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Intestinal co-colonization with different carbapenemase-producing *Enterobacterales* isolates is not a rare event in an OXA-48 endemic area

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ABSTRACT

Background: The current spread of carbapenemase-producing *Enterobacterales* (CPE) is a great concern.
Methods: We recovered 198 CPE from 162 patients admitted in our Hospital (March 2014-March 2016) during the R-GNOSIS European Project. Microbiological features and plasmid characteristics of CPE recovered from patients co-colonized with multiple CPE were studied.
Findings: Thirty patients (18.5%; CI 95%= 12.5%–24.5%) presented co-colonization with multiple CPE producing the same (CPE-SC) (15.4%) or a different carbapenemase (CPE-DC) (4.3%). OXA-48 (83.3%) was the most frequent carbapenemase, followed by VIM-1 (26.7%), NDM-1 (10%) and KPC-3 (3.3%). CPE-DC-patients had longer admissions [63 days (20–107)] than the other patients. Moreover, hospital stay until CPE detection was lower [9 days (5–14)] ($p = 0.0052$) in CPE-SC-patients than in those with a single colonization; 56% showed co-colonization in the first positive sample, although most of them had previous admissions and had received multiple antibiotic treatments. CPE were more frequently recovered in clinical samples from co-colonized [CPE-DC (28.6%), CPE-SC (24%)] patients than from patients with a single CPE (15.2%). Among CPE-SC–OXA-48 [80% ($p = 0.11$)], *K. pneumoniae* [88% ($p = 0.006$)] and *E. coli* [84% ($p < 0.001$)] were the most frequent species. In 60% of patients, *K. pneumoniae* and *E. coli* species were simultaneously recovered, frequently after a single OXA-48-*K. pneumoniae* colonization. High-risk clones (ST11, ST15, ST307) were detected in OXA-48-*K. pneumoniae* but a higher clonal diversity was found among *E. coli*. A frequent *in-vivo* cross-species plasmid transmission was shown, due to a dominant plasmid (IncL-pOXA-48), but also involving related or unrelated bla_{VIM-1}-, bla_{NDM-1}- and bla_{KPC-3}-encoding plasmids.
Interpretation: CPE co-colonization status should be monitored during epidemiological surveillance cultures, as these patients might be at a higher risk for infection.
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Research in context

Evidence before this study

The increasing prevalence of carbapenemase-producing *Enterobacterales* (CPE) has become a major threat to healthcare facilities. A higher incidence of OXA-48-producing *E. coli* has been reported in our geographic area, including our Hospital. The role of epidemic high-risk clones of *Klebsiella pneumoniae* as ST11, ST307 or ST405 seems to be critical in the maintenance and spread of highly transferable plasmids such as the worldwide disseminated IncL/M-pOXA-48a.

Added value of this study

During the R-GNOSIS European Project (March 2014-April 2016), we detected 30 patients (18.5%) co-colonized with multiple CPE species (or clones of the same species) producing the same or a different carbapenemase. According to our results, patients co-colonized with isolates producing different carbapenemases had longer admissions and patients carrying multiple CPE producing

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the same carbapenemase enzyme had lower hospital stays until CPE detection. Moreover, CPE isolates were more frequently recovered in samples from infective sites in both subgroups of co-colonized patients than among patients with a single CPE. Major finding revealed that co-colonization with OXA-48 producers was the most frequent event and that *K. pneumoniae* and *Escherichia coli* were the predominant species. Our results highlight a successful horizontal inter-species dissemination of bla_{OXA-48} though a dominant plasmid (IncL-pOXA-48), likely from hospital-adapted *K. pneumoniae* high-risk clones to unrelated *E. coli* clones.

Implications of all the available evidence

Intestinal colonization is the key step in CPE epidemiology and most clinical cases are secondary to the selection of CPE in the gut. Our results suggest that multiple colonization with CPE might also increase the risk of infection with these multidrug-resistant microorganisms. Moreover, CPE co-colonization is more frequently due to cross-species transfer of an IncL-pOXA-48 plasmid than to the sequential acquisition of different CPE isolates. In addition, the increased prevalence of OXA-48-producing *E. coli* could contribute in the endemicity of OXA-48 carbapenemase in the hospital setting, but also in the community, as previously happened with CTX-M-15-producing *E. coli*. Implementation of active surveillance cultures are crucial for detect CPE co-colonization status and reduce the risk for infections.

1. Introduction

The occurrence and increased dissemination of carbapenemase-producing *Enterobacterales* (CPE) has been reported worldwide in the last decade, predominantly in hospital setting and more rarely in the community [1,2]. Resistance to carbapenems can be due to loss or decreased expression of porins combined with plasmid-mediated extended-spectrum β-lactamases (ESBLs) or overexpression of chromosomal cephalosporinases (AmpC) and overproduction of efflux pumps, although the most frequent resistance mechanism is the carbapenemase production. Almost all carbapenemase-producers (except certain class D enzymes) show resistance to most β-lactam antibiotics and frequently exhibit co-resistance to other antimicrobial drugs. The fact that carbapenemase genes are usually contained on transmissible mobile genetic elements, such as plasmids, facilitate their spread among different bacterial species [2,3].

In the last years, several nosocomial outbreaks of *Enterobacterales* harboring plasmid-acquired carbapenemases have been described in Spain, initially due to VIM and KPC groups, and more recently linked to OXA-48 [4–8]. NDM enzymes have been sporadically detected in our country, although are recently increasing [9,10]. The epidemiology of OXA-48 enzyme has been largely associated with both global expansion of *Klebsiella pneumoniae* hospital-adapted high-risk clones and the dissemination of plasmids related to IncL/M-pOXA-48a [11–13]. Nevertheless, the current spread of bla_{OXA-48}-encoding plasmids into other Gram-negative bacteria, especially *E. coli*, is contributing to the bla_{OXA-48} endemicity in health-care settings and depicts a great public health threat [8,14–16].

The penetration and establishment of successful carbapenem-resistant clones into the human intestinal microbiota has become a matter of great concern. The improvement of surveillance screening programs for detecting multidrug-resistant microorganisms, the promotion of rapid microbiological diagnosis, as well as the reduction of excessive consumption of antimicrobials are necessary actions to prevent the emergence and global dissemination of CPE, including the community setting.

During an active surveillance-screening program for detecting ESBLs-carriers (R-GNOSIS European Project), we detected a relatively high number of patients co-colonized during the hospital stay with multiple CPE. The aim of our study was to define the microbiological features, the population structure and the plasmid content of CPE strains recovered from co-colonized patients and to associate these findings with their clinical characteristics.

2. Methods

2.1. Study design and patient's data

A total of 15,556 rectal swabs from 8209 patients admitted at Ramon y Cajal University Hospital (Madrid, Spain) were collected from March 4th, 2014 through March 31st, 2016, as part of an active surveillance-screening program for detecting ESBL-carriers (R-GNOSIS-FP7-HEALTH-F3-2011-282,512, www.r-gnosis.eu/). During this project, the CPE incidence was 2% (162/8209; CI 95%= 1.7%−2.3%) and 198 CPE isolates were recovered [16]. Most of the patients were colonized with carbapenemase-producing *K. pneumoniae* (102/162; 62.9%) and *E. coli* (37/162; 22.8%), and OXA-48 (104/162; 64.2%) was the most frequent carbapenemase followed by VIM-1 (50/162; 30.9%), NDM-1 (9/162; 5.6%) and KPC-3 (6/162; 3.7%).

In this work we have focused in the subset of patients colonized with multiple CPE. Accordingly, co-colonization was defined as the detection of ≥2 different species or clones within the same species of CPE in faecal cultures from the same host along their hospital stay [17]. Therefore, two subgroups were identified: patients co-colonized with multiple CPE producing the same carbapenemase (CPE-SC) and patients co-colonized with multiple CPE producing a different carbapenemase (CPE-DC).

According to the guidance of prevention and control against infection with CPE, infection control measures were implemented immediately after identification of each new case of colonization and/or infection [18].

Clinical and epidemiological characteristics were retrospectively reviewed and the following data were included: age, gender, patient location (hospital ward), length of stay (LOS), LOS until the first (LOS-1) and second (LOS-2) positive culture for carbapenemase production, infection and colonization sites, antibiotic treatment and underlying diseases. The study was approved by the Ramón y Cajal University Hospital Ethics Committee (Reference 251/13).

2.2. Screening protocol and bacterial identification

Rectal samples were obtained from 8209 patients within 72 h of ward admission and at discharge in those with a hospital stay ≥3 days. Additional samples were recovered weekly in patients hospitalized ≥7 days. Samples were directly plated on ChromoID-ESBL and –CARB/OXA-48 selective agar media (BioMérieux, Marcy l'Etoile, France) and growing colonies were identified by MALDI-TOF MS (Bruker Daltonics, Bremen, Germany). Carbapenemase production was confirmed with KPC/MBL/OXA-48 Confirm Kit test (Rosco Diagnostica, Taastrup, Denmark) and the Modified Hodge Test (MHT) [16].

2.3. Susceptibility testing

Antimicrobial susceptibility testing was determined with the MicroScan automated system (Beckman Coulter, CA, USA). Results were interpreted according to EUCAST guidelines (EUCAST breakpoint v7.1, www.eucast.org). Isolates categorized as intermediate were considered as resistant.

2.4. Resistance genes characterization

Presence of the bla_{VIM}, bla_{KPC}, bla_{NDM}, bla_{OXA-48} and bla_{ESBL} genes (bla_{SHV}, bla_{TEM} and bla_{CTX-M}) was demonstrated by multiplex PCR [16]. All PCR products were sequenced and compared with available sequences in the GenBank database.

2.5. Clonal relatedness and diversity analysis

Epidemiological relatedness among all CPE isolates was established by PFGE-*Xba*I digestion following standard procedures and clustering analysis of genotyping was carried out with the BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). *K. pneumoniae* and *E. coli* PFGE clusters were also studied by MLST. The Simpson Diversity Index (SDI) based on clones identified by the PFGE method was used to evaluate the *K. pneumoniae* and *E. coli* populations diversity. The SDI is expressed between the values 0 (maximum clonality) and 1 (maximum diversity).

2.6. Transferability of carbapenemase genes and plasmid characterization

In order to characterize the carbapenemase-containing plasmids, conjugation and transformation assays were performed in non-duplicated bacterial isolates per patient. *In vitro* genetic transfer of carbapenemase genes was tested by filter mating assays using the azide-resistant *E. coli* J53 as recipient strain at a donor:recipient ratio of 1:2. Transconjugants were selected in MacConkey agar plates containing sodium azide (100 µg/ml) and ertapenem (0.5 µg/ml). Plasmid DNA purification with Qiagen Plasmid Midi Kit (Qiagen, Düsseldorf, Germany) and subsequent heat shock transformation using DH5-α *E. coli* as recipient were also performed in those strains in which conjugation was not effective. Transconjugants and transformants were confirmed by PCR and sequencing. The plasmid size was studied by S1 nuclease-digestion. Southern-blot DNA transfer and hybridization were also performed. Plasmid DNA in all transconjugants was also extracted using Qiagen Plasmid Midi Kit. Plasmid incompatibility groups were investigated by the PCR-based replicon typing (PBRT) scheme. The *repA, traU* and *parA* genes were detected by PCR to relate OXA-48-encoding plasmids to the IncL/M-pOXA-48a-plasmid backbone. Plasmid similarity was assessed comparing *Dra*I- and *Hpa*I-digested plasmid DNA profiles in all transconjugants/transformants.

2.7. Statistical analysis

Differences concerning patient characteristics were determined using the Kruskal-Wallis, ANOVA, Student's T and Fisher's Exact tests. Fisher's Exact test was also used to estimate differences in co-colonization rates of different carbapenemase types and the prevalence of different CPE species. Student's T and Z-tests were used to calculate 95% confidence intervals. All tests were performed using R software (RStudio, Boston, MA, http://www.rstudio.com/) and *P*-value <0.05 was considered statistically significant. Each patient contributed to the study once and the statistical analysis included the episode in which co-colonization with CPE was detected.

3. Results

3.1. Frequency of co-colonization with CPE and characteristics of patients

During the studied period, 30 patients (18.5%, CI 95% = 12.5% – 24.5%) showed co-colonization with multiple CPE. Twenty-five patients (25/162; 15.4%, CI 95%= 10%–21%) (patients 1–25) were co-colonized with two (21/25, 84.0%) or three (4/25, 16.0%) different species [or clones within the same species (2/25, 8.0%)] of CPE producing the same carbapenemase (CPE-SC). Additionally, co-colonization with multiple CPE each of which produced a different carbapenemase (CPE-DC) was also demonstrated in seven patients (7/162; 4.3%, CI 95%= 1.2%–7.4%) (patients 24–30). It should be noted that both co-colonization events were identified in two cases (patients 24 and 25). Among CPE isolates recovered from co-colonized patients, OXA-48 (25/30, 83.3%) was the most frequent carbapenemase, followed by VIM-1 (8/30, 26.7%), NDM-1 (3/30, 10%) and KPC-3 (1/30, 3.3%) producers (Table 1).

Even though the low number of cases precludes statistical significance ($p = 0.11$), overall, patients co-colonized with CPE-DC had longer LOS [63 days, CI 95% (20–107 days)] than CPE-SC-patients [35 days, CI 95% (20–51 days)] ($p = 0.20$) and patients colonized with a single CPE [32 days, CI 95% (25–38 days)] ($p = 0.09$). Conversely, LOS-1 was lower in CPE-SC-patients [9 days, CI 95% (5–14 days)] than among patients with CPE-DC [33 days, CI 95% (3–62 days)] ($p = 0.15$) or with a single CPE [22 days, CI 95% (17–26 days)] ($p = 0.0052$). Furthermore, LOS-2 was longer in CPE-DC-patients [47 days, CI 95% (16–79 days)] than in CPE-SC-patients [27 days, CI 95% (15–39 days)] ($p = 0.19$). It should be noted that CPE were more frequently recovered in samples from infective sites in both co-colonized subgroups [CPE-DC (28.6%) ($p = 0.35$; odds ratio = 0.51; CI 95%= 0.08–5.59) and CPE-SC (24%) ($p = 0.39$; odds ratio = 0.61; CI 95%= 0.20–2.08] than among patients with a single CPE (15.2%) ($p = 0.11$; odds ratio=0.46; CI 95%= 0.17–1.38) (Table 2). Overall, urine samples were the most frequent (6/9, 66.7%).

A sample at admission was recovered in most of the CPE-SC-patients (20/25) and 65% (13/20) of them had a positive CPE culture. Most of them had a previous admission in our hospital (18/25, 72%) or in other health care centers (3/25, 12%) and had been previously treated with multiple antibiotics. Quinolones (65.2%) and β-lactam-β-lactamase inhibitor combinations (65.2%) were the antimicrobials most frequently used, followed by carbapenems (43.5%) and cephalosporins (43.5%). Finally, most of the CPE-DC-patients (71.4%, 5/7) had a negative CPE result in the admission sample. All of them received multiple antibiotics during hospitalization until the CPE detection and the β-lactam-β-lactamase inhibitor combinations (60%), carbapenems (40%) and cephalosporins (40%) were the groups most frequently used (Table 1).

3.2. Events and dynamics of co-colonization

Among CPE-SC-patients, *K. pneumoniae* (22/25, 88%) and *E. coli* (21/25, 84%) were the predominant species. In 20 cases (20/25, 80%), both species were detected growing together (16/20) or in subsequent positive cultures (4/20) along the hospital stay (Table 1). *K. pneumoniae* (88%) or *E. coli* (84%) colonization was significantly more frequent in these CPE-SC patients than among the rest of the patients [*K. pneumoniae*: 82/137, 59.8%; ($p = 0.006$) (odds ratio = 0.20; CI 95% 0.40–0.73) *E. coli*: 17/137, 12.4%; ($p < 0.001$), (odds ratio = 0.03; CI 95% 0.01–0.09)] (Fig. 1a). OXA-48-co-colonization (20/25, 80%) was predominant with respect to other carbapenemases ($p = 0.11$) (odds ratio = 0.39; CI 95% 0.11–1.18) (Fig. 1b). Interestingly, OXA-48-co-colonization with *K. pneumoniae* and *E. coli* was the most frequent combination (15/20) and colonization with OXA-48-*K. pneumoniae* in a previous admission was demonstrated in four of these cases (4/15). It was remarkable that both species producing OXA-48 were persistently isolated in most patients in whom subsequent cultures were available [*K. pneumoniae* (10/11, 90.9%) and *E. coli* (7/8, 87.5%)]. *K. pneumoniae* and *E. coli* were also recovered coexisting along with other OXA-48 producing species but in a lower proportion (Table 1). The presence of ESBLs in CPE was demonstrated in 21 patients (84%). CTX-

Table 1
Data of patients co-colonized with carbapenemase-producing *Enterobacterales*.

Patient	Age	Gender	Ward	Underlying diseases	Previous admissions/CPE	ATB in the previous year	AS	LOS (days)	First positive culture				Second positive culture				
									Previous ATB	Bacterial ID	CP	LOS-1 (days)	Previous ATB	Bacterial ID	CP	LOS-2 (days)	Clinical sample
1	81	M	P	COPD	NO	–	NG	9	AMC, CLR	*Ka + Ro*	OXA-48	9	–	–	–	–	–
2	98	M	U	Pneumonia, CRD	YES	LEV, AMC, CXM	PS	15	AMC	*Kp + Ec*	OXA-48	2	AMC	*Kp + Ec*	OXA-48	8	*Kp* (Urine and BS)
3	40	F	N	Spondylodiscitis	YES	VAN	NG	35	CTX, VAN	*Kp*	OXA-48	13	CTX, VAN	*Kp + Ec*	OXA-48	19	–
4	88	F	G	Diverticulitis	YES	LEV, CIP, FOS	PS	28	MTR, CIP	*Ec*	OXA-48	3	MTR, VAN	*Kp*	OXA-48	14	*Kp* (Urine)
5	80	M	P	Pneumonia	YES	AMC, TZP	PS	29		*Ka*	OXA-48	1	TZP, VAN, MER, MTR	*Kp + Ec*	OXA-48	15	–
6	65	F	P	COPD	YES	LEV	NR	161	CRO, CD, CXM, AMC	*Kp*	OXA-48	15	TZP	*Kp + Ec*	OXA-48	26	*Kp + Ec* (Wound)
7	83	M	P	Pulmonary fibrosis	YES	GEN, MET, LEV, ERT, AZM, FEP	NG	81	GEN, MTR	*Kp + Ko*	OXA-48	17	MER	*Kp + Cf*	OXA-48	81	–
8	38	M	P	Alveolar proteinosis	NO*	MER, CIP	PS	9	–	*Kp + Ec*	OXA-48	2	MER	*Kp + Ec*	OXA-48	9	–
9	78	M	P	Pulmonary neoplasia	YES/*Ec*	LEV	NR	30	TZP, AMC	*Kp + Cf*	OXA-48	16	AMC	*Ec + Cf*	OXA-48	23	–
10	52	F	G	ERCP(SI)	YES/*Ec*	LEV	PS	1	–	*Ec + Ko*	OXA-48	1	–	–	–	–	–
11	85	F	G	COPD	YES	CRO, AMC, AMP, LEV, FOS, CIP, MTR	PS	20	–	*Ec*	OXA-48	1	LEV	*Kp + Ec*	OXA-48	11	–
12	86	F	P	Pneumonia	NO*	–	PS	3	CRO y CD	*Kp + Ec*	OXA-48	2	–	–	–	–	–
13	94	F	U	CRD	YES	MER, COL, CXM, LEV, AMC	PS	6	AMC	*Kp + Ec*	OXA-48	2	–	*Kp + Ec*	OXA-48	6	–
14	84	M	G	ARD	NO	–	NG	23	MET, CIP, AZT, TIG, DAP	*Kp + Ec*	OXA-48	21	–	–	–	–	–
15	87	M	U	Bladder tumor infiltration	YES/*Kp*	CZ, AMC, CRO, CIP, TZP	PS	19	CRO	*Kp + Ec*	OXA-48	1	AMC	*Kp + Ec*	OXA-48	4	–
16	68	M	N	Brain aneurysm	NO	–	NG	84	AMC	*Kp*	OXA-48	45	AMC	*Kp + Ec*	OXA-48	53	*Kp* (Urine)
17	49	M	U	Cholangitis	YES / *Kp + Ec*	AMC, TZP, MER, AMK	PS	5	AMC, TZP	*Kp + Ec*	OXA-48	3	–	–	–	–	*Kp* (prosthesis)
18	87	M	U	Cholangitis	YES	CIP, MTR	PS	20	AMC, GEN, CD	*Kp + Ec*	OXA-48	1	–	*Kp + Ec*	OXA-48	6	–
19	71	F	P	Pneumonia	YES	CZ, AMC	PS	11	LEVO	*Kp + Ec*	OXA-48	5	LEV	*Kp + Ec*	OXA-48	11	–
20	90	F	G	Cholangitis	YES/ *Kp + Ec*	MER, ERT, AMK, FEP, AMC, CIP	NR	9	TZP	*Kp + Ec*	OXA-48	9	–	–	–	–	–
21	72	M	G	Septic shock of biliary origin	YES	TZP, MER, LIN, CIP	NR	41	LZD, MER	*Kp*	VIM-1	14	LZD	*Ec*	VIM-1	14	–
22	64	M	U	SI (Endoscopy)	YES	CZ, AMC, CX, TZP, CIP LZD, MER	NR	79	TZP	*Cf*	VIM-1	27		*Ko*	VIM-1	79	*Ko* (Urine)
23	36	M	N	Cervical pain	NO*	–	NG	58	AMC, CZ	*Kp*	NDM-1	27	AMC, CIP	*Kp*	NDM-1	58	–
24	66	M	P	Pneumonia	YES	TZP	PS	14	–	*Ec*	KPC-3	1	MER	*Kp Ec*	KPC-3 VIM-1	14	–
25	49	M	N	Subarachnoid hemorrhage	NO	–	NG	88	VAN, MER, LZD	*Kp*	NDM-1 VIM-1	29	–	*Kp + Ec*	VIM-1	79	–
26	92	M	N	Cranioencephalic trauma	YES	TZP	NG	92	CIP	*Kp*	NDM-1	41	TZP	*Kp*	NDM-1 OXA-48	52	*Kp* NDM-1 (Urine)
27	80	F	G	Esophageal prosthesis (SI)	YES/ *Kp + Ec*	TZP, CZ, ERT, MER, VAN, LZD, SXT	PS	6	–	*Ec*	VIM-1	1	–	*Kp*	OXA-48	2	–
28	75	M	N	Cerebral cavernoma	NO	–	NG	114	VAN, CIP, AMK, CD, AZT	*Ec*	OXA-48	68	CIP, AZT	*Kp*	VIM-1	71	–
29	76	M	P	Pulmonary adenocarcinoma	YES	–	NG	106	CXM, AMC, MER, LZD, AMK, TZP, VAN	*Ecl*	VIM-1	80	–	*Kp*	OXA-48	88	*Ecl* (Urine)
30	44	M	P	Pulmonary adenocarcinoma	NO	–	NG	24	AMC, CX	*Kp*	VIM-1	10	TZP	*Ec*	OXA-48	24	–

CPE = Carbapenemase producing *Enterobacterales*; ATB = Antibiotics; AS = Admission sample; CP = Carbapenemase; F = Female; M = Male; G = Gastroenterology; P = Pneumology; N = Neurosurgery; U = Urology; LOS = Length of stay; LOS-1 = Length of stay until first positive CPE culture; LOS-2 = Length of stay until second positive CPE culture; NG = Negative; PS = Positive; NR = Not registered; SI = Scheduled income; COPD = Chronic obstructive pulmonary disease; CRD = Chronic renal disease; ARD = acute renal disease; ERCP = Endoscopic retrograde cholangiopancreatography; *Ka = Kluyvera* spp.; *Ro = R. ornithinolytica; Kp = K. pneumoniae; Ec = E. coli; Ecl = E. cloacae; Ko = K. oxytoca; Cf = C. freundii;* AMC = Amoxicillin-clavulanic acid; AMK = Amikacin; AMP = Ampicillin; AZM = Azithromycin; AZT = Aztreonam; CD = Clindamycin; CIP = Ciprofloxacin; CLR = Clarithromycin; COL = Colistin; CRO = Ceftriaxone; CTX = Cefotaxime; CX = Cloxacillin; CXM = Cefuroxime; CZ = Cefazoline; DAP = Daptomycin; ERT = Ertapenem; FEP = Cefepime; FOS = Fosfomycin; GEN = Gentamicin; LEV = Levofloxacin; LZD = Linezolid; MER = Meropenem; MTR = Metronidazol; STX = Trimethoprim sulfamethoxazole;TIG = Tigecycline; TZP = Piperacillin-tazobactam; VAN = Vancomycin.

* Previous admission in other health care center.

Table 2
Patient characteristics during the R-GNOSIS Project.

	TOTAL		Co-colonization with CPE-SC		Co-colonization with CPE-DC		Colonization with a single CPE		*P-value*
	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%	Mean	CI 95%	
Age (y)[a]	71	69–74	73	66–80	69	53–85	71	69–73	0.744
LOS (d)[a]	33	28–39	35	20–51	63	20–107	32	25–38	0.113
LOS-1 (d)[b]	20	16–25	9*	5–14	33	3–62	22*	17–26	0.016
LOS-2 (d)[c]	–	–	27	15–39	47	16–79	–	–	0.194
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**	
Males[d]	97	59.9	16	64.0	6	85.7	77	58.3	>0.05
Clinical sample[d]	28	17.3	6	24.0	2	28.6	20	15.2	>0.05
TOTAL	162	2.0	25	15.4	7	4.3	132	81.5	

CPE-SC = CPE producing the same carbapenemase.
CPE-DC = CPE producing a different carbapenemase.
[a] ANOVA Test was used to determine the *P-value*.
[b] Kruskal-Wallis Test was used to determine the *P-value*.
[c] Student's T-Test was used to determine the *P-value*.
[d] Fisher's Exact Test was used to determine the *P-value*.
* *P-value* (Mann Whitney Test) ≤ 0.0052.

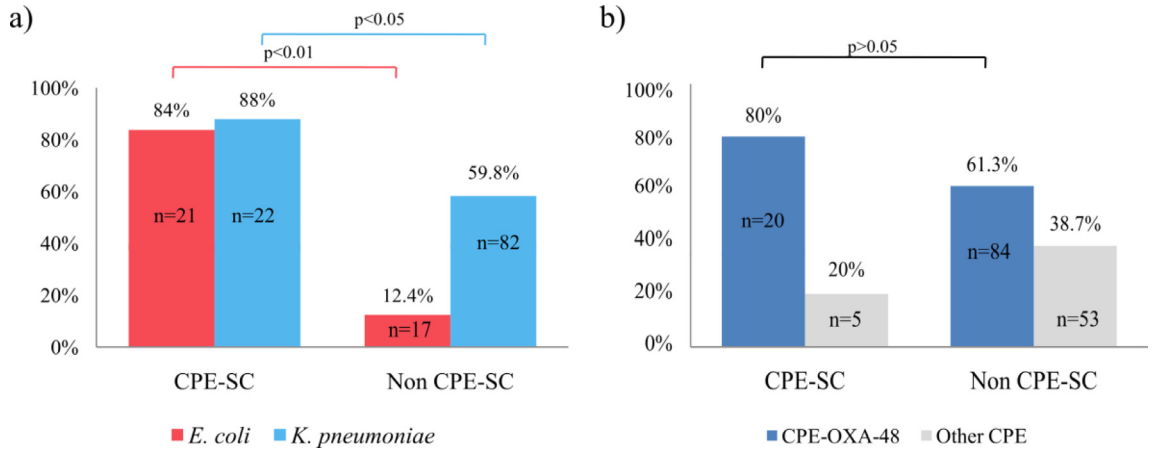

Fig. 1. (a) Frequency of intestinal recovery of *E. coli* or *K. pneumoniae* in patients co-colonized with carbapenemase-producing-*Enterobacterales* (CPE). CPE producing the same carbapenemase (CPE-SC), and non-CPE-SC patients (co-colonized with CPE producing different carbapenemases and with a single CPE); (b) Frequency of intestinal recovery of OXA-48 carbapenemase strains/clones compared with other carbapenemases, in CPE-SC and non-CPE-SC patients. *P-value* was calculated using the Fisher's Exact Test.

M-15 was the most frequent ESBL (18/21, 85.7%) and was mainly associated with OXA-48-*K. pneumoniae* (13/18, 72.2%) (Table S1).

Three co-colonization patterns were identified among CPE-SC-patients: (i) co-colonization detected in the first positive culture (pattern A), (ii) co-colonization detected in the second positive culture, after the first CPE detection (pattern B) and (iii) colonization with different CPE in the first and subsequent cultures (pattern C). Pattern A was detected in 14/25 (56%) patients and all CPE isolates were OXA-producers. Most of them had previous admissions in our hospital (10/14, 71.4%) or in other health care centers (2/14, 14.3%). Pattern B was identified in six patients, five of whom were OXA-48 carriers. In three cases, OXA-48-*K. pneumoniae* strains were recovered in the first positive culture. Finally, pattern C was mostly identified among patients co-colonized with VIM-1, NDM-1 and KPC-3 producers (Fig. 2).

On the other hand, *K. pneumoniae* isolates were found in all CPE-DC-patients, while *E. coli* strains were identified in 71.4% (5/7) of them. Both species were also significantly more prevalent in this subgroup of co-colonized patients than among the rest of the patients [*K. pneumoniae*: 95/155, 61.3% ($p = 0.047$) (odds ratio = 0.0; CI 95% 0.0–1.15), *E. coli*: 32/155, 20.6% ($p = 0.007$) (odds ratio = 0.11; CI 95% 0.01–0.68)]. Each of these species was detected in the first positive culture in three cases, but persistence along subsequent cultures was not demonstrated (Table 1).

3.3. Molecular typing and clonal diversity

Among the CPE-SC patients, two cases of co-colonization with two different *K. pneumoniae* clones were identified (patients 23 and 25). In the remaining patients, identical PFGE profiles were observed among CPE isolates of the same bacterial species recovered in subsequent positive cultures. Among patients co-colonized with *K. pneumoniae*, ST11 (8/22, 36.4%), ST15 (3/22, 13.6%), ST101 (2/22, 9%) and ST307 (2/22, 9%) high-risk clones were identified. Conversely, high-genetic diversity was found among *E. coli* isolates (19 PFGE profiles) and clonal complex 10 (clone A) was the most frequent (4/21, 19%) (table S1). Differences were observed in terms of clonal diversity between *K. pneumoniae* (SDI = 0.89) and *E. coli* (SDI = 0.98). For the other species, PFGE-*Xba*I revealed a single *Kluyvera* spp. clone; two *Citrobacter freundii* clones [A ($n = 2$) and B ($n = 1$)] and three distinct clones of *Klebsiella oxytoca* (A-C) (Table S1).

Among CPE-DC-patients, *K. pneumoniae* ST11 (2/7, 28.6%) and ST101 (2/7, 28.6%) high-risk clones were also detected. Additionally, the *K. pneumoniae* ST54-VIM-1 clone was also identified (3/7, 42.8%). Conversely, all *E. coli* isolates were grouped into different PFGE patterns and STs (Table S1).

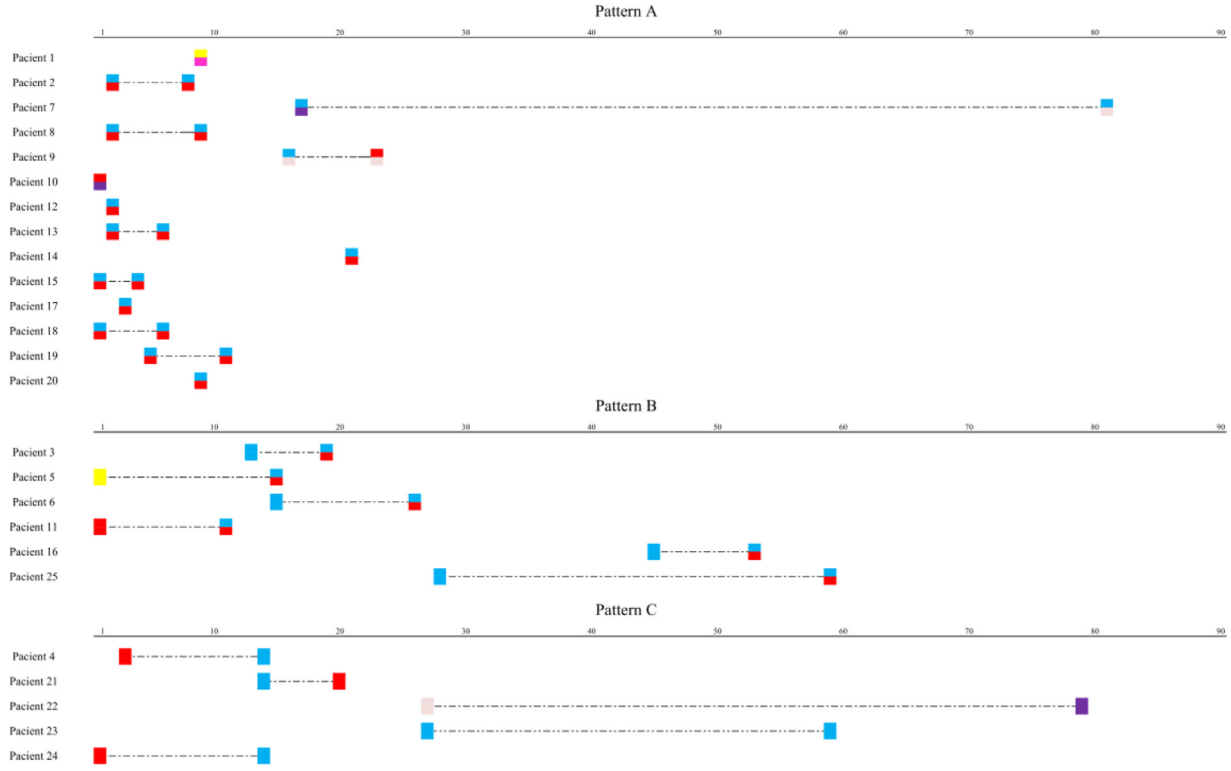

Fig. 2. Dynamics of patients' co-colonization with different carbapenemase producing *Enterobacterales* (CPE), producing the same carbapenemase during the R-GNOSIS Project. Numbers scale indicates the colonization or co-colonization day from the admission. Dashed line represents the LOS (length of hospital stay).

3.4. Transferability of carbapenemase genes and plasmid characterization

All $bla_{\text{OXA-48}}$, $bla_{\text{VIM-1}}$ and $bla_{\text{KPC-3}}$ genes were successfully transferred to the azide-resistant *E. coli* J53 strain, while $bla_{\text{NDM-1}}$ transconjugants were only obtained from a single clone (ST101). From *Kluyvera* spp., two transformants carrying $bla_{\text{OXA-48}}$ were obtained (Table S1).

$bla_{\text{OXA-48}}$, $bla_{\text{KPC-3}}$ and most $bla_{\text{VIM-1}}$ were located on a *ca.* 60 kb transferable plasmid in clinical isolates and transconjugant/transformant strains. All *ca.* 60 kb $bla_{\text{OXA-48}}$- and $bla_{\text{VIM-1}}$- encoding plasmids were assigned to the broad host-range IncL group. RFLP patterns showed comparable restriction profiles (profile A) and *repA, parA* and *traU* genes were amplified, indicating a close relation with the IncL/M-pOXA-48a plasmid previously described. [12] The $bla_{\text{CTX-M-15}}$ gene was also located on these plasmids in both clinical and transconjugant isolates in only three *K. pneumoniae* isolates (cases 4, 13 and 26). In the *K. pneumoniae* ST54 clone a *ca.* 90 kb pVIM-1 + SHV-12 plasmid, nontypeable by PBRT, was identified. Concerning the $bla_{\text{KPC-3}}$-harboring plasmid, the incompatibility group IncN was assigned following the PRBT scheme. Moreover, the restriction profiles (profile B) were different from those of $bla_{\text{OXA-48}}$- and $bla_{\text{VIM-1}}$-containing plasmids. Amplification for *rep*A, *tra*U and *par*A (genes related to the IncL/M-pOXA-48a backbone) was not demonstrated in $bla_{\text{VIM-1}}$- (*ca.* 90 kb), $bla_{\text{KPC-3}}$- (*ca.* 60 kb) and $bla_{\text{NDM-1}}$- (*ca.* 120 kb) containing plasmids (Table S1).

Overall, OXA-48-harboring transconjugants/transformants exhibited different levels of non-susceptibility to carbapenems (58% ertapenem, 25% imipenem and 17% meropenem). In contrast to clinical isolates, these transconjugants/transformants showed high susceptibility to broad-spectrum cephalosporins and aztreonam. Moreover, all transconjugants carrying pNDM-1, pVIM-1 or pKPC-3 showed high resistance to carbapenems and broad-spectrum cephalosporins (Table 3).

4. Discussion

In recent years, emergence and occurrence of CPE has been increasing worldwide, including Spain [2,19]. During the R-GNOSIS Project, the incidence of CPE intestinal carriage was 2% and was mainly due to OXA-48 producers [16]. According to previous publications, prolonged stays in healthcare institutions is recognized as a risk factor for rectal CPE colonization [20,21]. In the present study, we retrospectively observed a high prevalence of co-colonization involving CPE producing different carbapenemases (4.3%), particularly in those patients with longer hospital stays. Nevertheless, our work indicates that intra-patient transmission of the same carbapenemase gene among different CPE species is probably the most frequent event resulting in co-colonization (15.4%). In subgroup CPE-SC-patients, the mean LOS until the detection of CPE (LOS-1) was significantly lower (9 days) than in patients carrying a single CPE. It should be noted that CPE-SC co-colonization was directly detected in the first positive sample in 56% of these patients, since most of them had previous admissions in our hospital (71.4%) or in other healthcare centers (14.3%) and had received multiple antibiotic treatments. Therefore, our results suggest that previous hospital admissions, prolonged LOS and antibiotic treatments could be a risk factor for co-colonization with CPE producing the same or a different carbapenemase. A recent study has evidenced the inter-species transmission of plasmid-encoding carbapenemases under antibiotic pressure [22]. Colonization with carbapenemase producing isolates is also considered a risk factor for infection [20,21,23]. In our study, CPE in infective sites were more frequently detected in co-colonized patients than in those colonized with a single CPE. Although further studies are required to support this finding, our results suggest that coloniza-

Table 3
Antibiotic non-susceptibility (resistant plus intermediate).

	OXA-48		VIM-1		KPC-3		NDM-1	
	CI No. (%)	TC No. (%)	CI No. (%)	TC No. (%)	CI No. (%)	TC No. (%)	CI No. (%)	TC No. (%)
Amoxicillin-Clavulanate	48 (100)	48 (100)	12 (100)	12 (100)	2 (100)	2 (100)	4 (100)	3 (100)
Piperacillin- tazobactam	48 (100)	48 (100)	12 (100)	12 (100)	2 (100)	2 (100)	4 (100)	3 (100)
Cefotaxime	34 (70.8)	10 (20.8)	12 (100)	12 (100)	2 (100)	2 (100)	4 (100)	3 (100)
Ceftazidime	30 (62.5)	10 (20.8)	12 (100)	12 (100)	2 (100)	2 (100)	4 (100)	3 (100)
Cefepime	27 (56.2)	6 (12.5)	12 (100)	12 (100)	2 (100)	2 (100)	4 (100)	3 (100)
Aztreonam	28 (58.3)	8 (16.7)	5 (41.7)	3 (25)	2 (100)	2 (100)	4 (100)	0 (0)
Imipenem	9 (18.7)	12 (25)	6 (50)	5 (41.7)	2 (100)	2 (100)	4 (100)	3 (100)
Meropenem	31 (64.6)	8 (16.7)	7 (58.3)	2 (16.7)	2 (100)	2 (100)	4 (100)	3 (100)
Ertapenem	48 (100)	28 (58.3)	8 (66.7)	3 (25)	2 (100)	2 (100)	4 (100)	3 (100)
Gentamicin	25 (52.1)	2 (4.2)	4 (33.3)	0 (0)	0 (0)	0 (0)	4 (100)	3 (100)
Tobramycin	25 (52.1)	1 (2.1)	11 (91.6)	0 (0)	0 (0)	0 (0)	4 (100)	3 (100)
Amikacin	1 (2.1)	0 (0)	1 (8.3)	0 (0)	0 (0)	0 (0)	3 (75)	3 (100)
Ciprofloxacin	32 (66.7)	1 (2.1)	7 (58.3)	1 (8.3)	2 (100)	0 (0)	4 (100)	0 (0)
Sulfamethoxazole trimethoprim	31 (64.6)	1 (2.1)	8 (66.7)	1 (8.3)	2 (100)	0 (0)	1 (25)	0 (0)
Tigecycline	2 (4.2)	1 (2.1)	2 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Fosfomycin	36 (75)	0 (0)	7 (58.3)	0 (0)	2 (100)	0 (0)	3 (75)	0 (0)
Colistin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

(CI = Clinical isolates; TC = Transconjugant).

tion with more than one CPE could increase the risk of infection with these multidrug-resistant microorganisms. A limitation of this retrospective study was the lack of a full set of swabs in each patient (admission, weekly and discharge samples). In fact, we cannot rule out that a higher number of cases involving patients co-colonized with multiple CPE could have occurred.

During our study, the incidence of *K. pneumoniae* and *E. coli* in patients co-colonized with CPE producing the same or a different carbapenemase was significantly higher than among the rest of the patients. Moreover, we observed that co-colonization with different OXA-48-producing *Enterobacterales* species was the most frequent event. Overall, *K. pneumoniae* was the main reservoir of $bla_{\text{OXA-48}}$, although the number of studies reporting cases due to other OXA-48 producing *Enterobacterales* species is increasing worldwide, including those performed in our hospital [16,24–27]. In our study, OXA-48-producing *K. pneumoniae* and *E. coli* were recovered simultaneously in the same sample in most patients but, recurrently, after a prior colonization with a single OXA-48-*K. pneumoniae*. According to our results, we suggest that a diversity of *E. coli* clones act as the recipients of $bla_{\text{OXA-48}}$ gene which is usually transferred by well-adapted hospital clones of *K. pneumoniae*. In Spain, the main candidates acting as $bla_{\text{OXA-48}}$ donors are the high-risk *K. pneumoniae* clones ST11-OXA-48 and ST15-OXA-48 [8,25]. Efficient *in-vivo* conjugative transfer of $bla_{\text{OXA-48}}$ from *K. pneumoniae* to *E. coli* and the spread among different *E. coli* clones, might explain the early detection and high frequency of co-colonization with both *Enterobacterales* species during hospital stay. In our collection, the successful intra-patient horizontal transfer of $bla_{\text{OXA-48}}$ was due to the spread of a dominant and highly transmissible *ca.* 60 kb IncL plasmid related to the previously described and worldwide disseminated IncL/M-pOXA-48a [12,28]. The conjugation efficiency of IncL/M-pOXA-48a has been related to the insertion of the composite transposon Tn*1999* containing $bla_{\text{OXA-48}}$ into the *tir* gene [29,30]. Note that most *K. pneumoniae* strains also carried bla_{ESBLs} genes (76.2%) but transfer to *E. coli* was non frequent if compared with carbapenemases genes, particularly $bla_{\text{OXA-48}}$, suggesting that probably genes encoding-ESBL are chromosomally located (data not shown). Intra-patient lateral transmission of related pOXA-48 plasmids from high-risk clones of *K. pneumoniae* to unrelated *E. coli* isolates has also been occasionally described in other studies [4,31–33]. It was remarkable that the cross-species transfer of related IncL VIM-1-containing plasmids was also detected over time during our study. Additionally, strains harboring these IncL pVIM-1 and pOXA-48 plasmids co-existed with other *Enterobacterales* containing unrelated IncN plasmids carrying $bla_{\text{KPC-3}}$ gene and non-typeable plasmids carrying $bla_{\text{NDM-1}}$ gene, which could contribute to exchange genetic material and facilitate the acquisition of other resistance genes. A recent study suggested that the rate of *in vivo* horizontal transfer of $bla_{\text{OXA-48}}$ is underestimated and the development of *in vivo* models rather than *in vitro* experiments could help to highlight the real magnitude of acquisition and spread of carbapenemase-encoding plasmids [32].

Although, co-colonization with different CPE isolates in our study might be driven by our local epidemiological scenario dominated by OXA-48-producing *Enterobacterales,* our results suggest that CPE co-colonization is more frequently due to horizontal gene transfer between species than to the sequential acquisition of different CPE isolates. Under antibiotic exposure, CPE co-colonization probably increases in absolute numbers in the intestinal compartment. This fact might facilitate translocation or migration to the urinary tract and we found that co-colonized patients have CPE more frequently in infective sites [23]. Moreover, CPE co-colonization status should be monitored during epidemiological surveillance cultures, as these patients might be at a higher risk for systemic or local (mostly urinary tract) infections. Limitation of the use of antibiotics, shorter antibiotic treatments and reduction of hospital stay might also help to limit intestinal co-colonization with different CPE isolates.

Contributors

MH-G undertook the investigation, methodology, data curation and writing the original draft. PR-G and RC performed the funding acquisition, conceptualization, supervision and writing, reviewing and editing the manuscript. BP-V provided technical support to MH-G during the methodology. CN-S contributed to the data curation, writing, reviewing and editing the draft. FB and MM contributed to the supervision and writing, reviewing and editing the final version of the manuscript. All authors have read and approved the final version of this manuscript.

Declaration of Competing Interest

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

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Ethics committee approval

This study was approved by the Ramón y Cajal University Hospital Ethics Committee (Reference 251/13).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.eclinm.2019.09.005.

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