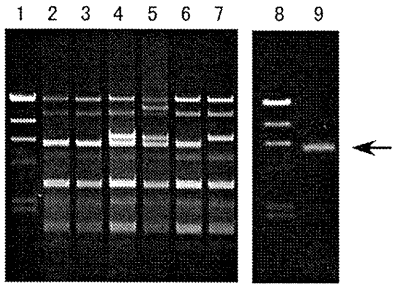

FIG. 1. Agarose gel electrophoresis of EcoRI-digested plasmid DNAs of wild-type VRE strains and pHY. Lanes: 1 and 8, HindIII-digested lamda DNA; 2 to 7, strains VRE34, VRE35, VRE36, VRE37, VRE38, and VRE39, respectively; 9, pHY harbored by the bacteriocinogenic VRE38–BM4105SS transconjugant. The arrow indicates the 6.0-kbp plasmid DNA fragment.

exhibited bacteriocin activity against *E. faecalis*, *E. faecium*, *E. hirae*, *E. durans*, *E. raffinosus*, *E. gallinarum*, and *L. monocytogenes*, and one strain, designated VRE35, exhibited bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*. These strains contained several plasmids (Fig. 1). Since it was possible that the bacteriocin determinant was encoded on a plasmid, the cotransferability of the bacteriocin activities with drug resistance was examined. The transferability of the gentamicin resistance of all strains except VRE36 to the recipient strain *E. faecium* BM4105RF was examined by filter mating. The gentamicin resistance of four strains was transferred to the recipient strain at a frequency of about 10^{-4} per donor cell. Fifty gentamicin resistance transconjugants from each of the four strains were examined for bacteriocin activity. About 75% to 95% of the gentamicin transconjugants showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*, suggesting that the bacteriocin activity was cotransferred. A DNA plasmid of about 6.0 kbp was found in the bacteriocinogenic transconjugants of each strain and was not found in nonbacteriocinogenic transconjugants. The experiment to transfer bacteriocin production was repeated by using the initial bacteriocinogenic *E. faecium* BM4105RF transconjugant of each strain to transfer the bacteriocin to *E. faecium* BM4105SS by filter mating. About 80 to 90% of the gentamicin-resistant transconjugants were bacteriocinogenic and showed bacteriocin activity identical to that of the *E. faecium* BM4105RF donor strain. The bacteriocinogenic transconjugants harbored only the 6.0-kbp plasmid, implying that this plasmid might encode the bacteriocin. The 6.0-kbp plasmid itself is not conjugative, but it is presumed to be mobilized by a resident plasmid of unknown identity. The transconjugant of *E. faecium* VRE38 was used as the representative strain for further analyses. The 6.0-kbp plasmid DNA identified in the bacteriocinogenic transconjugants of *E. faecium* VRE38 was designated pHY.

DNA sequence of the pHY plasmid. The DNA sequence of the pHY plasmid was determined and was found to be 6,037 bp long. Computer analysis revealed the presence of nine open reading frames (ORFs) in pHY, all of which were oriented in the same direction. Figure 2a shows the ORFs that had a good ribosome binding site within a 20-base region upstream of the predicted start codon. Homology analyses of the deduced amino acid sequences and the nucleotide sequence of each ORF of pHY were performed. The deduced proteins encoded on ORF3, ORF4, ORF5, ORF7, and ORF8 showed significant homology to those encoded by the reported genes *mobC* (1, 23), *mobA* (1, 23), ORF6 of plasmid pTI1 (23), *repA* (23, 30), and ORF10 of plasmid pTI1 (23), respectively. Mobilization plasmids usually carry mobilization genes (*mob*) that encode specific relaxasome components, as well as the origin of transfer (*oriT*) (3, 14). The nicking of *oriT* is an essential initial step in the mobilization of plasmid DNA. The MobA relaxase makes a reversible site- and strand-specific nick at a specific sequence within *oriT* (3, 14). MobC is an accessory protein of MobA for DNA-nicking activity. Rep protein has replication origin binding and nicking-closing activities (24). The deduced proteins of ORF1, ORF2, ORF6, and ORF9 showed no significant homology with reported proteins. The analysis of ORF1 and ORF2 is described below.

Cloning of pHY into pMW119 (Spc^r). The attempt to clone pHY into the cloning vector pAM401 failed. The *E. coli* cloning vector pMW119 was tagged with the spectinomycin resistance gene (Spc^r) for use as a selective marker in enterococci as described in Materials and Methods, and the resultant plasmid was designated pMW119 (Spc^r). pHY has a single EcoRI site, which was cut and used to clone it into the EcoRI site of pMW119 (Spc^r). *E. faecalis* OG1S or *E. hirae* ATCC 9790 was transformed by the cloned pMW119 (Spc^r)::pHY plasmid. The

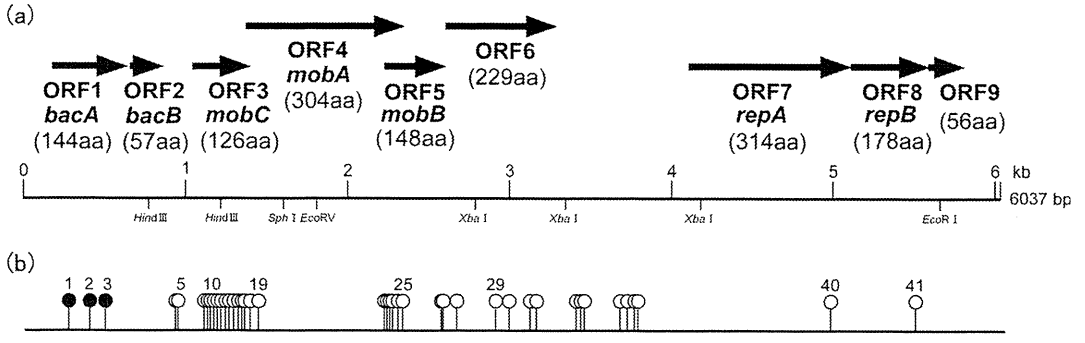

FIG. 2. Physical map of pHY (6.0 kb) showing the deduced ORFs and mapping of the transposon insertions. (a) Physical map of pHY and deduced ORFs. Thick horizontal arrows indicate the deduced ORFs encoded on pHY and the direction of transcription. (b) Map of Tn*5* insertions into pMW119 (Spc^r)::pHY. Lollipops indicate the points of Tn*5* mutant insertion; filled circles, mutants that did not express bacteriocin; open circles, bacteriocinogenic mutants.

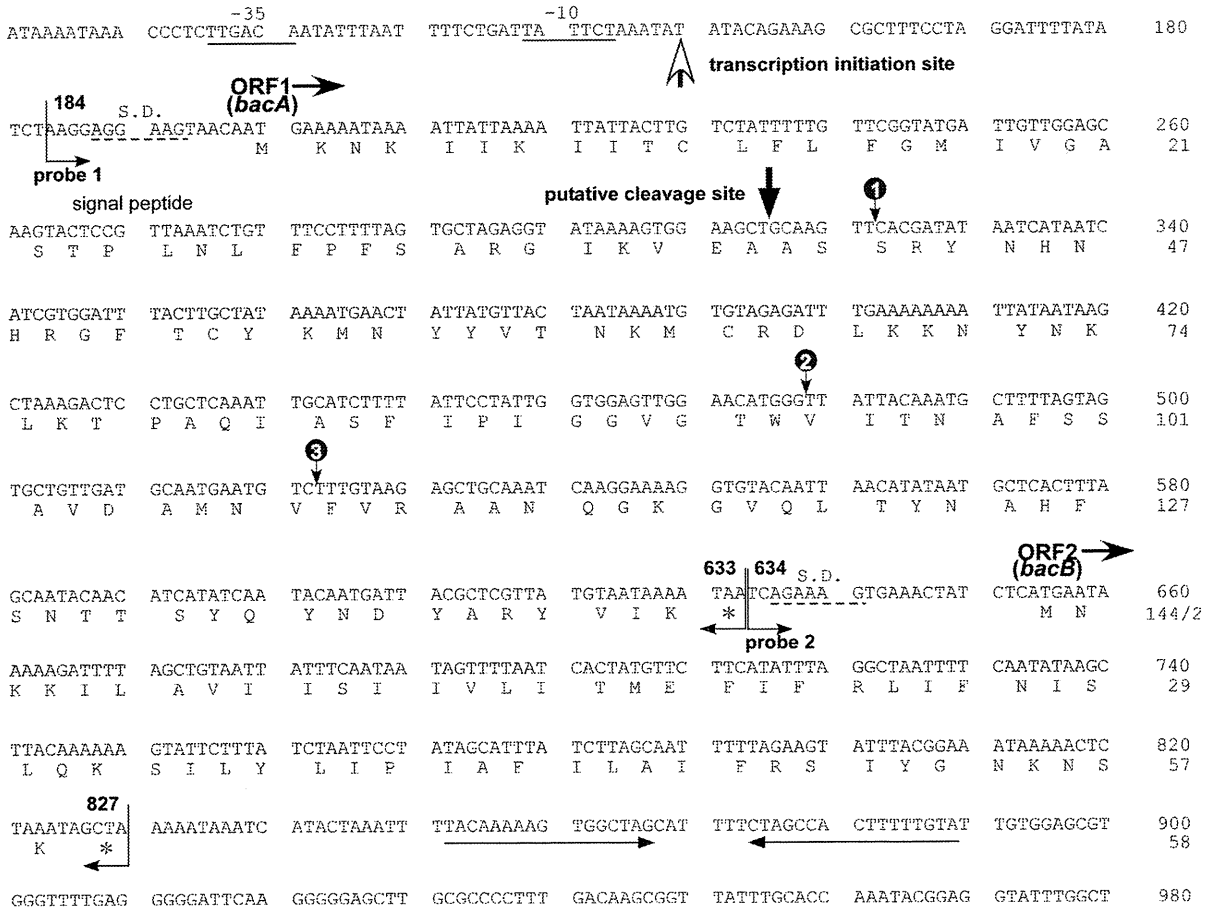

FIG. 3. Nucleotide sequences of *bacA* and *bacB* for bacteriocin 51 and deduced amino acid sequences. Potential promoters (−10 and −35) are underlined, and the Shine-Dalgarno (S.D.) ribosome binding sequences are marked by dashed underlines. The potential signal peptidase cleavage site is indicated by a filled vertical arrow. The transcription initiation site of bacteriocin 51 is indicated by an open vertical arrow. Horizontal arrows under the nucleotide sequence indicate inverted repeats, which are explained in the text. Three numbered circles indicate the points of Tn*5* insertion that abolished bacteriocin expression. Probe 1, which corresponds to a 450-base sequence in *bacA* (bases 184 to 633), and probe 2, which corresponds to a 194-base sequence in *bacB* (bases 634 to 827), were used for Northern hybridization analysis.

spectinomycin-resistant transformants of *E. faecalis* OG1S and *E. hirae* ATCC 9790 showed bacteriocin activity against *E. faecium*, *E. hirae*, and *E. durans*, indicating that pMW119 (Spc^r)::pHY transformed both *E. faecalis* OG1S and *E. hirae* ATCC 9790. *E. hirae* ATCC 9790 was sensitive to the bacteriocin of the bacteriocinogenic transformant *E. faecalis* OG1S. However, the bacteriocinogenic *E. hirae* ATCC 9790 strain was resistant to the bacteriocin activity of the bacteriocinogenic *E. faecalis* OG1S strain (data not shown). These results indicate that plasmid pHY encodes the bacteriocin and the immunity function for its own bacteriocin. The bacteriocin encoded on pHY was designated bacteriocin 51 (Bac 51).

Analysis of Tn*5* insertion mutants. Tn*5* insertion mutants of pMW119 (Spc^r)::pHY were generated in *E. coli* TH688::Tn*5*. A total of 41 insertions into pHY were examined; the location of each insertion is shown in Fig. 2b. Three of the insertions resulted in the loss of bacteriocin activity, while activity was retained by the remaining insertion mutants. The three non-bacteriocinogenic mutants, designated pMG701, pMG702, and pMG703, were mapped at bp 322, bp 478, and bp 522 from the first adenine nucleotide of the pHY map within ORF1 (Fig. 3). *E. hirae* ATCC 9790 harboring pMG701, pMG702, or pMG703 did not show bacteriocin activity or resistance (immunity) to the bacteriocin activity of *E. faecalis* OG1S or *E. hirae* ATCC 9790 harboring pMW119 (Spc^r)::pHY. These results suggested that ORF1 and ORF2, which we designated *bacA* and *bacB*, were bacteriocin determinants.

Detailed sequence analysis of ORF1 (*bacA*) and ORF2 (*bacB*). *bacA* encoded a 144-amino-acid protein. The ATG start codon was preceded by a potential ribosome binding site located 5 bp upstream. Computer analysis suggested that the deduced *bacA* protein had a signal peptide sequence, and a potential signal peptidase-processing site corresponding to the V-E-A sequence was located at positions 37 to 39 (Fig. 3). The predicted mature BacA protein consisted of 105 amino acids. BacB encoded a 55-amino-acid protein. The ATG start codon was preceded by a potential ribosome binding site located 12 bp upstream. There was no obvious promoter sequence upstream of the ribosome binding site. A putative transcription terminator signal was identified downstream of *bacB*. There was no obvious promoter sequence between *bacA* and *bacB*. These results suggested that *bacA* was the structural gene and *bacB* was the immunity gene for bacteriocin 51.

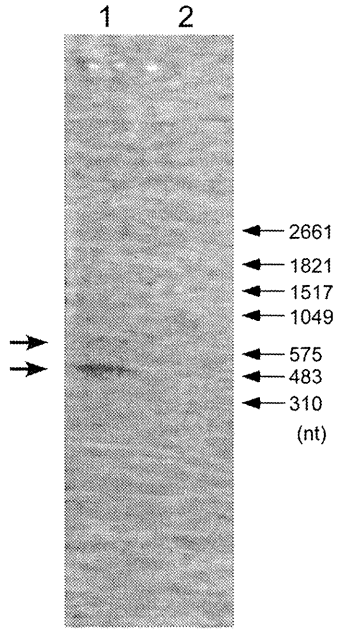

FIG. 4. Northern blot analysis of the *bacA* and *bacB* genes. Lanes; 1, *E. faecium* BM4105SS cells harboring pHY; 2, BM4105SS plasmid-free cells. Arrows on the left indicate mRNAs of about 500 nt and 700 nt. The positions of the RNA markers are noted on the right.

Analysis of transcripts of *bacA* and *bacB*. DNA sequence analysis revealed that *bacA* and *bacB* were transcribed in the same direction. The transcripts of these genes were analyzed by Northern hybridization (Fig. 4). Probe 1, which corresponds to a 450-nucleotide (nt) sequence in *bacA* (Fig. 3), detected two transcripts of about 700 and 500 nucleotides, corresponding to the molecular sizes of *bacA* and *bacB* combined and *bacA* alone, respectively. The quantity of transcript corresponding to the combined *bacA* and *bacB* nucleotide sequence was much smaller than that corresponding to the *bacA* sequence.

Probe 2, which corresponds to a 194-nucleotide sequence in *bacB* (Fig. 3), did not detect any transcript (data not shown) from *bacB* alone, implying that the small quantity of transcript that was detected corresponded to combined *bacA* and *bacB* (Fig. 4). The transcription start site upstream of *bacA* was examined by the RACE method and was determined to be the T nucleotide located 6 nucleotides downstream from the −10 promoter sequence (Fig. 3). These results indicated that transcription initiated from the transcription start site upstream of *bacA*.

Construction of the plasmid producing C-terminally His tagged mature Bac 51 protein. Plasmid DNA from *E. faecium* BM4105SS(pHY) was used to amplify the DNA sequence of the predicted mature BacA protein of Bac 51 by PCR. The PCR product was cloned into the *E. coli* vector pET22b(+). The resulting cloned plasmid, pET22b(+)::*bac51*, was used to transform *E. coli* BL21 as described in Materials and Methods.

Purification of His-tagged Bac 51 protein from *E. coli* BL21[pET22b(+)::*bac51* (*bacA*)]. The procedure for the purification of His-tagged Bac 51 is described in Materials and Methods. Analysis by SDS-PAGE showed that elution with either 35 mM, 50 mM, 100 mM, or 200 mM imidazole gave rise to one band, which corresponded to a molecular mass of approximately 12 kDa (see Fig. S1 in the supplemental material). The eluates obtained using a 100 mM, 200 mM, or 300 mM concentration of imidazole were examined for bacteriocin activity against *E. hirae* ATCC 9790 by the metal-cup method as described in Materials and Methods. The eluates obtained with 100 mM or 200 mM imidazole gave rise to a 12-kDa protein band by SDS-PAGE analysis and produced a complete bacterial growth inhibition zone with *E. faecium*, *E. hirae*, or *E. durans* below and around the metal cup (see Fig. S2 in the supplemental material). However, no inhibition zone was observed with the eluate obtained with 300 mM imidazole, which did not gave rise to a 12-kDa protein band in SDS-PAGE analysis. These results indicated that the mature His-tagged Bac 51 protein was purified, showed the same activity as the Bac 51 protein of the wild-type strain, and had a molecular mass of about 12 kDa.

Construction of the plasmid producing C-terminally His tagged Bac 32 protein. *bac32* consists of the *bacA* and *bacB* genes (23). *bacA* is the structural gene for Bac 32, and *bacB* is the immunity gene for its own bacteriocin. The *bacA* protein is composed of 89 amino acids with a putative signal peptide at the N terminus. The predicted signal peptide could be cleaved after the V-E-A residues (amino acids 33 and 35) by computer analysis using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), and the *bacA* product would consist of a 35-amino-acid signal peptide and a 54-amino-acid mature BacA protein. Plasmid DNA from FA2-2(pTI1) was used for PCR amplification of the DNA sequence of the predicted mature BacA protein of Bac 32. The plasmid producing C-terminally His tagged Bac 32 protein was constructed by using pET-22b(+) as described above.

Purification of His-tagged Bac 32 protein from *E. coli* BL21[pET22b(+)::*bac32* (*bacA*)]. SDS-PAGE analysis showed that elution with imidazole at concentrations between 35 mM and 400 mM gave rise to a single band corresponding to a molecular mass of approximately 6.4 kDa (data not shown). Each of these eluates showed complete inhibition of bacterial growth by *E. faecium*, *E. hirae*, and *E. durans* below and around the metal cup containing the eluate. These results indicated that the mature His-tagged Bac 32 protein had been purified, that it showed the same activity as the Bac 32 protein of the wild-type strain, and that its molecular mass was about 6.4 kDa.

Liquid bacteriocin assay. The results of the liquid bacteriocin assay are shown in Fig. 5. The bacteriocin activity of purified Bac 51 BacA-His was examined using the liquid bacteriocin assay as described in Materials and Methods. The number of cells of the indicator strain in the culture containing Bac 51 BacA-His remained unchanged in the 5-h period of the experiment (data not shown). On the other hand, the survival of indicator strain cells in cultures containing the bacteriocin 31 culture filtrate or purified Bac 32 BacA-His was reduced to <1/10,000 after 30 min. The survival of indicator strain cells in the culture containing the bacteriocin 43 culture filtrate was also reduced logarithmically. The cell counts of the indicator strain in the culture without bacteriocin increased logarithmically during incubation.

Thermostability of the bacteriocin. Purified BacA-His was examined for bacteriocin activity after heat treatment. The

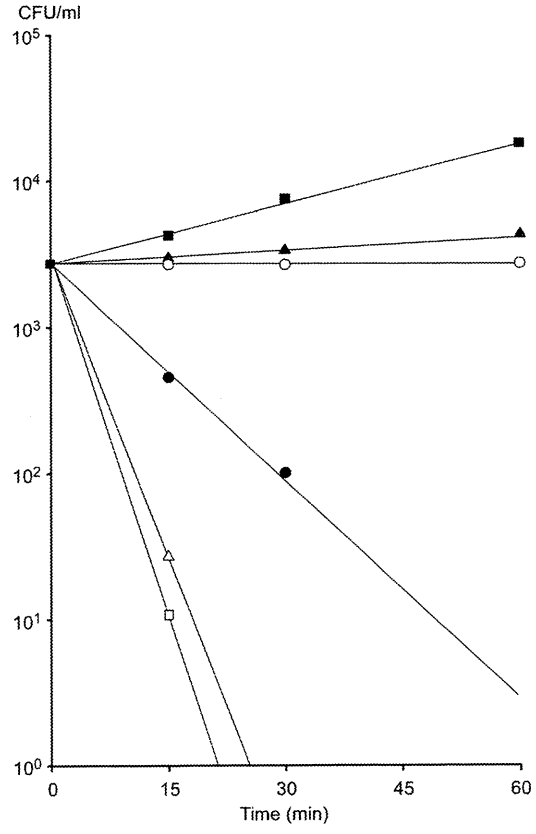

FIG. 5. Liquid bacteriocin assay. The assay was performed by adding 0.2 ml of the test sample to 1.8 ml of the *E. hirae* indicator strain. Symbols for test samples: ○, purified His-tagged Bac 51 (2.5×10^3 BU/ml) from elution with 200 mM imidazole; △, FA2-2(pYI17 *bac31*) culture filtrate (8.0×10^2 BU/ml); □, purified His-tagged Bac 32 (2.5×10^3 BU/ml) from elution with 200 mM imidazole; ●, FA2-2(pMG502 *bac43*) culture filtrate (8.0×10^2 BU/ml); ▲, *E. faecalis* FA2-2 culture filtrate; ■, fresh THB broth.

bacteriocin was stable at temperatures below 100°C for 5 min or at 70°C for 10 min. It was inactivated when incubated at 72°C for 10 min. These results indicated that the bacteriocin was heat stable.

Identification of Bac 51 in *E. faecium* isolates. One hundred thirty-eight vancomycin-sensitive *E. faecium* isolates, 87 vancomycin-resistant *E. faecium* isolates, 120 vancomycin-sensitive *E. faecalis* isolates recovered in Japanese hospitals, and 662 vancomycin-resistant *E. faecium* isolates recovered in the University of Michigan hospital in Ann Arbor (23, 35) were examined for the presence of the Bac 51 determinant by PCR analysis with a primer specific for Bac 51 *bacA*. Two (1.4%) of the 138 vancomycin-sensitive *E. faecium* strains, 2 (2.3%) of the 87 VRE strains isolated in Japanese hospitals, and 2 (0.3%) of the VRE strains isolated in the University of Michigan hospital gave rise to the expected 365-bp PCR product with the *bacA*-specific primer. No *bacA*-specific PCR products were detected in *E. faecalis* isolates. Plasmid DNAs isolated from the *bacA*-positive strains were examined for the presence of the Bac 51 gene by PCR analysis with the primer and by Southern blot analysis with the *bacA* probe. The results showed that the Bac 51 determinant was encoded on the plasmids of these strains.

DISCUSSION

A new bacteriocin, designated Bac 51, was identified in a VanA-type vancomycin-resistant *E. faecium* strain, VRE38. Bac 51 exhibited a relatively narrow spectrum of activity and was active against *E. faecium*, *E. hirae*, and *E. durans* strains. Bac 51 was encoded on plasmid pHY (6.0 kbp), which was efficiently mobilized for transfer to a recipient *E. faecium* strain at a frequency of 10^{-5} to 10^{-7} per donor cell with the coresident conjugative gentamicin resistance (35). The Bac 51 determinant consisted of the bacteriocin structural gene, *bacA*, and the immunity gene, *bacB*. The *bacA* gene encoded a deduced 144-amino-acid protein with a putative signal sequence of 39 amino acid residues at the N terminus that was predicted to give rise to a 105-amino-acid mature protein. The *bacB* gene encoded a deduced 55-amino-acid protein without a putative signal sequence. The deduced BacA and BacB proteins had no homology to known bacteriocins, indicating that Bac 51 was a new type of bacteriocin from *E. faecium*. The results of Northern blot analysis of *bacA* and *bacB* suggest that there is significant transcription termination after *bacA*, but with some extension through *bacB*. The transcription start site was determined to be the T nucleotide located 6 nucleotides downstream from the −10 promoter sequence. These results indicated that *bacA* and *bacB* constituted an operon; *bacA* was the bacteriocin structural gene, and *bacB* was the immunity gene.

The purified C-terminally His tagged BacA (BacA-His) of Bac 51 inhibited bacterial cell growth in *E. faecium*, *E. hirae*, and *E. durans* but did not inhibit cell growth in *L. monocytogenes*, as shown by the metal-cup bacteriocin assay. Bac 31, originally isolated from *E. faecalis* (32), and Bac 32 (23) and Bac 43 (30), originally isolated from *E. faecium* clinical isolates, are pore-forming bacteriocins. Purified Bac 32 BacA-His and the culture filtrates of *E. faecalis* strain FA2-2, which produces each of these pore-forming bacteriocins, were active against the indicator strains and inhibited cell growth by the metal-cup method. In the liquid bacteriocin assay, purified Bac 51 BacA-His did not kill the indicator strain. On the other hand, purified Bac 32 BacA-His and the culture filtrates of the Bac 31- and Bac 43-producing *E. faecalis* strain FA2-2 killed the indicator strain shortly after it was exposed to the bacteriocin. These results indicated that in contrast to the bactericidal effect produced by the pore-forming bacteriocins Bac 31, Bac 32, and Bac 43, the mode of action of Bac 51 is bacteriostatic.

In our previous study of Bac 32, we showed that the Bac 32 *bacA* product comprised 89 amino acids with a putative signal peptide at the N terminus, based on genetic and DNA sequence analysis (23). We carried out computer analysis using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) in the present study. The *bacA* product may consist of a 35-amino-acid signal peptide and a 54-amino-acid mature BacA protein, and the signal peptide may be cleaved after the V-E-A residues (positions 33 to 35). The DNA primer for amplification of the mature *bacA* protein was designed to produce a DNA fragment for the 54-amino-acid mature BacA protein. The purified BacA-His protein was active against the indicator strain. These data indicated that the signal peptide could be cleaved after the V-E-A residues (positions 33 to 35). Computer analysis using Genetyx, version 7, was described in our previous report (23), where we predicted that the deduced

bacA product consisted of a 19-amino-acid signal peptide and a 70-amino-acid mature protein, and that the signal peptide was cleaved after the L-L-A residues (positions 17 to 19). The results of the present study of the bacteriocin activity of the purified 54-amino-acid BacA mature protein of Bac 32 showed that the previous prediction of the signal peptidase cleavage site could be wrong (23).

The well-characterized *E. faecium* bacteriocins (i.e., enterocins) are produced by food-grade organisms and show strong bacteriocin activity against *L. monocytogenes* (27). In contrast to the bacteriocins of food-grade *E. faecium* strains, Bac 32 or Bac 32-type bacteriocins, which are frequently (about 40% of clinical isolates) identified in *E. faecium* clinical isolates, are active against *E. faecium*, *E. hirae*, and *E. durans* but show no activity against *L. monocytogenes* (23). Bac 51 was also identified in VRE clinical isolates and was active against *E. faecium*, *E. hirae*, and *E. durans* but not against *L. monocytogenes*. However, there was no sequence homology between the predicted Bac 51 and Bac 32 proteins. Bac 51-type bacteriocins were identified in *E. faecium* clinical isolates at a frequency of about 2% but were not identified in *E. faecalis* clinical isolates, indicating that Bac 51 is specific to *E. faecium* clinical isolates.

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Antimicrobial Ointments and Methicillin-Resistant *Staphylococcus aureus* USA300

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We tested 259 methicillin-resistant *Staphylococcus aureus* isolates and 2 USA300 ATCC type strains for susceptibility to bacitracin and neomycin contained in over-the-counter antibacterial ointments. Resistance to both bacitracin and neomycin was found only in USA300. The use of over-the counter antimicrobial drugs may select for the USA300 clone.

Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is rapidly spreading worldwide. MRSA USA300 is a clone of increasing public health concern among rapidly disseminating CA-MRSA strains in the United States (*1*). MRSA USA300 is designated as sequence type (ST) 8 by multilocus sequence typing (MLST) and possesses staphylococcal cassette chromosome *mec* (SCC*mec*) type IVa. Although the rapid dissemination of the USA300 clone may occur because of a high virulence level that arises from the production of Panton-Valentine leukocidin (PVL) or an existing arginine catabolic mobile element (ACME) (*2*), there is no conclusive evidence to support this hypothesis (*3*). Furthermore, the hypothesis cannot account for the rapid dissemination of MRSA in countries where USA300 clones are not the dominant clones (most European countries, South Korea, and Japan) (*4–6*). In most European countries, the dominant CA-MRSA clone is the European clone (ST80, SCC*mec* type IV, PVL positive and ACME negative) (*1,4*). In South Korea, only 1 isolate was a USA300 clone among 138 MRSA isolates collected from patients with bacteremia and soft tissue infection (*5*). In Japan, the MRSA USA300 clone is rare (*6*).

In many cases, soft tissue infection acquired in communities was treated by using over–the-counter (OTC) drugs called triple-antibiotic ointment (TAO), e.g., Neosporin (polymyxin B [PL-B] sulfate, 5,000 units/g; bacitracin, 400 units/g; and neomycin, 3.5 mg/g) and Polysporin triple ointment (PL-B sulfate, 10,000 units/g; bacitracin, 500 units/g; and gramicidin 0.25 mg/g). These ointments contain antimicrobial drugs at concentrations far exceeding their MICs among *S. aureus* strains (16–32 μg/mL [equivalent to 124–248 unit/mL] for PL-B, <1–64 units/mL for bacitracin, and <1–>128 μg/mL for neomycin) (*7,8*). It is hypothesized that CA-MRSA cases in the United States were under the selective pressure of TAOs.

In this study, we tested the susceptibilities of MRSA isolates, including the USA300 clone, to the antimicrobial drugs in TAOs. We also considered the possible role of TAOs in spreading the USA300 clone.

The Study

We selected 222 MRSA isolates that were not classified as the New York/Japan (NY/JP) clone on the basis of the absence of SCC*mec kdpC* (*9*). In addition, 37 NY/JP clone-like isolates were used. A total of 259 MRSA isolates were tested in our study. Of these 259 isolates, 227 were collected during 2004–2010 at Nagoya Medical Center, and 32 isolates were collected in 2006–2009 at Kyoto University Hospital, including 9 USA300 outbreak isolates (*6*). Details of isolates used in this study are shown in Table 1. ATCC BAA1556 (USA300 FPR3757) (American Type Culture Collection, Manassas, VA, USA) and ATCC BAA1717 (USA300-HOU-MR TCH1516) strains were also used in our study. Susceptibilities to bacitracin and neomycin were tested by the Kirby-Bauer disk diffusion method (Becton Dickinson, Franklin Lakes, NJ, USA). MICs of bacitracin, neomycin, and PL-B for USA300 strains were determined by the agar dilution method according to the Clinical and Laboratory Standard Institute M07-A8 guidelines (*10*). To observe interaction among these 3 antimicrobial drugs, a double-disk synergy test was performed with modification by using ATCC BAA1717 (*11*).

SCC*mec* were determined according to the method of Hisata et al. (*12*). Isolates possessing both PVL and *arcA* (*13*) were analyzed by pulsed-field gel electrophoresis as described in our previous study (*9*). Moreover, USA300 isolates were genotyped by MLST (www.mlst.net) and staphylococcal protein A (*spa*) typing (www.spaserver.ridom.de).

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Table 1. Source of methicillin-resistant *Staphylococcus aureus* isolates, Japan, 2004–2010

Source	Outpatients		Inpatients		Health care workers
	No. community-acquired infections*	No. hospital-acquired infections	No. community-acquired infections*	No. hospital-acquired infections	
Skin and soft tissue	23	7	4	23	0
Bloodstream	0	2	1	19	0
Respiratory tract	0	2	1	17	0
Urinary tract	0	2	2	3	0
Ear	8	0	0	1	0
Eye	3	0	0	3	0
Others	1	1	2	3	0
Carriage	14	10	3	29	0
Screening	50	0	2	20	3
Total	99	24	15	118	3

*Community-acquired infections were determined on the basis of patients' histories according to Centers for Disease Control and Prevention (Atlanta, GA, USA) guidelines (www.cdc.gov/mrsa/diagnosis/index.html).

Nineteen of the 259 isolates harbored both the PVL and the *arcA* gene. Of these 19 isolates, 18 had been collected from Kyoto University Hospital and 1 from Nagoya Medical Center (Table 2). All 19 PVL- and ACME-positive isolates were determined to be ST8 by MLST. These isolates showed USA300 PFGE patterns identical to ATCC BAA1556 and were of SCC*mec* type IVa. SCC*mec* elements of other isolates were determined as type I (n = 4), IIa (n = 37), IIb (n = 52), II untypeable (n = 14), IV (n = 104), and V (n = 9). The SCC*mec* element of the remaining 20 isolates could not be identified.

The 18 USA300 isolates collected from Kyoto University Hospital showed the same *spa* type (t008). However, the 1 USA300 isolate collected from Nagoya Medical Center was of *spa* t190.

ATCC BAA1717 and 9 USA300 isolates collected during 2007–2009 at Kyoto University Hospital were resistant to both bacitracin and neomycin. The USA300 isolate detected at Nagoya Medical Center in 2004 was bacitracin resistant and neomycin susceptible. The other 9 USA300 isolates and ATCC BAA1556 were susceptible to both drugs (Table 2). Highlander et al. (*14*) found that the bacitracin- and aminoglycoside-resistant genes were located on pUSA300-HOU-MR, a plasmid typically observed in the USA300 strain TCH1516. The resistance to bacitracin and neomycin may depend on the presence of the plasmid and may be absent in some USA300 clones.

On the other hand, nearly all MRSA isolates that were determined to be a type other than USA300 were susceptible to bacitracin. One isolate was determined to have intermediate resistance to bacitracin. Also, 11 (4.5%) of the 240 MRSA isolates not deemed to be USA300 were resistant to neomycin, while 132 (55%) demonstrated intermediate resistance (Table 2). A study performed in the 1990s reported that most MRSA strains were susceptible to bacitracin, and many were resistant to neomycin (*8*). Our findings were consistent with the previous study.

MICs of bacitracin, neomycin, and PL-B were 400 units/mL, 128 μg/mL, and 400 units/mL, respectively, among most USA300 isolates with resistance to both bacitracin and neomycin (Table 2). The concentrations of neomycin and PL-B in the TAOs were ≈10 to 30× higher than the MICs of both drugs. In addition, neomycin and PL-B were observed to be weakly synergistic (Figure). However, Bearden et al. reported that despite containing antimicrobial drugs at concentrations far exceeding their MICs among MRSA, PL-B and neomycin ointment, or PL-B and gramicidin ointment exhibited deficient bactericidal activity in time-kill assays (*15*). Bacitracin may thus be required for sufficient bactericidal activity. Acquiring resistance to bacitracin and neomycin may be essential for survival under the selective pressure of TAOs. If so, bacitracin resistance should be considered a key characteristic of the USA300 clone.

Table 2. Bacitracin and neomycin susceptibility of MRSA USA300 and other MRSA isolates*

Bacitracin/neomycin	MRSA USA300 (MICs of bacitracin, neomycin, and polymyxin B)†			Other MRSA
	Kyoto University Hospital	Nagoya Medical Center	ATCC type strains	
R/R	9 (400, 128, 200–400)	0	BAA1717 (400, 128, 400)	0
R/S	0	1 (400, 0.25, 400)		0
S/R	0	0		11
I/S	0	0		1
S/I	0	0		132
S/S	9 (6.25–12.5, 0.25, 400)	0	BAA1556 (6.25, 0.25, 400)	96
Total	18	1	2	240

*MRSA, methicillin-resistant *Staphylococcus aureus*; ATCC, American Type Culture Collection (Manassas, VA, USA); R, resistant; S, susceptible; I, intermediate resistance.
†MICs are expressed as units/mL for bacitracin and polymyxin B and in μg/mL for neomycin.

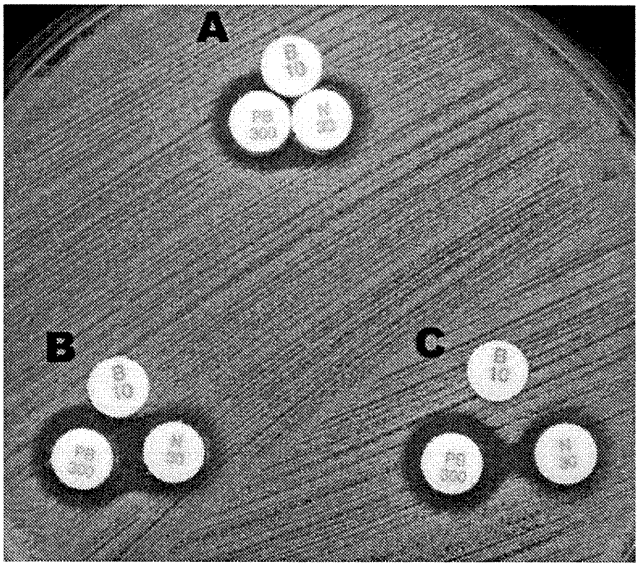

Figure. Double-disk synergy test with 3 disks, bacitracin (B10 disk), neomycin (N30 disk), or polymyxin B (PL-B, PB300 disk) was performed with USA300 strain ATCC BAA1717. Disks were placed at 6 mm (A), 9 mm (B), and 11 mm (C) distance from disk centers. Neomycin and PL-B were found to be weakly synergistic.

TAOs containing bacitracin, neomycin, and PL-B are widely used in the United States; thus, bacitracin- and neomycin-resistant strains may be selected by the selective pressure of the TAOs. Although bacitracin and neomycin ointments are also available as OTC drugs in Japan, use of the ointments is not widespread. As a result, the selective pressure that leads to bacitracin and neomycin resistance is weak in Japan.

Conclusions

The emergence of MRSA USA300 depends partly on the virulence of MRSA USA300, but it may be influenced by usage of OTC drugs. In each country, susceptibilities of MRSA USA300 to bacitracin and neomycin should be thoroughly investigated, and relationships between the dissemination of MRSA USA300 and the usage of OTC drugs should be clarified. Such an investigation will provide valuable information regarding the emergence of organisms resistant to OTC topical antibiotics and likely a warning against the indiscriminate use of antimicrobial drugs. Further studies are required to validate these findings.

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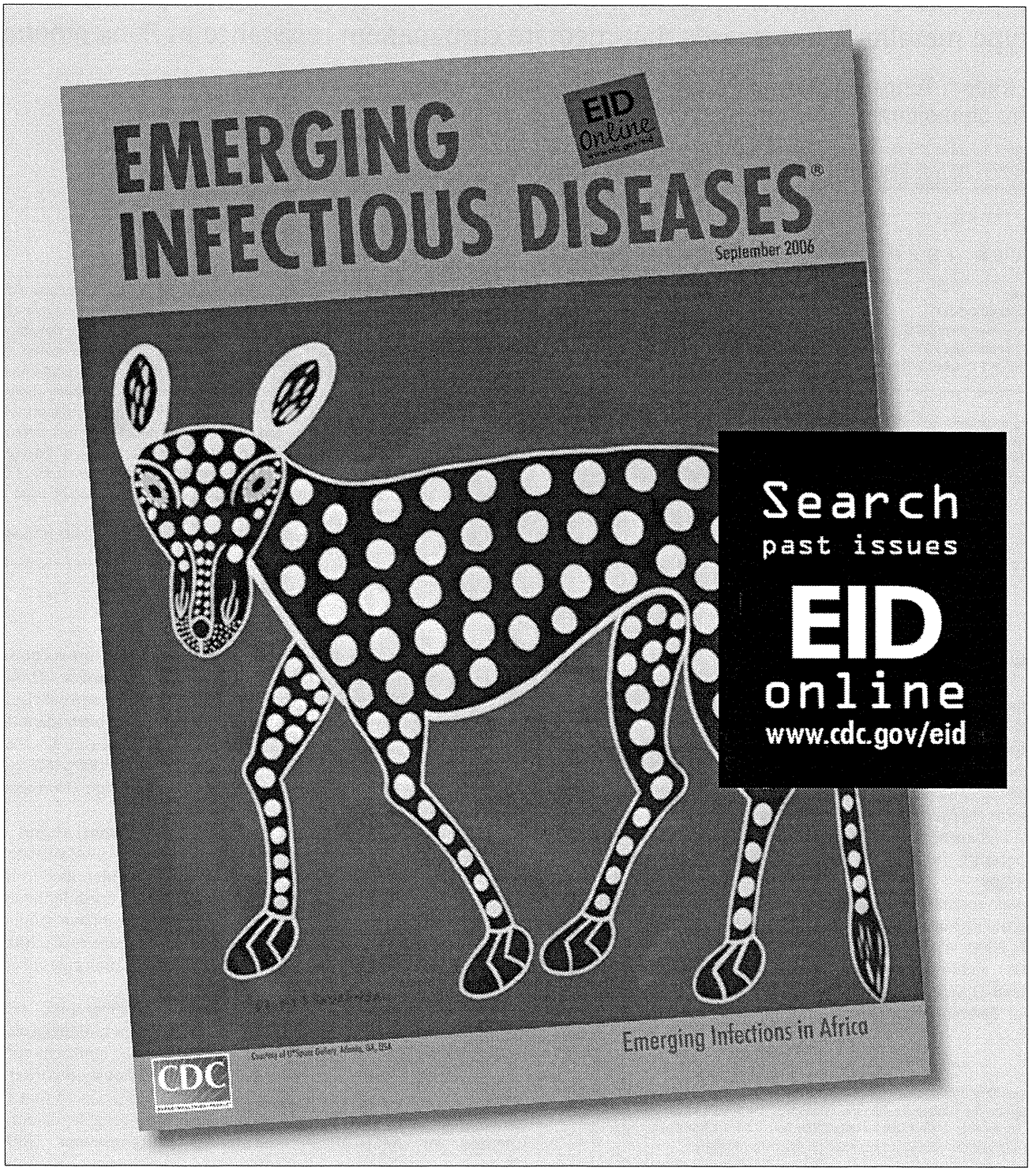

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Development of an immunochromatographic assay for diagnosing the production of IMP-type metallo-β-lactamases that mediate carbapenem resistance in *Pseudomonas*

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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.

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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–O113S	IMP-R110Q–P113S_F	TGGCTTAATTCTCAATCTATCtCCACGTATG CATCT
R110Q–O113S	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	CATAGCGACAGCtCGGGCGGAATAGAGTGG
T101S	IMP-T101S_R	CCACTCTATTCCGCCCGAGCTGTCGCTATG
E122D	IMP-E122D_F	GAATTAACAAATGACCTGCTTAAAAAAGAC
E122D	IMP-E122D_R	GTCTTTTTTAAGCAGGTCATTTGTTAATTC

Table 2
The combination of mAbs used to construct the 9 prototypes and their performance.

Prototype no.	mAb immobilized to membrane	mAb labbeled with colloidal gold	Detection result[a]		
			Extraction buffer	IMP-1 (100 ng/test)	IMP-1 (1 ng/test)
No.1	1H11-C/F5	1H11-C/F5	−	−	−
No.2	1H11-C/F5	4C9-C/F6	−	+ +	−
No.3	1H11-C/F5	4E7-C/F6	−	+ +	−
No.4	4C9-C/F6	1H11-C/F5	−	+ +	−
No.5	4C9-C/F6	4C9-C/F6	−	+ + +	−
No.6	4C9-C/F6	4E7-C/F6	−	+ + +	+ +
No.7	4E7-C/F6	1H11-C/F5	−	+ +	−
No.8	4E7-C/F6	4C9-C/F6	−	+ + +	+
No.9	4E7-C/F6	4E7-C/F6	−	+ + +	+ +

[a] The intensity of test line was evaluated by visual inspection on four scale of − to +++.

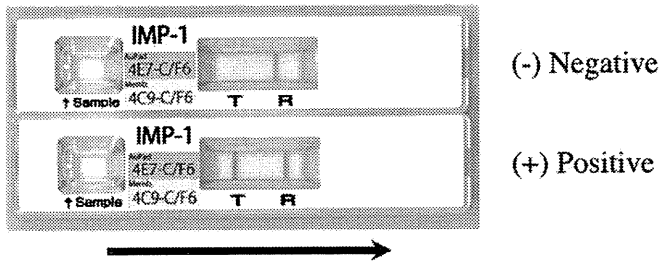


Fig. 1. Immunochromatography developed using mAbs 4E7-C/F6 and 4C9-C/F6. In the case of negative results, a single line appears at the position of the reference line (R) only. In the case of positive results, another line also appears at the position of the test line (T) in addition to the reference line. The arrow indicates the direction of lateral flow.

To investigate the reliability of the assay, all strains were analyzed by PCR detection of bla_{IMP} using specific primer sets as previously described (Poirel et al., 2011).

2.9. Analytical sensitivity testing of the assay

The detection limit of the assay was determined using *P. aeruginosa* NCGM2.S1 strain and purified recombinant IMP-1 protein. The procedure was same as previously described (Kitao et al., 2010).

3. Results

3.1. Development of the assay

In the screening of mAbs generated by hybridoma clones, we found 3 mAbs, 1H11-C/F5, 4E7-C/F6, and 4C9-C/F6, having high reactivity with recombinant IMP-1 from *P. aeruginosa* NCGM2.S1 in ELISA.

To determine the best combination of mAbs to detect of IMP-1, the 9 prototypes of the immunochromatographic assay were constructed using the 3 identified mAbs (Table 2). In the test using 100 ng of IMP-1 per plate, the test line appeared in the prototypes except for prototype no. 1, which consisted of mAb 1H11-C/F6. In the test using 1 ng of IMP-1 per plate, the test line appeared in prototype nos. 6, 8, and 9. The intensity of the test line was highest in prototype no. 6, consisting of 4C9-C/F6 immobilized to the membrane and 4E7-C/F6 labeled with colloidal gold in both tests. Therefore, these mAbs were utilized in the development of a novel immunochromatographic assay for the rapid detection of IMP-1 (Fig. 1).

3.2. Identification of epitopes recognized by mAbs

To determine the region in IMP-1 recognized by 4E7-C/F6 and 4C9-C/F6, we synthesized 22 different 15-mer peptides (≥70% purity) covering the 21 to the 246-end region of IMP-1 without the N-terminal signal sequences (Table 3). In ELISA using these peptides, both mAbs bound to peptides 51–65, 81–95, 131–145, 141–155, and 161–175, as well as rat IgG, prior to immunization, indicating that these peptides are not epitopes (Fig. 2A). The 4C9-C/F6 bound to peptides 101–115, 191–205, and 201–215. The 4E7-C/F6 bound to peptides 111–125, 191–205, and 201–215.

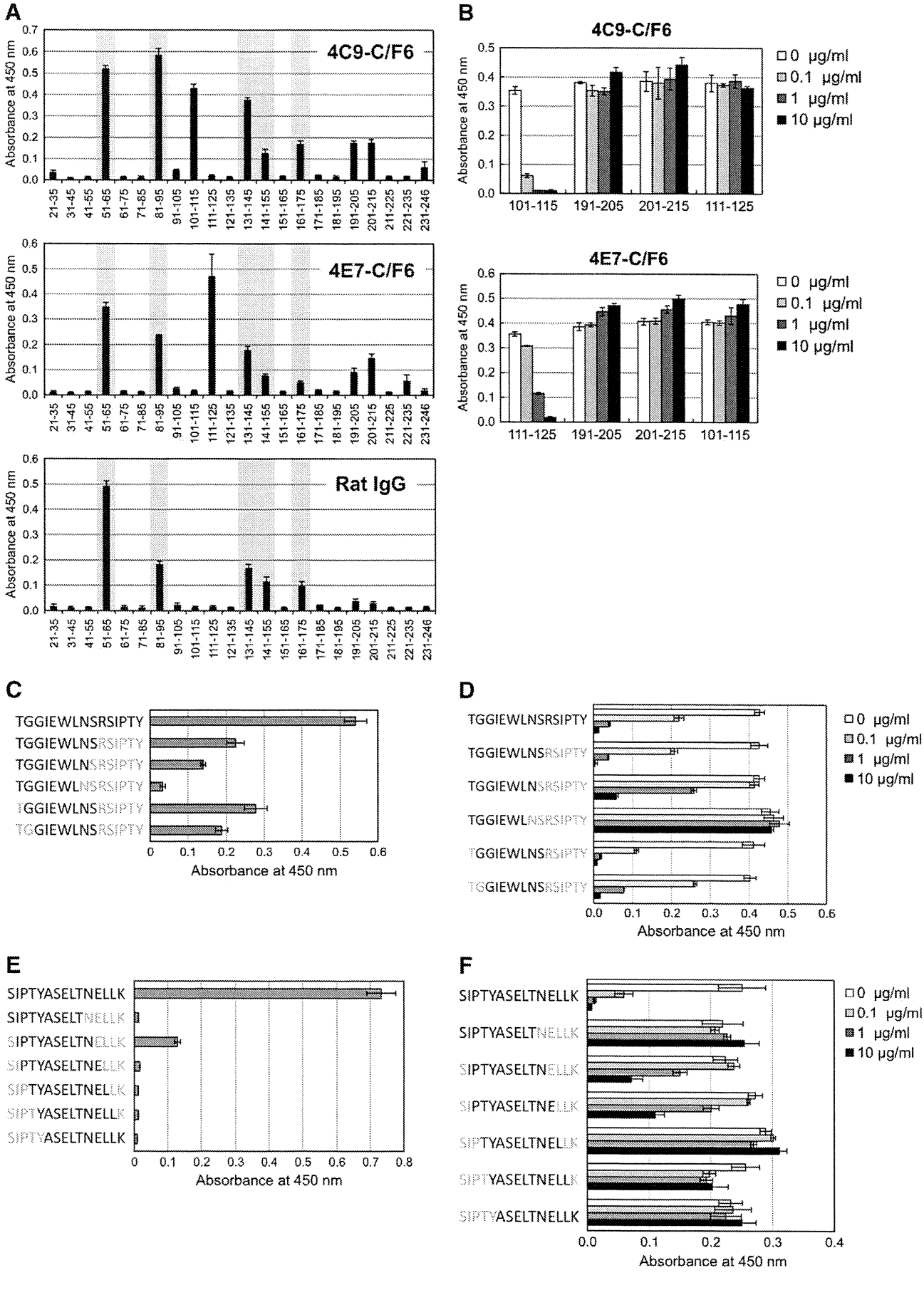
A competitive assay using the candidate peptides was also performed (Fig. 2B). The recognition of IMP-1 by 4C9-C/F6 was significantly inhibited by only peptide 101–115 inhibited in a dose-dependent manner. The recognition of IMP-1 by 4E7-C/F6 was significantly inhibited by only peptide 115–125 in a dose-dependent manner. Due to a sharing a 5-mer amino acid sequence, SIPTY, in both peptide 111–125 and 101–115, the epitope region recognized by both mAbs was assumed to overlap. Peptide 111–125 recognized by 4E7-C/F6 did not inhibit the recognition of IMP-1 by 4C9-C/F6, while peptide 101–115, recognized by 4C9-C/F6 also did not inhibit the recognition of IMP-1 by 4E7-C/F6. These results indicate that the epitopes of 4C9-C/F6 and 4E7-C/F6 were independently included in peptides 101–115 and 111–125, respectively.

To narrow the range of peptide 101–115 or 111–125 required for mAb recognition, 10-mer truncated peptides 101–115 and 111–125 were also synthesized. The 4C9-C/F6 captured 4 kinds of peptide except for TGGIEWL, in addition to peptide 101–115 consisting of TGGIEWLNSRSIPTY (Fig. 2C). These 4 kinds of peptide also inhibited the recognition of IMP-1 by 4C9-C/F6 in a dose-dependent manner (Fig. 2D). These results indicate that TGGIEWLN is a region required for IMP-1 recognition by 4C9-C/F6, and that the C-terminal end of the asparagine residue (N) in the TGGIEWLN peptide plays a key role in antigen recognition. In contrast, the 4E7-C/F6 captured only

Table 3
Peptides used in epitope mapping.

Number	Region in IMP-1	Sequence
1	21–35	SLPDLKIEKLDEGVY
2	31–45	DEGVYVHTSFEEVNG
3	41–55	EEVNGWGVVPKHGLV
4	51–65	KHGLVVLVNAEAYLI
5	61–75	EAYLIDTPFTAKDTE
6	71–85	AKDTEKLVTWFVERG
7	81–95	FVERGYKIKGSISSH
8	91–105	SISSHFHSDSTGGIE
9	101–115	TGGIEWLNSRSIPTY
10	111–125	SIPTYASELTNELLK
11	121–135	NELLKKDGKVQATNS
12	131–145	QATNSFSGVNYWLVK
13	141–155	YWLVKNKIEVFYPGP
14	151–165	FYPGPGHTPDNVVVW
15	161–175	NVVVWLPERKILFGG
16	171–185	ILFGGCFIKPYGLGN
17	181–195	YGLGNLGDANIEAWP
18	191–205	IEAWPKSAKLLKSKY
19	201–215	LKSKYGKAKLVVPSH
20	211–225	VVPSHSEVGDASLLK
21	221–235	ASLLKLTLEQAVKGL
22	231–246	AVKGLNESKKPSKPSN

Fig. 2. Determination of epitope by ELISA. (A, C, and E) The peptides were immobilized onto an EIA plate, and the interaction between peptides and mAb was detected with anti-rat IgG goat antibody. The shaded regions in panel A show the nonspecific regions captured by rat IgG prior to immunization of IMP-1. (B, D, and F) Recombinant IMP-1 was immobilized onto an EIA plate, and the interaction between IMP-1 and mAb was inhibited using serially diluted peptides. In the peptide sequences presented in C, D, E, and F, the truncated amino acids are indicated by gray letters.


A
4C9-C/F6
4E7-C/F6
Rat IgG
Absorbance at 450 nm
21-35
31-45
41-55
51-65
61-75
71-85
81-95
91-105
101-115
111-125
121-135
131-145
141-155
151-165
161-175
171-185
181-195
191-205
201-215
211-225
221-235
231-246
B
4C9-C/F6
101-115
191-205
201-215
111-125
4E7-C/F6
111-125
191-205
201-215
101-115
0 μg/ml
0.1 μg/ml
1 μg/ml
10 μg/ml
C
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
Absorbance at 450 nm
D
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
TGGIEWLNSRSIPTY
0 μg/ml
0.1 μg/ml
1 μg/ml
10 μg/ml
Absorbance at 450 nm
E
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
Absorbance at 450 nm
F
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
SIPTYASELTNELLK
0 μg/ml
0.1 μg/ml
1 μg/ml
10 μg/ml
Absorbance at 450 nm

by peptide IPTYASELTN of the 6 peptides in addition to peptide 111–125 consisting of SIPTYASELTNELLK (Fig. 2E). The recognition of IMP-1 by 4E7–C/F6 was inhibited by peptides IPTYASELTN and PTYASELTNE in a dose-dependent manner (Fig. 2F). These results indicate that IPTYASELTNE is a region required for IMP-1 recognition by 4E7–C/F6. The peptide PTYASELTNE that inhibited the recognition of IMP-1 by 4E7–C/F6 in Fig. 2F was not captured by 4E7–C/F6 in Fig. 2E, probably due to differences in peptide condition between the 2 assays because the N-terminal end of the peptide was not free in the solution in the experiment in Fig. 2E.

We also analyzed the location of the region recognized by 4C9–C/F6 and 4E7–C/F6 in the three-dimensional structure of IMP-1 (PDB ID: 2DOO) (Kurosaki et al., 2006). The region recognized by both mAbs was found to be located around the active center of IMP-1 consisting of α-β-α motif (Wang et al., 1999) (Fig. 3A). The epitope region showed considerable sequence diversity in other types of MBLs, despite the α–β–α motif was shared (Fig. 3B).

3.3. Potential of the assay for broad reactivity with IMP-type MBLs

To date, 24 subtypes of IMP-type MBL have been submitted to GenBank. The amino acid sequence alignment indicated that the 101–125 region on IMP-1 including the epitopes of 4E7–C/F6 and 4C9–C/F6 shows high similarity among these known IMP-type MBLs (Fig. 4). Moreover, the region among IMP-type MBLs corresponding to the 101–125 region on IMP-1 is of 8 types according to sequence alignment.

To examine whether the 4E7–C/F6 and 4C9–C/F6 could capture the 7 types of 101–125 regions on IMP-1, as well as type 1 epitope (WT), we created IMP mutants. The interactions between IMP mutants and mAbs were analyzed by ELISA (Fig. 5A). The binding ability of 4C9–C/F6 to IMP mutant with type 6 epitope (G102A–R110Q–P113S) decreased by about 30% compared with WT, whereas the binding abilities of 4E7–C/F6 to IMP mutants with type 2 epitope (R110Q), type 3 epitope (E105G), type 4 epitope (R110Q–E122D), type 5 epitope (T101S–R110Q), type 7 epitope (G102A–R110Q), and type 8 epitope (R110Q–E118V) were similar to those of WT. The binding ability of 4E7–C/F6 to IMP mutant with type 6 epitope (G102A–R110Q–P113S) also decreased by about 60% compared with WT, whereas the binding abilities of 4E7–C/F6 to other 6 kinds of IMP mutant were similar to those of WT. Considering that both mAb bound to IMP mutant with type 7 epitope (G102A–R110Q) and that the epitope of 4C9–C/F6 does not include the proline residue at position 113 in IMP-1, the amino acid substitution from proline to serine at position 113 in IMP-1 might affect the stability of the three-dimensional conformation of IMP-1, resulting in a decrease in the binding between the IMP mutant with type 6 epitope (G102A–R110Q–P113S) and mAbs.

The IMP mutants were further tested using the immunochromatographic assay (Fig. 5B). The positive line clearly appeared when 100 ng of protein was used per test. This result indicated that the assay could detect all the IMP mutants as well as WT IMP-1, suggesting that the assay constructed using 4C9–C/F6 and 4E7–C/F6 has the potential to capture all subtypes of IMP-type MBLs.

3.4. Evaluation of the assay

To investigate the reliability of the assay, the developed assay was evaluated using 248 clinical isolates of *P. aeruginosa*. Bacterial colonies grown on BHI (brain heart infusion) agar plates were assessed using the developed assay, and the results were compared with those of PCR detection of bla_{IMP} genes.

As shown in Table 4, the immunochromatographic assay identified the production of IMP-type MBLs in 191 (77%) of the 248 strains tested. These results were fully consistent with those of PCR analysis for bla_{IMP} gene, showing 100% specificity and 100% sensitivity. The developed

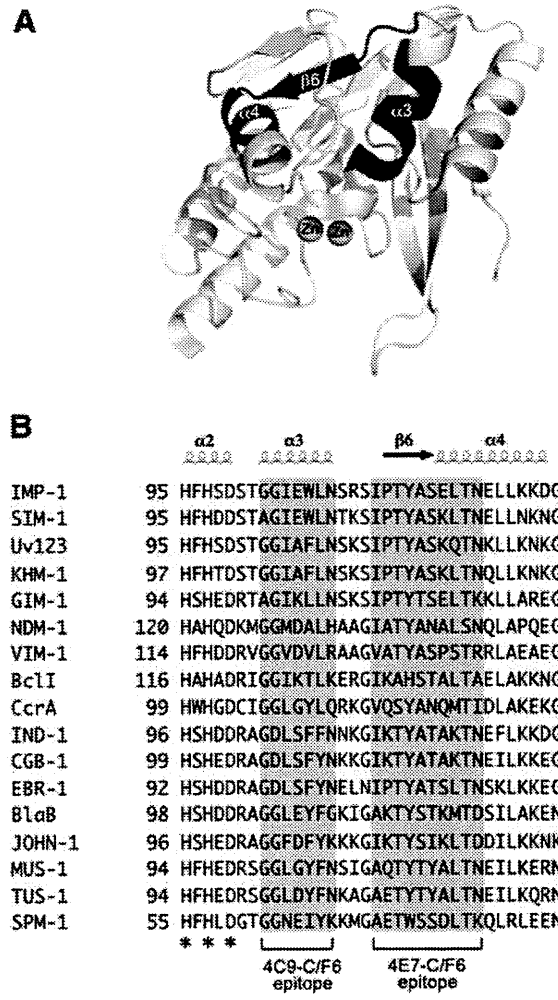


Fig. 3. Sequence alignment of the epitope region among MBLs. (A) Epitope region on the three-dimensional structure of IMP-1. The figure was created with MacPymol software according to the IMP-1 structure (PDB ID: 2DOO) determined by Dr. Kurosaki (Kurosaki et al., 2006). The black part presents the region recognized by 4C9–C/F6 and 4E7–C/F6. The 2 zinc molecules indicate the location of active center. (B) Sequence alignment of the epitope region among MBLs. The GenBank accession numbers of MBLs used in the alignment analysis are as follows: IMP-1, AAB30289; SIM-1, AAX76774; Uvs123, AAP70377; KHM-1, BAH16555; GIM-1, CAF05908; NDM-1, ADP20459; VIM-1, CAB46686; BclI, P04190; CcrA, P25910; IND-1, AAD20273; CGB-1, AAL55263; EBR-1, AAN32638; BlaB, CAA65601; JOHN-1, AAK38324; MUS-1, AAN63647; TUS-1, AAN63648; and SPM-1, CAD37801. Sequence alignment was performed using the ClustalW2 program (Larkin et al., 2007). The two-dimensional information was analyzed with ESPript (Gouet et al., 2003). The parts corresponding to the regions recognized by 4C9–C/F6 and 4E7–C/F6 are shaded.

assay therefore yielded no false-positives or false-negative results, indicating that is reliable. All positive strains showed carbapenem resistance (MIC ≥ 16 μg/ml). The sequencing analyses of DNA amplicons indicated that the 101–125 amino acid region in the IMP-type MBLs produced by the positive strains are type 1, 3, and 4 epitopes (data not shown). Additionally, the assay also identified the production of IMP-type MBLs in 2 strains of *P. putida*, 2 strains of *A. baumannii*, and 4 strains of *A. xylosoxidans*, which were carrying bla_{IMP} genes.

The detection limit of the assay was determined by using diluted culture of *P. aeruginosa* NCGM2.S1 strain and diluted recombinant IMP-1 protein. The intensity of the test line was correlated with the number of NCGM2.S1 in the range of 5.2×10^4 to 5.2×10^5 cfu, indicating a high degree of linearity ($r^2 = 0.9992$) (data not shown). The theoretical detection limit for bacteria was 5.7×10^4 cfu per test. At protein level, the intensity of the test line was correlated with

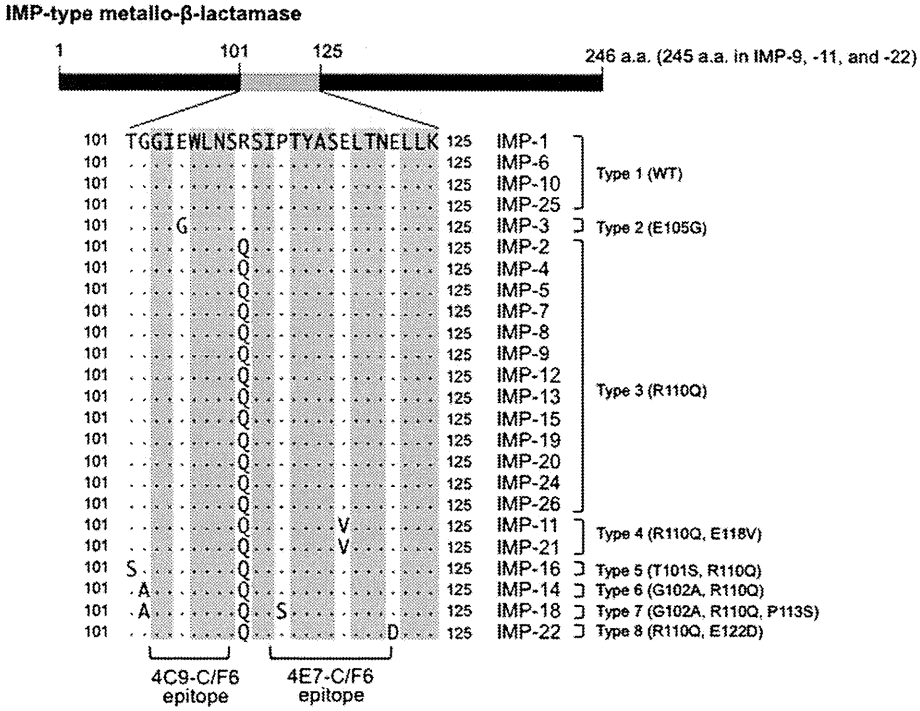


Fig. 4. Comparison of amino acid sequences in the epitope regions of IMP-type MBLs. The regions in the 24 subtypes of IMP-type MBLs corresponding to the 101–125 region in IMP-1 were compared. The dots indicate the residues identical to the IMP-1 sequence. The completely conserved amino acids are shaded. The amino acids are shown as single letters.

the amount of IMP-1 in the range of 0.1–10 ng, indicating a high degree of linearity ($r^2 = 0.9407$) (data not shown). The theoretical detection limit for antigens was 0.3 ng per test.

Taken together, the data strongly suggest that the developed assay meets the requirements for the rapid detection of IMP-type MBLs produced by Gram-negative bacteria in the clinical laboratory.

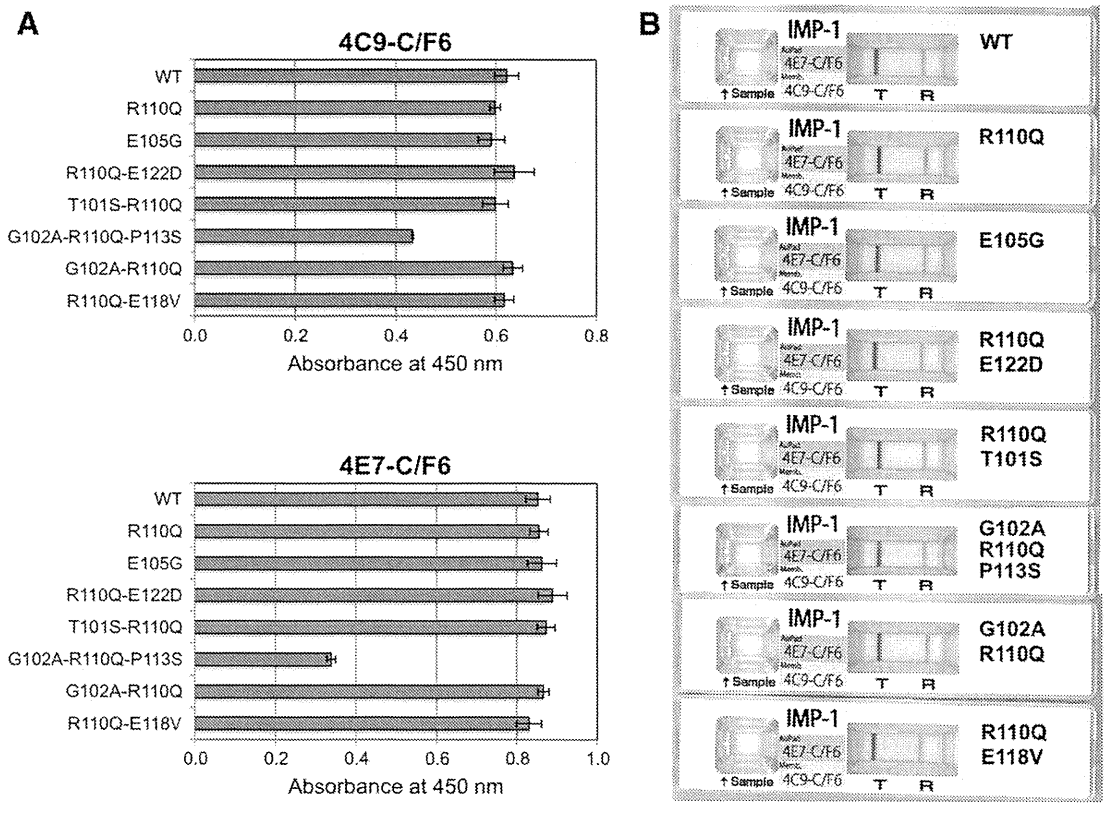


Fig. 5. Evaluation of mAbs and the assay using IMP mutants. (A) Interaction between mAbs and IMP mutants by ELISA. The purified IMP mutant proteins were immobilized onto an EIA plate, and the interaction between mutants and mAb was detected with anti-rat IgG goat antibody. (B) Immunochromatographic detection of IMP mutants. One hundred nanograms of protein of mutant per test were analyzed using the developed assay.

Table 4
Diagnostic performance of the IMP-immunochromatographic assay compared with PCR detection of bla_{IMP}.

Result of PCR detection	No. of isolates ($N=248$)	Result of IMP-immunochromatographic assay		Specificity (%)	Sensitivity (%)
		Positive	Negative		
Positive	191	191	0	100	100
Negative	57	0	57		

4. Discussion

Immunodiagnostic assays rely on specific antigen–antibody interactions for accurate results. ELISA, immunofluorescent antibodies, and immunochromatographic assay are often employed in the diagnosis of several infectious diseases. Of these, immunochromatographic assay is the most commonly used diagnostic method owing to its ease of handling.

In this study, we focused on the production of exogenous enzymes that mediate antibiotic resistance in bacteria, and designed a novel immunochromatographic assay to detect IMP-type MBLs produced by nosocomial pathogens such as *P. aeruginosa*. To our knowledge, this is the first report on an immunological detection assay using antibodies specific for IMP-type MBLs.

The assessment of the assay using clinical isolates of *P. aeruginosa* showed that the assay yielded no false positives and negatives. Additionally, all positive strains were resistant to imipenem (MIC≥16 μg/mL). These results indicate that the immunochromatographic assay developed in this study is a reliable technique for rapid detection of IMP-type MBLs. Although PCR is a reliable detection method, it requires special equipment such as micropipettes, thermal cycler, and agarose gel electrophoresis apparatus. Given that immunochromatographic detection does not require the aforementioned equipment, it may be more suitable for clinical laboratory use compared with PCR detection. Nonetheless, the PCR method will remain to be a powerful technique for the identification of novel mutations in target genes.

The tested samples were prepared using a buffer containing nonionic detergent from bacterial colonies. Given that the developed assay was able to detect the IMP-type MBLs produced by *P. putida*, *A. baumannii*, and *A. xylosoxidans* in addition to *P. aeruginosa*, the sample extraction strategy with detergent is considered efficient for lysing Gram-negative bacterial cells. The antibiotic resistance gene can be transferred across bacteria through plasmids, transposons, and integrons. Indeed, many reports have shown the carriage of bla_{IMP} genes in Gram-negative bacteria. Thus, the data presented in this study support that the assay could be widely adopted in routine work in the screening for carbapenem-resistant bacteria producing IMP-type MBLs.

The analyses using IMP mutants suggested that the assay constructed using 4E7–C/F6 and 4C9–C/F6 has a potential to detect all known IMP-type MBLs. However, it could not detect all carbapenem-resistant bacteria because carbapenem resistance in bacteria is mediated by several classes of MBLs. Although the amino acid sequence analyses have shown that the IMP epitope region recognized by 4E7–C/F6 and 4C9–C/F6 is antigenically distinct from other classes of MBLs such as NDM-1, GIM-1, and SIM-1, further evaluation including clinical trials will be required to elucidate the cross-reactivity of the assay with other types of MBLs.

In recent years, nosocomial infections caused by antibiotic-resistant bacteria have become more complex. The occurrence of MDR nosocomial pathogens, in particular, is currently regarded as a serious medical problem. The determinant of antibiotic resistance in nosocomial bacteria varies with geographical location. The immunochromatographic assay can adopt multiple test lines using various antibodies against different antigens of interest; it therefore has potential for wide use in infection-control measures and epidemiological researches. In Japan, *P. aeruginosa* isolate showing resistance to carbapenem (MIC≥16 μg/ml), amikacin (MIC≥32 μg/ml), and fluoroquinolone (MIC≥4 μg/ml) is defined as MDR *P. aeruginosa* according to the criteria established by the Japanese Ministry of Health, Labor, and Welfare (Kirikae et al., 2008). In our laboratory, further work is in progress to design an immunochromatographic assay recognizing both AAC(6′)-Iae and IMP-type MBLs to survey the prevalence of these enzymes among the MDR clinical strains in Japan.

5. Conclusion

The findings presented in this study indicate that the newly developed assay is a highly sensitive, reliable, easy-to-use, and rapid immunological method for diagnosing the production of IMP-type MBLs in *Pseudomonas*. It will be useful for infection-control measure and a crucial aspect of antimicrobial chemotherapy, and may also useful to investigate epidemiological information about carbapenem-resistant Gram-negative bacteria.

Supplementary materials related to this article can be found online at doi:10.1016/j.mimet.2011.09.011.

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Genome Sequence of Multidrug-Resistant *Pseudomonas aeruginosa* NCGM1179

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We report the annotated genome sequence of multidrug-resistant *Pseudomonas aeruginosa* strain NCGM1179, which is highly resistant to carbapenems, aminoglycosides, and fluoroquinolones and is emerging at medical facilities in Japan.

Pseudomonas aeruginosa is a Gram-negative rod bacterium of the *Pseudomonadaceae* family of bacteria. It is an opportunistic pathogen, causing urinary tract infections, respiratory system infections, dermatitis, bacteremia, and a variety of systemic infections, particularly in immunosuppressed patients (11). *P. aeruginosa* is intrinsically resistant to many antibiotics and has a remarkable capacity for acquiring new resistance mechanisms under selective pressure of antibiotics; therefore, the emergence of multidrug-resistant (MDR) *P. aeruginosa* with resistance to aminoglycosides, beta-lactams, and fluoroquinolones poses serious problems for medical facilities in various countries (2, 3, 6, 7, 12), including Japan (4, 9, 10).

MDR *P. aeruginosa* NCGM1179 was isolated from the respiratory tract of an inpatient in Japan in 2010. A further 16 isolates with identical patterns of pulsed-field gel electrophoresis were obtained from respiratory tracts of hospitalized patients among 10 prefectures in the same year, indicating that the NCGM1179 strain was emerging at medical facilities throughout Japan. The strain was highly resistant to carbapenems, aminoglycosides, and fluoroquinolones, with MIC_{90}s of more than 64 μg/ml, and produced IMP-type metallo-β-lactamase and aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)]-Iae (5, 8).

The genome of strain NCGM1179 was sequenced using a GS FLX Titanium sequencer using Pyrosequencing technology. We obtained a total of 863,079 reads, covering a total of 232,282,665 bp. The number of contigs (over 100 bp) was 290, and the number of bases was 6,735,052 bp. The number of contigs (over 500 bp) was 258, and the number of bases was 6,727,128 bp. The number of scaffolds was 25, and that of bases was 7,014,004. The largest scaffold size was 6,910,294 bp. The genome of strain NCGM1179 has a G+C content of 66.0%, and the draft assemblies contained 6,213 potential protein-coding sequences, 61 tRNA and 1 transfer messenger RNA (tmRNA). Primary coding sequence extraction and initial functional assignment were performed by the RAST (Rapid Annotation using Subsystem Technology) automated annotation servers (1). Their results were compared to verify the annotation and were corrected manually by *in silico* molecular cloning (In Silico Biology, Inc., Kanagawa, Japan).

Nucleotide sequence accession numbers. Nucleotide sequences of the chromosome of *P. aeruginosa* NCGM1179 have been deposited in the DNA Database of Japan under accession no. DF126593 to DF126613.

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Complete Genome Sequence of Highly Multidrug-Resistant *Pseudomonas aeruginosa* NCGM2.S1, a Representative Strain of a Cluster Endemic to Japan

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We report the completely annotated genome sequence of *Pseudomonas aeruginosa* NCGM2.S1, a representative strain of a cluster endemic to Japan with a high level of resistance to carbapenem (MIC ≥ 128 μg/ml), amikacin (MIC ≥ 128 μg/ml), and fluoroquinolone (MIC ≥ 128 μg/ml).

The emergence of multidrug-resistant (MDR) *P. aeruginosa* strains is a serious problem in Japan (2, 6, 7). The MDR *P. aeruginosa* strain NCGM2.S1 caused an outbreak of urinary tract infection at a hospital in Miyagi Prefecture, northern Japan (6). NCGM2.S1 harbors a metallo-β-lactamase gene, *blaIMP-1*, and an aminoglycoside 6′-*N*-acetyltransferase gene, *aac(6′)-Iae*, in the class I integron In113 (6). Epidemiological studies indicated that clonal expansion of NCGM2.S1 occurred in hospitals in this area (7) as well as other areas in Japan (4, 8). We developed kits to detect *aac(6′)-Iae* and AAC(6′)-Iae, which were used to survey MDR *P. aeruginosa* strains (3, 7).

The genome of *P. aeruginosa* was sequenced using a Roche FLX Titanium genome sequencer. We obtained a total of 532,063 reads, covering a total of 6,697,230 bp, or 28.9-fold coverage. Sequences were assembled into a total of 270 contigs. Gaps were filled by Sanger sequencing of PCR products by brute force amplification of the regions between contigs. Primary CDS extraction and initial functional assignment were performed using the RAST automated annotation servers (1). The results were compared to verify the annotation and were corrected manually by *in silico* molecular cloning (In Silico Biology, Inc., Kanagawa, Japan). The *P. aeruginosa* NCGM2.S1 genome consists of a single circular chromosome of 6,764,661 bp, with an average GC content of 66.1%. The chromosome was shown to contain a total of 6,271 protein-coding genes, 77 tRNA genes, 1 tmRNA for all amino acids, and 4 *rrn* operons. In addition, the chromosome harbors 6 prophage-like elements.

Although *P. aeruginosa* NCGM2.S1 is a representative strain of an endemic cluster showing a high level of multidrug resistance in Japan, it does not have any plasmids. Instead, the chromosome was shown to harbor the class I integron In113 carrying *aac(6′)-Iae* and *blaIMP-1*, which are responsible for high levels of resistance to aminoglycosides and β-lactams, respectively. Of note, In113 is inserted into the middle of *oprD*, resulting in complete disruption of the gene. OprD is responsible for sensitivity to imipenem, and its reduced expression increases the level of resistance (5). Analysis of the complete NCGM2.S1 genome sequence strongly suggested that *P. aeruginosa* acquires drug resistance not only by obtaining drug resistance genes but also by disrupting the genes involved in drug sensitivity.

Nucleotide sequence accession number. The nucleotide sequence of the chromosome of *P. aeruginosa* NCGM2.S1 has been deposited in the DNA Database of Japan under accession no. AP012280.

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Short communication

Emergence of a novel multidrug-resistant *Pseudomonas aeruginosa* strain producing IMP-type metallo-β-lactamases and AAC(6′)-Iae in Japan

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ABSTRACT

The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases (MBLs) and aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] has become a serious problem in medical settings in Japan. A total of 217 MDR *P. aeruginosa* isolates were obtained from August 2009 to April 2010 from patients at 144 hospitals in Japan, of which 145 (66.8%) were positive for IMP-type MBLs and AAC(6′)-Iae when tested with an immunochromatographic assay. Polymerase chain reaction (PCR) showed that these isolates were also positive for *bla*IMP and *aac(6′)-Iae* genes. When these IMP-type MBL- and AAC(6′)-Iae-producing isolates were analysed by pulsed-field gel electrophoresis (PFGE), two clusters (I and II) were detected. Most of the isolates (88.3%; 128/145) were grouped under cluster I and had multilocus sequence type ST235 and serotype O11, except for one isolate that was ST991 and serotype O3. The isolates were mainly isolated from the urinary tract (82/145; 56.6%) and respiratory tract (58/145; 40.0%). The epidemiological properties of the isolates belonging to cluster I were similar to those of MDR *P. aeruginosa* isolates that have been previously reported in Japan. The remaining 16 isolates belonged to cluster II, had identical PFGE patterns and were multilocus sequence type ST991 and serotype O18; all of these isolates were isolated from the respiratory tract. The properties of isolates belonging to cluster II have not been previously described, indicating that a novel IMP-type MBL- and AAC(6′)-Iae producing *P. aeruginosa* strain is emerging in Japan. Isolates belonging to both clusters were isolated from different parts of the country.

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1. Introduction

The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates, which are resistant to all β-lactams, aminoglycosides and fluoroquinolones, is a serious medical problem in Japan. MDR *P. aeruginosa* are defined as strains showing resistance to carbapenems [minimum inhibitory concentration (MIC) ≥ 16 μg/mL], amikacin (AMK) (MIC ≥ 32 μg/mL) and fluoroquinolones (MIC ≥ 4 μg/mL) based on the criteria specified by the Ministry of Health, Labour, and Welfare of Japan [1].

MDR *P. aeruginosa* isolates in Japan frequently produce IMP-type metallo-β-lactamases (MBLs) and aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] [2–4]. Therefore, we recently designed immunochromatographic assay kits for the detection of IMP-type MBL- [4] and AAC(6′)-Iae producing *P. aeruginosa* [3].

In this study, 145 isolates of MDR *P. aeruginosa* were randomly obtained from 89 medical settings to perform a nationwide epidemiological study on IMP-type MBL- and AAC(6′)-Iae producing MDR *P. aeruginosa* in Japan.

2. Materials and methods

2.1. Bacterial strains

A total of 217 clinical isolates of *P. aeruginosa* resistant to imipenem (IPM) (MIC ≥ 16 μg/mL), AMK (MIC ≥ 32 μg/mL) and ciprofloxacin (CIP) (MIC ≥ 4 μg/mL) were obtained from 144 hospitals located in 31 of the 47 prefectures in Japan from August 2009 to April 2010. The strains were isolated from the urinary tract (n = 111), respiratory tract (n = 94) and other systems of patients (n = 12). MDR *P. aeruginosa* strains NCGM2.S1 [5] and NCGM1179 were used as reference strains. NCGM1179 [6] strain was one of the 217 isolates.

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