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Clonal spread of *Klebsiella pneumoniae* producing OXA-1 betalactamase in a Spanish hospital

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Summary. Multi-drug resistant *Klebsiella pneumoniae* isolates are associated with nosocomial infections, in which colonized patients act as a reservoir and source of cross-infection for other patients. In this study, the antimicrobial susceptibility of *K. pneumoniae* was tested by microdilution using the commercial method MicroScan (Siemens). The genetic relatedness of *K. pneumoniae* strains was determined by pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PCR experiments were carried out to obtain primer sets and positive PCR products were purified and sequenced. From May 2007 until December 2009, 98 clonally related *K. pneumoniae* isolates were detected from clinical samples of 38 patients admitted to the University Hospital of Bellvitge, Barcelona, Spain, including 27 admitted to the intensive care unit (ICU). The most important sources of the isolates were: lower respiratory tract (n = 12), urine (n = 12), and blood (n = 11). The strains were resistant to amoxicillin/clavulanic acid, piperacillin/tazobactam, tobramycin, amikacin, and ciprofloxacin, and had diminished susceptibility to cefepime. All the isolates shared a common PFGE pattern related to sequence type 14 after MLST analysis. In *K. pneumoniae* isolates and their transconjugants, the $bla_{\text{OXA-1}}$ gene was located in the variable region of a class I integron that also contains the *aac(6')Ib-cr* gene. Sequencing of the quinolone resistance determinant regions of *gyrA* and *parC* revealed a S83F change in GyrA and no changes in ParC. [**Int Microbiol** 2013; 16(4):227-233]

Keywords: *Klebsiella pneumoniae* · sequence type ST14 · gene *bla*OXA-1 · integrons · nosocomial outbreaks

Introduction

Klebsiella pneumoniae is a human pathogen that often colonizes the skin and mucosae of hospitalized patients, producing respiratory and urinary tract infections and bacteremia. Multi-drug resistant isolates are associated with nosocomial infections, with colonized patients acting as a reservoir and source of cross-infection for other patients [3,16]. However, the genetic elements that encode resistance, especially betalactamases, are now spreading outside the hospital setting such that resistant isolates may also be recovered from patients with community-acquired infections [6]. The virulence of *K. pneumoniae* strains is related to several bacterial factors, including the polysaccharidic capsule, lipopolysaccharide, iron scavenging systems, and fimbrial and non-fimbrial adhesins [6]. While isolates causing severe community-acquired infections are usually associated with a restricted number of *K. pneumoniae*

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serotypes and clones, nosocomial isolates are highly diverse, suggesting that the bacterium is an opportunistic pathogen. Since the clonal complex is a good predictor of the virulence of *K. pneumoniae* isolates, the use of multilocus sequence typing (MLST) of the strains would improve investigations of nosocomial outbreaks [8].

Multidrug resistance patterns of *K. pneumoniae* and other enterobacteria are usually associated with the most frequently occurring integrons, those of class I, which carry different resistance determinants in the variable region and can be horizontally transferred intra- and inter-species [11,15]. The structure of class I integrons includes two conserved regions, 5´CS and 3´CS, that flank the gene cassette. The 5´CS conserved segment contains a gene coding for an integrase (*intI*), a recombination site (*attI*), and a promoter of the gene cassette. The 3´CS conserved segment contains the *qacEΔ1* and *sulI* genes, which confer resistance to quaternary ammonium compounds and sulfamethoxazole, respectively [11]. The variable region of the integrons is located between these two conserved segments and integrates the resistance genes. The variability of the gene cassettes causes the high diversity of antibiotic resistance [4,11]. Because of the easy bacterial acquisition of gene cassettes and their exchanges under different pressure conditions, many antibiotic resistance combinations are possible. The prolonged use of certain antibiotics also aids in the selection of certain resistance elements and promotes the persistence of multidrug-resistant (MDR) bacteria [4,11].

In our hospital, an active surveillance program against MDR *K. pneumoniae* started in 1993, after a nosocomial outbreak. Resistance was ascribed to extended-spectrum betalactamase (ESBL)-producing *K. pneumoniae* [16]. In May 2007, a possible new MDR strain of *K. pneumoniae* was isolated from a patient admitted to the intensive care unit (ICU). This strain spread in the following years, causing clustered infections throughout the hospital. Here we describe and characterize the *K. pneumoniae* isolates obtained during the subsequent surveillance program.

Materials and methods

Study design and sampling. The present study was performed at the Hospital Universitari de Bellvitge, L'Hospitalet de Llobregat (Barcelona), Spain, a university teaching hospital for adults, with an average yearly admission of 26,000 patients. The hospital has three 12-bed medical-surgical ICUs. The surveillance of MDR *K. pneumoniae* began in May 2007, after the detection of several MDR non-ESBL *K. pneumoniae* isolates, and continued until December 2009. Clinical charts of patients were reviewed and assessment was determined using previously described methods [16]. Clinical samples collected within a month of each other from the same patient and that yielded isolates were considered as representative of different episodes. The isolation of *K. pneumoniae* from a patient without related signs or symptoms of infection was considered as a colonization. When *K. pneumoniae* was detected from a patient during an ICU stay or during the first week after ICU discharge, the infection was considered to be ICU-acquired. All other *K. pneumoniae* cases were considered as non-ICU-acquired.

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Fig 1. Double disk synergy test of OXA-1-producing *Klebsiella pneumoniae*. AMP: ampicillin; TIC: ticarcillin; CFO: cefoxitin: NAL: nalidixic acid; AZT: aztreonam; CAZ: ceftazidime; CXM: cefuroxime; CIPR: ciprofloxacin; CTX: cefotaxime; AMC: amoxicillin/clavulanic acid; FO: fosfomycin; NI: nitrofurantoin; IMI: imipenem; FEP: cefepime; GEN: gentamicin; SXT: trimethoprim-sulfamethoxazole. There are synergy of FEP and CTX with AMC.

Antimicrobial susceptibility and molecular typing. Antimicrobial susceptibility was tested by microdilution using a commercial method MicroScan (Siemens). The multidrug resistance pattern was assessed by the disk diffusion method. The presence of an ESBL was screened by disk diffusion (Fig. 1) [1]. The genetic relatedness of *K. pneumoniae* strains was tested by pulsed field gel electrophoresis (PFGE). The entire DNA content was digested with *XbaI*. DNA-band analysis was performed by visual inspection following the criteria described by Tenover [18]. Strains differing in three or less bands were considered subtypes. Seven isolates were selected for multilocus sequence typing (MLST): two isolates with a common PFGE pattern (one isolated in August 2007 and the other in 2008) and one of each PFGE subtype pattern. MLST was performed following Protocol 2, which recommends universal sequencing primers, as described on the MLST web site of the Institut Pasteur [http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html]. The allele's number and sequence type (ST) were assigned according to the recommendations of this web site.

Characterization of the multidrug resistance pattern. Isoelectric focusing of crude extracts was performed in accordance with previous procedures [1]. Strains of *K. pneumoniae* or their transconjugants were grown for 4 h in lysogeny broth (LB). The cells were then harvested by centrifugation, resuspended in distilled water, and sonicated. The resulting extracts were purified by ultracentrifugation. Isoelectric focusing was

Table 1. Primers used in this study

Gene	Primer		Size PCR product	Ref.
bla_{SHV}	SHV fw	5′CTTTATCGGCCCTCACTCAA3′	273 bp	[9]
	SHV rev	5′AGGTGCTCATCATGGGAAAG3′		
bla_{TEM}	TEM fw	5′CGCCGCATACACTATTCTCAGAATGA3′	445 bp	[9]
	TEM rev	5′ACGCTCACCCGCTCCAGATTTAT3′		
bla_{CTX-M}	CTX-M fw	5′ATGTGCAGYACCAGTAARGTKATGGC3′	593 bp	[9]
	CTX-M rev	5′TGGGTRAARTARGTSACCAGAAYCAGCGG3′		
bla_{OXA}	OXA fw	5′ACACAATACATATCAACTTCGC3′	813 bp	[9]
	OXA rev	5′AGTGTGTTTAGAATGGTGATC3′		
aac(6')	aac(6')-Ia	5′TAATTGCTGCATTCCGC3′	654 bp	[11]
	aac(6')-Ib	5′TGTGACGGAATCGTTGC3′		
	5′CS	5′GGCATCCAAGCAGCAAG3′	Variable	[11]
	3′CS	5′AAGCAGACTTGACCTGA3′	Variable	[11]
	IntI1-F	5′GGTCAAGGATCTGGATTTCG3′	Variable	[12]
	IntI1-R	5′ACATGCGTGTAAATCATCGTC3′	Variable	
	sulI	5′TGAAGGTTCGACAGCAC3′	Variable	[11]
parC	parC-up	5′CTGAATGCCAGCGCCAAATT3′	319 pb	[5]
	parC-dn	5′TGCGGTGGAATATCGGTCGC3′		
gyrA	gyrA-up	5′CGCGTACTATACGCCATGAACGTA3′	589 pb	[5]
	gyrA-dn	5′ACCGTTGATCACTTCGGTCAGG3′		

performed using the PhastSystem apparatus and polyacrylamide gels with a pH range of 3–9. The gels were then stained with 500 mg nitrocefin/ml, and the isoelectric points (pIs) were obtained by comparison with a set of different betalactamases of known pIs.

Conjugation. These experiments were performed in LB using *Escherichia coli* J53-2 (rifampicin MIC ≥ 100 µg/ml) as recipient. Transconjugants were selected in trypticase soy agar plates containing both rifampicin (100 µg/ml) and tobramycin (16 µg /ml) and tested for antimicrobial susceptibility by disk diffusion and microdilution.

PCRs and characterization of integrons. A multiplex PCR assay, which includes detection of the TEM, SHV, OXA, CTX-M9 and CTX-M10 families of betalactamases, was used to characterize the betalactamase gene family members carried in *K. pneumoniae* strains and their transconjugants [9]. PCR experiments were repeated separately for primer sets that yielded a positive result and the PCR products were purified and sequenced (Table 1).

Characterization of ciprofloxacin resistance. Quinolone-resistance-determining regions (QRDR) of GyrA and ParC were amplified and sequenced using the primers and conditions described previously [5]. A PCR assay was performed to detect the presence of the *aac(6′)-Ib-cr* gene. The PCR products were sequenced to detect the quinolone-modifying variant of this enzyme. The detection of class I integrons and the characterization of their variable region were carried out using previously described procedures (Table 1) [11].

Results and Discussion

Description of the outbreak. During the study period (2007–2009), 366 *K. pneumoniae* strains were isolated from 222 patients. Of these, 98 isolates obtained from the clinical samples of 38 patients shared a multidrug resistance pattern (see below). The mean age of the patients was 60.0 (SD ±18.4; range 19–85 years), and 23 patients (60.5 %) were men. Among the 38 patients studied, the infectious episodes in order of frequency were respiratory tract infection (bronchial aspirate, 7; bronchoalveolar lavage, 3; and tracheal aspirate, 1), bacteremia (9), and urinary tract infection (8). In the remaining ten patients, the isolates were collected from catheter-related samples (4), abdominal samples (3), wounds (2) and cerebrospinal fluid (1).

Klebsiella pneumoniae infection was acquired in the ICU in 27 patients. Figure 2 shows the temporal detection pattern in

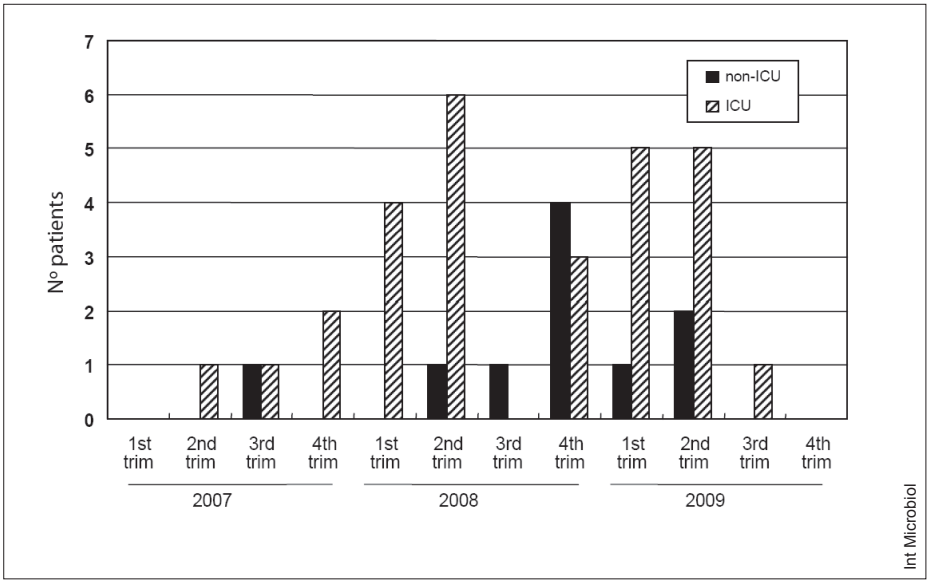

Fig. 2. Temporal distribution of newly admitted patients infected with *Klebsiella pneumoniae*. Striped bars represent patients with ICU-acquired *K. pneumoniae*, and black bars those patients with non-ICU-acquired *K. pneumoniae.*

newly admitted patients with clonally related *K. pneumoniae* infection over the study period.

Antibiotic susceptibility, molecular typing, and virulence profile. The isolates showed resistance to aminopenicillins, ureidopenicillins, amoxicillin/clavulanic acid, and piperacillin/tazobactam, and diminished susceptibility to cefepime (MIC range 2–4 μg/ml). They also showed resistance to tobramycin, amikacin, and ciprofloxacin (Table 2).

A common PFGE pattern with five different subtypes was observed in all *K. pneumoniae* strains analyzed. This pattern was different from those determined in other MDR and non-MDR *K. pneumoniae* strains isolated in the same period (Fig. 3) and was associated with ST14, as confirmed by MLST of seven representative strains (allelic profile: *gapA* 1, *infB* 6, *mdh* 1, *pgi* 1, *phoE* 1, *rpoB* 1, and *tonB* 1). This ST is the founder of clonal complex 14 (CC14), originally described as nosocomial, which has been detected in many countries [8]. The CC14/K2 clone is associated with reduced lethality in mice, most probably because of the absence of virulence determinants [6].

CC14/K2 strains have been implicated in nosocomial outbreaks related to the global spread of antibiotic resistance [8]. For instance, the Hungarian epidemic clone producing CTX-M-15 belongs to CC14, as does an epidemic carbapenemase-producing clone isolated in various facilities in the Midwestern United States [10]. These epidemic clones, in analogy to methicillin-resistant *Staphylococcus aureus* (MRSA) USA300, have been called the "new MRSAs" [7]. However, our results highlight the need for surveillance of all MDR *K. pneumoniae* strains, not only those that produce carbapenemase or ESBL.

Characterization of resistance mechanisms. All strains were PCR-positive for the bla_{OXA} gene, identified as bla_{OXA-1} after sequencing. On isoelectric focusing, the OXA-1 betalactamase produced a band with a pI of 7.3. This enzyme is widely disseminated in Enterobacteriaceae and is a common cause of amoxicillin/clavulanic acid resistance, especially in *E. coli* and *Salmonella enterica* [12,15]. OXA-1 has also been associated with diminished susceptibility to cefepime in *E. coli* and *Pseudomonas aeruginosa* [2,4]. The transfer of this betalactamase by conjugation in *E. coli* J53-2 yielded transconjugants that also exhibited the MDR pattern, with the exception of ciprofloxacin MIC, in which resistance was diminished in the recipient strain (Table 2). PCR followed by sequencing confirmed the presence of the *aac(6')Ib-cr* gene in both MDR *K. pneumoniae* strains and their transconjugants. The resistance to tobramycin and amikacin was due to *aac(6')Ib*, encoding an aminoglycoside-modifying enzyme [14]. A new variant of this enzyme, encoded by *aac(6')Ib-cr*, has been recently shown to modify quinolones, thus conferring a low level of resistance [4]. QRDR sequencing of GyrA and ParC identified a S83F substitution in GyrA [7]. The presence of a

Table 2. Minimal inhibitory concentration to several antimicrobials of *Klebsiella pneumoniae* strain and its transconjugants

	K. pneumoniae strain 8260	*K. pneumoniae* strain 8260 TC1
Antimicrobial	MIC (μg/ml)	MIC (μg/ml)
Ampicillin	≥ 32	≥ 32
Piperacillin	≥ 128	≥ 128
Ticarcillin	≥ 128	≥ 128
Amox/ clavulanic	≥ 32/16	≥ 32/16
Piper/ tazobactam	32	32
Cefuroxime	16	16
Cefotaxime	≤ 2	≤ 2
Ceftacidime	≤ 1	≤ 1
Cefepime	2	2
Aztreonam	≤ 1	≤ 1
Imipenem	≤ 2	≤ 2
Gentamicin	≤ 4	≤ 4
Tobramycin	≥ 16	≥ 16
Amikacin	32	32
Cotrimoxazole	≥ 4/76	≥ 4/76
Ciprofloxacin	2	0.5

GyrA mutation in the strain described here supports the role of the enzyme encoded by *aac(6′)-Ib-cr* in achieving clinically relevant resistance levels to quinolones, by selection of mutations in QRDR (Table 1) [4]. Since bla_{OXA-1} is usually integron-located, specific amplifications of antibiotic resistance genes and integron-specific primers were performed (Table 3). These amplifications suggested the presence of a class I integron whose variable region contained two resistance genes: *aac(6′) Ib-cr* in the first part of the cassette and bla_{OXA-1} in the second.

Nosocomial outbreaks due to MDR enterobacteria have become a serious problem worldwide, especially given the spread of ESBL- or carbapenemase-producing strains [3,16]. The OXA-1 betalactamase is widespread among enterobacteria and it has been described in isolates from hospitalized patients, from patients with community-acquired infections, and from sewage. The widespread distribution of the bla_{OXA-1} gene is associated with integrons that frequently carry other determinants, such as *aac(6′)Ib*, ESBLs of the CTX-M family, and carbapenemases. Moreover, class I integrons harboring

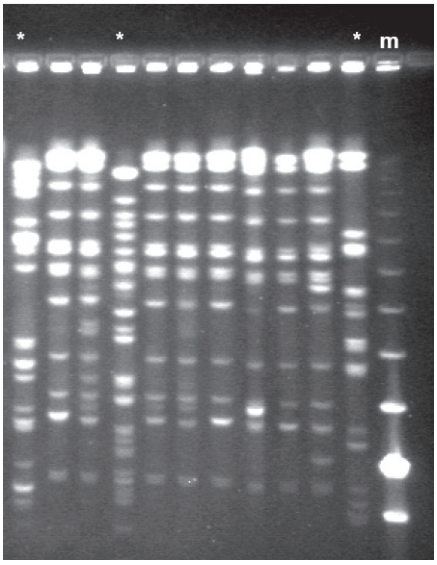

Fig. 3. PFGE patterns of OXA-1-producing *Klebsiella pneumoniae* isolates after restriction with *Xba*I**.** Asterisks (*) indicate contemporary non-clonally related *K. pneumoniae* strains. m: molecular weight marker.

Table 3. Results of PCR reactions performed to characterize the class I integron

Primer combination	Size of amplified fragment[a]
5´CS and 3´CS	2 kb
aac 6´Ib-cr-fw and OXA-rev	1655 pb
OXA-fw and aac 6´Ib-cr-rev	No amplification
5´CS and aac 6´Ib-cr-rev	500 pb
OXA-fw and 3´CS	700 pb
Intl-fw and aac 6´Ib-cr-rev	2,3 kb
OXA-fw and Sul3	500 pb

[a]Size is approximate

bla_{OXA-1}-*aac(6')Ib-cr* in the variable region are frequently described in MDR *E. coli* and *Salmonella enteritidis* isolates [4,5]. *Klebsiella pneumoniae* strains of this CC14 have been detected among ESBL-producing isolates in Spain; however, to the best of our knowledge, ours is the first report of clustered infections caused by a non-ESBL-producing *K. pneumoniae* strain harboring bla_{OXA-1} and *aac(6')Ib-cr*. Moreover, the strain belonged to ST14 and was detected in a hospital setting in Spain [15]. In our studies of other non-MDR non-ESBL-producing *K. pneumoniae* strains we noted the high variability of these strains, a product of their multiclonality. In addition, the MDR strain belonged to ST14, which has been detected in European countries and in Tanzania, and Argentina [13,15,17]. The detection of these MDR strains in the community is a matter of concern because, in the clinical setting, the prolonged hospitalization of a colonized or infected patient in a high risk unit such as the ICU may allow their spread. These strains are not resistant to the majority of betalactam antibiotics used in clinical practice, and the problem is not as widespread as in the case of ESBL; however, since this multiresistance pattern is integron-located, new resistance determinants could be incorporated that threaten the effectiveness of the therapeutic options currently in use.

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Competing interests. None declared.

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