid-mediated acquisition of bla_{OXA-48} among other sporadic clinical multidrug-resistant pathogens, such as the above-described *Kluyvera* species clone, highlight the successful diversification of this carbapenemase in the hospital setting and in the patients' microbiomes. Due to the wide spread of OXA-48-producing *Enterobacterales* in the nosocomial environment, the occurrence of this enzyme is reaching dramatic levels and leaves a complex epidemiological scenario, which could also facilitate its dissemination into the community through epidemic and nonepidemic multidrug-resistant clones.

MATERIALS AND METHODS

Sample collection and bacterial strains. A total of 15,556 rectal swabs from 8,209 patients were collected from four different hospital wards in our hospital (gastroenterology, pneumology, neurosurgery, and urology) between March 2014 and March 2016. The samples were recovered as part of a surveillance screening program intended for the reduction of carriage, infection, and spread of multidrug-resistant Gram-negative bacteria (MDR-GNB), included in the European collaborative research project R-GNOSIS (Resistance in Gram-Negative Organisms: Studying Intervention Strategies) (http://www.r-gnosis.eu/). According to international and Spanish guidelines, prevention and infection control measures, including contact isolation (CI), were implemented for all patients both colonized and infected with CPE (40). Clinical records of colonized patients were retrospectively reviewed, and epidemiological data were included. The study was approved by the ethical committee (refence number 251/13). The swabs were seeded onto Chromo ID-ESBL and Chromo-CARB/OXA-48 agar plates (bioMérieux, Marcy l'Etoile, France).

Bacterial identification. Bacterial identification was performed by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Partial 16S rRNA amplification and sequencing were also carried out.

Resistance mechanism characterization and clonal relatedness. Antibiotic susceptibility testing was performed by microdilution (MicroScan; Beckman Coulter, CA), and results were interpreted according to EUCAST criteria (http://www.eucast.org/). A KPC/MBL Confirm kit, extended-spectrum β-lactamase (ESBL) and AmpC screen kit tests (Rosco Diagnostica, Taastrup, Denmark), and a modified Hodge test (MHT) were used to phenotypically confirm carbapenemase and/or ESBL production. In addition, PCR and sequencing were performed by using primers and conditions reported previously (41, 42). Clonal relatedness was established by XbaI-PFGE, and a dendrogram based on a Dice coefficient algorithm was elaborated by using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium).

Transformation assay, plasmid typing, and antibiotic susceptibility testing. For all *K. ascorbata* isolates, plasmid DNA purification was carried out with the Qiagen plasmid midi kit (Qiagen, GmbH, Hilden, Germany) (25) according to instructions provided by the manufacturer. Plasmids carrying carbapenemase genes were transferred into *E. coli* DH5α cells by heat shock, and transformants were confirmed by PCR. The MICs of imipenem, ertapenem, and meropenem for all OXA-48 transformants were determined by using MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy). Coresistance to other antimicrobial agents was studied by a disk diffusion read with the Adagio automated system (Bio-Rad, Hercules, CA, USA) and interpreted according to EUCAST criteria (http://www.eucast.org). S1 nuclease digestion-PFGE and Southern blot hybridization were performed according to standard procedures. OXA-48-encoding plasmids were typed by PCR (*rep*A, *par*A, and *tra*U genes) (43) and categorized by the PCR-based replicon typing scheme described previously by Carattoli et al. (44, 45). Relationships between plasmids were established by comparison of restriction fragment length polymorphisms (RFLPs) using DraI and HpaI as restriction enzymes.

Cloning of $bla_{CTX-M-213}$ and site-directed mutagenesis. $bla_{CTX-M-213}$ was amplified by PCR using primers CTX-M-9-Seq F (5′-TGAGATGGTGACAAAGAGAATGC-3′) and CTX-M-9-Seq R (5′-CCATTATTGAGA GTTACAGCCC-3′) and cloned directly into the pCR-Blunt II-TOPO vector (Zero Blunt TOPO PCR cloning kit; Invitrogen, Cergy-Pontoise, France) according to instructions provided by the manufacturer. The constructed vector, pCTX-M-213, was transformed into competent *E. coli* cells (NEB 5-alpha competent *E. coli*; New England BioLabs Inc., Ipswich, MA, USA) and then selected on Luria broth agar medium supplemented with kanamycin (50 μg/ml), ampicillin (30 μg/ml), and IPTG (isopropyl-β-D-thiogalactopyranoside)-Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg/ml). In order to generate a plasmid encoding CTX-M-13 β-lactamase (pCTX-M-13), the recombinant plasmid pCTX-M-213 was used as a template for site-directed mutagenesis. pCTX-M-213 plasmid purification was performed by using the QIAprep spin miniprep kit high-yield protocol (Qiagen), and PCR was performed using primers CTX-M-213-K56E-F (5′-CCAAAGAT AATACGCAGGTGCTTTATCGCGGTGATGAACGCTTTCC-3′) and CTX-M-213-K56E-R (5′-TATCGATGAGCGCG ACGCCCAGCCGCCCTCCGCTGC-3′) (Q5 site-directed mutagenesis kit; New England BioLabs Inc.). Transformation into *E. coli* DH5α cells was performed, and subsequent PCR and sequencing were carried out. To search for the role of lysine 56 in the CTX-M-213-hydrolytic spectrum, MIC values for different β-lactam antibiotics were tested in triplicate for both CTX-M-213 and CTX-M-13 enzymes by using MIC test strips.

Whole-genome sequencing and bioinformatics analysis. Total DNA from 3 ml of cultures grown overnight was extracted by using a Wizard genomic DNA purification kit (Promega, Madison, WI, USA), and the DNA concentration was measured by using a Qubit fluorometer and a Nanodrop 1000 instrument (Thermo Scientific, Waltham, MA, USA). The *K. ascorbata* KA2 genome was fully sequenced by using a standard 2- by 100-base protocol with the Genome Analyzer IIx HiSeq-2500 platform (Illumina, San Diego, CA). The paired-end reads were *de novo* assembled by using the SPAdes genome assembler (v.3.5.0), and quality was evaluated by using the QUAST tool (46, 47). The assembled genome of the *Kluyvera* species KA2 isolate was screened for antibiotic resistance genes and plasmid diversity using gene databases and *in silico* genomic tools (ARG-ANNOT and PlasmidFinder, respectively).

Core genomes were obtained from the KA2 isolate and 9 *Kluyvera* species reference strains available in the GenBank database. Variant calling and alignment of single nucleotide polymorphisms (SNPs) were performed by using the Snippy v3.1 tool (https://github.com/tseemann/snippy). IQ-tree software and the iTOL application were used to generate and trace a maximum likelihood tree (48, 49).

Plasmid reconstruction from short reads was performed by using the PLACNET (Plasmid Constellation Network) tool combined with plasmidSPAdes (v.3.5.0) (50, 51). Contigs were annotated with Prokka v1.12, and their gene functions were classified according to the Clusters of Orthologous Groups of Proteins (COG) database (http://www.ncbi.nlm.nih.gov/COG) (52). Comparison of contigs with known sequences in the NCBI database was performed by using BLASTN and BLASTP analyses. Vector NTI v10.3.0 and NuRIG were used for the final assembly and drawing of plasmid figure schemes and comparisons.

Accession number(s). The $bla_{CTX-M-213}$ gene and the *Kluyvera* sp. strain KA2 genome were submitted to the GenBank database under accession numbers MH094805 and PYHO01000000, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01238-18.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

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We declare no conflict of interest with the content of this article.

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