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First Detection of the Ambler Class C 1 AmpC β-Lactamase in *Citrobacter freundii* by a New, Simple Double-Disk Synergy Test▽

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We report on the first detection of an AmpC-type Ambler class C 1 (ACC-1) β-lactamase in *Citrobacter freundi* isolated from a patient also harboring ACC-1-producing *Escherichia coli* and *Klebsiella pneumoniae*. We propose a simple cefoxitin-based double-disk synergy test (DDST) for the specific detection of ACC-1 in members of the family *Enterobacteriaceae*, including natural AmpC producers, in association with a cloxacillin-based DDST as a first-line AmpC-type β-lactamase screening test.

β-Lactamase production is the main mechanism underlying resistance to β-lactam antibiotics in gram-negative bacteria. β-Lactamase genes are often located on plasmids or transposons, making them readily transmissible among different species. During the last decade plasmid-mediated AmpC-type β-lactamases have been increasingly isolated worldwide and have been linked to treatment failure (27, 33). Plasmid-mediated AmpC β-lactamases are derived from the chromosomally encoded AmpC β-lactamases produced by various bacteria, such as *Aeromonas* (5, 18, 19), *Citrobacter freundii* (4, 10, 35), *Enterobacter* (9, 28), and *Morganella morganii* (2, 14). In 1999 a novel plasmid-borne AmpC β-lactamase was isolated from *Klebsiella pneumoniae* in Germany (3). Unlike other plasmidic AmpCs, it had low levels of activity against cephamycins. The authors proposed the name Ambler class C 1 (ACC-1). It was subsequently shown that ACC-1 was derived from the chromosome-borne AmpC of *Hafnia alvei* (25). ACC-1 has so far been isolated only in Germany (3), France (6, 16, 25, 26), Spain (24), and Tunisia (21); the French isolates originated from Tunisia (6, 16, 25, 26). ACC-1 is carried by *K. pneumoniae* (3, 6, 25, 26), *Escherichia coli* (6, 16, 24), *Proteus mirabilis* (16), and *Salmonella enterica* serotypes Mbandaka and Livingstone (21, 31). Here we describe the simultaneous isolation of three bacterial species, each of which carries ACC-1, from the same stool sample of a 17-year-old patient. To our knowledge this is the first time that the ACC-1 β-lactamase has been found in *Citrobacter freundii*. We also describe a new cefoxitin-based double-disk synergy test (DDST) for specific ACC-1 detection.

The patient, a teenage boy, was admitted to the Robert-Debré Hospital (Paris, France) in November 2005 for surgical treatment of a leaking jejuno-jejunal anastomosis after intestinal grafting. He had received multiple courses of antibiotics, including broad-spectrum cephalosporins.

The same stool culture yielded three different ceftazidime-resistant members of the family *Enterobacteriaceae*: 10^8 CFU/g *Escherichia coli* (strain Ec1), 10^9 CFU/g *Klebsiella pneumoniae* (strain Kp1), and 10^6 CFU/g *Citrobacter freundii* (strain Cf1). Antimicrobial susceptibility was determined by the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France), as recommended by the Clinical and Laboratory Standards Institute (formerly NCCLS). The three isolates were susceptible to cefoxitin and cefepime but had decreased susceptibilities to ceftazidime and cefotaxime. No synergy was observed between clavulanic acid and ceftazidime, cefotaxime, or cefepime in the double-disk synergy test. The MICs of cefoxitin, cefotaxime, ceftriaxone, ceftazidime, cefepime, imipenem, piperacillin, and piperacillin-tazobactam were determined by the Etest diffusion method (AB Biodisk, Solna, Sweden) (Table 1), as recommended by the manufacturer.

Strains Ec1, Kp1, and Cf1 were conjugated with rifampin-resistant, amoxicillin-susceptible *Escherichia coli* strain J53, as described previously (1). The transconjugants (TCs; TC Ec, TC Kp, and TC Cf, respectively) displayed the same patterns of β-lactam resistance as the donor strains. PCR and sequencing were used to assess the three isolates and their transconjugants for the presence of $bla_{\text{ACC-1}}$ and $bla_{\text{TEM-1}}$, as described previously (6, 25). Pulsed-field gel electrophoresis showed that strains Ec1 and Kp1 were unrelated to previous French isolates harboring $bla_{\text{ACC-1}}$ (6, 23) (data not shown).

The cefoxitin susceptibilities of the ACC-1-producing strains were recently attributed to ACC-1 β-lactamase inhibition by cefoxitin itself (17). We therefore determined the ceftazidime and cefepime MICs for strains Ec1, Kp1, and Cf1 and also for 15 isolates of the *Enterobacteriaceae* expressing a broad range of AmpC-type β-lactamases by using the Etest diffusion method (AB Biodisk) on agar plates containing cefoxitin concentrations of 0.25, 1, and 4 μg/ml (2, 6, 13, 20, 21, 23, 30, 36). The results confirmed the powerful inhibitory action of cefoxitin and its specificity for ACC-1, as cefoxitin did not reduce the ceftazidime or cefepime MICs for the other strains tested. Cefoxitin concentrations below 0.25 μg/ml were not inhibitory. We used these results to develop a DDST with cefoxitin and ceftazidime or cefotaxime for the detection of ACC-1. We used a distance of only 2 cm between the centers of the two disks, as in previous studies, cefoxitin showed no particular

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TABLE 1. In vitro susceptibilities (MICs) of the three stool isolates, the *E. coli* recipient strain (J53), and the resulting transconjugants

Antimicrobial(s)	MIC (μg/ml)						
	E. coli (Ec1)	*K. pneumoniae* (Kp1)	*C. freundii* (Cf1)	J53	TC Ec	TC Kp	TC Cf
Cefoxitin	6	6	8	2	2	2	2
Cefotaxime	12	24	16	0.032	3	4	4
Ceftriaxone	12	16	16	0.023	4	4	4
Ceftazidime	24	24	24	0.047	4	4	12
Cefepime	0.5	1	0.38	0.016	0.125	0.19	0.19
Imipenem	0.25	0.25	0.19	0.125	0.125	0.125	0.125
Piperacillin	192	>256	64	1	32	32	32
Piperacillin + tazobactam	32	64	16	1	6	6	6

synergy with broad-spectrum cephalosporins against ACC-1-expressing isolates at a distance of 3 cm, possibly owing to inadequate cefoxitin concentrations (Table 2).

A DDST with 500-μg cloxacillin disks (Rosco Diagnostica, Denmark) was applied to the 15 strains mentioned above, as recommended by the manufacturer. Cloxacillin was synergistic with both ceftazidime and cefotaxime against all the strains (Fig. 1). In contrast, cefoxitin was synergistic with cefotaxime and ceftazidime only against ACC-1-expressing strains (Fig. 1A). As expected (15), cefoxitin induced resistance to ceftazidime for the DHA-1-expressing strain (Fig. 1C).

Detection of plasmidic AmpC is an emerging challenge for clinical laboratories, especially in bacteria that naturally expressing AmpC, like *Citrobacter freundii*. Various homemade detection tests have been proposed, but none has been widely adopted (7, 8, 11, 12, 22, 32, 34).

Previous reports of ACC-1-expressing isolates (3, 6, 16, 21, 24–26) mention no particular interactions between antibiotic disks. Indeed, since no *ampR* motif was identified on bla_{ACC-1} (3), cefoxitin was not an inducer by the double-disk test (3, 25). The cefoxitin DDST that we have described here is specific for the ACC-1 β-lactamase, which, to our knowledge, is the only plasmidic AmpC to be inhibited by cefoxitin (29). This cefoxitin DDST method readily identified ACC-1 even in natural AmpC producers like *Citrobacter freundii*. As ACC-1-expressing strains have an atypical resistance profile (notably, cefoxitin susceptibility, ceftazidime resistance, and cefepime susceptibility), the cefoxitin DDST can be used as a second-line test to confirm the expression of ACC-1. We did not test this DDST with strains carrying both ACC-1 and other wide-spectrum β-lactamases (e.g., extended-spectrum β-lactamases, other plasmid-mediated AmpC β-lactamases, or derepressed *ampC*). In such strains, the synergy between cefoxitin and ceftazidime provided by DDST may be more difficult to visualize.

The DDST with a 500-μg cloxacillin disk successfully identified AmpC expression in all 15 positive control strains tested here. However, whereas the manufacturer recommends one disk-to-disk distance (1 cm edge to edge), we adapted this distance using the inhibition diameter of the cephalosporin (center-to-center distance = inhibition radius + 1 cm) tested to obtain the most obvious synergy picture possible (Fig. 1).

In conclusion, we report on the first isolation of a *Citrobacter freundii* strain expressing the ACC-1 β-lactamase and propose a cefoxitin DDST in association with the DDST with a 500-μg cloxacillin disk for identifying this enzyme in isolates of the family *Enterobacteriaceae*, including natural *ampC* carriers. The synergy between cefoxitin and ceftazidime or cefotaxime reveals ACC-1 β-lactamase expression, whereas induction is observed with inducible AmpC β-lactamases such as DHA-1. This DDST can easily be performed on the same agar plate by the DDST with a 500-μg cloxacillin disk, which is specific for AmpC-type β-lactamases. These two associated DDSTs may

TABLE 2. Ceftazidime and cefepime MICs determined by Etest diffusion method on agar plates with or without cefoxitin

Organism	Reference	AmpC type	Ceftazidime MIC (μg/ml) with cefoxitin at concn (μg/ml) of:				Cefepime MIC (μg/ml) with cefoxitin at concn (μg/ml) of:			
			4	1	0.25	0	4	1	0.25	0
Klebsiella pneumoniae (Kp1)	This study	ACC-1	0.094	0.75	24	24	0.023	0.38	1	1
Escherichia coli (Ec1)	This study	ACC-1	No growth	0.25	16	24	No growth	0.032	0.25	0.5
Citrobacter freundii (Cf1)	This study	ACC-1	No growth	0.25	12	24	No growth	0.032	0.19	0.38
Salmonella serotype Mbandaka	21	ACC-1	No growth	2	256	>256	No growth	0.047	0.25	1
Escherichia coli	6	ACC-1	No growth	8	64	64	No growth	0.38	0.75	0.75
Klebsiella pneumoniae	6	ACC-1	No growth	1	48	64	No growth	0.125	0.5	0.75
Klebsiella pneumoniae	26	ACC-1	1	24	64	256	0.125	0.38	0.5	0.75
Klebsiella pneumoniae	13	CMY-2	3	4	3	4	0.047	0.064	0.047	0.064
Salmonella serotype Senftenberg	20	CMY-2b	24	24	24	24	0.19	0.25	0.25	0.25
Klebsiella pneumoniae	36	DHA-1	32	16	8	4	0.094	0.064	0.064	0.064
Klebsiella pneumoniae	30	MOX-2	48	48	48	48	0.5	0.38	0.5	0.5
Klebsiella pneumoniae	23	FOX-3	32	48	32	32	0.25	0.25	0.25	0.25
Hafnia alvei	This study	Derepressed *ampC*	No growth	2	4	16	No growth	0.016	0.032	0.064
Citrobacter freundii	This study	Derepressed *ampC*	>256	>256	>256	>256	0.38	0.38	0.38	0.38
Enterobacter cloacae	This study	Derepressed *ampC*	>256	>256	>256	>256	0.032	0.032	0.032	0.032

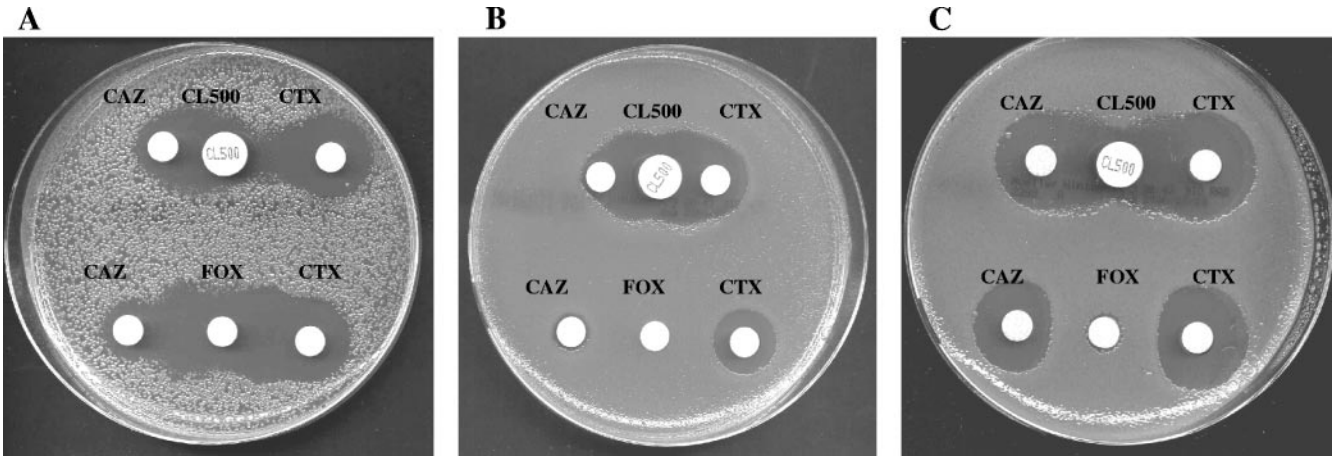

FIG. 1. Double-disk synergy test on Mueller-Hinton agar plates with broad-spectrum cephalosporins and either cloxacillin (upper set of disks) or cefoxitin (lower set of disks) applied to three AmpC-carrying strains (CAZ, ceftazidime; CTX, cefotaxime; FOX, cefoxitin; CL500, cloxacillin at 500 μg). The distances between the disks were optimized according to the inhibition diameters, as described in the text. Cloxacillin is synergistic with both ceftazidime and cefotaxime for the three strains. (A) *Citrobacter freundii* harboring ACC-1 (strain Cf1) for which cefoxitin is synergistic with cefotaxime and ceftazidime; (B) *Citrobacter freundii* with chromosomal *ampC* derepression for which no synergy is observed between cefoxitin and either cefotaxime or ceftazidime; (C) *Klebsiella pneumoniae* harboring DHA-1 (36) for which cefoxitin induces resistance to cefotaxime and ceftazidime.

be simple and cost-effective first-line tests for AmpC-type β-lactamase determination, indicating the presence of an AmpC-type β-lactamase, and further providing a specific means of detection of the ACC-1 β-lactamase.

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