研究成果の刊行に関する一覧表

雑誌

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齋藤昭彦	インフルエンザワクチンの効果.	インフルエンザ	40(11)	211-222	2010
庄司健介,齋藤昭彦	新型インフルエンザワクチン 小児.	臨牀と研究	87(12)	1692-1695	2010
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加藤達夫,山口晃史,菅原美絵,石井由美子,栗山猛,中村秀文,齋藤昭彦	新型インフルエンザワクチン10ml バイアル使用時における死腔の少ない新しいシリンジの有用性.	日本医師会雑誌	139(9)	1904-1906	2010

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Yasuhito Iwashima, Atsushi Nakamura, Haru Kato, Hideaki Kato, Yukio Wakimoto, Naoki Wakiyama, Chiharu Kaji, and Ryuzo Ueda.	A retrospective study of the epidemiology of *Clostridium difficile* infection at a university hospital in Japan: genotypic features of the isolates and clinical characteristics of the patients.	J Infect Chemother	16	329-33	2010
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Development of an immunochromatographic assay for the rapid detection of AAC(6′)-Iae-producing multidrug-resistant *Pseudomonas aeruginosa*

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

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

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

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

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

Introduction

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

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

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

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

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

Materials and methods

Bacterial strains

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

Purification of recombinant proteins

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

Preparation of monoclonal antibodies

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

Assembly of the assay

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

Analytical sensitivity testing of the assay

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

Assessment of the assay

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

Antimicrobial susceptibility testing

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

PFGE

PFGE assays were performed as described previously.[3]

PCR amplification and DNA sequencing

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

Results

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

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

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

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

Assessment of the assay using clinical isolates

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

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

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

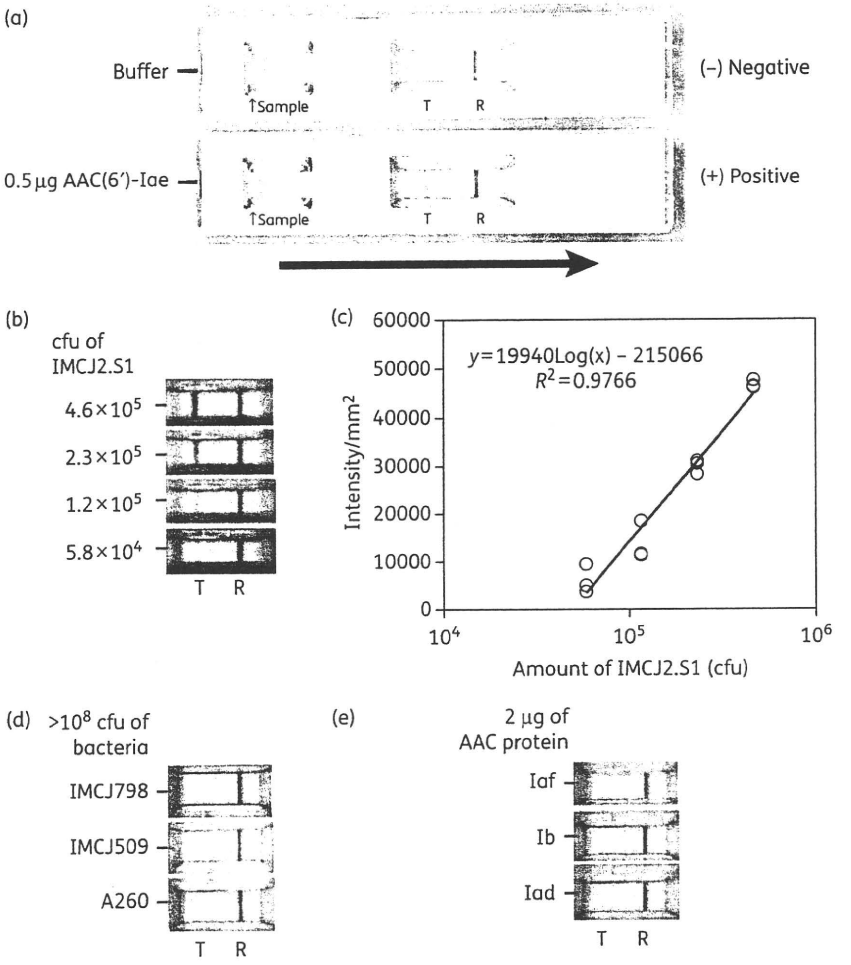

Figure 1. (a) Immunochromatography using MAbs 1H7 and 3F12. In negative cases, a single line appears at the position of the reference line (R) only. In positive cases, another line also appears at the position of the test line (T) in addition to the reference line. The arrow indicates the direction of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

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

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

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

All positive strains showed serotype O:11.

Discussion

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

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

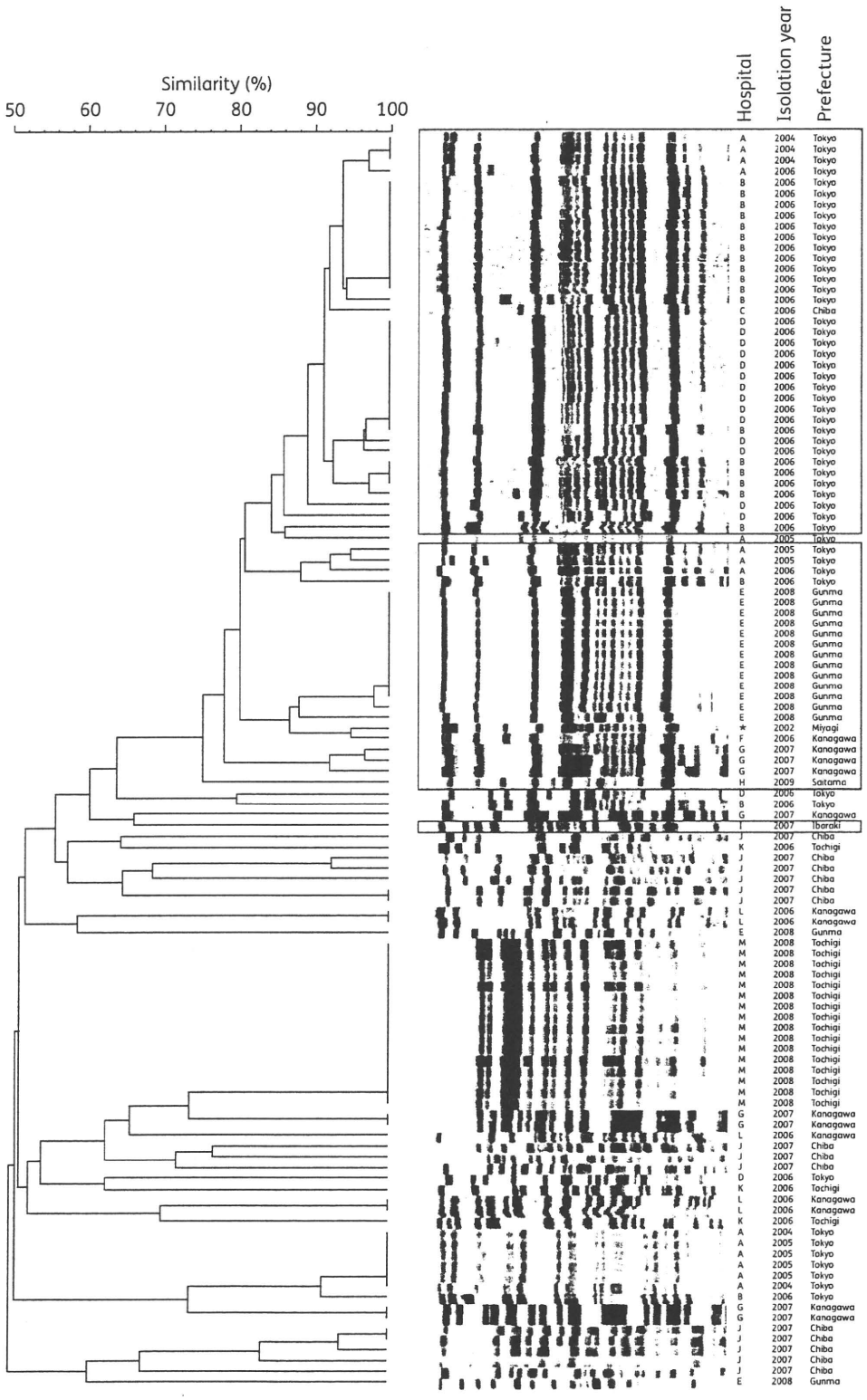

Figure 2. PFGE assay of 116 *P. aeruginosa* clinical isolates used in the assessment of the developed immunochromatography assay. The asterisk indicates the *P. aeruginosa* IMCJ2.S1 strain previously found in Miyagi. Areas enclosed within squares indicate the positive isolates detected.

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

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

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

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Transparency declarations

None to declare.

Supplementary data

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

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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.

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.

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.

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

4. Conclusion

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

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Multicenter prospective evaluation of a novel rapid immunochromatographic diagnostic kit specifically detecting influenza A H1N1 2009 virus

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ABSTRACT

Background: Definitive diagnosis is crucial in reducing morbidity and mortality from pandemic influenza A H1N1 2009 (A/H1N1/2009), especially in high-risk populations. We recently developed a rapid diagnosis kit (RDK) capable of specifically detecting A/H1N1/2009.
Objectives: To evaluate the diagnostic capability of the RDK in a multicenter, prospective trial.
Study design: Samples were obtained by nasal swab from patients with suspected influenza. The diagnostic capability of the RDK was compared with that of the standard, real-time reverse transcription-polymerase chain reaction (RT-PCR) method.
Results: Of 266 patients who met the criteria, 122 and 92 were positive for A/H1N1/2009 influenza by PCR and by the newly developed RDK, respectively. The sensitivity, specificity and positive and negative predictive values of the RDK were 73.0%, 97.9%, 96.7% and 81.0%, respectively. A/H1N1/2009 detection rates by the RDK were significantly lower in samples obtained from patients more than 3 days after onset than in samples obtained between 1 and 2 days.
Conclusions: The A/H1N1/2009-specific RDK is a reliable test that can be used easily at a patient's bedside for rapid diagnosis of A/H1N1/2009. This test will be of key importance in the control of A/H1N1/2009.

1. Background

The World Health Organization (WHO) has reported patients with laboratory-confirmed pandemic influenza A H1N1 2009 (A/H1N1/2009) in more than 209 countries and overseas territories or communities worldwide (http://www.who.int/csr/don/2010_07_09/en/index.html). Certain populations, including pregnant women, children younger than 2 years old, and people with chronic lung disease, are at high risk for the rapid development of severe pneumonia associated with A/H1N1/2009, with failure of other organs, or marked worsening of underlying diseases.[1–3] The clinical picture in severely affected individuals seems to be strikingly different from the disease pattern observed during epidemics of seasonal influenza. To promptly initiate adequate treatment, especially for high-risk populations, definitive diagnosis of A/H1N1/2009 is crucial. Currently new types of influenza are definitively diagnosed using methods based on viral genome analyses. Although these methods are highly sensitive, they usually take more than 2–6 h to complete and require well-equipped laboratories with virologists or well-trained medical technicians and specialized tools for virus genome isolation and amplification.[4–6]

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Table 1
Characteristics of the study population.

	Percentage	Number
Age (year)		
<1	10.00%	27
1–2	28.50%	76
3–5	21.90%	58
6–11	27.70%	74
12–17	6.90%	18
≥17	5.00%	13
Average (mean ± SE)	6.2 ± 0.5	
Gender		
Male	58.20%	155
Female	41.80%	111
Period from symptom onset to testing (days)		
<1	42.10%	112
1–2	39.80%	106
2–3	10.50%	28
>3	7.50%	20
Average (mean ± SE)	0.93 ± 0.08	

Rapid diagnostic kits (RDKs) based on immunochromatography consist of combinations of antibodies against pathogen components of interest—nucleoprotein (NP) in the case of influenza. These RDKs are widely used in clinical practice to diagnose seasonal influenza A/B, both because of their ease of use and because they usually provide results within 15 min. Although RDKs may suggest infection with A/H1N1/2009, the RDKs for seasonal influenza are unable to distinguish A/H1N1/2009 from the seasonal viruses.[7]

2. Objectives

Recently, we developed an RDK capable of specifically detecting A/H1N1/2009 virus (RDK(A/H1N1/2009)).[8] To validate its diagnostic capability in clinical practice, we tested it in a multicenter prospective study from December 2009 to January 2010.

3. Study design

3.1. Study oversight

This clinical trial was registered with UMIN-CTR Clinical Trials (https://lippmann.umin.ac.jp/cgi-open-bin/ctr/ctr.cgi?function=brows&action=brows&recptno=R000004018&type=summary&language=E) under the registration number UMIN000003318. From December 1, 2009, through January 4, 2010, 8 centers located in central and southern Japan participated in a prospective clinical trial to validate the diagnostic capability of the RDK(A/H1N1/2009).

3.2. Patients

A patient was defined as an individual with a body temperature >38 °C and at least one influenza-like symptom, including headache, arthralgia, myalgia, cough, pharyngalgia, nasal secretion, and systemic symptoms such as fatigue. We excluded results from patients enrolled in this trial and suspected of being infected with A/H1N1/2009 but administered anti-influenza drugs before clinical samples could be obtained. Patients' backgrounds are summarized in Table 1. The study was conducted in accordance with the Declaration of Helsinki.[9] The protocol was approved by each center's institutional review board, and informed consent was obtained from all patients or their guardians before any study-related procedures were performed.

3.3. RDK

The RDK capable of specifically detecting A/H1N1/2009 virus (RDK(A/H1N1/2009)) was assembled as described previously.[8] Briefly, monoclonal antibody (mAb) 1 recognizing NP from AH1pdm, seasonal H1N1 and H3N2 but not from HPAI, and mAb2 recognizing NPs from AH1pdm and HPAI but not other NPs were used for specific detection of A/H1N1/2009. The composition of the RDK used for immunochromatographic detection of A/H1N1/2009 virus (RDK(A/H1N1/2009)) and the diagnostic procedure are shown in Fig. 1. For comparisons, we utilized a commercial RDK for seasonal influenza A/B (RDK(conv)) (Quick Chaser Flu A,B, MIZUHO MEDY, Co., Ltd.), which can distinguish influenza A from B but cannot distinguish A/H1N1/2009 from seasonal influenza A (Fig. 1).

3.4. Specimens

Two nasal swabs were taken from each patient. One swab was immediately analyzed with the RDKs for A/H1N1/2009 and seasonal influenza in the examination room, while the second swab was stored in viral transport medium (VTM) for real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis. VTM consisted of MEM containing 0.5% of bovine serum albumin, 300 U/mL of penicillin, 300 μg/mL of streptomycin, 100 μg/mL of gentamycin and 2 μg/mL of amphotericin B.

3.5. RDK test

Viral antigens were extracted from each swab using sample extraction tubes containing sample extraction buffer (0.4 M Tris buffer containing 1% non-ionic detergent and 0.09% sodium azide) (MIZUHO MEDY, Co., Ltd.). The extract was transferred directly from the extraction tube to the RDK, and the appearance of a line was assessed visually.

3.6. Real-time RT-PCR

Detection of A/H1N1/2009 or seasonal influenza A by real-time RT-PCR with LightCycler 1.5 (Roche Diagnostics Japan, Tokyo, Japan) was used as the gold standard against which we compared the results of RDKs. All swab samples were stored in VTM stored at −30 °C until analysis. RNA was extracted from each sample using QIAamp Viral RNA Mini Kits (Qiagen, Hilden, Germany) and assayed for virus using *artus* Infl./H1 LC/RG RT-PCR Kits (Qiagen). Samples giving crossing points at less than 35 cycles were defined as positive, whereas samples showing no amplification by 40 cycles were defined as negative. Samples showing amplification after 35 cycles were considered marginal. For these samples, we utilized two analytical protocols for influenza A/H1N1/2009 and seasonal influenza A based on WHO recommendations (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf (protocol 3, a one step conventional RT-PCR for pandemic (H1N1) 2009 HA gene, page 13 for A/H1N1/2009; Annex 1, conventional RT-PCR analyses for the matrix gene of influenza type A viruses, page 6, for seasonal A)) for further identification. Both protocols are based on one-step realtime PCR using TaqMan probes.

3.7. Seasonal influenza viruses

As no patients confirmed with seasonal influenza were included in this clinical trial, stored nasal swab samples collected between 2008 and 2009 and confirmed positive for seasonal influenza A or B by RT-PCR were used to evaluate the specificity of the RDK(A/H1N1/2009). These samples had been stored in extraction solution at −20 °C. Experiments using these clinical samples

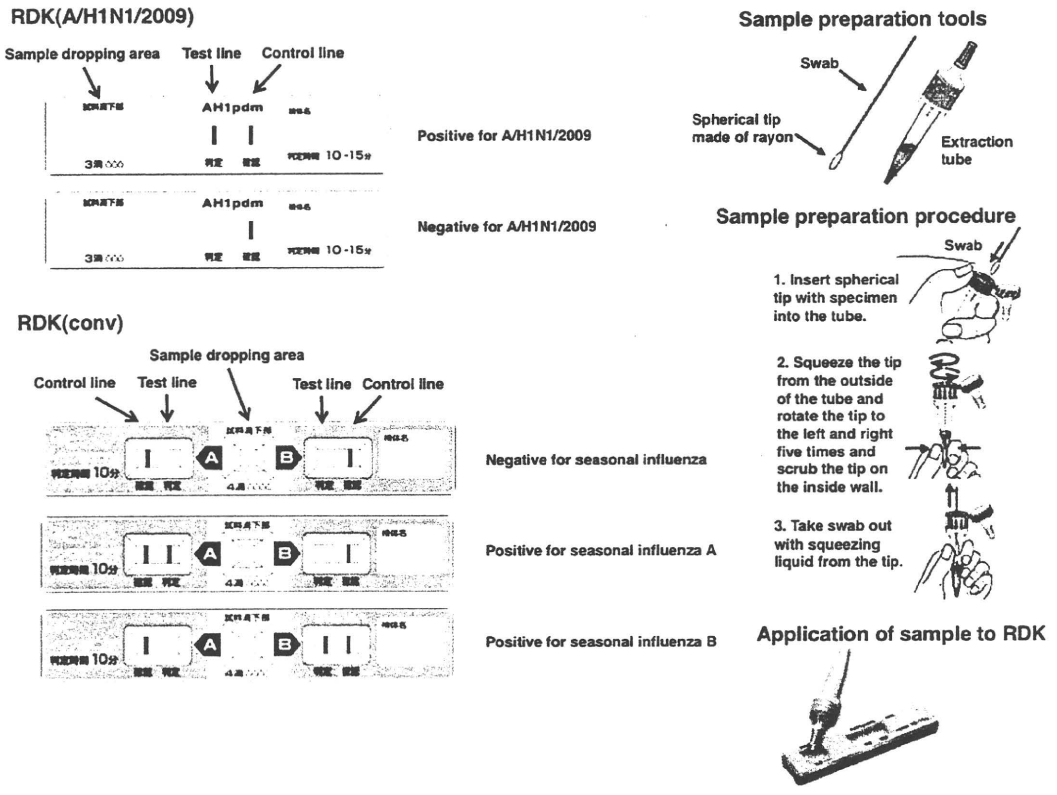

Fig. 1. Composition of RDK(A/H1N1/2009) and RDK(conv) used in the clinical trial and interpretation of the results. Each RDK consists of a sample dropping area and detection area(s) containing a test line and a control line. The control line confirms sample flow. RDKs are supplied with a swab made of rayon and an extraction tube. After squeezing the nasal swab in the extraction tube to obtain the sample, three drops of the liquid inside the tube (approximately 110 μL) is applied to the sample dropping area of the RDK. Diagnosis is based on the appearance of purple lines in the detection area 15 min later at ambient temperature (15–30 °C). A sample is considered positive for A/H1N1/2009 when both the test line and control line are present (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

were reviewed and approved by the Institutional Review Board of Mizuho Medy. All patients provided written informed consent for use of their sample to evaluate RDKs for influenza, and all clinical samples were assayed anonymously.

3.8. Statistical analyses

Statistical analyses were performed with computer software (R, http://www.r-project.org/). The agreement between RDKs and real-time RT-PCR was assessed by determining the percentage of concordant results and the Kappa coefficient with confidence interval. The correlation between RDK and real-time RT-PCR results was assessed by Kendall's correlation coefficient. The negative and positive predictive values (NPV and PPV, respectively) of the RDK were calculated by Bayes' theorem. Fisher's exact test was used to compare the rates of detection of A/H1N1/2009 by real-time RT-PCR and the RDK in the stratified patient data.

4. Results

4.1. Study population

A total of 329 patients (82, 156 and 91 from Tokyo, Miyazaki and Chiba centers, respectively) were enrolled into the trial based on their influenza-like symptoms and body temperature >38 °C. Patients who had taken anti-influenza medication, including oseltamivir, zanamivir, or amantadine, and those for whom nasal swab specimens were not available, were excluded from analysis. The gender, age distribution, and time from onset of symptoms to testing of the remaining 266 patients are summarized in Table 1. Nearly 90% of patients were less than 12 years old, and 81.9% had visited the hospital within 2 days of symptom onset.

4.2. Comparison of RDK(A/H1N1/2009) and real-time RT-PCR in detection of A/H1N1/2009

Of the 266 patients, 122 were positive for A/H1N1/2009 by real-time RT-PCR, whereas none was positive for seasonal influenza. The RDK(A/H1N1/2009) identified 92 patients as positive for A/H1N1/2009 (Table 2). Thus, the sensitivity and specificity of this RDK were 73.0% (95% confidence interval (CI): 65.1–80.8%, $P < 0.0001$) and 97.9% (95% CI: 95.6–100.2%), respectively. Results obtained with real-time RT-PCR and RDK(A/H1N1/2009) were highly concordant (86.5%), agreeing for 230 of the 266 specimens tested and showed a strong correlation (Kendall's correlation coef-

Table 2
Detection of A/H1N1/2009 by RDK(A/H1N1/2009).

		RDK(A/H1N1/2009)		
		Positive	Negative	Total
RT-PCR (A/H1N1/2009)	Positive	89	33	122
	Negative	3	141	144
	Total	92	174	266

Agreement between RDKs and real-time RT-PCR was based on the percentage of concordant results and the Kappa coefficient with confidence interval. The correlation between RDK and real-time RT-PCR results was assessed by Kendall's correlation coefficient. The negative and positive predictive values (NPV and PPV, respectively) of the RDK were calculated using Bayes' theorem.

Table 3
Comparison of RDK(A/H1N1/2009) and RDK(conv).

		RDK(A/H1N1/2009)		
		Positive	Negative	Total
RDK(conv)	Positive	82	0	82
	Negative	10[a,b]	174	184
	Total	92	174	266

Agreement between RDK(A/H1N1/2009) and RDK(conv) was based on the percentage of concordant results and the Kappa coefficient with confidence interval. The correlations between results of the two RDKs were assessed by Kendall's correlation coefficient. One of the 3 false-positive samples in RDK(A/H1N1/2009) in Table 2 was also positive in RDK(conv) while the other 2 samples were negative in RDK(conv).

[a] Eight of 10 samples were positive and 2 were negative for A/H1N1/2009 by PCR analyses.

[b] Of the 10 samples that tested negative on the RDK(conv), 8 were found to be false negatives by PCR.

ficient $r = 0.871$, $P < 0.0001$). Kappa was 0.72 (95% CI: 0.64–0.81%, $P < 0.001$). The calculated PPV and NPV were 96.7% (95% CI: 93.4–100.4%) and 81.0% (72.8–89.2%), respectively. These results indicated that RDK(A/H1N1/2009) is highly specific and sufficiently sensitive to diagnose patients with A/H1N1/2009 in a clinical setting. The 89 samples negative by RDK but positive by real-time PCR had C_t-values in real-time RT-PCR of 30.9 ± 4.0 (89 samples), while the samples positive by both tests had C_t-values in real-time RT-PCR of 25.9 ± 4.0 ($P < 0.001$). Thus, on average, the viral load of the samples negative by RDK but positive by real-time PCR was roughly 10-fold lower than the viral load of the samples positive by both assays.

When we compared the results of RDK(A/H1N1/2009) with those of RDK(conv) (Table 3), we found that they showed a high degree of concordance (96.2%), agreeing for 256 of the 266 specimens tested and were strongly correlated (Kendall's correlation coefficient $r = 0.959$, $P < 0.001$). Kappa was 0.91 (95% CI: 0.86–0.97%; $P < 0.001$). Ten samples were positive on the RDK(A/H1N1/2009) and real-time RT-PCR assays but not on RDK(conv), suggesting that RDK(A/H1N1/2009) is at least as sensitive as RDK(conv) in detecting A/H1N1/2009 infected individuals.

4.3. Reactivity of RDK(A/H1N1/2009) against specimens positive for seasonal influenza

As no patients were positive for seasonal influenza during the period of this clinical trial, we evaluated the specificity of RDK(A/H1N1/2009) using 71 stored samples known to be positive for seasonal influenza A (57 for AHI1 and 14 for AH3) and 50 positive for seasonal influenza B, as shown initially by RDK(conv) and validated by RT-PCR. RDK(A/H1N1/2009) yielded negative results in all of these samples (data not shown).

4.4. Comparison of the detection rate with stratified patient data

To elucidate the factors affecting the performance of RDK(A/H1N1/2009), we analyzed the detection rate using stratified patient data. Patients were stratified by time from symptom onset to testing, by age, and by body temperature at the time of testing, and those in each stratum positive for A/H1N1/2009 by real-time RT-PCR and RDK(A/H1N1/2009) were compared (Fig. 2). Since we observed no significant differences between RDK(A/H1N1/2009) and real-time RT-PCR in the rate of the detection of A/H1N1/2009 (Supplemental Table), stratified patient data were analyzed by comparing the percentage of positive samples in each stratum.

We found that the percentages of patients positive for A/H1N1/2009, by both real-time RT-PCR and RDK(A/H1N1/2009), tended to decline with increasing time from onset of symptoms to testing. In particular, statistical analysis showed that the percentage of positive results on RDK(A/H1N1/2009) was slightly higher in samples tested 1–2 days after symptom onset than in samples tested 2–3 days after onset (not significant).

We also found that the percentage of positive results tended to be higher in older than in younger patients, especially when we compared patients 1–2 years old with those 12–17 years old, by both real-time RT-PCR ($P = 0.0086$) and RDK(A/H1N1/2009) ($P = 0.0033$), with both strata containing comparable numbers of patients.

We found no significant differences in percentage of positive results according to patient body temperature, although patients with body temperatures below 38 °C had been excluded from evaluation.

5. Discussion

The newly developed RDK(A/H1N1/2009) kit that we evaluated in this clinical trial may be used for the rapid, noninvasive, and cost-effective diagnosis of A/H1N1/2009 in infected individuals, both by clinicians at the bedside and by healthcare workers in remote sites. Although we found that the sensitivity of RDK(A/H1N1/2009) was lower than that of real-time RT-PCR, RDK(A/H1N1/2009) is a much more rapid assay. Further, viral gene-based methods of detecting new-type influenza viruses utilize as target the viral hemagglutinin, which is highly mutagenic.[10] In contrast, the amino acid sequences of the NPs, which are targeted by the RDK, are well conserved.[8] Thus, utilization of RDK(A/H1N1/2009) may overcome the antigenic drift of A/H1N1/2009.

The sensitivity of RDK(conv)s has been found to range from 10% to 70%.[7,11–15] Almost all studies evaluating the performance of these RDK(conv)s have analyzed specimens that are collected at remote sites, stored in VMT and transferred to central laboratories. Thus the sensitivity of these kits may be affected by many factors, including specimen collection methods, RDK(conv) performance, and testing of adequate patients, all of which may vary among studies. Specimen handling in Japan differs strikingly between these clinical studies and actual clinical situations. In Japan, almost all samples are tested immediately within the same hospital. Our trial, which mirrors actual clinical settings in Japan, showed that sensitivity and PPV of RDK(A/H1N1/2009) were 73.0% and 96.7%, respectively. Although we utilized RDK(A/H1N1/2009) and RT-PCR to test two different samples and we had to use frozen samples to test the specificity of RDK(A/H1N1/2009) for seasonal influenza, our results indicate that RDK(A/H1N1/2009) is sufficient for definite diagnosis of A/H1N1/2009.

This clinical trial allowed us to analyze the factors affecting the performance of RDK. We found that patient age and time from the onset of influenza-like symptoms to testing were critical factors. Diagnoses based on interview are difficult in children less than 3 years old. Furthermore, parents or caretakers of these children may be worried about the possibility of influenza and may present their children to doctors as a precaution. In contrast, the time from symptom onset to testing would influence the shedding of viral antigen, with higher percentages of positive specimens being those obtained during the first 2 days. Thus, by understanding the characteristic features of the RDK, we found that this RDK(A/H1N1/2009) is sufficient for diagnosis of A/H1N1/2009 in clinical settings.

When used together with RDK(conv), the RDK(A/H1N1/2009) evaluated in this trial was able to differentiate between influenza A virus subtypes (seasonal A or A/H1N1/2009). Due to the rapidity of the RDK(A/H1N1/2009) assay, clinical management plans can be implemented immediately. This is especially important when both seasonal influenza and A/H1N1/2009 are circulating concomitantly. Certain populations, such as pregnant women and younger children, are at high risk of developing serious

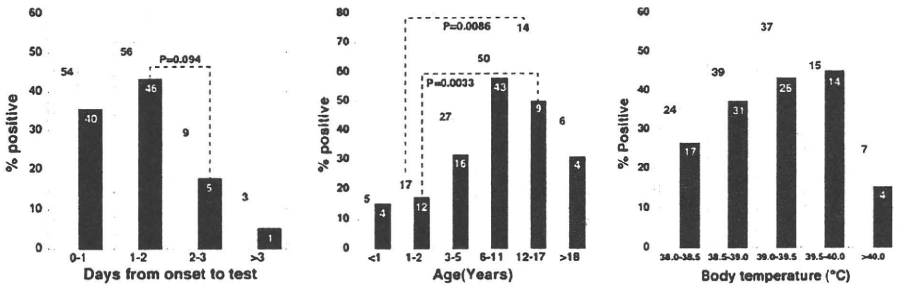

Fig. 2. Percentage of patients positive for A/H1N1/2009 by real-time RT-PCR and RDK(A/H1N1/2009). Patients were stratified by time from onset of influenza-like symptoms to specimen acquisition, by patient age, and by body temperature at the time of sampling. Statistical comparisons were performed using pairwise proportion tests with Fisher's exact tests. White bars and black bars indicate the percent of patients in each stratum positive by RT-PCR and RDK(A/H1N1/2009), respectively. The numbers in the bars indicate the absolute numbers of patients positive for each test in the stratum. The sensitivities of the RDK(A/H1N1/2009) relative to RT-PCR in patients aged <1, <3, <6, <12, <18 and >18 years were 80.0%, 72.2%, 66.7%, 85.1%, 64.3% and 66.7%, respectively, although the difference was not statistically significant.

illnesses when infected with A/H1N1/2009 but not with seasonal influenza.[1,2,16–19] More than 99% of the current seasonal H1 strains are oseltamivir-resistant, whereas little resistance has been reported in A/H1N1/2009 (http://www.flu.gov/individualfamily/prevention/medicine/antiviralsrecommend.html). A definitive diagnosis of A/H1N1/2009 would therefore ensure the selection of appropriate antiviral treatment regimens. As the specificity and PPV of RDK(A/H1N1/2009) are excellent, positive results on this assay would enable immediate intervention in patients at risk. However, the diagnostic ability of RDK(A/H1N1/2009) in adults should be evaluated in larger numbers of patients. The RDK(A/H1N1/2009) was approved by the Japanese Government as an extracorporeal diagnostic agent on July 5, 2010, and is now commercially available.

Conflict of interest

Kenji Naranara and Hirotake Kitajima are employees of MIZUHO MEDY. The other authors do not believe that any conflicts of interest are likely to arise in future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.01.007.

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Evaluation of a line probe assay for the rapid detection of *gyrA* mutations associated with fluoroquinolone resistance in multidrug-resistant *Mycobacterium tuberculosis*

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The aim of this study was to establish the importance of detecting fluoroquinolone (FQ) resistance in multidrug resistant (MDR) *Mycobacterium tuberculosis*, and to show the usefulness of a hybridization-based line probe assay (LiPA) for detecting *gyrA* mutations. Thirty-three MDR *M. tuberculosis* isolates were collected from a total of sixty MDR isolates identified in Japan over 6 months during a national surveillance study in 2002. Seventeen MDR isolates were collected by the National Center for Global Health and Medicine in Japan over 6 years from 2003 to 2008. These 50 isolates were examined for FQ susceptibility, and analysed by LiPA and *gyrA* sequencing. Among them, 22 (44 %) showed FQ resistance. All FQ-resistant isolates had at least one mutation in *gyrA*. The results of the LiPA were fully consistent with the DNA sequencing results. Given that on the basis of our results almost half of the MDR *M. tuberculosis* isolates in Japan might have resistance to FQ, it is important to monitor FQ resistance in patients with MDR tuberculosis (TB), as well as with drug-susceptible TB, prior to commencing treatment. For the detection of FQ resistance, LiPA is useful and can rapidly and efficiently assess FQ resistance.

INTRODUCTION

The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* are a serious threat to the control of tuberculosis (TB), and constitute an increasing public-health problem (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*, 2009; Van Deun *et al.*, 2010a). Multidrug resistant (MDR) strains are defined as strains resistant to both rifampicin and isoniazid, and extensively drug-resistant strains are defined as MDR plus resistance to any fluoroquinolone (FQ) and to at least one of the second-line injectable drugs. Patients infected with these strains are difficult to cure and are more likely to remain infected for a longer period of time than patients with drug-susceptible strains (Caminero, 2010; Johnston *et al.*, 2009; Sotgiu *et al.*, 2009). With the emergence of MDR and extensively drug-resistant TB, the need for rapid drug susceptibility testing is now apparent (Van Deun *et al.*, 2010a).

FQ is a class of antimicrobial drug with strong activity against *M. tuberculosis* and is one of the most important second-line antituberculosis drugs used in the treatment of MDR TB (Yew *et al.*, 2010). Newer FQs, including gatifloxacin (GFLX) and moxifloxacin (MFLX), have been developed. A prospective study (Van Deun *et al.*, 2010b) indicated that a treatment regimen including GFLX was highly effective for MDR TB. A phase II trial study (Conde *et al.*, 2009) showed that a regime including MFLX improved culture conversion in the initial treatment of TB.

The main target of FQ in *M. tuberculosis* is the DNA gyrase encoded by *gyrA* and *gyrB*, which is essential for DNA supercoiling (Guillemin *et al.*, 1998). The genetic mechanism of FQ resistance is mainly due to alterations in the DNA gyrase, especially mutations in a short sequence called the quinolone-resistance-determining region (QRDR) of the *gyrA* (Guillemin *et al.*, 1998).

Abbreviations: CPFX, ciprofloxacin; FQ, fluoroquinolone; GFLX, gatifloxacin; JATA, Japan Anti-Tuberculosis Association; LiPA, line probe assay; LVFX, levofloxacin; MDR, multidrug resistant; MFLX, moxifloxacin; NCGM, National Center for Global Health and Medicine; QRDR, quinolone-resistance-determining region; SPFX, sparfloxacin; TB, tuberculosis; TRC, Tuberculosis Research Committee.

In this study, 50 MDR *M. tuberculosis* isolates collected in Japan were examined for FQ susceptibility and analysed by *gyrA* sequencing. Furthermore, we developed and evaluated a hybridization-based line probe assay (LiPA) for the rapid detection of *gyrA* mutations, which can easily be used in clinical applications. The whole procedure takes only 9 h, and the estimated cost per sample is £22 (US $35).

We found that nearly half of our Japanese MDR *M. tuberculosis* isolates had resistance to FQ. Therefore, it is important to detect FQ resistance in patients with MDR TB, as well as with drug-susceptible TB, prior to commencing treatment.

METHODS

Bacterial strains. Among the 50 MDR *M. tuberculosis* isolates, 33 were obtained from patients with TB in 9 hospitals in Japan located in various regions – 1 in Hokkaido, 1 in Tohoku, 4 in Kanto, 2 in Kinki and 1 in Kyushu – during a national surveillance study [conducted from June to November 2002 by the Tuberculosis Research Committee (Ryoken), Japan] (TRC, 2007). The remaining 17 were obtained from patients diagnosed as MDR TB in the National Center for Global Health and Medicine (NCGM) from 2003 to 2008. During the national surveillance study, 60 MDR *M. tuberculosis* isolates were obtained (TRC, 2007). Of them, 33 were recovered from bacterial stocks and used in this study. *M. tuberculosis* strain H37Rv (ATCC 27294) and a *Mycobacterium bovis* strain (BCG Japanese strain 172), which are susceptible to FQ, were used in this study. To determine the species specificity of the LiPA, the following 18 bacterial strains were also used: *Mycobacterium avium* (ATCC 25291), *Mycobacterium chelonae* (ATCC 19237), *Mycobacterium fortuitum* (RIMD 1317004, ATCC 15754), *Mycobacterium intracellulare* (JCM 6384, ATCC 13950), *Mycobacterium kansasii* (JCM 6379, ATCC 124878), *Mycobacterium nonchromogenicum* (JCM 6364, ATCC 124878), *Mycobacterium scrofulaceum* (JCM 6381, ATCC 19981), *Mycobacterium smegmatis* (ATCC 19420), *Mycobacterium terrae* (GTC 623, ATCC 15755), *Escherichia coli* (ATCC 8739), *Haemophilus influenzae* (IID 984, ATCC 9334), *Klebsiella pneumoniae* (IID 5209, ATCC 15755), *Legionella pneumophila* (GTC 745), *Mycoplasma pneumoniae* (IID 817), *Pseudomonas aeruginosa* (ATCC 27853), *Rhodococcus equi* (ATCC 33710), *Staphylococcus aureus* strain N315 and *Streptococcus pneumoniae* (GTC 261).

FQ susceptibility testing. Drug susceptibility testing was performed at two institutions [33 isolates at the Japan Anti-Tuberculosis Association (JATA) and 17 at the NCGM]. Susceptibility to levofloxacin (LVFX), sparfloxacin (SPFX) and ciprofloxacin (CPFX) was determined at JATA, and susceptibility to LVFX was determined at the NCGM. A broth dilution method (BrothMIC MTB-I; Kyokuto) was used at JATA, and an agar proportion method with egg-based Ogawa medium (Vit Spectrum-SR; Kyokuto) was used at the NCGM. The proportion method is based on a slight modification of the World Health Organization protocol (Aziz *et al.*, 2003) and is recommended by the Japanese Society for Tuberculosis (Aziz *et al.*, 2003; Fujiki, 2001). The results are shown in Table 1.

Isolation of genomic DNA. Genomic DNA was extracted from bacteria as described previously (Sekiguchi *et al.*, 2007b).

PCR and DNA sequencing. The QRDR of *gyrA* was amplified and sequenced by a previously described method (Sekiguchi *et al.*, 2007a). DNA sequences were compared with H37Rv using GENETYX-MAC, version 14.0.2 (Genetyx).

LiPA. The LiPA was performed as described previously (Ando *et al.*, 2010; Sekiguchi *et al.*, 2007b). Seven oligonucleotide probes were designed for the LiPA to detect *gyrA* mutations (Table 2). Two oligonucleotide probes, S1 and S2, were designed to cover the entire QRDR of *gyrA* of H37Rv. S2 also contained another probe to compensate for a neutral mutation of G to C at nucleotide position 284 (S95T) (Zhang & Telenti, 2000). The remaining five oligonucleotide probes, R1–R5, were designed to detect five mutations that were reported previously in FQ-resistant isolates obtained in Japan (Sekiguchi *et al.*, 2007a). These probes were immobilized on strips of nitrocellulose membrane. The QRDR of *gyrA* was amplified by nested PCR. Immobilized probes were hybridized with the biotinylated PCR products, and then incubated with streptavidin labelled with alkaline phosphatase. The results were visualized using colour development after incubation with 5-bromo-4-chloro-3′-indolyl-phosphate *p*-toluidine and nitro blue tetrazolium. The presence or absence of bands on all strips was determined visually. Among the 20 bacterial strains described above, 2 strains of *M. tuberculosis* and *M. bovis* were positive for PCR, but the other 18 strains were negative. The PCR products from the two strains hybridized with the S probes, but not the R probes (data not shown). These data indicated that the LiPA is specific for *M. tuberculosis* and *M. bovis*. The sensitivity of the LiPA was 24.2 fg *M. tuberculosis* DNA, equivalent to five copies of *gyrA* (data not shown).

RESULTS AND DISCUSSION

Fifty MDR *M. tuberculosis* isolates were collected. Of them, 33 were collected from a total of 60 MDR isolates identified in Japan over 6 months during the national surveillance study in 2002, and 17 were collected in the NCGM in Japan over 6 years from 2003 to 2008. These isolates and H37Rv were examined for FQ susceptibility, and 22 (44 %) of the isolates were resistant to FQ (Table 1). Among the 33 MDR isolates analysed at JATA, 14 (42 %) were resistant to all three FQs used, i.e. LVFX, SPFX and CPFX. None of the isolates were resistant to only one or two of these FQs (Table 1). The MICs of the three FQs for FQ-susceptible isolates ranged from 0.06 to 0.5 mg l^{-1} (median 0.125 mg l^{-1}) for LVFX, from 0.03 to 0.25 mg l^{-1} (median 0.125 mg l^{-1}) for SPFX, and from 0.06 to 0.5 mg l^{-1} (median 0.25 mg l^{-1}) for CPFX. The MICs for FQ-resistant isolates ranged from 2 to 16 mg l^{-1} (median 4 mg l^{-1}) for LVFX, from 1 to 8 mg l^{-1} (median 4 mg l^{-1}) for SPFX, and from 2 to 16 mg l^{-1} (median 8 mg l^{-1}) for CPFX. Among the 17 MDR isolates analysed at the NCGM, 8 (47 %) were resistant to LVFX (Table 1). There were no differences in rates of FQ resistance in MDR isolates between the two institutions (P=0.4).

We examined whether the MDR isolates had mutations in the QRDR of *gyrA* by LiPA and DNA sequencing analysis. Hybridization signals visualized as violet bands on the strips were strong and readily discernible with low background (data not shown). As shown in Table 1, regarding the FQ-resistance profile, the LiPA yielded results that were 100 % in agreement with those obtained by culture-based susceptibility testing. Of the 50 isolates, 28 were positive for both S1 and S2, and the remaining 22 were negative for one of the S probes (ΔS1 or ΔS2 in Table 1). Of the 22, 4 were positive for R1 indicating that isolates

Table 1. FQ susceptibility, LiPA profiles and *gyrA* mutations among 50 MDR *M. tuberculosis* isolates

Source of isolate	Strain	FQ MIC (mg l^{-1})			LiPA profile*	Mutation	
		LVFX†	SPFX	CPFX		Nucleotide change	Amino acid change
Control strain	H37Rv	0.25	0.125	0.25	WT (S1, S2)	–	–
JATA	2A-3-14	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-47	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-84	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-3-142	0.25	0.125	0.25	WT (S1, S2)	–	–
	2A-4-30	0.06	0.03	0.06	WT (S1, S2)	–	–
	2A-4-138	0.25	0.125	0.25	WT (S1, S2)	–	–
	2B-7-38	0.5	0.25	0.5	WT (S1, S2)	–	–
	2C-1-3	0.25	0.125	0.25	WT (S1, S2)	–	–
	2E-1-3	0.25	0.125	0.25	WT (S1, S2)	–	–
	2E-1-93	0.25	0.125	0.25	WT (S1, S2)	–	–
	2G-2-5	0.25	0.125	0.25	WT (S1, S2)	–	–
	2G-3-24	0.125	0.06	0.125	WT (S1, S2)	–	–
	2I-11-4	0.25	0.125	0.25	WT (S1, S2)	–	–
	2O-2-16	0.125	0.06	0.125	WT (S1, S2)	–	–
	2P-1-120	0.25	0.125	0.25	WT (S1, S2)	–	–
	2P-5-113	0.25	0.25	0.25	WT (S1, S2)	–	–
	2P-5-253	0.5	0.25	0.5	WT (S1, S2)	–	–
	2R-1-48	0.25	0.125	0.25	WT (S1, S2)	–	–
	2V-5-5	0.125	0.06	0.25	WT (S1, S2)	–	–
	2A-3-11	4	2	4	ΔS1, R1(A90V)	C269T	A90V
	2B-7-33	8	4	8	ΔS2, R4(D94G)	A281G	D94G
	2O-4-41	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2O-5-21	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-1-57	2	1	2	ΔS2, R5(D94A)	A281C	D94A
	2P-1-114	4	2	8	ΔS2, R4(D94G)	A281G	D94G
	2P-1-118	2	2	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-58	8	4	8	ΔS1, R1(A90V)	C269T	A90V
	2P-5-65	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-108	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-167	2	1	4	ΔS2, R5(D94A)	A281C	D94A
	2P-5-230	4	4	8	ΔS2, R4(D94G)	A281G	D94G
	2P-5-233	4	4	8	ΔS2, R5(D94A)	A281C	D94A
	2P-5-254	2	1	4	ΔS2, R5(D94A)	A281C	D94A
NCGM	NCGM2819	S	ND	ND	WT (S1, S2)	–	–
	NCGM2825	S	ND	ND	WT (S1, S2)	–	–
	NCGM2847	S	ND	ND	WT (S1, S2)	–	–
	NCGM2861	S	ND	ND	WT (S1, S2)	–	–
	NCGM2862	S	ND	ND	WT (S1, S2)	–	–
	NCGM2864	S	ND	ND	WT (S1, S2)	–	–
	NCGM2929	S	ND	ND	WT (S1, S2)	–	–
	NCGM2931	S	ND	ND	WT (S1, S2)	–	–
	NCGM2933	S	ND	ND	WT (S1, S2)	–	–
	NCGM2803	R	ND	ND	ΔS2	G280T	D94Y
	NCGM2822	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2834	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V
	NCGM2835	R	ND	ND	ΔS2, R5(D94A)	A281C	D94A
	NCGM2863	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2888	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2930	R	ND	ND	ΔS2, R4(D94G)	A281G	D94G
	NCGM2932	R	ND	ND	ΔS1, R1(A90V)	C269T	A90V

ND, Not determined; R, resistant to LVFX; S, sensitive to LVFX; WT, wild-type.

*Δ indicates a negative signal for the probe.

†S and R results were determined by the agar proportion method.