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First Outbreak of a Plasmid-Mediated Carbapenem-Hydrolyzing OXA-48 β-Lactamase in *Klebsiella pneumoniae* in Spain[▽]

Cristina Pitart,*† Mar Solé,† Ignasi Roca, Anna Fàbrega, Jordi Vila, and Francesc Marco

Department of Clinical Microbiology, Hospital Clinic IDIBAPS, School of Medicine, University of Barcelona, and Barcelona Centre for International Health Research, Barcelona, Spain

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Twenty *Klebsiella pneumoniae* isolates producing OXA-48 were collected from April 2009 to September 2010. Strains were clonally related and coproduced a CTX-M-15 β-lactamase. A conjugative plasmid of circa 70 kb carrying bla_{OXA-48} was identified. Eleven isolates showed low-level resistance to carbapenems, whereas nine showed high-level resistance. Decreased expression of OmpK36 was related to high-level resistance to carbapenems. The isolates belonged to sequence type 101 (ST101). This is the first outbreak caused by an OXA-48-producing *K. pneumoniae* strain in Spain.

Carbapenems currently represent the drugs of choice for treatment of serious infections caused by multidrug-resistant strains of *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs). Recently, however, the emergence of carbapenem resistance has been increasingly reported among *Enterobacteriaceae* and is a matter of major clinical concern. The most common mechanism for carbapenem resistance in *Klebsiella pneumoniae* is the production of carbapenemases belonging to Ambler class A, B, or D (21). Acquired class D carbapenemases have previously been reported mainly in *Acinetobacter* spp. and occasionally in *Enterobacteriaceae*. The oxacillinase OXA-48 was first identified from a *K. pneumoniae* isolate in Istanbul, Turkey (24), and spread of OXA-48-producing *Enterobacteriaceae* throughout the Mediterranean area has been observed (3, 12, 16, 19). OXA-48-producing *Enterobacteriaceae* have also been reported in Senegal (22), Belgium (11), Argentina (8), and India (2), and several outbreaks have been described in Turkey (6), the United Kingdom (27), and more recently in France (13).

The bla_{OXA-48} gene is part of the Tn*1999* composite transposon made of two copies of the insertion sequence IS*1999* (1) and is located in a conjugative plasmid of circa 70 kb (7). Modification of outer membrane proteins (OMPs) has also been shown to play an additional role in increasing carbapenem resistance in *K. pneumoniae* producing KPC carbapenemase (18), as well as in strains bearing plasmid-mediated AmpC cephalosporinases and ESBLs showing resistance to ertapenem (5, 20).

In this study we describe the first detection as well as the first outbreak of a *K. pneumoniae* strain producing OXA-48 and CTX-M-15 in Spain. The role of the expression of outer membrane proteins in the increased carbapenem resistance phenotype of these isolates is also analyzed.

On 7 April 2009, a male (patient 2) was transferred to the neurosurgical intensive care unit (SICU) at the Hospital Clínic of Barcelona, Spain, from the ICU of a hospital in Marrakesh, Morocco, where he had been admitted after head trauma and had stayed for 24 days with several episodes of fever and pneumonia. Two days later the first *K. pneumoniae* isolate producing OXA-48 carbapenemase was found from a patient in the same SICU (patient 1), whereas an OXA-48-producing *K. pneumoniae* isolate from patient 2 (presumably the index case) was not detected until 14 April 2009. Active surveillance rectal cultures were collected from all patients from the SICU. Screening was performed using the chromogenic medium chromID ESBL (bioMérieux) and the Hodge test to confirm carbapenemase production. During the subsequent period of time (April 2009 to September 2010), 18 more OXA-48-producing *K. pneumoniae* isolates were recovered. Rectal colonization was found in each of all tested patients who were infected, and one additional patient was also colonized (patient 6). The implementation of barrier precautions as well as promotion of hand hygiene and reinforcement of room cleaning measures led to the successful control of the outbreak. Table 1 summarizes the characteristics of the patients and the origin of the isolates.

Antimicrobial susceptibility testing was performed by using the Phoenix system (Becton Dickinson, Franklin Lakes, NJ), and MICs of β-lactam antibiotics and tigecycline were also evaluated by Etest (AB bioMérieux, Solna, Sweden). Eleven strains collected from April 2009 to February 2010 were resistant to all the antibiotics tested, except cefoxitin, amikacin, fosfomycin, tigecycline, and colistin, and showed low-level resistance to carbapenems, with three being susceptible to imipenem and meropenem according to CLSI breakpoints updated in June 2010 (9). The remaining nine strains, collected from April 2010 to September 2010, showed the same resistance pattern except that they were resistant to cefoxitin and showed high-level resistance to carbapenems (Table 2).

PCR and sequence analysis for carbapenemases, ESBLs, and plasmid-mediated AmpC cephalosporinase-encoding genes were performed (4, 23–25). All isolates were positive for bla_{OXA-48} (carbapenemase), $bla_{CTX-M-15}$ (ESBL), and bla_{SHV-1}, the latter being the chromosomally encoded β-lactamase usually found in this microorganism.

The analysis of the isolates by pulsed-field gel electrophoresis (PFGE) profiles of XbaI-digested genomic DNA (New

* Corresponding author. Mailing address: Department of Clinical Microbiology, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain. Phone: 34-93-2275522. Fax: 34-93-2279372. E-mail: cristinapitart@gmail.com.

† C. Pitart and M. Solé contributed equally to this work.

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TABLE 1. Characteristics of 20 patients carrying *K. pneumoniae* isolates producing OXA-48 and CTX-M-15

Patient no.	Hospital unit	Isolate no.	Date of isolation	Site of isolation	Underlying disease	Final antibiotic therapy[a]	Outcome
1	SICU	5837	9 April 2009	Bronchoaspirate	Brain trauma	AMK + MEM	Cure
2	SICU	5836	14 April 2009	Catheter blood culture	Head trauma	TGC + TMP-SXT + AMK	Cure
3	SICU	5834	16 April 2009	Urine	Subdural hemorrhage	TGC + AMK	Cure
4	SICU	5839	16 April 2009	Catheter blood culture	Subarachnoidal hemorrhage	AMK + CST	Cure
5	SICU	5835	20 April 2009	Urine	Subarachnoidal hemorrhage	TGC + AMK	Cure
6	SICU	6082	3 June 2009	Rectal swab		None	
7	SICU	6083	5 June 2009	Urine	Vertebral fracture	TGC + AMK	Cure
8	SICU	6168	25 June 2009	Catheter blood culture	Subarachnoidal hemorrhage	None	Exitus
9	SICU	6167	25 June 2009	Bronchoaspirate	Subarachnoidal hemorrhage	TGC + AMK	Exitus
10	SICU	6440	20 August 2009	Urine	Subdural hematoma	TGC + FOF	Cure
11	SICU	7310	16 February 2010	Catheter	Severe pneumonia	None	Exitus
12	SICU	7591	7 April 2010	Urine	Subarachnoidal hemorrhage	TGC + FOF + AMK	Cure
13	Hepatology ward	7619	19 April 2010	Urine	Liver transplant	TGC + FOF	Cure
14	SICU	7680	5 May 2010	Catheter blood culture	Septic shock	TGC	Cure
15	Hepatology ward	7745	18 May 2010	Urine	Liver transplant	TGC + FOF	Cure
16	SICU	7911	25 June 2010	Blood culture	Nosocomial pneumonia	TGC + AMK + CST	Exitus
17	Hepatology ward	7951	5 May 2010	Catheter blood culture	Liver transplant	TGC + FOF	Cure
18	SICU	8037	24 July 2010	Urine	Liver cancer	TGC + FOF	Cure
19	Hepatology ward	8064	26 July 2010	Catheter blood culture	Liver transplant	TGC + FOF	Cure
20	Hepatology ward	8268	20 September 2010	Urine	Liver cirrhosis	TGC + FOF	Cure

[a] TMP-SXT, trimethoprim-sulfamethoxazole; AMK, amikacin; TGC, tigecycline; CST, colistin; MEM, meropenem; FOF, fosfomicin.

England BioLabs, Beverly, MA) revealed that all isolates were clonally related (Fig. 1).

Transferability of the bla_{OXA-48} gene was studied by conjugation experiments between the *K. pneumoniae* 7680 isolate and the recipient strain, *Escherichia coli* J53 AziR, in broth medium. Transconjugants were selected on MacConkey agar plates supplemented with 1 μg/ml of imipenem and 100 μg/ml of sodium azide (Sigma Chemical Co., St. Louis, MO) and were screened for bla_{OXA-48} and $bla_{CTX-M-15}$. PCR analysis was only positive for the bla_{OXA-48} gene. *E. coli* J53 7680T producing OXA-48 was susceptible to all the β-lactam antibiotics except ampicillin, amoxicillin-clavulanate, and piperacillin-tazobactam and showed higher MICs of carbapenems than the original recipient strain (Table 2).

TABLE 2. *In vitro* susceptibilities of the low- and high-level carbapenem-resistant *K. pneumoniae* 5837 and *K. pneumoniae* 7680 isolates, respectively, and the *E. coli* transconjugant expressing OXA-48 carbapenemase

β-Lactam(s)	MIC (μg/ml)			
	K. pneumoniae 5837 (OXA-48, CTX-M-15, OmpK36$^+$)	*K. pneumoniae* 7680 (OXA-48, CTX-M-15, OmpK36$^-$)	*E. coli* J53 7680T (OXA-48)	*E. coli* J53
Amoxicillin	>256	>256	>256	4
Amoxicillin + clavulanate[a]	>256	>256	256	4
Piperacillin + tazobactam[b]	>256	>256	64	1
Cefoxitin	6	96	2	2
Cefotaxime	>256	>256	0.75	0.094
Ceftazidime	96	>256	0.125	0.125
Cefepime	128	>256	0.38	0.25
Imipenem	1 (0.75–4)[c]	>32 (12 to >32)[d]	0.5	0.19
Meropenem	2 (0.75–4)[c]	>32 (12 to >32)[d]	0.125	0.023
Doripenem	1.5 (1–2)[c]	>32 (8 to >32)[d]	0.125	0.032
Ertapenem	24 (3 to >32)[c]	>32 (>32)[d]	0.5	0.008
Aztreonam	>256	>256	0.064	0.064

[a] Clavulanate was used at a fixed concentration of 2 μg/ml.
[b] Tazobactam was used at a fixed concentration of 4 μg/ml.
[c] Carbapenem MIC range for isolates collected from April 2009 to February 2010 (low-level resistance to carbapenems).
[d] Carbapenem MIC range for isolates collected from April 2010 to September 2010 (high-level resistance to carbapenems).

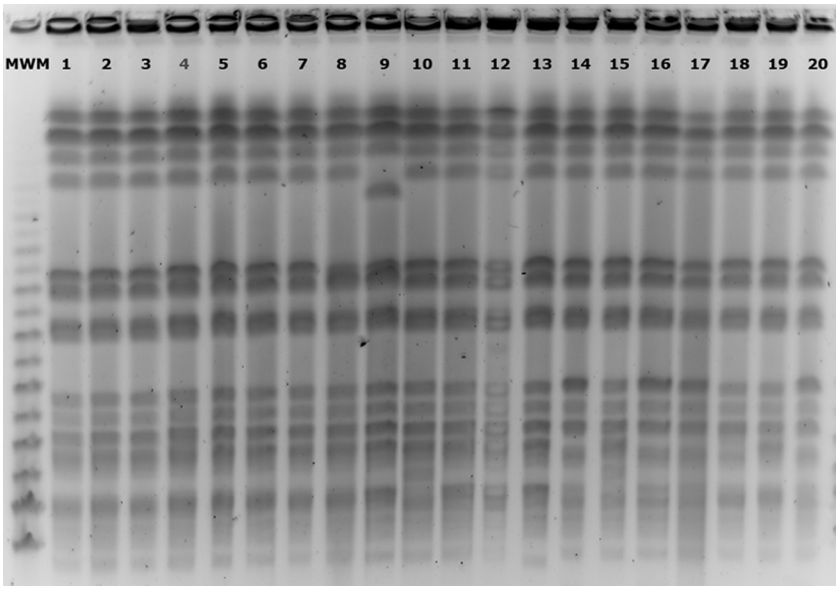

FIG. 1. XbaI PFGE patterns of OXA-48-producing *K. pneumoniae* isolates. Lane 1, isolate 5837; lane 2, isolate 5836; lane 3, isolate 5834; lane 4, isolate 5839; lane 5, isolate 5835; lane 6, isolate 6083; lane 7, isolate 6168; lane 8, isolate 6167; lane 9, isolate 6440; lane 10, isolate 7310; lane 11, isolate 6082; lane 12, isolate 7591; lane 13, isolate 7619; lane 14, isolate 7680; lane 15, isolate 7745; lane 16, isolate 7951; lane17, isolate 8037; lane 18, isolate 8064, lane 19; isolate 8268; lane 20, isolate 7911. MWM, molecular weight marker.

Extraction of plasmid DNA from *K. pneumoniae* strain 7680 and its transconjugant, *E. coli* J53 7680T, was performed by the method of Kado and Liu (17) and showed the presence of a plasmid of circa 70 kb in both strains (data not shown). Plasmids of similar sizes have already been described in previous OXA-48 carbapenemase producers (7).

The genetic environment of the $bla_{\text{OXA-48}}$ gene was determined by PCR using primers matching the insertion sequence IS*1999* as well as primers matching either the upstream or the downstream region of the $bla_{\text{OXA-48}}$ gene to confirm the presence of the Tn*1999* transposon (1). The presence of an IS*1R* element truncating the IS*1999* insertion sequence upstream from $bla_{\text{OXA-48}}$ allowed identification of a Tn*1999.2*-type transposon as previously described for Turkish isolates (6).

Taking cefoxitin resistance and the level of resistance to carbapenems into account, two resistance patterns were defined, as previously mentioned. In order to investigate the differences between the two groups, the outer membrane proteins of one representative isolate from each group (*K. pneumoniae* 5837 and *K. pneumoniae* 7680, respectively) (Table 1) were extracted and analyzed by SDS-PAGE together with protein extracts from known control strains (15) (Fig. 2). SDS-PAGE analysis indicated that the high-level carbapenem- and cefoxitin-resistant isolate presented decreased expression of the protein band corresponding to OmpK36 in the control strains. Matrix-assisted laser desorption ionization–time of flight–time of flight mass spectrometry of the gel-purified band correctly identified this protein as OmpK36, thereby confirming that cefoxitin-susceptible isolates produced OmpK36, whereas cefoxitin-resistant isolates showed decreased expression of this protein (Fig. 2). The sequences of the o*mpK36* gene and its promoter were also analyzed in both isolates, and no differences were found.

Until the isolation of the present *K. pneumoniae* isolate harboring OXA-48 and CTX-M-15, carbapenem resistance in *K. pneumoniae* associated with carbapenemases in Spain was exclusively attributed to VIM-1 and KPC-3 (10, 26). This is the first *Enterobacteriaceae* strain identified in Spain that carries a carbapenem-hydrolyzing oxacillinase. Multilocus sequence typing was performed as previously described (14) and revealed that the *K. pneumoniae* isolate belonged to sequence type 101 (ST101), which has previously been found in an OXA-48-producing *K. pneumoniae* isolate from Tunisia (13).

In this report we describe the first outbreak in Spain involving 20 patients at our hospital that was caused by a single clone of *K. pneumoniae* carrying OXA-48 and CTX-M-15. The outbreak was divided into two periods of time in which the level of resistance to carbapenems increased, apparently due to a decrease in the expression of OmpK36. The facts that these strains contained a plasmid of similar size to that of previously identified OXA-48-producing *K. pneumoniae* isolates (6) and that all were clonally related and belonged to the same se-

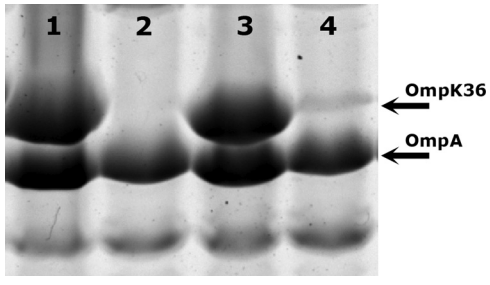

FIG. 2. SDS-PAGE of OMPs from *K. pneumoniae* isolates. Lane 1, CSUB 10S control strain expressing OmpK36; lane 2, CSUB 10R control strain not expressing OmpK36; lane 3, *K. pneumoniae* 5837; lane 4, *K. pneumoniae* 7680. Only the relevant parts of the gel are shown.

quence type (ST101) as OXA-48-producing *K. pneumoniae* isolates from Tunisia (even though the presumable index case of this outbreak, patient 2, had returned from Morocco) suggest their spread among Mediterranean countries.

It is worth mentioning that three of the isolates were susceptible to imipenem and meropenem according to CLSI guidelines, further complicating the detection of OXA-48-producing *Enterobacteriaceae* isolates, which, in turn, may delay the administration of appropriate antimicrobial therapy as well as the enforcement of infection control measures.

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