ing are significant, or when a specific antimicrobial agent is prescribed.

Staphylococcus aureus bacteraemia causes considerable morbidity and mortality in the hospital setting, and strategies to improve the management and outcome of this condition are needed [1,9,10]. Jenkins *et al.* [10,11] reported that mandatory intervention by IDPs improves adherence, although they did not demonstrate a statistically significant improvement in outcomes, possibly as a result of inadequate statistical power.

In the present study, we assessed the impact of 7 years of systematic intervention and report distinct improvements with respect to evaluation, treatment and outcome in patients with *S. aureus* bacteraemia.

Materials and methods

Study setting and population

The study took place at Kyoto University Hospital, which is a 1240-bed tertiary hospital that admits 340 000 patients/year. Approximately 400–600 nosocomial BSIs are treated at this hospital annually and *S. aureus* is the second most common cause (after coagulase-negative staphylococci) accounting for approximately 10% of BSI.

This retrospective cohort study compared the outcomes of all patients with *S. aureus* bacteraemia between the initial and the latter halves of an intervention period. Patients were included if they had proven *S. aureus* bacteraemia, which was defined as at least one *S. aureus*-positive blood culture plus a systemic inflammatory response. Patients with actual re-infection (rather than relapse) could be included several times. Data were excluded from analysis when patient survival or death could not be confirmed by medical records. The study periods comprised initial and later intervention periods from 2002–2005, and from 2006–2008, respectively. Data included age, sex, underlying conditions, location of acquisition, primary focus, timing of antibiotic initiation, complications and survival at 30 days after detection of *S. aureus* in blood cultures.

Intervention

Mandatory intervention began in 2002. Six IDPs from the Department of Infection Control and Prevention at Kyoto University Hospital were immediately informed of a positive blood culture. An IDP immediately assumed responsibility for a patient with clusters of Gram-positive cocci in blood cultures and provided recommendations directly to the attending physician regarding appropriate antibiotic therapy (i.e. choice of drug including glycopeptides, dose regimen and treatment period) and optimal management of the infection. The content and validity of all interventions were discussed at weekly meetings involving all IDPs with an independent assessor. Catheter-related infection was defined if the catheter tip grew $>1 \times 10^3$ colonies of *S. aureus* in the absence of an alternative source of infection. The modified Duke criteria were applied to all suspected cases of infective endocarditis [12,13].

Standards for the management of *S. aureus* bacteraemia

We developed key standards of care to evaluate and manage *S. aureus* bacteraemia, which comprised: obtaining blood for follow-up cultures within 5 days; administration of at least 14 days of antibiotic therapy for the bacteraemia; and investigation for infective endocarditis by echocardiography. Further evaluations, such as radiographic studies, were recommended to the attending physicians if clinical resolution was delayed regardless of appropriate antibiotic therapy. Practical 14- and 28-day regimens for uncomplicated and complicated patients, respectively, were determined on the basis of current guidelines and recent literature [14–16]. However, because the optimal treatment and appropriate classification of *S. aureus* bacteraemia remain undefined, we established 14 days as the minimal duration of therapy.

Assessment of general effects of the mandatory intervention in cases of bacteraemia

We reviewed the numbers of blood cultures received by the laboratory from any patients during the study period and the trends of all consultations recorded by the Department of Infection Control and Prevention in a database, as indicators of general effects on laboratory use and liaison with IDPs. Each consultation record was classified as 'consultation with attending physician', 'significant laboratory results', 'antibiotic prescription' and 'other' when the intervention started.

Statistical analysis

For bivariate analyses, categorical variables were compared by Fisher's exact test and an unpaired *t*-test where appropriate. The cumulative survival time between the day of the first blood culture results that were positive for *S. aureus* and death or the last outpatient clinic visit during the study period was calculated by the Kaplan–Meier method for all patients. The difference in 30-day cumulative survival of patients was tested by the Mantel–Cox test. The potential factors associated with 30-day mortality of patients were examined by the Cox proportional hazards regression analysis. All covariates that differed significantly between the initial intervention period and the later intervention period in the bivariate analysis were considered for model entry into the

above mentioned multivariate analyses. Data were analyzed with PASW for windows, version 18.0 (SPSS Inc., Chicago, IL, USA). All p values were two-tailed and p <0.05 was considered statistically significant.

Results

We reviewed the results from 346 patients with initially *S. aureus*-positive blood cultures. The initial and the latter intervention periods included 194 and 152 patients, respectively. Table 1 shows that the patients' demographic characteristics and comorbidities were generally similar between the two periods, although the proportion of methicillin-resistant *S. aureus* (MRSA) was lower during the later intervention period (56.2% vs. 43.3%; p 0.02), and the proportion of patients who received immunosuppressants was higher during the later period (19.6% vs. 28.9%; p 0.05).

Table 2 compares the two periods in terms of details of the infection process, the clinical management and the 30-day mortality. Echocardiography was applied more frequently (37.1% vs. 64.5%; p <0.001), which led to the discovery of more valvular vegetations (seven vs. ten patients) during the later period. Infective endocarditis or early metastatic infection was identified more frequently (10.8% vs. 20.4%; p 0.01). Follow-up blood samples for culture were obtained more regularly (52.1% vs. 73.7%; p <0.001), and therapy was more frequently administered for at least 14 days (47.4% vs. 82.2%; p <0.001). More patients with MRSA bacteraemia received anti-MRSA drugs (vancomycin, teicoplanin or arbekacin) within 2 days of blood cultures being obtained (64.2% vs. 89.4%; p <0.001).

The number of blood cultures increased annually to 1.7-fold more that that obtained at the beginning of the study period and the number of consultations also increased by approximately 1.6-fold compared to 2002. The growth rate in the number of consultations was higher for 'consultation with attending physicians' than for 'significant laboratory results' (Fig. 1).

The 30-day mortality decreased from 25.8% during the initial intervention period to 16.4% during the later intervention period (p 0.04 by the Mantel–Cox test) (Fig. 2). The results of Cox multivariate regression analysis suggested that appropriate timing of anti-MRSA drug, follow-up blood culture obtained, echocardiogram obtained and later intervention period remained as a predictor for 30-day mortality (Table 3).

Discussion

The present study has demonstrated that the setting up of a system with mandatory involvement of IDPs can improve the management and outcome in patients with *S. aureus* bacteraemia. Although, throughout the period of the investigation, IDPs were informed of cases by the laboratory and were therefore able to intervene with advice, including details of the approved regimen, they do not themselves order investigations or prescribe antibiotics. Therefore, the effectiveness of their mandatory involvement depends on building a relationship of confidence and trust. We consider that this contributed to the improved results obtained in the second period. The detailed evaluation of patients probably increased the recognition of infective endocarditis and metastatic infection [10]. This suggests that instances of endocarditis and metastatic infection remained undiagnosed and were treated as uncomplicated infections before consistent consultation was established. The rate of detected complica-

TABLE 1. **Microbiological characteristics of causative isolates and clinical background of patients with *Staphylococcus aureus* bacteraemia according to intervention period**

	2002–2005 (*n* = 194) Initial intervention period		2006–2008 (*n* = 152) Later intervention period		p
Age	62.1 ± 18.2		63.2 ± 16.8		0.78
Female sex	73	37.6%	63	41.4%	0.50
Risk factor					
Diabetes mellitus	42	21.6%	24	15.8%	0.21
Immunosuppressant	38	19.6%	44	28.9%	0.05
Haemodialysis	12	6.2%	9	5.9%	>0.99
Malignancy	35	18.0%	29	23.6%	0.88
Post transplantation	28	14.4%	13	8.6%	0.97
Hospitalized in intensive care unit	18	9.3%	19	12.5%	0.38
Management provider at time of bacteraemia					
Surgical	100	51.5%	72	47.4%	0.45
Medical	75	38.7%	62	40.8%	0.73
Paediatric	17	8.8%	24	15.8%	0.06
Obstetrics and gynaecology	2	1.0%	3	2.0%	0.65
Methicillin-resistant isolates	109	56.2%	66	43.4%	0.02

TABLE 2. Evaluation and classification of *Staphylococcus aureus* bacteraemia and complications according to intervention period

	2002–2005 (*n* = 194) Initial intervention period		2006–2008 (*n* = 152) Later intervention period		p
Primary source of infection					
Intravascular catheter	64	33.0%	60	39.5%	0.26
Skin and/or soft tissue	42	21.6%	33	21.7%	>0.99
Respiratory tract	9	4.6%	12	7.9%	0.26
Other	10	5.2%	6	3.9%	0.80
Unknown	69	35.6%	41	27.0%	0.10
Infective endocarditis	7	3.6%	10	6.6%	0.22
Metastatic infection	14	7.2%	21	13.8%	0.05
Endocarditis or metastatic infection	21	10.8%	31	20.4%	0.01
Vertebral osteomyelitis	7	3.6%	12	7.9%	0.09
Deep-tissue infection or abscess	2	1.0%	3	2.0%	0.66
Septic pulmonary emboli	3	1.5%	4	2.6%	0.70
Septic arthritis	2	1.0%	2	1.3%	>0.99
Appropriate timing of anti-MRSA drug within 2 days	70/109	64.2%	59/66	89.4%	<0.001
Follow-up blood culture obtained	101	52.1%	112	73.7%	<0.001
Days of therapy ≥ 14	92	47.4%	125	82.2%	<0.001
Echocardiogram obtained	72	37.1%	98	64.5%	<0.001
30-Day mortality	50	25.8%	25	16.4%	0.04
MSSA	14/85	16.5%	11/86	12.8%	0.52
MRSA	36/109	33.0%	14/66	21.2%	0.12

MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-susceptible *Staphylococcus aureus*.

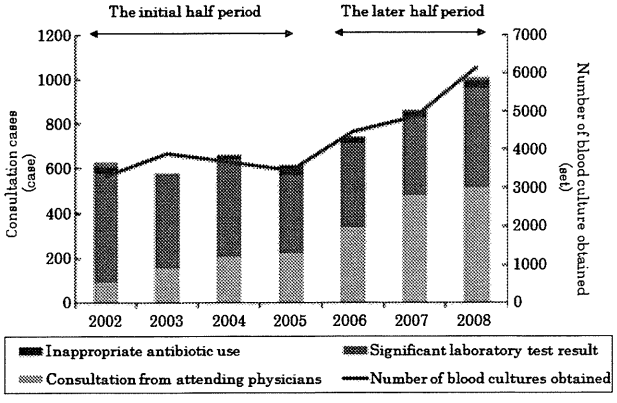

FIG. 1. Trends of characteristics of consultations (bars) and number of blood cultures obtained (solid line). The number of blood cultures increased annually to 1.7-fold more than that obtained at the beginning of the study period, and the number of consultations also increased by approximately 1.6-fold compared to 2002. Growth rate in number of consultations was higher for 'consultation with attending physicians' than for 'significant laboratory results'.

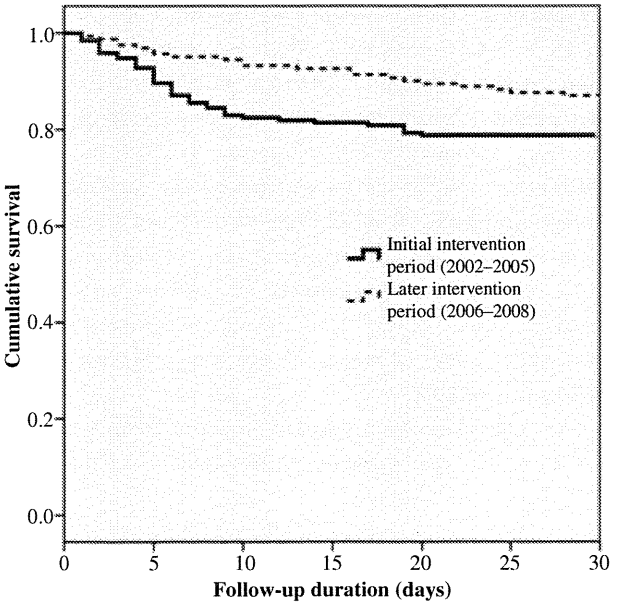

FIG. 2. Kaplain–Meier survival curves (at 30 days) for patients in the first period and the second period (Mantel–Cox test: p 0.04).

tions during the initial intervention period was low. If the actual incidence of complications did not differ during the whole study period, it is likely that one reason for the improved prognosis during the second phase is that more complications were recognized, and thus more patients were appropriately treated during this latter intervention period.

Another reason for the improved prognosis could be the lower proportion of methicillin-resistant isolates. One study has suggested that the mortality of MRSA bacteraemia is higher than that of methicillin-susceptible *S. aureus* (MSSA) bacteraemia [17]. However, the better overall prognosis cannot be fully explained only by a reduction in the numbers of resistant pathogens. In addition, 30-day mortality improved, despite the higher proportion of patients receiving immunosuppressants during the latter period.

The improved outcome in patients with MRSA bacteraemia might be derived from the earlier administration of optimal antibiotics during the second period. Several prospective studies have shown that inadequate antibiotic

TABLE 3. **Predictors of 30-day mortality (Cox multivariate analysis)**

Predictor	Adjusted hazards ratio (95% CI)	p
Pediatrics	0.69 (0.27–1.74)	0.43
Immunosuppressant	1.37 (0.75–2.49)	0.31
Methicillin resistant	1.27 (0.79–2.07)	0.33
Appropriate timing of anti-MRSA drug within 2 days	0.79 (0.31–1.36)	0.08
Follow-up blood culture obtained	1.12 (0.35–1.93)	0.09
Days of therapy ≥ 14	0.57 (0.30–0.98)	0.08
Echocardiogram obtained	0.65 (0.33–1.26)	0.33
Calendar year	0.99 (0.78–1.12)	0.85
Later period (2006–2008)	0.60 (0.30–0.89)	0.02

MRSA, methicillin-resistant *Staphylococcus aureus*.

therapy is a significant risk factor for mortality resulting from *S. aureus* bacteraemia, with inadequate therapy being more frequent in MRSA than in MSSA bacteraemia [18,19]. Lodise *et al.* [20] reported that a delay in administering correct therapy beyond a breakpoint as late as 45 h after obtaining blood cultures is an independent predictor of infection-related mortality. We essentially recommend the use of glycopeptides when Gram stains of positive blood cultures reveal Gram-positive clusters of cocci because over 60% of clinical staphylococcal isolates are methicillin-resistant at our hospital. Aggressive treatment and optimal management strategies are central to the management of *S. aureus* bacteraemia [21,22] and the earlier initiation of anti-MRSA drugs may have substantially contributed to the improved prognosis in the present study. The value of intervention by IDPs can be more readily assessed in patients with staphylococcal bacteraemia as a result of the crtical therapeutic decision to administer glycopeptides in this situation, whereas the impact of IDP intervention on antibiotic use against Gram-negative bacteraemia may be difficult to demonstrate because broad-range antibiotics are empirically administered to many septic patients.

Our policy of active intervention resulted in a general increase in the number of consultations with attending physicians. The significant increase in the number of blood cultures and changes in consultation trends suggest that attending physicians have become more cognisant of the concept of optimal therapies for infectious diseases and the usefulness of IDP advice. The present study demonstrates that the increased acceptance of such an intervention by attending physicians can improve subsequent outcomes of patients with *S. aureus* bacteraemia.

Limitations that are generally inherent in historical cohort studies apply to the present study. A potential confounding effect exists because we compared findings from two consecutive periods, and there may have been other factors influencing the differences observed. However, no major changes such as the introduction of a new anti-MRSA drug for treating *S. aureus* bacteraemia occurred during the study period. We eliminated selection bias by including all patients with *S. aureus* bacteraemia who presented at our hospital during the study. Possible changes in practice that were not evaluated in the present study, such as the management of therapeutic drug monitoring for anti-MRSA drugs or the administration of optimal antimicrobial therapy against concomitant infection as a result of our intervention, might have affected patient prognosis in some way. The effects of such changes, however, could be considered part of the benefit derived from active IDP involvement.

Proactive intervention by IDPs raised awareness of the optimal management of bacteraemia and improved adherence to the standard of care for patients with *S. aureus* bacteraemia, which subsequently resulted in an improved outcome.

Transparency Declaration

All authors report no conflicts of interest relevant to this article.

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AAC(6′)-Iaf, a Novel Aminoglycoside 6′-*N*-Acetyltransferase from Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Isolates[▽]

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We report here the characterization of a novel aminoglycoside resistance gene, *aac(6′)-Iaf*, present in two multidrug-resistant (MDR) *Pseudomonas aeruginosa* clinical isolates. These isolates, IMCJ798 and IMCJ799, were independently obtained from two patients, one with a urinary tract infection and the other with a decubitus ulcer, in a hospital located in the western part of Japan. Although the antibiotic resistance profiles of IMCJ798 and IMCJ799 were similar to that of MDR *P. aeruginosa* IMCJ2.S1, which caused outbreaks in the eastern part of Japan, the pulsed-field gel electrophoresis patterns for these isolates were different from that for IMCJ2.S1. Both IMCJ798 and IMCJ799 were found to contain a novel chromosomal class 1 integron, In123, which included *aac(6′)-Iaf* as the first cassette gene. The encoded protein, AAC(6′)-Iaf, was found to consist of 183 amino acids, with 91 and 87% identity to AAC(6′)-Iq and AAC(6′)-Im, respectively. IMCJ798, IMCJ799, and *Escherichia coli* transformants carrying a plasmid containing the *aac(6′)-Iaf* gene and its upstream region were highly resistant to amikacin, dibekacin, and kanamycin but not to gentamicin. The production of AAC(6′)-Iaf in these strains was confirmed by Western blot analysis. Thin-layer chromatography indicated that AAC(6′)-Iaf is a functional acetyltransferase that specifically modifies the amino groups at the 6′ positions of aminoglycosides. Collectively, these findings indicate that AAC(6′)-Iaf contributes to aminoglycoside resistance.

Pseudomonas aeruginosa is a nosocomial pathogen that exhibits a remarkable ability to acquire resistance to several antibiotics. The most serious problem has been the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains with resistance to all β-lactams, aminoglycosides, and quinolones (39, 40). In Japan, MDR *P. aeruginosa* is defined as having resistance to carbapenem (MIC ≥ 16 μg/ml), amikacin (AMK; MIC ≥ 32 μg/ml), and fluoroquinolone (MIC ≥ 4 μg/ml).

Bacterial resistance to aminoglycosides can result from three causes (44): decreased membrane permeability (13), the modification of 16S RNA (14, 16, 17, 49) or ribosomal proteins (13), and the enzymatic modification of aminoglycosides. In *P. aeruginosa* isolates, resistance to aminoglycosides is due primarily to the production of aminoglycoside-modifying enzymes (4, 47). The aminoglycoside acetyltransferases (AACs) are aminoglycoside-modifying enzymes that transfer acetyl groups to the amino groups of aminoglycosides. The AACs can be grouped into four classes, AAC(1), AAC(2′), AAC(3′), and AAC(6′), based on the acetylation sites of the aminoglycosides (22, 44). N-acetylation at the 6′ position catalyzed by AAC(6′) is one of the most prevalent forms of modification of aminoglycosides (32). AAC(6′)-I confers resistance to AMK but not to gentamicin (GEM) (41). To date, at least 27 AAC(6′)-I enzymes, designated AAC(6′)-Ia to AAC(6′)-Iae, have been identified and characterized (15, 22, 38, 44). In contrast, only two AAC(6′)-II enzymes, which confer resistance to GEM but not to AMK, have been identified (41). The *aac* genes are often found in class 1 integrons (21). These integrons possess two conserved segments at each end, separated by a variable region that includes integrated antibiotic resistance gene cassettes (19, 20). The 5′-conserved segment (5′-CS) contains the *intI* gene, and the 3′-conserved segment (3′-CS) contains the *qacEΔ1* and *sul1* genes (19).

We previously described a nosocomial outbreak of catheter-associated urinary tract infection with an MDR *P. aeruginosa* strain, IMCJ2.S1, in a hospital in the Tohoku region in the eastern part of Japan (39). IMCJ2.S1 was found to harbor an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, in a chromosomal integron. We developed kits to detect the *aac(6′)-Iae* gene and the AAC(6′)-Iae protein and used these kits to survey MDR *P. aeruginosa* strains in hospitals throughout Japan (27, 39). During surveillance in the western part of Japan, two MDR *P. aeruginosa* clinical isolates negative for *aac(6′)-Iae* were identified. Each of these isolates contained a novel aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iaf*. We report here the structure of this gene and the properties of its product.

MATERIALS AND METHODS

Bacterial strains and plasmids. Two *P. aeruginosa* clinical isolates, IMCJ798 and IMCJ799, were individually obtained from two patients, one with a urinary tract infection and the other with a decubitus ulcer. *P. aeruginosa* ATCC 27853 was obtained from the American Type Culture Collection (Manassas, VA) and used as a reference strain for antibiotic susceptibility testing. *Escherichia coli* strains DH5α (Takara Bio, Shiga, Japan) and JM109 (Stratagene, La Jolla, CA) were used as hosts for recombinant plasmids. *E. coli* BL21(DE3)(pLysS) (Invitrogen, Carlsbad, CA) was used for the expression of recombinant *aac(6′)-Iaf*. A rifampin-resistant mutant of *P. aeruginosa*, ATCC 27853 Rfp^r, was used for conjugation. *P. aeruginosa* GN17203, carrying plasmid pMS350 containing bla_{IMP-1} (46), was kindly provided by S. Iyobe (Kitasato University, Sagamihara, Japan).

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TABLE 1. PCR primers used in this study

Primer	Sequence[a] (5′ to 3′)	Label in Fig. 2	Description	Reference
5′-CS	GGCATCCAAGCAGCAAG	B	5′-End common segment of class 1 integrons	29
3′-CS	AAGCAGACTTGACCTGA	F	3′-End common segment of class 1 integrons	29
intI-R	TGCGTGTAAATCATCGTCGT	A	Positions 196–177 in *intI1*	38
qacEdelta-R	GCAATTATGAGCCCCATACC	G	Positions 287–268 in *qacEΔ1*	38
sul1-R	GGGTTTCCGAGAAGGTGATT	H	Positions 787–768 in *sul1*	38
aac(6′)Iaf-F	TTGGACTATTCAATATGCGA	C	Positions 1–20 in *aac(6′)-Iaf*	This study
aac(6′)Iaf-R	CTAGCTAATATCTTTCCACA	D	Positions 552–533 in *aac(6′)-Iaf*	This study
blaIMP-1-F	GAAGTTAACGGGTGGGGCG		Positions 124–142 in bla_{IMP-1}	This study
blaIMP-1-R	CTTTAACCGCCTGCTCTAAT	E	Positions 700–681 in bla_{IMP-1}	This study
16S-rRNA-F	ATGCAAGTCGAGCGGATGAAGGGAG		Positions 55–79 in 16S rRNA gene	This study
16S-rRNA-R	TAGTCGACATCGTTTACGGCGTGGA		Positions 822–798 in 16S rRNA gene	This study
23S-rRNA-F	CGAGGACAGTGTATGGTGGGCAGT		Positions 2207–2231 in 23S rRNA gene	This study
23S-rRNA-R	CTCAACGCCTCACAACGCTTACACA		Positions 2856–2832 in 23S rRNA gene	This study
PstI-aac-F	aactgcagGGCTTGTTATGACTGTTTTT		Sequence in the 185- to 166-bp upstream region of *aac(6′)-Iaf* with PstI site	This study
EcoRI-aac-R	ggaattcCTAGCTAATATCTTTCCACA		Positions 552–533 in *aac(6′)-Iaf* with EcoRI site	This study
SphI-aac-F	aaagcatgcgATGGACTATTCAATATGCGA		Positions 1–20 in *aac(6′)-Iaf* with SphI[b]	This study
PstI-aac-R	aactgcagCTAGCTAATATCTTTCCACA		Positions 552–533 in *aac(6′)-Iaf* with PstI site	This study

[a] Lowercase letters represent restriction enzyme recognition sites attached on the 5′ ends of primers.
[b] The initiation codon TTG in *aac(6′)-Iaf* was replaced with ATG.

Antimicrobial agents. Amikacin (AMK) and imipenem (IPM) were obtained from Banyu Pharmaceutical Co. (Tokyo, Japan), arbekacin (ABK) and dibekacin (DIB) were purchased from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), aztreonam (ATM) was obtained from Eizai (Tokyo, Japan), ceftazidime (CAZ) was acquired from GlaxoSmithKline K.K. (Tokyo, Japan), gentamicin (GEM) and neomycin B and C mixtures (NEO) were obtained from Nacalai Tesque, Inc. (Kyoto, Japan), isepamicin (ISP), netilmicin (NET), and sisomicin (SIS) were from Schering-Plough K.K. (Osaka, Japan), kanamycin A (KAN) and polymyxin B (PMB) were purchased from Sigma-Aldrich (St. Louis, MO), meropenem (MEM) was obtained from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), ofloxacin (OFX) was acquired from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), piperacillin (PIP) and piperacillin-tazobactam (TZP) were obtained from Tomiyama Pure Chemical Industries, Ltd. (Tokyo, Japan), and tobramycin (TOB) was purchased from Towa Pharmaceutical Co., Ltd. (Osaka, Japan).

In vitro susceptibility tests. MICs were determined using a microdilution method according to the protocols recommended by the Clinical and Laboratory Standards Institute (10).

Serotyping. The O serotypes of isolates were determined with a slide agglutination test kit (Denka Seiken Co., Tokyo, Japan).

Detection of MDR *P. aeruginosa* using a LAMP method and an agglutination test. The *aac(6′)-Iae* gene was assessed using a loop-mediated isothermal amplification (LAMP) method, and the AAC(6′)-Iae protein was evaluated using an agglutination test, as described previously (39).

PFGE. DNA plugs were prepared as described previously (18) and digested overnight at 37°C with SpeI and XbaI (Takara Bio). Pulsed-field gel electrophoresis (PFGE) analysis was performed as described previously (38).

PCR amplification of class 1 integrons. Genomic DNA was extracted as described previously (36) and used as PCR templates. Class 1 integrons were detected by PCR using 5′-CS and 3′-CS primers as described previously (11, 29) and genetically mapped using the primers listed in Table 1. An Expand high-fidelity PCR system (Roche Diagnostics GmbH, Penzberg, Germany) was used for all PCR amplifications. All PCR products were sequenced to identify genes and their orders in the integrons.

DNA sequencing. DNA sequences were determined using an ABI PRISM 3100 sequencer (Applied Biosystems). Homology searches of nucleotide and translated protein sequences were performed using BLAST (2, 3). Multiple-sequence alignments and searches for open reading frames (ORFs) were performed using the Clustal W2 program (28) and GENETYX software (Genetyx, Tokyo, Japan). The dendrogram for AACs was determined with the Clustal W2 program (28).

Plasmid extraction. The methods of Kado and Liu (25) and Casse et al. (8), modified as follows, were used to extract plasmid DNA from *P. aeruginosa*. The bacterial pellet was lysed by the addition of 2 ml of lysis buffer (50 mM Tris-Cl, 20 mM EDTA, 4% sodium dodecyl sulfate [SDS], pH 12.6), followed by gentle shaking for 30 min at 37°C. The lysate was neutralized by adding 400 μl of 1 M Tris-Cl (pH 7.5), and the proteins were precipitated by adding 250 μl of 5 M NaCl. The solution was extracted with an equal volume of phenol-chloroform solution (1:1, vol/vol). The plasmid DNA in the aqueous phase was precipitated by adding a twofold volume of 100% ethanol. The DNA pellet was collected. Plasmid DNA preparations were analyzed by electrophoresis on 0.7% agarose gels in 0.5× Tris-borate-EDTA buffer at 4°C.

Transformation using plasmid preparations from *P. aeruginosa* IMCJ798 and IMCJ799. Plasmid preparations from *P. aeruginosa* strains were used to transform *E. coli* DH5α and *P. aeruginosa* PAO1 by electroporation using a Gene Pulser Xcell system (Bio-Rad Laboratories, Hercules, CA). The transformants were cultured on Luria-Bertani (LB) agar plates containing 20 μg/ml AMK for 24 h at 37°C.

Transfer of aminoglycoside resistance. Drug resistance was transferred from *P. aeruginosa* clinical isolates to a rifampin-resistant mutant of *P. aeruginosa*, ATCC 27853 Rfp^r, using the broth mating method (26). The transconjugants were selected on Mueller-Hinton agar plates containing rifampin (200 μg/ml) and IPM (16 μg/ml) or AMK (20 μg/ml).

Genome typing by I-CeuI digestion and Southern blot hybridization. DNA plugs containing total genomic DNA from isolates were digested overnight with I-CeuI. DNA fragments were separated by PFGE. Southern hybridization was performed using an enhanced chemiluminescence direct nucleic acid-labeling and detection system according to the instructions of the manufacturer (GE Healthcare, Tokyo, Japan), as described previously (24, 30, 34), to determine whether the novel class 1 integron identified in the *P. aeruginosa* isolates, designated In123, has a chromosomal location. Probes for *aac(6′)-Iaf*, bla_{IMP-1}, 16S rRNA, and 23S rRNA genes from IMCJ798 were amplified by PCR using the primer sets aac(6′)Iaf-F/aac(6′)Iaf-R, blaIMP-1-F/blaIMP-1-R, 16S-rRNA-F/16S-rRNA-R, and 23S-rRNA-F/23S-rRNA-R, respectively (Table 1).

Cloning of *aac(6′)-Iaf* gene. The ORF of *aac(6′)-Iaf* and 185 bp of the upstream region of the gene, which includes the promoter, were PCR amplified from *P. aeruginosa* IMCJ798 by using the primer set PstI-aac-F and EcoRI-aac-R (Table 1). The PCR products were digested with EcoRI and PstI and ligated into the PstI and EcoRI sites of pSTV28, at a polarity opposite the transcriptional direction of the promoter on the vector. The plasmids were used to transform DH5α, and transformants were selected on LB agar containing 30 μg/ml of chloramphenicol. The resulting plasmid was designated pSTV-*aac*WT. To determine MICs, *E. coli* JM109 was transformed with pSTV-*aac*WT, which represses transcription driven by the promoter on the pSTV28 vector.

Site-directed mutagenesis. The putative initiation codon on pSTV-*aac*WT, TTG, was replaced by ATG by using a QuikChange site-directed mutagenesis kit (Stratagene). The resulting plasmid was designated pSTV-*aac*(TTG→ATG). To determine MICs, *E. coli* JM109 was transformed with this plasmid.

Construction of AAC(6′)-Iaf-overexpressing strains. The *aac(6′)-Iaf* gene from *P. aeruginosa* IMCJ798 was PCR amplified using the primer set SphI-aac-F and PstI-aac-R (Table 1), and the product was digested with SphI and PstI and

TABLE 2. Antimicrobial susceptibility parameters of IMCJ798, IMCJ799, IMCJ2.S1, and ATCC 27853 for various antibiotics[a]

Isolate name	MIC (μg/ml) of:										
	PIP	TZP	CAZ	IPM	MEM	ATM	AMK	ABK	GEM	OFX	PMB
IMCJ798	256	256	512	128	>512	64	128	8	4	>128	4
IMCJ799	256	256	512	128	>512	64	128	16	2	>128	4
IMCJ2.S1	256	256	512	128	512	128	128	2	16	128	2
ATCC 27853	<4	4	<1	4	1	2	2	<0.5	<1	<0.5	2

[a] PIP, piperacillin; TZP, piperacillin-tazobactam; CAZ, ceftazidime; IPM, imipenem; MEM, meropenem; ATM, aztreonam; AMK, amikacin; ABK, arbekacin; GEM, gentamicin; OFX, ofloxacin; PMB, polymyxin B.

ligated into pQE2 (Invitrogen), which had been digested with the same restriction enzymes. The plasmid was used to transform DH5α, and the transformants were selected on LB agar containing 100 μg/ml of ampicillin. The resulting plasmid, pQE-*aac(6′)-Iaf*, was used to transform *E. coli* BL21(DE3)(pLys), which was used for recombinant protein purification.

Purification of recombinant AAC(6′)-Iaf. *E. coli* BL21(DE3)(pLysS) carrying plasmid pQE2-*aac(6′)-Iaf* was grown in LB medium containing 200 μg/ml ampicillin at 37°C until the A_{600} reached 0.3. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to a concentration of 0.1 mM to induce the expression of AAC(6′)-Iaf, and the culture was incubated for 4 h at 37°C. The hexahistidine-tagged AAC(6′)-Iaf was purified from the soluble fraction using Ni-nitrilotriacetic acid agarose according to the instructions of the manufacturer (Qiagen, Tokyo, Japan). The final concentration of protein was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Purification of native AAC(6′)-Iaf from *P. aeruginosa*. Rabbits were immunized with recombinant AAC(6′)-Iaf protein emulsified in Freund's adjuvant. The animal experiments were approved by the ethical committee for animal experiments at the Research Institute of the International Medical Center of Japan. Anti-AAC(6′)-Iaf immunoglobulin G (IgG), purified from the rabbit sera on protein G-Sepharose (GE Healthcare), was coupled to NHS-activated Sepharose according to the instructions of the manufacturer (GE Healthcare). Bacterial cells from overnight cultures of *P. aeruginosa* IMCJ798 were disrupted by sonication, and the cleared lysate was applied to the IgG-coupled Sepharose column. After the column was washed with phosphate-buffered saline containing 0.05% Tween 20, protein was eluted with 0.1 M glycine-HCl (pH 2.5). The purified protein was dialyzed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 8.0) and separated by SDS–15% polyacrylamide gel electrophoresis (SDS–15% PAGE), and the N-terminal sequence was analyzed by a commercial service (Nippi, Inc., Tokyo, Japan).

Western blotting. *E. coli* JM109 bacteria carrying pSTV28, pSTV-*aac*WT, or pSTV-*aac*(TTG→ATG) were cultivated for 16 h at 37°C in LB broth containing 30 μg/ml chloramphenicol. *P. aeruginosa* isolates IMCJ798 and IMCJ799 were cultivated for 16 h at 37°C in LB broth containing 20 μg/ml AMK. One milliliter of each culture was collected by centrifugation, and whole-cell lysates in 200 μl of SDS-PAGE sample buffer were prepared. A 5-μl aliquot of each cell lysate was separated on an SDS–15% PAGE gel, and the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in a mixture of 20 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 and incubated with rabbit polyclonal anti-AAC(6′)-Iaf antibodies, obtained by immunization with His–AAC(6′)-Iaf. After the incubation of the membranes with secondary horseradish peroxidase-linked anti-rabbit IgG (GE Healthcare), bands were detected by chemiluminescence. The intensity of each band was quantified using Quantity One software (Bio-Rad Laboratories).

TLC analysis of acetylated aminoglycosides. Mixtures containing 2 mM aminoglycoside, 2 mM acetyl coenzyme A (acetyl-CoA), and 50 μg/ml AAC(6′)-Iaf in 20 μl of phosphate buffer (pH 7.4) were incubated for 16 h at 37°C, 3 μl of each aminoglycoside mixture was spotted onto the surface of a silica gel 60 thin-layer chromatography (TLC) plate containing a fluorescence indicator with a 254-nm excitation wavelength (Merck Ltd., Japan), and the results were developed with 5% phosphate potassium solution. The aminoglycosides and their acetylated products were detected with 0.5% ninhydrin in acetone (50).

Nucleotide sequence accession number. The nucleotide sequence of In123 determined in this study has been deposited in the EMBL and GenBank databases and the DDBJ and assigned accession number AB462903.

RESULTS AND DISCUSSION

Characterization of *P. aeruginosa* IMCJ798 and IMCJ799. We obtained two *P. aeruginosa* clinical isolates, IMCJ798 and IMCJ799, from two patients, one with a urinary tract infection and the other with a decubitus ulcer, from a hospital in the Chugoku region of western Japan in 2007. After the implementation of various infection control measures, no other patients with such infections were detected.

The genotypic and phenotypic properties of IMCJ798 and IMCJ799 were compared with those of the MDR *P. aeruginosa* strain IMCJ2.S1, which had been reported previously to be found in Japan (38). The MICs for *P. aeruginosa* IMCJ798, IMCJ799, IMCJ2.S1, and ATCC 27853 are shown in Table 2. Multidrug resistance phenotypes were observed in IMCJ798 and IMCJ799. These isolates were resistant to all antibiotics except for GEM. In particular, they showed high levels of resistance to β-lactams, AMK, and OFX. These results were similar to those for IMCJ2.S1, except for ABK and GEM (Table 2). IMCJ798, IMCJ799, and IMCJ2.S1 also had the same serotype, O:11. Although IMCJ798 and IMCJ799 seemed to be derived from IMCJ2.S1, both were negative for the *aac(6′)-Iae* gene by the LAMP method (data not shown) and for AAC(6′)-Iae protein by the agglutination test (data not shown), whereas IMCJ2.S1 was positive for *aac(6′)-Iae* (39). The PFGE patterns of SpeI- and XbaI-digested fragments from the IMCJ798 and IMCJ799 isolates were identical but differed from those of fragments from IMCJ2.S1 (Fig. 1). The PFGE patterns for IMCJ798 and IMCJ799 showed similarities of 56.4% (SpeI) and 70.5% (XbaI), respectively, to that for IMCJ2.S1. Thus, the genotypic properties of IMCJ798 and IMCJ799 differed from those of IMCJ2.S1, although these strains had similar phenotypes. Further nationwide, hospital-based surveillance of MDR *P. aeruginosa* is required.

***aac(6′)-Iaf* in the class 1 integron.** To identify the drug resistance genes of IMCJ798 and IMCJ799, the variable regions of class 1 integrons were amplified with primers 5′-CS and 3′-CS (Table 1). Amplicons of 1.7 kbp generated from both strains were found to be identical by DNA sequencing. Sequence analysis revealed a variable region containing two cassettes, one carrying a novel *aac(6′)* gene and the other carrying a bla_{IMP-1} metallo-β-lactamase gene (Fig. 2). The novel *aac(6′)* gene comprised an ORF of 552 bp, starting with a TTG codon, and its sequence showed 94 and 91% identity to those of *aac(6′)-Iq* from *Klebsiella pneumoniae* (9) and *aac(6′)-Im* from *Citrobacter freundii* (23). Based on the standard nomenclature (45), we named this ORF *aac(6′)-Iaf*.

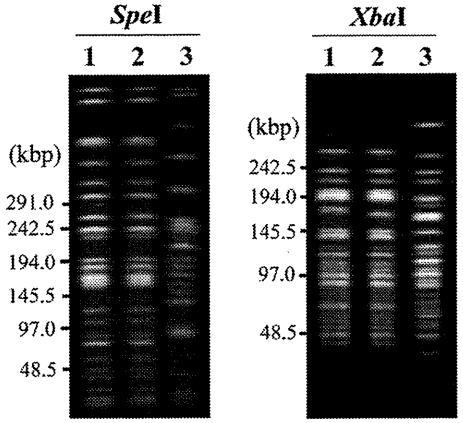

FIG. 1. PFGE patterns for SpeI- and XbaI-digested genomic DNA from MDR *P. aeruginosa* strains IMCJ798 and IMCJ799. The DNA fragments were detected by ethidium bromide staining. Results for IMCJ798 (lanes 1), IMCJ799 (lanes 2), and IMCJ2.S1 (lanes 3) are shown.

The 5′ CS and 3′ CS of the integron were further mapped with PCR cartography using external primers (Table 1; Fig. 2). Typical 59-base elements (42) were observed in both cassettes. These results supported the idea that the *aac(6′)-Iaf* gene in *P. aeruginosa* IMCJ798 and IMCJ799 is localized within the class 1 integron. The sequence of the integron was not found in any database; we therefore named the integron In123.

In addition, the *aac(6′)-Iaf* gene has a G+C content of 34.4%; in contrast, the average G+C contents of the *P. aeruginosa* PAO1 and *K. pneumoniae* MGH78578 genomes are 66.6 and 57.1%, respectively (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi?view=1). These findings suggested that *aac(6′)-Iaf* may be derived from species with intrinsically low G+C contents, not from *Pseudomonas* or *Klebsiella* species.

Location of In123. Class 1 integrons are frequently located on plasmids, and they can be transferred among bacteria (5). Plasmid preparation, transformation, conjugation, and Southern hybridization using genomic DNA digested by I-CeuI were carried out to determine the locations and transmission ability of In123 in IMCJ798 and IMCJ799. *P. aeruginosa* GN17203, which harbors pMS350 containing bla_{IMP-1}, was used as the positive control (46). Initially, we prepared plasmid DNA as described in Materials and Methods. No plasmid in IMCJ798 or IMCJ799 was detected by electrophoresis, whereas pMS350 was detected in *P. aeruginosa* GN17203 (data not shown). *E. coli* DH5α and *P. aeruginosa* PAO1 were transformed with the plasmid DNA preparations by electroporation. No transformants were obtained on LB agar plates containing AMK. In conjugation tests using *P. aeruginosa* ATCC 27853 Rfp^r as a recipient strain, the AMK resistance was not transferred from

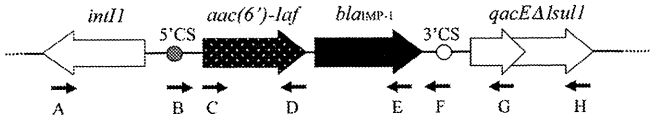

FIG. 2. Genetic structure of In123. Primers labeled A, B, C, D, E, F, G, and H are described in Table 1. Arrows indicate primer locations and directions.

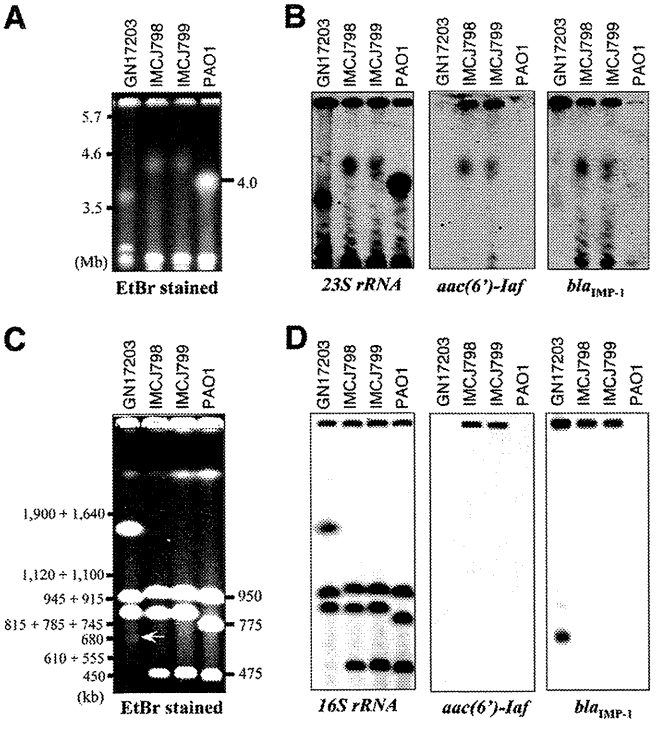

FIG. 3. PFGE patterns (A and C) and Southern hybridization analyses (B and D) of *P. aeruginosa* genomic DNA digested by I-CeuI. PFGE analysis of *P. aeruginosa* genomic DNA digested with I-CeuI was done under the following two different sets of conditions: condition set 1 for the separation of the largest, 4.0-Mb fragment of PAO1 (A) consisted of a 106° angle, 0.8% agarose, and linear switching times of 20 to 30 min for 48 h with a voltage gradient of 2 V/cm, and condition set 2 for the separation of the 950-, 775-, and 475-kb fragments of PAO1 (C) consisted of a 120° angle, 1% agarose, and nonlinear switching times of 5.3 to 120 s for 19.5 h with a voltage gradient of 6 V/cm. The molecular standards were *Schizosaccharomyces pombe* (A) and *Saccharomyces cerevisiae* YPH80 (C). An arrow in panel C indicates the location of an extrachromosomal band that may correspond to pMS350. DNA fragments for which results are shown in panels A and C were transferred onto membranes and were used for the hybridization analyses presented in panels B and D, respectively. Southern hybridization was performed with probes for rRNA genes, *aac(6′)-Iaf*, and bla_{IMP-1}, as shown in panels B and D. EtBr, ethidium bromide.

IMCJ798 and IMCJ799 to *P. aeruginosa* ATCC 27853 Rfp^r whereas carbapenem resistance was transferred from *P. aeruginosa* GN17203 to ATCC 27853 Rfp^r. In order to confirm that In123 is located on the chromosome, PFGE analysis and Southern hybridizations using *P. aeruginosa* genomic DNA digested by I-CeuI were performed. In all strains, four chromosomal fragments of various sizes (PAO1, 4,063, 950, 775, and 475 kb; GN17203, ca. 3,600, 1,500, 945, and 900 kb; and IMCJ798 and IMCJ799, ca. 4,500, 950, 900, and 480 kb) were detected by the rRNA gene probes (Fig. 3A and C and left panels in B and D). The *aac(6′)-Iaf* probe detected the 4,500-kb fragments from IMCJ798 and IMCJ799. The band hybridized by the *aac(6′)-Iaf* probe was also recognized by the rRNA gene probe. The bla_{IMP-1} probe detected the same fragments in the IMCJ798 and IMCJ799 clinical isolates as the *aac(6′)-Iaf* and rRNA gene probes. Additionally, the bla_{IMP-1} probe detected a 700-kbp extrachromosomal fragment, which may correspond to pMS350 in GN17203 (46), that was not

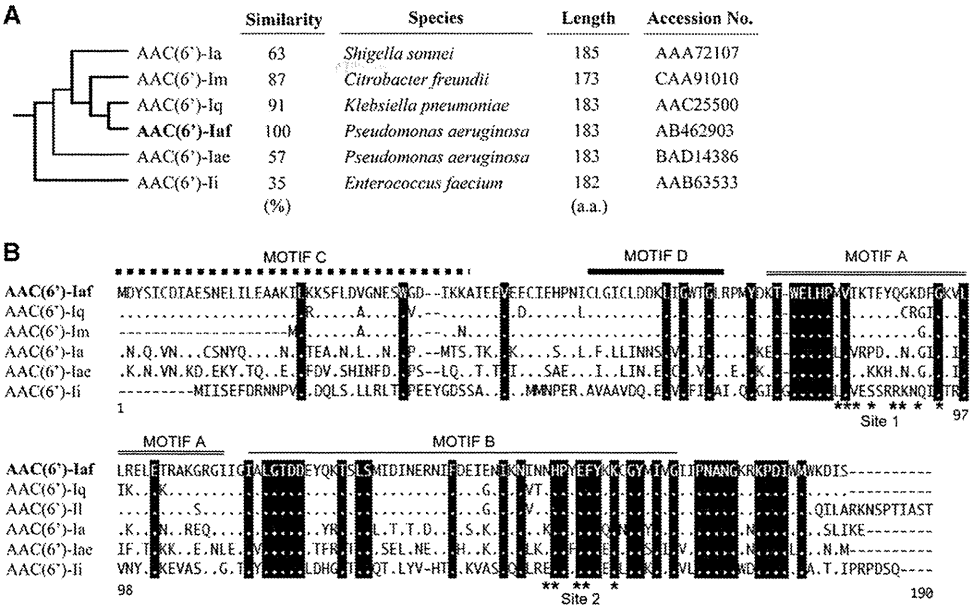

FIG. 4. Phylogenic tree and amino acid sequence alignments for the AAC(6′)-I subfamily. (A) Phylogenic relationships were determined with the Clustal W2 program. aa, amino acids. (B) Dots indicate amino acids identical to those of AAC(6′)-Iaf. Black-highlighted amino acids are conserved among this AAC(6′)-I subfamily. Dashes represent gaps introduced to optimize similarity.

detected by the rRNA gene probe. Another smaller bla_{IMP-1} probe-specific band detected in the IMCJ798 and IMCJ799 clinical isolates, as shown in Fig. 3B, was not observed in the analysis presented in Fig. 3D, probably due to differences in electrophoretic conditions (see the legend to Fig. 3). It is likely that this band was not resolved under the conditions used in the analyses presented in Fig. 3C and D.

Collectively, these results strongly suggest that In123, which carries *aac(6′)-Iaf*, is located on a chromosome, not on a plasmid, in *P. aeruginosa* IMCJ798 and IMCJ799. This arrangement is similar to those for other class 1 integrons, including *aac(3)-Ib*, *aac(3)-Ic*, and *aac(6′)-Iae* integrons in *P. aeruginosa* (35, 37) and the *aac(3)-Id* integron in *Vibrio fluvialis* (1).

Comparison of AAC(6′)-Iaf with other AAC(6′)-I enzymes. AAC(6′)-Iaf, encoded by the first cassette gene in In123, consists of 183 amino acids. The amino acid sequence of AAC(6′)-Iaf was compared to those of other AAC(6′)-I enzymes. The deduced molecular phylogeny of these sequences suggests that all the AAC(6′)-I enzymes can be classified into three subfamilies (44), the first containing AAC(6′)-Ib and AAC(6′)-Ie, the second containing AAC(6′)-Ic, AAC(6′)-Id, and AAC(6′)-Ih, and the third containing AAC(6′)-Ia, AAC(6′)-Iae, and AAC(6′)-Iq. It was found that AAC(6′)-Iaf belonged to the third subfamily, whose members show considerable phylogenetic distance from those of the other two subfamilies, which include AAC(6′)-Ib or AAC(6′)-Iad (15, 38, 44). Using multiple-sequence alignments, AAC(6′)-Iaf was found to have 91, 87, 63, 57, and 35% identity to AAC(6′)-Iq from *K. pneumoniae* (9), AAC(6′)-Im from *C. freundii* (23), AAC(6′)-Ia from *Shigella sonnei* (43), AAC(6′)-Iae from *P. aeruginosa* (38), and AAC(6′)-Ii from *Enterococcus faecium* (12), respectively (Fig. 4A). Moreover, four motifs (C, D, A, and B) of GCN5-related *N*-acetyltransferases (33) were also observed in AAC(6′)-Iaf, as well as most other AAC(6′)-I enzymes (Fig. 4B). Additionally, the crystal structure of AAC(6′)-Ii, which also belongs to the third subfamily, has been resolved, and two acetyl-CoA binding sites have been reported (6, 7). Putative sites required for acetyl-CoA binding, sites 1 and 2, were also found in AAC(6′)-Iaf (Fig. 4B).

Effects of *aac(6′)-Iaf* on aminoglycoside resistance. Both *P. aeruginosa* IMCJ798 and IMCJ799 were resistant to AMK, DIB, ISP, KAN, NET, and TOB but were sensitive to GEM

TABLE 3. MICs of various aminoglycosides for *P. aeruginosa* strains and *E. coli* strains transformed with *aac(6′)-Iaf*

Strain[a]	MIC[b] (μg/ml) of:									
	AMK	ABK	DIB	GEM	ISP	KAN	NET	SIS	TOB	NEO
P. aeruginosa IMCJ798	128	8	>128	4	>128	>128	>128	32	32	8
P. aeruginosa IMCJ799	128	16	>128	2	>128	>128	>128	32	32	8
E. coli JM109/pSTV28	1	2	1	0.5	1	2	0.5	0.5	0.125	2
E. coli JM109/pSTV-*aac*WT	16	4	16	1	4	64	8	1	4	4
E. coli JM109/pSTV-*aac*(TTG→ATG)	32	4	32	1	16	128	8	2	4	4

[a] The MICs for *E. coli* strains were determined with Mueller-Hinton broth preparations containing chloramphenicol (30 μg/ml) and individual aminoglycosides.
[b] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEM, gentamicin; ISP, isopamicin; KAN, kanamycin; NET, netilmicin; SIS, sisomicin; TOB, tobramycin; NEO, neomycin.

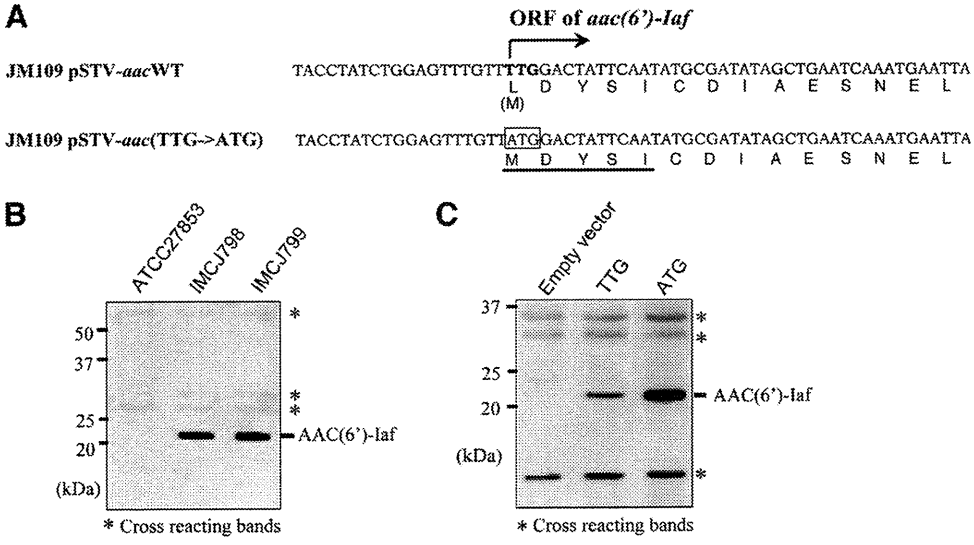

FIG. 5. Production of AAC(6′)-Iaf in *P. aeruginosa* clinical isolates and *E. coli* transformants used in aminoglycoside susceptibility tests. Each lysate was separated by SDS–15% PAGE and analyzed by Western blotting using polyclonal anti-AAC(6′)-Iaf antibodies. The positions of nonspecifically reacting protein bands serving as loading controls are indicated by asterisks. (A) Partial sequences of the *aac(6′)-Iaf* gene and its promoter region on pSTV-*aac*WT and pSTV-*aac*(TTG→ATG) and of the corresponding peptides. The putative wild-type initiation codon is shown in boldface type. The initiation codon altered to ATG is boxed. The N-terminal sequence of native AAC(6′)-Iaf, as determined by the Edman degradation method, is underlined. (B) Expression of AAC(6′)-Iaf in *P. aeruginosa* clinical isolates IMCJ798 and IMCJ799. (C) Expression of AAC(6′)-Iaf in *E. coli* JM109 carrying pSTV28 (lane 1; empty vector), pSTV-*aac*WT (lane 2; TTG), and pSTV-*aac*(TTG→ATG) (lane 3; ATG).

(Table 3). To determine whether *aac(6′)-Iaf* mediates aminoglycoside resistance, the MICs of various aminoglycosides were assessed using *E. coli* JM109 transformants carrying pSTV-*aac*WT or pSTV-*aac*(TTG→ATG). pSTV-*aac*WT includes both *aac(6′)-Iaf* and its 185-bp upstream region from IMCJ798. By using *E. coli* JM109, the *aac(6′)-Iaf* gene was expressed utilizing its native promoter and initiation codon. In pSTV-*aac*(TTG→ATG), the putative initiation codon TTG was replaced with ATG, as shown in the nucleotide sequences in Fig. 5A, to solve the problem of low-level expression in *E. coli* caused by a rare initiation codon, as described previously (9, 31). As shown in Table 3, the aminoglycoside resistance profile for *E. coli* JM109 carrying pSTV-*aac*WT or pSTV-*aac*(TTG→ATG) was correlated with those for IMCJ798 and IMCJ799. For the *E. coli* transformants, effective increases of MICs of the same aminoglycosides to which IMCJ798 and IMCJ799 exhibited resistance, AMK, DIB, ISP, KAN, NET, and TOB, were observed. Furthermore, MICs of five of the six above-listed aminoglycosides increased more than twofold for JM109 carrying pSTV-*aac*(TTG→ATG) compared those for JM109 carrying pSTV-*aac*WT. To confirm whether the initiation codon of *aac(6′)-Iaf* is TTG, N-terminal sequencing of the native AAC(6′)-Iaf protein was performed. The native protein was purified from IMCJ798 by using an affinity column with polyclonal anti-AAC(6′)-Iaf rabbit IgG coupled to Sepharose. The molecular mass of purified native AAC(6′)-Iaf, as determined by 15% PAGE, corresponded to the estimated size of approximately 21 kDa. The N-terminal sequence of AAC(6′)-Iaf was found to be MDYSI (Fig. 5A). Additionally, AAC(6′)-Iaf production in IMCJ798, IMCJ799, and *E. coli* JM109 transformants was confirmed by Western blot analysis using polyclonal anti-AAC(6′)-Iaf IgG (Fig. 5B and C). The production level of AAC(6′)-Iaf in JM109 carrying pSTV-*aac*(TTG→ATG) increased 2.4-fold compared to that in JM109 carrying pSTV-*aac*WT, whereas the intensities of cross-reacting protein bands were unchanged (Fig. 5B). This increase was similar to results observed previously for the 6-hydroxy-D-nicotine oxidase gene carrying TTG as an initiation codon (31). These results demonstrate that the ORF of *aac(6′)-Iaf* initiates from TTG and that *aac(6′)-Iaf* plays a crucial role in aminoglycoside resistance in *P. aeruginosa* IMCJ798 and IMCJ799.

Acetylation activity of AAC(6′)-Iaf. To examine the biochemical properties of AAC(6′)-Iaf in aminoglycoside resistance, acetylation activities against various aminoglycosides were assayed by TLC using native AAC(6′)-Iaf. TLC analyses showed that all aminoglycosides with an amino group at the 6′ position were acetylated by native AAC(6′)-Iaf in the presence of acetyl-CoA (Fig. 6). Commercially available GEM is a mixture of derivatives of GEM, such as GEM C1, C1a, C2, and

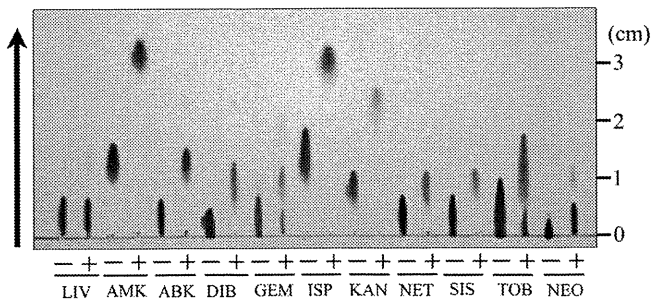

FIG. 6. Analysis of acetylated aminoglycosides by TLC. Native AAC(6′)-Iaf and various aminoglycosides were incubated in the absence (−) or presence (+) of acetyl-CoA. LIV, lividomycin A. The arrow indicates the direction of development.

C2b (41, 44). NEO also consists of derivatives of NEO B and C. In these two reagent mixtures, partially acetylated reagents were observed. Surprisingly, lividomycin A, which has a hydroxyl group at the 6′ position, was also a substrate for AAC(6′)-Iaf, although only an extremely small amount of AAC activity was detected. This partial acetylation of lividomycin A suggests that AAC(6′)-Iaf may have acetylation activities for an alternate amino group in the aminoglycoside molecule. These findings indicate that *aac(6′)-Iaf* encodes a functional aminoglycoside 6′-*N*-acetyltransferase that effectively modifies the amino groups at the 6′ positions of aminoglycosides in vitro. However, *E. coli* JM109 carrying pSTV-*aac*WT, expressing exogenous AAC(6′)-Iaf, did not show reduced susceptibility to ABK, GEM, or NEO (Table 3). *E. faecium* producing AAC(6′)-Ii is susceptible to NEO even though AAC(6′)-Ii acetylates NEO (48). ABK and NEO were shown previously to retain their antibiotic effects on an ABK-resistant actinomycete strain, even after they were acetylated at the 6′ positions by AAC(6′) enzymes (50). These results suggest that the acetylation of ABK and NEO at the 6′ positions does not affect the antimicrobial activities of these drugs. The antimicrobial activity retained after treatment with AAC(6′)-Iaf may be due to residual unacetylated ABK or NEO. GEM derivatives C1 and C2b carry methyl groups at the 6′ positions, and they may be refractory to AAC(6′)-I enzymes. Further work is needed to determine the detailed biochemical properties of AAC(6′)-Iaf.

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Development of an immunochromatographic assay for the rapid detection of AAC(6′)-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*

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Objectives: To develop an easy-to-use method for the rapid detection of antibiotic-resistant bacteria. Here, a new immunochromatographic assay specific for aminoglycoside 6′-*N*-acetyltransferase AAC(6′)-Iae was designed. AAC(6′)-Iae is a significant marker molecule for multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates in Japan.

Methods: Monoclonal antibodies specific for AAC(6′)-Iae were used to construct the assay. The assessment of the assay was performed using 116 *P. aeruginosa* clinical isolates obtained from hospitals in the Kanto area of Japan where little was known about AAC(6′)-Iae producers. PCR analyses of the *aac(6′)-Iae* and class 1 integron, antimicrobial susceptibility testing and PFGE analysis were performed to characterize positive strains.

Results: The detection limit of the assay was 1.0×10^5 cfu. Of 116 clinical isolates, 60 were positive for AAC(6′)-Iae using the assay. The results of assessment with clinical isolates were fully consistent with those of *aac(6′)-Iae* PCR analyses, showing no false positives or negatives. All positive strains detected by the assay showed MDR phenotypes that were resistant to several classes of antibiotic. PFGE analysis showed that 59 of 60 positive strains tightly clustered, and these included clonal expansions.

Conclusions: The developed assay is an easy-to-use and reliable detection method for AAC(6′)-Iae-producing MDR *P. aeruginosa*. This approach may be applicable for screening and investigation of antibiotic-resistant bacteria as an alternative to PCR analysis.

Keywords: aminoglycoside 6′-*N*-acetyltransferase, molecular epidemiology, rapid diagnosis

Introduction

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* often cause nosocomial outbreaks, and result in life-threatening infections in compromised patients. Hence, the rapid detection of such bacteria is crucial to early infection control to prevent nosocomial infection.

Patterns of bacterial antibiotic resistance are becoming more complex with multiple mechanisms.[1] In *P. aeruginosa* isolates, antibiotic resistance is often due to the production of exogenous enzymes including antibiotic-modifying or -degrading enzymes. Most of these enzyme genes are found in class 1 integrons,[2] and they complicate bacterial antibiotic resistance. Integrons can be transmitted between bacteria via plasmids and transposons; integron carriers therefore often lead to nosocomial outbreaks.

Previously, we identified an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, from MDR *P. aeruginosa* IMCJ2.S1.[3] Subsequent studies have revealed that *aac(6′)-Iae*-carrying MDR *P. aeruginosa* were isolated in Miyagi, Tokyo and Hiroshima.[3–5] Given that all identified *aac(6′)-Iae* have been linked with bla_{IMP} and *aadA1* in integron In113, AAC(6′)-Iae (responsible for amikacin resistance) might be a significant marker molecule for MDR *P. aeruginosa* in Japan. Two diagnostic methods were developed to detect these strains:[3] a loop-mediated isothermal amplification (LAMP) system using *aac(6′)-Iae*-specific primers; and an agglutination method using anti-AAC(6′)-Iae polyclonal antibody (pAb). Although these methods are highly sensitive, LAMP was time consuming and required specialized tools and well-trained medical technicians. The agglutination assay was found to cause some problems such as inter-rater errors in routine work with many samples.

In this study, we designed a new rapid detection method for AAC(6′)-Iae-producing *P. aeruginosa* using an

immunochromatographic assay. Here, we report its effectiveness in clinical screening and molecular epidemiology as an alternative to PCR analysis.

Materials and methods

Bacterial strains

P. aeruginosa IMCJ2.S1 was used as positive control strain of *aac(6′)-Iae*. *P. aeruginosa* IMCJ798 carrying *aac(6′)-Iaf*,[6] *P. aeruginosa* IMCJ509 carrying *aac(6′)-Ib* and *Acinetobacter baumannii* A260 carrying *aac(6′)-Iad*[7] were used in the specificity tests of the assay.

Purification of recombinant proteins

aac(6′)-Iae, *-Iaf*, *-Ib* and *-Iad* were amplified with their specific primers from IMCJ2.S1, IMCJ798, IMCJ509 and A260, respectively [Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)]. Cloning and protein purification were performed as described previously.[6]

Preparation of monoclonal antibodies

Anti-AAC(6′)-Iae monoclonal antibodies (MAbs) were prepared as described previously.[8] His-AAC(6′)-Iae [Figure S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)] was used for immunization and screening of hybridomas by ELISA. The animal experiments were approved by the Ethics Committee for Animal Experiments at the Research Institute of the International Medical Center of Japan.

Assembly of the assay

The assay was assembled as described previously.[8] The composition and principle of the assay are described in Figure S2 [available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/)].

Analytical sensitivity testing of the assay

In the tests using bacteria, 100 μL serial 2-fold dilutions of overnight culture were suspended with 900 μL of extraction buffer (20 mM Tris–HCl, pH 7.5, 1.0% Triton X-100). Then, 100 μL aliquots were applied onto the sample area. In parallel, equivalent bacterial dilutions were also spread onto Mueller–Hinton (MH) agar plates to determine the cfu. The line intensity was quantified using QuantityOne software (Bio-Rad). The mean intensity of triplicate measurements at each point was plotted. The detection limit (*y*-axis) was defined as the intensity greater than the sum of the average values and 3× standard deviation of the endpoint values in the linear standard curve. The cfu (*x*-axis) corresponding to the theoretical detection limit was calculated by the equation in Figure 1(c).

Assessment of the assay

To assess the assay, 116 *P. aeruginosa* clinical isolates were obtained from 13 hospitals located in the Kanto area of Japan where little was known about AAC(6′)-Iae producers. These isolates were associated with nosocomial infections from 2004 to 2009; they include 14 strains from a Tokyo hospital (hospital A in Figure 2) in our previous work.[4] In the assessment, colonies on MH agar were directly picked up with a swab, and were suspended in a soft test tube containing extraction buffer. After lysing cells physically and chemically, four drops of lysate were dropped onto the assay. The results were determined by visual inspection 10 min after applying the samples.

Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed by the broth micro-dilution method recommended by the CLSI.[9] In this study, MDR *P. aeruginosa* was defined as showing resistance to imipenem (MIC ≥ 16 mg/L), amikacin (MIC ≥ 32 mg/L) and ofloxacin (MIC ≥ 4 mg/L) based on the criteria of the Ministry of Health, Labor, and Welfare of Japan.[10]

PFGE

PFGE assays were performed as described previously.[3]

PCR amplification and DNA sequencing

The *aac(6′)-Iae* and class 1 integrons were amplified with the specific primer sets (Table S1). All amplicons were sequenced to identify their contents with primers listed in Table S1.

Results

Development of immunochromatography specific for AAC(6′)-Iae

We obtained three MAbs with high reactivity to the recombinant His-AAC(6′)-Iae from *P. aeruginosa* IMCJ2.S1. Western blotting analysis and ELISA using the subtype AAC(6′)-I proteins of AAC(6′)-Iaf, -Ib and -Iad identified in clinical isolates in Japan showed that two MAbs, 1H7 and 3F12, specifically recognized AAC(6′)-Iae (Figure S1). Thus, these MAbs were utilized for the assembly of the immunochromatography assay (Figure S2). The assembled assay worked in the preliminary test using 0.5 μg of His-AAC(6′)-Iae prepared in PBS (Figure 1a).

Sensitivity testing using IMCJ2.S1 indicated that a clear line appeared with $>1.2\times10^5$ cfu of bacteria (Figure 1b), whereas reference lines appeared in all cases. The intensity of the test line was correlated with the number of bacteria in the range 5.8×10^4 to 4.6×10^5 cfu, indicating a high degree of linearity ($r^2=0.9766$) (Figure 1c). The theoretical detection limit for bacteria was 1.0×10^5 cfu per test.

The assay was also evaluated using strains carrying the subtype *aac* gene (Figure 1d) and the subtype proteins of AAC(6′)-Iaf, -Ib and -Iad (Figure 1e). Test lines did not appear with any proteins or bacteria, whereas reference lines appeared in all cases.

Assessment of the assay using clinical isolates

The assessment of the assay using 116 *P. aeruginosa* clinical isolates revealed that 60 (52%) of the 116 isolates were positive. These results were fully consistent with those of *aac(6′)-Iae* PCR analyses, indicating that the developed assay has no false positives or negatives. As for the source of positive strains, isolates from urine were the most frequent, followed by those from sputum.

Characterization of detected AAC(6′)-Iae-positive isolates

Antimicrobial susceptibility testing indicated that all positive strains showed an MDR phenotype. In addition to imipenem, amikacin and ofloxacin, effective increases in MICs of piperacillin,

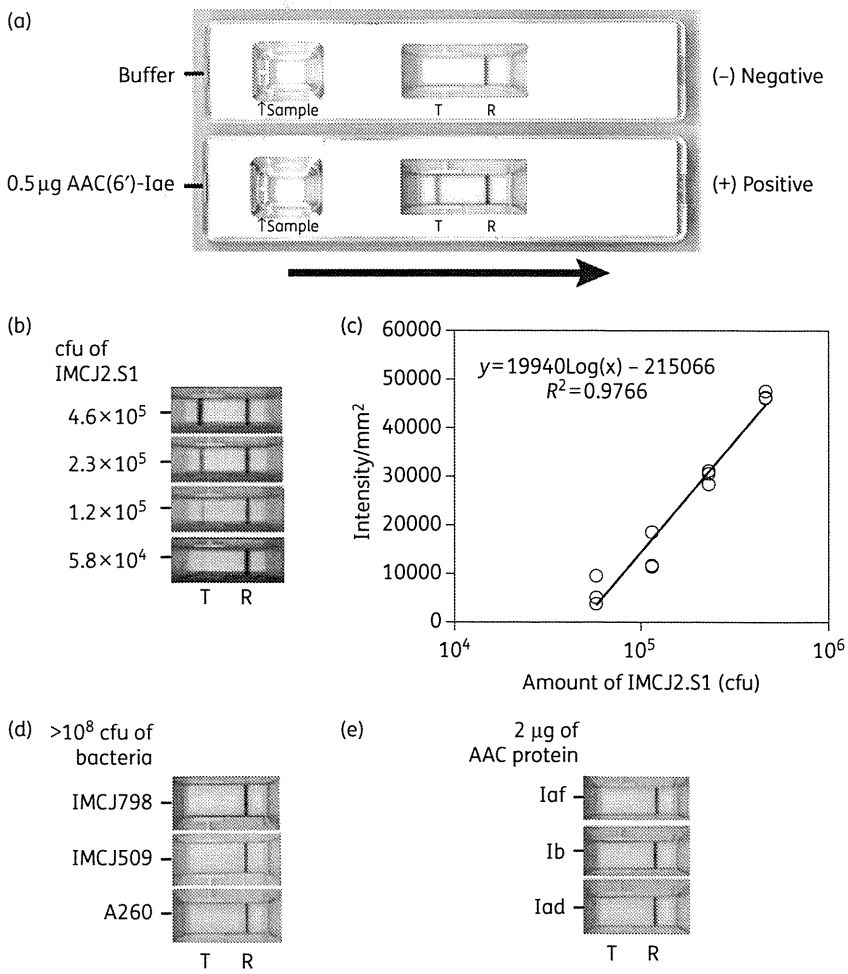

Figure 1. (a) Immunochromatography using MAbs 1H7 and 3F12. In negative cases, a single line appears at the position of the reference line (R) only. In positive cases, another line also appears at the position of the test line (T) in addition to the reference line. The arrow indicates the direction of lateral flow. (b) Sensitivity testing using positive control strain IMCJ2.S1. (c) Relationship between IMCJ2.S1 amount and the intensity of each test line. (d) Specificity testing using an excess amount of bacteria carrying the subtype *aac* gene. (e) Specificity testing using an excess amount of the subtype AAC(6′) proteins.

piperacillin/tazobactam, ceftazidime, aztreonam and meropenem were observed for most positive strains. In contrast, arbekacin, gentamicin and polymyxin B showed relative antibiotic potency towards positive strains.

PFGE assay of the tested 116 isolates revealed that the genetic lineages of AAC(6′)-Iae-positive strains were relatively similar to the IMCJ2.S1 strain previously isolated in Miyagi and outbreak-associated strains in a Tokyo hospital (hospital A, Figure 2).

The genetic environments of *aac(6′)-Iae* were determined by PCR and DNA sequencing. Forty-eight (80%) of 60 strains carried bla_{IMP-1}, *aac(6′)-Iae* and *aadA1* in their integron; these were identical to In113 in IMCJ2.S1. In the other 12 (20%) positive strains, bla_{IMP-1} was replaced with bla_{IMP-10} due to substitution of guanine by thymine at position 145 in bla_{IMP-1}. However, the 59 bp element of the bla_{IMP-10} cassette was identical to bla_{IMP-1} in In113.

All positive strains showed serotype O:11.

Discussion

Several immunochromatographic assays have been developed to identify various infectious agents such as influenza virus.[8] Most of these target their secretory proteins and cellular components. In this study, we designed an immunochromatographic assay to detect AAC(6′)-Iae-producing MDR *P. aeruginosa*. To our knowledge, this is the first report of immunochromatography using antibodies specific for a molecule that confers antibiotic resistance to bacteria. Immunological diagnosis can utilize antibodies against antigens of interest. Therefore, this approach could serve as a model for other molecules involved in antibiotic resistance.

The analytical sensitivity of the assay was 10^5 cfu/test when bacterial lysate of the positive control strain IMCJ2.S1 was used. This is a sufficient detection limit, because the colony counts of the used samples ranged from 10^8 to 10^9/test when the nearly equal amounts of colonies were analysed to determine cfu (data not shown). Most AAC(6′)-Iae-positive strains

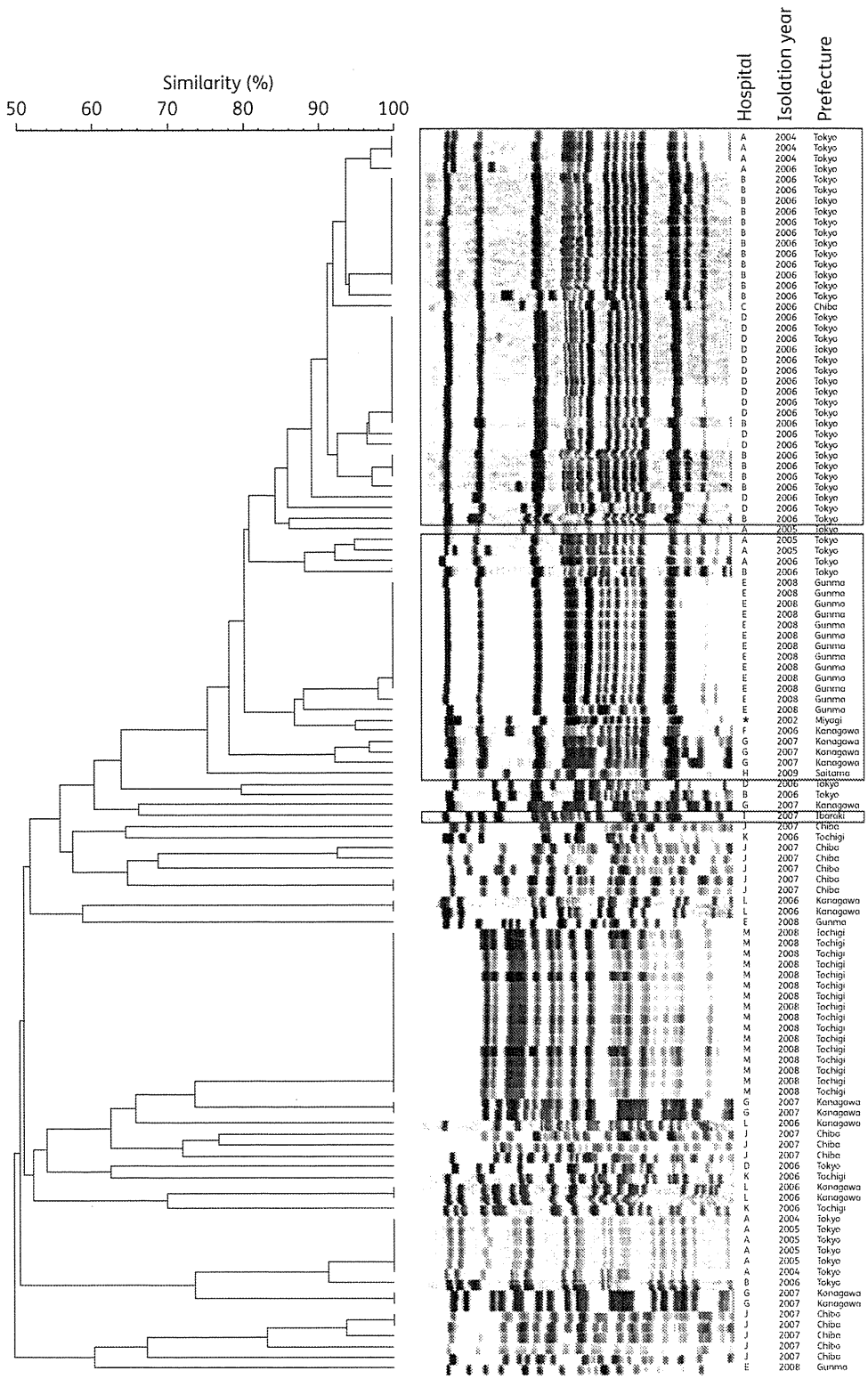

Figure 2. PFGE assay of 116 *P. aeruginosa* clinical isolates used in the assessment of the developed immunochromatography assay. The asterisk indicates the *P. aeruginosa* IMCJ2.S1 strain previously found in Miyagi. Areas enclosed within squares indicate the positive isolates detected.

detected were isolated from urine and sputum. Further work is needed to evaluate the assay using such clinical specimens. Additionally, considering that antibiotic resistance genes can be transmitted between different kinds of Gram-negative bacteria via plasmids and transposons, the developed assay might also allow the detection of AAC(6′)-Iae in other Gram-negative species involved in nosocomial infections.

AAC(6′)-Iae producers were found to be newly detected from hospitals in five prefectures of Gunma, Saitama, Kanagawa, Chiba and Ibaraki, following the previous reports of *P. aeruginosa* carrying *aac(6′)-Iae* in Miyagi, Tokyo and Hiroshima.[3–5] These AAC(6′)-Iae producers showed a similar genetic background; some of them were spread clonally. But it must also be noted that PFGE patterns of some negative strains were similar to those of positive strains (Figure 2). These observations suggest that strains with similar genetic backgrounds acquired resistance via a small mobile element. As such, the analysis of class 1 integrons indicated that all positive strains carried an In113 or In113-derived integron. However, the mode of transmission of In113 is still unknown. Further analysis is required to examine whether In113 is plasmid encoded.

All positive isolates showed MDR phenotypes. These data strongly demonstrate that AAC(6′)-Iae plays a crucial role as a marker molecule for MDR *P. aeruginosa* in Japan. But not all MDR *P. aeruginosa* isolates could be detected using the present assay. Actually, 21 (37%) of 56 negative strains showed MDR phenotypes; the number was coincident with amikacin resistance (data not shown). Furthermore, 16 (76%) of 21 negative strains were positive for both *aac(6′)-Ib* and bla_{IMP-1}; these strains were found to have caused the clonal nosocomial infection in hospital M (Figure 2). Immunochromatography can adopt multiple test lines in the current assay. Further work is in progress to design an immunochromatography assay targeting AAC(6′)-Ib and metallo-β-lactamase IMP.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-β-lactamases that mediate carbapenem resistance in *Pseudomonas*

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ABSTRACT

Rapid and reliable detection of carbapenem-resistant bacteria is an important infection-control measure and a crucial aspect of antimicrobial chemotherapy. IMP-type metallo-β-lactamase (MBL) is an emzyme that mediate carbapenem resistance in bacteria. Here, an immunochromatographic assay was newly developed using novel monoclonal antibodies (mAbs) recognizing IMP-type MBL. Epitope mapping of mAbs and mutational analysis of the epitope region in IMP antigen suggested that the mAbs could react to all known subtypes of IMP-type MBL. Evaluation of the assay using *Pseudomonas aeruginosa* strains (n = 248) showed that the results of the immunochromatographic detection of the IMP-type MBLs were fully consistent with those of the PCR analysis for bla_{IMP} genes, showing false positives and negatives. All positive strains were resistant to carbapenem (MIC ≥ 16 μg/ml). The assay also accurately distinguished the production of IMP-type MBLs in *Pseudomonas putida*, *Acinetobacter baumannii*, and *Alcaligenes xylosoxidans*. The detection limit of the assay was 5.7×10^4 cfu per test. Taken together, these data suggest that the developed assay can be used for rapid and reliable diagnosis of the production of IMP-type MBLs in Gram-negative bacteria.

1. Introduction

Carbapenems are key agents to treat life-threatening bacterial infections (Rahal, 2008). However, the emergence of carbapenem resistance in nosocomial pathogens, including *Serratia marcescens*, those of Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, constitutes a serious problem for the continued use of carbapenems (Masterton, 2009). Therefore, the rapid and reliable detection of carbapenem-resistant bacteria has become of urgent and vital importance in infection-control measures and antimicrobial chemotherapy.

Bacterial resistance to carbapenems is a complex process, including the loss or reduced expression of OprD porin (Hancock and Brinkman, 2002; Wolter et al., 2004), hyperproduction of AmpC (Tam et al., 2009), and/or overexpression of intrinsic efflux systems such as MexA–MexB–OprM (Aeschlimann, 2003; Li et al., 1995). Among clinical isolates of *Enterobacteriaceae* and *Pseudomonas* spp., resistance to carbapenems has been found to be mainly due to the production of the carbapenem-hydrolyzing enzymes, metallo-β-lactamases (MBLs) (Queenan and Bush, 2007; Walsh et al., 2005). To date, several classes of MBLs, such as IMP, VIM, GIM, KHM, and SPM, have been identified in clinical pathogens (Castanheira et al., 2004; Lauretti et al., 1999; Osano et al., 1994; Poirel et al., 2004; Sekiguchi et al., 2008). IMP-type MBLs are the most common and are found worldwide (Nordmann and Poirel, 2002).

IMP-1 MBL has been identified primarily from strains of *P. aeruginosa* and *S. marcescens* in Japan (Osano et al., 1994; Watanabe et al., 1991). In addition, 24 types of bla_{IMP} have been identified from a variety of clinical isolates and submitted to GenBank. Considering that approximately 1.9% of clinical isolates of *P. aeruginosa* have acquired MBL, and most of these are IMP-1-type MBLs (Kimura et al., 2005). IMP-type MBLs are thought to be significant marker molecules of carbapenem resistant *P. aeruginosa* in Japan.

Previously, we developed an immunochromatographic assay using monoclonal antibodies (mAbs) recognizing an aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] responsible for amikacin resistance in MDR *P. aeruginosa* strain NCGM2.S1 (previously reported as IMCJ2.S1) (Kitao et al., 2010; Sekiguchi et al., 2005). Given that the developed assay was a rapid, easy-to-use, and reliable detection method for AAC(6′)-Iae-producing multidrug-resistant (MDR)

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P. aeruginosa, the assay based on the antigen–antibody reaction could serve as a model for the development of a molecular diagnosis method for the screening and investigation of antibiotic-resistant bacteria as an alternative to PCR analysis.

In this study, an immunochromatographic assay using novel mAbs that recognize IMP-type MBLs has been developed. We report here the properties of mAbs used to construct the assay and the evaluation of the assay using clinical isolates.

2. Materials and methods

2.1. Construction and purification of IMP-1 mutants

The bla_{IMP-1} gene was PCR amplified from *P. aeruginosa* NCGM2.S1 strain using the primer sets *Nde*I-bla_{IMP1}(55–74)-F (5′-gcacgccatATGG-CAGAGTCTTTGCCAGATTT-3′) and *Bam*HI-bla_{IMP1}-R (5′-cgcggatccT-TAGTTGCTTGGTTTTGA-3′). The amplicon was digested with *Nde*I and *Bam*HI and then ligated into pET28a (Novagen) digested with the same restriction enzymes. The ligation products were used to transform DH5α, and the transformants were selected on LB agar containing 50 μg/mL kanamycin. The resulting plasmid pET28-bla_{IMP-1} was transformed into *E. coli* BL21(DE3) (TaKaRa) for recombinant protein expression. Protein purification was performed as described previously (Kitao et al., 2010).

2.2. Preparation of mAbs

Anti-IMP mAbs were prepared as previously described (Kishiro et al., 1995). The purified His-IMP-1 was used for immunization and screening of hybridomas by enzyme-linked immunosorbent assay (ELISA). The animal experiments were approved by the Ethical Committee for Animal Experiments at the Research Institute of the National Center for Global Health and Medicine (NCGM).

2.3. Assembly of the assay

The assay was assembled according to the instructions for a commercially available rapid diagnosis kit, Quick Chaser™ Flu A, B (Mizuho Medy, Saga, Japan) as previously described (Miyoshi-Akiyama et al., 2010). To prepare the test lines, 0.76 mg of rat mAb per test was coated onto nitrocellulose membranes (Millipore, Billerica, MA) at a position of 30 mm from the sample application area. To prepare the reference lines, 0.2 mg of anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) per test was coated onto the membranes at a position of 39 mm from the sample application area. Pads were prepared by soaking glass filters with rat mAb and rabbit IgG, each conjugated with colloidal gold. The membranes and pads were assembled within a plastic housing. The assembled assays were stored in a waterproof bag with a desiccant at room temperature until use.

2.4. Determination of the epitope region recognized by mAbs

The peptides (10 μg/mL) (Sigma-Aldrich Co.) were immobilized onto the wells of a 96-well enzyme immunoassay (EIA) plate (Corning) by incubation in 50 mM carbonate buffer (pH 9.0) containing 1 mM of the chemical cross-linker disuccinimidyl suberate (Pierce) at 4 °C for 16 h. After blocking with Superblock (Pierce), the plate was incubated for 1 h with 10 μg/mL rat mAb diluted with PBST (phosphate buffer saline containing 0.05% Tween) and washed 3 times with PBST. The binding of mAb to each peptide was detected with HRP (horseradish peroxidase)-goat anti-rat IgG (GE Healthcare) and TMB (3,3′,5,5′-tetramethylbenzidine) (Bio-Rad).

In the competitive assay, the purified IMP-1 prepared in 50 mM carbonate buffer (pH 9.0) was immobilized onto the wells of a 96-well EIA plate (Corning) at 4 °C for 16 h. After blocking, the plate was incubated for 1 h with 10 μg/mL of rat mAb preincubated with serially diluted peptides and washed 3 times with PBST. The binding of mAb to immobilized IMP-1 was detected with HRP-goat anti-rat IgG (GE Healthcare) and TMB (Bio-Rad).

2.5. Site-directed mutagenesis

Site-directed mutagenesis was performed using QuickChange Mutagenesis Kit according to the instructions of the manufacturer (Stratagene). IMP mutants were created by site-directed mutagenesis in the genetic region encoding amino acid residues 101–125 of the IMP-1 antigen. The primers used in the mutagenesis are listed in Table 1. The pET28–blaIMP-1 was used as a template plasmid.

2.6. Analysis of interaction between IMP mutants and mAbs

Purified IMP-1 protein and mutants (2 μg/mL) prepared in 50 mM carbonate buffer (pH 9.0) were immobilized onto the wells of a 96-well microtiter plate (Corning) by incubation at 4 °C for 16 h. After blocking with Superblock (Pierce), the plate was incubated for 1 h with 10 μg/mL mAb diluted with PBST and washed 3 times with PBST. Binding of mAb to each peptide was detected with HRP-goat anti-rat IgG (GE Healthcare) and TMB (Bio-Rad).

2.7. Bacterial strains

A total of no duplicate 248 strains of *P. aeruginosa* were obtained from BML Inc. to evaluate the assay. *P. aeruginosa* NCGM2.S1 was used as a positive strain for bla_{IMP-1} (Sekiguchi et al., 2005). One of two *Acinetobacter baumannii* strains, a strain of *A. baumannii* NCB0211-439 carrying bla_{IMP-2} was obtained from National Institute of Infectious Diseases in Japan. Another *A. baumannii* strain AB-NCGM112 carrying bla_{IMP-1} was clinically isolated from single inpatient at NCGM. Two strains of *Pseudomonas putida* (PP-NCGM265 and PP-NCGM266) carrying bla_{IMP-1} and four strains of *Alcaligenes xylosoxidans* (AX-NCGM1, AX-NCGM2, AX-NCGM3, and AX-NCGM4) carrying bla_{IMP-1} were obtained from inpatients at NCGM.

2.8. Assessment of the assay using bacterial strains

As shown in Supplementary Fig. 1, bacterial colonies on Muellar–Hinton agar (Gibco) were picked with a swab and were suspended in a soft test tube containing extraction buffer with nonionic detergent. After lysing the cells physically and chemically, three drops of bacterial lysate were added onto the test plate. The results were analyzed by visual inspection 15 min after the addition of the sample.

Table 1
Primers used in mutagenesis.

Mutants	Primer name	Sequence (5′ to 3′ orientation)
R110Q	IMP-R110Q_F	GAGTGGCTTAATTCTCAATCTATCCCCACG
R110Q	IMP-R110Q_R	CGTGGGGATAGATTGAGAATTAAGCCACTC
E105G	IMP-E105G_F	ACGGGCGGAATAGGGTGGCTTAATTCTCGA
E105G	IMP-E105G_R	TCGAGAATTAAGCCACCCTATTCCGCCCGT
R110Q–Q113S	IMP-R110Q–P113S_F	TGGCTTAATTCTCAATCTATCtCCACGTATG CATCT
R110Q–Q113S	IMP-R110Q–P113S_R	AGATGCATACGTGGAGATAGATTGAGAATT AAGCCA
E118V	IMP-E118V_F	ACGTATGCATCTGTATTAACAAATGAACTG
E118V	IMP-E118V_R	CAGTTCATTTGTTAATACAGATGCATACGT
G102A	IMP-G102A_F	AGCGACAGCACGGCCGGAATAGAGTGGCTT
G102A	IMP-G102A_R	AAGCCACTCTATTCCGGCCGTGCTGTCGCT
T101S	IMP-T101S_F	CATAGCGACAGCtCGGCCGGAATAGAGTGG
T101S	IMP-T101S_R	CCACTCTATTCCGCCCGAGCTGTCGCTATG
E122D	IMP-E122D_F	GAATTAACAAATGACCTGCTTAAAAAAGAC
E122D	IMP-E122D_R	GTCTTTTTTAAGCAGGTCATTTGTTAATTC