

In Vivo Transfer of Plasmid-Encoded ACC-1 AmpC from *Klebsiella pneumoniae* to *Escherichia coli* in an Infant and Selection of Impermeability to Imipenem in *K. pneumoniae*

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We describe in vivo selection of a *Klebsiella pneumoniae* strain with diminished imipenem susceptibility attributable to plasmid-encoded ACC-1 β -lactamase production and loss of a 36-kDa major outer membrane protein, together with transfer of this plasmid from *K. pneumoniae* to *Escherichia coli* in a Tunisian infant.

Resistance to carbapenems in gram-negative bacteria may be due either to carbapenemase production or to combination of chromosomal or plasmid-mediated class C β -lactamase overproduction and impermeability (20). Among plasmid-mediated AmpC proteins, only ACT-1 and CMY-2 types have been reported to confer resistance to imipenem in association with porin loss (4, 5, 12).

We isolated a *Klebsiella pneumoniae* strain with diminished imipenem susceptibility from a Tunisian infant. This resistance was related to a combination of plasmid-mediated AmpC ACC-1 expression and impermeability acquired under antimicrobial pressure.

A 1-year-old boy from Tunisia with spina bifida and cloacal extrophy was admitted to Robert-Debré hospital (Paris, France) for reconstruction, enterocystoplasty (Mitrofanoff procedure), and pelvic osteotomy. Surgery was complicated by suppuration and disunion of the pubic symphysis. Pus culture yielded ampicillin-resistant *Escherichia coli* and ceftazidime-resistant *Pseudomonas aeruginosa*. Imipenem and amikacin were given for 20 days postoperatively. Before surgery, fecal analysis showed carriage of a cefotaxime-resistant, imipenem-susceptible *K. pneumoniae* strain (isolate K1, 10⁵ CFU/g). At the end of antibacterial chemotherapy (postoperative day 20), the ileal flora grew a cefotaxime-resistant *K. pneumoniae* strain with diminished imipenem sensitivity (isolate K2, 10⁵ CFU/g). The child subsequently developed two urinary tract infections due to an ampicillin-resistant *E. coli* strain (isolate E1), on postoperative days 30 and day 45, both episodes being treated with cefotaxime and gentamicin. A third culture of the enteric flora on day 45 grew both a cefotaxime-resistant *E. coli* strain (isolate E2, 10⁸ CFU/g) and the previous *K. pneumoniae* isolate, K2 (10⁵ CFU/g).

K. pneumoniae and *E. coli* were identified using commercial

kits (API 20E and ID32GN; Biomerieux, Marcy l'Etoile, France). Antimicrobial susceptibility was tested by the disk diffusion method on Mueller-Hinton agar (Sanofi-Diagnostics Pasteur, Marnes-La-Coquette, France), as recommended by the Clinical and Laboratory Standards Institute. The MICs of amoxicillin, cefoxitin, cefotaxime, ceftazidime, cefepime, and imipenem were determined by the E-test method (Biodisk, Solna, Sweden). The imipenem and cefepime MICs were also determined by the E-test method on Mueller-Hinton agar containing 250 μ g/ml cloxacillin (17).

Resistance transfer experiments from *E. coli* E1 and *K. pneumoniae* K1 and K2 were performed by conjugation with *E. coli* J53-2 as previously described (1). The MICs of selected β -lactam antibiotics and associated resistance markers for the clinical isolates and transconjugants are shown in Table 1.

Clinical isolates E2, K1, and K2 were resistant to amoxicillin, amoxicillin-clavulanic acid, ticarcillin, ticarcillin-clavulanic acid, cephalothin, cefixime, aztreonam, ceftazidime, and cefotaxime. The double-disk diffusion test showed no synergy between clavulanic acid and the cephalosporins (cefepime, ceftazidime, and cefotaxime). K2 was also resistant to cefoxitin (MIC, 64 μ g/ml) and cefepime (MIC, 16 μ g/ml) and intermediate to imipenem (MIC, 8 μ g/ml). E2, K1, and K2 were also resistant to kanamycin, gentamicin, tobramycin, netilmicin, and cotrimoxazole. The J53-2 transconjugants of E2, K1, and K2 had the same resistance pattern as isolate E2, except that they were sensitive to cotrimoxazole. The β -lactam susceptibility patterns of K1 and E2, together with an inhibition diameter of 15 mm for ticarcillin-clavulanic acid and 6 mm for ticarcillin, suggested the presence of both class C and class A β -lactamases, while the association of ceftazidime resistance and cefoxitin susceptibility suggested the presence of an ACC-1 β -lactamase.

For genetic studies, total DNA was extracted with the QIAamp DNA Mini Kit (QIAGEN, Courtaboeuf, France). Strains E2, K1, and K2 were screened by PCR for the following β -lactamase genes as previously described (14): *bla*_{TEM}, *bla*_{VEB}, *bla*_{CMY-2} type, *bla*_{DHA}, *bla*_{KPC}, *bla*_{GES}, *bla*_{NMCA},

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*bla*_{VIM-1}, *bla*_{VIM-2}, *bla*_{IMP-1}, *bla*_{IMP-2}, *bla*_{SPM-1}, and *bla*_{GIM-1}. The *bla*_{ACC-1} and *bla*_{TEM} genes were amplified by PCR and directly sequenced with PCR primers described elsewhere (14). Strains K1, K2, and E2 produced both ACC-1b and TEM-1 β-lactamases, whereas strain E1 produced only TEM-1. The contribution of ACC-1 to cefepime and imipenem resistance was demonstrated by using cloxacillin-containing Mueller-Hinton agar plates (Table 1): the MICs of cefepime and imipenem fell from 16 to 1 and from 8 to 0.25 μg/ml, respectively. When we tested the combination of imipenem and EDTA on strain K2, the MIC was 4 μg/ml, thus displaying the absence of class B carbapenemase in this strain.

As *K. pneumoniae* strains K1 and K2 had the same β-lactamases expression and as the K1, K2, and E2 transconjugants had similar susceptibility patterns, we suspected in vivo transfer of the *bla*_{ACC-1} gene from K1 to E1, generating strain E2. We thus investigated the epidemiological links between K1 and K2 and between E1 and E2, respectively, and analyzed the plasmid encoding ACC-1 in strains K1, K2, and E2. Pulsed-field gel electrophoresis (PFGE) analysis was performed as previously described (3), using the SpeI restriction enzyme for *K. pneumoniae* and NotI for *E. coli*. Isolates K1 and K2 were genetically related and differed from a control *K. pneumoniae* strain harboring ACC-1 (SLK54) isolated in Paris in 1998 from a Tunisian patient (14) (Fig. 1A). Isolates E1 and E2 also had identical PFGE patterns that differed from those of unrelated control strains (Fig. 1B). Plasmid extraction from the three transconjugants by the Takahashi method (19) yielded the same large plasmid after digestion by EcoRI and BamHI (data not shown).

Some *K. pneumoniae* clinical isolates have been reported to develop imipenem resistance through a combination of a plasmid-mediated cephalosporinase (ACT-1 or CMY-4) and altered permeability. We thus tested strain K2 for this combination. Outer membrane protein expression by isolates K1 and K2 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described elsewhere (5), which showed that a 36-kDa protein present in strain K1 was absent from strain K2 (Fig. 2). The absence of a protein of similar size has previously been described in *K. pneumoniae* isolates resistant to cefoxitin and broad-spectrum cephalosporins (6, 12).

K. pneumoniae strains producing plasmid-mediated AmpC β-lactamases such as MIR-1 (15), ACT-1 (4), and more recently ACC-1 (14) have been implicated in numerous nosocomial outbreaks (16). AmpC ACC-1 is a plasmid-encoded class C β-lactamase originating from *Hafnia alvei* and now found in various members of the family *Enterobacteriaceae* in North Africa and Europe (2, 7, 8, 11, 13, 14, 18). Combination of a plasmid-mediated AmpC β-lactamase and loss of an outer membrane protein can lead to imipenem resistance in *K. pneumoniae* (4, 5); the two incriminated enzymes were ACT-1 and CMY-4, which are closely related to their chromosomal counterparts in *Enterobacter cloacae* and *Citrobacter freundii*, respectively (16). Clinical isolates of *E. cloacae* and *C. freundii* can become resistant to carbapenems through a combination of impermeability and overproduction of their chromosomal enzymes (9, 10). However, this is the first time that a combination of an ACC-1 β-lactamase, originating from *Hafnia alvei*, and the loss of a major outer membrane protein has been

TABLE 1. MICs of β-lactam drugs and strains characteristics

Isolate, strain or transconjugants	Species	Origin	β-Lactam resistance mechanism		MIC (μg/ml) ^a								Associated resistance ^e
			AMX	FOX	CTX	CAZ	CPM	CPM (+ CLX) ^b	IPM	IPM (+ CLX)			
K1	<i>K. pneumoniae</i>	Digestive flora	>256	4	32	64	0.75	0.25	0.125	0.125	KTGNt + Sxt		
K2	<i>K. pneumoniae</i>	Digestive flora	>256	64	>32	96	16	1	8	0.25	KTGNt + Sxt		
E1	<i>E. coli</i>	UTT ^d	>256	4	0.064	0.25	0.094	ND ^e	0.125	ND			
E2	<i>E. coli</i>	Digestive flora	>256	4	32	64	0.75	ND	0.125	ND	KTGNt + Sxt		
J53	<i>E. coli</i>	Recipient strain	2	ND	0.032	0.047	0.016	ND	0.125	ND	Rif		
J53-K1, J53-K2, J53-E2	<i>E. coli</i>	Transconjugants	>256	4	3	6	0.125	ND	0.125	ND	KTGNt + Rif		

^a MICs were determined by the E-test method (BioDisk, Solna, Sweden). Abbreviations: AMX, amoxicillin; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime; CPM, cefepime; IPM, imipenem.
^b MICs were determined on Mueller-Hinton agar containing cloxacillin (CLX) 250 μg/ml.
^c KTGNt, resistance to kanamycin, tobramycin, gentamicin, and netilmicin; Sxt, resistance to cotrimoxazole (trimethoprim-sulfamethoxazole); Rif, resistance to rifampin.
^d UTT, urinary tract infection.
^e ND, not determined.

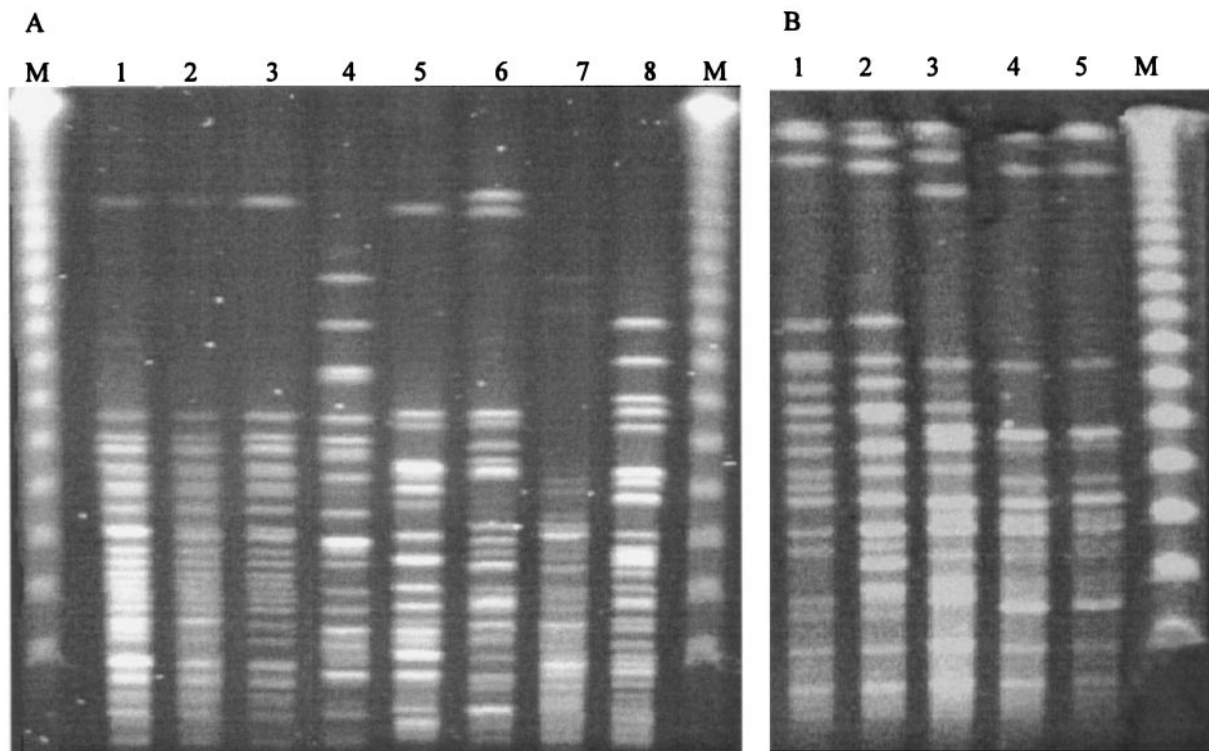


FIG. 1. PFGE analysis. (A) PFGE patterns of SpeI-restricted DNA from *K. pneumoniae*. Lane 1, isolate K1; lanes 2 and 3, isolate K2; lane 4, outbreak isolate (SLK54) producing ACC-1 β -lactamase (14); lanes 5 to 8, unrelated strains of *K. pneumoniae*; lane M, molecular weight marker (lambda ladder; Bio-Rad). (B) PFGE patterns of NotI-restricted DNA from *E. coli*. Lanes 1 to 3, unrelated strains of *E. coli*; lane 4, isolate E1; lane 5, isolate E2; lane M, molecular weight marker (lambda ladder; Bio-Rad).

shown to reduce imipenem activity. This phenomenon has not been described in clinical isolates of *H. alvei*.

In the case we describe, the chronology of strain isolation and antimicrobial therapy and the results of molecular studies suggest the following scenario. The boy first acquired a *K. pneumoniae* strain producing the plasmid-mediated β -lactamase ACC-1 AmpC in Tunisia (unrelated to the strain previ-

ously responsible for a nosocomial outbreak) (14). Then, under imipenem pressure, a spontaneous mutation led to the loss of an outer membrane protein and increased the imipenem MIC. Finally, ACC-1 AmpC was transferred in vivo to an ampicillin-resistant *E. coli* strain that had caused a urinary tract infection in the same infant after cefotaxime therapy. This case emphasizes the need to monitor the enteric flora, both on hospital admission and after antimicrobial chemotherapy, in children who receive numerous antibiotics for complex syndromes.

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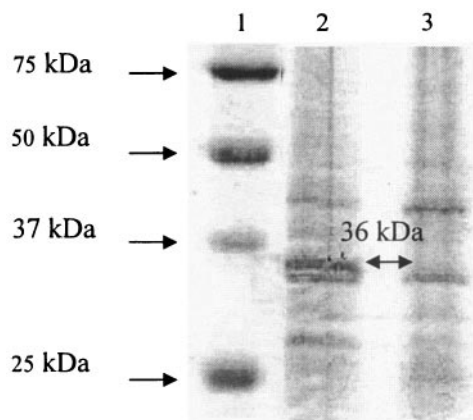


FIG. 2. Outer membrane protein profiles of *K. pneumoniae* clinical isolates K1 and K2 expressing ACC-1. Outer membrane proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% acrylamide. Lane 1, molecular size standard; lane 2, isolate K1 (imipenem susceptible); lane 3, isolate K2 (imipenem resistant).

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