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Original Article

# Discrimination of influenza A subtype by antibodies recognizing host-specific amino acids in the viral nucleoprotein

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**Background** Nucleoprotein (NP) of influenza viruses is utilized to differentiate between the A, B, and C viral serotypes. The availability of influenza genome sequence data has allowed us to identify specific amino acids at particular positions in viral proteins, including NP, known as "signature residues," which can be used to discriminate human influenza A viruses from H5N1 highly pathogenic avian influenza in human cases (HPAI) and pandemic H1N1(2009) (H1N1/2009) viruses.

**Methods** Screening and epitope mapping of monoclonal antibodies (mAb) against NP of influenza A, which reacted differently with NP from human influenza A virus from HPAI and H1N1/2009 A virus. To identify the epitope(s) responsible for the discrimination of viral NP by mAbs, we prepared mutant NP proteins in the 293 cell expression system because some of the mAbs reacted with non-linear epitopes.

**Results and Conclusions** In the present study, we identified 3 mAbs. The results of epitope mapping showed that the epitopes were located at the signature residues. These results indicated that signature residues of NP could discriminate influenza A viruses from different origin.

**Keywords** Epitope, influenza, monoclonal antibody, nucleoprotein.

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## Introduction

The recent pandemic of swine-origin H1N1 2009 influenza A (H1N1/2009) virus[1,2] and outbreaks of human cases of H5N1 highly pathogenic avian influenza (HPAI) infection[3] (http://gamapserver.who.int/mapLibrary/app/searchResults.aspx) have prompted the development of diagnosis methods, which specifically detects new-type influenza. In clinical practice, rapid diagnostic kits (RDKs) based on immunochromatography utilizing antibodies against nucleoprotein (NP) of influenza virus are used to diagnose influenza, allowing the immediate initiation of antiviral drug administration.[4,5] We developed an RDK capable of distinguishing H1N1/2009 viruses from seasonal influenza viruses [6] and evaluated its diagnostic efficacy in a prospective multicenter clinical trial.[7] During the course of these studies, we obtained a unique monoclonal antibody (mAb) that reacted with the NP proteins from H1N1/2009 virus and HPAI, but not those from human seasonal H1N1 and H3N2 viruses. Epitope mapping experiments showed that the mAb recognizes a specific sequence of amino acids found in the NP proteins from H1N1/2009 virus and HPAI viruses located at residues 16–18 of these NPs.[6] These findings prompted us to identify amino acids that distinguish human influenza viruses from HPAI and H1N1/2009 viruses at specific positions in the NP proteins. Such residues are known as "signature" residues.[8–11]

In the present study, we identified 5 and 9 signature residues in the NP proteins of HPAI viruses from human cases and H1N1/2009 influenza viruses, respectively. During the screening of monoclonal antibodies (mAbs) against the NP proteins, we identified 3 mAbs that reacted differently to NP from human influenza A virus compared to those of HPAI and H1N1/2009. Epitope mapping indicated that these mAbs recognized residues identified as signature amino acids in each NP. These results indicated that host-



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specific amino acids of NP could discriminate influenza A viruses from different origin.

## Materials and methods

### Identification of signature residues in influenza A virus nucleoprotein (NP) and prediction of accessibility for antibody binding

A total of 1182 of NP sequences of H1N1/2009 viruses in addition to HPAI and human viruses based on the data from January 1, 2007 to September 11, 2009 registered as human cases were retrieved from the Influenza Virus Resources in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FLU/) and analyzed to identify signature amino acids that distinguish human influenza viruses from HPAI and H1N1/2009 viruses based on an alignment obtained using the blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). To predict the accessibility of the signature residues for antibodies, the locations of the signature residues based on the crystal structure of the NP proteins[12,13] *in silico* using Cn3D (http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml), a crystal structure viewing software.

To prepare an anti-NP mAb, recombinant NPs of influenza A virus (A/Viet Nam/VL-020/2005(H5N1)) (accession number: AAZ72762), a virus isolated from a patient infected with HPAI, and H1N1/2009 (A/California/04/2009) (accession number: ACP44151.1) were prepared from *Escherichia coli* BL21 (DE3) CodonPlus-RIPL (Stratagene, La Jolla, CA, USA) and used to immunize 7–9-week-old female WKY rats (Oriental Yeast Co. Ltd., Tsukuba, Japan), and rat mAbs were prepared as described.[6] The mAb 3G2 was prepared by immunization of GANP Mice™ (TransGenic Inc., Kumamoto, Japan).

### ELISA analysis of mAbs

Reactivity of the mAbs with NPs derived from seasonal influenza, H1N1/2009, and H5N1 was analyzed by conventional ELISA using microplates coated with NPs or by sandwich ELISA using microplates coated with polyclonal antibodies prepared from rabbits immunized with recombinant NPs as described previously.[6]

Sources of NP proteins for the sandwich ELISA included cultured human A/New York/55/2004(H3N2) and A/New Caledonia/20/1999 (H1N1) viruses in tissue culture, and recombinant NPs from HEK293 cells transfected with cytomegalovirus (CMV) promoter-driven plasmids[14,15] encoding an NP gene with the sequence of H1N1/2009 (A/California/04/2009(H1N1)) and that of HPAI (A/Viet Nam/VL-020/2005(H5N1).

The concentration of each NP was normalized by conventional Western blotting with rabbit anti-NP polyclonal antibody. To perform sandwich ELISA, 250 ng of rabbit anti-NP polyclonal Ab dissolved in 50 mm sodium carbonate buffer (pH 9·0) was fixed to each well of a 96-well microtiter plate (Corning Inc., Corning, NY, USA) at room temperature for 1 h. After washing with phosphate-buffered saline containing 0·02% Tween-20 (PBS-T) and blocking with SuperBlock (Pierce, Rockford, IL, USA), 10 ng of the NP proteins dissolved in PBS-T was added to each well. Following incubation and washing, the wells were incubated with 50 ng/well of mAbs indicated. In conventional ELISA, 50 ng/wells of antigens were fixed onto the plates directly. Binding of mAbs was detected with the HRP-goat anti-rat IgG (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and TMB (Bio-Rad, Hercules, CA, USA).

### Epitope mapping of mAb using recombinant NP fragments or synthetic peptides

Eight NP fragments (Table S1) derived from NP (A/California/04/2009(H1N1) Accession No. ACP44151) were prepared in *E. coli* as described above and used for epitope mapping of mAbs based on the ELISA results. Synthetic peptides prepared by a commercial service (500 ng each, > 70% purity; Invitrogen, Carlsbad, CA, USA) were fixed to the plates by incubation in 50 mm carbonate buffer (pH 9·0) containing 1 mm of the chemical cross-linker disuccinimidyl suberate (DSS; Pierce) at room temperature for 1 h, followed by epitope mapping using 500 ng/well of mAb indicated.

### Epitope mapping of mAb using mutant NP proteins expressed in HEK293 cells

The NP proteins containing signature amino acids in the background of HPAI or H1N1/2009 viruses were produced by conventional PCR using mega-primers to introduce codons corresponding to each amino acid. Primer sequences are available on request. The resultant NP constructs were expressed in HEK293 cell as described above and used for epitope mapping using 50 ng/well of M322211.

### Phylogenetic analysis of NP

The full-length amino acid sequence data of NP registered at NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) dated 2005 to 2011 were retrieved. Number of sequences retrieved was 1917. Sequence alignment and phylogenetic analysis were performed using MUSCLE[16] and MEGA5,[17] respectively, on our own server.

## Results

During the development of a rapid diagnostic kit specific for H1N1/2009 viruses using mAbs against the NP proteins of influenza A viruses, we obtained a mAb that reacted with NPs from H1N1/2009 and H5N1 HPAI viruses but

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not with those from seasonal H1N1 and seasonal H3N2 viruses. The epitope of the mAb, designated 6G6, was located at residues 16–18 of NP.[6] The corresponding region of NPs from H1N1/2009 and H5N1 HPAI had the sequence GGE, while those of seasonal H1N1 and H3N2 were DGE and DGD, respectively. These findings prompted us to analyze the reactivity of mAbs against NPs of H1N1/2009, H5N1 HPAI, seasonal H1N1, or seasonal H3N2 prepared in our laboratory and from commercial sources.

## Signature amino acids found in NPs from H1N1/2009 and HPAI viruses

Large-scale sequence analyses revealed signature amino acids at specific positions in viral proteins including NPs that distinguish human influenza viruses from avian viruses.[9–11] We analyzed the 1182 of NP sequences of H1N1/2009 viruses in addition to HPAI and human viruses based on the data from January 1, 2007 to September 11, 2009 registered as human cases at Influenza Virus Resources in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FLU/) and found 5 and 9 signature residues specific for HPAI viruses and H1N1/2009 viruses, respectively (Table 1). These residues may be the keys to distinguish human seasonal viruses from HPAI and H1N1/2009 viruses using mAbs recognizing the viral NP proteins.

## Characterization of mAbs that reacted differently with NP in an origin-dependent manner

### *3A4*

The mAb 3A4 is one of the mAbs that was generated by immunization of recombinant NP from H1N1/2009 viruses (A/California/04/2009) and screening of mAbs that reacted with H1N1/2009 NP but not human or HPAI NPs. The results indicated that 3A4 reacted with NP from H1N1/2009 virus only (Figure 1A). To further narrow down the epitope of 3A4, we prepared 7 overlapping fragments of H1N1/2009 virus NP (Table S1). The mAb 3A4 reacted with fragments containing residues 1 – 56 (Full, F1, F1-1, and F1-1-1)(Figure 1B). Then, we used a 15-mer overlapping synthetic peptide corresponding to residues 1–71 of H1N1/2009 NP. Although a peptide corresponding to residues 31–45 reacted with 3A4, it also reacted to the positive control mAb and negative control IgG indicating that this peptide recognition was non-specific and 3A4 reacted specifically with a peptide corresponding to residues 11–25 (Figure 1C). Comparison of the corresponding sequences in human, HPAI, and H1N1/2009 viruses

**Table 1.** Signature amino acids specific for HPAI and H1N1/2009 viruses

| Signature amino acids | | Human | | HPAI | H1N1/2009 | Accessibility prediction |
|---|---|---|---|---|---|---|
| Position | Residues | H1N1 | H3N2 | | | |
| | Specific for human HPAI | | | | | |
| 33 | V | 0/372 | 0/252 | 14/14 | 0/542 | Poor |
| 100 | R | 0/372 | 0/252 | 14/14 | 0/542 | Good |
| 136 | L | 0/372 | 0/252 | 14/14 | 0/542 | Poor |
| 305 | R | 0/372 | 0/252 | 14/14 | 0/542 | Good |
| 357 | Q | 0/372 | 0/252 | 14/14 | 0/542 | Good |
| | Specific for H1N1/2009 | | | | | |
| 21 | D | 0/372 | 1/252 | 0/14 | 542/542 | Good |
| 53 | D | 0/372 | 0/252 | 0/14 | 542/542 | Good |
| 190 | A | 0/372 | 1/252 | 0/14 | 542/542 | Poor |
| 313 | V | 0/372 | 0/252 | 0/14 | 542/542 | Good |
| 316 | M | 0/372 | 0/252 | 0/14 | 542/542 | Good |
| 350 | K | 0/372 | 1/252 | 0/14 | 542/542 | Poor |
| 371 | V | 0/372 | 1/252 | 0/14 | 542/542 | Good |
| 433 | N | 0/372 | 1/252 | 0/14 | 542/542 | Good |
| 456 | L | 0/372 | 1/252 | 0/14 | 542/542 | Poor |

A total of 1182 of NP sequence (372, 252, 14, and 542 of human H1N1, H3N2, HPAI in human cases, and H1N1/2001 registered as human cases at Influenza Virus Resources at NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/) were analyzed to identify signature amino acids that distinguish human influenza viruses from HPAI and H1N1/2009 viruses. Prediction of the signature residues is based on the quatemary structure of the NP proteins.



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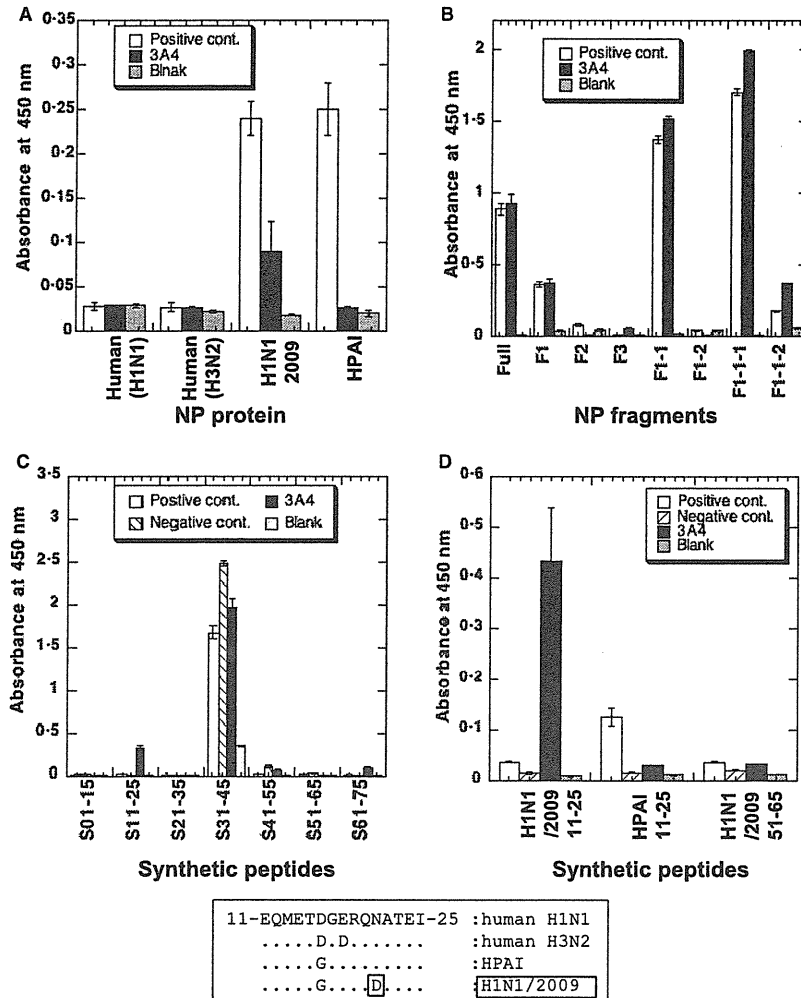


**Figure 1.** Comparison of the reactivity of 3A4 with NPs from human, H1N1/2009, and HPAI viruses and the results of epitope mapping. mAb reactive with NP of H1N1/2009 and HPAI viruses[6] was used as a positive control. (A) Reactivity of 3A4 with NPs of human, H1N1/2009, and HPAI viruses were analyzed by sandwich ELISA using polyclonal anti-NP antibodies as the capture antibodies. (B) Dissection of the 3A4 epitope using recombinant NP fragments by direct ELISA. Corresponding amino acids for each fragment are shown in Table S1. (C) Mapping of the epitope of 3A4 using 15-mer overlapping peptides with direct ELISA. (D) Dissection of the critical residues to discriminate the NP protein from viruses of different origin by 3A4 using synthetic peptides shown in the insert. Data are presented as means ± SD.

indicated that only N21D varied specifically in NP from H1N1/2009 viruses, and this was identified as a signature residue specific for NPs from H1N1/2009 viruses (Table 1). Thus, we tested the reactivity of a peptide corresponding to residues 11–25 of HPAI against 3A4. The results showed that 3A4 reacted the H1N1/2009 version of peptide but not the one of HPAI version (Figure 1D). These results indicated that variation of 21D is responsible for the specific recognition of NP from H1N1/2009 viruses by 3A4.

*3G2*

The mAb 3G2 was also raised against recombinant NP from H1N1/2009 viruses by a method similar to that used for 3A4. 3G2 reacted with NP from H1N1/2009 virus and to a lesser extent with NP from HPAI virus, but did not react with those from human seasonal viruses (Figure 2A). Reactivity against NP fragments showed that the epitope of 3G2 was located in residues 383 – 498 (Full and F3) (Figure 2B). Dissection of the epitope using 15-mer overlapping synthetic peptide corresponding to residues 415–469 of H1N1/2009 NP showed that 3G2 reacted specifically with a peptide corresponding to residues 455–469 (Figure 2C). We also analyzed the reactivity of 3G2 against synthetic peptides corresponding to residues 455–469 of NPs from human and HPAI viruses. The results showed that 3G2 reacted with H1N1/2009-type peptide and to a

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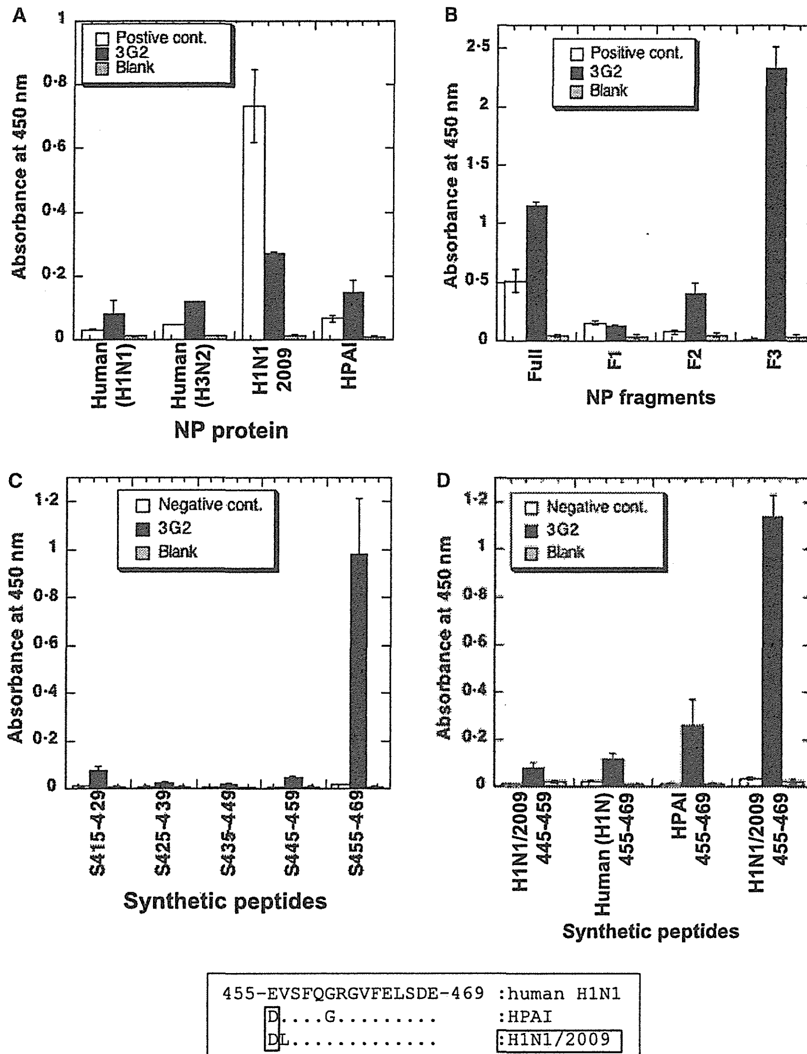


**Figure 2.** Comparison of the reactivity of 3G2 with NPs from human, H1N1/2009, and HPIA viruses and the results of epitope mapping. mAb 3A4 (mentioned above), which reacts only with H1N1/2009 NP, was used as a positive control. (A) Reactivity of 3G2 with NPs of human, H1N1/2009, and HPAI viruses were analyzed by sandwich ELISA using polyclonal anti-NP antibodies as the capture antibodies. (B) Dissection of the 3G2 epitope using recombinant NP fragments by direct ELISA. (C) Mapping of the epitope of 3G2 using 15-mer overlapping peptides by direct ELISA. (D) Dissection of the critical residues to discriminate the NP protein from viruses of different origin using synthetic peptides shown in the insert. A peptide corresponding to residues 445–459 of the NP protein of H1N1/2009 virus was used as a negative control. Data are presented as means ± SD.

lesser extent with HPAI-type peptide but not with the human-type peptide (Figure 2D). Comparison of the corresponding sequences in human, HPAI, and H1N1/2009 viruses indicated that the only residue that varied specifically in both NPs from H1N1/2009 and HPAI viruses was E455D. This residue, E455D, is not a signature residue specific for NPs from H1N1/2009 virus, although it is adjacent to 456L, which is a signature residue specific for NPs from H1N1/2009 viruses (Table 1). These results indicated that it is likely that variation of 455D is responsible for the discrimination of NPs from H1N1/2009 and HPAI viruses by 3G2, and that the signature residue 456L also contributes to the discrimination between NP from H1N1/2009 and HPAI viruses.

*M322211*

M322211 is a commercial mAb that was previously found to react with NPs from human and H1N1/2009 viruses but



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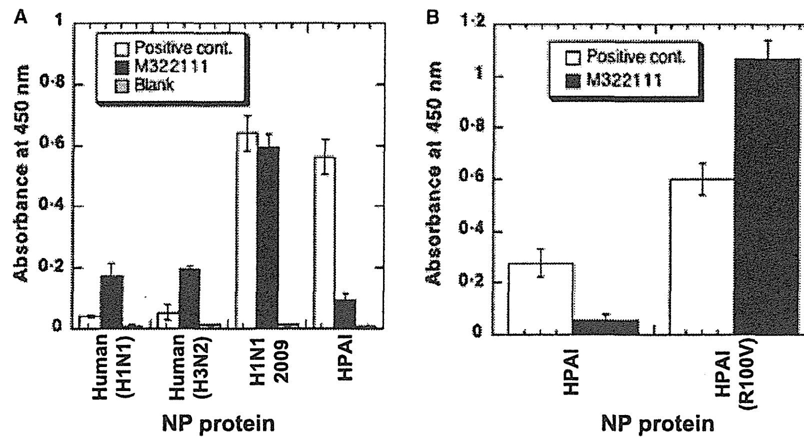


**Figure 3.** Comparison of the reactivity of M322111 with NPs of human, H1N1/2009, and HPAI viruses and the results of epitope mapping. mAb reactive with NP of H1N1/2009 and HPAI viruses[6] was used as a positive control. (A) Reactivity of M322111 with NPs of human, H1N1/2009, and HPAI viruses were analyzed by sandwich ELISA using polyclonal anti-NP antibodies as the capture antibodies. (B) Reactivity of M322111 with NPs of wild-type avian virus and that with the introduction of R100V mutation. Wild-type and R100V mutants of avian NP proteins were expressed in HEK293 cells and used for the analysis by sandwich ELISA format. Data are presented as means ± SD.

not with that from HPAI virus.[6] M322211 did not react with recombinant NP fragments (data not shown), suggesting that the mAb recognizes a structural rather than a linear epitope. In a previous study, we used a series of chimeras consisting of NPs from human and avian viruses to locate the epitope of M322211, and the results indicated that the epitope was located within residues 1–188.[6] In this study, we expressed recombinant avian NP carrying R100V mutation, which reverted a signature mutation in NP of HPAI virus to that of H1N1/2009 virus, in HEK293 cells and performed sandwich ELISA. The results showed that M322211 gained reactivity against avian NP by the introduction of R100V mutation (Figure 3B). R100 was shown to be responsible for the discrimination between NPs from human and H1N1/2009 viruses and that from HPAI viruses by M322211.

## Discussion

In the present study, we characterized three antibodies that can discriminate between the NP proteins from human influenza viruses and those of H1N1/2009 or HPAI origin. By dissection of the epitope of each antibody, we identified the mechanism of discrimination underlying the origin-dependent specific "signature residues" present in the amino acid sequence of NP.

As large amounts of NP protein sequence data are now available, we performed phylogenetic analysis of the NP proteins registered between 2006 and 2011 (Figure 4). The results indicated that the NP proteins of seasonal influenza, HPAI, and H1N1/2009 belong to different clades, even though all of these clades contain swine viral NP. Sequence identity among the NP proteins is around 90% (data not shown) and NP consists of about 500 amino acids, suggesting that about 50 amino acids could be different between two NP proteins selected at random. As these variations could be shared among the viruses, we compared the NP sequences and identified the specific residues in H1N1/2009 (nine residues) and HPAI isolated from human cases (five residues), which are different from the NP proteins of human seasonal viruses (Table 1). As mentioned above, the signature residues are responsible for the discrimination of NP among viruses of different origin by particular antibodies.

To evaluate the accessibility of the signature residues identified by the antibodies, we predicted the locations of the signature residues based on the crystal structure of the NP proteins[12,13] *in silico* (Table 1). The NP proteins form oligomers in solution. Thus, in addition to residues not located on the surface of NPs, it would be difficult for antibodies to access those located at the interface of NP oligomers. The results of *in silico* analysis suggested that signature residues of HPAI at positions 33 and 136, and those of H1N1/2009 NP at positions 190, 350, and 456 are unlikely to be exposed to the external environment, while those of HPAI at positions 100, 305, and 357, and those of H1N1/2009 NP at positions 21, 53, 313, 316, 371, and 433 are likely candidates for recognition by antibodies. Indeed, the antibodies characterized in the present study only recognized the candidate signature residues, suggesting that these residues may be useful for the discrimination of influenza A virus based on the NP sequence.

In conclusion, we identified key amino acid residues in the NP protein, which could discriminate influenza A

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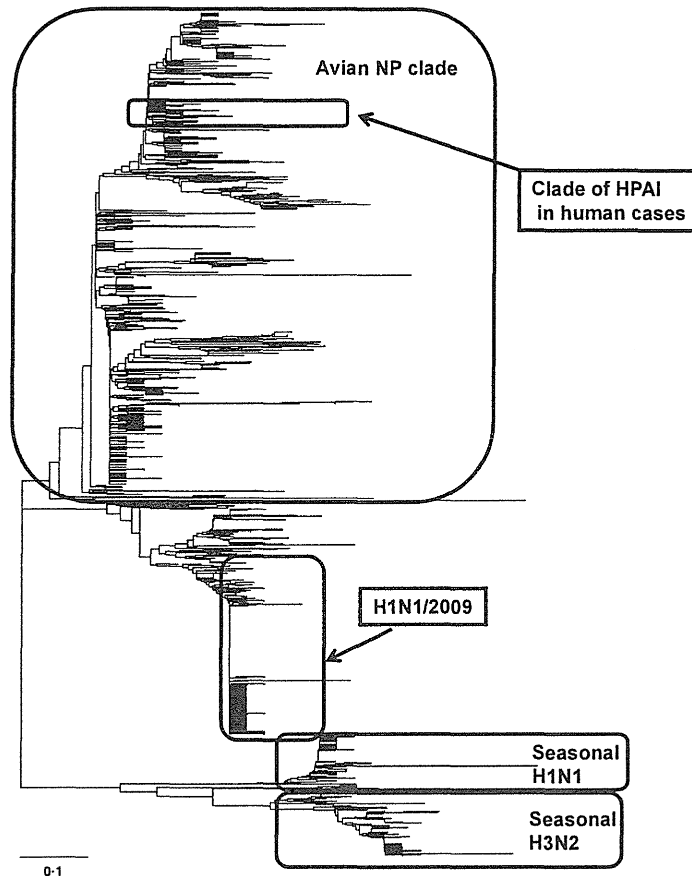


**Figure 4.** Phylogenetic analysis of NP based on their amino acid sequence. The NP sequences of human-, avian-, and swine-origin influenza viruses reported from 2005 to 2011 were included into the analysis. Full-length amino acid sequence data of NP registered in NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database) dated 2005 to 2011 were retrieved. Number of the sequences used in the analysis was 1917. Clades containing NPs of human-, avian-, and H1N1/2009 virus are indicated. All clades contained NP proteins of swine viruses.

viruses based on the viral origin and verified the feasibility of their utilization to determine the origin of NP. As NP protein is the target of rapid influenza diagnostic kits, which have been implemented in clinical settings, the information presented here could be easily translated to clinical practice.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Recombinant NP proteins of H1N1/2009 used for the epitope mapping.

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GENOME ANNOUNCEMENT

# Complete Genome Sequence of *Streptococcus pyogenes* M1 476, Isolated from a Patient with Streptococcal Toxic Shock Syndrome

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**Here, we report the completely annotated genome sequence of *Streptococcus pyogenes* M1 476 isolated from a patient with streptococcal toxic shock syndrome (STSS) during pregnancy. The genome sequence will provide new insights into the mechanisms underlying STSS.**

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Group A streptococci (GAS) cause a wide variety of infectious diseases that range from relatively benign to life-threatening, including streptococcal toxic shock syndrome (STSS). GAS can be subtyped according to the genotype of *emm*, which encodes the M protein expressed on the bacterial cell surface. Particular *emm* types of GAS have been associated with certain diseases (2, 4). *emm1* GAS has been found predominantly in patients with STSS. *Streptococcus pyogenes* M1 GAS 476 was isolated from a patient with STSS during pregnancy in 1994 and showed the strongest virulence in a mouse STSS model (designated M1-d in reference 5).

Here, an 8-kb pair-end library of the *S. pyogenes* M1 476 genome was prepared and used for sequence analysis with a GS junior titanium sequencer (Roche). This generated 185,092 reads, covering 40,467,919 bp (22.2-fold coverage), which were assembled into contigs and scaffolds by using a GS De Novo Assembler 2.6 (Newbler; Roche). Gap filling among the contigs and scaffolds was then performed by conventional Sanger sequencing of the PCR fragments based on brute-force PCR. Finally, the 5,968,488 pair-end reads determined using a Genome Analyzer IIx (Illumina) were added to the draft genome sequence. Primary coding segment extraction was performed using MetaGeneAnnotator (6). Initial functional assignment and manual correction were carried out by *in silico* molecular cloning. Prophage regions and clustered regularly interspaced short palindromic repeats (CRISPRs) were identified by Prophage Finder (1) and CRISPRFinder (3), respectively. The *S. pyogenes* M1 GAS 476 genome consists of a single circular chromosome of 1,813,709 bp with an average GC content of 38.5%. The chromosome was shown to contain a total of 1,848 protein-coding genes, 57 tRNA genes for all amino acids, and 5 *rrn* operons. In addition, the chromosome harbors 5 prophage-like elements. The prophage regions contain genes corresponding to superantigen (two genes), streptodornase, and mitogenic factor. The chromosome also contains five putative CRISPRs.

**Nucleotide sequence accession number.** The nucleotide sequence of the chromosome of *S. pyogenes* M1 GAS 476 has been deposited in the DNA Database of Japan under accession no. AP012491.

## ACKNOWLEDGMENTS

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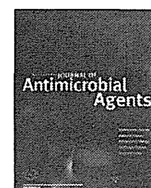


Short communication

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

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ABSTRACT

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

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

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

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

## 2. Materials and methods

### 2.1. Bacterial strains

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

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*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), 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 ($MIC_{50}$ and $MIC_{90}$, 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 *SpeI* (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 housekeeping 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

**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].

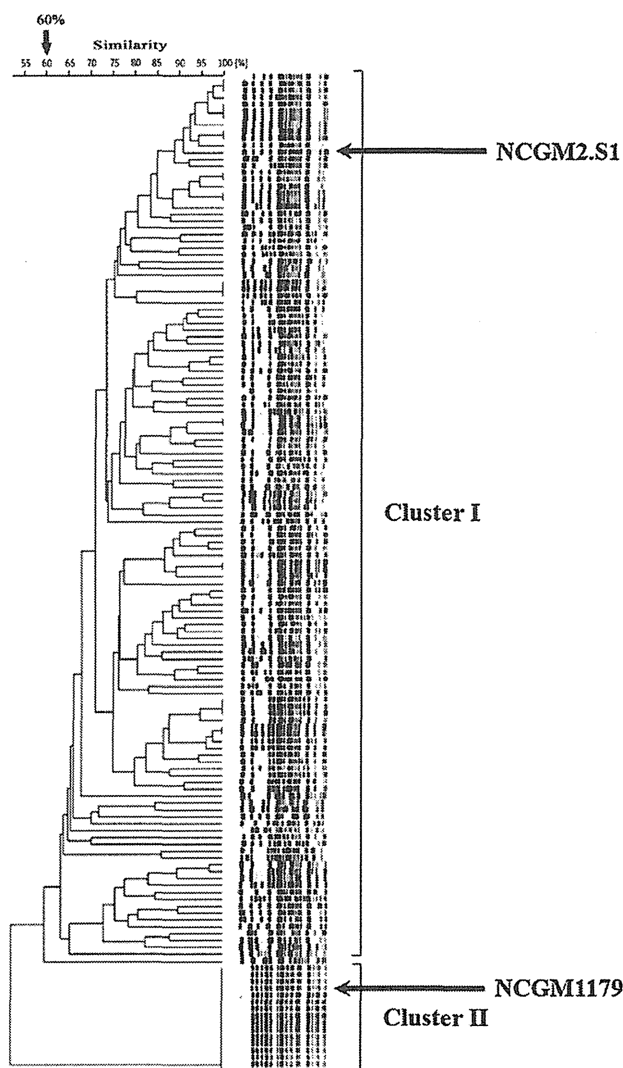


**Fig. 1.** Dendrogram of pulsed-field gel electrophoresis (PFGE) patterns of 145 multidrug-resistant *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae. Two clusters (I and II) were detected. Of the 128 isolates belonging to cluster I, 127 isolates were ST235 and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

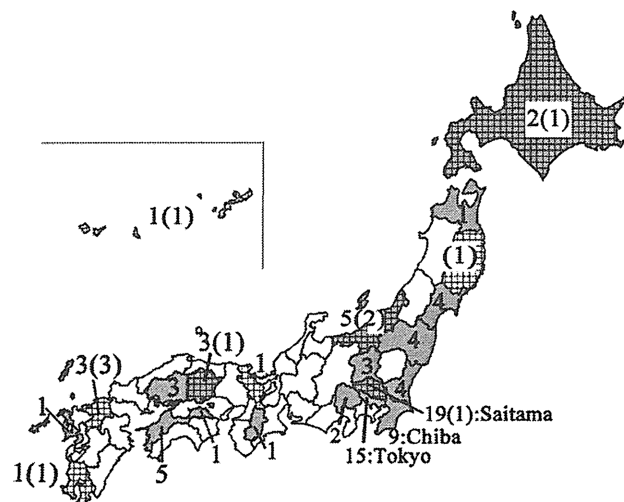


**Fig. 2.** Geographical distribution of multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates producing IMP-type metallo-β-lactamases and AAC(6′)-Iae in Japan. Isolates belonging to cluster I (Fig. 1) were obtained from prefectures marked in grey; isolates belonging to cluster II were obtained 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.

transposase 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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*Competing interests*: None declared.

*Ethical approval*: This study was approved in 2010 by the Biosafety Committee, National Center for Global Health and Medicine (Tokyo, Japan) (approval no. 23-M-58).

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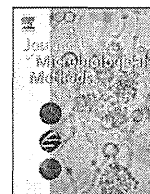

Note

# Development of an immunochromatographic assay for rapid detection of AAC(6′)-Ib-producing *Pseudomonas aeruginosa*

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ABSTRACT

To detect aminoglycoside 6′-*N*-acetyltransferase-Ib [AAC(6′)-Ib]-producing, *Pseudomonas aeruginosa* isolates which are a frequent cause of nosocomial infections in Japan, an immunochromatographic assay was developed using two kinds of monoclonal antibodies (mAbs) recognizing AAC(6′)-Ib. The results of the assessment were fully consistent with those of *aac(6′)-Ib* PCR analyses.

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The emergence of multidrug-resistant (MDR) *Pseudomonas aeruginosa* strains, defined as having resistance to all carbapenems, amikacin, and fluoroquinolones, has become a serious problem in medical settings in Japan (Kirikae et al., 2008; Sekiguchi et al., 2007; Sekiguchi et al., 2005; Kitao et al., 2012).

The major mechanism of aminoglycoside resistance is the production of aminoglycoside-modifying enzymes (Shaw et al., 1993). The aminoglycoside 6′-*N*-acetyltransferases [AAC(6′)s] are of interest because they can modify a number of clinically important aminoglycosides.

More than 20% of MDR *P. aeruginosa* isolates in Japan produced the aminoglycoside-modifying enzyme 6′-*N*-acetyltransferase-Ib [AAC(6′)-Ib], which will be reported elsewhere. Approximately 70% of MDR *P. aeruginosa* isolates in Japan produced AAC(6′)-Iae (Kitao et al., 2012). We first developed an immunochromatographic assay to detect AAC(6′)-Iae (Kitao et al., 2010). In this study, an immunochromatographic assay using mAbs to AAC(6′)-Ib was developed.

*P. aeruginosa* NCGM509 producing AAC(6′)-Ib was obtained from a respiratory tract of a patient in a hospital located in Tokyo in 2005. NCGM2.S1 producing AAC(6′)-Iae (Sekiguchi et al., 2005), NCGM798 producing AAC(6′)-Iaf (Kitao et al., 2009) and NCGM1588 producing AAC(6′)-Iaj (Tada et al., unpublished results) were obtained. Two hundred seventeen *P. aeruginosa* isolates were obtained from BML. Of those, 98 were PCR-positive for *aac*(6′)-*Ib*, and the remaining 119 were PCR-negative.

The *aac(6′)-Ib* was PCR amplified from *P. aeruginosa* NCGM509 using the primer sets SphI-aac(6′)-Ib-F (5′-ccccgcatgcgATGACCAACAGCACCGATTCCGTCACA-3′) and NotI-aac(6′)-Ib-R (5′-gggggcggccgcTTAGGCATCACTGCGTGTTCGCTCGAA-3′). Recombinant His-tagged AAC(6′)-Ib, AAC(6′)-Iae (Sekiguchi et al., 2005), AAC(6′)-Iaf (Kitao et al., 2009), and AAC(6′)-Iaj (Tada et al., unpublished results), purified by Protein G Sepharose 4 Fast Flow (GE Healthcare Bio-Science, Tokyo, Japan) were prepared as described previously (Kitao et al., 2010). Anti-AAC(6′)-Ib monoclonal antibodies (mAbs) were prepared as described previously (Kitao et al., 2010). The animal experiments were approved by the Ethics Committee for Animal Experiments at NCGM (approval number: NCGM23-C-1).

The immunochromatographic assay was assembled as described previously (Miyoshi-Akiyama et al., 2010). Nitrocellulose membranes were coated with 0.76 mg of rat mAbs and 0.2 mg of anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) per test at positions 30 and 39 mm from the sample application area, respectively. Pads were prepared by soaking glass filters with rat mAb and rabbit IgG, each conjugated with colloidal gold.

To determine the epitopes for anti-AAC(6′)-Ib antibody, 3F9 and 3A9; recombinant peptides having the same amino acid sequences of regions in AAC(6′)-Ib were prepared using PCR cloning procedure. We designed 4 long peptides consisting of 55–61-mers covering from

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positions 1 to 184-end region of AAC(6′)-1b, and 13 short peptides consisting of 14–15-mers covering from the positions 51 to 184-end region (Table 1).

The immunochromatographic assay was evaluated using 217 clinical isolates of *P. aeruginosa*, and NCGM2.S1 producing AAC(6′)-Iae, NCGM798 producing AAC(6′)-Iaf, NCGM1588 producing AAC(6′)-Iaj, and *Escherichia coli* harboring pQE2-*aac(6′)-Ib*. Bacterial colonies grown on Mullar–Hinton agar plates were picked with a swab and suspended in soft test tubes containing 0.4 M Tris–HCl buffer containing 1.0% Triton X-100 (pH 7.5). Three drops of the bacterial lysate were added to the test plate. The results were analyzed by visual inspection 15 min after addition of the sample.

The *aac(6′)-Ib* was amplified with primers as follows; 5′-ATGACTGAGCATGACCTTGCGAT-3′ and 5′-GGCATCACTGCGTGTTCGCTCGAAT-3′. PCR amplification started at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 52 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The PCR procedure took 90 min.

We obtained 8 mAbs with reactivities to recombinant His-AAC(6′)-Ib NCGM509 in ELISA. Fifty-six immunochromatographic assay prototypes were constructed using these 8 mAbs and examined for reactivities to His-AAC(6′)-Ib (data not shown). The strongest intensity of the test line was obtained with the prototype consisting of 3F9/F1 immobilized on the membrane and 3A9/F1 labeled with colloidal gold (data not shown). Therefore, these two mAbs were utilized in the development of an immunochromatographic assay for detection of AAC(6′)-Ib (Fig. 1A). The sensitivity was 8 ng per test or $7.6 \times 10^6$ cfu per test (Fig. 1B). Other aminoglycoside acetyltransferases, including AAC(6′)-Iae, AAC(6′)-Iaf and AAC(6′)-Iaj, were not detected using the assay (data not shown).

Competition assays using 4 long peptides covering the whole region of AAC(6′)-Ib indicated that 3A9/F1 bound to two peptides, 91–145 and 130–184, whereas 3F9/F1 bound to peptide 45–105 (Fig. 2A). Further competition assays were performed using 13 short peptides. As shown in Fig. 2B, 3A9/F1 bound to peptide 171–184 consisting of VQTRQAFERTRSDA, whereas 3F9/F1 bound to peptide 61–75 consisting of TPYIAMLNGEPIGYA.

Of all 217 *P. aeruginosa* isolates tested, 98 were positive by the immunochromatographic assay. These results were fully consistent with those of PCR for *aac(6′)-Ib* showing 100% specificity and 100% sensitivity.

Here, we demonstrated that the newly developed immunochromatographic assay was a rapid and easy-to-use kit to detect AAC(6′)-Ib-producing *P. aeruginosa* isolates. The sensitivity is sufficient to use for bacteria isolated in clinical laboratories, although the sensitivity was lower than that of PCR (≤10 cfu, data not shown).

The 3A9/F1 and 3F9/F1 antibodies were likely to bind to the opposite sites of AAC(6′)-Ib and capture the molecule effectively on the immunochromatographic assay. The three-dimensional structure of AAC(6′)-Ib (PDB ID: 2PRB) (Vetting et al., 2008) indicated that AAC(6′)-Ib is composed of 7 β-strands and 5 α-helices (Fig. 2C). The aminoglycoside binding site is constructed as a pocket with β2-4 (Vetting et al., 2008). The 3F9/F1 seems to bind to β2, β3, and a loop formed by both β strands, whereas 3A9/F1 seems to bind to α5 in the C-terminal region (Fig. 2C).

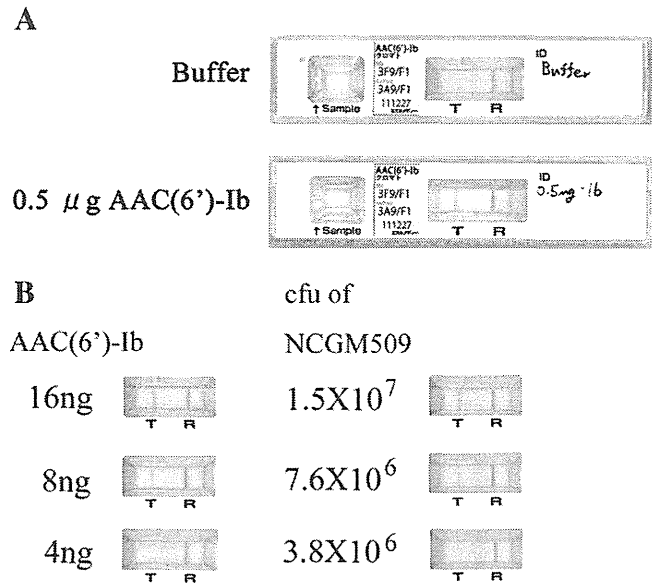


**Fig. 1.** (A) Immunochromatography using mAbs 3A9/F1 and 3F9/F1. In negative cases, a line appears only at the reference line (R). In positive cases, another line also appears at the positive test line (T) in addition to the reference line. The arrow indicates the direction of lateral flow. (B) Sensitivity testing using AAC(6′)-Ib or the positive control strain NCGM509.

The immunochromatographic assay to detect AAC(6′)-Ib will be useful when applied together with immunochromatographic assays to detect isolates of AAC(6′)-Iae (Kitao et al., 2010) and IMP (Kitao et al., 2011), to detect MDR *P. aeruginosa* isolates effectively in medical settings in Japan.

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**Table 1**
Peptides used in epitope mapping were expressed and purified recombinant peptides.

| Number | Region in AAC(6′)-Ib | Sequence |
|---|---|---|
| 1 | 1–60 | VTNSTDSVTLRLMTEHDLAMLYEWLNRSHIVEWWGGEEARPTLADVQEQYLPSVLAQESV |
| 2 | 45–105 | DVQEQYLPSVLAQESVTPYIAMLNGEPIGYAQSYVALGSGDGWWEEETDPGVRGIDQLLAN |
| 3 | 91–145 | ETDPGVRGIDQLLANASQLGKGLGTKLVRALVELLFNDPEVTKIQTDPSPSNLRA |
| 4 | 130–184 | VTKIQTDPSPSNLRAIRCYEKAGFERQGTVTTPDGPAVYMVQTRQAFERTRSDA |
| 5 | 51–65 | LPSVLAQESVTPYIA |
| 6 | 61–75 | TPYIAMLNGEPIGYA |
| 7 | 71–85 | PIGYAQSYVALGSGD |
| 8 | 81–95 | LGSGDGWWEEETDPG |
| 9 | 91–105 | ETDPGVRGIDQLLAN |
| 10 | 101–115 | QLLANASQLGKGLGT |
| 11 | 111–125 | KGLGTKLVRALVELL |
| 12 | 121–135 | LVELLFNDPEVTKIQ |
| 13 | 131–145 | VTKIQTDPSPSNLRA |
| 14 | 141–155 | SNLRAIRCYEKAGFE |
| 15 | 151–165 | KAGFERQGTVTTPDG |
| 16 | 161–175 | TTPDGPAVYMVQTRQ |
| 17 | 171–184 | VQTRQAFERTRSDA |

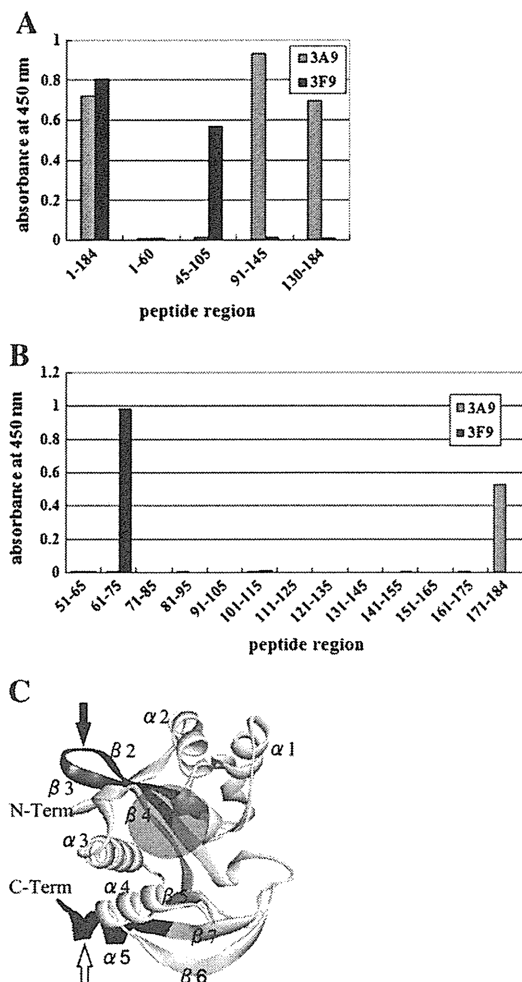


**Fig. 2.** Determination of epitopes by ELISA. (A) Competition assays using 4 long peptides covering the whole region of AAC(6′)-Ib revealed that 3A9/F1 bound to two peptides, 91–145 and 130–184, whereas 3F9/F1 bound to peptide 45–105. (B) Competition assays using 13 short peptides covering the region of peptide 51–184 of AAC(6′)-Ib revealed that 3A9/F1 bound to peptide 171–184, whereas 3F9/F1 bound to peptide 61–75. (C) Epitope regions of 3A9/F1 and 3F9/F1 on AAC(6′)-Ib crystal structure (PDB ID: 2PRB) (Vetting et al., 2008). The white arrow indicates the epitope region of 3A9/F1, which consists of amino acid residues corresponding to positions 171–184 of AAC(6′)-Ib. The black arrow indicates the epitope region of 3F9/F1, which consists of amino acid residues corresponding to positions 61–75 of AAC(6′)-Ib. The gray disk indicates the location of the aminoglycoside binding pocket.

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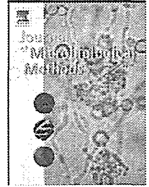

# Loop-mediated isothermal amplification assays for identification of antiseptic- and methicillin-resistant *Staphylococcus aureus*

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ABSTRACT

A method for rapid identification of antiseptic- and methicillin-resistant *Staphylococcus aureus* (MRSA) based on 3 loop-mediated isothermal amplification (LAMP) assays was developed. LAMP targeting the *femB* gene identified *S. aureus* with 100% specificity, and LAMP targeting the *mecA* gene associated with methicillin resistance identified methicillin-resistant staphylococci with 100% specificity. LAMP targeting the *qacA/B* gene encoding an efflux pump responsible for antiseptic resistance identified high-acriflavine-resistant (MIC≥100 mg/L) MRSA (92.5% positive) and acriflavine-susceptible (MIC<25 mg/L) MRSA (100% negative). They were performed under the same reaction conditions within 60 min at 63 °C. The combined LAMP assays will be useful for rapid identification of *S. aureus* isolates and determination of their antibiotic and antiseptic resistance patterns with regard to methicillin and organic cationic substrates.

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## 1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most important hospital-associated (nosocomial) bacterial pathogens worldwide. Preventive measures against MRSA infection in hospitals include the topical application of antiseptics to living tissue, such as skin and mucous membranes, and the use of disinfectants for medical appliances. The use of antiseptics and disinfectants, including quaternary ammonium compounds (QAC), is also considered to prevent the development of infections, which will minimize antibiotic use. However, overuse of antiseptic agents has resulted in the emergence of antiseptic-resistant MRSA (Noguchi et al., 2005; Sekiguchi et al., 2004). It was reported that more than a half of MRSA isolates obtained from 11 Asian countries, including South Korea, China, the Philippines, Singapore, Vietnam, Thailand, Indonesia, India, Sri Lanka, Saudi Arabia and Japan, were resistant to QAC (Noguchi et al., 2005). MRSA strain HPV107, a representative of the MRSA Iberian clone that is currently disseminated throughout several European countries and the USA in medical settings were also resistant to QAC (Costa et al., 2010). Therefore, for nosocomial infection control, it is important to obtain epidemiological information regarding antiseptic susceptibility as well as antibiotic susceptibility of MRSA.

Methicillin-resistance in *S. aureus* is primarily mediated by the low-affinity penicillin-binding protein 2a or 2' (PBP2a or PBP2'), encoded by the chromosomal structural gene *mecA* (Hartman and Tomasz, 1984; Matsuhashi et al., 1986). However, *mecA* is also widely distributed among coagulase-negative staphylococci (CNS), and is associated with methicillin-resistance of CNS (Ubukata et al., 1990). Thus, polymerase chain reaction (PCR) assay targeting *mecA* alone cannot discriminate between MRSA and methicillin-resistant CNS. The *femB* gene locus is distant from the *mecA* gene on the chromosome, and it is involved in cell wall pentaglycine side chain and interpeptide bridge formation (Berger-Bächi et al., 1992; Henze et al., 1993). *femB* is highly conserved in *S. aureus*, but it is not found in CNS (Kobayashi et al., 1994). Therefore, both *mecA* and the *femB* are employed for the rapid detection of MRSA by PCR (Kobayashi et al., 1994; Jonas et al., 1999, 2002; Pérez-Roth et al., 2001; Towner et al., 1998).

MRSA, which is highly resistant to QAC, has efflux pumps that are membrane-bound and proton-motive force-dependent cation export proteins (Chapman, 2003). The proteins are encoded by *qacA* and *qacB* genes located on plasmids carrying various antibiotic resistance genes (Paulsen et al., 1996; Tennent et al., 1989). *qacA* confers resistance to various organic cationic substrates, including monovalent cations, such as acriflavine, ethidium bromide, QAC, and benzalkonium chloride, and divalent cations, such as chlorhexidine and pentamidine. The nucleotide sequence of *qacA* is almost identical to that of *qacB* (Paulsen et al., 1996). The QacA protein encoded by *qacA* and the QacB protein encoded by *qacB* differ in only one amino acid at residue 323

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(Asp in QacA and Ala in QacB) (Paulsen et al., 1996). However, QacA protein confers greater resistance to divalent cations than QacB protein. *qacA* is also found in CNS (Leelaporn et al., 1994).

Loop-mediated isothermal amplification (LAMP), auto-cycling and strand displacement DNA synthesis, have been reported for 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. Recently, LAMP assays have been employed as replacements for PCR because of the rapidity, higher specificity, and equal or greater sensitivity (Cai et al., 2008; Goto et al., 2010; Hong et al., 2004).

Here, we report combination use of three LAMP assays for identification of antiseptic-resistant MRSA: the assay targeting *femB* for identification of *S. aureus*, an assay targeting *mecA* for identification of methicillin resistance, and an assay targeting *qacA/B* for identification of antiseptic resistance.

## 2. Materials and methods

### 2.1. Bacterial strains and DNA extraction

The specificity of the LAMP assays was evaluated with DNA extracted from *S. aureus* N315, 192 clinical isolates of MRSA, 3 clinical isolates of methicillin-sensitive *S. aureus* (MSSA), 6 clinical isolates of methicillin-resistant *Staphylococcus epidermidis* (MRSE), 4 clinical isolates of methicillin-susceptible CNS (MSCNS), and 27 non-staphylococcal bacteria listed in Table 1. Each clinical isolates of MRSA was obtained from single inpatient at National Center for Global Health and Medicine (NCGM) in October 2003, October 2004 and October 2005. Each clinical isolate of MSSA, MRSE, MSCNS was obtained from single inpatient at NCGM in October 2005. These isolates were biochemically identified and analyzed for drug susceptibility by the MicroScan WalkAway system (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Unless otherwise stated, they were stocked at NCGM. Genomic DNA was extracted by a simple boiling method from respective bacteria. A loopful of cultured bacteria was suspended in 100 μL of distilled water and boiled for 10 min. The bacterial suspension was then centrifuged at 12,000 × *g* for 5 min, and the supernatant was used as the DNA sample for subsequent LAMP and PCR assays.

### 2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) of acriflavine, determined using the twofold serial dilution method in Mueller–Hinton broth, was used as an index of antiseptic susceptibility. The mixture, consisting of one of 260 MRSA isolates, nutrient medium, and acriflavine, was incubated at 35 °C for 24 h. The lowest concentration of acriflavine at which no visible growth occurred was defined as the MIC (breakpoint ≥ 25 μg/mL).

**Table 1**
The *femB*-, *mecA*-, and *qacA/B*-LAMP and PCR assays among various bacterial species.

| Species | Resource/strain[a] | *femB*- | | *mecA*- | | *qacA/B* | |
|---|---|---|---|---|---|---|---|
| | | LAMP | PCR | LAMP | PCR | LAMP | PCR |
| Staphylococci | | | | | | | |
| *Staphylococcus aureus* | N315 | + | + | + | + | − | − |
| MRSA[b] | NCGM342 + 135 isolates | + | + | + | + | + | + |
| MRSA[c] | NCGM962 + 55 isolates | + | + | + | + | − | − |
| MSSA[c] | NCGM1665 + 2 isolates | + | + | − | − | − | − |
| MRSE[b] | NCGM1 + 5 isolates | − | − | + | + | + | + |
| MSCNS[c] | NCGM425 + 3 isolates | − | − | − | − | − | − |
| Non-staphylococci | | | | | | | |
| *Enterococcus faecalis* | NCGM1 | − | − | − | − | − | − |
| *E. faecium* | NCGM1 | − | − | − | − | − | − |
| *E. gallinarum* | NCGM1 | − | − | − | − | − | − |
| *E. hirae* | NCGM1 | − | − | − | − | − | − |
| *Streptococcus pneumoniae* | GTC261 | − | − | − | − | − | − |
| *S. pyogenes* | NCGM1 | − | − | − | − | − | − |
| *Listeria monocytogenes* | EGD | − | − | − | − | − | − |
| *Acinetobacter baumannii* | NCB0211-439 | − | − | − | − | − | − |
| *Bacteroides fragilis* | NCTC10581 | − | − | − | − | − | − |
| *Enterobacter cloacae* | NCGM1 | − | − | − | − | − | − |
| *Escherichia coli* | ATCC8739 | − | − | − | − | − | − |
| *Haemophilus influenzae* | IID944 (ATCC9334) | − | − | − | − | − | − |
| *Klebsiella oxytoca* | NCGM1 | − | − | − | − | − | − |
| *K. pneumoniae* | IID5209 (ATCC15380) | − | − | − | − | − | − |
| *Proteus mirabilis* | NCGM1 | − | − | − | − | − | − |
| *Pseudomonas aeruginosa* | ATCC27853 | − | − | − | − | − | − |
| *Salmonella* Enteritidis | No. 11 | − | − | − | − | − | − |
| *S.* Typhimurium | rbf388 | − | − | − | − | − | − |
| *Serratia marcescens* | NCGM13 | − | − | − | − | − | − |
| *Stenotolophonorus maltophilia* | NCGM1 | − | − | − | − | − | − |
| *Legionella pneumophila* | GTC745 | − | − | − | − | − | − |
| *Rhodococcus equi* | ATCC33710 | − | − | − | − | − | − |
| *Mycoplasma pneumoniae* | IID817 | − | − | − | − | − | − |
| *Mycobacterium avium* | ATCC25291 | − | − | − | − | − | − |
| *M. intracellulare* | JCM6384 (ATCC13950) | − | − | − | − | − | − |
| *M. kansasii* | JCM6379 (ATCC124878) | − | − | − | − | − | − |
| *M. tuberculosis* | H37Rv (ATCC27294) | − | − | − | − | − | − |

[a] ATCC, American Type Culture Collection, Rockville, MD, USA; GTC, Gifu Type Culture Collection, Department of Microbiology, Gifu University School of Medicine, Gifu, Japan; IID, Institute of Medical Science, The University of Tokyo, Tokyo, Japan; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan; NCGM strains and clinical isolates were collected from patients visiting the hospital, National Center for Global Health and Medicine.

[b] *QacA/B*-PCR positive.

[c] *qacA/B*-PCR negative.

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**Table 2**
LAMP primer sets for *femB*-, *mecA*-, and *qacA/B*-genes.

| Primers | Sequences (5′ to 3′) |
|---|---|
| *femB* | |
| F3 | TGTTTAAATCACATGGTTACGAG |
| B3 | TCACGTTCAAGGAATCTGA |
| FIP | TACCTTCAAGGTTTAATACGCCCATCATCATGGCTTTACAACTGAG |
| BIP | ACACCCGAAACATTGAAAAAGACACTTTAACACCATAGTTTATCGCTT |
| *mecA* | |
| F3 | GCGACTTCACATCTATTAGGT |
| B3 | GCCATCTTTTTTCTTTTTCTCT |
| FIP | TCCCTTTTTACCAATAACTGCATCATATGTTGGTCCCATTAACTCT |
| BIP | AAGCTCCAACATGAAGATGGCCGATTGTATTGCTATTATCGTCAA |
| *qacA/B* | |
| F3 | GAAAGGGCCACTGCATTA |
| B3 | ATCCCACGAGTGAGACTT |
| FIP | CTCAAGTAAAGCTCCTCCGATAATTTATGGTCAATCGCTTCATCG |
| BIP | AATGTACCGTTTGCGATAATAGCATCTTTTGATAACTTAGACTCTGG |

Underlined nucleotides in the *qacA/B* FIP primer show mismatch nucleotides in a *qacB* gene (GenBank, accession no. GQ900490).

### 2.3. LAMP primers

The respective oligonucleotide primer sets for the detection of *femB*, *mecA*, and *qacA* were designed using the PrimerExplorer V3 software (http://primerexplorer.jp/e/). Both *femB* and *mecA* were taken from the genome sequence of *S. aureus* N315 (GenBank ID: BA000018), and *qacA* was taken from DNA sequence of the plasmid pSA1379 isolated from *S. aureus* IMCJ1379 (GenBank ID: AB255366). Each set of 4 primers consisted of 2 outer primers (F3 and B3) and 2 inner primers (FIP and BIP). The sequences of primers used for the 3 LAMP assays are listed in Table 2.

### 2.4. LAMP assay

The LAMP assays were carried out with a 12.5-μL reaction mixture containing 1 μL (approximately 100 ng) of extracted DNA, 1 of 3 sets of primers (0.2 μM each of F3 and B3, and 1.6 μM each of FIP and BIP), 1.4 mM of each dNTP, 4 U of the large fragment of *Bst* DNA polymerase (New England BioLabs, Sumida, Tokyo, Japan), and 8 μg monomeric cyanine (Invitrogen, Carlsbad, CA) in 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]. They were incubated at 63 °C for 60 min in a real-time thermal cycling system Roter-Gene 2000 (Corbett Research, Mortlake, New South Wales, Australia). DNA amplification was monitored at 510 nm during incubation.

### 2.5. PCR assay

Duplex PCR for simultaneous detection of *femB* and *mecA* was performed as described previously (Jonas et al., 1999; Jonas et al., 2002). The 50-μL PCR mixture consisted of 1 μL of genomic DNA, 0.3 μM of both FemB1 (5′-TTA CAG AGT TAA CTG TTA CC-3′) and FemB2 (5′-ATA CAA ATC CAG CAC GCT CT-3′) primers, 0.1 μM of both MecA1 (5′-GTA GAA ATG ACT GAA CGT CCG ATA A-3′) and MecA2 (5′-CCA ATT CCA CAT TGT TTC GGT CTA A-3′) primers, 0.2 mM of each dNTP, and 1.25 U of *Ex Taq* DNA polymerase (TaKaRa Bio, Shiga, Japan) in 1× *Ex Taq* buffer. Amplification was performed using an initial denaturation step of 4 min at 94 °C followed by 30 cycles of 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 60 s, with a final extension step of 2 min at 72 °C. PCR for detection of the *qacA/B* gene was also performed as described previously (Sekiguchi et al., 2004). The reaction composition was the same as described above except for the primers used (forward, 5′-TCC TTT TAA AGC TGG CTT ATA CC-3′; reverse, 5′-AGC CKT ACC AGC TCC AAC TA-3′). Amplification was performed using an initial denaturation step of 1 min at 95 °C followed by 30 cycles of 94 °C for 15 s, 56 °C for 15 s, and 72 °C for 20 s, with a final extension step of 1 min at 72 °C. PCR products were analyzed by electrophoresis on 1.5% agarose gels.

## 3. Results and discussion

Various bacterial species were tested in the LAMP and PCR assays for detection of *femB*, *mecA*, and *qacA/B*, and the results are summarized in Table 1. The results of LAMP showed 100% agreement with those of PCR. All *S. aureus* strains tested, including *S. aureus* N315, 192 MRSA isolates, and 3 MSSA isolates, were positive for *femB*-LAMP. Other bacterial species tested were negative for *femB*-LAMP. All methicillin-resistant *Staphylococcus* spp., including *S. aureus* N315, 192 MRSA isolates, and 6 MRSE isolates, were positive for *mecA*-LAMP. Other strains tested, including MSSA, MSCNS, and 27 non-staphylococcal bacterial species, were negative for *mecA*-LAMP. Antiseptic-resistant *Staphylococcus* spp., defined as those resistant to acriflavine, including 136 antiseptic-resistant MRSA isolates and 6 antiseptic-resistant MRSE isolates, were positive for *qacA/B*-LAMP. Other bacterial strains, including *S. aureus* N315, 56 antiseptic-susceptible MRSA isolates, 3 antiseptic-susceptible MSSA isolates, 4 antiseptic-susceptible MSCNS isolates, and 27 non-staphylococcal bacterial species, were negative for *qacA/B*-LAMP. These results indicated that the three LAMP assays were highly specific for *femB*, *mecA*, and *qacA/B*, respectively, and that *femB*-, *mecA*-, and *qacA/B*-LAMP detected antiseptic methicillin-resistant *S. aureus*.

To examine whether acriflavine resistance is related to *qacA/B*, the MIC of acriflavine was determined in 260 MRSA isolates. As shown in Table 3, of 147 MRSA isolates highly resistant to acriflavine (defined as MIC≥100 mg/L), 136 (92.5%) were positive for *qacA/B*-LAMP and PCR. Of 57 MRSA isolates with low-level acriflavine resistance (defined as MIC 50–25 mg/L), 16 (28.1%) were positive for *qacA/B*-LAMP and PCR. The results of PCR-restriction fragment length polymorphism analysis (Sekiguchi et al., 2004) revealed that 3 of the 16 isolates harbored *qacA* and the remaining 13 harbored *qacB* (data not shown). None of 56 acriflavine-susceptible MRSA isolates (MIC<25 mg/L) were negative for *qacA/B*-LAMP and PCR. Acriflavine resistance was closely associated with harboring *qacA/B* in MRSA.

PCR-based methods targeting *qacA/B* and/or *qacC* were developed to detect antiseptic-resistant MRSA isolates (Mayer et al., 2001; Noguchi et al., 1999, 2005; Sekiguchi et al., 2004). PCR-based methods require the use of an expensive real-time thermal cycler or a conventional thermal cycler with time-consuming and labor-intensive gel electrophoresis analysis for confirmation of DNA amplification. In contrast, LAMP requires no special equipment because the reaction can be carried out at a constant temperature. DNA amplification in the LAMP assay can be judged easily by the naked eye by simply adding a metal ion indicator before the reaction (Goto et al., 2009). However, LAMP is not a suitable technique for multiplex gene amplification, because LAMP produces various DNA amplification products differing in size in the presence of the target gene (Iseki et al., 2007; Notomi et al., 2000). Therefore, we developed three LAMP assays targeting *femB*, *mecA*, and *qacA* as replacements for a single or multiplex PCR assay. These assays could be carried out simultaneously in a conventional incubator at 63 °C within 60 min (data not shown).

**Table 3**
Diagnostic performance of *qacA/B*-LAMP assay compared with *qacA/B*-PCR assay.

| MIC (mg/L) | No. of MRSA isolates (*N*=260) | No. of positives | | Predictive value[a] (%) | Correlation[b] (%) |
|---|---|---|---|---|---|
| | | LAMP | PCR | | |
| ≥100 | 147 | 136 | 136 | 92.5 | 100 |
| 50–25 | 57 | 16 | 16 | 28.1 | 100 |
| 25> | 56 | 0 | 0 | 0 | 100 |

[a] Positive predictive value by *qacA/B*-LAMP assay.
[b] Correlation between *qacA/B*-LAMP assay and *qacA/B*-PCR assay.