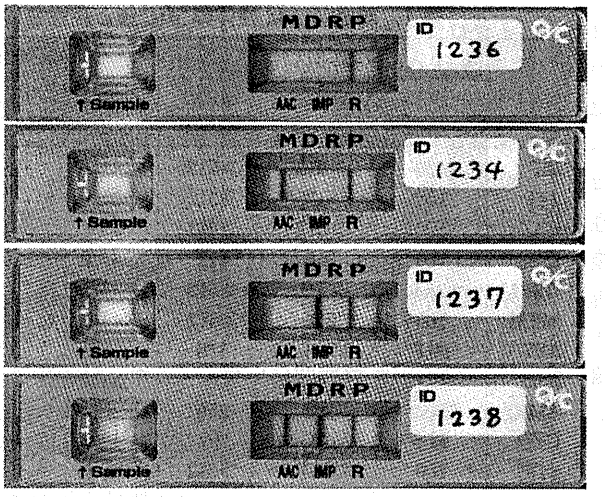

図2　AAC(6′)-IaeおよびIMP-1イムノクロマトキット(文献[9]より改変)
実際に開発したAAC-Iae/IMP-1検出用のダブルテストラインのキット．かなり高感度で検出できる．
AAC：アミノグリコシド耐性遺伝子産物AAC(6′)-Iae，IMP：メタロ-β-ラクタマーゼ遺伝子産物IMP-1，R：陽性対照．

パターンがほぼ同一であった[6]．これは，ある特定のMDRP株が病院内で院内感染起因菌として伝播し，更に他施設へも拡大していることを意味している．恐らく患者などの移動に伴って，施設間の伝播が起きていたのだろう．この地域的なMDRP多発事例は，この地域の感染制御ネットワークフォーラムの様々な感染対策プログラムによって収束に向かいつつある．

このように施設を越えて医療施設間で伝播拡大していることが，宮城県以外でも，広島県[7]や関東地方[8]などの地域でも報告されており，特定の高度多剤耐性緑膿菌株が全国の医療施設に伝播拡大されていることが危惧された．

4. 高度多剤耐性緑膿菌株の遺伝的特徴と迅速診断キットの開発

一般的に，緑膿菌は薬剤耐性因子をプラスミドによって獲得することが知られている．しかし，現在日本の医療機関で問題となっている高度多剤耐性緑膿菌株は，主要な薬剤耐性遺伝子がプラスミドではなくゲノム上に存在し，同じような遺伝子バックグラウンドを有する菌株が医療機関で伝播拡大していると考えられる．この高度多剤耐性緑膿菌株の多くはアミノグリコシド耐性遺伝子*aac(6′)-Iae*とメタロ-β-ラクタマーゼ遺伝子bla_{IMP-1}をもつ．これらの遺伝子は，ゲノムに存在するインテグロンと呼ばれる遺伝子構造上に存在する．したがって，この2種類の遺伝子産物をイムノクロマト法のように簡便に検出することができれば，高度多剤耐性緑膿菌株迅速モニタリングやスクリーニング，高度多剤耐性緑膿菌感染症の迅速診断に利用でき，迅速診断はもとより，高度多剤耐性緑膿菌株の感染拡大の防止に極めて有効であろう．そこで，アミノグリコシド耐性遺伝子およびメタロ-β-ラクタマーゼ遺伝子産物AAC(6′)-IaeおよびIMP-1を簡便に検出するイムノクロマト法をミズホメディー社と開発した(**図2**)．IMP-1には，現時点で24種類(2つの欠番を含めてIMP-1～IMP-26)の亜型が存在することが知られている．このキットではこれらすべての亜型，言い換えるとIMPを検出できる．

株式会社BML総合研究所が全国規模で分離した248株のMDRP臨床分離株を対象に，AAC(6′)-IaeおよびIMPイムノクロマト試験を実施した．248株のMDRPを対象にAAC

(6′)-Iae/IMPイムノクロマト試験を実施した結果，AAC(6′)-IaeおよびIMPの両因子が陽性を示す株は150株(60.5％)であった．これら陽性株はすべて高度多剤耐性緑膿菌株であり，本イムノクロマト試験により，MDRPのうち，高度多剤耐性緑膿菌株を特異的に迅速検出できることが示唆された．地理的分布を解析した結果，47都道府県中24都道府県において検出され，AAC(6′)-IaeおよびIMPを産生する高度多剤耐性緑膿菌株が，既に全国の医療機関に伝播していることが明らかとなった．

5. 新たな高度多剤耐性緑膿菌株の新興

最近の解析から，これまで報告のあったMDRP株とは，パルスフィールド電気泳動のパターンが異なるAAC(6′)-IaeおよびIMP-1産生株が少数検出されることがわかった．この菌株も全国の医療施設から分離されており，分離検査材料はすべて呼吸器由来の検体であった．このクローンの詳細な分子疫学解析が必要であるが，MDRPが日本の医療機関で大きく進化している可能性が示唆されている．今後とも，我が国の医療施設で分離されるMDRPを注意深く監視する必要がある．

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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.
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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

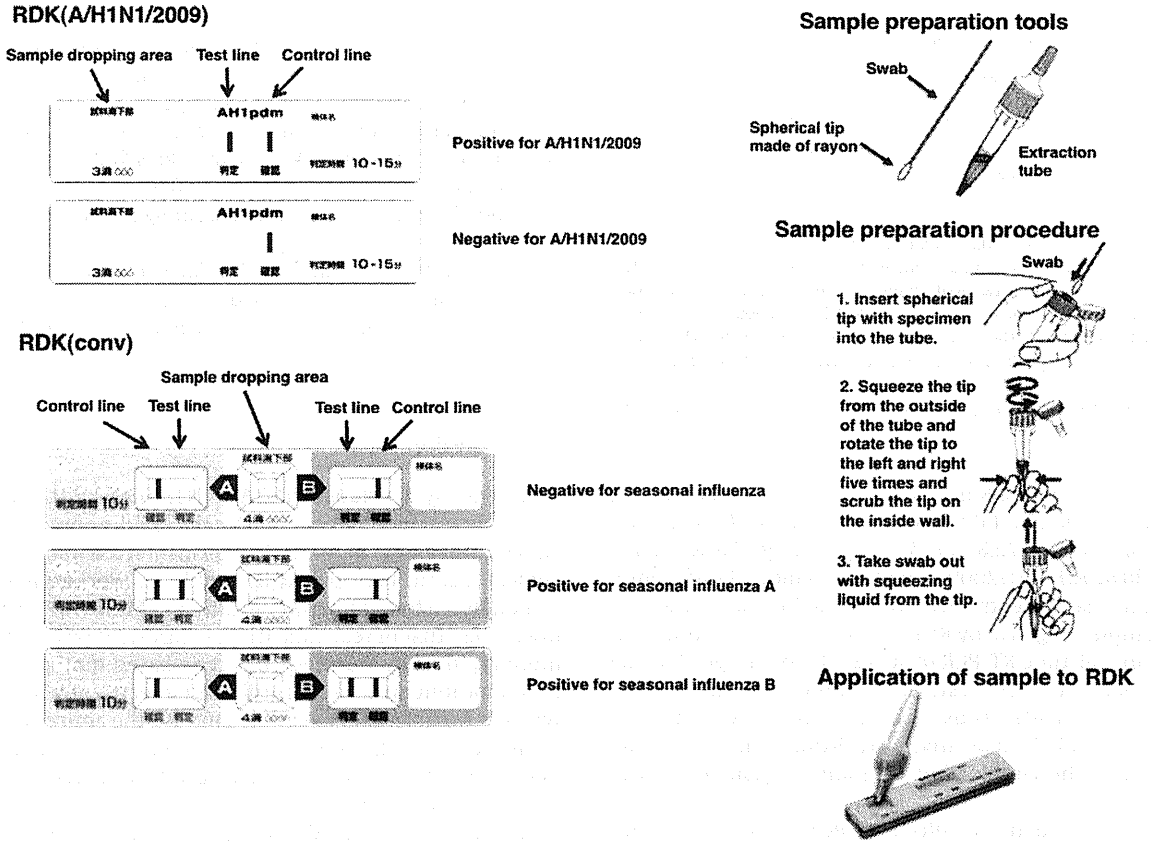

Fig. 1. Composition of RDK(A/H1N1/2009) and RDK(conv) used in the clinical trial and interpretation of the results. Each RDK consists of a sample dropping area and detection area(s) containing a test line and a control line. The control line confirms sample flow. RDKs are supplied with a swab made of 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

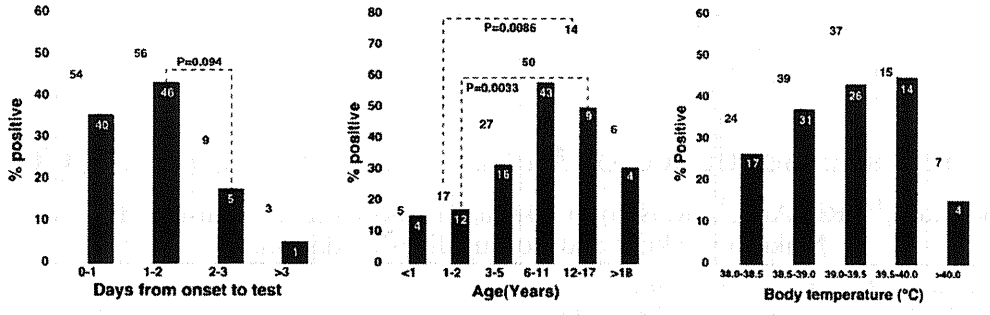

Fig. 2. Percentage of patients positive for A/H1N1/2009 by real-time RT-PCR and RDK(A/H1N1/2009). Patients were stratified by time from onset of influenza-like symptoms to specimen acquisition, by patient age, and by body temperature at the time of sampling. Statistical comparisons were performed using 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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Complete Genome Sequence of *Melissococcus plutonius* ATCC 35311[▽]

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We report the first completely annotated genome sequence of *Melissococcus plutonius* ATCC 35311. *M. plutonius* is a one-genus, one-species bacterium and the etiological agent of European foulbrood of the honeybee. The genome sequence will provide new insights into the molecular mechanisms underlying its pathogenicity.

Melissococcus plutonius, the etiological agent of European foulbrood of the honeybee (10), was first cultured and characterized in detail by Bailey (2). Although selective media have been used to cultivate *M. plutonius* (5), the relative complexity of its culture procedure makes it extremely difficult to isolate. Although it can be detected using a PCR method targeting its 16S rRNA sequence (6, 8), lack of genome information hinders further development of a rapid and reliable diagnostic method for European foulbrood. The strain selected for sequencing (*M. plutonius* ATCC 35311) was the type strain of *M. plutonius*, which was originally deposited at the National Collection of Dairy Organisms by Bailey and Collins (3).

The genome of *M. plutonius* was sequenced using the Roche genome sequencer FLX Titanium. We obtained a total of 246,722 reads, covering a total of 90,529,838 bp, or 45.1-fold coverage. Sequences were assembled into a total of 47 contigs. Gaps were filled by Sanger sequencing of PCR products by brute force amplification of the regions between each pair of contigs. Primary coding sequence (CDS) extraction and initial functional assignment were performed by the automated annotation servers RAST (1) and ISGA (7). Their results were compared to verify the annotation and were corrected manually by *in silico* molecular cloning (*In Silico* Biology, Inc., Kanagawa, Japan).

The *M. plutonius* ATCC 35311 genome consists of a single circular chromosome of 1,891,014 bp, with an average GC content of 31.4%, and a plasmid of 177,718 bp, with an average GC content of 29.2%. The chromosome contained a total of 1,773 protein-coding genes, 61 tRNA genes for all amino acids, and four *rrn* operons, while the plasmid contained a total of 150 protein-coding genes. In addition, the chromosome harbors 1 prophage-like element. Whole-chromosome comparison using the BLAST algorithm showed that the closest organism to *M. plutonius* ATCC 35311 was *Enterococcus faecalis* V583 (9), with 12% genome coverage.

M. plutonius requires potassium for efficient growth. Interestingly, we found that *M. plutonius* ATCC 35311 harbors only two genes, one encoding a potassium efflux system KefA protein and the other encoding a high-conductance mechanosensitive channel, that are putatively involved in potassium homeostasis. *M. plutonius* ATCC 35311 also lacks a tricarboxylic acid (TCA) cycle, an electron transport system, and a sugar alcohol utilization system, although it likely has a glycolysis system and a pentose phosphate pathway. *M. plutonius* ATCC 35311 harbors clustered regularly interspaced short palindromic repeats (CRISPR), which confer resistance to infection by phages (4). The plasmid of *M. plutonius* ATCC 35311 encodes a number of transporters, sugar chain-modifying enzymes, and, interestingly, an NADH dehydrogenase.

Nucleotide sequence accession numbers. Nucleotide sequences of the chromosome and plasmid of *M. plutonius* ATCC 35311 have been deposited in the DNA Database of Japan database under accession no. AP012200 and AP012201, respectively.

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Genome Sequence of Multidrug-Resistant *Pseudomonas aeruginosa* NCGM1179

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

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

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

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

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

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Genome Sequence of Clinical Isolate *Mycobacterium tuberculosis* NCGM2209

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We report the annotated genome sequence of a clinical isolate, *Mycobacterium tuberculosis* strain NCGM2209, which belongs to the "Beijing family" and was isolated in Japan.

Mycobacterium tuberculosis strains with the Beijing genotype, the "Beijing family," are disseminated worldwide and particularly prevalent in East Asia (5), including Japan (4, 6). Approximately 75% of *M. tuberculosis* isolates in Japan are Beijing family strains (4, 6). The Beijing family strains are classified phylogenetically into the ancient (atypical) and modern (typical) sublineages based on the absence or presence of insertion sequence (IS) *6110* in the NTF genome region, respectively (3). The ancient Beijing strains are predominant in Japan; i.e., 76% of the Beijing family isolates are the ancient ones (2). Nevertheless, the modern strains are prevalent worldwide (5).

M. tuberculosis NCGM2209 was obtained from a foreign-born patient in his 20s in a Tokyo hospital. We will report elsewhere that 28.6% (26/91) of *M. tuberculosis* isolates from foreign-born patients in Japan showed a cluster in analysis with pulsed-field gel electrophoresis, and most of them were the ancient Bejiing strains. There may exist a population of foreign-born patients who have been infected with *M. tuberculosis* in Japan.

The genome of strain NCGM2209 was sequenced using a GS FLX Titanium sequencer with pyrosequencing technology. We obtained a total of 237,550 reads, covering a total of 105,423,123 bp. The number of contigs over 100 bp was 317, and the size was 4,280,431 bp. The number of contigs over 500 bp was 248, and the size was 4,261,581 bp. The number of scaffolds was 2, and that of bases was 4,467,000. The largest scaffold size was 4,464,184 bp. The genome of NCGM2209 strain has a G+C content of 61.8%, and the draft assemblies contained 3,728 potential protein-coding sequences, 51 tRNA genes, and 1 tmRNA gene. Primary coding sequence extraction and initial functional assignment were performed by the automated annotation server RAST (Rapid Annotation using Subsystem Technology) (1). The results were compared to verify the annotation and were corrected manually by *in silico* molecular cloning (In Silico Biology, Inc., Kanagawa, Japan).

Nucleotide sequence accession numbers. Nucleotide sequences of the chromosome of *M. tuberculosis* strain NCGM2209 have been deposited in the DNA Database of Japan under accession no. DF126614 and DF126615.

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Comprehensive Multicenter Evaluation of a New Line Probe Assay Kit for Identification of *Mycobacterium* Species and Detection of Drug-Resistant *Mycobacterium tuberculosis*

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We evaluated a new line probe assay (LiPA) kit to identify *Mycobacterium* species and to detect mutations related to drug resistance in *Mycobacterium tuberculosis*. A total of 554 clinical isolates of *Mycobacterium tuberculosis* ($n = 316$), *Mycobacterium avium* ($n = 71$), *Mycobacterium intracellulare* ($n = 51$), *Mycobacterium kansasii* ($n = 54$), and other *Mycobacterium* species ($n = 62$) were tested with the LiPA kit in six hospitals. The LiPA kit was also used to directly test 163 sputum specimens. The results of LiPA identification of *Mycobacterium* species in clinical isolates were almost identical to those of conventional methods. Compared with standard drug susceptibility testing results for the clinical isolates, LiPA showed a sensitivity and specificity of 98.9% and 97.3%, respectively, for detecting rifampin (RIF)-resistant clinical isolates; 90.6% and 100%, respectively, for isoniazid (INH) resistance; 89.7% and 96.0%, respectively, for pyrazinamide (PZA) resistance; and 93.0% and 100%, respectively, for levofloxacin (LVX) resistance. The LiPA kit could detect target species directly in sputum specimens, with a sensitivity of 85.6%. Its sensitivity and specificity for detecting RIF-, PZA-, and LVX-resistant isolates in the sputum specimens were both 100%, and those for detecting INH-resistant isolates were 75.0% and 92.9%, respectively. The kit was able to identify mycobacterial bacilli at the species level, as well as drug-resistant phenotypes, with a high sensitivity and specificity.

The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis*, resistant to at least rifampin (RIF) and isoniazid (INH), markedly hinders the control of tuberculosis (8). Nontuberculous mycobacteria (NTM) are also associated with pulmonary diseases (2, 16). Drug resistance in *M. tuberculosis* is due to mutations, including *rpoB* mutations, associated with RIF resistance; mutations in *katG*, the promoter region of the *fabG1-inhA* operon, *fabG1*, *furA*, and *inhA*, associated with INH resistance; *pncA* mutations, associated with pyrazinamide (PZA) resistance; and *gyrA* mutations, associated with resistance to fluoroquinolones (FQ) (47). Hybridization-based line probe assays (LiPAs) detect mutations associated with resistance to RIF (12, 21, 33, 38), INH (3), PZA (42), and FQ (15).

A new LiPA kit was recently developed to identify clinically important *Mycobacterium* species and to detect drug resistance mutations in *M. tuberculosis*. Evaluation of this kit in six independent hospitals in Japan showed that this assay is promising for the rapid detection of drug-resistant tuberculosis and for identification of major NTM.

MATERIALS AND METHODS

Clinical isolates. A total of 554 clinical isolates of *M. tuberculosis* and NTM were obtained between January 2005 and December 2009 from 554 patients with pulmonary tuberculosis or NTM-related disease in the following six hospitals in Japan: Japan Anti-Tuberculosis Association Fukujuji Hospital (hospital A), National Hospital Organization (NHO) Tokyo Hospital (hospital B), NHO Kinki-Chuo Chest Medical Center (hospital C), NHO Ibaraki Higashi Hospital (hospital D), Tokyo Metropolitan Tama Medical Center (hospital E), and Osaka Prefectural Medical Center for Respiratory and Allergic Diseases (hospital F). Each participating hospital provided 79 to 109 isolates, all of which were subjected to species identification and drug susceptibility testing (DST). The *M. tuberculosis* isolates included 160 that were susceptible to all drugs tested and 156 that were resistant to at least one of the drugs tested (see Table S1 in the supplemental material). Of the drug-resistant isolates, 88 were resistant to RIF, 138 were resistant to INH, 58 were resistant to PZA, and 57 were resistant to levofloxacin (LVX) (data not shown). Other isolates included *Mycobacterium avium* ($n = 71$), *Mycobacterium intracellulare* ($n = 51$), and *Mycobacterium kansasii* ($n = 54$), as well as other NTM ($n = 62$) (see Table S1).

Clinical specimens. A total of 163 sputum specimens were obtained from patients suspected to have or previously diagnosed with tuberculosis or NTM disease (one specimen each) in the hospitals during the period from June 2009 to April 2010. These specimens were transported to the National Reference Laboratory of Tuberculosis (RIT) and stored at −80°C until tested. Each specimen was smeared and stained according to the Ziehl-Neelsen method, followed by treatment with an *N*-acetyl-L-cysteine–NaOH solution as described previously (41). Each pretreated specimen was resuspended in 1.5 ml of phosphate buffer (pH 6.8). Ali-

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quots of 0.2 ml of each suspension were transferred to 1.5-ml tubes and subjected to LiPA. Further aliquots of 0.2 ml of each suspension were transferred to fresh 1.5-ml tubes and subjected to the PCR-based Cobas Amplicor MTB test (Roche Diagnostics, Basel, Switzerland) (9, 26, 31) and the Cobas Amplicor *M. avium* and *M. intracellulare* tests (Roche Diagnostics). Aliquots of 0.1 ml and 0.5 ml of each specimen were inoculated into egg-based modified Ogawa medium (27) containing 2% (wt/vol) KH_2PO_4 and into MGIT broth (Bactec MGIT 960; BD Biosciences, Sparks, MD), respectively, for mycobacterial examination. Aliquots of 1 ml of the suspension for LiPA were centrifuged for 15 min at 13,000 × *g*, and the supernatant was removed with a pipette. Tris-EDTA (TE) buffer (100 μl) was added to the pellet, and the solution was again centrifuged for 15 min at 13,000 × *g*. The pellet was suspended in 50 μl of TE buffer and incubated at 95°C for 30 min. Aliquots of the supernatant (5 μl) were used for each LiPA. The total time for all procedures was about 3 h.

Species identification. *M. tuberculosis* was identified at hospitals A and B by use of TRCRapid M.TB kits (Tosoh Bioscience, Tokyo, Japan), based on the transcription-reverse transcription concerted reaction (13, 44), and at hospitals C to F and RIT by use of the Cobas Amplicor MTB test. *M. avium* and *M. intracellulare* were identified at hospitals C to F and RIT by use of the Cobas Amplicor *M. avium* and *M. intracellulare* tests, respectively. The *M. avium* complex (MAC) was identified at hospitals A and B by use of TRCRapid MAC kits (Tosoh Bioscience); isolates identified as MAC species were heat-killed and transported to RIT for species identification. The other NTM were identified at hospitals A, B, and D to F and at RIT by use of the DNA-DNA hybridization technique (DDH Mycobacteria Kyokuto; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) (28) and at hospital C by using AccuProbe (Gen-Probe, San Diego, CA) (17, 18). NTM isolates that were not identified by commercial kits were subjected to 16S rRNA gene sequencing at RIT.

DST and pyrazinamidase activity assay. DSTs for RIF, INH, PZA, and LVX were performed at each participating hospital. At hospitals A and B, the MGIT AST (BD Biosciences) test was performed to detect RIF, INH, and PZA resistance, and an egg-based Ogawa medium (24) (1% KH_2PO_4) method (Welpack S test; Nihon BCG Inc., Tokyo, Japan) was used to detect RIF, INH, and LVX resistance. At hospital C, MGIT AST, Welpack S, and a broth microdilution method (broth MIC MTB-I; Kyokuto Pharmaceutical Industrial Co.) were performed to detect RIF, INH, and LVX resistance. At hospital D, the egg-based Ogawa medium (1% KH_2PO_4) method (Bit Spectre-SR; Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) was performed to detect RIF, INH, and LVX resistance, and a broth method was used to detect PZA resistance (PZA broth; Kyokuto Pharmaceutical Industrial Co.). At hospital E, the MGIT AST test was used to test for RIF, INH, and PZA resistance (LVX resistance was not tested at this hospital). At hospital F, the broth MIC MTB-I test was used to test for RIF, INH, and LVX resistance, and PZA broth (Kyokuto) was used to detect PZA resistance. At RIT, the standard proportion method using Ogawa medium (1% KH_2PO_4) was used to test for RIF, INH, and LVX resistance, and the MGIT AST test was used to test for PZA resistance. Isolates showing discordant results between phenotypic and genotypic DSTs for PZA were transferred to RIT and their pyrazinamidase activities tested (45), except for six isolates that had not been stored at the hospital. The INH resistance levels were as follows: isolates were considered resistant to INH at 0.2 μg/ml when they were resistant to INH at 0.2 μg/ml and susceptible to INH at 1.0 μg/ml; isolates were considered resistant to INH at 1.0 μg/ml when they were resistant to INH at 1.0 μg/ml. All kits for identification of mycobacteria and DSTs used in this study were recommended by the Japanese Society for Tuberculosis and approved as diagnosis reagents by the Ministry of Health, Labor and Welfare, Japan.

LiPA. LiPA was performed as described previously (3, 42), using 121 oligonucleotide probes (see Table S2 in the supplemental material) immobilized onto four strips, called the NTM/MDR-TB, INH, PZA, and FQ strips (Nipro Co., Osaka, Japan). All clinical isolates and all sputum specimens were tested by LiPAs using all four strips, regardless of the results of any particular strip. The NTM/MDR-TB strip was designed to identify four *Mycobacterium* species—*M. tuberculosis*, *M. avium*, *M. intracellulare*, and *M. kansasii*—and to detect mutations associated with RIF and INH resistance in *M. tuberculosis*. The INH, PZA, and FQ strips were designed to detect mutations associated with INH, PZA, and FQ resistance of *M. tuberculosis*, respectively. The corresponding regions and mutations for each probe are shown in Table S2. A probe designed to detect the wild-type sequence of *M. tuberculosis* was designated as S probe, whereas a probe designed to detect a mutant sequence frequently found in drug-resistant *M. tuberculosis* was designated as R probe. On the INH strip, 46 S probes covered various regions of the following *M. tuberculosis* genes: $P_{fabG1\text{-}inhA}$ (*inhA*-1), *inhA* (*inhA*-2), *fabG1* (*fabG1*-1 and -2), *furA* (*furA*-1 and -2), and *katG* (*katG*-1 to -40) (3). The *katG* probes covered 90 mutations related to INH resistance. On the PZA strip, 47 S probes covered regions of *M. tuberculosis pncA* (*pncA*-1 to -47), with 2 probes (*pncA*-16 and -17) containing a silent mutation in *pncA* (42). Probes *inhA*-S6 and -S7 and *katG*-S8 to -S11 on the NTM/MDR-TB strip were the same as *inhA*-1 and -2 and *katG*-20, -22, -23, and -24 on the INH strip, respectively.

Using biotinylated primers, the following products were obtained by nested PCR: *rpoB* (290 bp), $P_{fabG1\text{-}inhA}$ (477 bp), and *katG* (248 bp) for the NTM/MDR-TB strip; $P_{fabG1\text{-}inhA}$ (477 bp), *fabG1* (209 bp), *furA* (256 bp), and *katG* (612 bp, 698 bp, and 907 bp) for the INH strip; *pncA* (641 bp) for the PZA strip; and *gyrA* (379 bp) for the FQ strip. The immobilized probes on each strip were hybridized with the PCR products at 62°C for 30 min and then incubated with streptavidin labeled with alkaline phosphatase at room temperature for 30 min. Color was developed by incubation with 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and nitroblue tetrazolium.

The presence or absence of bands, i.e., hybridization signals, on all strips was judged independently by three different observers. The results of LiPA were interpreted as follows. For identification of *Mycobacterium* species, when a signal was observed on the NTM/MDR-TB strip with any of the four probes (*rpoB*-AVI, *rpoB*-INT, *rpoB*-KAN, and *rpoB*-TB), the sample was thought to contain the corresponding *Mycobacterium* species. Conversely, when no signals were observed, the sample contained none of these four species. For detection of drug-resistant *M. tuberculosis*, when no signal was observed with any of the S probes, *M. tuberculosis* in the sample was considered resistant to the corresponding drug. In addition, when a signal(s) was observed with any of the R probes, the samples contained drug-resistant *M. tuberculosis* with the corresponding mutation(s). It took about 7 h to complete all procedures of the LiPA method.

DNA sequencing. The PCR products were sequenced. The sequenced samples were as follows: 1 isolate and 1 clinical specimen that showed discrepancies in species identification between conventional methods and LiPA and 40 isolates and 4 clinical specimens that showed discrepancies in drug susceptibility between phenotypic DST and LiPA. DNA sequences were compared with the sequence of *M. tuberculosis* H37Rv by using Genetyx-Mac, version 14.0.2 (Genetyx Corporation, Tokyo, Japan). We also sequenced the 16S rRNA genes of NTM isolates when they could not be identified by conventional identification kits. The sequences of the 16S rRNA genes were analyzed with software for DNA sequence-based diagnosis, published by the Ribosomal Differentiation of Microorganisms Project (RIDOM) (19), or with the Basic Local Alignment Search Tool (BLAST) to identify the species.

Ethical considerations. The study protocol was carefully reviewed and approved by the ethics committee of each participating hospital (hospital A approval date, 29 January 2009; hospital B approval date, 30 April 2009 [approval number 21-02-Da]; hospital C approval date, 14 November 2008 [approval number 20-18]; hospital D approval date, 18 September 2008; hospital E approval date, 28 November 2008; hospital F approval date, 28 March 2009 [approval number 5-84]). All clinical sputum specimens were collected after obtaining written informed consent from the participants.

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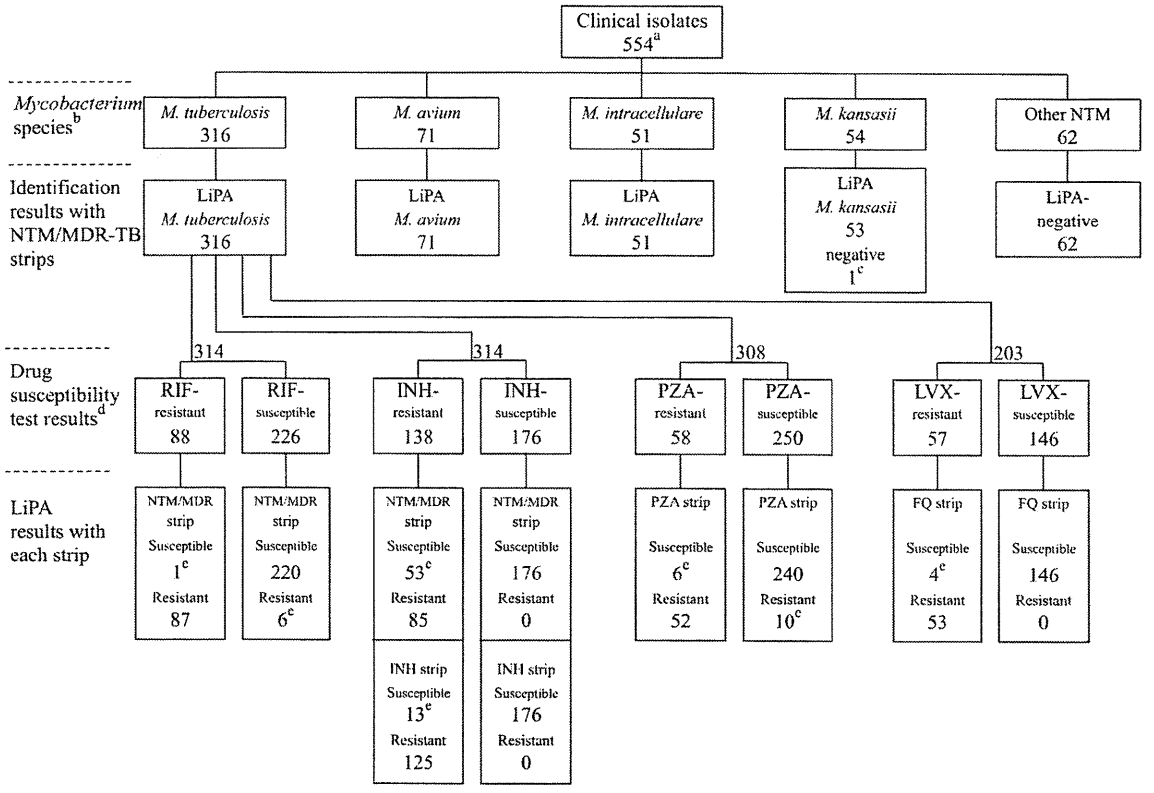

FIG 1 Distribution of LiPA results for 554 clinical isolates. [a], number of clinical isolates. [b], *Mycobacterium* species identified by conventional methods. [c], *M. kansasii* subtype III. [d], drug susceptibility testing and assays for pyrazinamidase activity were performed at each hospital (see the supplemental material). Of 316 *M. tuberculosis* isolates, 314 were subjected to RIF and INH susceptibility testing, 308 to PZA susceptibility testing, and 203 to LVX susceptibility testing. [e], some isolates showed different results between DST and LiPA. DNA sequences of each target gene were determined for these discrepant isolates (see the footnote in Table S4 in the supplemental material).

RESULTS

Identification of clinical isolates. Among 554 isolates, LiPA results for species identified as *M. tuberculosis*, *M. avium*, and *M. intracellulare* showed 100% agreement with those of conventional genetic methods (Fig. 1; see Table S3 in the supplemental material). Of 54 *M. kansasii* isolates, 53 were identified as *M. kansasii* by the LiPA kit. The one discrepant isolate of *M. kansasii* carried an *rpoB* sequence identical to that of subtype III of the seven subtypes of *M. kansasii*, defined by sequence polymorphisms in *hsp65* (1, 43). LiPA results were negative for 62 isolates of other NTM species.

Correlation between conventional DST and LiPA results. LiPA results were compared with those of DST (Table 1; see Table S4 in the supplemental material).

(i) RIF resistance and *rpoB* mutations. LiPA identified 98.9% (87/88 isolates) of RIF-resistant isolates and 97.3% (220/226 isolates) of RIF-susceptible isolates when *M. tuberculosis* isolates were tested using NTM/MDR-TB strips designed to detect *rpoB*

TABLE 1 Diagnostic performance of LiPA in comparison with drug susceptibility testing

Antituberculosis drug (strip used in LiPA[c])	Clinical isolates		Clinical samples (sputa)	
	Sensitivity[a]	Specificity[b]	Sensitivity[a]	Specificity[b]
RIF (NTM/MDR-TB strip)	98.9 (87/88)	97.3 (220/226)	100 (3/3)	100 (52/52)
INH (INH strip)	90.6 (125/138)	100 (176/176)	75.0 (3/4)	92.9 (39/42)
INH (NTM/MDR-TB strip)	61.6 (85/138)	100 (176/176)	50.0 (3/6)	97.8 (45/46)
PZA (PZA strip)	89.7 (52/58)	96.0 (240/250)	100 (4/4)	100 (52/52)
LVX (FQ strip)	93.0 (53/57)	100 (146/146)	100 (7/7)	100 (48/48)

[a] Data are percentages (no. of drug-resistant samples by LiPA/no. of drug-resistant samples by DST).

[b] Data are percentages (no. of drug-susceptible samples by LiPA/no. of drug-susceptible samples by DST).

[c] LiPA was performed using four strips, namely, NTM/MDR-TB, INH, PZA, and FQ strips (see the supplemental material). The NTM/MDR-TB strip was designed to identify four *Mycobacterium* species and to detect mutations associated with RIF resistance and INH resistance (C-15T and T-8C mutations in $P_{fabG1\text{-}inhA}$ and S315T and S315N mutations in *katG*). The INH, PZA, and FQ strips were designed to detect mutations associated with INH, PZA, and FQ resistance of *M. tuberculosis*, respectively. The corresponding regions and mutations for each probe are shown in Table S1 in the supplemental material. The INH strip covered 46 regions of the following *M. tuberculosis* genes: $P_{fabG1\text{-}inhA}$, *inhA*, *fabG1*, *furA*, and *katG* (3). The PZA strip covered *pncA* (40), and the FQ strip covered *gyrA* (4).

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mutations (Table 1). Of all the isolates tested, seven showed discrepancies between DST and LiPA for RIF susceptibility testing. One isolate, identified as RIF resistant by DST but RIF susceptible by LiPA, had an I572F substitution. The remaining six were identified as RIF susceptible by DST but RIF resistant by LiPA. Of these, three had an H526S substitution, while the other three had an L511P mutation, a D516Y mutation, and a silent mutation at codon 516 (GAC → GAT). Of these six isolates, the three with the H526S mutation and the one with the L511P mutation were reported by hospital C as RIF susceptible by the MGIT AST and Welpack S tests but as RIF "intermediate" (MICs of 0.25 mg/liter and 0.5 mg/liter, respectively) by the broth MIC MTB-I test.

(ii) INH resistance and mutations of $P_{fabG1\text{-}inhA}$, *fabG1*, *furA*, and *katG*. The INH strip was designed to detect mutations associated with INH resistance in *M. tuberculosis*, including mutations in $P_{fabG1\text{-}inhA}$ (C-15T and T-8C), *fabG1* (G609A [L203L]), *furA* (C41T [A14V]), and *katG* (see Table S2 in the supplemental material). The strips identified 90.6% (125/138 isolates) of INH-resistant isolates and 100% (176/176 isolates) of INH-susceptible isolates (Table 1). Thirteen isolates were found to be INH resistant by DST but INH susceptible by LiPA (see Table S4). Of these, 10 had no mutations in the amplified regions for the INH strip, while the other 3 had S17N, G206S, and E340Q substitutions in *katG*.

The NTM/MDR-TB strip was designed to detect mutations of $P_{fabG1\text{-}inhA}$ (C-15T and T-8C) and *katG* (S315T and S315N) (see Table S2 in the supplemental material) which are frequently detected in INH-resistant clinical isolates (41, 47). NTM/MDR-TB strips identified 61.6% (85/138 isolates) of INH-resistant isolates and 100% (176/176 isolates) of INH-susceptible isolates (Table 1). Fifty-three isolates were identified as INH resistant by DST but INH susceptible by LiPA using NTM/MDR-TB strips. Of these, 13 isolates and the remaining 40 isolates were identified as INH susceptible and INH resistant, respectively, by LiPA using INH strips (see Table S4). Of these 40 INH-resistant isolates, 21 were resistant to INH at 1.0 μg/ml, and the remaining 19 isolates were resistant to INH at 0.2 μg/ml. Of the 21 isolates resistant to INH at 1.0 μg/ml, 5 showed no hybridization with the *fabG1*-1 probe (G609A [L203L]); 12 showed no hybridization with any of the *katG* probes, including *katG*-1 (1 isolate with a Δ152A mutation [frameshift]), *katG*-5 (1 isolate with an A338C [Y113S] mutation), *katG*-6 (2 isolates with a Δ367G mutation [frameshift]), *katG*-8 (1 isolate with a G412T [N138Y] mutation), *katG*-9 (1 isolate with an A425G [D142G] mutation), *katG*-10 (1 isolate with an A454C [K152Q] mutation), *katG*-11 (1 isolate with a G487A [D163N] mutation), *katG*-15 (1 isolate with a T571G [W191G] mutation), *katG*-29 (1 isolate with an A1382C [Q461P] mutation), *katG*-37 (1 isolate with a G1795T [G599stop] mutation), and *katG*-39 (1 isolate with a T2093C [F698S] mutation); 2 showed no hybridization with two *katG* probes, either *katG*-21 and *katG*-25 (A922C [T308P] and G1037C [S346T] mutations) or *katG*-39 and *katG*-40 (Δ1991-2173 [frameshift] mutation); 1 showed no hybridization with *katG*-26 to -40 (the DNA sequence was not determined); and 1 showed no hybridization with the *fabG1*-1 (G609A [L203L] mutation) and *katG*-6 (G378T [M126I] mutation) probes. Of the 19 isolates resistant to INH at 0.2 μg/ml, 12 showed no hybridization with the *fabG1*-1 probe (G609A [L203L] mutation), 6 showed no hybridization with the *katG*-28 probe (G1255C [D419H] mutation), and 1 showed no hybridization with the *katG*-28 and -34 probes (sequence not determined).

(iii) PZA resistance and *pncA* mutations. The PZA strip was designed to detect *pncA* mutations associated with PZA resistance in *M. tuberculosis* (42). The LiPA test identified 89.7% (52/58 isolates) of PZA-resistant and 96.0% (240/250 isolates) of PZA-susceptible isolates (Table 1). Sixteen isolates showed discrepancies between DST and LiPA results (see Table S4 in the supplemental material). Six isolates found to be PZA resistant by DST but PZA susceptible by LiPA had no mutations in *pncA*. Ten other isolates were PZA susceptible by DST but PZA resistant by LiPA. Of these 10 isolates, 4 had G162S, 2 had G17D, 2 had T168I, 2 had G132D, and 2 had V147I substitutions.

(iv) FQ resistance and *gyrA* mutations. The FQ strip was designed to detect *gyrA* mutations associated with FQ resistance in *M. tuberculosis* (see Table S2 in the supplemental material). FQ strips identified 93.0% (53/57 isolates) of LVX-resistant and 100% (146/146 isolates) of LVX-susceptible isolates (Table 1). Four isolates found to be LVX resistant by DST but LVX susceptible by LiPA had no mutations in *gyrA*. These isolates also had no mutations in *gyrB*.

Direct identification of *Mycobacterium* species and detection of drug-resistant *M. tuberculosis* in sputum specimens. A total of 163 sputum specimens were collected from patients who had been diagnosed with or were suspected to have pulmonary tuberculosis or NTM diseases.

(i) Detection and identification of *Mycobacterium* species in sputum specimens. Direct application of the LiPA kit to sputum samples for species identification showed high degrees of consistency and efficiency that were comparable with those of conventional methods. The sensitivity of LiPA with NTM/MDR-TB strips was 90.2% (74/82 specimens) for *M. tuberculosis*, 84.6% (11/13 specimens) for *M. avium*, 54.5% (6/11 specimens) for *M. intracellulare*, and 80.0% (4/5 specimens) for *M. kansasii* (see Table S5 in the supplemental material). One specimen, which was misidentified by LiPA, was found to be *Mycobacterium rhodesiae* by DNA sequence analysis. The overall sensitivity of LiPA with NTM/MDR-TB strips for detection of target species was 85.6% (95/111 specimens) (see Table S5). Eighteen samples were LiPA negative despite being PCR and/or culture positive (7 smear-positive and 11 smear-negative samples) (Fig. 2), whereas 14 samples were LiPA positive despite being PCR and culture negative (6 smear-positive and 8 smear-negative samples) (Fig. 2).

(ii) Correlation of conventional DST and LiPA results for sputum specimens. Among 163 samples, 49 smear-positive and 10 smear-negative samples were culture positive for *M. tuberculosis* (Fig. 3). For the 49 smear-positive samples, LiPA results for any drug susceptibility ranged from 89.8% (44/49 specimens) to 100% (49/49 specimens); for the 10 smear-negative samples, LiPA results ranged from 20% (2/10 specimens) to 70% (7/10 specimens). LiPA results were even obtained for some culture-negative samples, although these results could not be compared with those of DST. LiPA results were obtained for 11 to 16 of 45 smear-positive and *M. tuberculosis* culture-negative samples and for 7 to 11 of 59 smear-negative and *M. tuberculosis* culture-negative samples (Fig. 3). Direct application of the LiPA kit to sputum samples showed high sensitivities and specificities for detection of resistance to RIF, PZA, and LVX, whereas LiPA for detection of INH resistance showed a relatively low sensitivity (Table 1; see Table S6 in the supplemental material). However, its sensitivity and specificity were improved by using the INH strip.

Eight specimens showed discordance between DST and LiPA results (see Table S6 in the supplemental material). Four showed

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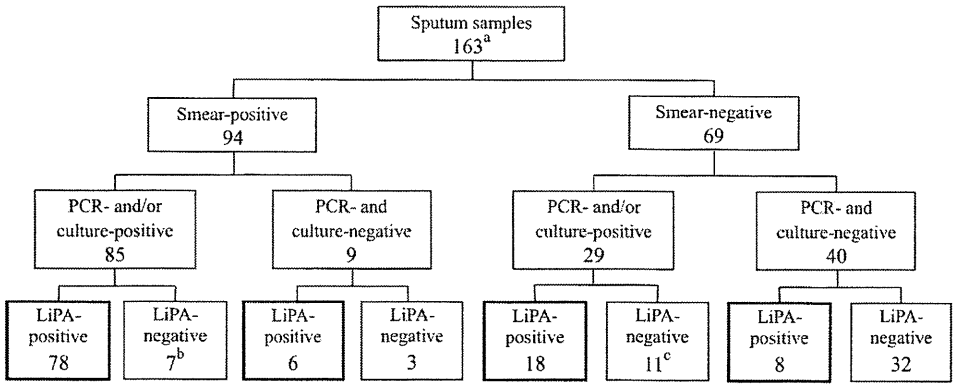

FIG 2 Distribution of LiPA results obtained with NTM/MDR-TB strips for the detection of target species in 163 sputum samples. [a], number of clinical samples. [b], one of these isolates was *Mycobacterium fortuitum*. [c], one of these isolates was *Mycobacterium abscessus*.

discordance between DST results for INH susceptibility and LiPA results obtained using INH strips. Of these, isolates from two specimens that were INH resistant by LiPA had a *fabG1* (G609A [L203L]) mutation, and one had a $P_{fabG1\text{-}inhA}$ (C-15T) mutation. Four specimens showed discordance between DST results for INH susceptibility and LiPA results obtained using NTM/MDR-TB strips (see Table S6). Of these, two specimens indicated as INH susceptible with NTM/MDR-TB strips were identified as INH resistant with INH strips, and isolates from these two specimens had *katG* mutations, i.e., G1795T (G599stop) and T2093C (F698S) mutations. One specimen was also identified as INH susceptible with the INH strip, and DNA sequencing revealed that an isolate from the specimen had two *katG* mutations (T571C [W191R] and G1079A [G360D]). One specimen showing INH resistance with the NTM/MDR-TB strip had a $P_{fabG1\text{-}inhA}$ (C-15T) mutation.

As shown in Fig. 3, for the culture-negative specimens, LiPA results were obtained for 26 specimens (15 smear-positive and 11 smear-negative specimens) for RIF susceptibility, 18 specimens (11 smear-positive and 7 smear-negative specimens) for INH susceptibility with INH strips, 23 specimens (15 smear-positive and 8 smear-negative specimens) for INH susceptibility with NTM/MDR-TB strips, 23 specimens (15 smear-positive and 8 smear-negative specimens) for PZA susceptibility, and 24 specimens (16 smear-positive and 8 smear-negative specimens) for LVX susceptibility. Of these, no specimens were found to be RIF resistant, three were INH resistant, one was PZA resistant, and none were LVX resistant (data not shown).

DISCUSSION

The newly developed LiPA kit successfully identified important *Mycobacterium* species, including *M. kansasii*, except for subtype III of *M. kansasii*. The *rpoB*-KAN probe on the NTM/MDR-TB strip is compatible with the *rpoB* genes of subtypes I, II, IV, and V but not III and VI, perhaps explaining why this LiPA kit was unable to identify a subtype III *M. kansasii* isolate (Fig. 1; see Table S3 in the supplemental material). Among the isolates of *M. kansasii* obtained in four European countries, the majority belonged to subtypes I (68%) and II (31%), with only 1% belonging to subtype III (1). Similar distributions of subtypes were reported in Switzerland (subtype I, 67%; subtype II, 21%; subtype III, 8%; and other subtypes, 4%) (43) and in Catalonia, Spain (subtype I, 98%; subtype VI, 2%) (39). These epidemiological results indicate that subtype III of *M. kansasii* causes significantly fewer human infections than subtypes I and II. Although the LiPA kit showed a signifi-

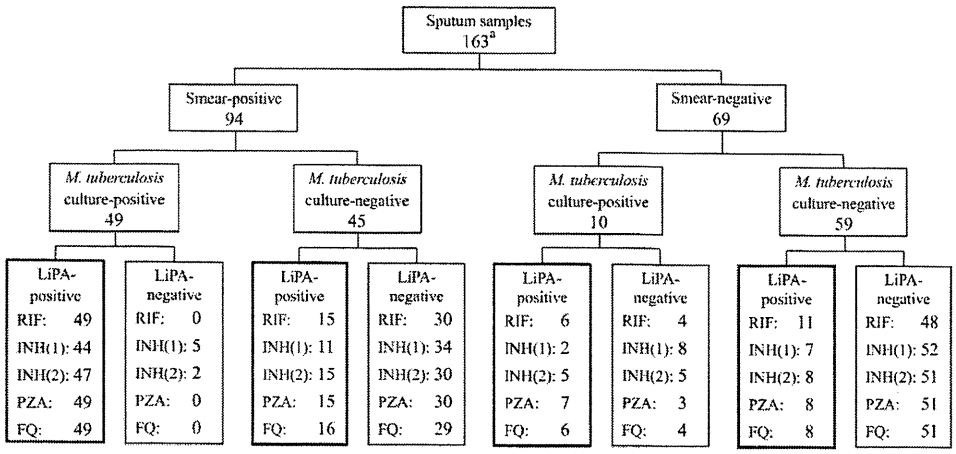

FIG 3 Distribution of LiPA results obtained with four strips for detection of a mutation(s) associated with drug resistance in 163 sputum samples. [a], number of clinical samples. RIF, RIF susceptibility with NTM/MDR-TB strips; INH(1), INH susceptibility with INH strips; INH(2), INH susceptibility with NTM/MDR-TB strips; PZA, PZA susceptibility with PZA strips; FQ, LVX susceptibility with FQ strips.

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cantly efficient performance, improvements are required for detection of subtype III.

Seven isolates showing discrepancies between DST and LiPA for detection of RIF resistance had an I572F, D516D (a silent mutation), L511P, D516Y, or H526S mutation in *rpoB*. With the exception of the D516D mutation, these mutations are associated with RIF resistance. The I572F mutation has been reported to be associated with RIF resistance (46), although this mutation was not covered by the probes on the NTM/MDR-TB strip. The L511P and D516Y mutations have been reported to be associated with RIF resistance (25). RIF-resistant isolates have been reported to possess at least 11 mutations in codon 526, resulting in amino acid mutation of H to C, D, E, G, L, N, P, R, Q, T, or Y but not to S (47). An H526S mutation would be associated with RIF resistance.

LiPA using NTM/MDR-TB strips to detect mutations associated with INH resistance showed a low sensitivity (61.6%) among the isolates, although the strips were able to detect the most frequent mutations found in INH-resistant isolates, including S315T and S315N mutations in *katG* and C-15T and T-8C mutations in the promoter region of *inhA* (34, 35, 47). The Genotype MTBDRplus kit (Hain Lifescience, Nehren, Germany), a commercially available LiPA kit that uses a strip to detect these mutations, showed various degrees of sensitivity to INH-resistant *M. tuberculosis* isolates, including MDR isolates, in several countries, i.e., 92% in Germany (20), 82% in Taiwan (23), 73% in Spain (29), 67% in Italy (30), and 66% in Japan (10). The frequency of INH-resistant clinical isolates with S315T and S315N *katG* mutations and C-15T and T-8C mutations in the promoter region of *inhA* depends on the geographical origin of isolates. INH-resistant isolates with these mutations make up relatively small populations in Japan and Italy.

LiPA using INH strips, which covered more mutations, showed greater sensitivity than that with NTM/MDR-TB strips. Thirteen isolates were found to be INH resistant by DST but INH susceptible by LiPA using INH strips (see Table S4 in the supplemental material). No mutations were detected in 10 isolates, indicating that mutations in other genes may be associated with INH resistance. One isolate had a mutation of *katG* (S17N) which has been reported to confer INH resistance (11) but which is located away from the target sites. Of the remaining two isolates, one each had *katG* G206S and *katG* E340Q mutations, neither of which has been reported previously, to our knowledge. Both may be associated with INH resistance.

LiPA using PZA strips to detect mutations associated with PZA resistance showed high sensitivity and specificity. However, discrepancies between LiPA and DST were observed for 16 isolates. Six isolates were identified as PZA resistant by DST but PZA susceptible by LiPA, with none of these having a mutation in *pncA*, although one was positive in the pyrazinamidase test. Pyrazinamidase-positive but PZA-resistant strains are very rare and usually show a low level of resistance (36). The PZA resistance of the pyrazinamidase-positive strain may have been due to a mechanism other than *pncA* mutation (37). Ten isolates were identified as PZA susceptible by DST but PZA resistant by LiPA. Of these, two had *pncA* mutations causing T168I substitution, and one had a V147I mutation. These three isolates were positive in the pyrazinamidase test, suggesting that these mutations are not related to PZA resistance. Four isolates had a *pncA* G162S mutation, two had a G17D mutation, and one had a G132D mutation. These isolates were PZA susceptible by DST, but they were not tested for pyrazinamidase activity. To our knowledge, the G162S mutation has not been reported previously. The G17D and G132D mutations have been reported to confer PZA resistance (22). These discrepancies may have been due to the limited efficiency of DST methods (6).

LiPA with FQ strips for detection of mutations associated with FQ resistance showed high sensitivity and specificity, with only four isolates showing discordant results. None of these four had a mutation in *gyrA*, indicating that the FQ strips could detect all known mutations associated with FQ resistance. These four isolates had no mutation in *gyrB*, which was recently reported to confer FQ resistance in clinical isolates without *gyrA* mutations (7, 14, 32). Alternatively, the results of DST may show false resistance.

Of 163 sputum samples, 14 were LiPA positive but PCR and culture negative (Fig. 2). The results of LiPA for these 14 samples are likely correct assignments, as all came from patients previously diagnosed by culture methods or PCR as having tuberculosis or NTM diseases, showing 100% agreement. However, many of these results could have come from the shedding of nonviable bacilli from previously treated patients. Therefore, nucleic acid amplification methods, including LiPA, need to be interpreted carefully for previously treated tuberculosis patients. LiPA was always performed with a negative control and repeated when the results were in discordance with those of conventional methods. However, the discrepancies may be explained by cross-contamination during LiPA procedures.

The LiPA kit might be useful for rapid diagnosis of MDR tuberculosis, especially in Asian countries, where the genetic characteristics of INH resistance are unique (36). It is also important to detect resistance to PZA and LVX in MDR tuberculosis, as the majority of MDR *M. tuberculosis* isolates have been reported to be resistant to either PZA or LVX in Japan (4, 5).

The LiPA kit reported here is the first genetic diagnosis kit that can simultaneously identify the major clinical isolates of *Mycobacterium* species and detect mutations associated with resistance to INH, RIF, PZA, and FQ. The present study provides a unique perspective for assessing the overall reliability, specificity, and sensitivity of this kit in comparison with conventional tests. The LiPA kit may also be useful in laboratories in developing countries where mycobacterial culture cannot be performed. However, a follow-up culture-based DST is recommended where resources permit.

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

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ABSTRACT

Rapid and reliable detection of carbapenem-resistant bacteria is an important infection-control measure and a crucial aspect of antimicrobial chemotherapy. IMP-type metallo-β-lactamase (MBL) is an emzyme that mediate carbapenem resistance in bacteria. Here, an immunochromatographic assay was newly developed using novel monoclonal antibodies (mAbs) recognizing IMP-type MBL. Epitope mapping of mAbs and mutational analysis of the epitope region in IMP antigen suggested that the mAbs could react to all known subtypes of IMP-type MBL. Evaluation of the assay using *Pseudomonas aeruginosa* strains (n=248) showed that the results of the immunochromatographic detection of the IMP-type MBLs were fully consistent with those of the PCR analysis for bla_{IMP} genes, showing false positives and negatives. All positive strains were resistant to carbapenem (MIC≥16 μg/ml). The assay also accurately distinguished the production of IMP-type MBLs in *Pseudomonas putida*, *Acinetobacter baumannii*, and *Alcaligenes xylosoxidans*. The detection limit of the assay was 5.7×10^4 cfu per test. Taken together, these data suggest that the developed assay can be used for rapid and reliable diagnosis of the production of IMP-type MBLs in Gram-negative bacteria.

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1. Introduction

Carbapenems are key agents to treat life-threatening bacterial infections (Rahal, 2008). However, the emergence of carbapenem resistance in nosocomial pathogens, including *Serratia marcescens*, those of Enterobacteriaceae, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, constitutes a serious problem for the continued use of carbapenems (Masterton, 2009). Therefore, the rapid and reliable detection of carbapenem-resistant bacteria has become of urgent and vital importance in infection-control measures and antimicrobial chemotherapy.

Bacterial resistance to carbapenems is a complex process, including the loss or reduced expression of OprD porin (Hancock and Brinkman, 2002; Wolter et al., 2004), hyperproduction of AmpC (Tam et al., 2009), and/or overexpression of intrinsic efflux systems such as MexA–MexB–OprM (Aeschlimann, 2003; Li et al., 1995). Among clinical isolates of *Enterobacteriaceae* and *Pseudomonas* spp., resistance to carbapenems has been found to be mainly due to the production of the carbapenem-hydrolyzing enzymes, metallo-β-lactamases (MBLs) (Queenan and Bush, 2007; Walsh et al., 2005). To date, several classes of MBLs, such as IMP, VIM, GIM, KHM, and SPM, have been identified in clinical pathogens (Castanheira et al., 2004; Lauretti et al., 1999; Osano et al., 1994; Poirel et al., 2004; Sekiguchi et al., 2008). IMP-type MBLs are the most common and are found worldwide (Nordmann and Poirel, 2002).

IMP-1 MBL has been identified primarily from strains of *P. aeruginosa* and *S. marcescens* in Japan (Osano et al., 1994; Watanabe et al., 1991). In addition, 24 types of bla_{IMP} have been identified from a variety of clinical isolates and submitted to GenBank. Considering that approximately 1.9% of clinical isolates of *P. aeruginosa* have acquired MBL, and most of these are IMP-1-type MBLs (Kimura et al., 2005). IMP-type MBLs are thought to be significant marker molecules of carbapenem resistant *P. aeruginosa* in Japan.

Previously, we developed an immunochromatographic assay using monoclonal antibodies (mAbs) recognizing an aminoglycoside 6′-*N*-acetyltransferase [AAC(6′)-Iae] responsible for amikacin resistance in MDR *P. aeruginosa* strain NCGM2.S1 (previously reported as IMCJ2.S1) (Kitao et al., 2010; Sekiguchi et al., 2005). Given that the developed assay was a rapid, easy-to-use, and reliable detection method for AAC(6′)-Iae-producing multidrug-resistant (MDR)

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P. aeruginosa, the assay based on the antigen–antibody reaction could serve as a model for the development of a molecular diagnosis method for the screening and investigation of antibiotic-resistant bacteria as an alternative to PCR analysis.

In this study, an immunochromatographic assay using novel mAbs that recognize IMP-type MBLs has been developed. We report here the properties of mAbs used to construct the assay and the evaluation of the assay using clinical isolates.

2. Materials and methods

2.1. Construction and purification of IMP-1 mutants

The bla_{IMP-1} gene was PCR amplified from *P. aeruginosa* NCGM2.S1 strain using the primer sets *Nde*I-bla_{IMP1}(55–74)-F (5′-gcacgccatATGG-CAGAGTCTTTGCCAGATTT-3′) and *Bam*HI-bla_{IMP1}-R (5′-cgcggatccT-TAGTTGCTTGGTTTTGA-3′). The amplicon was digested with *Nde*I and *Bam*HI and then ligated into pET28a (Novagen) digested with the same restriction enzymes. The ligation products were used to transform DH5α, and the transformants were selected on LB agar containing 50 μg/mL kanamycin. The resulting plasmid pET28-bla_{IMP-1} was transformed into *E. coli* BL21(DE3) (TaKaRa) for recombinant protein expression. Protein purification was performed as described previously (Kitao et al., 2010).

2.2. Preparation of mAbs

Anti-IMP mAbs were prepared as previously described (Kishiro et al., 1995). The purified His-IMP-1 was used for immunization and screening of hybridomas by enzyme-linked immunosorbent assay (ELISA). The animal experiments were approved by the Ethical Committee for Animal Experiments at the Research Institute of the National Center for Global Health and Medicine (NCGM).

2.3. Assembly of the assay

The assay was assembled according to the instructions for a commercially available rapid diagnosis kit, Quick Chaser™ Flu A, B (Mizuho Medy, Saga, Japan) as previously described (Miyoshi-Akiyama et al., 2010). To prepare the test lines, 0.76 mg of rat mAb per test was coated onto nitrocellulose membranes (Millipore, Billerica, MA) at a position of 30 mm from the sample application area. To prepare the reference lines, 0.2 mg of anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA) per test was coated onto the membranes at a position of 39 mm from the sample application area. Pads were prepared by soaking glass filters with rat mAb and rabbit IgG, each conjugated with colloidal gold. The membranes and pads were assembled within a plastic housing. The assembled assays were stored in a waterproof bag with a desiccant at room temperature until use.

2.4. Determination of the epitope region recognized by mAbs

The peptides (10 μg/mL) (Sigma-Aldrich Co.) were immobilized onto the wells of a 96-well enzyme immunoassay (EIA) plate (Corning) by incubation in 50 mM carbonate buffer (pH 9.0) containing 1 mM of the chemical cross-linker disuccinimidyl suberate (Pierce) at 4 °C for 16 h. After blocking with Superblock (Pierce), the plate was incubated for 1 h with 10 μg/mL rat mAb diluted with PBST (phosphate buffer saline containing 0.05% Tween) and washed 3 times with PBST. The binding of mAb to each peptide was detected with HRP (horseradish peroxidase)-goat anti-rat IgG (GE Healthcare) and TMB (3,3′,5,5′-tetramethylbenzidine) (Bio-Rad).

In the competitive assay, the purified IMP-1 prepared in 50 mM carbonate buffer (pH 9.0) was immobilized onto the wells of a 96-well EIA plate (Corning) at 4 °C for 16 h. After blocking, the plate was incubated for 1 h with 10 μg/mL of rat mAb preincubated with serially diluted peptides and washed 3 times with PBST. The binding of mAb to immobilized IMP-1 was detected with HRP-goat anti-rat IgG (GE Healthcare) and TMB (Bio-Rad).

2.5. Site-directed mutagenesis

Site-directed mutagenesis was performed using QuickChange Mutagenesis Kit according to the instructions of the manufacturer (Stratagene). IMP mutants were created by site-directed mutagenesis in the genetic region encoding amino acid residues 101–125 of the IMP-1 antigen. The primers used in the mutagenesis are listed in Table 1. The pET28–blaIMP-1 was used as a template plasmid.

2.6. Analysis of interaction between IMP mutants and mAbs

Purified IMP-1 protein and mutants (2 μg/mL) prepared in 50 mM carbonate buffer (pH 9.0) were immobilized onto the wells of a 96-well microtiter plate (Corning) by incubation at 4 °C for 16 h. After blocking with Superblock (Pierce), the plate was incubated for 1 h with 10 μg/mL mAb diluted with PBST and washed 3 times with PBST. Binding of mAb to each peptide was detected with HRP-goat anti-rat IgG (GE Healthcare) and TMB (Bio-Rad).

2.7. Bacterial strains

A total of no duplicate 248 strains of *P. aeruginosa* were obtained from BML Inc. to evaluate the assay. *P. aeruginosa* NCGM2.S1 was used as a positive strain for bla_{IMP-1} (Sekiguchi et al., 2005). One of two *Acinetobacter baumannii* strains, a strain of *A. baumannii* NCB0211-439 carrying bla_{IMP-2} was obtained from National Institute of Infectious Diseases in Japan. Another *A. baumannii* strain AB-NCGM112 carrying bla_{IMP-1} was clinically isolated from single inpatient at NCGM. Two strains of *Pseudomonas putida* (PP-NCGM265 and PP-NCGM266) carrying bla_{IMP-1} and four strains of *Alcaligenes xylosoxidans* (AX-NCGM1, AX-NCGM2, AX-NCGM3, and AX-NCGM4) carrying bla_{IMP-1} were obtained from inpatients at NCGM.

2.8. Assessment of the assay using bacterial strains

As shown in Supplementary Fig. 1, bacterial colonies on Muellar–Hinton agar (Gibco) were picked with a swab and were suspended in a soft test tube containing extraction buffer with nonionic detergent. After lysing the cells physically and chemically, three drops of bacterial lysate were added onto the test plate. The results were analyzed by visual inspection 15 min after the addition of the sample.

Table 1
Primers used in mutagenesis.

Mutants	Primer name	Sequence (5′ to 3′ orientation)
R110Q	IMP-R110Q_F	GAGTGGCTTAATTCTCAATCTATCCCCACG
R110Q	IMP-R110Q_R	CGTGGGGATAGATTGAGAATTAAGCCACTC
E105G	IMP-E105G_F	ACGGGCGGAATAGGGTGGCTTAATTCTCGA
E105G	IMP-E105G_R	TCGAGAATTAAGCCACCCTATTCCGCCCGT
R110Q–O113S	IMP-R110Q–P113S_F	TGGCTTAATTCTCAATCTATCtCCACGTATG CATCT
R110Q–O113S	IMP-R110Q–P113S_R	AGATGCATACGTGGAGATAGATTGAGAATT AAGCCA
E118V	IMP-E118V_F	ACGTATGCATCTGTATTAACAAATGAACTG
E118V	IMP-E118V_R	CAGTTCATTTGTTAATACAGATGCATACGT
G102A	IMP-G102A_F	AGCGACAGCACGGCCGGAATAGAGTGGCTT
G102A	IMP-G102A_R	AAGCCACTCTATTCCGGCCGTGCTGTCGCT
T101S	IMP-T101S_F	CATAGCGACAGCtCGGGCGGAATAGAGTGG
T101S	IMP-T101S_R	CCACTCTATTCCGCCCGAGCTGTCGCTATG
E122D	IMP-E122D_F	GAATTAACAAATGACCTGCTTAAAAAAGAC
E122D	IMP-E122D_R	GTCTTTTTTAAGCAGGTCATTTGTTAATTC