

Detection of two simultaneous outbreaks of *Klebsiella pneumoniae* coproducing OXA-48 and NDM-I carbapenemases in a tertiary-care hospital in Valencia, Spain

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Abstract

Klebsiella pneumoniae is one of the most common hospital-acquired Gram-negative pathogens. During the last decade, the emergence of strains with reduced susceptibility or resistance to carbapenems is becoming a therapeutic challenge. This study takes place after the isolation of 14 strains of carbapenem-resistant *K. pneumoniae* with similar susceptibility patterns and carriage of OXA-48 and NDM-I carbapenemases genes. Fourteen patients were found to be colonized (faecal carriage) and/or infected by two different clones of carbapenemase-coproducing *K. pneumoniae* during a 1-year period of time. Some of the patients had shared a hospital ward and continued to be colonized several months after the outbreak.

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Introduction

Carbapenemase production among *Enterobacteriaceae* is becoming a major problem worldwide. One of the species closely related to the spread of such enzymes is *Klebsiella pneumoniae*, an important pathogen involved in serious nosocomial infections [1] and whose expansion by means of high-risk clones harbouring plasmid resistance genes has been widely reported [2–5]. OXA-48, NDM-I and KPC are the most frequent carbapenemases detected nowadays in *K. pneumoniae* [6]. Their increase favours the emergence of carbapenemase-coproducing isolates. In the present study, we report the outbreak detection of two different clones of *K. pneumoniae* coproducing NDM-I, OXA-48 and CTX-M-

15 enzymes in the same institution in a short period of time.

Consorcio Hospital General Universitario de Valencia is a healthcare facility complex with a reference population of almost 360 000 inhabitants in Valencia, Spain. The first cases of carbapenemase production in *Enterobacteriaceae*—mainly *K. pneumoniae* carrying OXA-48—were detected in 2014. At that time no carbapenemase production had been reported in our area, although we had implemented surveillance for carbapenemase production in carbapenem-resistant isolates according to Clinical and Laboratory Standards Institute (CLSI) guidelines [7]. Since then the number of such isolates has raised dramatically in our healthcare setting, especially in 2016 (unpublished data). An imported case of NDM-I carbapenemase-producing *K. pneumoniae* in November 2015 marked the emergence of these metallo- β -lactamases in our hospital. In October 2016, coproducing OXA-48, NDM-I and CTX-M-15 *K. pneumoniae* was initially isolated in the urine sample from a patient who arrived in the emergency ward from home but who had been discharged from the hospital 10 days before. During the following 10 months, another 13 patients were found to be faecal carriers and/or infected by this carbapenemase-coproducing microorganism.

Since then, no more patients have been found to carry this multidrug-resistant carbapenemase-coproducing *K. pneumoniae*. Further, interestingly, NDM-I carbapenemase is at present detected in a very low rate compared to OXA-48 enzyme, which is by far the predominant carbapenemase identified in *Enterobacteriaceae* in our setting (unpublished data).

The objective of this study was to retrospectively analyse whether the strains coproducing both carbapenemases were clonal and carried related plasmids or not; and to establish a possible epidemiologic relationship between the patients infected and/or colonized by these microorganisms.

Materials and methods

Bacterial identification and broth microdilution susceptibility testing were performed using the commercial platform MicroScan Walkaway Plus system (Beckman Coulter, Brea, CA, USA); when necessary, MIC confirmation was performed by gradient test (Liofilchem, Waltham, MA, USA). The MICs of ampicillin, amoxicillin clavulanate, piperacillin/tazobactam, cefuroxime, cefoxitin, cefotaxime, ceftazidime, cefepime, ertapenem, imipenem, meropenem, norfloxacin, ciprofloxacin, levofloxacin, gentamicin, tobramycin, amikacin, fosfomicin, tigecycline, colistin and trimethoprim/sulfamethoxazole were determined. Some of the antibiotics being tested varied depending on the origin of the sample, such as norfloxacin and fosfomicin in urine samples. Susceptibility breakpoints were interpreted according to the recommendations of the CLSI [7], except for colistin and tigecycline, also tested using MicroScan Walkaway Plus system [8], but interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoints [9].

Carbapenemase production and phenotypic characterization were assessed when nonsusceptibility to ertapenem, meropenem or imipenem was detected following CLSI breakpoints [7]. Firstly, screening of carbapenemase with the β -CARBA test (Bio-Rad, Hercules, CA, USA) was performed, followed by the 'KPC, MBL and OXA-48 confirmation kit: carbapenemases' (Rosco Diagnostica, Taastrup, Denmark) in case of positive results for the β -CARBA test. Carbapenemase genes were detected by the Xpert-carba-R (Cepheid, Sunnyvale, CA, USA), BDMax CRE (Becton Dickinson, San Diego, CA, USA) or either Eazyplex SuperBug CRE (Amplex Biosystems, Giessen, Germany) and sequenced by in-house Sanger PCR. The primers used in order to obtain PCR products for Sanger sequencing are summarized in Table 1.

Clonal diversity of isolates was performed by both pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE was performed according to the

TABLE 1. Specific primers used for detection and sequencing of OXA-48, NDM-I and CTX-M β -lactamases

Gene	Name	Sequence	Reference
<i>bla</i> _{OXA-48}	OXA-48F	TTGGTGGCATCGATTATCGG	[10]
	OXA-48R	GAGCACTTCTTTTGTGATGGC	
<i>bla</i> _{NDM-I}	NDM-IF	CCAATATTATGCACCCGGTCC	[11]
	NDM-IR	ATGCGGGCCGTATGAGTGATTG	
<i>bla</i> _{CTX-M-I}	CTX-M-I-SEQ-F	CCCATGGTTAAAAAATCACTGC	[11]
	CTX-M-I-SEQ-R	CAGCGCTTTTGCCGCTAAG	

F, forward; R, reverse.

procedure described by Gautom [12]. Restriction patterns were analysed and interpreted visually by two people according to the criteria of Tenover et al. [13]. Sequence types were determined on the basis of the genetic variation of *gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB* and *tonB* housekeeping genes, as described online (<https://bigsdbs.pasteur.fr/klebsiella/klebsiella.html>).

Conjugation experiments with azide-resistant *Escherichia coli* J53 used as recipient and selection on Müller-Hinton plates containing sodium azide (100 μ g/mL) and imipenem (4 μ g/mL) were performed in order to characterize the plasmid or plasmids carrying *bla*_{OXA-48}, *bla*_{NDM-I} and/or *bla*_{CTX-M-15}. Transconjugants were later investigated for extended-spectrum β -lactamase (ESBL) and carbapenemase production by PCR using the Eazyplex SuperBug CRE kit (Amplex Biosystems, Giessen, Germany). Plasmid typing was performed with the PBRT kit (Diateva, Fano, Italy) in every transconjugant [14].

Results

We found that all the strains harboured OXA-48 and NDM-I carbapenemases, as well as a CTX-M-15 ESBL, by means of commercial kits followed by sequencing confirmation of the abovementioned *bla* genes. We obtained two different clones according to results of both PFGE and MLST, which correlated perfectly. Twelve isolates belonged to PFGE pattern A and sequence type (ST) 101 and two strains to PFGE pattern B and ST437. Fig. 1 shows a PFGE gel image of four strains belonging to pattern A.

In the conjugation experiment, different transconjugants were detected: one harbouring OXA-48 only, another harbouring NDM-I and CTX-M-15, and another harbouring OXA-48 and CTX-M-15. Regarding plasmid typing, OXA-48 was found in an IncL/M plasmid without other resistance determinants, and NDM-I was found in an IncFIB plasmid with other resistance determinants such as CTX-M-15 β -lactamase. Both OXA-48 and NDM-I carbapenemases were never found together in the same plasmid after conjugation experiments.

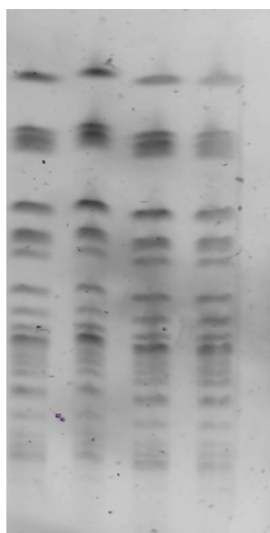


FIG. 1. Pulsed-field gel electrophoresis image showing results of four strains belonging to pattern A.

Susceptibility was assessed phenotypically: all the strains showed resistance to all β -lactams, including meropenem, imipenem and ertapenem; additionally, they were resistant to ciprofloxacin, gentamicin and tobramycin, but they had different susceptibility patterns to trimethoprim/sulfamethoxazole, fosfomycin, amikacin, tigecycline and colistin. After the detection of the two different clones, the following differences in the susceptibility patterns between both clones were realized: pattern A isolates were always susceptible to trimethoprim/sulfamethoxazole and resistant to amikacin, whereas pattern B isolates were resistant to trimethoprim/sulfamethoxazole and susceptible to amikacin. Table 2 provides a summary of the antimicrobial susceptibility pattern of each clone, its sequence type, its plasmid incompatibility group and the resistance genes detected.

Epidemiologic information showed a possible relationship among ten patients with pattern A strains due to ward sharing

(ward 1) in a short period of time (at the same time or within a 2-month difference). It was not possible to find relationship between two of the 12 patients of pattern A, even though the isolates belonged to the same ST and were clonal to the others. The two isolates of pattern B were found in two patients who were hospitalized in another ward (ward 2) than those of pattern A. The first case of pattern B occurred in a patient who had been previously discharged from another hospital in the same city. Time and space sharing between the different patients involved in the outbreaks is shown in Fig. 2.

Information about clinical, demographic and microbiologic features of the patients and microorganisms is shown in Table 3. It should be noted that during the study period, some patients were discharged from the hospital, then readmitted. It was in the second (or following) hospitalization episode when the coproducing isolate was detected, which complicates the discovery and follow-up of the outbreaks. After recompilation of epidemiologic data, no other relationship but sharing a room during the same period of time could explain the transmission of the two clones among the patients involved in the two outbreaks. Because we could not find any obvious epidemiologic relationship in patients 11 and 14 and they accounted for various admissions before the coproducing *K. pneumoniae* was isolated from one of their samples, we are not sure of the time these patients had been previously colonized (Table 3).

Discussion

Dissemination of *K. pneumoniae* isolates harbouring carbapenem resistance genes continues to increase. NDM-1 or OXA-48 harbouring *K. pneumoniae* isolates has been identified worldwide [4–6]. However, finding the coproduction of both carbapenemases in the same isolate has not been frequently reported in outbreaks of two or more patients. The first *K. pneumoniae* strain coproducing NDM-1 and OXA-48 was isolated from the urine sample of an elderly man in Morocco

TABLE 2. Clones, susceptibility profiles and resistance genes detected

Strain pattern	ST	Plasmid incompatibility group	Susceptibility profile	Resistance genes detected
A	ST101	IncL/M, IncFIB	S: SXT/TMP, ^a TIG, FOS, COL R: AK, ^a AMX/CLV, CPE, PIP/TZB, CTX, CAZ, CFX, ERT, MER, IMI, LEV, CIP, GEN, TOB.	<i>bla</i> _{OXA-48} , <i>bla</i> _{NDM-1} , <i>bla</i> _{CTX-M-15}
B	ST437	IncL/M, IncFIB	S: TIG, FOS, COL, AK ^a SXT/TMP, ^a AMX/CLV, CPE, PIP/TZB, CTX, CAZ, CFX, ERT, MER, IMI, LEV, CIP, GEN, TOB.	<i>bla</i> _{OXA-48} , <i>bla</i> _{NDM-1} , <i>bla</i> _{CTX-M-15}

AK, amikacin; AMX/CLV, amoxicillin/clavulanate; CIP, ciprofloxacin; COL, colistin; CPE, cefepime; CTX, cefotaxime; ERT, ertapenem; GEN, gentamicin; IMI, imipenem; LEV, levofloxacin; MER, meropenem; PIP/TZB, piperacillin/tazobactam; R, resistant; S, susceptible; ST, sequence type; SXT/TMP, trimethoprim/sulfamethoxazole; TIG, tigecycline; TOB, tobramycin.

^aAntibiotics with susceptibility that changed depending on ST and pattern.

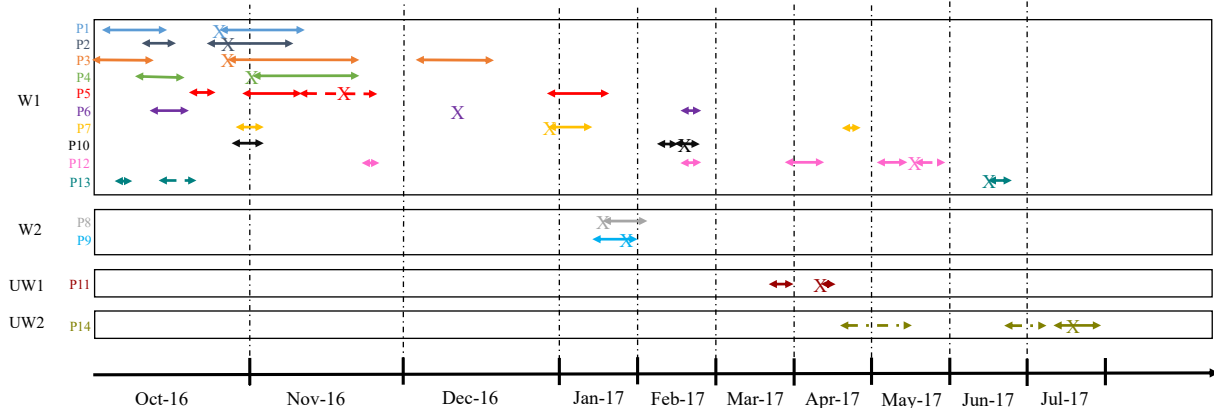


FIG. 2. Epidemiologic relationship of patients involved in two outbreaks. Solid arrows indicate periods of hospital stay for each patient; broken arrows, hospital stay in different ward; and X, moment when first isolate was obtained from each patient. P, patient, UW, unrelated ward, W, ward.

TABLE 3. Clinical, demographic and microbiologic features of 14 patients infected and/or colonized with *Klebsiella pneumoniae*

Patient No.	Date of first hospital admission	Date of first isolate	Time elapsed from first admission to carbapenemase detection (days)	Sample source	Hospital ward	ST (pattern)
1 ^a	03.10.2016	25.10.2016	22	Urine	W1	101 (A)
2 ^a	11.10.2016	26.10.2016	15	Blood, urine, faecal carriage	W1	101 (A)
3 ^a	01.10.2016	26.10.2016	25	Blood, urine, faecal carriage	W1	101 (A)
4 ^a	09.10.2016	26.10.2016	15	Urine, faecal carriage	W1	101 (A)
5 ^a	20.10.2016	19.11.2016	30	Blood, urine, faecal carriage	W1	101 (A)
6 ^a	12.10.2016	11.12.2016	60	Urine	W1	101 (A)
7 ^a	30.10.2016	30.12.2016	61	Urine	W1	101 (A)
8	17.01.2017	17.01.2017	0	Urine	W2	437 (B)
9	16.01.2017	27.01.2017	10	Urine, faecal carriage	W2	437 (B)
10 ^a	28.10.2016	17.02.2017	112	Urine	W1	101 (A)
11 ^a	25.03.2017	13.04.2017	19	Urine, faecal carriage, blood	UW	101 (A)
12 ^a	23.11.2016 or 21.02.2017 or 27.03.2017	21.05.2017	179 or 89 or 55	Urine, faecal carriage	W1	101 (A)
13 ^a	05.10.2016	19.06.2017	257	Urine	W1	101 (A)
14 ^a	22.04.2017 or 23.06.2017 or 13.07.2017	19.07.2017	88 or 26 or 6	Ulcer, faecal carriage	UW	101 (A)

UW, unrelated ward; W1, ward 1; W2, ward 2.

^aThese patients were discharged and then readmitted during study period.

in 2012 [15]. The second was reported in Tunisia, a country where OXA-48 producers are already endemic, as in Turkey [16]. The third was detected in the screening rectal swab of a patient transferred from the intensive care unit of a hospital located in Belgrade, Serbia, to Bern University Hospital in Switzerland, belonging to ST101, as did some of the strains in our study [17]. The present study reveals two outbreaks of carbapenemase-coproducing *K. pneumoniae* isolates in a tertiary-care hospital at the same time. In our case, the sequence types detected were 101 and 437, which have already been found to harbour NDM-1 and OXA-48 carbapenemases in Switzerland and Slovenia, where they also caused nosocomial outbreaks [17,18]. ST101 has already been identified in Spain as a carbapenemase carrier, and it is one of the sequence types widely distributed across Europe, having been submitted from 15 countries [19]. It is considered to be a high-risk clone that is often associated with outbreaks

[20,21]. It probably possesses particular characteristics that increase its tenacity, transmissibility and population size, thereby providing a greater opportunity for the acquisition of antibiotic resistance genes [4]. Regarding ST437, it has already been found to be involved in the interhospital spread of carbapenemase genes in Spain [22], although not coproducing carbapenemases.

In the last few years, *K. pneumoniae* has developed resistance to several antibiotic classes, leading to an increase in the life-threatening infections and to a major interest in the most appropriate treatments [5,23]. The strains in our study were considered to be multidrug resistant because they were found to be resistant to at least one agent in more than three antimicrobial families [24]. All the strains remained susceptible to colistin, fosfomycin and tigecycline, considered to be last-resort antimicrobial agents used to fight multidrug-resistant *K. pneumoniae* infections [25].

Regarding plasmid typing, all three types detected in our study have already been reported in the literature as carbapenemase carriers [26]. This shows the high risk of intra- and interspecies horizontal transmission of carbapenemase genes among successful genetic platforms, enhancing dissemination and persistence [27]. IncFIB has been found to carry NDM-I and CTX-M-15 ESBL in *K. pneumoniae* [27,28]. Although some other widespread carbapenemase determinants, such as the *bla*_{KPC} and *bla*_{NDM} genes, have been shown to disseminate through different plasmid scaffolds [29], the current spread of the *bla*_{OXA-48} gene is linked to the wide diffusion of an identical IncL/M plasmid scaffold. Previous studies indicated that plasmids carrying the *bla*_{OXA-48} gene from different enterobacterial isolates, different clones and different countries may share very similar features [30].

Regarding epidemiologic relatedness, it is important to mention the fact that some of the patients who were discharged and then readmitted after many months (more than 8 months in patient 13) were still either colonized, or both colonized and infected. It takes a long time for the patient to be uncolonized or for the bacteria to lose the carbapenemase-carrying plasmids, as previously described [31].

The first isolate of pattern B was found in a patient who had been previously discharged from another hospital in the same city, which would explain the coexistence of the two different clones in the same period of time and the lower number of pattern B isolates compared to pattern A. This is consistent with the idea that the co-carriage of different carbapenemases in a same clone is a frequent phenomenon, favoured by the coexistence in similar quantity of strains that carry one enzyme or the other.

It is also remarkable that during the outbreaks there were patients who were infected or colonized by different isolates of *K. pneumoniae*, some coproducing both carbapenemases and others carrying only OXA-48. This is of great interest because we found isolates carrying OXA-48 on its own, but there were no isolates carrying NDM-I without OXA-48. This may be because the plasmid carrying OXA-48 confers more stability to the bacteria, as it is a small and self-transferable plasmid that does not carry any additional resistance gene [32]. This would also explain the disappearance of NDM-I from our hospital and the continuous spread, even in other sequence types, of OXA-48 (unpublished data).

To our knowledge, our description of carbapenemase coproduction is the first to be reported in the area of Valencia. Moreover, the occurrence of two simultaneous different outbreaks of coproducing *K. pneumoniae* is exceptional in the literature. Further and deeper studies on this subject are needed, as these findings emphasize the importance of systematic detection in order to contain the spread of these multidrug-resistant organisms.

Conflict of Interest

None declared.

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