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Wide Dissemination of a Carbapenemase Plasmid among Gram-Negative Bacteria: Implications of the Variable Phenotype

Broad-spectrum carbapenem antibiotics are the last bastion of empirical therapy for resistant gram-negative bacterial infections, and outbreaks of carbapenem-resistant bacterial infection may be associated with greatly increased mortality in the critically ill (4). Carbapenems (e.g., imipenem, meropenem, ertapenem) are increasingly threatened by dissemination of metallo-β-lactamases (MβLs), the most important of which are the integron-borne members of the VIM and IMP families. Integrons are genetic elements able to capture and express antibiotic resistance determinants in the form of gene cassettes via site-specific recombination (7). Class 1 integrons are dominant among the four classes associated with antibiotic resistance, and plasmids are important vectors for their transmission between bacteria in the hospital environment. While transmissible MβLs are increasingly reported throughout Asia, Europe, and South America, Australia has only recently observed the emergence of an MβL, IMP-4 (5). We found the bla_{IMP-4} gene cassette as the first of four in a class 1 integron-associated array (GenBank accession no. AJ609296), which also includes an *aacA4* gene cassette conferring resistance to gentamicin and tobramycin. This array was present on a large conjugative plasmid (pJIBE401) in a clinical isolate of *Klebsiella pneumoniae* (Kp1239) referred to our laboratory (2).

With the arrival of a new gene or set of genes in the gram-negative microflora, it is necessary to determine both its transmissibility characteristics and the resulting phenotype to understand the threat it poses. This means assessing the efficiency of transmission and the host range of the transmissible trait, as well as defining the relevant antibiotic resistance phenotype and any potential disadvantage to the organism in terms of growth characteristics or biological fitness (that is, the chance of being segregated out of the microflora by failing to compete in the absence of selection pressure). We therefore mated Kp1239 with rifampin-resistant recipient strains generated from a range of wild-type enteric bacteria, performing conjugation experiments as previously described (1). Transmission of bla_{IMP-4}-bearing plasmid pJIBE401 from Kp1239 into *Escherichia coli* (EcUB5201*Rf*) and *Enterobacter aerogenes* (Ea13048*Rf*) occurred at a frequency of 10^{-2}, while transmission into *Citrobacter freundii* (Cf4000*Rf*) and *K. pneumoniae* (Kp13883*Rf*) occurred at frequencies of 10^{-6} and 10^{-7}, respectively. *Serratia marcescens* (Sm1002*Rf*) transconjugants were also generated, at frequencies of $\leq 10^{-9}$ (Table 1). Attempts to introduce pJIBE401 from Kp1239 into *Acinetobacter baumannii* and *Pseudomonas aeruginosa* were repeatedly unsuccessful.

Since the detection of Kp1239, we have observed bla_{IMP-4} in a number of other enteric bacteria isolated from patients within our region. The clinical isolates *K. pneumoniae* Kp1239 and Kp2730, *Enterobacter cloacae* El3518, and *Citrobacter amalonaticus* Ca3927 are all temporally and geographically distinct and readily separated by DNA fingerprinting (enterobacterial repeat intergenic consensus sequence typing; data not shown) (6). These isolates were used for conjugation experiments into EcUB5201*Rf* (Table 2). All appeared to have a common high-molecular-weight plasmid of the same size as pJIBE401 (>150 kb; data not shown) carrying identical gene cassettes, consistent with that plasmid's widespread dissemination.

MICs were determined by Vitek 2 AST-N019 gram-negative susceptibility card (Vitek AMS; BioMérieux Vitek Systems Inc., Hazelwood, Mo.), except for imipenem, which was determined by Etest (AB BIODISK, Solna, Sweden). Most bla_{IMP-4}-bearing transconjugants expressed resistance below the Clinical and Laboratory Standards Institute clinical breakpoint for imipenem sensitivity (Table 1), and we therefore tested whether we could induce higher-level resistance in these strains upon antibiotic exposure in vitro. Kp2730, an imipenem-intermediate (MIC, 8 μg ml^{-1}) clinical isolate, was compared with Kp13883*Rf*/pJIBE401 transconjugants, which were imipenem sensitive (MIC, 3 μg ml^{-1}; Table 1). Kp2730 (but not Kp13883*Rf*/pJIBE401) grew as isolated colonies on nutrient agar (Difco) containing 16 μg ml^{-1} of imipenem, occurring at a frequency of 10^{-1} to 10^{-2}. A loopful of these colonies was amplified in nutrient broth (Difco) with 16 μg ml^{-1} of imipenem and further subcultured on 64 μg ml^{-1} of imipenem in nutrient agar with a 10^{-1} to 10^{-2} yield of isolated colonies again (compared with growth on nonselective medium). A loopful of the highly resistant Kp2730-derived mutants (Kp2730^R, obtained from the plates containing 64 μg ml^{-1} of imipenem) was resuspended in nutrient broth, and 100 CFU each of these and of Kp1239, Kp2730, Kp13883*Rf*, and KP13883*Rf*/pJIBE401, at log phase, was used to inoculate separate aerobic Bactec 9000 blood culture bottles (BD Diagnostics). All bottles signaled as positive after overnight incubation, with no significant difference in growth rates (Kp1239 and Kp2730 bottles signaled positive at 10 h; Kp13883*Rf*, KP13883*Rf*/pJIBE401, and Kp2730^R at 11 h), and the high imipenem MICs (≥64 μg ml^{-1}) persisted on subculture.

Decreased outer membrane permeability is one logical explanation for the high carbapenem MICs for some *Klebsiella* and *Enterobacter* strains (Tables 1 and 2), but the Kp2730^R

TABLE 1. MICs of various drugs for the isolates studied

Drug[a]	MIC (μg/ml)[b] for:								
	Kp1239 (+)	Cf4000*Rf*		Ea13048*Rf*		Kp13883*Rf*		Sm1002*Rf*	
		−	+	−	+	−	+	−	+
AMK	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
GEN	≥16	≤1	≥16	≤1	≥16	≤1	≥16	≤1	≥16
TOB	8	≤1	8	≤1	≥16	≤1	8	≤1	≥16
CEF	≥64	≤4	≥64	≥64	≥64	4	≥64	≥64	≥64
FOX	≥64	≥64	≥64	≥64	≥64	≤4	≥64	≤4	≥64
CRO	≥64	≤1	≥64	≤1	≥64	≤1	32	≤1	8
CAZ	≥64	≤1	≥64	≤1	≥64	≤1	≥64	≤1	≥64
AMP	≥32	4	≥32	16	≥32	≥32	≥32	≥32	≥32
AMX	≥32	≤2	≥32	≥32	≥32	≤2	≥32	≥32	≥32
PIP	≥128	≤4	32	8	≥128	≤4	64	≤4	32
TZP	32	≤4	≤4	8	8	≤4	8	≤4	≤4
TIM	≥128	≤8	≥128	≤8	≥128	≤8	≥128	≤8	≥128
IPM	≥32	0.19	1.5	0.5	≥32	0.25	3	0.5	2

[a] Abbreviations: AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CEF, cephalothin; FOX, cefoxitin; CRO, ceftriaxone; CAZ, ceftazidime; AMP, ampicillin; AMX, ampicillin-clavulanate; PIP, piperacillin; TZP, piperacillin-tazobactam; TIM, ticarcillin-clavulanate; IPM, imipenem.

[b] Minus and plus signs indicate the absence and presence, respectively, of pJIBE401.

TABLE 2. MICs of various drugs for clinical isolates and transconjugants

Drug[a]	MIC (μg/ml)[b] for:								
	Kp1239		Kp2730		El3518		Ca3927		EcUB5201*Rf* (−)[c]
	WT	TX	WT	TX	WT	TX	WT	TX	
AMK	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2	≤2
GEN	≥16	≥16	≥16	≥16	≥16	≥16	≥16	≥16	≤1
TOB	8	8	≥16	8	≥16	8	≥16	8	≤1
CEF	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	8
FOX	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≤4
CRO	≥64	16	≥64	16	≥64	16	≥64	≥32	≤1
CAZ	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≤1
AMP	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	4
AMX	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	4
PIP	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≤4
TZP	32	≤4	32	8	32	8	8	≤4	≤4
TIM	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≥128	≤8
IPM	≥32	1	8	2	≥32	1	2	1	0.19

[a] For definitions of abbreviations, See Table 1, footnote *a*.
[b] WT and TX indicate each clinical isolate and its transconjugant into EcUB5201*Rf*, respectively.
[c] The minus sign indicates the absence of pJIBE401.

mutants have not yet been examined in detail. The integron promoter remained unchanged in Kp2730^R, and EcUB5201*Rf* transconjugants displayed little difference in carbapenem MICs as determined by Etests (data not shown), indicating the lack of plasmid involvement in the increased resistance seen. However, *ompK36* encodes a porin of known relevance to carbapenem resistance in *Klebsiella* (3) and we have found this to be disrupted by an insertion sequence in Kp1239 (our unpublished data). Thus, we conclude that a broad-host-range plasmid carrying bla_{IMP-4} and *aacA4* is efficiently transmitted to a wide variety of gram-negative bacteria, that efficiency presumably varying both within and between species, resulting in an elevated MIC of carbapenems which is often still in the sensitive range, but from which stable high-level resistance can be readily selected, at least in some *K. pneumoniae* strains. This occurs without an obvious growth defect in optimal media and is likely to be clinically important.

The efficient horizontal transfer of resistance genes between gram-negative bacteria in the face of intensive selection pressure in hospitals poses a great problem. The conditional nature of resistance phenotypes such as we describe further complicates infection control measures. Detection of these independent bacterial isolates over the past year suggests dissemination throughout our microflora of a newly arrived panresistance to the main β-lactams and aminoglycosides. This is a reminder that we need to manage gram-negative resistance surveillance thoughtfully and that we need to think of outbreaks in terms of the transmitted unit (typically a plasmid) and the vagaries of its host range and phenotype.

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