Acriflavine was used to define antiseptic resistance, because antiseptics other than acriflavine show narrow MIC ranges against MRSA (Noguchi et al., 2005). The *qacA/B*-LAMP assay failed to show 100% specificity for antiseptic-resistant MRSA isolates, although the combination of *femB*-LAMP and *mecA*-LAMP achieved 100% specificity to MRSA isolates. These observations can be explained by the presence of antiseptic resistance genes other than *qacA* and *qacB*, such as *qacC* (Mayer et al., 2001), *qacG* (Heir et al., 1999), *qacH* (Heir et al., 1998), *qacJ* (Bjorland et al., 2003), and *smr* (Noguchi et al., 1999). In addition, antiseptic resistance gene-independent acriflavine-resistant MRSA has been reported previously (Kawai et al., 2009). The resistance is supposed to be related to cell wall thickness of MRSA (Kawai et al., 2009). Nevertheless, a survey of *qacA/B* using the *qacA/B*-LAMP assays will provide useful information about expansion of antiseptic-resistant MRSA isolates, because a positive predictive value of 92.5% was obtained in a high-level acriflavine-resistant group and a negative predictive value of 100% was obtained in an acriflavine susceptible group.

4. Conclusion

Combined use of *femB*-, *mecA*-, and *qacA/B*-LAMP assays will be useful to assess the control and efficacy of preventive measures against antiseptic-resistant MRSA in medical settings, and will also be useful to investigate epidemiological information about staphylococci with regard to both antibiotic and antiseptic resistance.

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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	CATAGCGACAGCtCGGCGGAATAGAGTGG
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 + + +.

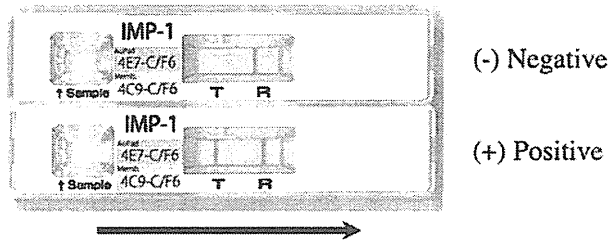


Fig. 1. Immunochromatography developed using mAbs 4E7-C/F6 and 4C9-C/F6. In the case of negative results, a single line appears at the position of the reference line (R) only. In the case of positive results, another line also appears at the position of the test line (T) in addition to the reference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

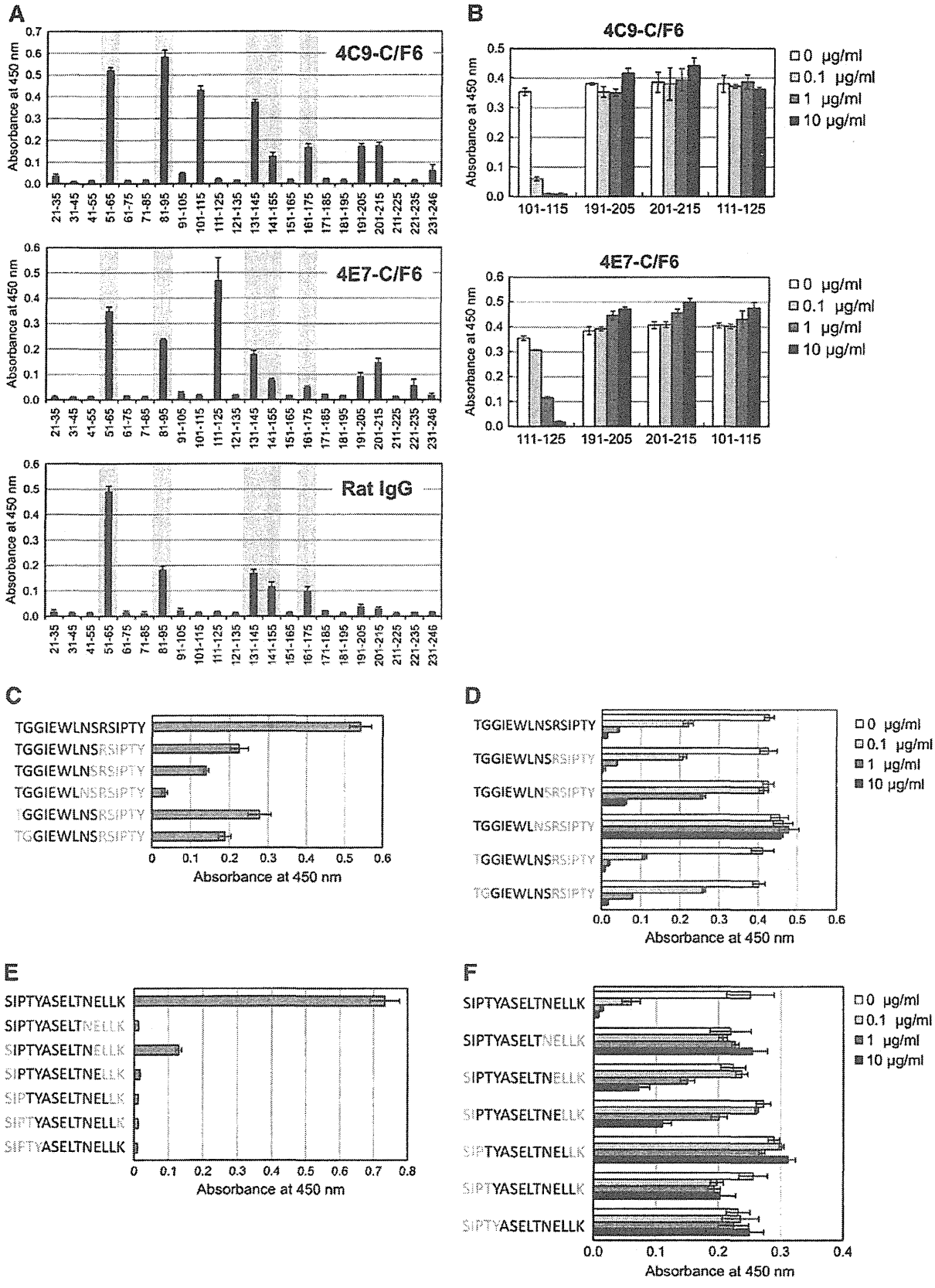
A competitive assay using the candidate peptides was also performed (Fig. 2B). The recognition of IMP-1 by 4C9-C/F6 was significantly inhibited by only peptide 101–115 inhibited in a dose-dependent manner. The recognition of IMP-1 by 4E7-C/F6 was significantly inhibited by only peptide 115–125 in a dose-dependent 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.



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 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

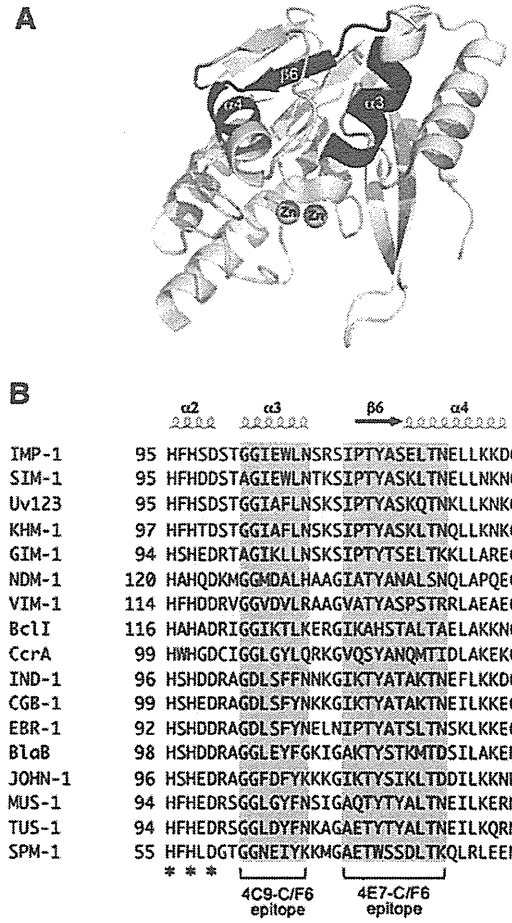


Fig. 3. Sequence alignment of the epitope region among MBLs. (A) Epitope region on the three-dimensional structure of IMP-1. The figure was created with MacPymol software according to the IMP-1 structure (PDB ID: 2DOO) determined by Dr. Kurosaki (Kurosaki et al., 2006). The black part presents the region 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.

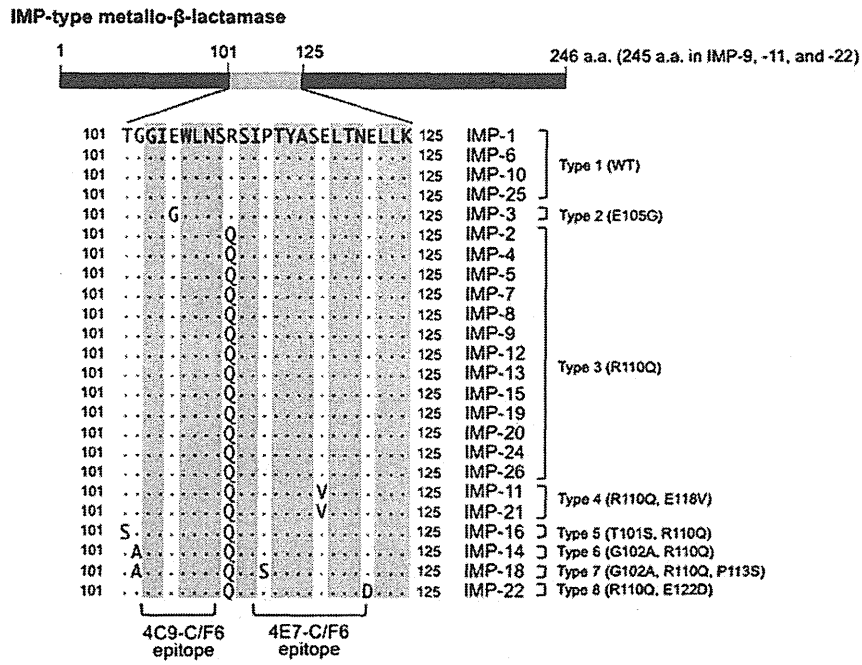


Fig. 4. Comparison of amino acid sequences in the epitope regions of IMP-type MBLs. The regions in the 24 subtypes of IMP-type MBLs corresponding to the 101–125 region in IMP-1 were compared. The dots indicate the residues identical to the IMP-1 sequence. The completely conserved amino acids are shaded.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

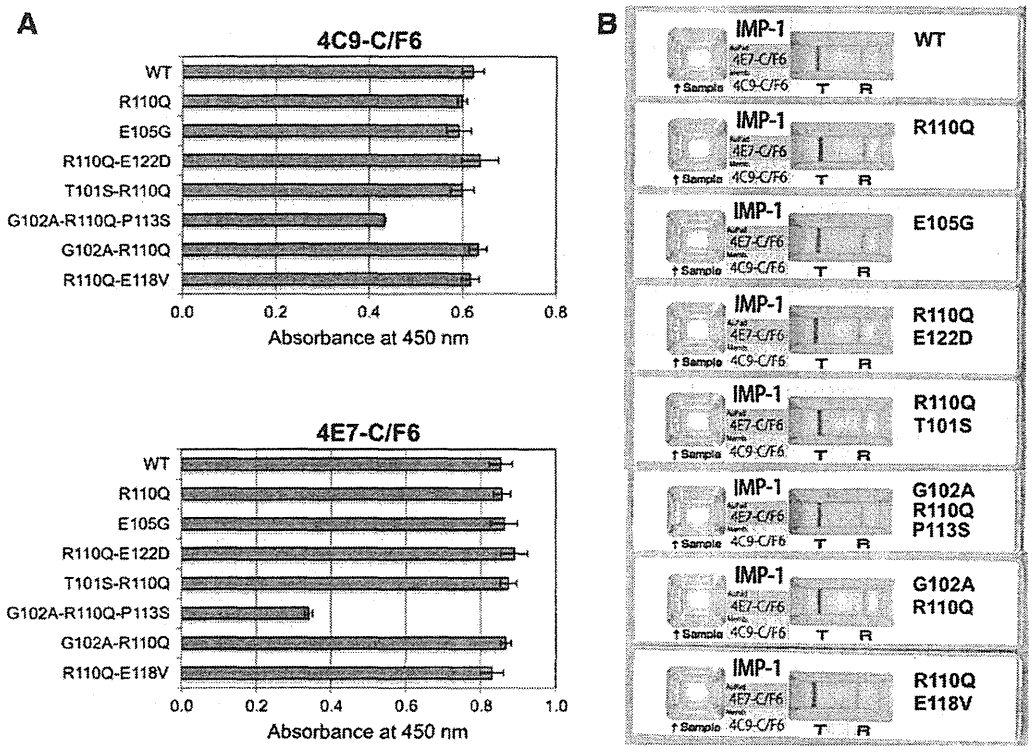


Fig. 5. Evaluation of mAbs and the assay using IMP mutants. (A) Interaction between mAbs and IMP mutants by ELISA. The purified IMP mutant proteins were immobilized onto an EIA plate, and the interaction between mutants and mAb was detected with anti-rat IgG goat antibody. (B) Immunochromatographic detection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

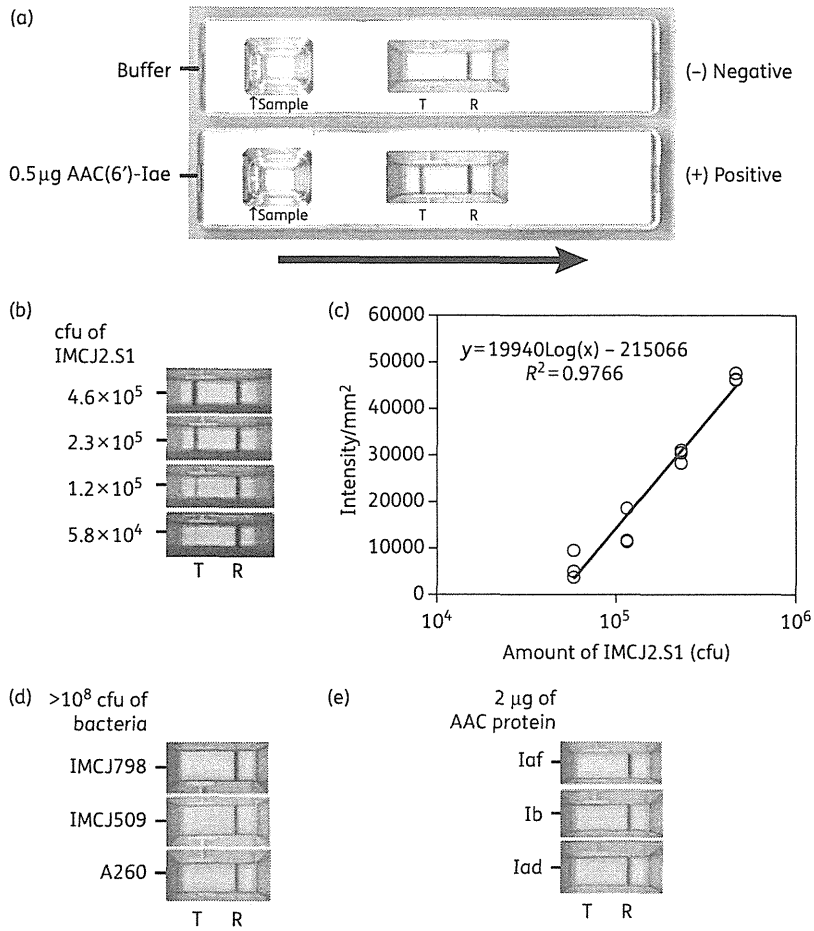

Figure 1. (a) Immunochromatography using MAbs 1H7 and 3F12. In negative cases, a single line appears at the position of the reference line (R) only. In positive cases, another line also appears at the position of the test line (T) in addition to the reference line. The arrow indicates the direction of 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

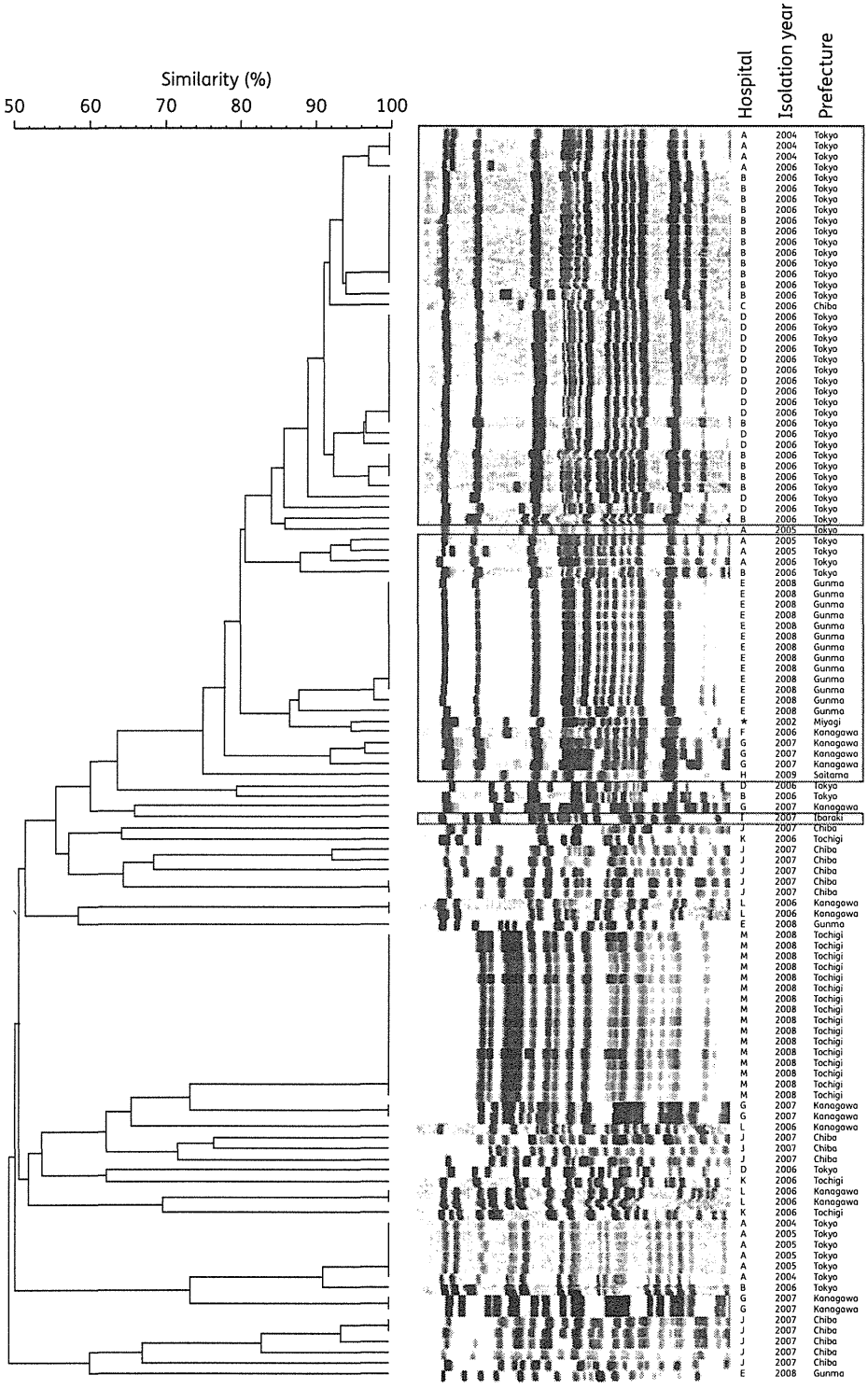

Figure 2. PFGE assay of 116 *P. aeruginosa* clinical isolates used in the assessment of the developed immunochromatography assay. The asterisk indicates the *P. aeruginosa* IMCJ2.S1 strain previously found in Miyagi. Areas enclosed within squares indicate the positive isolates detected.

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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Rapid detection of *Pseudomonas aeruginosa* in mouse feces by colorimetric loop-mediated isothermal amplification

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ABSTRACT

A colorimetric loop-mediated isothermal amplification (LAMP) assay with hydroxy naphthol blue was designed to amplify a region in the outer membrane lipoprotein (*oprL*) gene of *Pseudomonas aeruginosa*. The LAMP assay showed 100% specificity for the serogroup and other bacteria, and the sensitivity was 10-fold higher than that of the PCR assays. The LAMP assay could detect *P. aeruginosa* inoculated in mouse feces at 130 colony-forming units (CFU)/0.1 g feces (3.25 CFU/reaction). The assay was completed within 2 h from DNA extraction. In a field trial, the LAMP assay revealed that none of the 27 samples was obtained from 2 specific pathogen-free (SPF) mouse facilities that were monitoring infection with *P. aeruginosa*; 1 out of 12 samples from an SPF mouse facility that was not monitoring infection with *P. aeruginosa* and 2 out of 7 samples from a conventional mouse facility were positive for *P. aeruginosa*. In contrast, *P. aeruginosa* was not detected in any of the samples by a conventional culture assay. Thus, this colorimetric LAMP assay is a simple and rapid method for *P. aeruginosa* detection.

1. Introduction

Pseudomonas aeruginosa, a gram-negative obligate aerobe, is found in many natural and man-made environments; it has been isolated from plants (Walker et al., 2004); soils (Green et al., 1974); water (Pellett et al., 1983; Blasco et al., 2008); and warm, moist environments containing very low levels of organic material (van der Kooij et al., 1982). This organism is an opportunistic pathogen that infects humans and animals, and it is resistant to many antibiotics. In humans, *P. aeruginosa* is a common cause of nosocomial infections (Sekiguchi et al., 2007) in burn patients (Church et al., 2006) and other immunocompromised patients, including transplant (Johnson et al., 2009), cancer (Elting et al., 1997), and acquired immune deficiency syndrome patients (Meynard et al., 1999). In addition, *P. aeruginosa* is a major cause of morbidity and subsequent mortality in patients with cystic fibrosis (Cheng et al., 1996; Jones et al., 2001). *P. aeruginosa* is an infectious agent sometimes found in specific pathogen-free (SPF) laboratory rodents. Inbred mouse strains show different susceptibility to experimental *P. aeruginosa*-induced lung infection: BALB/c mice are resistant, C57BL/6 and A/J mice are relatively susceptible with low mortality, and DBA/2 mice are extremely susceptible with high mortality (Stotland et al., 2000). Immunodeficient rodents such as athymic nude mice, severe combined deficiency mice, and rodents immunosuppression caused by X-ray irradiation and steroid treatment are also susceptible to *P. aeruginosa* infection. Affected rodents show clinical signs of *P. aeruginosa* infection, including the hunchback posture, ruffled coat, apathy, shortness of breath, oblique head posture, circus movement, and emaciation (Baker, 1998; Cryz et al., 1983; Dietrich et al., 1996; Taffs, 1974). Spread of *Pseudomonas* infection to multiple organs causes tissue lesions. Necrotic foci, abscess formation, and suppuration are observed in the liver, lung, and kidney of infected mice (Gaydos et al., 1975). It is possible that natural *P. aeruginosa* infections affect the findings of researches that use SPF rodents.

Identification of *P. aeruginosa* in the clinical laboratory is generally performed by growing the bacteria on either cetrimide agar or nalidixic acid-cetrimide (NAC) agar. Although this method is reliable, the time required for performing it is up to 48 h. Polymerase chain reaction (PCR) methods, which allow for more rapid identification of *P. aeruginosa* by DNA amplification, have been reported (da Silva Filho et al., 1999; De Vos et al., 1997; Jaffe et al., 2001; Khan and Cerniglia, 1994; Lavenir et al., 2007; McIntosh et al., 1992; Spilker et al., 2004; Xu et al., 2004).

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However, the PCR method has some drawbacks: (i) the device used is expensive; (ii) the amplification specificity is highly influenced by the primer design and reaction conditions; (iii) gel electrophoretic analysis of the DNA product is required for confirming DNA amplification.

Loop-mediated isothermal amplification (LAMP), auto-cycling, and strand displacement DNA synthesis have been reported as possible replacements for PCR, and used for the detection of specific gene sequences (Notomi et al., 2000). The LAMP reaction is carried out with a set of 4 oligonucleotide primers, which recognize 6 distinct regions on the target DNA, at a constant temperature ranging from 60 °C to 65 °C. Nagamine et al. (2002) reported that addition of 1 or 2 loop primers accelerates the reaction. Insoluble magnesium pyrophosphate as well as DNA is synthesized during the LAMP reaction, and a large amount of Mg^{2+} is consumed in the reaction. As a result, the concentration of Mg^{2+} in the solution decreases. We reported that hydroxy naphthol blue, a metal ion indicator, is a useful indicator for the LAMP reaction; it allows easy visualization of the reaction—the color changes from violet to sky blue when the reaction is positive (Goto et al., 2009). The sensitivity of LAMP for gene sequence detection is equal to or higher than that of PCR, and this method requires less time (Kudo et al., 2007; Yoneyama et al., 2007).

This paper is the first to report the use of the LAMP assay for the detection of *P. aeruginosa*. A specific primer set was designed to detect the outer membrane peptidoglycan-associated lipoprotein (*oprL*) gene of *P. aeruginosa*. This colorimetric LAMP assay can detect *P. aeruginosa* DNA in mouse feces within 70 min.

2. Materials and methods

2.1. Bacterial strains and DNA preparation

The bacterial strains used in this study are listed in Table 1. Unless otherwise stated, they were stocked in the Department of Infectious Diseases, Research Institute, International Medical Center of Japan (IMCJ). Between June 2006 and December 2009, we obtained 47 isolates of *P. aeruginosa*, 16 of *Acinetobacter baumannii*, 1 of *Enterococcus faecium*, 1 of *Proteus mirabilis*, 1 of *Serratia marcescens*, and 2 of *Staphylococcus aureus* from inpatients at Toyama Hospital, IMCJ. In addition, 2 isolates of *Pseudomonas pseudoalcaligenes* and 3 of *P. putida* were obtained from surface environments in the same hospital. *P. aeruginosa*, *P. alcaligenes*, *P. fluorescens*, *P. pseudoalcaligenes*, and *P. putida* were cultured aerobically overnight at 37 °C on brain heart infusion (BHI) agar. *Pseudomonas citronellolis* and 5 strains of *P. syringae* were cultured at 30 °C aerobically overnight on BHI agar. *Pseudomonas resinovorans* was cultured at 30 °C aerobically overnight on Luria-Bertani agar. *A. baumannii*, *E. faecium*, *Escherichia coli*, *P. mirabilis*, *Salmonella* Enteritidis, *Salmonella* Typhimurium, *S. marcescens*, and *S. aureus* were also used in order to determine the specificity of the assay. *A. baumannii* was cultured at 44 °C aerobically overnight on BHI agar. *Mycobacterium kansasii* was cultured at 37 °C aerobically on Ogawa medium for 2 weeks. All the other bacteria were cultured at 37 °C aerobically on BHI agar. A loopful of cultured cells was suspended in 0.5 mL of 10 mM Tris–EDTA (TE) buffer (pH 7.5), and 0.1 mL of the suspension was boiled for 10 min. The cellular debris were precipitated at 13,000×*g* for 5 min, and the supernatant containing the genomic DNA was used for both the LAMP and PCR assays.

Table 1
Specificity of LAMP and PCR assays on panels of *P. aeruginosa* strains, species of pseudomonads and other bacteria.

Species	Source (strain)	No.	No. of positive strains		
			LAMP	*ecfX* PCR	*oprL* PCR
Pseudomonas strains					
P. aeruginosa	ATCC 47085	1	1	1	1
	ATCC 27853	1	1	1	1
	Clinical isolates	47	47	47	47
P. alcaligenes	JCM20561	1	0	0	0
P. citronellolis	JCM21587	1	0	0	1
P. fluorescens	JCM5963	1	0	0	0
P. pseudoalcaligenes	Clinical isolates	2	0	0	0
P. putida	Clinical isolates	3	0	0	0
P. resinovorans	LMG2274	1	0	0	0
P. syringae	Pathovars[a]	5	0	0	0
Non-Pseudomonas strains					
Acinetobacter baumannii	NCB0211-439	1	0	0	0
	Clinical isolates	16	0	0	0
Enterococcus faecium	Clinical isolate	1	0	0	0
Escherichia coli	ATCC 8739	1	0	0	0
Mycobacterium kansasii	JCM6379	1	0	0	0
Proteus mirabilis	Clinical isolate	1	0	0	0
Salmonella Enteritidis	No. 11	1	0	0	0
S. Typhimurium	ATCC 15277	1	0	0	0
Serratia marcescens	Clinical isolate	1	0	0	0
Staphylococcus aureus	Clinical isolates	2	0	0	0

ATCC, American Type Culture Collection, Rockville, MD, USA.
Clinical isolates were collected from patients visiting the hospital or nosocomical environments, IMCJ.
JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan.
[a] *P. syringae* pv. *coronafaciens*; *P. syringae* pv. *maculicola*; *P. syringae* pv. *phaseolicola*; *P. syringae* pv. *pisi*; *P. syringae* pv. *syringae*.

2.2. Limiting dilution experiment

A loopful of *P. aeruginosa* PAO1 was suspended in 500 µL distilled water. The resulting solution was diluted to 10^{-7}. A volume of 100 µL of the mixture was spread on each of the 3 NAC agar plates. The plates were then incubated under aerobic conditions overnight at 37 °C. The resulting colonies were counted in order to estimate the number of colony-forming units (CFU) per dilution tube. Another 100 µL of the original suspension was mixed with 1 µL of 1% Triton X-100, boiled for 5 min, and then diluted to 10^{-7} in TE buffer. Each dilution was used as a template for the LAMP and PCR assays described below. The detection limit of LAMP and PCR was estimated from the serial dilutions.

2.3. Inoculation test

P. aeruginosa-free fecal samples that had been inoculated with known numbers of CFU of *P. aeruginosa* PAO1 were used in the inoculation test. A 0.1-g sample of dried feces (about 10–13 lumps of feces) from an 8-week-old C.B-17/Icr-*scid*/*scid* mouse (CLEA, Tokyo, Japan) was inoculated with 50 µL of a suspension containing 10-fold serial dilutions of *P. aeruginosa* ranging from 1.3 to 1.3×10^6 CFU. Distilled water was used as a negative control; 50 µL of it was dropped on 0.1 g of dried feces. DNA was extracted from the fecal samples with a ZR Fecal DNA kit (Zymo Research, CA, USA). We followed the manufacturer's instructions, but with the following modifications: 0.1 g of dried feces was incubated in 750 µL of the lysis solution at room temperature for 30 min, and the sample was lysed by subjecting it to bead beating for 5 min. The DNA was eluted in 40 µL of DNA elution buffer, which was supplied with the kit.

2.4. DNA extraction and culturing of fecal samples

Dried fecal samples obtained from 46 mouse cages were collected from 3 SPF facilities and 1 conventional mouse facility. The samples were weighed and divided into 0.1-g portions. DNA was then extracted from these portions with the ZR Fecal DNA kit as described above. These portions were cultured in NAC medium at 37 °C aerobically for 48 h, and then each 100 µL of the supernatant was further cultured on a NAC agar plate at 37 °C for 24 h.

2.5. Design of LAMP oligonucleotide primers

The oligonucleotide primers used for the LAMP assay were designed using the *oprL* gene (GenBank, accession no. Z50191) of *P. aeruginosa*. A set of 6 primers consisting of 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP), which recognized 6 distinct regions on the target sequence, and 2 loop primers (LF and LB) were designed with the PrimerExplorer V4 software (http://primerexplorer.jp/e/). The LAMP primer sequences were as follows: F3, 5′-GCG TTG CCG CCA ACA ATG-3′; B3, 5′-CAT GCG GGC AAC CTC TC-3′; FIP, 5′-GTT GTC ACC CCA CCT CCG GGC GGC AAC GTT CCT CC-3′; BIP, 5′-CTC CGT GCA GGG CGA ACT GCA GGC GAG CCA ACT C-3′; LF, 5′-ACC TGC CGT GCC ATA CC-3′; LB, 5′-GTT CAT GCA GCT CCA GCA G-3′ (Fig. 1). All the primers used in this study were synthesized by Invitrogen (Tokyo, Japan).

2.6. LAMP assay

The LAMP assays were carried out with a 25-μL reaction mixture containing 1 μL of 1:100 diluted DNA extracted from the culture fluids or DNA extracted from fecal samples, 1.6 μM each of FIP and BIP, 0.2 μM each of F3 and B3, 0.8 μM each of LF and LB, 1.4 mM of each dNTP, 8 U of the large fragment of *Bst* DNA polymerase (New England BioLabs, Tokyo, Japan), and 120 μM hydroxy napthol blue trisodium salt (HNB, CAS No. 63451-35-4; Dojindo, Kumamoto, Japan) in the LAMP buffer (20 mM Tris–HCl [pH 8.8], 8 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, and 0.8 M betaine). The DNA extracts from the fecal samples were boiled for 5 min before use. Distilled water was used instead of the DNA extract in the negative control. All the reaction mixtures were incubated at 63 °C for 1 h and were then heated at 80 °C for 2 min in a Mastercycler ep gradient S thermocycler (Eppendorf, Hamburg, Germany) to terminate the reaction. The results were considered to be negative when the color of the solution was violet and positive when the color was sky blue.

2.7. PCR assay

Two PCR methods were used to detect *P. aeruginosa*. One method (*oprL* PCR) was originally reported by De Vos et al. (1997) and targeted the *oprL* gene. The following primers were used in this method: PAL1, 5′-ATG GAA ATG CTG AAA TTC GGC-3′; PAL2, 5′-CTT CTT CAG CTC GAC GCG ACG-3′ (Fig. 1). The other method (*ecfX* PCR) was originally reported by Lavenir et al. (2007) and targeted the *ecfX* gene. The following primers were used in *ecfX* PCR: ECF1, 5′-ATG GAT GAG CGC TTC CGT G-3′; ECF2, 5′-TCA TCC TTC GCC TCC CTG-3′. PCR was performed with a 25-μL reaction mixture containing 1 μL of the DNA extract, 0.5 μM each of the primers, 0.2 mM of each dNTP, and 1.25 U of *Taq* DNA polymerase (New England BioLabs) in a ThermoPol reaction buffer (20 mM Tris–HCl [pH 8.8], 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 10% DMSO [for the *ecfX* target]). The samples were placed in a thermocycler and were subjected to the following thermocycling process in the given order: an initial denaturation step at 94 °C for 5 min; 35 cycles at 94 °C for 20 s, 57 °C (for *oprL* PCR) or 58 °C (for *ecfX* PCR) for 20 s, 72 °C for 45 s; and a final elongation step at 72 °C for 5 min. In the negative control, the DNA extract was substituted with distilled water. The PCR products were electrophoresed on 2% agarose gel along with a 1 kb Plus DNA Ladder (Invitrogen). The gel was stained with ethidium bromide, and the amplicons were visualized under an ultraviolet transilluminator. Each PCR was carried out in triplicate.

3. Results

3.1. Specificity of the LAMP assay compared to the PCR assays

The specificity of the LAMP and PCR assays was examined with genomic DNA from various species of bacteria. The results of the LAMP assay were positive for all *P. aeruginosa* strains and isolates tested, whereas they were negative for 7 other *Pseudomonas* species and all non-*Pseudomonas* bacteria tested (Table 1). The same results were obtained in *ecfX* PCR (Table 1), and essentially similar results were obtained in *oprL* PCR; however, *P. citronellolis* was detected by *oprL* PCR but not by LAMP and *ecfX* PCR (Table 1).

3.2. Sensitivity and rapidity of the LAMP assay compared to the PCR assays

The stock solution of *P. aeruginosa* PAO1 genomic DNA (1.2×10^9 CFU/mL) was serially diluted in 10-fold increments, and each dilution was assayed by LAMP and PCR (Table 2). Genomic DNA was detected up to a dilution of 10^{-5} by LAMP; this corresponded to

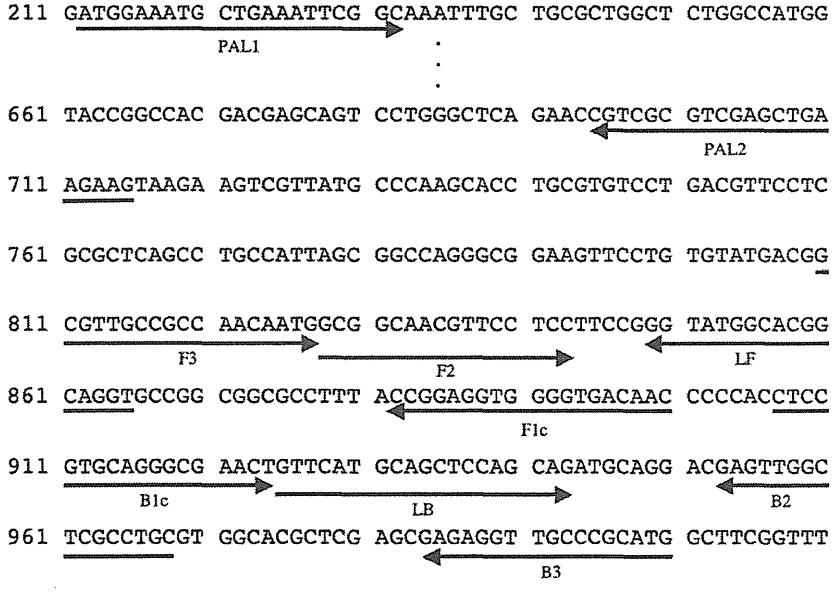

Fig. 1. Nucleotide sequence of the *oprL* gene of *P. aeruginosa* used for designing the LAMP and PCR primers. Numbers on the left correspond to the positions in the *oprL* gene (GeneBank accession no. Z50191). The locations of the target sequences are underlined with an arrow. FIP consists of the F1c and F2 sequences, and BIP consists of the B1c and B2 sequences.

Table 2
Comparison of the detection sensitivity among LAMP and PCR assays with 10-fold serially diluted *P. aeruginosa* genomic DNA.

P. aeruginosa (CFU/reaction)	1.2×10^4	1.2×10^3	120	12	1.2	0.12
LAMP	3/3	3/3	3/3	3/3	1/3	0/3
oprL PCR	3/3	3/3	3/3	0/3	0/3	0/3
ecfX PCR	3/3	3/3	3/3	1/3	0/3	0/3

The assays were performed in triplicate, and the results were shown as 'Number of positive reactions/number of reactions tested'.

12 CFU of *P. aeruginosa* per reaction. The LAMP reaction followed by colorimetric analysis can be completed within 70 min. In contrast, genomic DNA was detected up to a dilution of 10^{-4} by both *oprL* PCR and *ecfX* PCR. However, PCR followed by gel electrophoresis required about 2 h for completion.

3.3. Detection of *P. aeruginosa* in mouse fecal samples inoculated with a varying number of *P. aeruginosa* organisms

P. aeruginosa DNA was purified from fecal samples inoculated with a known number of *P. aeruginosa* organisms that were cultured at 37 °C for 6 h in NAC medium. Then LAMP and PCR assays were performed. Color changes in the LAMP reaction mixture were observed in samples containing as little as 130 CFU/0.1 g feces of *P. aeruginosa* (which corresponded to 3.25 CFU/reaction) (Fig. 2A). DNA amplified by the PCR was detected on ethidium bromide–stained agarose gel in samples containing as little as 1300 CFU/0.1 g feces (which corresponded to 32.5 CFU/reaction) (Fig. 2B).

3.4. Screening of *P. aeruginosa*-infected mice in mouse facilities

A total of 46 fecal samples were collected from 3 SPF facilities (2 facilities are excluded *P. aeruginosa* from the barriers and 1 is not excluded *P. aeruginosa* from the barriers) and 1 conventional mouse facility. The samples were analyzed by both LAMP and NAC medium culture assays (Table 3). According to the results of LAMP, the 27 samples taken from 2 of the SPF facilities that were *P. aeruginosa*-free were negative for *P. aeruginosa*, and 1 of the 12 samples from the third SPF facility and 2 of the 7 samples from the conventional mouse facility were positive for *P. aeruginosa*. However, *P. aeruginosa* was not detected in any of the field samples in the culture assay.

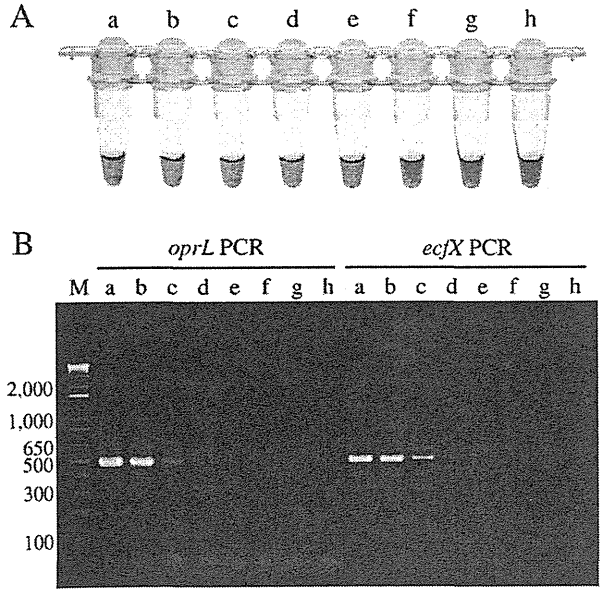

Fig. 2. Comparison of the sensitivities of the LAMP and PCR assays by using *P. aeruginosa*-inoculated fecal samples. A, Sensitivity of LAMP for *P. aeruginosa* as determined by the change in the color of HNB. A positive reaction is indicated by a color change from violet to sky blue. B, Agarose gel electrophoretic analysis of PCR products obtained by the 35-cycle reaction. The expected sizes of the PCR product obtained by *oprL* PCR and *ecfX* PCR were 504 bp and 528 bp, respectively. Lanes (tubes) a, 1.3×10^6; b, 1.3×10^5; c, 1.3×10^4; d, 1.3×10^3; e, 130; f, 13; g, 1.3 CFU; h, no *P. aeruginosa*; M, DNA size marker.

4. Discussion

We designed LAMP primers for the region between nt 801 and nt 1000 of the *oprL* gene; the LAMP assay showed 100% specificity for *P. aeruginosa* and 10-fold higher sensitivity than the presently used PCR assays for the detection of *P. aeruginosa*. Nucleic acid amplification assay for detecting the *oprL* gene of *P. aeruginosa* was first described by De Vos et al. (1997). This assay detected *P. aeruginosa* DNA directly in clinical samples obtained from patients with burns and those with cystic fibrosis. Xu et al. (2004) demonstrated the clinical feasibility of using the *oprL* PCR assay for the early detection of *P. aeruginosa*: in their study, DNA was directly detected in the sputum samples obtained from cystic fibrosis patients. However, Lavenir et al. (2007) reported that the *oprL* PCR assay did not show sufficient specificity for *P. aeruginosa* DNA in the case of soil and water samples (Table 1). A homology search with the BLASTn software with the sequences currently available in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the *oprL* gene sequence between the PAL1 and PAL2 primers (501 bp) was similar to that found in the pseudomonads (≥99%) *Pseudomonas entomophila* (accession no. CT573326.1), *P. fluorescens* (accession no. AM181176.4), *P. mendocina* (accession no. CP000680.1), *P. putida* (accession no. CP000712.1, CP000926.1), *P. stutzeri* (accession no. CP000304.1), *P. syringae* pv. *phaseolicola* (accession no. CP000058.1), and *Pseudomonas syringae* pv. *syringae* (accession no. CP000075.1). In the PAL1 primer region, only the 3′ terminal nucleotide was different between *P. aeruginosa* (C) and other *Pseudomonas* species (T). In the PAL2 primer region, the sequence was 100% identical in all the *Pseudomonas* species listed above. Homology analysis indicated that the LAMP assay targeting the *oprL* gene sequence between the PAL1 and PAL2 primers may not be suitable for detecting *P. aeruginosa* DNA. Unlike the *oprL* gene sequence present between the PAL1 and PAL2 primers, the *oprL* gene sequence from nt 801 to nt 1000 is not similar to that found in other pseudomonads: 81% homology with *P. mendocina*, 52% homology with *P. putida* and *P. syringae* pv. *phaseolicola*, and 43% homology with *P. fluorescens*. These results strongly suggest that the region from nt 801 to nt 1000 is the best target for the LAMP primers for the detection of *P. aeruginosa*.

The LAMP assay provides correct results in about 1 h when purified DNA is used as the template. However, for amplification efficiency, it is important that the template DNA is denatured before adding the large fragment of *Bst* DNA polymerase. LAMP assays that used non-denatured *P. aeruginosa* DNA were 10–100 times less sensitive than those that used denatured DNA (data not shown), although LAMP was often carried out

Table 3
LAMP and culture results for field samples from mice bred in 3 SPF and 1 conventional facilities.

Facilities	No. of samples	No. of positives	
		LAMP assay	Culture assay
A	11	0	0
B	16	0	0
C	12	1	0
D	7	2	0

Facilities A and B, SPF excluding *P. aeruginosa*; C, SPF containing *P. aeruginosa*; D, conventional.