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 + + +.

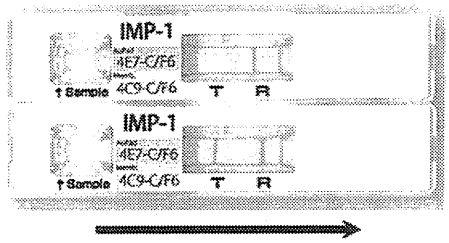


Fig. 1. Immunochromatography developed using mAbs 4E7-C/F6 and 4C9-C/F6. In the case of negative results, a single line appears at the position of the reference line (R) only. In the case of positive results, another line also appears at the position of the test line (T) in addition to the reference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

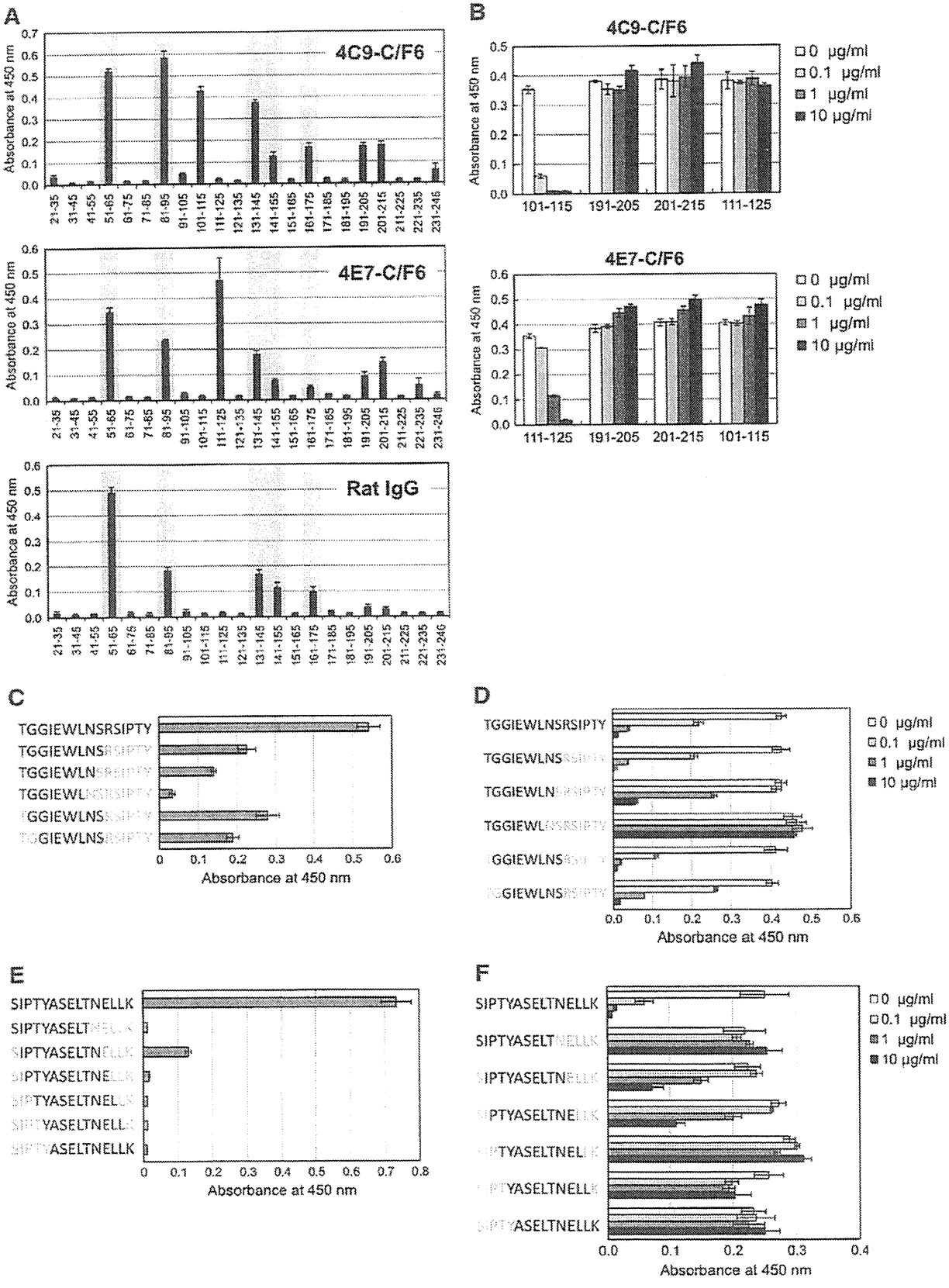
A competitive assay using the candidate peptides was also performed (Fig. 2B). The recognition of IMP-1 by 4C9-C/F6 was significantly inhibited by only peptide 101–115 inhibited in a dose-dependent manner. The recognition of IMP-1 by 4E7-C/F6 was significantly inhibited by only peptide 115–125 in a dose-dependent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

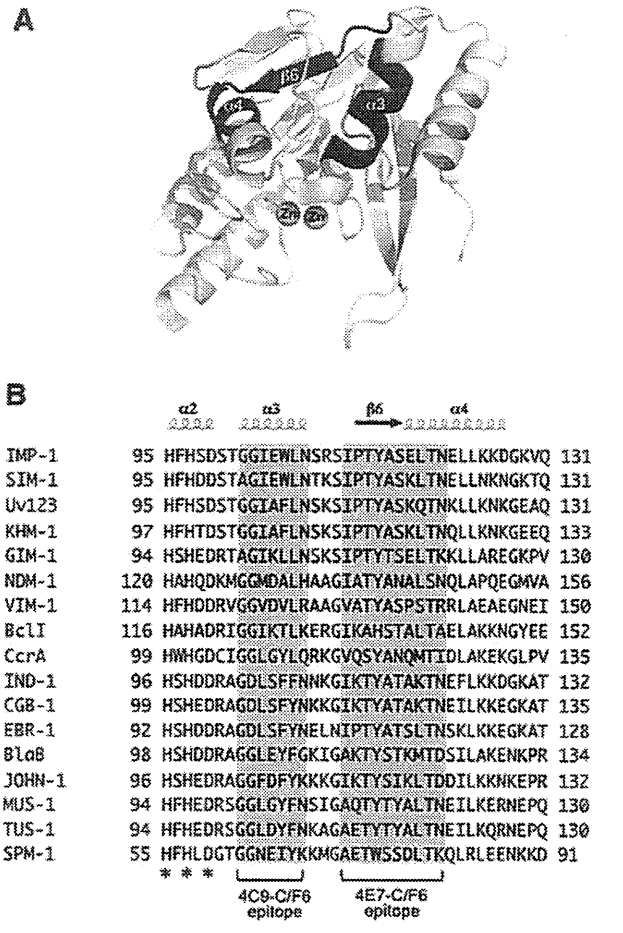


Fig. 3. Sequence alignment of the epitope region among MBLs. (A) Epitope region on the three-dimensional structure of IMP-1. The figure was created with MacPymol software according to the IMP-1 structure (PDB ID: 2DOO) determined by Dr. Kurosaki (Kurosaki et al., 2006). The black part presents the region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

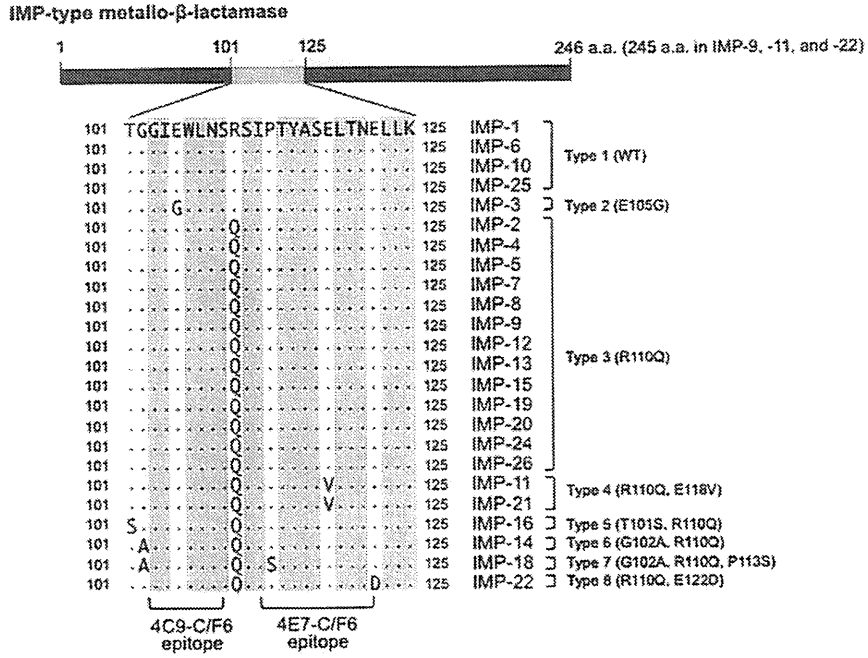


Fig. 4. Comparison of amino acid sequences in the epitope regions of IMP-type MBLs. The regions in the 24 subtypes of IMP-type MBLs corresponding to the 101–125 region in IMP-1 were compared. The dots indicate the residues identical to the IMP-1 sequence. The completely conserved amino acids are shaded.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

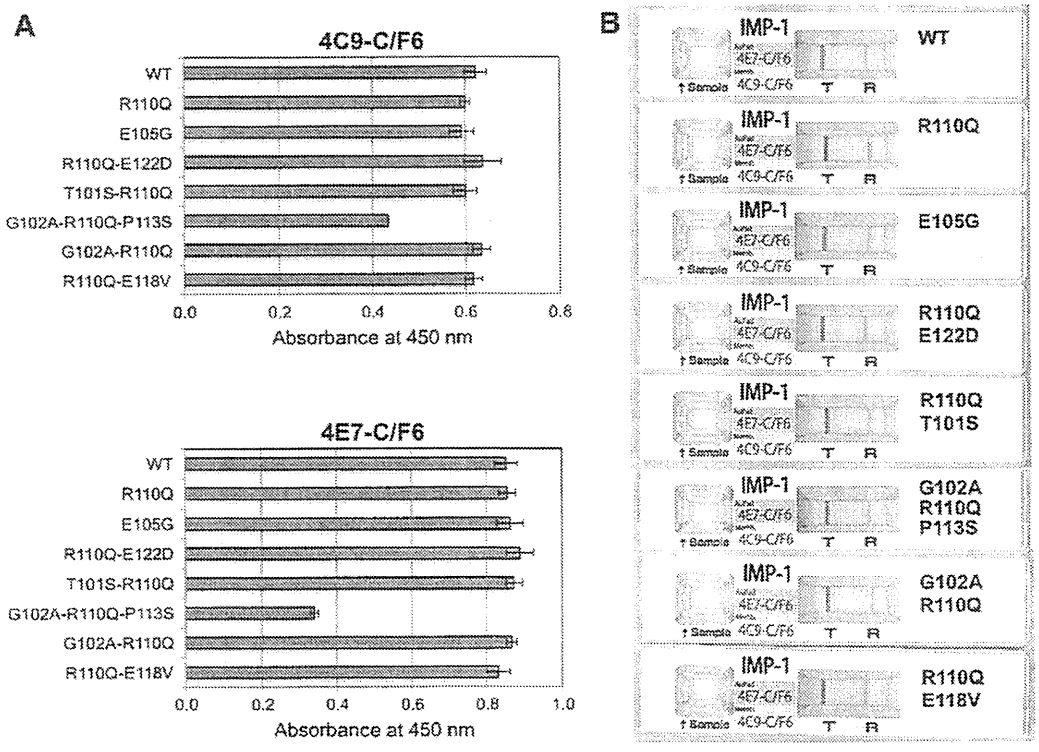


Fig. 5. Evaluation of mAbs and the assay using IMP mutants. (A) Interaction between mAbs and IMP mutants by ELISA. The purified IMP mutant proteins were immobilized onto an EIA plate, and the interaction between mutants and mAb was detected with anti-rat IgG goat antibody. (B) Immunochromatographic detection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

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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2.2. Detection of IMP-type metallo-β-lactamases and AAC(6′)-Iae

IMP-type MBLs and AAC(6′)-Iae were detected using an immunochromatographic assay kit (Mizuho Medy Co., Saga, Japan) designed for the detection of these enzymes [3,4].

2.3. Antimicrobial susceptibility

MICs of IPM (Banyu Pharmaceutical Co., Tokyo, Japan), AMK (Banyu Pharmaceutical ~~Co.~~), CIP (Daiichi Pharmaceutical Co., Tokyo, Japan) and colistin (Sigma-Aldrich, St Louis, MO) were determined using the microdilution method as per the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [7]. Values of MICs at which 50% and 90% of the isolates were inhibited (MIC50 and MIC90, respectively) were determined. Isolates were tested for the presence of MBL by a double-disk synergy test with disks containing sodium mercaptoacetic acid (SMA) as described previously [8].

2.4. Detection of antibiotic resistance genes

The bla_{IMP} and *aac(6′)-Iae* genes were amplified using polymerase chain reaction (PCR) primers as described previously [9]. All of the PCR products were sequenced using an ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA). The class 1 integron was amplified using the PCR primer set of 5′CS and 3′CS. All of the PCR products were sequenced to identify the contents of the genes [10].

2.5. Pulsed-field gel electrophoresis (PFGE)

DNA plugs were prepared and digested overnight at 37 °C with *Spe*I (Takara Bio, Otsu, Japan). PFGE analysis was performed as described previously [8]. Fingerprinting patterns were analysed by the unweighted pair-group method using Molecular Analyst Fingerprinting Plus software (Bio-Rad Laboratories, Hercules, CA) to create an average linkage-based dendrogram.

2.6. Multilocus sequence typing (MLST)

MLST was performed according to the protocols described on the *P. aeruginosa* MLST Database website (http://pubmlst.org/paeruginosa/). PCR and sequencing were performed for seven chromosomal genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA* and *trpE*). The nucleotide sequences of these genes were compared with the sequences submitted to the MLST database to determine the allelic numbers and sequence types (STs).

2.7. Serotyping

Serotypes of the isolates were determined using a slide agglutination test kit (Denka Seiken Co., Tokyo, Japan). Serotype O18 was determined using a sequence-based method [11].

3. Results

3.1. Screening of multidrug-resistant Pseudomonas aeruginosa producing IMP-type metallo-β-lactamases and AAC(6′)-Iae

In total, 217 MDR *P. aeruginosa* isolates were screened for production of IMP-type MBLs and AAC(6′)-Iae using an immunochromatographic assay. Of these, 145 isolates (66.8%) produced both IMP-type MBLs and AAC(6′)-Iae, 29 (13.4%) produced IMP-type MBLs but did not produce AAC(6′)-Iae and 6 (2.8%) produced AAC(6′)-Iae but did not produce IMP-type MBLs. The six isolates producing AAC(6′)-Iae but not IMP-type MBLs were negative for MBL by the SMA double-disk synergy test. Results of the immunochromatographic assay were consistent with those of the PCR for bla_{IMP} and *aac(6′)-Iae* genes.

3.2. Genetic context of the IMP-type metallo-β-lactamases and AAC(6′)-Iae

DNA sequencing showed that the 145 isolates producing both IMP-type MBLs and AAC(6′)-Iae did not have a mutation in the *aac(6′)-Iae* gene. Of these, 125, isolates had bla_{IMP-1}, 6 had bla_{IMP-6} and 14 had bla_{IMP-10}.

PCR showed that of the 145 isolates producing IMP-type MBLs and AAC(6′)-Iae, 142 were positive for a class I integron. Of these 142 isolates, 124 had integron In*113*, which carried bla_{IMP-1} [9]; the remaining 18 isolates had In*113*-like integrons, which have the same structure as integron In*113* but the bla_{IMP-1} is replaced by IMP-6 (4 isolates) and IMP-10 (14 isolates).

3.3. Pulsed-field gel electrophoresis analysis, multilocus sequence typing and serotyping

The 145 isolates of MDR *P. aeruginosa* producing both IMP-type MBLs and AAC(6′)-Iae were analysed by PFGE. Analysis showed two clusters with >60% similarity (clusters I and II) (Fig. 1). Cluster I comprised 128 isolates and cluster II comprised 16 isolates; 1 isolate did not belong to any cluster. Cluster I included the NCGM2.S1 strain, which was the first reported MDR *P. aeruginosa* strain producing IMP-type MBLs and AAC(6′)-Iae [9]. The PFGE patterns of all of the isolates belonging to cluster II were identical (Fig. 1).

Of the 128 isolates belonging to cluster I, 127 were ST235 (STs: *acsA* 38, *aroE* 11, *guaA* 3, *mutL* 13, *nuoD* 1, *ppsA* 2 and *trpE* 4) and serotype O11, and 1 isolate was ST991 (STs, *acsA* 6, *aroE* 3, *guaA* 12, *mutL* 3, *nuoD* 3, *ppsA* 6 and *trpE* 7) and serotype O3. All 16 isolates belonging to cluster II were ST991 and serotype O18. ST991 does not appear to be related to ST235 because all the STs of the house keeping genes are different.

3.4. Antimicrobial susceptibility

All of the isolates belonging to clusters I and II were highly resistant to IPM, AMK and CIP; there was no difference in the MIC profiles of these two groups (Table 1). Of the 16 isolates belonging to cluster II, 15 were susceptible to colistin (MIC = 2 μg/mL) and 1 was intermediately susceptible (MIC = 4 μg/mL). One isolate belonging to cluster I and one isolate not belonging to any cluster were susceptible to colistin (MIC = 2 μg/mL).

3.5. Geographical distribution

MDR *P. aeruginosa* isolates producing IMP-type MBLs and AAC(6′)-Iae were obtained from 89 medical settings located in 22 prefectures in Japan (Fig. 2). Of these, isolates belonging to cluster I were obtained from 17 prefectures distributed along the northern to southern region of Japan. Isolates belonging to cluster II were obtained from nine prefectures that were also distributed along the northern to southern region of Japan (Fig. 2). The MDR *P. aeruginosa* isolates were obtained from relatively many medical settings in the Kanto area of Japan, e.g. 19 in Saitama, 15 in Tokyo and 9 in Chiba (Fig. 2). These findings suggest that MDR *P. aeruginosa* isolates belonging to both the clusters were spread throughout Japan.

4. Discussion

This study showed that IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa* ST235, serotype O11, which belong to cluster I (Fig. 1), have undergone clonal expansion in medical settings in Japan. NCGM2.S1 strain, which belongs to cluster I, was

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Table 1
Minimum inhibitory concentrations (MICs) and percent antimicrobial resistance for IMP-type metallo-β-lactamase- and AAC(6′)-Iae-producing *Pseudomonas aeruginosa* isolates belonging to clusters I and II.

Antimicrobial agent	Breakpoint for resistance (μg/mL)	Cluster I (n = 128)				Cluster II (n = 16)			
		%R	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	%R	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
IPM	≥16	100	32 to >128	128	>128	100	128	128	128
AMK	≥32	100	32 to >128	128	>128	100	64	64	64
CIP	≥8	100	8 to >128	64	>128	100	>128	>128	>128

%R, percent resistance; $MIC_{50/90}$, MIC at which 50% and 90% of the isolates were inhibited, respectively; IPM, imipenem; AMK, amikacin; CIP, ciprofloxacin.

determined to be the cause of an outbreak of catheter-associated urinary tract infections in the neurosurgery ward of a hospital in Miyagi [8], Japan. Further epidemiological studies found that clonal expansion of this strain had also occurred in community hospitals in Kanto region [3] and Hiroshima [2]. Clonal expansion of MBL-producing *P. aeruginosa* ST235, serotype O11 has also been reported in South Korea [12] and Scandinavia [13].

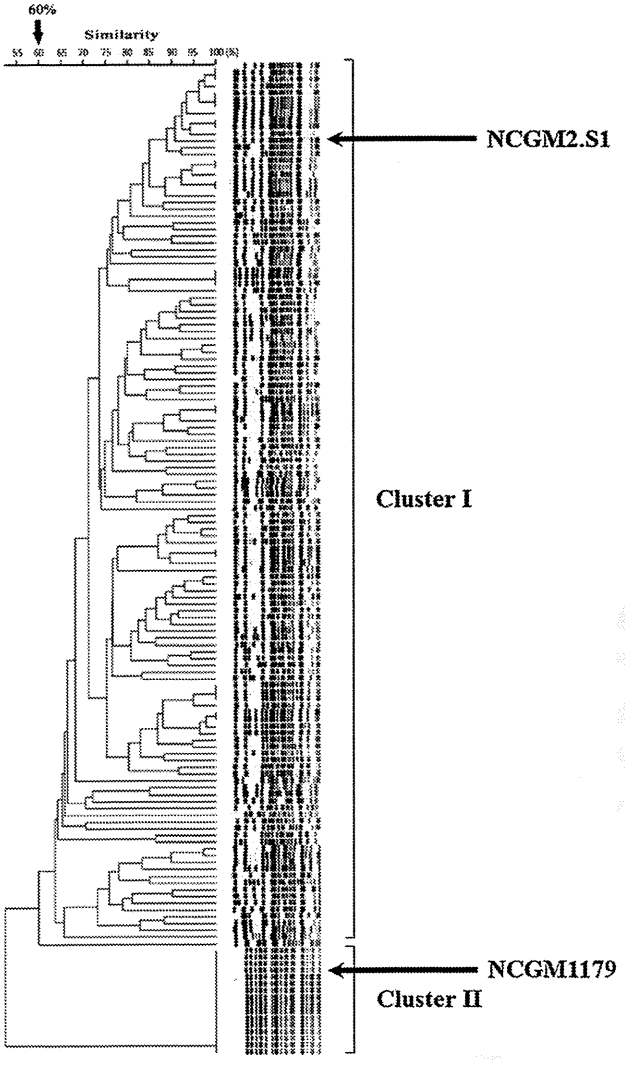

Fig. 1. Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of 145 multidrug-resistant *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae. Two clusters (I and II) were detected. Of the 128 isolates belonging to cluster I, 127 isolates were ST235 and serotype O11 and 1 isolate was ST991 and serotype O3. All of the 16 isolates belonging to cluster II were ST991 and serotype O18.

The isolates belonging to cluster I were mainly obtained from the urinary and respiratory tracts; the percentage of isolates from the urinary tract was markedly higher. A surveillance study of *P. aeruginosa* clinical isolates with and without multidrug resistance showed that MDR isolates were particularly increased in the urinary tract of Japanese individuals [1]. The increase in the number of MDR isolates in the urinary tract may be related to the epidemic of IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa* in Japan.

This is the first report describing MDR *P. aeruginosa* ST991, serotype O18, which belonged to cluster II (Fig. 1) and is a recent emerging strain in medical settings in Japan. ST991 was originally registered by C. Giske at Karolinska University Hospital, Sweden in 2010 in the *P. aeruginosa* MLST Database (http://pubmlst.org/paeruginosa/). However, to the best of our knowledge, there are no reports on the association of ST991 and multidrug resistance in *P. aeruginosa*. All of the isolates belonging to cluster II were obtained from the respiratory tract. In contrast, 32.8% of the isolates belonging to cluster I (42/128) were obtained from the respiratory tract. MDR *P. aeruginosa* ST991 dominantly causes respiratory infections. MDR isolates of *P. aeruginosa* serotype O18 have not been previously reported. Most of the MDR clinical isolates of *P. aeruginosa* exhibit serotype O11 or O12 [11].

We have reported the complete genome sequences of NCGM2.S1 [5] and NCGM1179 [6]. Integron In*113* was inserted in the *oprD* gene and disrupted it in NCGM2.S1; integron In*113* was located downstream of the *tnpA* gene that codes for transposase

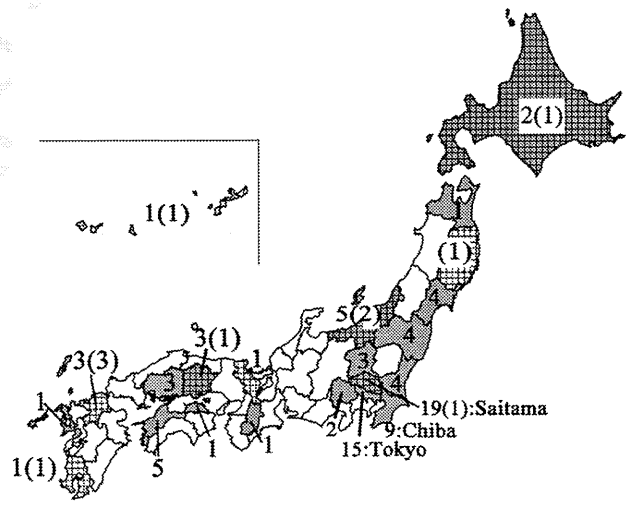

Fig. 2. Geographical distribution of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae in Japan. Isolates belonging to cluster I (Fig. 1) were obtained from prefectures marked in grey; isolates belonging to cluster II were obtained from prefectures marked in a checked pattern. Isolates belonging to both clusters were obtained from prefectures marked in a grey checked pattern. The number and the number in parenthesis represent the number of medical settings in the prefecture where MDR *P. aeruginosa* isolates belonging to cluster I and cluster II, respectively, were obtained.

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of Tn*4380* of the mercury transposon Tn*3* family and the *tnpR* gene that codes for serine-base site-specific recombinase of Tn*6050*. However, the *oprD* was found to be intact in the NCGM1179 strain. *oprD* codes for a specialised pore protein, OprD, which allows selective permeation of basic amino acids and their structural analogues such as carbapenems, including IPM and meropenem [14]. It is unclear whether OprD affects the MIC of carbapenems in IMP-type MBL- and AAC(6′)-Iae-producing MDR *P. aeruginosa*. The details of the comparative genome analysis of the two MDR strains will be reported elsewhere.

Of the 217 MDR *P. aeruginosa* isolates tested in this study, 72 did not produce IMP-type MBLs and/or AAC(6′)-Iae. At present, we are looking for genes conferring high resistance to all β-lactams, aminoglycosides and fluoroquinolones.

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Isolation rates of multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter* spp. at medical facilities in Japan

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Abstract

Background: The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* strains is a serious problem at medical facilities in Japan.

Methods: We distributed questionnaires to assess the isolation rates of multidrug-resistant (MDR) *P. aeruginosa* and *Acinetobacter* spp. at all medical facilities with more than 200 beds throughout Japan from 2007 to 2009.

Results: Completed questionnaires were received from 771 medical facilities. The total numbers of *P. aeruginosa* and MDR isolates obtained at the medical facilities were 684 982, and 19 911 (2.9% of *P. aeruginosa* isolates), respectively; MDR isolates were found nationwide. One or more MDR *P. aeruginosa* isolates were found at approximately 53% of the medical facilities each year. The percentages of MDR isolates decreased significantly from 2007 to 2009. MDR *P. aeruginosa* strains were obtained mainly from the urinary and respiratory tracts. The total numbers of *Acinetobacter* spp. and MDR isolates obtained at the medical facilities were 94 012 and 558 (0.6% of *Acinetobacter* spp. isolates), respectively. Of these MDR isolates, 82.1% were *Acinetobacter baumannii.* The percentages of MDR isolates increased significantly from 2007 to 2009. One or more MDR *Acinetobacter* spp. isolates were found at approximately 5% of the medical facilities each year. MDR *Acinetobacter* spp. strains were obtained mainly from the respiratory tract.

Conclusions: MDR *P. aeruginosa* was prevalent nationwide in Japan, but its incidence decreased significantly after 2007. MDR *Acinetobacter* spp. is an emerging problem in medical facilities in Japan.

Keywords: nationwide surveillance, retrospective questionnaire, laboratory-based surveillance

48 BACKGROUND

49 The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* and
50 *Acinetobacter baumannii* strains is a serious problem at medical facilities [1-5].
51 Outbreaks of MDR *P. aeruginosa* infection have become problematic in hospitals in
52 various countries [6-10], including Japan[2, 11, 12]. Nosocomial outbreaks of MDR *A.*
53 *baumannii* infection have been major issues in many countries [14], including the UK
54 [15], the USA [16], and Korea [3, 17]. There has been only one previous report of an
55 outbreak of MDR *Acinetobacter* spp. in Japan [18]. Recently, there was an outbreak of
56 *A. baumannii* infection at a university hospital in Fukuoka prefecture, Japan, in 2009.
57 The index case of the outbreak received medical treatment in another country (data not
58 shown). During the present study period, another large outbreak of *A. baumannii*
59 infection occurred at a university hospital in Tokyo, Japan, in 2010.

60 Previously, we reported that the isolation rate of MDR *P. aeruginosa* strains was
61 2.4% in medical facilities in Japan during the period January 2003 through June 2006
62 [19]. The percentages of MDR isolates increased significantly from 2003 to 2005 [19].
63 Here, we performed a surveillance study of clinically isolated MDR *P. aeruginosa* in
64 Japan to determine whether the rate of MDR isolates has increased or decreased at
65 medical facilities in Japan after the first surveillance [19]. We also investigated
66 clinically isolated strains of MDR *Acinetobacter* spp. in Japan. This is the first
67 surveillance study of clinically isolated MDR *Acinetobacter* spp. in Japan.
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74 METHODS

75 Information was gathered by a questionnaire survey. Questionnaires were sent on 16
76 March 2010 to all medical facilities with 200 or more beds in Japan (2 719 facilities). *P.*
77 *aeruginosa* isolates that were resistant to carbapenems (imipenem or meropenem),
78 amikacin, and fluoroquinolones (clinafloxacin, gatifloxacin, levofloxacin, lomefloxacin,
79 norfloxacin, or ofloxacin) were defined as MDR isolates. *P. aeruginosa* isolates that
80 were resistant to two of these drugs were defined as two-drug-resistant (TDR) isolates.
81 *Acinetobacter* spp. isolates that were resistant to carbapenems (imipenem or
82 meropenem), amikacin, and fluoroquinolones (levofloxacin or clinafloxacin) were
83 defined as MDR isolates. Drug resistance was assessed by determining the minimum
84 inhibitory concentration (MIC) in culture medium containing the drugs or by
85 determining the diameter of the growth inhibition zone (DGIZ) on culture agar with the
86 use of drug-sensitivity discs. Breakpoints were determined in accordance with the
87 criteria for MDR isolates specified by the Japanese Nosocomial Infection Surveillance
88 System (JANIS), Japanese Ministry of Health, Labour and Welfare. MIC breakpoints of
89 *P. aeruginosa* for carbapenems (imipenem or meropenem), amikacin, and
90 fluoroquinolones (clinafloxacin, gatifloxacin, levofloxacin, lomefloxacin, norfloxacin,
91 or ofloxacin) were ≥ 16, ≥ 16, ≥ 32, ≥ 4, ≥ 8, ≥ 8, ≥ 8, ≥ 16, and ≥ 8 mg/L, respectively;
92 DGIZ breakpoints for these drugs were ≤ 13, ≤ 13, ≤ 14, ≤ 15, ≤ 14, ≤ 13, ≤ 18, ≤ 12,
93 and ≤ 12 mm, respectively. MIC breakpoints of *Acinetobacter* spp. for carbapenems
94 (imipenem or meropenem), amikacin, and fluoroquinolones (levofloxacin or
95 clinafloxacin) were ≥ 16, ≥ 16, ≥ 64, ≥ 4 and ≥ 8 mg/L, respectively; DGIZ breakpoints
96 for these drugs were ≤ 13, ≤ 13, ≤ 14, ≤ 15, and ≤ 13 mm, respectively.

97 The questionnaire solicited information about: 1) the number of beds; 2) the total

number of *P. aeruginosa* isolates obtained each year with or without TDR or MDR; 3) the number of patients with TDR or MDR *P. aeruginosa* isolates; 4) the tissue sources of the *P. aeruginosa* isolates; 5) the total number of *Acinetobacter* spp. isolates obtained each year with or without MDR; 6) the number of patients with MDR *Acinetobacter* spp. isolates; 7) the tissue sources of *Acinetobacter* spp. isolates; and 8) the number of isolates of each *Acinetobacter* spp. each year when they were identified at the facilities.

Isolates were obtained from inpatients and outpatients with suspected *P. aeruginosa* or *Acinetobacter* spp. infection and subjected to drug susceptibility testing. Repeat testing of single patients was assumed when repeat examinations were ordered. Isolates for analysis in this study were not from the environment, carriers, nonsymptomatic patients, or healthy staff.

Chronological trends in the proportions of TDR and MDR isolates were assessed by Friedman's test. The numbers of isolates from various tissue sources and populations of drug-resistant isolates were analyzed by χ^2 test. In all analyses, $P < 0.0001$ was taken to indicate statistical significance.

The contents of the questionnaires are considered to be exempt from the ethical guidelines for epidemiological research, 5 December 2008, by Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour and Welfare, Japan.

RESULTS

Completed questionnaires were returned by 771 medical facilities (collection rate, 28.4%) as of 30 April 2010. The average number of beds in these medical facilities was 410 ± 208 (median, 346; range, 50 – 1 494). The investigation was performed from fiscal year 2007 to 2009 (April 2007 through March 2010).

As shown in Table 1, during the study period, a total of 684 982 *P. aeruginosa* isolates were obtained at the 771 medical facilities. The numbers of TDR and MDR isolates were 41 392 (6.0% of the number of *P. aeruginosa* isolates) and 19 911 (2.9% of the number of *P. aeruginosa* isolates), respectively. The total numbers of isolates, as well as the adjusted numbers (number of isolates/1 000 beds), neither increased nor decreased between years during the study period.

The total numbers of TDR isolates and the adjusted numbers decreased gradually from 2007 to 2009 ($P = 0.0003$ and $P = 0.03$, respectively). The percentage of TDR isolates decreased significantly during the study period ($P < 0.0001$). The number of patients with TDR isolates and the number of patients per 1 000 beds/year decreased gradually from 2007 to 2009 ($P = 0.0002$ and $P = 0.03$, respectively).

TDR isolates were obtained at 583 of the 771 facilities (75.6%) during the study period: 521 (67.6%) in 2007, 537 (69.6%) in 2008, and 553 (71.7%) in 2009. The numbers of patients with TDR isolates at each facility from 2007 to 2009 (per 1 000 beds/year) were as follows: median values, 12.0, 12.5, and 11.4, respectively; maximum values, 255.4, 338.7, and 383.9, respectively; and 90th percentile, 53.3, 48.9, and 44.7, respectively.

Facilities with values higher than the 90th percentile were distributed throughout Japan but the distribution was uneven (data not shown). There were no facilities with

146 greater than the 90th percentile in 16 of all prefectures (47) in Japan.

147 The total numbers of MDR isolates and the adjusted numbers decreased gradually
148 from 2007 to 2009 ($P = 0.0001$ and $P = 0.02$, respectively). The percentage of MDR
149 isolates decreased significantly during the study period ($P < 0.0001$). The number of
150 patients with MDR isolates and the number of patients per 1 000 beds/year decreased
151 gradually from 2007 to 2009 ($P = 0.0001$ and $P = 0.003$, respectively). MDR isolates
152 were obtained at 545 of the 771 facilities (70.7%) during the study period: 411(53.3%)
153 in 2007, 405(52.5%) in 2008, and 409 (53.0%) in 2009.

154 The numbers of patients with MDR at each facility from 2007 to 2009 (per 1 000
155 beds/year) were as follows: median values, 1.9, 1.9, and 1.8, respectively; maximum
156 values, 502.5, 275.6, and 373.3, respectively; and 90th percentile values, 21.7, 19.6, and
157 18.7, respectively. The numbers of patients with MDR per 1,000 beds/year in each
158 group of medical facilities categorized by number of beds are shown in Figure 1. In
159 large-scale facilities with ≥ 600 beds and those of medium-scale with 300 – 599 beds,
160 the numbers decreased markedly from 2007 to 2009 ($P = 0.05$ for both groups), whereas
161 no changes were observed in small-scale facilities with < 300 beds ($P = 0.6$).

162 Facilities with values higher than the 90th percentile were distributed throughout
163 Japan but the distribution was uneven (data not shown). There were no facilities with
164 values greater than the 90th percentile in 18 of all 47 prefectures. There were no
165 significant differences in these values between geographic regions (data not shown).

166 The tissue sources and percentages of the total *P. aeruginosa* isolates obtained at the
167 771 medical facilities over the study period and those of TDR and MDR strains are
168 shown in Figure 2. The percentages for each year were similar to those for the entire
169 study period (data not shown). These results indicated that *P. aeruginosa*, including

TDR and MDR isolates, affected mainly the respiratory and urinary tracts. However, it is notable that the percentages of TDR and MDR isolates in the urinary tract were significantly greater than those of the total isolates ($P < 0.0001$) and that the percentages of MDR isolates in the urinary tract surpassed those in the respiratory tract.

A total of 94 012 *Acinetobacter* spp. isolates were obtained from 690 of the 771 medical facilities (89.5%) during the study period. As shown in Table 2, the total numbers of isolates, as well as the adjusted numbers (number of isolates/1 000 beds), decreased slightly during the study period ($P = 0.002$ and $P = 0.002$, respectively).

The total numbers of MDR isolates were 558 (0.6% of the numbers of isolated *Acinetobacter* spp.) during the study period. The total numbers of MDR and the adjusted numbers were small compared to those of *Acinetobacter* spp. in each year, but they increased markedly from 2007 to 2009 ($P = 0.06$ and $P = 0.06$, respectively) (Table 2). The percentage of MDR isolates increased significantly during the study period ($P < 0.0001$). The number of patients with MDR isolates and the number of patients per 1 000 beds/year increased markedly from 2007 to 2009 ($P = 0.2$ and $P = 0.2$, respectively).

MDR isolates were obtained at 92 of the 771 facilities (11.9%) during the study period: 39 (5.1%) facilities in 2007, 37 (4.8%) in 2008, and 49 (6.4%) in 2009. The numbers of patients with MDR isolates at each facility from 2007 to 2009 (per 1 000 beds/year) were as follows: median values, 0 in all of these years; maximum values, 10.7, 31.6, and 18.4, respectively; and 99th percentile values, 4.1, 4.1, and 5.5, respectively.

Facilities with *Acinetobacter* spp. were distributed throughout Japan but the distribution was uneven (data not shown). There were facilities without *Acinetobacter*