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Detection of Carbapenemase-Producing *Acinetobacter baumannii* in a Hospital

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Acinetobacter baumannii **strains resistant to both imipenem (IPM) and ceftazidime (CAZ) were isolated from 1994 through 1996 at Gunma University Hospital. Nine isolates from different inpatients were examined for carbapenem-hydrolyzing activity and for the carbapemase gene** ***bla*$_{IMP}$ by the PCR method. All nine isolates were carbapenemase-producing strains that hydrolyzed IPM and that harbored *bla*$_{IMP}$. The *bla*$_{IMP}$ gene was transmissible by conjugation to an IPM-susceptible recipient strain of *A. baumannii* and conferred resistance to IPM, CAZ, cefotaxime (CTX), ampicillin (AMP), and piperacillin (PIP). Either intermediate or high-level resistance to amikacin (AMK) was transferred from two and five strains, respectively, concomitantly with *bla*$_{IMP}$, and gentamicin (GEN) resistance was also transferred in one instance of high-level AMK resistance. Comparative examination of clinical isolates for resistance patterns to nine drugs, IPM, CAZ, CTX, aztreonam, AMP, PIP, AMK, GEN, and norfloxacin, in addition to pulsed-field gel electrophoresis patterns with *Not*I-digested genomic DNA, confirmed nosocomial transmission of infections involving carbapenemase-producing *A. baumannii* strains.**

Acinetobacter baumannii is a glucose-nonfermentative gram-negative bacillus that is widely distributed in hospital environments and is one of the nosocomial pathogens that often causes serious infections, especially in immunocompromised inpatients (1, 8). Extensive use of antimicrobial chemotherapy against bacterial infections has contributed to the emergence and increase in the number of multidrug-resistant strains, especially among opportunistic pathogens such as members of the family *Enterobacteriaceae* and glucose-nonfermentative bacteria. Antimicrobial susceptibility testing and biological and genomic typing of *Acinetobacter* isolates have revealed the dissemination of drug-resistant strains in various hospitals (5, 13, 14, 20, 22, 24, 26).

We have detected *A. baumannii* strains resistant to imipenem (IPM) and various other β-lactam antibiotics including ceftazidime (CAZ). IPM is a potent β-lactam, partly because of its resistance to hydrolysis by most β-lactamases except carbapenemase (2). The latter enzyme, a metallo-β-lactamase belonging to molecular class B, is capable of hydrolyzing both IPM and CAZ, in addition to most β-lactam antibiotics, and confers resistance to these agents in pathogenic bacteria (11, 19). The nucleotide sequence of the gene encoding the carbapenem-hydrolyzing metallo-β-lactamase identified on a plasmid in *Pseudomonas aeruginosa* was completely identical to that of the *Serratia marcescens* gene named *bla*$_{IMP}$ (7, 16, 27). Furthermore, surveillance for the identification of *bla*$_{IMP}$ by PCR techniques revealed that the gene was disseminated among various gram-negative pathogens, especially in *P. aeruginosa* and *S. marcescens* (6, 15). Although IPM-resistant *Acinetobacter* species have been isolated, strains that produce the carbapenemase have not yet been reported (3, 17, 18, 21).

We detected the carbapenemase gene, *bla*$_{IMP}$, in clinical isolates of IPM- and CAZ-resistant *A. baumannii* strains and investigated the strains for their dissemination mode in a single hospital.

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MATERIALS AND METHODS

Bacterial strains. IPM- and CAZ-resistant isolates of *A. baumannii* were obtained from different inpatients in Gunma University Hospital from 1994 through 1996.

Media. Sheep blood agar (BBL) was used for isolation and purification of *A. baumannii* strains. Sensitivity testing (ST) agar and ST broth (Nissui Pharmaceutical Co., Ltd.) were used routinely for cultivation and drug susceptibility testing.

Antibacterial agents. The abbreviations and sources of the antibacterial agents used in the study are as follows: amikacin (AMK) and IPM, Banyu Pharmaceutical Co., Ltd.; aztreonam (ATM), Bristol-Myers Squibb, Inc.; CAZ, Nippon Glaxo Co., Ltd.; cefotaxime (CTX), Hoechst Japan, Ltd.; gentamicin (GEN), Schering Corp.; norfloxacin (NOR), Kyorin Seiyaku Co., Ltd.; piperacillin (PIP), Toyama Chemical Co., Ltd.; and rifampin (RIF), Daiichi Pharmaceutical Co., Ltd.

Drug susceptibility tests. MICs were determined by an agar dilution method with an inoculum size of 10^4 CFU per spot (27).

Assay of β-lactamase activity. Exponentially growing cells of *A. baumannii* in ST broth were washed with 50 mM phosphate buffer (pH 7.0) and were disrupted by sonication. The supernatant obtained after the cellular debris was removed by centrifugation (15,000 × *g*, 15 min, 4°C) was used as the crude enzyme extract.

β-Lactamase activity was determined spectrophotometrically by previously described methods (27). One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min at 30°C.

PCR amplification. The PCR consisted of 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and amplification at 72°C for 1.5 min, followed by an additional 7 min at 72°C, with the Premix *Taq* reagent (Takara Shuzo Co., Ltd.) used with the Program Template Control System PC-700 (ASTEC Co., Ltd.).

The nucleotide sequences of the forward and reverse primers used for detection of the carbapenemase gene, *bla*$_{IMP}$, were those constructed by Senda et al. (23). The PCR product was 587 bp, was derived from within the 741-bp *bla*$_{IMP}$ sequence, and was detected by agarose gel electrophoresis.

Conjugation experiment. Conjugal transmissibility of *bla*$_{IMP}$ was examined with the recipient strain *A. baumannii* TY44Rp, a RIF-resistant mutant of strain TY44, which was a clinical isolate that lost IPM resistance after storage in Casitone medium agar.

The membrane filter method was used for conjugation. A mixture of donor and recipient cells in broth cultures at an early stationary phase of growth was filtered through membranes (pore size, 0.45 μm), and those bacterial cells that were retained were placed on ST agar plates overnight at 30°C. The transmissibility of *bla*$_{IMP}$ was determined by transfer of CAZ resistance rather than IPM resistance, because the level of CAZ resistance expressed by *bla*$_{IMP}$ was high

TABLE 1. Origins of imipenem-resistant *A. baumannii* strains

Strain	Isolation		
	Date (yr, mo)	Ward	Source
TY7	1994, February	1st internal medicine	Urine
TY8	1994, April	1st internal medicine	Oral fluid
TY9	1994, July	1st internal medicine	Sputum
TY10	1994, August	Dermatology	Bedsore
TY11	1994, September	2nd surgery (ICU)	Blood
TY12	1994, September	2nd surgery (ICU)	Pus
TY15	1995, February	Otolaryngology (ICU)	Drainage
TY40	1996, June	2nd internal medicine	Sputum
TY42	1996, May	2nd internal medicine	Sputum

TABLE 2. Properties of IPM- and CAZ-resistant *A. baumannii* strains

Strain	Hydrolysis of IPM (sp act)[a]	Detection of bla_{IMP} gene	Conjugal transferability of IPM and CAZ resistance[b]
TY7	0.51	+	10^{-3}
TY8	0.36	+	10^{-3}
TY9	0.28	+	10^{-4}
TY10	0.38	+	10^{-3}
TY11	0.39	+	10^{-2}
TY12	0.59	+	10^{-3}
TY15	0.63	+	10^{-3}
TY40	1.43	+	10^{-2}
TY42	0.58	+	10^{-3}
TY44[c]	<0.01	−	Not done

[a] See Materials and Methods for description of specific activity. One unit of enzyme activity is the amount of enzyme that hydrolyzed 1 μmol of substrate per min at 30°C.
[b] Transfer frequency was expressed as the ratio of the number of transconjugant cells to the number of recipient cells.
[c] Susceptible strain.

enough to distinguish transconjugants from the recipient. Transconjugant cells were selected on agar plates containing 8 μg of CAZ per ml and 100 μg of RIF per ml. The transfer frequency was expressed as the ratio of the number of transconjugants to the number of donor cells in the conjugation mixture.

Pulsed-field gel electrophoresis (PFGE). Genomic DNA was prepared in agarose plugs that had been treated with lysozyme and proteinase K by using the GenePath Reagent kit (Bio-Rad) by the procedures recommended by the manufacturer. The DNA was then digested with 12.5 U of the restriction endonuclease *Not*I. The DNA segments generated were separated in a 1% agarose gel and were run in Tris-borate-EDTA buffer on the pulsed-field apparatus (Gene Path System; Bio-Rad) at 6.0 V/cm for 19.7 h, with pulse times ranging from 5.3 to 34.9 s.

RESULTS

Isolation of IPM-resistant *A. baumannii*. A total of 251 *A. baumannii* strains were isolated from different inpatients during the 3 years from 1994 to 1996 in Gunma University Hospital, and 28 of these were resistant to both IPM (MICs, ≧8 μg/ml) and CAZ (MICs, ≧16 μg/ml). However, the resistance was not stable, because 19 of the 28 strains lost resistance to both IPM and CAZ after storage for a year or more in Casitone medium. The remaining nine strains that harbored IPM and CAZ resistance are listed in Table 1. The biochemical properties of these isolates were examined with the API NE20 system and were found to be the same. They were isolated from various sites from different inpatients on six wards at different times. Three of the nine isolates were obtained from patients on intensive care units (ICUs) after they were moved there from other wards.

Detection of carbapenemase activity and bla_{IMP} gene. Nine IPM- and CAZ-resistant isolates and one susceptible strain (TY44; MICs of IPM and CAZ, <0.5 μg/ml), whose resistance had been lost after storage, were examined for IPM-hydrolyzing activity with crude enzyme extracts and for the bla_{IMP} gene by the PCR method. All of the strains except for TY44 were capable of hydrolyzing IPM and were PCR positive, indicating that they harbored bla_{IMP} (Table 2).

Transfer of carbapenemase gene by conjugation. The IPM- and CAZ-resistant strains were mated with TY44Rp on membrane filters, and transconjugants were selected on ST agar containing both CAZ and RIF. Transconjugants were obtained at frequencies of 10^{-2} to 10^{-4} per donor cell from all nine strains (Table 2). For all transconjugants, the MICs of IPM were higher than those for the recipient strain and the gene bla_{IMP} was detectable by PCR (data not shown).

Susceptibilities of IPM-resistant *A. baumannii* strains to various chemotherapeutic agents. The drug susceptibilities of the clinical isolates and their transconjugants were determined and are shown in Table 3. The IPM MICs for nine clinical isolates were 8 to 16 μg/ml, and IPM resistance was accompanied by resistance to CAZ, CTX, and PIP but not always by resistance to ATM. Seven strains were highly resistant (MICs, >128 μg/ml) or intermediately resistant (MICs, 32 to 64 μg/ml) to AMK, and six strains were resistant to GEN (MICs, 8 to 16 μg/ml). Four strains were obviously resistant to NOR (MICs, 8 to 16 μg/ml). Identical drug resistance patterns were observed between TY11 and TY12 and between TY40 and TY42. The latter two strains, which were isolated relatively late in the study, showed characteristic resistance to multiple potent chemotherapeutic agents such as β-lactams, aminoglycosides, and quinolones.

In all cases, resistance to the β-lactam antibiotics IPM, CAZ, CTX, ampicillin (AMP), and PIP was transferred concomitantly, whereas neither ATM nor NOR resistance was transmissible. Transconjugants obtained from strains TY11 and TY12 or from strains TY40 and TY42 had almost identical drug resistance patterns. High-level resistance to AMP and PIP was transferred from the latter two strains. Transfer of high-level or intermediate resistance to AMK was observed from five strains, strains TY7, TY8, TY9, TY10, and TY15. Resistance to GEN was transferred from TY10.

PFGE patterns of *A. baumannii* strains. The PFGE patterns after digestion with restriction endonuclease *Not*I are shown in Fig. 1, in which strains with identical patterns were placed in adjacent lanes. The following five different patterns were obtained for nine TY strains: pattern 1, strains TY11 and TY12; pattern 2, strains TY40 and TY42; pattern 3, strains TY8 and TY9; pattern 4, strains TY7 and TY10; and pattern 5, strain TY15. The two strains with pattern 1 (TY11 and TY12) and those with pattern 2 (TY40 and TY42) also had identical drug resistance patterns and transmissible drug resistance patterns (Table 3) and were isolated from the same ward and in the same month of 1994 and within a 3-month period in 1996, respectively (Table 1). The two strains with pattern 3 (TY8 and TY9) were isolated from the same ward in 1994 within 3 months of each other (Table 1), but the levels of resistance to PIP and AMK were higher for the latter strain. The drug resistance patterns of the transconjugants of these strains were identical (Table 3). The two strains with pattern 4 (TY7 and TY10) were isolated from different wards in 1994, and the AMP, AMK, and GEN MICs for the strains were different. Resistance to GEN was transferred from TY10 along with the gene bla_{IMP}.

The presence of strains with identical PFGE and drug resistance patterns or with identical PFGE patterns and similar

TABLE 3. Susceptibilities of *A. baumannii* strains to various drugs

Strain	MIC (μg/ml)								
	IPM	CAZ	CTX	ATM	AMP	PIP	AMK	GEN	NOR
Clinical isolate									
TY11	8	>128	128	16	128	64	2	16	8
TY12	8	>128	128	16	128	64	2	16	8
TY40	8	>128	>128	32	>128	128	32	16	16
TY42	8	>128	>128	32	>128	>128	32	16	16
TY8	8	128	128	8	64	32	64	8	2
TY9	8	>128	128	4	128	128	>128	16	<1
TY7	8	>128	128	16	128	32	32	4	4
TY10	16	>128	128	16	32	32	>128	16	2
TY15	16	>128	128	8	64	64	32	8	2
Transconjugant from:									
TY11	2	16	32	<1	32	16	1	1	<1
TY12	4	32	64	<1	64	16	1	1	<1
TY40	4	16	64	<1	>128	128	8	2	<1
TY42	4	16	64	<1	>128	128	4	1	<1
TY8	8	32	128	<1	32	8	32	<1	<1
TY9	8	32	128	<1	32	16	64	<1	<1
TY7	4	64	64	<1	64	16	64	<1	<1
TY10	8	16	128	<1	32	8	64	8	<1
TY15	4	32	32	<1	32	8	64	<1	<1
Recipient, TY44Rp	<1	<1	4	<1	8	1	1	<1	<1

drug resistance patterns strongly suggested that different inpatients in the same or different wards were infected with the same strain or with derivatives of the same strain.

DISCUSSION

Acinetobacter spp. are important causes of nosocomial infections, and it has been reported that they acquire resistance to chemotherapeutic agents in various hospitals (1, 13, 20, 22, 26). Although β-lactam antibiotics are potent chemotherapeutic agents, *Acinetobacter* strains resistant to multiple β-lactam antibiotics, mainly due to hydrolysis by β-lactamases, have been increasing (14, 25). Some β-lactamases in *A. baumannii* were reported to be involved in resistance to IPM, but their catalytic activities for IPM were very low or were barely detectable (4, 17).

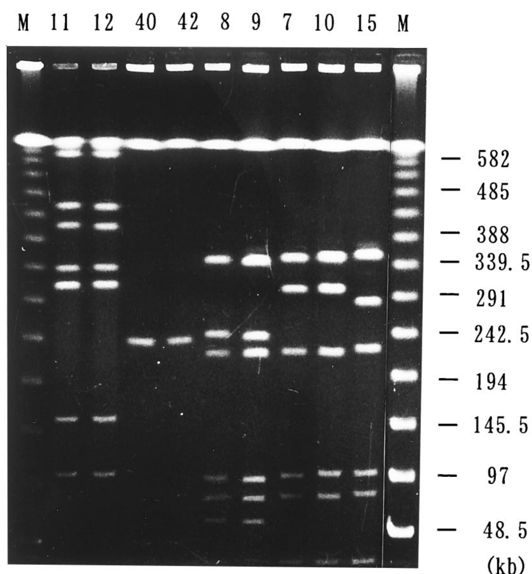

FIG. 1. PFGE patterns of genomic DNAs from *A. baumannii* isolates. The TY numbers of 9 *A. baumannii* isolates are indicated above the lanes. The size markers of the DNA segments are indicated in lanes M, with sizes shown on the right.

We isolated *A. baumannii* strains resistant to multiple β-lactam antibiotics, including IPM and CAZ, and in those strains we detected IPM-hydrolyzing activity and the carbapenemase gene bla_{IMP}.

The bla_{IMP}-bearing strains of *A. baumannii* isolated during a 3-year period in our hospital were typed according to their patterns of resistance to nine drugs and by PFGE analysis of the genome. PFGE pattern analysis has been reported to be the most discriminatory method for the genomic typing of nosocomial *Acinetobacter* spp. (1, 12). The restriction enzyme *Not*I was chosen for digestion of DNA, because the number of cutting sites associated with *Not*I is fewer than that associated with *Apa*I or *Sma*I, simplifying discrimination of digestion patterns.

Among nine isolates, two types each consisted of two strains from the same ward, and the strains had identical resistance and PFGE patterns. They were all isolated from different inpatients, indicating nosocomial transmission. Strains of one type, strains TY40 and TY42, were resistant to six β-lactams, IPM, CAZ, CTX, ATM, AMP, and PIP, as well as two aminoglycosides, AMK and GEN, and a quinolone. The resistance of these strains to all β-lactams except ATM was transmissible, resulting from the transfer of bla_{IMP}, and intermediate resistance to AMK and GEN was transferred concomitantly. Transfer of high-level AMK resistance accompanied by transfer of bla_{IMP} was observed in five other isolates, two of which, TY8 and TY9, were from the same ward and had identical PFGE patterns, although the MICs of PIP and AMK were slightly different. The drug resistance patterns that were transferred from these two strains were identical. They were probably derived from the same strain with some additional changes in the MICs for strains from different hosts.

IPM resistance encoded by bla_{IMP} appeared to be readily lost after prolonged storage in Casitone medium. Furthermore, this resistance was transmissible by conjugation to susceptible strains. These findings strongly suggest that bla_{IMP} is a foreign gene introduced from another species of bacteria and is retained only in environments supplied with IPM.

The frequencies of conjugal transfer of bla_{IMP} were rather high in our study. This was mainly because of the availability of the proper recipient. We used a RIF-resistant mutant of a bla_{IMP}-negative strain, strain TY44, which was a clinical isolate that lost the gene after storage in Casitone medium agar. It was suggested that such a strain like TY44 would be a good recipient for the bla_{IMP} gene.

We attempted to detect plasmid DNA in our isolates, but the physical identification was unsuccessful.

In *Acinetobacter* spp. from various geographic areas in France, dissemination of an AMK resistance gene was reported. The gene was conjugally transmissible in some strains, although the plasmids that carried the AMK resistance gene were not revealed (9).

On the other hand, one transmissible plasmid of 63 kb and another of ca. 45 kb were reported to confer resistance to AMK and IPM, respectively, in *A. baumannii* strains (10, 21). The resistance to IPM was due to a β-lactamase but not a carbapenemase, since hydrolysis of IPM was not demonstrated by enzyme assay but was demonstrated only by microbiological methods.

Acquisition of bla_{IMP} and nosocomial transmission of *A. baumannii* are important considerations for chemotherapy, especially in instances of multidrug resistance to aminoglycosides and quinolones as well as to β-lactams.

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