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Molecular epidemiology and drug-resistance mechanisms in carbapenem-resistant *Klebsiella pneumoniae* isolated in patients from a tertiary hospital in Valencia, Spain

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ABSTRACT

Objectives: The aim of this study has been to characterize carbapenem-resistant *Klebsiella pneumoniae* isolates and to determine the resistance mechanisms involved, the clonal relationship between strains and clinical and demographical data of the infected patients.
Methods: Clinical and demographical data from patients were collected and statistically analysed. Antimicrobial susceptibility testing was performed and resistance genes were detected both phenotypically and genotypically. Conjugation assays were performed to show horizontal transferability of resistance genes. Clonal relationship was also studied. Next-generation sequencing (NGS) was performed to obtain information regarding resistance genes, sequence types, virulence factors and plasmid types.
Results: Statistical significance was shown by the presence of an infection if there had been a previous hospital stay; urinary catheter carriage and chronic renal disease also indicated higher probabilities of being infected. More than 95% of the isolates were non-susceptible to third-generation cephalosporins, and more than 90% were non-susceptible to quinolones. Phenotypic and genotypic methods for resistance detection were concordant and later confirmed by NGS. This is the first detection of OXA-48, NDM-1 and CTX-M-15 co-production in the area. No plasmid-mediated colistin resistance was found. Tetracycline, sulfonamides and aminoglycoside resistance genes were found in almost all the isolates studied. No virulence factors were detected. Multilocus sequence typing showed more than 15 different sequence types, with ST101, ST307 and ST11 being the most prevalent.
Conclusions: This study is the first to report such a large group of OXA-48 carbapenemases with clonal dissemination among carbapenem-resistant *K. pneumoniae* in Valencia. This is also the first detection of OXA-48, NDM-1 and CTX-M-15 co-production in the area.

1. Introduction

Klebsiella pneumoniae, a member of the Enterobacteriaceae family, is naturally present in the human and animal gastrointestinal tract [1]. However, it is the most common aetiological agent of nosocomial and community-acquired infections. Pathogenic *K. pneumoniae* strains have the potential to cause a wide variety of infectious diseases, including urinary tract, respiratory tract and blood infections [2].

Furthermore, the emergence and dissemination of carbapenem-resistant *K. pneumoniae* (CRKP) strains has posed an urgent threat to public health on a global scale. Carbapenem resistance in *K. pneumoniae* is mainly owing to the acquisition of carbapenemase genes associated with mobile elements such as plasmids. In fact, CRKP strains are characterized by multiantibiotic-resistance profiles that involve most β-lactams, including carbapenems, along with other non β-lactam antibiotics, limiting therapeutic options and affecting the clinical outcome of infections, with high mortality rates reported in some categories of patients [3].

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Specific virulence factors associated with CRKP have not been reported. However, risk factors associated with the acquisition of CRKP include prolonged hospitalization, intensive care unit (ICU) stay, invasive devices, immunosuppression and multiple antibiotic treatment before initial infection [4].

Since 2015, we noticed a marked increase in the number of clinical isolates of *K. pneumoniae* resistant to carbapenems in our hospital, which is a university hospital located in Valencia (eastern part of Spain), with a total capacity of 500 beds. Since then, an active surveillance programme for carbapenemase-producing Enterobacteriaceae, using axillar-rectal and pharyngeal swabs for patients admitted to the ICU has been ongoing. The objective of this study has been to investigate the clinical and microbiological characteristics of CRKP isolates collected in our hospital from 2015 until 2018, either from clinical samples and/or the active surveillance programme.

2. Methods

We conducted a retrospective study from 2015 until 2018 at the microbiology department of the Valencia General Hospital, including isolates of *K. pneumoniae* that were found to be non-susceptible to any of the carbapenems tested. Only one sample per patient was included in this study, usually the first one.

2.1. Demographical and clinical data of patients infected and/or colonized with CRKP

The following information about the patients was collected and saved in a database for further statistical analysis with MedCalc online software [5]:

- Hospital ward
- Previous hospitalization (less than 6 months)
- Type of infection:
  - Healthcare associated: occurring in a patient after 48 h or more after hospitalization or within 30 days after having received healthcare [6].
  - Community-acquired infection: diagnosed within 48 h of admission in patients without any previous encounter with healthcare [7].
- Recent antibiotic therapy (less than 30 days)
- Underlying conditions
  - Diabetes mellitus
  - Chronic renal disease
  - Cirrhosis
  - Chronic pulmonary diseases
  - Functional or structural uropathology
  - Urinary catheter
  - Previous colonization (pharyngeal or axillar-rectal)
- Acute events during hospitalization:
  - Sepsis
  - Pneumonia (associated or not to mechanical ventilation)
  - Surgical event or complication (less than 30 days)
- Treatment adequacy: directed treatment considering in vitro susceptibilities, source of infection and adequacy to guidelines.

2.2. Identification, antimicrobial susceptibility testing and phenotypical detection of carbapenemase production

2.2.1. Identification and antimicrobial susceptibility testing

All isolates were initially identified by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) Bruker (Billerica, Massachusetts, United States) and biochemical testing (MicroScan Walkaway, Beckman Coulter (Brea, California, United States)).

The minimum inhibitory concentrations (MICs) of ampicillin, amoxicillin and clavulanate, amikacin, cefepime, cefotaxime, cefoxitin, ceftazidime, cefuroxime, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, norfloxacin, piperacillin–tazobactam, tigecycline, trimethoprim–sulfamethoxazole, meropenem and levofloxacin were obtained using the microdilution automated system MicroScan Walkaway (Beckman Coulter) [8]. The antibiotics tested varied depending on the origin of the sample. Susceptibility breakpoints were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [9]. *Escherichia coli* ATCC 25922 was used for quality control. All isolates were frozen for preservation at −80 °C in an adequate medium for posterior analyses.

2.2.2. Phenotypic detection of carbapenemases

2.2.2.1. β-Carbatest (BioRad). The β-Carbatest was performed on all strains that were found to be non-susceptible to at least one carbapenem. It consists of a qualitative colorimetric test based on colour change when hydrolysis of the imipenem β-lactam ring occurs. When there is a change in colour from yellow to orange/red (Fig. 1), the test is considered positive [10].

2.2.3. Inhibitor-based approach

The inhibitor-based tests were also performed on all strains found to be intermediate or resistant to at least one carbapenem. These tests rely on the ability of certain compounds to inhibit the carbapenemase activity specifically. Thus, metallo-β-lactamase activity is inhibited by ethylenediaminetetraacetic acid and dipicolinic acid (which are chelating agents) and boronic acid inhibits *K. pneumoniae* carbapenemase (KPC) enzymes. Cloxacillin, however, is used to differentiate AmpC β-lactamase production. The addition of a temocillin disk has been proposed as a surrogate marker of OXA-48 and functionally related variants. Examples of these tests are shown in Fig. 2 [11].

2.2.4. Phenotypic detection of hypervirulent strains: string test

The string test is a widely used marker for hypervirulent *K. pneumoniae* strains. It is considered positive when it is possible to generate a viscous string >5 mm in length from a bacterial colony using an inoculation loop [12]. The test was performed on all the strains that were found to be non-susceptible to at least one carbapenem.

2.2.5. Molecular detection of resistance genes

Carbapenemase genes (Class A [bla_{KPC}], Class B [bla_{IMP}, bla_{VIM}, bla_{NDM}]), plasmid-encoding AmpC (bla_{CMY}, bla_{DHA} and bla_{MOX}) and extended-spectrum β-lactamase (ESBL) genes (bla_{CTX-M}) were detected by a loop-mediated isothermal amplification (LAMP)-

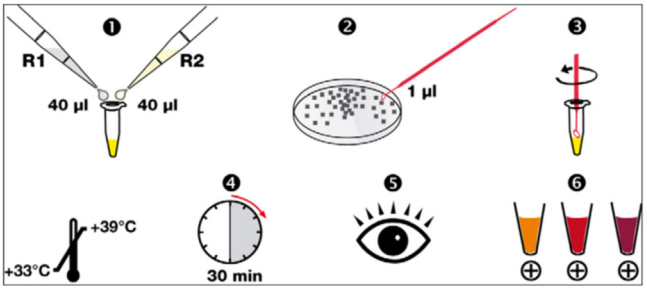

Fig. 1. Procedure for the detection of carbapenemase activity [10]. R1: Reactive 1; R2: Reactive 2.

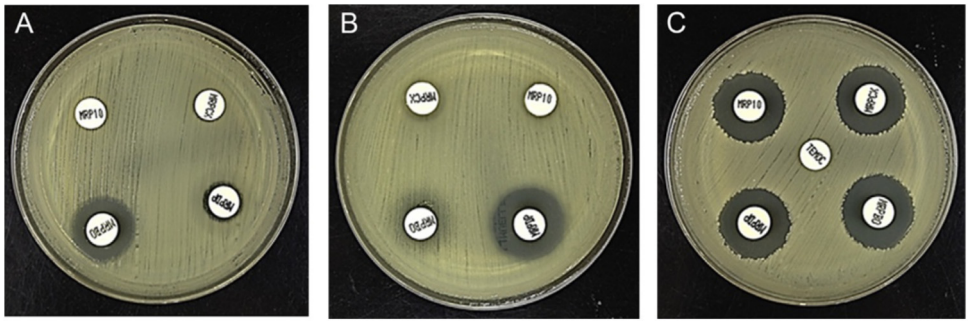

Fig. 2. (A) Positive for KPC production. (B) Positive for metallo-β-lactamase activity. (C) Positive for OXA-48 production [10]. KPC, *Klebsiella pneumoniae* carbapenemase.

based system named Eazyplex (Amplex® Biosystems GmbH, Giessen, Germany).

The Eazyplex SuperBug CRE system is a qualitative genotypic diagnostic test consisting of a freeze-dried, ready-to-use mixture for an isothermal amplification reaction that covers carbapenemase variants of the VIM (-1 to -37), NDM (-1 to -7) and KPC (-2 to -15) families, part of the OXA-48 family (-48, -162, -204 and -244), OXA-181 and the CTX-M ESBL families (CTX-M-1 group [CTX-M-1, CTX-M-3 and CTX-M-15] and CTX-M-9 group) from Gram-negative bacteria. Amplification products that generated LAMP were visualized by real-time fluorescence measurement of a fluorescent dye bound to double-stranded DNA using the GENIE II instrument (OptiGene, Horsham, UK) [13].

2.2.6. Conjugation assays

The conjugation transfer assay was performed in broth culture using azide-resistant *E. coli* J53 as the recipient. The recipient cells were grown at 37 °C in fresh brain–heart infusion broth (5 mL) containing sodium azide (100 μg/mL) for 24 h. In this case, the donor strains were two isolates of *K. pneumoniae* NDM-1 and CTX-M-1 producer. The donor cells were grown overnight at 37 °C in brain–heart broth. Donor and recipient cells were mixed at a ratio of 1:10 (donor/recipient) and incubated at 37 °C for at least 4 h. The cell suspension was inoculated in agar plates containing ertapenem (1 μg/mL) and supplemented with sodium azide (100 μg/mL). The colonies grown on the selection media were selected and the species were identified using MALDI-TOF/MS as previously described. Manual disk-diffusion antimicrobial susceptibility testing of β-lactams and non-β-lactams such as trimethoprim–sulfamethoxazole, ciprofloxacin and colistin [14] was performed, together with the isothermal amplification for the detection of *bla* genes to confirm horizontal resistance gene transfer as previously described.

2.2.7. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed on 150 isolates, which were selected following sequence-type analysis. The preparation of the plugs and digestion with *Xba*I Thermo Fischer Scientific (Waltham, Massachusetts, United States) was performed according to Romesh K. Gautom protocol [15]. The DNA fragments were separated by electrophoresis using a CHEF-DR III apparatus (Bio-Rad Laboratories, Hercules, CA). DNA samples from *Saccharomyces cerevisiae* (Bio-Rad Laboratories) were used as molecular size markers. The PFGE patterns were analysed using Bionumerics 7.6 software (Applied Maths, Sint-Martens-Latem, Belgium). PFGE patterns were assigned to clusters according to the criteria proposed by Tenover et al. [16].

2.2.8. Next-generation sequencing applied to clinical microbiology

Genomic DNA was purified from all strains using a DNA extraction kit, MagCore® Automated Extraction System (RBC Bioscience (New Taipei City, Taipei)). DNA libraries were prepared using the Nextera XT sample preparation method and sequenced with a NextSeq instrument (Illumina (San Diego, California, United States)) [17].

2.2.8.1. Multilocus sequence typing analysis. Multilocus sequence typing (MLST) was determined using a BLAST-based approach with ARIBA (Antimicrobial Resistance Identification from Assembly) [18] using the short reads (150 bp, paired-ends) derived for each strain.

2.2.8.2. Antimicrobial resistance genes, virulence factors and plasmid detection. Resistance to β-lactams and non-β-lactam antibiotics, virulence factors and replicon genes were detected using the short reads derived by next-generation sequencing (NGS). This analysis was also performed with ARIBA, which uses a combined mapping/alignment and targeted local assembly approach to identify antimicrobial resistance (AMR) genes and variants efficiently and accurately from paired sequencing reads. ARIBA includes support for a number of public databases. ResFinder [19], PlasmidFinder [20] and Virulence Finder database (VFDB) [21] were used for the identification of corresponding factors in our samples.

2.2.8.3. Statistical analyses. Bivariate analyses were performed to obtain the odds ratio of the different factors considered using MedCalc online software [5]. A confidence interval of 95% and a *P*-value < 0.05 was established as cut-off values.

3. Results

3.1. Demographics and clinical features

A total of 1013 CRKP strains (including clinical and surveillance isolates) corresponding to 234 patients were isolated from January 2015 to December 2018 at the Valencia General Hospital (Spain). Only patients at the ICU underwent active surveillance at the moment of admission and once per week (axillar-rectal and pharyngeal swab culture), according to the protocols established in our hospital.

Among the 234 patients enrolled, 135 were male (58%). The age range was 26–99 years (72.01 ± 14.11). The distribution according to the different wards of the hospital is shown in Fig. 3.

Of the 1013 CRKP isolates, 607 (60%) were obtained from surveillance cultures, whereas 406 (40%) were collected from clinical specimens (Fig. 4), including 58% (234 of 406) from urine, 7% (30 of 406) from respiratory samples and 7% (27 of 406) from blood samples.

A total of 150 CRKP-infected patients (64.1%) were detected. Previous colonization with CRKP was observed in 60 infected patients (40%), but only information about previous colonization

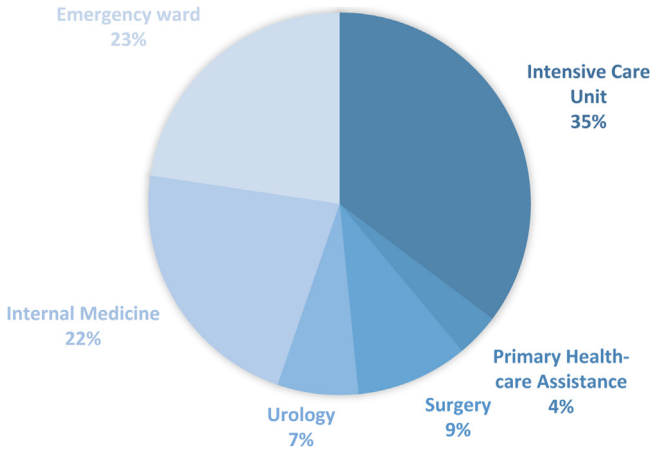

Fig. 3. Distribution of the patients colonized and/or infected by CRKP among the different wards. CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

was collected from patients who had had a previous stay in the ICU. Most of them had the same *K. pneumoniae* isolate in more than one sample; 49 had sepsis at some point (not all of them were microbiologically diagnosed), and 16 had pneumonia (50% were associated with assisted ventilation).

Among the CRKP-infected patients, almost 50% had previously been admitted to the hospital, and the ones who had not had other risk factors such as having a urinary catheter, previous antibiotic treatment and/or surgery complications.

A bivariate analysis was performed to find a significant difference in suffering an infection by CRKP (Table 1). The different risk factors were studied and the results showed that there was statistical significance with an odds ratio higher than 1 in suffering an infection if there had been a previous hospital stay. Regarding the risk factors related to the patient, there was also statistical significance for patients carrying a urinary catheter and patients with chronic renal disease, showing higher probability for suffering a CRKP infection. Regarding the other risk factor studies, although the odds ratio was higher than 1, it could not be statistically proven.

Regarding the classification of the infection-type distribution depending on its origin (Fig. 5), only 16% of the infections caused by CRKP in all 234 patients were acquired in the community (37/234), 84% being related to hospitalization (197/234).

3.2. Antimicrobial susceptibility testing and phenotypic detection of carbapenemase production

3.2.1. Antimicrobial susceptibility testing

Susceptibility testing of the 234 strains revealed that more than 95% were non-susceptible to third-generation cephalosporins

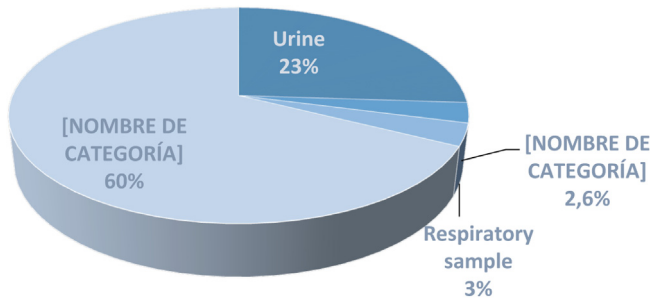

Fig. 4. Sample distribution.

Table 1
Bivariate analyses of risk factors associated with CRKP infection.

Risk factor	Odds ratio	95% CI	*P*-value
Previous antibiotic therapy	1.529	0.899–2.601	0.12
Previous hospital stays	3.071	1.677–5.626	<0.001
Surgery	1.615	0.927–2.815	0.09
Diabetes mellitus	1.491	0.819–2.715	0.19
Chronic renal disease	5.482	2.811–10.691	<0.001
Chronic pulmonary disease	1.038	0.511–2.108	0.92
Neoplasia	1.04	0.567–1.907	0.9
Structural or functional uropathology	2.34	0.643–8.568	0.19
Urinary catheter	2.57	1.323–4.994	0.0053

CRKP, carbapenem-resistant *Klebsiella pneumoniae*.

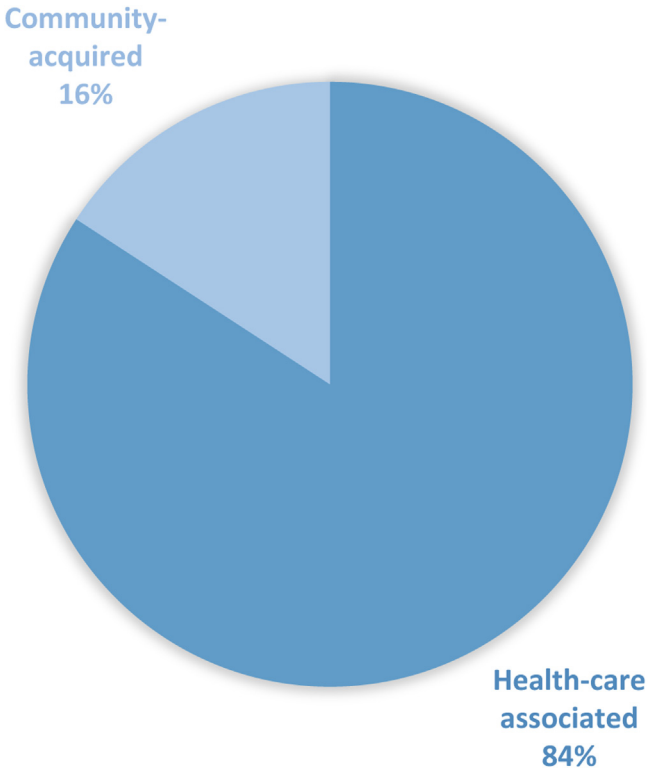

Fig. 5. Infection-type distribution.

(MICs > 4 mg/L), and more than 90% were also non-susceptible to quinolones. Resistance to imipenem, meropenem and ertapenem in all the isolates was variable (MICs ranging from 1 to >8 mg/L). A moderate frequency of resistance, around 50%, was shown to tigecycline (MICs > 2 mg/L), gentamicin (MIC > 4 mg/L) and fosfomycin (MICs > 32 mg/L). Circa 30% of the isolates presented resistance towards trimethoprim–sulfamethoxazole. Colistin turned out to be the antibiotic with the highest susceptibility rates (all MICs < 2 mg/L).

The distribution of antimicrobial susceptibility of all CRKP isolates is shown in Table 2.

3.3. Phenotypical detection of carbapenemases

3.3.1. β-Carbatest (BioRad)

The β-Carbatest was performed on the 234 strains. All the strains which were carbapenemase producers (199/234) were positive for the test. AmpC β-lactamase producers and ESBL-producer strains were β-Carbatest negative.

Table 2
Resistance rates against 20 antimicrobial agents assessed in 234 carbapenem-resistant *K. pneumoniae* isolates.

Antimicrobial family	Antimicrobial agent	Susceptibility (%)	Antimicrobial family	Antimicrobial agent	Susceptibility (%)
β-Lactams	Amoxicillin-clavulanate (*n* = 234)	0.85	Quinolones	Ciprofloxacin (*n* = 234)	5.5
	Cefepime (*n* = 185)	15.6		Levofloxacin (*n* = 233)	6
	Cefotaxime (*n* = 226)	4.4		Norfloxacin (*n* = 54)	3.7
	Cefoxitin (*n* = 193)	26.4	Aminoglycosides	Amikacin (*n* = 234)	70.1
	Ceftazidime (*n* = 234)	4.7		Gentamicin (*n* = 234)	41.0
	Cefuroxime (*n* = 231)	2.6		Tobramycin (*n* = 234)	28.2
	Ertapenem (*n* = 227)	3.1	Polymyxin	Colistin (*n* = 120)	86.6
	Imipenem (*n* = 234)	51.7	Tetracycline	Tigecycline (*n* = 179)	61.4
	Piperacillin/tazobactam (*n* = 234)	3	Sulfonamides	Trimethoprim–sulfamethoxazole (*n* = 233)	27.5
	Meropenem (*n* = 234)	52.6	Fosfomycin	Fosfomycin (*n* = 92)	46.7

3.3.2. Inhibitor-based approach

The test was performed on all the strains. One hundred and thirty-six strains were resistant to temocillin, suggesting OXA-48 production. Seventeen strains were resistant to temocillin and susceptible to meropenem + dipicolinic acid, suggesting co-production of OXA-48 and NDM; 15 strains were susceptible to cloxacillin being classified as AmpC producers, and 29 strains were susceptible to meropenem + dipicolinic acid, suggesting NDM-1 production.

3.4. String test

The test was applied to all of the 234 isolates, with only 2 positive results that were not later confirmed with NGS (see section 8).

3.5. Detection of resistance genes by PCR

All the antibiotic resistance genes found in the 234 isolates studied are shown in Table 3.

3.6. Conjugation assays

Selected carbapenemase-encoding plasmids were successfully transferred to a laboratory strain of azide-resistant *E. coli* J53. The carbapenemase-producing transconjugants expressed resistance to all β-lactams with carbapenemase phenotypes, similar to the one observed in the parental strains. Genotypic confirmation of the selected NDM-1 carbapenemase-positive (and CTX-M-1 producer) strain transconjugant was performed by isothermal amplification as previously described.

3.7. Pulsed-field gel electrophoresis

Macro-restriction analysis was performed with 130 CRKP isolates belonging to the main sequence-types detected (ST101, ST11 and ST307) and 20 CRKP isolates representing the other sequence types (STs) in this study. The analysis revealed 19 different profiles, which were grouped into 18 PFGE clusters. The predominant clusters were clone A (*n* = 22) corresponding to ST101, clones B1 (*n* = 22) and B2 (*n* = 27) belonging to ST11 but unrelated according to Tenover's criteria [16], and clones C1 (*n* = 37) and C2 (*n* = 22) belonging to ST307, which are possibly related according to band differences. The remaining PFGE clusters contained fewer than four isolates. In Fig. 6 different band patterns can be observed.

Table 3
Resistance genes detected by PCR in all 234 isolates.

Resistance genes		*n* (%)
β-Lactamase genes only	CTX-M-1	3 (1.3)
AmpC β-lactamase genes	ACC	5 (2.1)
	CMY-II	10 (4.2)
	MOX	1 (0.4)
	DHA	9 (4)
Carbapenemase genes +/- β-lactamase genes	NDM + CTX-M-1	29 (12.5)
	OXA-48 + CTX-M-1	85 (36.5)
	OXA-48	51 (22)
	OXA-48 + NDM + CTX-M-1	17 (7.5)
No resistance genes detected	—	19 (8.5)

3.8. Next-generation sequencing analysis

3.8.1. MLST analysis

Sixteen STs were identified using MLST. The major type was ST307, followed by ST11 and ST101. Other STs included six or fewer isolates in each ST. All isolates in the major PFGE clusters (clones B1 and B2, clones C1 and C2 and clone A) belonged to ST307, ST11 and ST101, respectively. Thirty-three isolates were typed as novel STs, with each one being different from the other (Fig. 7).

3.8.2. Distribution of ESBL, AmpC plasmid-mediated and carbapenemase genes

The analysis of the resistome of the 234 isolates accounted for most of their resistance traits. The presence of genes encoding ESBL, AmpC plasmid-mediated and carbapenemase genes was confirmed by next-generation sequencing. Two hundred and

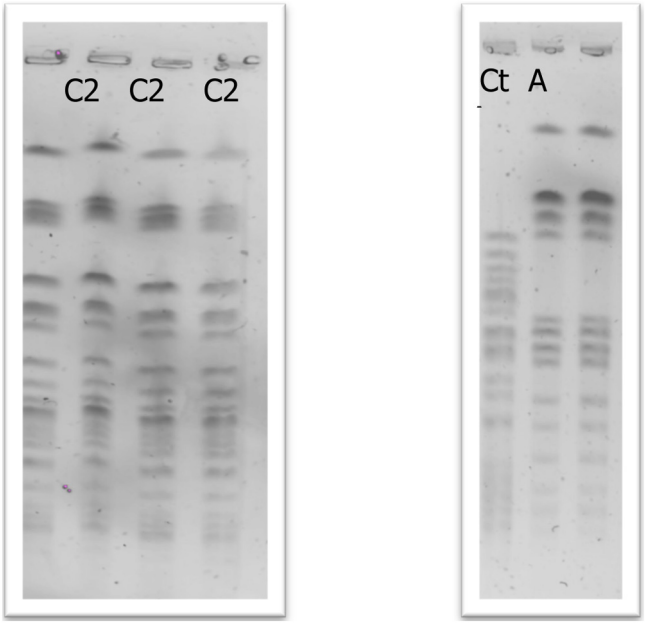

Fig. 6. Different band patterns obtained after performing PFGE. Ct (Control) = ATCC 25922 *Escherichia coli*. PFGE, pulsed-field gel electrophoresis.

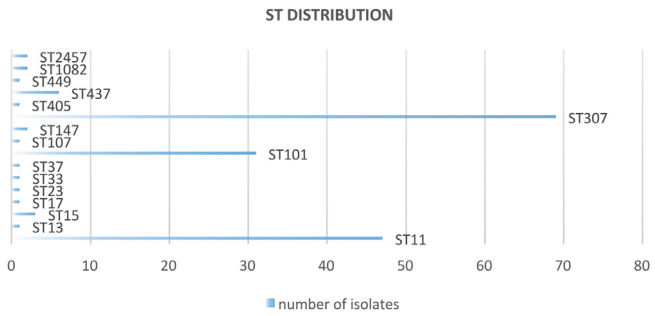

Fig. 7. Distribution of the sequence types found after performing MLST analysis. MLST, multilocus sequence typing.

thirteen isolates (91%) were positive for carbapenemase genes, including *K. pneumoniae* OXA-48 (n = 136, 60%), NDM-1 (n = 29, 12%) and co-production of OXA-48 and NDM-1 (n = 17, 7%). The prevalence of co-production of carbapenemases, ESBL and AmpC β-lactamases is detailed in Table 4.

3.8.3. Resistance to non-β-lactam antibiotics

Non-β-lactam resistance genes were also studied by applying NGS, and the results are shown in Table 4. In all 234 isolates resistance to quinolones was detected, mainly mediated by *Qnr* genes and efflux pumps such as Oqx. Regarding polymyxin resistance, no plasmid-mediated colistin resistance was found. Tetracycline resistance genes, together with sulfonamides and aminoglycoside resistance genes were also detected in almost all the isolates studied.

3.9. Virulence factors and plasmid analyses

No virulence factors were detected among the 234 isolates of CRKP. Regarding plasmid analysis, the different incompatibility groups found are shown in Table 4. In all the strains harbouring OXA-48, an IncL/M type replicon was detected, as previously described in the literature [22]. On the other hand, in those strains co-producing OXA-48 and NDM-1 carbapenemases, two replicon types were found, of types IncL/M and IncFIB. Other replicon types such as IncFII, IncQ, IncHI2, IncN and IncR were also detected in strains harbouring AmpC β-lactamase genes and other non-β-lactam resistance genes.

4. Discussion

CRKP strains pose a significant public health threat as they can quickly spread in hospital settings. Carbapenem resistance due to carbapenemase production is of serious concern due to the high level of resistance of these strains, not only to β-lactams but also to other antimicrobial drugs, such as aminoglycosides or quinolones. Infections caused by these multidrug-resistant microorganisms are associated with more adverse clinical outcomes and higher mortality rates [23].

This study describes the clinical epidemiology of the patients infected and/or colonized by CRKP, the phenotypic and genotypic features of the isolates and the molecular mechanisms involved in the acquisition and dissemination of CRKP at a tertiary hospital in Valencia (Spain). More than 80% of the isolates were recovered from hospitalized patients, which shows that CRKP infection and/or colonization is more likely to be nosocomial than community-acquired as it has already been described in the literature [24].

Regarding the clinical features of the patients in this study, suffering a CRKP infection was more likely when patients underwent surgery or a surgery complication, if patients had had previous hospital stays and if patients had chronic renal disease ($P < 0.05$). Other risk factors also showed higher probability but were not statistically significant, probably owing to the low number of patients.

Table 4
PFGE patterns, sequence types, replicon typing, presence of carbapenemase, *CTX-M*, *DHA-1*, *SHV-11* and other non-β-lactam resistance genes assessed for 135 carbapenem-resistant isolates of *K. pneumoniae* belonging to major circulating clones.

Strains	Year of isolation	PFGE	MLST	Carbapenemases found	CTX-M	DHA-1	SHV-11	Other resistance genes	Replicon typing
22	2016–2017	A	ST101	OXA-48 NDM-1 OXA-48 + NDM-1	CTX-M-1 CTX-M3 CTX-M15	DHA-1	SHV-11	*ArmA* *mphA* *TetR* *Qnr* *Dfr*	IncL/M IncQ IncFIB IncFII IncR
37	2015–2018	B1	ST307	OXA-48	CTX-M-15	–	SHV-28 SHV-106	*TetR* *Qnr* *Dfr*	IncL/M IncFI
22	2015–2018	B2	ST307	OXA-48	CTX-M-15	–	SHV-28 SHV-106	*TetR* *Qnr* *Dfr*	IncL/M IncN IncFI
22	2015–2018	C1	ST11	OXA-48	CTX-M-1 CTX-M3 CTX-M15	DHA-1	SHV-11	*ArmA* *mphA* *TetR* *Qnr* *Dfr*	IncL/M IncQ IncFIB IncFII IncR
27	2015–2018	C2	ST11	OXA-48	CTX-M-1 CTX-M3 CTX-M15	DHA-1	SHV-11	*ArmA* *mphA* *TetR* *Qnr* *Dfr*	IncL/M IncQ IncFIB IncFII IncR
3	2016–2017	D1	ST437	OXA-48 NDM-1	CTX-M-15 CTX-M32	–	SHV-11	*mphA* *TetR* *Qnr* *Dfr*	IncFIB IncFII IncL/M IncR
2	2016–2017	D2	ST437	OXA-48 NDM-1	CTX-M-15 CTX-M-32	–	SHV-11	*mphA* *TetR* *Qnr* *Dfr*	IncFIB IncFII IncL/M IncR

MLST, multilocus sequence typing; PFGE, pulsed-field gel electrophoresis.

Susceptibility testing showed co-resistance to non-β-lactam antibiotics, limiting treatment options to polymyxins, fosfomycin and amikacin in many cases. Interestingly, strains from the same cluster showed similar susceptibility patterns. Regarding the different STs found, ST101 was the only one harbouring both OXA-48 and NDM-1 carbapenemases. OXA-48 was found in ST11, ST437 and ST307, which were also among the most prevalent STs in our settings. In all NDM-1 producers, the *RmtF* gene that confers resistance to aminoglycosides was always detected, as previously described in the literature [25].

Phenotypic and genotypic methods showed 100% concordance: all the strains producing carbapenemase were correctly detected in the first instance by phenotypic methods and later confirmed by both isothermal amplification and next-generation sequencing.

Our results regarding epidemiological relationships show extensive clonal dissemination throughout the years. It is important to mention that the three major clones that have been circulating since 2015 or 2016 in our hospital, ST307, ST11 and ST101, have acquired and lost different plasmid-mediated resistance genes, and were still isolated in patients in 2018. However, in 2015 all the CRKP that were isolated belonged to ST11 and most of them harboured DHA-1 AmpC genes, whereas since 2016 there was a change in ST and carbapenemase genes: OXA-48 arose and NDM-1 appeared for the first time in our settings, coinciding with the arrival of a patient from Pakistan carrying an NDM-1-producing *K. pneumoniae*. From this moment, ST11 was replaced by ST307, ST437 and ST101, among others.

Data found in the literature suggest that CRE-producing OXA-48 are more prevalent in Europe and the Mediterranean region [26]. The prevalence of CRKP strains that produce the OXA-48-enzyme and NDM-1 metallo-β-lactamase is gradually increasing because of clonal dissemination and horizontal gene transfer.

After the conjugation assays were performed, the selected carbapenemase-producing transconjugants were detected that carried the gene and expressed carbapenemase activity, similar to their parental strains, suggesting that carbapenem resistance genes are located on self-transferable plasmids. In addition, OXA-48 is located on the IncL/M plasmid and NDM-1 is located on the IncF and IncX group, similar to that previously reported [27].

The co-existence of genes coding for at least two classes of carbapenemase in *K. pneumoniae* has been reported worldwide [28], but co-harbouring OXA-48 and NDM-1 metallo-β-lactamase is infrequently reported [29], with few reports worldwide and none in the area of the Valencian Community. To the best of our knowledge, this study is the first to report such a large group of OXA-48 carbapenemases with clonal dissemination among CRKP in Valencia (Spain). It is also the first detection of OXA-48, NDM-1 and CTX-M-15 co-production in the area.

In conclusion, *K. pneumoniae* with carbapenemases, ESBLs and AmpC beta-lactamases may be an emerging high-risk multidrug-resistant microorganism. It is important to mention that a single strain of ST307 CRKP harbouring two classes of carbapenemase genes (bla_{OXA-48} and bla_{NDM-1}) has not previously been identified in other hospitals in Valencia. The strains producing ESBLs genes plus only one carbapenemase, also described in this study, may already be far more prevalent than believed previously, especially regarding OXA-48 and CTX-M-1, which have already been widely reported in Spain.

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Conflict of interests

None declared.

Ethical approval

Not required.

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