field situations. In addition, many studies have demonstrated that MB patients have high titers of *M. leprae*-specific antibodies but PB patients have low or absent titers. For these reasons, the potential for serological diagnosis of low-BI patients, such as PB patients or MB patients who are developing disease, has not been thoroughly pursued.

In a recent small-scale study, we demonstrated that the ML0405 and ML2331 proteins were recognized by sera from MB leprosy patients presenting with high BI (20). In the current study we demonstrate that ML0405 and ML2331 are diagnostically relevant antigens by analyzing a large panel of MB leprosy patient sera from a variety of leprosy-affected regions (the Philippines, central and coastal Brazil, and Japan). We also examine the ability of *M. leprae* protein antigens to diagnose low-BI leprosy (PB patients and early MB patients) and show here the diagnostic potential of ML0405, ML2331, and a newly discovered *M. leprae* antigen, ML1556c. Based on the results, we construct and evaluate a fusion protein comprising ML0405 and ML2331 (designated *l*eprosy *I*DRI *d*iagnostic 1 [LID-1]) and demonstrate that this construct can be used to serologically diagnose leprosy patients among presymptomatic individuals, that is, before a clinical diagnosis is possible. Moreover, ML1556c may be a valuable adduct to LID-1 for the diagnosis of PB leprosy.

MATERIALS AND METHODS

Subjects and samples. Sera were obtained from patients with leprosy (MB and PB) or tuberculosis (TB), healthy household contacts of MB leprosy patients (HHC), and endemic and nonendemic controls (EC and NEC). MB and PB leprosy patient sera used in this study were derived from recently diagnosed, previously untreated individuals who did not have signs of reversal reactions. Leprosy was classified in each case by bacterial, histological, and clinical observations carried out by qualified personnel, with the BI recorded at the time of diagnosis. HHC were defined as adults living in the same house as an MB index case for at least 6 months. TB patients were included to evaluate potential antigen cross-reactivity with other mycobacterial infection. Sera from TB patients were obtained after drawing blood from *Mycobacterium tuberculosis* sputum-positive, human immunodeficiency virus-negative individuals with clinically confirmed pulmonary TB who were undergoing treatment. Normal sera (EC and NEC) were obtained after blood draws from volunteers with no history of leprosy or TB infection. In all cases, drawing of blood was carried out with informed consent (with local institutional review board approval or local ethics committee approval in Brazil, Japan, the Philippines, Seattle, and St. Louis). The composition of each study population is summarized in Table 1.

In Cebu City, leprosy and TB patients were recruited at the Cebu skin clinic and Leonard Wood Memorial Research Center in Cebu City, Cebu (Philippines) from 2003 to 2006. Between 1985 and 1991, sera were collected prospectively from individuals who resided with MB patients (BI > 2) for at least 2 years and were free of leprosy as determined by clinical dermato-neurological examination at the inclusion point of the study. Some of these individuals developed MB leprosy as the study progressed, and these sera have previously been described (11).

In Goiânia, the state capital of Goiás State (western central Brazil), leprosy and TB patients were recruited at the main outpatient clinics of Centro de Referência em Diagnóstico e Terapêutica and Hospital Anuar Auad in 2006. PB leprosy patients were selected from a cohort of leprosy patients with a single skin lesion recruited at Brazilian sites of endemicity from 1999 to 2001, as previously described (9).

In Salvador, the state capital of Bahia State (northeast coastal Brazil), leprosy patients were recruited at Hospital Dom Rodrigo de Menezes in 2006.

In Japan, leprosy patients were recruited at the National Sanatorium Oshimaseishoen, Kagawa.

In St. Louis, sera were collected from U.S.-based individuals at a variety of times following *Mycobacterium bovis* BCG immunization.

All serum specimens were aliquoted and stored at −20°C or −80°C prior to assay.

TABLE 1. Study populations

Site	Sample categorization (total no.)	BI (mean)	Sex ratio[a]	Mean age (yr) (range)
Cebu City, Philippines	MB (17)	2.8	2.4	30 (18–55)
	PB (54)	0.5	0.4	31 (15–45)
	TB (6)		5	45 (35–53)
	EC (8)		1	26 (19–38)
	HHC (10)		0.4	38 (18–60)
Goiânia, Brazil	MB (28)	2.4	1.5	44 (19–81)
	PB (83)	0	0.4	33 (7–76)
	TB (26)		2.7	39 (17–66)
	EC (30)		0.1	20 (19–26)
	HHC (11)		0.5	28 (18–51)
Salvador, Brazil	MB (10)	NA[b]	3.5	35.1 (20–70)
	PB (6)	0	5	31.6 (12–42)
	HHC (11)		0.1	48.5 (25–57)
Kagawa, Japan	MB (30)	NA	NA	60 (48–79)
	PB (30)	0	NA	70 (55–90)
	EC (26)		NA	54 (48–62)

[a] Male/female ratio.
[b] NA, not available.

Cloning and purification of target antigens. DNA encoding selected *M. leprae* proteins was PCR amplified from *M. leprae* Thai-53 genomic DNA using *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). PCR primers were designed to incorporate specific restriction enzyme sites 5′ and 3′ of the gene of interest and excluded in the target gene for directional cloning into the expression vector pET28a (Novagen, Madison, WI). After PCR amplification, purified PCR products were digested, ligated with vector DNA, and used to transform *Escherichia coli*, and individual clones were induced to produce recombinant proteins, as previously described (20). Recombinant proteins were quantified using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and quality was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The characteristics of each *M. leprae* protein evaluated are summarized in Table 2. The ML1556c protein was included because portions of the ML1556 protein were identified in four separate clones during serological expression screening with sera from PB leprosy patients (data not shown) (20). Recognition of the clones was derived from amino acids 58 to 256 of ML1556, which are only 47% identical to the *M. tuberculosis* protein Rv2839 (compared to 82% identity across the entire amino acid sequences of ML1556 and Rv2839).

Determining patient reactivity by ELISA. ELISAs were conducted independently at IDRI, Seattle, WA (Cebu and St. Louis sera); UFG, Goiânia, and UFB, Salvador, Brazil; and NIID, Tokyo, Japan. Polysorp 96-well plates (Nunc, Rochester, NY) were coated with 1 μg/ml recombinant protein or 200 ng/ml of natural disaccharide with octyl linkage (NDO), the synthetically derived B-cell epitope of PGL-I, conjugated to bovine serum albumin (NDO-BSA; kindly supplied by John Spencer, Colorado State University, under NIH contract N01 AI-25469), in bicarbonate buffer overnight at 4°C and blocked for 1 h at room temperature with phosphate-buffered saline-Tween with 1% BSA on a plate shaker. Serum diluted appropriately in 0.1% BSA was added to each well, and plates were incubated at room temperature for 2 h with shaking. Plates were washed with buffer only, and horseradish peroxidase-conjugated IgG or IgM (Rockland Immunochemicals, Gilbertsville, PA), diluted in 0.1% BSA, was added to each well and incubated at room temperature for 1 h with shaking. After washing, plates were developed with peroxidase color substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and the reaction was quenched by the addition of 1 N H_2SO_4. The optical density (OD) of each well was read at 450 nm. Positive responses were defined as an OD of >2× the mean OD of endemic control sera or an OD of >0.1, whichever was higher.

Statistics. *P* values were determined using Student's *t* test.

RESULTS

Recognition of *M. leprae* proteins by Filipino leprosy patient sera. The majority of MB leprosy patients are readily identified

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TABLE 2. Main characteristics of *M. leprae* antigens tested[a]

Gene accession no.	Functional classification[b]	Protein type	Length (bp)	Product size (kDa)	% Identity[c] with:				
					M. tuberculosis H37Rv[e]	*M. bovis* AF2122/97[e]	*M. avium* 104[d]	*M. marinum* ATCC BAA-535[e]	*M. smegmatis* MC2 155[d]
ML0091	II.C.2	28-kDa antigen precursor	711	23.7	53	53	54	54	48
ML0405	V	Conserved hypothetical	765	25.3	62	62	None	NA	None
ML1633	II.C.2	Possible secreted hydrolase	1,608	57.0	25	25	35	81	62
ML2055	IV.A	Probable cell surface protein	864	29.5	72	72	69	73	54
ML2331	II.C.2	Possible secreted protein	771	26.5	80	80	77	80	67
ML2346	VI	Hypothetical	906	33.9	None	None	None	None	None
ML1556	II.A.6	Translation initiation factor	2,775	96.6	84	82		90	

[a] Annotations for gene accession number, functional classification, and protein type are according to the Sanger database.
[b] Functional classifications: II.C.2, surface polysaccharides, lipopolysaccharides, proteins, and antigens; V, conserved hypotheticals; IV.A, virulence; VI, unknowns; II.A.6, protein translation and modification.
[c] BLAST reports were performed in September 2006; tBLASTn was used for comparisons of proteins versus translated DNA. NA, not applicable.
[d] From http://www.tigr.org.
[e] From http://www.sanger.ac.uk/Projects.

by ELISA and lateral flow tests, which assess the capacity of patient IgM to bind *M. leprae* PGL-I or its synthetic analogue (NDO) conjugated to a carrier protein (BSA). In comparison with MB leprosy patients, PB leprosy patients have low or no anti-PGL-I responses and are more difficult to diagnose serologically. We therefore sought to determine whether PB sera recognized protein antigens, expanding our previous analyses and comparing the potential of NDO-BSA, ML0405, and ML2331 to diagnose leprosy, and found that the protein antigens have a similar profile for leprosy diagnosis as that for NDO-BSA; all three test antigens were readily detected by MB patient sera, by some PB patient sera, and by few, if any, EC, HHC, or TB sera (Fig. 1). Thus, similar to NDO-BSA, ML0405 and ML2331 demonstrate good potentials for the diagnosis of leprosy.

Recognition of MB leprosy patient sera with refined ML0405 antigen constructs. To learn more regarding the seroreactivity of ML0405 and enhance recombinant ML0405 expression for purification, we expressed a variety of ML0405 polypeptide fragments and determined whether Filipino MB leprosy patient sera had similar binding capacities to these fragments and to full-length (ML0405FL) protein. All constructs were able to bind MB patient sera (Fig. 2) ($P < 0.01$ for MB versus EC). The reactivity of a truncated form (ML0405Tr) of the protein was equivalent to the reactivity of ML0405FL ($P = 0.885$ for MB patient sera), whereas the reactivity of the protein construct lacking the predicted membrane-spanning region (ML0405Tm) declined slightly (Fig. 2) ($P = 0.047$ and 0.060 for Tm versus FL and Tr forms, respectively, for MB). These data indicate that the majority, if not all, of the B-cell epitopes recognized by antibodies in patient sera are retained and accessible in the truncated form of the protein. Further testing was conducted using either ML0405FL or ML0405Tr.

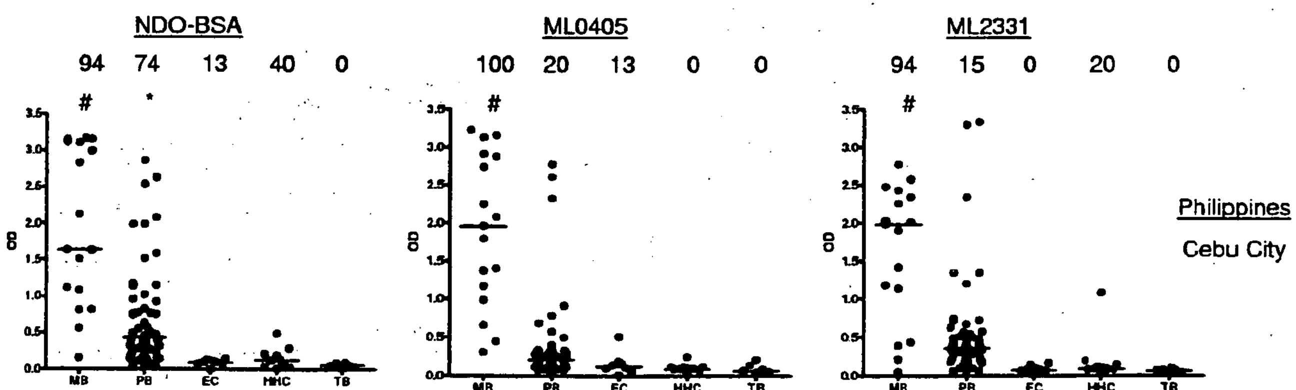

FIG. 1. Sera from Filipino leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, and ML2331. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC.

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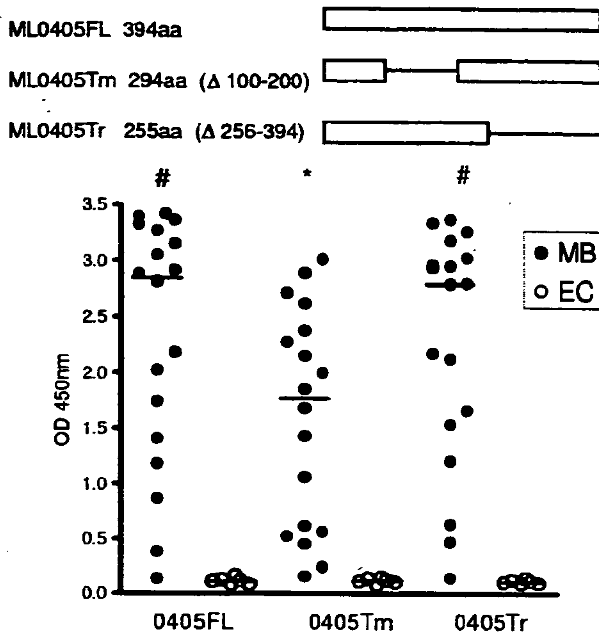

FIG. 2. ML0405 constructs react with MB leprosy patient sera. Different ML0405 constructs were created and expressed as recombinant proteins. The schematic diagram shows the sequence alignment of each of these constructs, with the deleted regions indicated by the line. Each construct was tested for IgG reactivity by ELISA with individual Filipino MB leprosy patient sera (n = 18) or EC sera (n = 6). *, $P < 0.05$; #, $P < 0.001$ versus EC.

Diagnosis of Filipino PB leprosy patients with *M. leprae* proteins. We then went on to more closely investigate the potential of *M. leprae* antigens for diagnosing PB leprosy. Sera from Filipino patients clinically diagnosed with PB leprosy and with a low BI were tested for reactivity with potential diagnostic *M. leprae* antigens (ML0405Tr, ML2331, ML1556c, and NDO-BSA). NDO-BSA was capable of identifying 57% (26 of 46) of these Filipino PB leprosy patients, but a substantial number of samples provided weak positive responses (Fig. 3). ML0405 and ML2331 also reacted with sera from some PB patients (Fig. 3A and B). Most of these Filipino sera that reacted with these proteins also demonstrated strong NDO-BSA responses, however, and so the added benefit of using these antigens for leprosy diagnosis within the Filipino population appeared minimal. In contrast, 4 of 20 sera that were weak positive/negative by NDO-BSA ELISA testing demonstrated strong reactivity to ML1556c (Fig. 3C). This result suggests that ML1556c may be useful as an adjunct to PGL-I testing, or other tests, to improve the sensitivity and clarity of leprosy diagnosis.

To test the specificity of ML1556c as a leprosy diagnostic reagent, we directly compared the reactivities of ML1556c with sera from PB leprosy patients, MB leprosy patients, TB patients, EC, and HHC of MB leprosy patients located in Cebu City, Philippines (Fig. 3D). Positive responses were observed in five of eight additional PB leprosy sera tested, with three of the sera yielding strong responses that could provide a clear diagnosis. Positive responses to ML1556c were also observed in two of seven MB leprosy sera tested in this experiment.

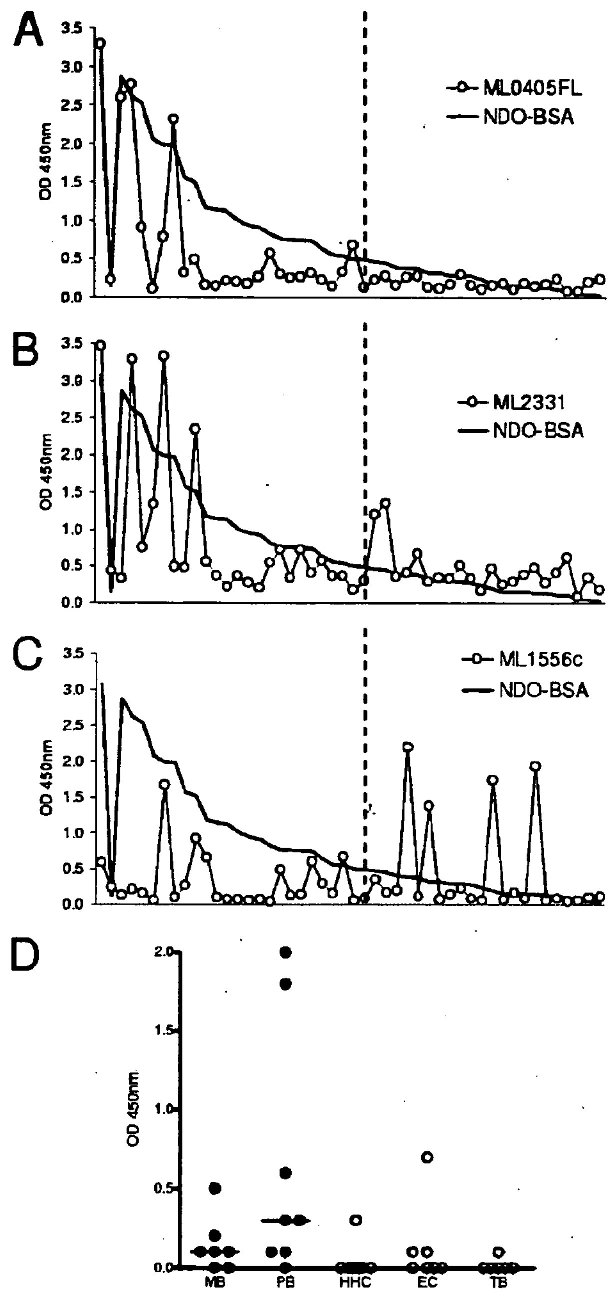

FIG. 3. *M. leprae* proteins react with PB leprosy patient sera. (A to C) Antibody reactivities of sera from a pool of clinically diagnosed MB leprosy patients, from a pool of negative control individuals, and from 46 clinically diagnosed PB leprosy patients were assessed against NDO-BSA and ML0405 (A), ML2331 (B), and ML1556c (C). NDO-BSA reactivity was assessed by IgM binding and, for reference, is shown in each plot. Recombinant protein reactivity was assessed by IgG binding. The first open circle represents the value obtained for pooled MB sera, while the next open circle represents the reactivity of pooled EC sera; individual PB sera are then arranged along the x axis according to their responsiveness versus NDO-BSA. The dashed line indicates the point at which diagnosis by NDO-BSA reactivity becomes unclear. ML1556c reacts with PB leprosy patient sera. (D) IgG reactivities of ML1556c with a small panel of individual sera from EC, leprosy patients (MB and PB), and TB patients were determined by ELISA using samples from Cebu City, Philippines. Each point represents an individual serum sample, and the median is represented by the line.

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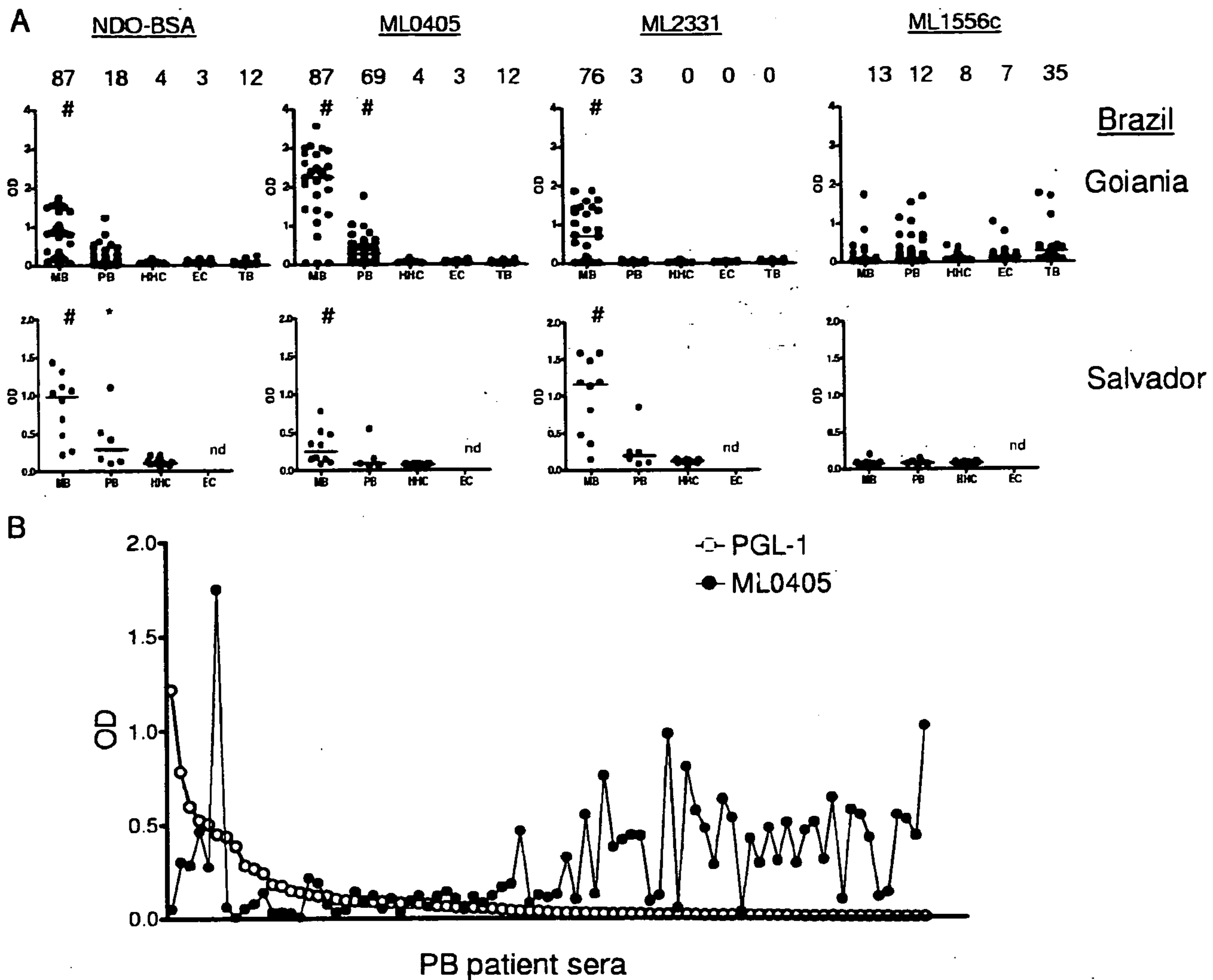

FIG. 4. Sera from Brazilian leprosy patients react with recombinant *M. leprae* antigens. Sera from clinically diagnosed MB and PB leprosy patients, EC individuals, and HHC of MB leprosy patients were assessed against NDO-BSA, ML0405, ML2331, and ML1556c. NDO-BSA reactivity was assessed by IgM binding, and protein reactivity was assessed by IgG binding. Sera were from Goiânia and Salvador (see Table 1). (A) Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC. (B) To demonstrate complementarity, the individual PB sera from Goiânia are arranged along the x axis according to their responsiveness versus NDO-BSA and overlaid with the response of each serum to ML0405.

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ML1556c did not react with any of the Filipino TB patient sera tested, was recognized by only one of eight HHC sera, and reacted with only one of six EC sera. Negative results were obtained upon further testing involving another 45 TB sera and 23 NEC sera (data not shown). Taken together, these results generated from sera from the Philippines suggested the utility of ML1556c to improve the diagnosis of PB leprosy.

Identification of leprosy patients in Brazil. We also examined the ability of recombinant *M. leprae* antigens to identify leprosy patients located around Goiânia, Brazil, and Salvador, Brazil. Within the clinically diagnosed leprosy population, PGL-I/NDO-BSA was capable of identifying 87% (33 of 38) of the MB patients (Fig. 4). In agreement with the results obtained by analysis of Filipino leprosy patient sera, ML0405 and ML2331 reacted with large proportions of Brazilian MB patient sera (87% [33 of 38] and 76% [29 of 38], respectively), and ML1556c reacted with only some MB patient sera (13%, 5 of 38) (Fig. 4). In Goiânia, positive responses were also observed against antigens ML0091 (71%, 20 of 28), ML1633 (32%, 9 of 28), ML2055 (75%, 21 of 28), and ML2346 (29%, 8 of 28) (data not shown). The clarity of MB leprosy diagnosis (strength of signal in positive samples versus negative samples) in Goiânia was greater when using ML0405 rather than NDO-BSA, but in Salvador it was greater when using ML2331 rather than NDO-BSA.

We also determined if these antigens were recognized by Brazilian PB patient sera. PGL-I/NDO-BSA was capable of identifying only 20% (18 of 89) of the PB patients, a level not appreciably higher than the proportion of positive responses observed with TB patients (12%, 3 of 26) (Fig. 4A). An IgG

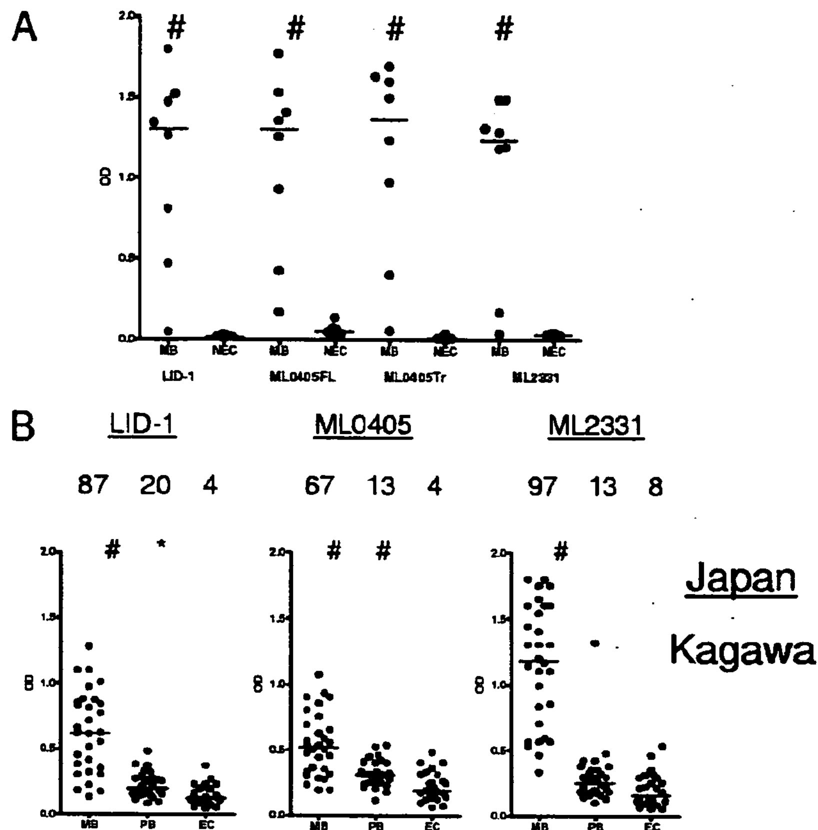

FIG. 5. LID-1 retains reactivity with leprosy patient sera. (A) LID-1 (a fusion construct of ML0405 and ML2331), ML0405FL, ML0405Tr, and ML2331 reactivities were assessed by IgG binding in an ELISA with eight MB leprosy patient serum samples from Salvador and eight NEC serum samples. (B) Sera from clinically diagnosed Japanese MB and PB leprosy patients, and Japanese EC individuals, were assessed for IgG reactivities with LID-1, ML0405, and ML2331. Each point represents an individual serum sample, and the median is represented by the line. The number above each data set is the percent positive responses. *, $P < 0.05$; #, $P < 0.001$ versus EC.

reactivity that permitted serologic diagnosis of an increased number of PB leprosy patients was observed for ML0405 (69%, 61 of 89), but responses to ML2331 were very weak, with very few positives (3%, 3 of 89) (Fig. 4A). The antigens ML0091 (6%, 5 of 83), ML1633 (17%, 14 of 83), ML2055 (13%, 11 of 83), and ML2346 (27%, 22 of 83) were recognized by some PB patient sera, but responses were generally weak (data not shown). Many of the PB patient sera that did not react with PGL-1 had a strong reactivity with ML0405 (Fig. 4B). ML1556c was recognized by only a minor subset of PB leprosy patient sera (12%, 11 of 89) and Brazilian EC individuals (6.7%, 2 of 30), but ML1556c reactivity was detected in a substantial number of Brazilian TB patients (35%, 9 of 26). These data indicate only a minor number of positive results in the Brazilian population if ML1556c is used for leprosy diagnosis, with a further complication of false-positive diagnosis in TB patients. Antigen ML0405, however, did not react with significant numbers of EC sera (3.3%, 1 of 30) or TB sera (12%, 3 of 26) (Fig. 4A). These results indicate that ML0405 can recognize some PB leprosy patients in the Brazilian population and could be used to augment leprosy diagnosis with PGL-1.

Construction of a fusion construct of ML0405-ML2331 (LID-1). Having extended our earlier observation that the single antigens ML0405 and ML2331 have the potential to diagnose leprosy (20), and given the observations that ML0405 appeared better for diagnosis in Goiânia and Cebu City but ML2331 appeared better for diagnosis in Salvador, we constructed a single fusion molecule incorporating both proteins. ML0405Tr was expressed at the C terminus of the molecule and ML2331 in the N terminus. Following recombinant expression, we validated the reactivity of the construct by assaying LID-1 versus a small panel of sera from Salvador that had bound each single component. These sera readily detected LID-1, ML0405FL, ML0405Tr, and ML2331 (Fig. 5A). Importantly, construction of the fusion protein did not introduce false-positive results with NEC sera (Fig. 5A).

We further extended our examination of sera from different geographic locations by assessing sera from Japanese leprosy patients for reactivity with ML0405, ML2331, and LID-1. Positive response were observed with MB patient sera (67% [20 of 30] for ML0405, 97% [29 of 30] for ML2331, and 87% [26 of 30] for LID-1) and PB patient sera (13% [4 of 30] for ML0405, 13% [4 of 30] for ML2331, and 20% [6 of 30] for LID-1), with few responses in EC sera (4% [1 of 26] for ML0405, 8% [2 of 26] for ML2331, and 4% [1 of 26] for LID-1) (Fig. 5B). Taken together, these data indicate that LID-1 is useful as a diagnostic antigen for leprosy.

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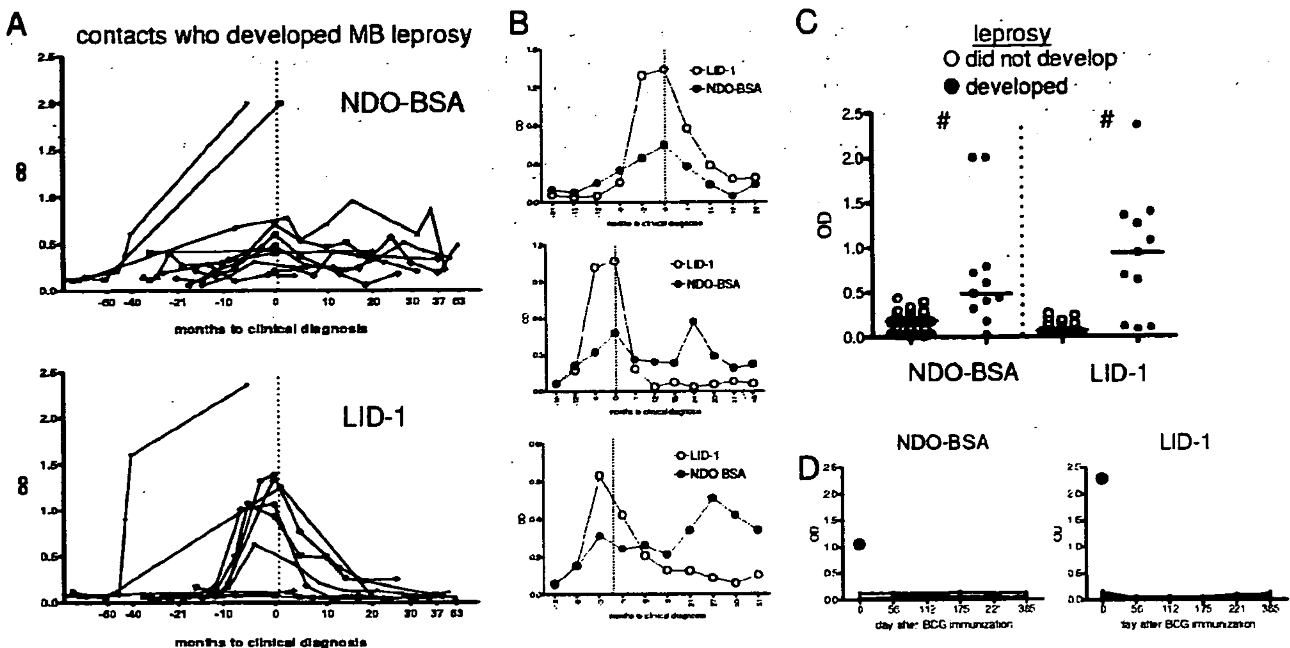

FIG. 6. LID-1 reactivity can diagnose leprosy before clinical symptoms. (A) LID-1 and NDO-BSA reactivities within sera from a prospective study conducted in Cebu City, Philippines, were assessed by either IgG or IgM binding in an ELISA. Sera were collected at a variety of times prior to the clinical diagnosis of MB leprosy in 11 patients and at a variety of times after the commencement of treatment. (B) Representative plots for individual patients are shown. (C) Sera were collected from 57 household contacts that did not develop clinical leprosy and were compared with single serum samples from each individual contact that developed leprosy (serum samples were collected within 3 months of clinical diagnosis). #, $P < 0.001$. (D) LID-1 and NDO-BSA reactivities within sera from a prospective study using 10 U.S.-based individuals who were immunized with BCG were assessed. Sera were collected at regular intervals following BCG immunization. The solid circle at day zero designates the reactivity of a leprosy patient serum sample that was included as a positive control.

LID-1 reactivity can diagnose leprosy before clinical symptoms. Having demonstrated that the LID-1 fusion molecule retained the ability to diagnose leprosy patients but lacked responses to EC sera, we obtained sera from a prospective study conducted in Cebu City, Philippines, between 1985 and 1991 (11). In that study, household contacts of leprosy patients were monitored over a prolonged period of time, and some developed clinical MB leprosy. In sera from the individuals who developed MB leprosy, as previously reported, anti-PGL-I levels increased before leprosy was diagnosed by clinical exam (Fig. 6A). Our data also indicate that anti-LID-1 antibody levels began to increase markedly as soon as 1 year prior to clinical diagnosis (Fig. 6A). For many of the patients (7 of 11, 64%) the increase in the anti-LID-1 IgG response was strikingly more obvious than the increase in the anti-PGL-I IgM response (Fig. 6B). Those patients that developed clinical leprosy had anti-PGL-1 antibody levels not dissimilar to many individuals who did not develop leprosy (Fig. 6C). The difference in anti-LID-1 antibody levels was much clearer, with a much larger differentiation between the positive responses of patients who developed leprosy compared with the extremely low levels of anti-LID-1 antibody in individuals who did not develop leprosy (Fig. 6C). Taken together, these data indicate that LID-1 is capable of providing an early serological diagnosis of leprosy.

LID-1 does not react with sera from individuals recently exposed to BCG. To examine in detail if leprosy diagnosis could be complicated by exposure to or infection with other mycobacteria, we also examined sera collected longitudinally from 10 U.S.-based individuals who were immunized with BCG. None of these BCG-immunized individuals developed positive serological responses against LID-1 or NDO-BSA (Fig. 6D). These data indicate that LID-1 can provide a clear diagnosis of *M. leprae* infection prior to the onset of signs that permit clinical leprosy diagnosis and that LID-1-based diagnostic tests could be used to expedite leprosy treatment.

DISCUSSION

Current diagnosis of leprosy is based on the appearance of clinical signs, and it is well established that the earlier a patient is identified the better their response to treatment. In addition, MB leprosy patient household contacts have a higher risk of developing clinical leprosy than contacts of PB leprosy patients (10, 12). This has been attributed to increased shedding and spreading of viable bacteria by MB leprosy patients (2). Accurate and early detection of *M. leprae*-infected individuals will open the possibility of earlier treatment that could both prevent disability and significantly reduce leprosy transmission.

We have evaluated the serological responses to a variety of *M. leprae* protein antigens in an attempt to discover antigens that can improve diagnosis of leprosy by detecting patients with a low BI (PB leprosy patients or early MB leprosy patients). We demonstrated that (i) ML0405 and ML2331 can be used to diagnose MB leprosy patients independently of geographic location; (ii) ML1556c can recognize some PB patients (al-

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though it is recognized by some TB sera as well); (iii) ML0405 and ML2331 can be used for diagnosis of some PB patients; (iv) a fusion construct of ML0405 and ML2331 (LID-1) retains diagnostic capability; and (v) LID-1 can provide a clear leprosy diagnosis before the onset of clinical symptoms. These findings will improve both leprosy diagnosis and patient care.

One approach for the early detection of *M. leprae* infection is through serological diagnosis. We have conducted screening to identify *M. leprae* antigens that have not previously been described, and we then evaluated the diagnostic potential of these antigens with leprosy patient sera. In this study, the diagnostic potential of select antigens was assessed in clinically disparate leprosy patient groups, ranging from MB patients who presented with large bacterial burdens and large skin lesions to PB patients who presented with low or absent bacterial burdens and a few, small skin lesions. As expected, MB leprosy patients were easier to identify by serological assays and typically yielded higher responses than PB patients. Unexpectedly, close examination of patients with a low BI from the Philippines indicated that some patients exhibited strong responses against the ML1556c protein. The responses of Filipino PB patients to ML1556c were often greater than those of MB patients. These results suggested the utility of this protein either as an adjunct to antigens that could identify MB patients to provide a cross-spectrum leprosy diagnosis or as a stand-alone protein for PB leprosy diagnosis. An objective and differential diagnosis of MB or PB leprosy could lead to better treatment of patients by guiding the multidrug therapy regimen provided to them.

We also analyzed the diagnostic potential of each antigen within geographically disparate groups of patients, from the Philippines and two sites in Brazil. In the Brazilian (Goiânia) PB leprosy patient group, ML1556c provided only a few positive responses; this dampened the enthusiasm for ML1556c to be a widely used diagnostic or prognostic leprosy antigen. Of interest, many PB leprosy patients in Brazil (both Goiânia and Salvador) could be diagnosed by ML0405 reactivity, and several PB patients (Salvador) could be diagnosed with ML2331 reactivity. It is unclear if the differences in the responses of patients from different geographic locations are related to differences in *M. leprae* strains or to regional variations in host genetics. These possibilities might be addressed by analysis of patient sera on fragments of ML1556c or by a survey of anti-ML1556c antibody on lysates of different *M. leprae* strains. Regardless, the observed differences indicate the importance of examining antigen-specific responses in several regions when considering their ability to diagnose leprosy globally.

Given that the ML0405Tr and ML2331 proteins could provide diagnosis of leprosy, we made a fusion protein (LID-1) of these individual components. After ensuring the fusion protein retained reactivity against leprosy sera from Salvador, Brazil, we tested the antigens against sera from Japan. As with results obtained using sera from Brazil, Japanese MB leprosy patient sera reacted as strongly with the fusion LID-1 as with the ML0405 and ML2331 components. In addition, some Japanese PB leprosy patient serum antibodies recognized these antigens.

Studies have argued that the presence of anti-PGL-I antibodies is an indicator of leprosy development, but this has been debated (5, 6, 14, 15). Many contacts of leprosy patients have anti-PGL-I antibodies but do not develop disease, limiting the capacity of PGL-I-based assays to predict disease development. Indeed, PGL-I-based tests are typically marketed as a support reagent to confirm clinical diagnosis and aid leprosy classification but are not recommended for use as a stand-alone for diagnosis (19). The differential in responses of sera from contacts that developed leprosy compared with contacts that did not develop leprosy was much greater for LID-1 than PGL-1. We demonstrated that LID-1 is capable of providing an early serological diagnosis of MB leprosy. A clear and early diagnosis was achieved in 7 of 11 contacts of leprosy patients who themselves went on to develop clinical leprosy. For the small panel of sera tested, the time benefit of a LID-1-based diagnosis over a clinical-based diagnosis was 6 to 8 months. Thus, screening for LID-1-reactive antibodies, either in the general population or within more focused at-risk populations, could significantly expedite treatment of leprosy patients and, also, affect transmission rates by reducing the number of individuals who develop large bacterial burdens. As another benefit, antibody levels against LID-1 dropped following the implementation of drug treatment in these individuals, and thus the reduction and disappearance of antibodies against LID-1 may be a useful measure of multidrug therapy efficacy.

We are currently evaluating additional antigens, diagnostic formats, and different geographic sources of patient sera with the objective of early and simple identification of leprosy patients regardless of incidence locality.

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REFERENCES

1. **Anderson, H., B. Stryjewska, B. L. Boyanton, and M. R. Schwartz.** 2007. Hansen disease in the United States in the 21st century: a review of the literature. Arch. Pathol. Lab. Med. **131:**982–986.
2. **Bakker, M. I., M. Hatta, A. Kwenang, P. Van Mosseveld, W. R. Faber, P. R. Klatser, and L. Oskam.** 2006. Risk factors for developing leprosy—a population-based cohort study in Indonesia. Lepr. Rev. **77:**48–61.
3. **Buhrer, S. S., H. L. Smits, G. C. Gussenhoven, C. W. van Ingen, and P. R. Klatser.** 1998. A simple dipstick assay for the detection of antibodies to phenolic glycolipid-I of Mycobacterium leprae. Am. J. Trop. Med. Hyg. **58:**133–136.
4. **Buhrer-Sekula, S., H. L. Smits, G. C. Gussenhoven, J. van Leeuwen, S. Amador, T. Fujiwara, P. R. Klatser, and L. Oskam.** 2003. Simple and fast lateral flow test for classification of leprosy patients and identification of contacts with high risk of developing leprosy. J. Clin. Microbiol. **41:**1991–1995.
5. **Cartel, J. L., S. Chanteau, J. P. Boutin, R. Plichart, P. Richez, J. F. Roux, and J. H. Grosset.** 1990. Assessment of anti-phenolic glycolipid-I IgM levels using an ELISA for detection of M. leprae infection in populations of the South Pacific Islands. Int. J. Lepr. Other Mycobact. Dis. **58:**512–517.
6. **Chanteau, S., P. Glaziou, C. Plichart, P. Luquiaud, R. Plichart, J. F. Faucher, and J. L. Cartel.** 1993. Low predictive value of PGL-I serology for the early diagnosis of leprosy in family contacts: results of a 10-year prospective field study in French Polynesia. Int. J. Lepr. Other Mycobact. Dis. **61:**533–541.
7. **Cho, S. N., R. V. Cellona, L. G. Villahermosa, T. T. Fajardo, Jr., M. V. Balagon, R. M. Abalos, E. V. Tan, G. P. Walsh, J. D. Kim, and P. J. Brennan.** 2001. Detection of phenolic glycolipid I of *Mycobacterium leprae* in sera from leprosy patients before and after start of multidrug therapy. Clin. Diagn. Lab. Immunol. **8:**138–142.
8. **Cho, S. N., D. L. Yanagihara, S. W. Hunter, R. H. Gelber, and P. J. Brennan.** 1983. Serological specificity of phenolic glycolipid I from *Mycobacterium leprae* and use in serodiagnosis of leprosy. Infect. Immun. **41:**1077–1083.
9. **Costa, M. B., P. F. Cavalcanti Neto, C. M. Martelli, M. M. Stefani, J. P. Maceira, M. K. Gomes, A. P. Schettini, P. F. Rebello, P. E. Pignataro, E. S.**

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Ueda, K. Narahashi, and D. M. Scollard. 2001. Distinct histopathological patterns in single lesion leprosy patients treated with single dose therapy (ROM) in the Brazilian Multicentric Study. Int. J. Lepr. Other Mycobact. Dis. **69:**177–186.

10. **Deps, P. D., B. V. Guedes, J. Bucker Filho, M. K. Andreatta, R. S. Marcari, and L. C. Rodrigues.** 2006. Characteristics of known leprosy contact in a high endemic area in Brazil. Lepr. Rev. **77:**34–40.
11. **Douglas, J. T., R. V. Cellona, T. T. Fajardo, Jr., R. M. Abalos, M. V. Balagon, and P. R. Klatser.** 2004. Prospective study of serological conversion as a risk factor for development of leprosy among household contacts. Clin. Diagn. Lab. Immunol. **11:**897–900.
12. **Fine, P. E., J. A. Sterne, J. M. Ponnighaus, L. Bliss, J. Saui, A. Chihana, M. Munthali, and D. K. Warndorff.** 1997. Household and dwelling contact as risk factors for leprosy in northern Malawi. Am. J. Epidemiol. **146:**91–102.
13. **Flower, C., D. Gaskin, and S. Marquez.** 2007. A case of recurrent rash and leg numbness mimicking systemic rheumatic disease: the occurrence of leprosy in a nonendemic area. J. Clin. Rheumatol. **13:**143–145.
14. **Gonzalez-Abreu, E., J. A. Pon, P. Hernadez, J. Rodriguez, E. Mendoza, M. Hernandez, E. Cuevas, and A. B. Gonzalez.** 1996. Serological reactivity to a synthetic analog of phenolic glycolipid I and early detection of leprosy in an area of low endemicity. Lepr. Rev. **67:**4–12.
15. **Hussain, R., S. Jamil, A. Kifayet, F. Firdausi, H. M. Dockrell, S. Lucas, and R. Hasan.** 1990. Quantitation of IgM antibodies to the M. leprae synthetic disaccharide can predict early bacterial multiplication in leprosy. Int. J. Lepr. Other Mycobact. Dis. **58:**491–502.
16. **Lockwood, D. N., and A. J. Reid.** 2001. The diagnosis of leprosy is delayed in the United Kingdom. QJM **94:**207–212.
17. **Lockwood, D. N., and S. Suneetha.** 2005. Leprosy: too complex a disease for a simple elimination paradigm. Bull. W. H. O. **83:**230–235.
18. **Meima, A., J. H. Richardus, and J. D. Habbema.** 2004. Trends in leprosy case detection worldwide since 1985. Lepr. Rev. **75:**19–33.
19. **Oskam, L., E. Slim, and S. Buhrer-Sekula.** 2003. Serology: recent developments, strengths, limitations and prospects: a state of the art overview. Lepr. Rev. **74:**196–205.
20. **Reece, S. T., G. Ireton, R. Mohamath, J. Guderian, W. Goto, R. Gelber, N. Groathouse, J. Spencer, P. Brennan, and S. G. Reed.** 2006. ML0405 and ML2331 are antigens of *Mycobacterium leprae* with potential for diagnosis of leprosy. Clin. Vaccine Immunol. **13:**333–340.
21. **Ridley, D. S., and W. H. Jopling.** 1966. Classification of leprosy according to immunity. A five-group system. Int. J. Lepr. Other Mycobact. Dis. **34:**255–273.
22. **Roche, P. W., W. J. Britton, S. S. Failbus, D. Williams, H. M. Pradhan, and W. J. Theuvenet.** 1990. Operational value of serological measurements in multibacillary leprosy patients: clinical and bacteriological correlates of antibody responses. Int. J. Lepr. Other Mycobact. Dis. **58:**480–490.
23. **Roche, P. W., S. S. Failbus, W. J. Britton, and R. Cole.** 1999. Rapid method for diagnosis of leprosy by measurements of antibodies to the M. leprae 35-kDa protein: comparison with PGL-I antibodies detected by ELISA and "dipstick" methods. Int. J. Lepr. Other Mycobact. Dis. **67:**279–286.
24. **Scollard, D. M.** 2004. Classification of leprosy: a full color spectrum, or black and white? Int. J. Lepr. Other Mycobact. Dis. **72:**166–168.
25. **Van Buynder, P., J. Eccleston, J. Leese, and D. N. Lockwood.** 1999. Leprosy in England and Wales. Commun. Dis. Public Health **2:**119–121.
26. **WHO.** 2005. Global leprosy situation, 2005. Wkly. Epidemiol. Rec. **80:**289–295.
27. **WHO.** 2007. Global leprosy situation, 2007. Wkly. Epidemiol. Rec. **82:**225–232.
28. **Young, D. B., and T. M. Buchanan.** 1983. A serological test for leprosy with a glycolipid specific for Mycobacterium leprae. Science **221:**1057–1059.

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Outbreaks of Multidrug-Resistant *Pseudomonas aeruginosa* in Community Hospitals in Japan[▽]

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We previously reported an outbreak in a neurosurgery ward of catheter-associated urinary tract infection with multidrug-resistant (MDR) *Pseudomonas aeruginosa* strain IMCJ2.S1, carrying the 6′-*N*-aminoglycoside acetyltransferase gene [*aac(6′)-Iae*]. For further epidemiologic studies, 214 clinical isolates of MDR *P. aeruginosa* showing resistance to imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 64 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml) were collected from 13 hospitals in the same prefecture in Japan. We also collected 70 clinical isolates of *P. aeruginosa* that were sensitive to one or more of these antibiotics and compared their characteristics with those of the MDR *P. aeruginosa* isolates. Of the 214 MDR *P. aeruginosa* isolates, 212 (99%) were serotype O11. We developed a loop-mediated isothermal amplification (LAMP) assay and a slide agglutination test for detection of the *aac(6′)-Iae* gene and the AAC(6′)-Iae protein, respectively. Of the 212 MDR *P. aeruginosa* isolates, 212 (100%) and 207 (98%) were positive in the LAMP assay and in the agglutination test, respectively. Mutations of *gyrA* and *parC* genes resulting in amino acid substitutions were detected in 213 of the 214 MDR *P. aeruginosa* isolates (99%). Of the 214 MDR *P. aeruginosa* isolates, 212 showed pulsed-field gel electrophoresis patterns with ≥70% similarity to that of IMCJ2.S1 and 83 showed a pattern identical to that of IMCJ2.S1, indicating that clonal expansion of MDR *P. aeruginosa* occurred in community hospitals in this area. The methods developed in this study to detect *aac(6′)-Iae* were rapid and effective in diagnosing infections caused by various MDR *P. aeruginosa* clones.

Pseudomonas aeruginosa causes nosocomial infections as a result of its ubiquitous nature, ability to survive in moist environments, and resistance to many antibiotics and antiseptics. A serious problem is the emergence of multidrug-resistant (MDR) *P. aeruginosa* strains resistant to β-lactams, aminoglycosides, and quinolones (34, 39, 46). Although intrinsically sensitive to β-lactams (e.g., ceftazidime [CAZ] and imipenem [IPM]), aminoglycosides (e.g., amikacin [AMK] and tobramycin), and fluoroquinolones (e.g., ciprofloxacin [CIP] and ofloxacin [OFX]), *P. aeruginosa* resistant to these antibiotics has emerged and is widespread (34, 39, 46).

We previously reported a nosocomial outbreak of catheter-associated urinary tract infection involving new MDR *P. aeruginosa* strain IMCJ2.S1, which occurred in a neurosurgery ward of a hospital located in the Tohoku area of Japan (46). This strain showed broad-spectrum resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, sulfonamide, and chlorhexidine. We found that IMCJ2.S1 harbored a novel class 1 integron, In113, containing an array of three gene cassettes of the metallo-β-lactamase (MBL) bla_{IMP-1} gene, aminoglycoside 6′-acetyltransferase *aac(6′)-Iae* gene, and aminoglycoside 3′-adenylyltransferase *aadA1* gene (46). This strain possessed mutations of the *gyrA* (83Thr→Ile) and *parC* (87Ser→Leu) genes involving amino acid substitutions, resulting in high-level resistance to fluoroquinolones.

In the geographic area where the MDR *P. aeruginosa* outbreak occurred (46), hospitals and a commercial clinical laboratory were surveyed for similar organisms. Because 99% of the MDR *P. aeruginosa* isolates analyzed were found to harbor the *aac(6′)-Iae* gene, we developed a loop-mediated isothermal amplification (LAMP) assay (31) and a slide agglutination assay to detect the *aac(6′)-Iae* gene and AAC(6′)-Iae protein, respectively. These methods were evaluated for their usefulness in detecting new MDR *P. aeruginosa* strains.

MATERIALS AND METHODS

Bacterial strains. Criteria for multidrug resistance of *P. aeruginosa* were in accordance with the Law Concerning the Prevention of Infections and Medical Care for Patients with Infections of the Japanese Ministry of Health, Labor, and Welfare; the criteria are resistance to imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 64 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml). The criterion for amikacin resistance (MIC ≥ 64 μg/ml) was different from that of a guideline of the Clinical and Laboratory Standards Institute (MIC ≥ 32 μg/ml) (4). Two

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hundred eighty-four clinical isolates of *P. aeruginosa* were obtained from 284 inpatients in 13 hospitals in Japan during the period October 2003 to September 2004: 214 isolates were MDR, and 70 were non-MDR. Information regarding the origins of the specimens was available for 99 of the 214 MDR isolates: 72 (73%) were from urine specimens, 18 (18%) were from respiratory tract specimens, 5 (5%) were from feces, 2 (2%) were from catheter tips, and 2 (2%) were from wounds. Of the 72 isolates from urine, 55 were from patients with urinary catheters. All *P. aeruginosa* isolates were originally identified by the submitting laboratories. Isolates that did not have typical characteristics (pigment and colony morphology) for *P. aeruginosa* were analyzed biochemically with an API 20NE kit (API-bioMerieux, La Balme les Grottes, France) to confirm identity as *P. aeruginosa*. *P. aeruginosa* M207 possessing bla_{IMP-1}, *P. aeruginosa* NCB326 possessing bla_{IMP-2}, and *Acinetobacter baumannii* NCB0211-439 possessing bla_{VIM-2} were provided by Y. Arakawa (National Institute of Infectious Diseases, Tokyo, Japan). *Escherichia coli* strain TOP10 (Invitrogen Corp., Carlsbad, CA) was used as the host for recombinant plasmids.

Serotyping. The O serotypes of the isolates were determined with a slide agglutination test kit containing three polyvalent antisera and 14 monovalent antisera (Denka Seiken Co., Tokyo, Japan). The kit was not in conformity with the International Antigenic Typing Scheme (IATS) (26) and was not applicable to some O types in the IATS. Therefore, we applied the standard classification of O types from A to N proposed by the Serotyping Committee for the Japan *Pseudomonas aeruginosa* Society (12).

Antimicrobial susceptibility. We obtained AMK and IPM from Banyu Pharmaceutical Co. (Tokyo, Japan), arbekacin [1-*N*-(*S*)-4-amino-2-hydroxybutyl dibekacin; ABK] from Meiji Seika Kaisha, Ltd. (Tokyo, Japan), aztreonam (AZL) from Eizai (Tokyo, Japan), CAZ from GlaxoSmithKline K. K. (Tokyo, Japan), CIP and OFX from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), doripenem (DRPM) from Shionogi & Co., Ltd. (Osaka, Japan), gentamicin (GEN) and streptomycin (STR) from Nacalai Tesque, Inc. (Kyoto, Japan), meropenem (MEM) from Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), piperacillin (PIP) and piperacillin-tazobactam (TZP) from Tomiyama Pure Chemical Industries, Ltd. (Tokyo, Japan), and polymyxin B (PL-B) from Sigma-Aldrich (St. Louis, MO). Arbekacin is an aminoglycoside antibiotic and has been used for the treatment of methicillin-resistant *Staphylococcus aureus* infections in Japan (51). Values for MICs at which 50% of isolates were inhibited (MIC_{50}) and MIC_{90} were determined by the microdilution method according to the Clinical Laboratory Standards Institute (CLSI, formally NCCLS; standard M7-A6) (4) except for ABK, PL-B, and STR, for which breakpoints ($\geq$4 μg/ml) were obtained from the published data (16, 30, 46).

Screening for MBL-producing *P. aeruginosa*. *P. aeruginosa* isolates were screened for the presence of MBL by a double-disk synergy test with disks containing sodium mercaptoacetic acid, according to the method of Arakawa et al. (2).

Immunologic detection of AAC(6′)-Iae. To detect AAC(6′)-Iae produced by *P. aeruginosa*, we developed a new method with AAC(6′)-Iae antibody-conjugated beads. Recombinant AAC(6′)-Iae was purified as reported previously (46) and used for immunization of Japanese white rabbits. Antibody against AAC(6′)-Iae was affinity purified from rabbit antisera with an *N*-hydroxysuccinimide–Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) conjugated to recombinant AAC(6′)-Iae. Purified antibody was coupled to Polybead carboxylated microspheres (2.022 μm in diameter; Polysciences, Inc., Warrington, PA) according to the manufacturer's instructions. Antibody-conjugated beads were suspended at 2.5% (vol/vol) in 0.1 M phosphate buffer (pH 7.4) containing 0.1% sodium azide. Agglutination tests were performed with *P. aeruginosa* isolates grown on *N*-acetyl-L-cysteine agar medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Bacterial cells suspended in distilled water were mixed with the antibody-conjugated beads. To confirm the specificity of the agglutination test, *P. aeruginosa* isolates were analyzed by conventional Western blotting with AAC(6′)-Iae antibody.

PCR of class 1 integrons. Class 1 integrons responsible for multidrug resistance in *P. aeruginosa* (21, 34, 46) were detected and characterized by PCR as described previously (24). Primer pairs designed to amplify the gene cassette of In113 (46) and three primer pairs specific for bla_{IMP-1}, bla_{IMP-2}, and bla_{VIM-2} (47) were used. Positive controls were *P. aeruginosa* IMCJ2.S1 for class 1 integron In113, *P. aeruginosa* M207 for bla_{IMP-1}, *P. aeruginosa* NCB326 for bla_{IMP-2}, and *A. baumannii* NCB0211-439 for bla_{VIM-2}. PCR was performed with a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). Genomic DNA was extracted as described by Sambrook et al. (44). When unexpected sizes of PCR products were obtained, the PCR products were cloned into cloning vector pCRII (Invitrogen Corp.) for DNA sequencing.

LAMP assay of *aac(6′)-Iae*. The LAMP assay amplifies DNA with high specificity under isothermal conditions (31). To identify *P. aeruginosa* isolates possessing *aac(6′)-Iae*, we designed four primers (FIP, 5′-CAA TAC AAA TGT TTT CGG CGC TAC GTC ACT CCA AAA GGC TAC-3′; BIP, 5′-TAA ACG ATG AAT TGT GTG GTT GGG TTG GAT GTA GTT CCC AAG TT-3′; F3, 5′-TCA CAC ATA AAT TTC GAT TCT TG-3′; and B3, 5′-ACC AAA TCC CTT ATT TTG ATG TT-3′) for the LAMP assay. To extract DNA from *P. aeruginosa* isolates, a colony on *N*-acetyl-L-cysteine agar medium was suspended in 100 μl distilled water and boiled for 5 min. The bacterial suspension was then centrifuged at 12,000 × *g* for 2 min, and DNA in the supernatant was used for the LAMP assay. The LAMP reaction was performed with a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The LAMP reaction mixture (12.5 μl), supplemented with 1.6 μM FIP and BIP primers, 0.2 μM F3 and B3 primers, 2× reaction mixture (6.25 μl), 4 U *Bst* DNA polymerase, 8 μg monomeric cyanine (YO-PRO-1), and 1.0 μl DNA sample, was incubated at 63°C for 45 min in a real-time thermal cycling system (Roter-Gene 2000; Corbett Research, Mortlake, New South Wales, Australia). Amplified DNA was monitored at 510 nm during the incubation. Alternatively, 25 μl of the reaction mixture was incubated at 63°C for 45 min on a block incubator (Advanced Science and Technology Enterprise Corp., Tokyo, Japan). After incubation, 10 μl of 1/100-diluted SYBR Green I nucleic acid gel stain (BioWhittaker Molecular Applications, Rockland, ME) was added to the reaction mixture. A change in color from orange to green indicated positive amplification.

PCR of QRDRs. The *gyrA*, *gyrB*, *parC*, and *parE* quinolone resistance-determining regions (QRDRs) were amplified by PCR with primers from and according to the methods described previously (1, 11, 20, 28). PCR products were sequenced with the same primers.

DNA sequencing. DNA sequences determined by the dideoxy chain termination method with an ABI PRISM 3100 sequencer (Applied Biosystems), and deduced protein sequences were subjected to homology searches in the DNA Data Bank of Japan (DDBJ), GenBank, and EMBL databases with FASTA and BLAST.

Pulsed-field gel electrophoresis (PFGE). Chromosomal DNA was prepared by the procedure of Grundmann et al. (10) and digested overnight with 10 U SpeI (Takara Bio, Inc., Shiga, Japan). The DNA fragments were separated on 1.0% agarose gels in 0.5× Tris-borate-EDTA buffer with a CHEF Mapper system (Bio-Rad Laboratories, Hercules, CA) at 6 V/cm for 20 h. The obtained fingerprinting patterns, normalized to the molecular weight markers, were analyzed by the unweighted-pair-group method with Molecular Analyst Fingerprinting Plus software, version 1.6 (Bio-Rad Laboratories, Inc.), to obtain average linkage-based dendrograms.

Statistical analysis. Results of a PCR assay, a LAMP assay, and an agglutination test were analyzed by chi-square test. A P value of <0.01 was considered statistically significant.

RESULTS

Distribution of MDR *P. aeruginosa* among hospitals. Nineteen hospitals and one clinical laboratory center from a single prefecture (population size, 2,360,000) participated in this study. MDR *P. aeruginosa* was isolated from 13 hospitals (Fig. 1). A total of 214 MDR *P. aeruginosa* isolates were obtained; 73 (34%), 38 (18%), and 22 (10%) were obtained from hospitals NA, CB, and CA, respectively, indicating that the spread of MDR *P. aeruginosa* was relatively limited. Seventy non-MDR *P. aeruginosa* isolates from the same hospitals were used for comparative analysis.

Serotyping. Ten serotypes were identified (Table 1): 222 were O11, 14 were O1, 10 were O10, 8 were B, 7 were M, 5 were O4, 4 were O3, 4 were O6, and 1 each was O9 and C. Six additional isolates showed agglutination with polyvalent antiserum but not with any of the monovalent antisera, i.e., they were nontypeable. A total of 212 of the 214 MDR *P. aeruginosa* isolates (99%) were serotype O11, whereas 70 of the non-MDR isolates were of a variety of serotypes, including O1, O3, O4, O6, O9, O10, O11, B, C, and M. These results indicated that serotype O11 was predominant for MDR *P. aeruginosa* in this prefecture.

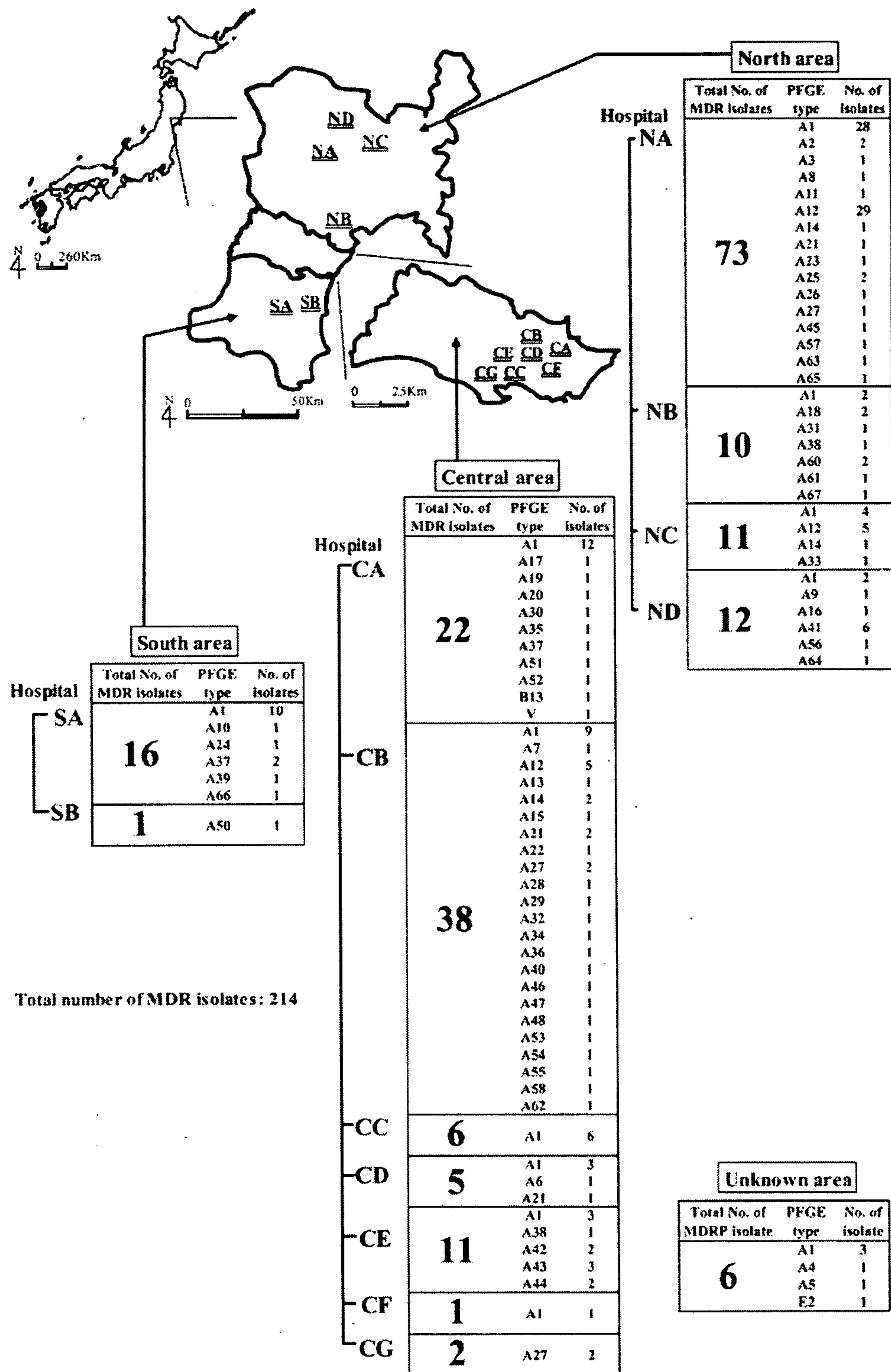

FIG. 1. Distribution of 214 isolates of MDR *P. aeruginosa* among 13 hospitals in Japan. Double capital letters indicate the locations of the hospitals that participated in this MDR *P. aeruginosa* survey.

Antimicrobial susceptibility tests. Most of the MDR *P. aeruginosa* isolates were resistant to all antimicrobials tested, except for GEN and PL-B (Tables 1 and 2). Rates of drug resistance were as follows: AMK, 100%; ABK, 91.6%; AZL, 99.5%; CAZ, 100%; CIP, 100%; DRPM, 99.1%; GEN, 57.5%; IPM, 100%; MEM, 100%; OFX, 100%; PIP, 100%; PL-B, 28%; STR, 100%; TZP, 100%. Rates of drug resistance among the non-MDR isolates were less than 63%, except that for

TABLE 1. Phenotypic and genotypic characterization of 284 clinical isolates of *P. aeruginosa*

No. of isolates	Susceptibility to:									Serotype	Gene cassette(s) of the class 1 integron	PFGE type(s)
	β-Lactams					Amino-glycosides		FQs[a]				
	PIP	TZP	CAZ	IPM	MEM	AMK	GEN	CIP	OFX			
MDR-*P. aeruginosa*[b]												
120	R	R	R	R	R	R	R	R	R	O11	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1, A2, A4, A5, A7, A8, A9, A10, A12, A14, A15, A16, A18, A20, A21, A24, A25, A27, A28, A30, A31, A32, A33, A38, A41, A42, A43, A44, A45, A46, A48, A51, A54, A56, A62, A64, E2
85	R	R	R	R	R	R	S	R	R	O11	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1, A2, A6, A11, A12, A13, A17, A18, A19, A21, A22, A23, A25, A26, A27, A34, A35, A36, A37, A39, A40, A41, A44, A47, A52, A53, A55, A58, A60, A61, A63, A65, A66, A67
1	R	R	R	R	R	R	R	R	R	O1	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A1
2	R	R	R	R	R	R	S	R	R	O1	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A38, A50
1	R	R	R	R	R	R	R	R	R	M	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A57
3	R	R	R	R	R	R	S	R	R	M	bla_{IMP-1}, *aac(6')-Iae*, *aadA1*	A3, A29, A37
1	R	R	R	R	R	R	R	R	R	O10	*aac(6')-31-like1*	B13
1	R	S	R	R	R	R	S	R	R	O1		V
Non-MDR-*P. aeruginosa*												
1	R	S	S	S	R	R	S	R	R	O11		A49
1	S	S	S	S	S	S	S	R	R	O11		A59
1	R	R	S	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B1
1	S	S	R	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B1
1	S	S	S	R	R	S	S	R	R	O1	*aac(6')-31-like2*	B1
1	R	S	S	R	R	S	R	R	R	O1	*aac(6')-31-like2*	B2
1	S	S	S	R	R	S	R	R	R	O1	*aac(6')-31*	B6
1	S	S	S	R	R	S	R	R	R	O1	*aac(6')-31-like1*	B8
1	R	S	S	R	R	S	S	R	R	O1	*aac(6')-31-like1*	B7
1	S	S	S	S	S	S	S	R	R	O6	*aac(6')-31-like1*	B3
1	S	S	S	S	S	S	S	R	R	O10	*aac(6')-31-like1*	B4
1	S	S	S	R	R	S	R	R	R	O10	*aac(6')-31-like1*	B5
1	S	S	S	R	R	S	S	R	R	O10	*aac(6')-31-like1*	B9
1	S	S	S	R	S	S	R	R	R	O10	*aac(6')-31*	B12
1	R	S	S	R	S	S	S	R	R	O10	*aac(6')-31-like1*	B14
1	S	S	S	S	S	S	S	R	R	NT	*aac(6')-31*	B10
1	R	S	S	R	R	S	S	R	R	M	*aac(6')-31-like1*	B11
2	R	R	R	R	R	S	S	R	R	NT		C1
1	R	R	R	R	R	S	S	R	R	O3		C2
2	R	R	R	R	R	S	S	R	R	O3		C4
1	S	S	S	R	R	S	S	R	R	O1		C3
1	S	S	R	R	R	S	S	R	R	O1		C7
1	R	R	R	R	R	S	S	R	R	B		C5
1	S	S	S	S	S	S	S	S	S	B		C6
1	R	R	R	R	R	S	S	R	R	O11		C8
1	S	S	S	S	S	S	S	S	R	O4		D1
1	S	S	S	S	S	S	S	R	R	O4		D2
1	S	S	S	S	S	S	S	R	S	O11		D3
1	S	S	S	R	R	S	S	R	R	O11		E1
1	R	S	S	R	R	S	S	R	R	M		F1
1	S	S	S	R	S	S	S	R	R	O4		F2
1	R	S	S	R	R	S	S	S	R	O11		G1
1	R	S	S	S	R	S	S	R	R	O11		G2
1	R	S	R	R	R	S	S	R	R	O11		H1
1	R	R	R	S	S	S	S	S	S	B		H2
2	S	S	S	R	R	S	S	S	S	O10		I
1	S	S	S	S	S	S	S	S	S	O4		J1
1	S	S	S	S	S	S	S	S	S	O3		J2
1	S	S	S	S	S	S	S	S	S	NT		K1
1	S	S	S	S	S	S	S	S	S	O6		K2
1	R	R	R	S	S	S	S	S	R	O9		L1
1	S	S	S	S	S	S	S	R	R	B		L2
1	R	S	S	S	S	S	R	R	R	O11	*aac(6')-31-like3*, *aadA6*, *orfD*	M
1	R	R	R	R	R	S	R	R	R	B	bla_{IMP-1}, *aadA1*	N
1	R	S	S	S	S	S	S	S	S	O1		O
1	R	S	R	R	R	S	S	S	S	O6		P
1	S	S	S	S	S	S	S	S	S	C		Q
1	R	R	S	R	R	S	S	S	R	O10		R
1	S	S	S	S	S	S	S	S	S	O4		S

Continued on facing page

TABLE 1—*Continued*

No. of isolates	Susceptibility to:									Serotype	Gene cassette(s) of the class 1 integron	PFGE type(s)
	β-Lactams					Amino-glycosides		FQs[a]				
	PIP	TZP	CAZ	IPM	MEM	AMK	GEN	CIP	OFX			
1	S	S	S	S	S	S	S	S	S	O11		T
1	S	S	S	S	S	S	S	S	S	O11		U
1	S	S	S	S	S	S	S	S	S	O11		W
1	S	S	S	S	S	S	S	S	S	O11		Z
1	S	S	S	S	S	S	S	S	S	O11		AA
1	S	S	S	S	S	S	S	S	S	O11		AJ
1	S	S	S	S	S	S	S	S	S	M		X
1	S	S	R	S	S	S	S	S	S	O1		Y
1	S	S	S	S	S	S	S	S	S	O10		AB
1	R	S	R	S	S	S	S	S	R	B		AC
1	S	S	S	S	S	S	S	S	S	O6		AD
1	R	R	R	S	S	R	S	S	S	O11		AE
1	S	S	S	S	S	R	R	S	S	O11		AF
1	R	R	S	S	S	S	S	S	S	NT		AG
1	R	S	S	S	S	S	S	S	S	B		AH
1	S	S	S	R	S	S	S	S	R	O1		AI
1	S	S	S	S	S	S	S	S	S	B		AK
1	S	S	S	S	S	S	S	S	S	NT		AL

[a] FQs, fluoroquinolones.
[b] Numbers of MDR isolates showing a respective PFGE type are shown in Fig. 1.
[c] NT, nontypeable.

STR, which was 98.6%. MIC_{50} and MIC_{90} values for MDR isolates were high, except those for ABK, GEN, and PL-B, and MIC_{50} and MIC_{90} values for non-MDR isolates were low, except those for AMK.

MBL production. MBL confers bacterial resistance to all β-lactams except AZL (53). Of the 284 isolates, 213 (75%) produced MBL and all except one were MDR isolates.

AAC(6′)-Iae production. AAC(6′)-Iae was first identified in MDR *P. aeruginosa* strain IMCJ2.S1 (46). We developed a slide agglutination test with AAC(6′)-Iae antibody-conjugated beads. *P. aeruginosa* IMCJ2.S1 showed a positive result within 30 s (Fig. 2, lane 2), whereas AAC(6′)-Iae-negative *P. aeruginosa* strain ATCC 27853 did not (Fig. 2, lane 4). Two hundred seventeen isolates were positive for the production of AAC(6′)-Iae in this test (Table 3). The results of the slide agglutination test were in complete agreement with Western blotting data obtained with AAC(6′)-Iae antibody (data not shown).

Detection of class 1 integrons. PCR assay with primers 5′-cs and 3′-cs (24), which are specific for the 5′ conserved segments (CS) (49) and the 3′ CS (49) of class 1 integrons, respectively, showed that 230 of the 284 isolates were positive. Amplified band sizes ranged from 0.8 kb to 2.5 kb (data not shown). All of these 230 isolates yielded a single band. Of these isolates, 212 yielded a 2.5-kb band, which is the same as that of the class 1 integron In113 (46). Sixteen isolates yielded a 0.8-kb band, and the remaining two yielded a 1.8-kb band and a 1.7-kb band. For the 212 isolates showing a 2.5-kb band, the presence of In113 was confirmed by PCR with specific primers, as described previously. MBL genes bla_{IMP-2} and bla_{VIM-2} are frequently found in Japan and are often associated with integrons (47). Therefore, we screened the 284 MDR *P. aeruginosa* iso-

TABLE 2. MIC_{50} and MIC_{90} values and percent antimicrobial resistance for 284 samples of *P. aeruginosa*

Antimicrobial agent	Breakpoint for resistance (μg/ml)	MDR isolates[a] (n = 214)				Non-MDR isolates (n = 70)			
		% Resistant	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistant	Range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
PIP	≥128	100	128–>512	>512	>512	41.4	1–>512	64	512
TZP	≥128/4	100	128–>512	512	>512	21.4	0.5–256	32	128
CAZ	≥32	100	32–>512	>512	>512	25.7	1–>512	8	64
IPM	≥16	100	32–>512	256	512	47.1	0.25–>512	8	32
DRPM	≥16	99.1	2–>512	>512	>512	34.3	<0.125–>512	8	32
MEM	≥16	100	32–>512	512	>512	44.3	<0.125–>512	4	32
AZT	≥32	99.5	16–>512	128	128	52.9	0.5–128	32	64
ABK	≥4	91.6	2–16	4	8	24.3	<0.125–16	1	8
AMK	≥32	100	32–256	128	256	2.9	0.25–256	2	16
GEN	≥16	57.5	0.25–>32	16	16	12.9	<0.125–>128	1	16
STR	≥4	100	512–>512	>512	>512	98.6	2–>512	32	128
CIP	≥4	100	16–>128	64	>128	51.4	<0.125–>128	4	64
OFX	≥8	100	32–>128	>128	>128	62.9	<0.125–>128	16	>128
PL-B	≥4	28.0	2–8	2	4	22.9	1–8	2	4

[a] Isolates defined as resistant to three antibiotics, imipenem (MIC ≥ 16 μg/ml), amikacin (MIC ≥ 32 μg/ml), and ciprofloxacin (MIC ≥ 4 μg/ml).

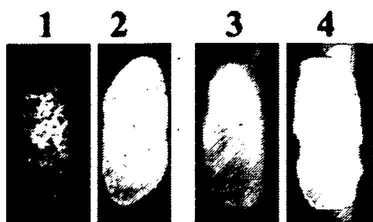

FIG. 2. Slide agglutination test with AAC(6')-Iae antibody-conjugated beads. Lane 1, AAC(6')-Iae positive control; lane 2, *P. aeruginosa* IMCJ2.S1 positive control; lane 3, 50 mM HEPES buffer negative control as solvent of AAC(6')-Iae; lane 4, *P. aeruginosa* ATCC 27853 negative control.

lates for bla_{IMP-2} and bla_{VIM-2} by PCR. None of the 284 isolates were positive for bla_{IMP-2} or bla_{VIM-2}.

The regions between the 5' CS and 3' CS of amplicons of unexpected sizes were sequenced, and the gene cassettes were identified (Table 1). Of 16 isolates showing an 0.8-kb band, three possessed a single gene cassette containing *aac(6')-31*, encoding 6'-*N*-aminoglycoside acetyltransferase type IV (R. E. Mendes, unpublished data; DDBJ/EMBL/GenBank accession no. AJ640197) (Table 1). This gene cassette was 639 nucleotides (nt) and contained a 65-nt 59-base-element (be) site, for site-specific cointegration events (35). Nine isolates possessed an *aac(6')-31-like1* cassette identical to *aac(6')-31*, with the exception of a C-to-T substitution at nt 269 in the coding region. Four isolates possessed an *aac(6')-31-like2* cassette identical to *aac(6')-31*, with the exception of a C-to-A substitution at nt 269. One isolate showing a 1.8-kb band possessed an array of three gene cassettes (Table 1). Of them, the first cassette was an *aac(6')-31-like3* cassette similar to *aac(6')-31* except for T-to-C and A-to-T substitutions at nt 57 and 266, respectively. The second cassette was 855 nt and contained the aminoglycoside adenylyltransferase gene *aadA6* (29) and a 60-nt 59-be site. The third cassette was 320 nt and contained open reading frame *orfD*, of unknown function (29). The *aadA6* and *orfD* cassettes were identical to those of In51 reported previously (29). One isolate showing a 1.7-kb band possessed two gene cassettes of bla_{IMP-1} (33) and *aadA1* (25) (Table 1).

Resistance to fluoroquinolones. Amino acid alterations to GyrA, GyrB, ParC, and ParE QRDRs of the 284 isolates are listed in Table 4. Amino acid replacement in the QRDR of GyrA (83Thr→Ile or 87Asp→Asn, Gly, or Tyr) was detected in 254 of the 284 isolates (89.4%). Of these 254 isolates, 8 possessed a mutation of GyrA alone. The remaining isolates possessed additional substitutions in GyrA, GyrB, ParC, and ParE. The 83Thr→Ile substitution in GyrA was the predominant replacement (251 of 284 isolates, 88.4%), in agreement with previous data on fluoroquinolone-resistant *P. aeruginosa* isolates (1, 22, 28). A double mutation of GyrA, 83Thr→Ile and 87Asp→Asn or Gly, was detected in nine isolates.

Amino acid replacement in the QRDR of ParC (87Ser→Leu or 91Glu→Lys) was detected in 244 of the 284 isolates (85.9%). All of these 244 isolates possessed additional mutations. The 87Ser→Leu substitution was the predominant replacement (242 of 284 isolates, 85.2%) and has been implicated in fluoroquinolone resistance of *P. aeruginosa* (1, 22, 28). A double mutation of ParC, 87Ser→Leu and 91Glu→Lys, was detected in three isolates. We found an 83Pro→Leu, 85Gly→Asp, and 88Ala→Pro alterations in one isolate each (Table 4).

Amino acid replacement in the QRDR of GyrB (468Glu→Asp) was detected in 70 of the 284 isolates (24.6%). No double mutations in GyrB were detected. Lee et al. (22) recently reported that 468Glu→Asp was a predominant alteration of GyrB, and isolates with this alteration, in addition to GyrA (83Thr→Ile) and ParC (87Ser→Leu) substitutions, showed a high level of resistance to CIP (MIC > 64 μg/ml). Our results were in accordance with their findings. We also found a 458Ala→Thr alteration in four isolates and a 496Ile→Val alteration in one isolate. These alterations are probably not associated with CIP resistance in *P. aeruginosa* because they were found in CIP-susceptible isolates.

Amino acid replacement in the QRDR of ParE (425Ala→Val or 459Glu→Asp or both) was detected in 30 of the 284 isolates (10.6%). All isolates possessed multiple mutations of ParE. Lee et al. (22) speculated that the 459Glu→Asp mutation of ParE is associated with moderate or high-level fluoroquinolone resistance in *P. aeruginosa*. The 425Ala→Val mutation has been reported in fluoroquinolone-resistant isolates of *P. aeruginosa* (1). Other mutations leading to amino acid changes were found at codons 419 (Asp→Asn, 1 isolate), 427 (Gln→Leu, 1 isolate), and 457 (Ser→Alg, 1 isolate). The fluoroquinolone

TABLE 3. Comparison of PCR, LAMP, and agglutination test results for the detection of MDR *P. aeruginosa* isolates belonging to genotype cluster A[a]

Isolates	No. of isolates with indicated result by:								
	PCR			LAMP			Agglutination test with AAC(6')-Iae antibody-conjugated beads		
	Positive	Negative	Total	Positive	Negative	Total	Positive	Negative	Total
MDR *P. aeruginosa*									
Cluster A	212	0	212	212	0	212	207	5	212
Other	0	2	2	0	2	2	0	2	2
Non-MDR *P. aeruginosa*									
Cluster A	0	2	2	0	2	2	0	2	2
Other	0	68	68	0	68	68	10	58	68
Total	212	72	284	212	72	284	217	65	284

[a] In all tests and combinations, the multidrug resistance of the isolates was positively associated with the positive results of *aac(6')* tests based on chi-square tests ($P < 0.0001$).

TABLE 4. Amino acid changes in *gyrA*, *gyrB*, *parC*, and *parE* genes in 284 clinical isolates of *P. aeruginosa*

No. of strains (n = 284)	MIC (μg/ml) of:		Replacement in QRDRs[j]									
			GyrA at position:		ParC at position:			GyrB at position:		ParE at position:		
	CIP	OFX	83Thr (ACC)	87Asp (GAC)	87Ser (TCG)	91Glu (GAG)	Other	468Glu (GAG)	Other	425Ala (GCG)	459Glu (GAG)	Other
MDR *P. aeruginosa*												
1	>128	>128	Ile (ATC)	—[a]	Leu (TTG)	—	83Pro→Leu[b]	Asp (GAT)	—	—	Asp (GAT)	—
25	128->128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	Asp (GAT)	—
1	128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	427Gln→Leu[g]
37	32–128	128->128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	—
1	>128	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	Lys (AAG)	—	—	—	—	—	—
1	16	32	Ile (ATC)	—	Leu (TTG)	—	85Gly→Asp[c]	—	—	—	—	—
147	16->128	32->128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	—	—	—
1	32	64	Ile (ATC)	—	—	—	—	—	—	—	—	457Ser→Alg[h]
Non-MDR *P. aeruginosa*												
5	64->128	>128	Ile (ATC)	—	Leu (TTG)	—	—	Asp (GAT)	—	—	—	—
4	32–128	64->128	Ile (ATC)	Asn (AAC)	Leu (TTG)	—	—	—	—	—	—	—
1	128	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	Lys (AAG)	—	—	—	—	—	—
1	>128	>128	Ile (ATC)	Asn (AAC)	—	Lys (AAG)	—	—	—	—	—	—
1	64	>128	Ile (ATC)	Asn (AAC)	Leu (TTG)	—	—	—	—	—	Asp (GAT)	—
1	64	128	Ile (ATC)	Gly (GGC)	Leu (TTG)	—	88Ala→Pro[d]	—	—	—	—	—
13	32–64	64->128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	—	—	—
2	16–32	32–128	Ile (ATC)	—	Leu (TTG)	—	—	—	—	Val (GTG)	—	—
1	16	128	Ile (ATC)	—	Leu (TTG)	—	—	—	458Ala→Thr[e]	—	—	—
1	16	128	Ile (ATC)	—	—	Lys (AAG)	—	—	—	—	—	—
1	16	128	Ile (ATC)	—	—	—	—	—	—	Val (GTG)	—	—
1	8	128	Ile (ATC)	—	—	—	—	—	458Ala→Thr	—	—	419Asp→Asn[i]
1	2	16	—	—	Leu (TTG)	—	—	—	458Ala→Thr	—	—	—
6	<0.25–0.5	1–8	—	—	—	—	—	—	458Ala→Thr	—	—	—
2	<0.25	0.25	—	—	—	—	—	—	496Ile→Val[f]	—	—	—
1	4	64	—	—	—	—	—	Asp (GAT)	—	—	—	—
5	0.5–4	8–16	Ile (ATC)	—	—	—	—	—	—	—	—	—
2	1–2	2–8	—	Tyr (TAC)	—	—	—	—	—	—	—	—
1	<0.25	0.25	—	—	—	—	—	—	—	—	—	—
20	<0.25–16	<0.25–64	—	Asn (AAC)	—	—	—	—	—	—	—	—

[a] —, no amino acid change.
[b] 83Pro→Leu, Pro at position 83 of ParC changed to Leu (CCG→CTG).
[c] 85Gly→Asp, Gly at position 85 of parC changed to Asp (GGC→GAC).
[d] 88Ala→Pro, Ala at position 88 of ParC changed to Pro (GCC→CCC).
[e] 458Ala→Thr, Ala at position 453 of GyrB changed to Thr (GCG→ACG).
[f] 496Ile→Val, Ile at position 496 of GyrB changed to Val (ATG→GTC).
[g] 427Gln→Leu, Gln at position 427 of ParE changed to Leu (CAG→CTG).
[h] 457Ser→Arg, Ser at position 457 of ParE changed to Arg (AGC→AGG).
[i] 419Asp→Asn, Asp at position 419 of ParE changed to Asn (GAC→AAC).
[j] Mutated nucleotides are underlined.

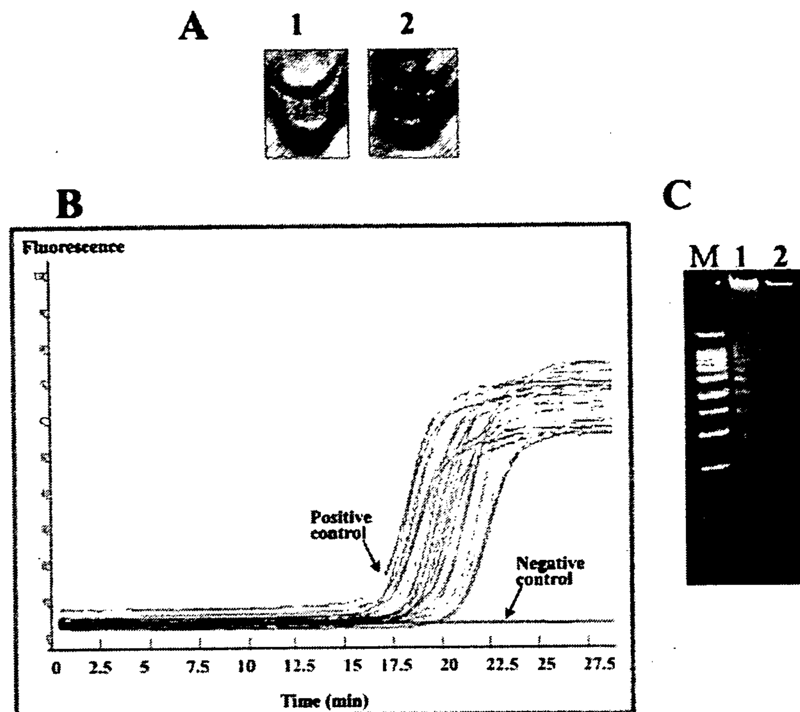

FIG. 3. LAMP assay to detect MDR *P. aeruginosa* isolates possessing the *aac(6')-Iae* gene encoding the aminoglycoside acetyltransferase AAC(6')-Iae. *P. aeruginosa* IMCJ2.S1 and ATCC 27853 were used as positive and negative controls, respectively. (A) Visual inspection analysis of LAMP products. Lane 1, *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853. (B) Real-time amplification monitoring of *aac(6')-Iae*-specific LAMP. The amplification signal was detected at an average of 18 min, as indicated by the continuous increase in fluorescence. Increased fluorescence was not observed in the negative control. (C) Acrylamide gel electrophoresis of LAMP product. Lane 1, LAMP product of the 204-bp target sequence of the *aac(6')-Iae* gene of *P. aeruginosa* IMCJ2.S1; lane 2, *P. aeruginosa* ATCC 27853 negative control; lane M, 1-kbp ladder.

resistance associated with these mutations remains to be determined.

Analysis of the *aac(6')-Iae* gene by the LAMP method. To detect *aac(6')-Iae*, we developed a gene-specific LAMP assay. The index strain IMCJ2.S1 was used to standardize the method. Visual inspection showed that the LAMP assay successfully amplified the target sequence of the *aac(6')-Iae* gene of *P. aeruginosa* IMCJ2.S1 (Fig. 3A). Real-time kinetics of the LAMP reaction showed that the amplification signal could be detected on average by 18 min; fluorescence increased in the positive samples, following a sigmoid curve (Fig. 3B). Agarose gel electrophoresis of the LAMP products (Fig. 3C) showed a ladder-like pattern on the gel due to the formation of a mixture of stem-loop DNAs of various stem lengths, which are characteristic of LAMP products.

A total of 284 isolates, including 214 MDR *P. aeruginosa* isolates, were tested by the LAMP assay (Table 3). A total of 212 isolates were positive by the LAMP assay (Table 3). The results of the LAMP assay were in complete concordance with the PCR data, indicating that the PCR can be replaced by the LAMP method for detection of *aac(6')-Iae*-carrying *P. aeruginosa*. These results, together with ones of the agglutination test (Table 3), indicate that multidrug resistance was strongly associated with the presence of *aac(6')-Iae* and AAC(6')-Iae production in the *P. aeruginosa* isolates ($P < 0.0001$).

Genotyping by PFGE. The 284 isolates, including 214 MDR isolates, were typed by PFGE. One hundred thirty-three different PFGE types, designated from A1 to AL, were distinguished (Table 1). Fourteen types, A1, A2, A12, A14, A18, A21, A25, A27, A37, A41, A42, A43, A44, and A60, were identified in more than 2 isolates (Fig. 1), and type A1, which represented 83 of the isolates (29%), was the most prevalent and widely disseminated (Fig. 1), suggesting prefecture-wide clonal dissemination. Types A1, A12, A14, A21, A27, A37, and A38 were identified at two or more hospitals. Cluster analysis of the PFGE restriction patterns showed three large clusters, A, B, and C, sharing ≥70% similarity (Fig. 4). Of the 214 MDR isolates, 211 belonged to cluster A, comprising types A1 to A67, indicating that multidrug resistance was associated with one genotype, cluster A (Fig. 4 and Table 3). Fifteen isolates belonged to cluster B comprising types B1 to B14, and 10 isolates belonged to cluster C, comprising types C1 to C8. The PFGE patterns of the 35 non-MDR isolates varied greatly.

DISCUSSION

A clonal expansion of *P. aeruginosa* resistant to three antibiotics, carbapenems, amikacin, and fluoroquinolones, has been reported (4, 14, 36, 37, 46). However, previous surveillance studies in Japan have not shown clonal expansion involving multiple hospitals (19, 52). The present study showed clonal expansion of MDR *P. aeruginosa* in hospitals in the Tohoku area of Japan. To our knowledge, this is the first description of a large-scale, community-wide outbreak of nos-

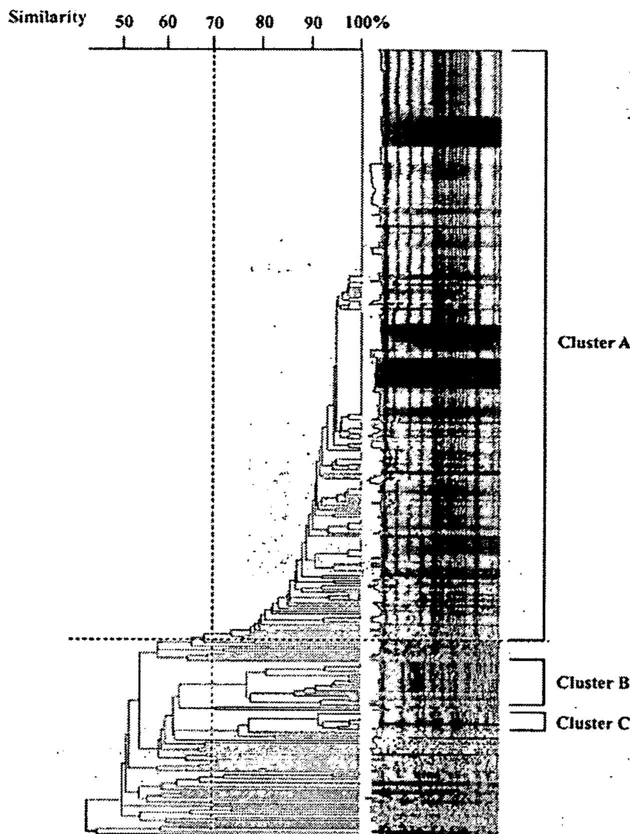

FIG. 4. Cluster analysis based on the PFGE patterns of 284 clinical isolates of *P. aeruginosa* from the 13 hospitals in the present study. Clustering was carried out with Molecular Analyst FingerprintingPlus software, version 1.6, as described in Materials and Methods.

ocomial infection caused by a single *P. aeruginosa* clone with high-level resistance to a large number of antibiotics. The routes of transmission of the MDR *P. aeruginosa* clone remain unclear. *P. aeruginosa* that can be recovered from the hospital environment could be a possible source of nosocomial infection (6, 42, 54). Patient-to-patient transmission has been documented among patients with cystic fibrosis (5, 42, 54). Catheter-associated urinary tract infections appeared widespread among the hospitals in our study; the majority of the isolates (approximately 70%) were obtained from urine specimens, and approximately 80% of these were from patients with urinary catheters.

Most MDR isolates tested (205 of 214; Table 1) showed a serotype of O11. This was not surprising because these isolates belonged to a single cluster, as revealed by PFGE analysis (Fig. 4). *P. aeruginosa* is categorized into 31 chemotypes, including 20 IATS serotypes and subtypes (48). Thus far, however, particular serotypes, such as serotypes O12 and O11, appear to have been preferentially associated with *P. aeruginosa* outbreaks (9, 23, 38, 41). A clone of *P. aeruginosa* belonging to serogroup O12, which was resistant to both carbenicillin and gentamicin, was predominant in outbreaks involving six hospitals in Athens in 1987 (23). Later, O12 isolates resistant to these two drugs were reported in European countries (9, 38, 41). *P. aeruginosa* O12 resistant to ciprofloxacin and ceftazidime and/or fosfomycin was implicated in hospital outbreaks in France during the period 1993 to 1994 (3). *P. aeruginosa* serotype O11 caused hospital outbreaks in the 1980s in the United States (8) and in 1994 and 1995 in Greece (50). *P. aeruginosa* O11 was implicated in folliculitis caused by the use of whirlpools and hot tubs in the 1970s and 1980s in the United States and Canada (40). More recently, hospital outbreaks caused by MDR *P. aeruginosa* serotype O11 occurred in Belgium (5) and in Japan (46). Different strains of serotype O11 were involved in the above-mentioned outbreaks because their PFGE profiles were quite different. In addition, the Japanese strains produced IMP-1 carbapenemase (46), but the Belgian strains did not (5). It is not known why *P. aeruginosa* strains belonging to particular serotypes of O12 and O11 were involved in these outbreaks.

We analyzed several features including serotype, antimicrobial susceptibility, MBL production, prevalence of *aac(6')-Iae*, structure of class 1 integrons, resistance to fluoroquinolones, and genotype based on PFGE analysis for MDR *P. aeruginosa* isolates. Results indicated that *aac(6')-Iae* is a good candidate marker for MDR *P. aeruginosa* infection. To detect the *aac(6')-Iae* gene and its product, we developed a LAMP-based detection assay and an agglutination assay. LAMP is a nucleic acid amplification method which relies on autocycling strand displacement DNA synthesis performed by the *Bst* DNA polymerase large fragment (31). The amplification products are stem-loop DNA structures with several inverted repeats of the target and cauliflower-like structures with multiple loops. LAMP assays are simple and short and do not require expensive equipment. LAMP assays have been applied to the analysis of various infectious agents such as hepatitis B virus (7), *Mycobacterium tuberculosis* (15), severe acute respiratory syndrome coronavirus (13), *E. coli* O157:H7 (27), *Clostridium difficile* (18), *Bordetella pertussis* (17), *Salmonella enterica* (32), *Mycoplasma pneumoniae* (43), and *Streptococcus pneumoniae* (45). The LAMP assay developed in this study was as sensitive and specific as PCR. Though less sensitive and specific than the LAMP assay, the agglutination assay for AAC(6')-Iae is sufficiently accurate to detect MDR *P. aeruginosa* (98% of MDR *P. aeruginosa* isolates were positive). The agglutination assay is simpler and cheaper than the LAMP assay and is also useful in detecting MDR *P. aeruginosa* in the clinical setting.

MDR *P. aeruginosa* may have spread across Japan as a result of the increasing use of carbapenems such as IPM, aminoglycosides such as AMK, and fluoroquinolones such as CIP. Nationwide surveillance for MDR *P. aeruginosa* is under way. At the hospital level, monitoring for environmental sources of bacteria, cleaning of contaminated surfaces of treatment rooms and bathrooms, review of infection control measures in the treatment of urine, and avoidance of unnecessary measurements of urine are considered effective in preventing *P. aeruginosa* nosocomial infections. Although the mode of transmission between hospitals is unknown, the movement of infected patients from one hospital to another is a possibility. Thirty-one patients infected with MDR *P. aeruginosa* had been transferred from other hospitals to the hospitals participating in the present study.

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REFERENCES

1. **Akasaka, T., M. Tanaka, A. Yamaguchi, and K. Sato.** 2001. Type II topoisomerase mutations in fluoroquinolone-resistant clinical strains of *Pseudomonas aeruginosa* isolated in 1998 and 1999: role of target enzyme in mechanism of fluoroquinolone resistance. Antimicrob. Agents Chemother. **45:** 2263–2268.
2. **Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto.** 2000. Convenient test for screening metallo-beta-lactamase-producing gram-negative bacteria by using thiol compounds. J. Clin. Microbiol. **38:**40–43.
3. **Bingen, E., S. Bonacorsi, P. Rohrlich, M. Duval, S. Lhopital, N. Brahimi, E. Vilmer, and R. V. Goering.** 1996. Molecular epidemiology provides evidence of genotypic heterogeneity of multidrug-resistant *Pseudomonas aeruginosa* serotype O:12 outbreak isolates from a pediatric hospital. J. Clin. Microbiol. **34:**3226–3229.
4. **Clinical and Laboratory Standards Institute.** 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 6th ed. Approved standard M07-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Deplano, A., O. Denis, L. Poirel, D. Hocquet, C. Nonhoff, B. Byl, P. Nordmann, J. L. Vincent, and M. J. Struelens.** 2005. Molecular characterization of an epidemic clone of panantibiotic-resistant *Pseudomonas aeruginosa*. J. Clin. Microbiol. **43:**1198–1204.
6. **Doring, G., S. Jansen, H. Noll, H. Grupp, F. Frank, K. Botzenhart, K. Magdorf, and U. Wahn.** 1996. Distribution and transmission of *Pseudomonas aeruginosa* and *Burkholderia cepacia* in a hospital ward. Pediatr. Pulmonol. **21:**90–100.
7. **Enomoto, Y., T. Yoshikawa, M. Ihira, S. Akimoto, F. Miyake, C. Usui, S. Suga, K. Suzuki, T. Kawana, Y. Nishiyama, and Y. Asano.** 2005. Rapid diagnosis of herpes simplex virus infection by a loop-mediated isothermal amplification method. J. Clin. Microbiol. **43:**951–955.
8. **Farmer, J. J., III, R. A. Weinstein, C. H. Zierdt, and C. D. Brokopp.** 1982. Hospital outbreaks caused by *Pseudomonas aeruginosa*: importance of serogroup O11. J. Clin. Microbiol. **16:**266–270.
9. **Grattard, F., O. G. Gaudin, B. Pozzetto, A. Ros, and A. D. Mbida.** 1993. Genotypic homogeneity of nosocomial *Pseudomonas aeruginosa* O12 strains demonstrated by analysis of protein profiles, DNA fingerprints and rRNA gene restriction patterns. Eur. J. Clin. Microbiol. Infect. Dis. **12:**57–61.
10. **Grundmann, H., C. Schneider, D. Hartung, F. D. Daschner, and T. L. Pitt.** 1995. Discriminatory power of three DNA-based typing techniques for *Pseudomonas aeruginosa*. J. Clin. Microbiol. **33:**528–534.
11. **Hocquet, D., X. Bertrand, T. Kohler, D. Talon, and P. Plesiat.** 2003. Genetic and phenotypic variations of a resistant *Pseudomonas aeruginosa* epidemic clone. Antimicrob. Agents Chemother. **47:**1887–1894.
12. **Homma, J. Y.** 1982. Designation of the thirteen O-group antigens of *Pseudomonas aeruginosa*: an amendment for the tentative proposal in 1976. Jpn. J. Exp. Med. **52:**317–320.
13. **Hong, T. C., Q. L. Mai, D. V. Cuong, M. Parida, H. Minekawa, T. Notomi, F. Hasebe, and K. Morita.** 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. **42:**1956–1961.
14. **Hsueh, P. R., L. J. Teng, P. C. Yang, Y. C. Chen, S. W. Ho, and K. T. Luh.** 1998. Persistence of a multidrug-resistant *Pseudomonas aeruginosa* clone in an intensive care burn unit. J. Clin. Microbiol. **36:**1347–1351.
15. **Iwamoto, T., T. Sonobe, and K. Hayashi.** 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium*, and *M. intracellulare* in sputum samples. J. Clin. Microbiol. **41:**2616–2622.
16. **Jones, R. N., T. R. Anderegg, J. M. Swenson, and The Quality Control Working Group.** 2005. Quality control guidelines for testing gram-negative control strains with polymyxin B and colistin (polymyxin E) by standardized methods. J. Clin. Microbiol. **43:**925–927.
17. **Kamachi, K., H. Toyoizumi-Ajisaka, K. Toda, S. C. Soeung, S. Sarath, Y. Nareth, Y. Horiuchi, K. Kojima, M. Takahashi, and Y. Arakawa.** 2006. Development and evaluation of a loop-mediated isothermal amplification method for rapid diagnosis of *Bordetella pertussis* infection. J. Clin. Microbiol. **44:**1899–1902.
18. **Kato, H., T. Yokoyama, and Y. Arakawa.** 2005. Rapid and simple method for detecting the toxin B gene of *Clostridium difficile* in stool specimens by loop-mediated isothermal amplification. J. Clin. Microbiol. **43:**6108–6112.
19. **Kimura, S., J. Alba, K. Shiroto, R. Sano, Y. Niki, S. Maesaki, K. Akizawa, M. Kaku, Y. Watanuki, Y. Ishii, and K. Yamaguchi.** 2005. Clonal diversity of metallo-β-lactamase-possessing *Pseudomonas aeruginosa* in geographically diverse regions of Japan. J. Clin. Microbiol. **43:**458–461.
20. **Kureishi, A., J. M. Diver, B. Beckthold, T. Schollaardt, and L. E. Bryan.** 1994. Cloning and nucleotide sequence of *Pseudomonas aeruginosa* DNA gyrase *gyrA* gene from strain PAO1 and quinolone-resistant clinical isolates. Antimicrob. Agents Chemother. **38:**1944–1952.
21. **Laraki, N., M. Galleni, I. Thamm, M. L. Riccio, G. Amicosante, J. M. Frere, and G. M. Rossolini.** 1999. Structure of In31, a bla_{IMP}-containing *Pseudomonas aeruginosa* integron phyletically related to In5, which carries an unusual array of gene cassettes. Antimicrob. Agents Chemother. **43:**890–901.
22. **Lee, J. K., Y. S. Lee, Y. K. Park, and B. S. Kim.** 2005. Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. Int. J. Antimicrob. Agents **25:**290–295.
23. **Legakis, N. J., N. Koukoubanis, K. Malliara, D. Michalitsianos, and J. Papavassiliou.** 1987. Importance of carbenicillin and gentamicin cross-resistant serotype 0:12 *Pseudomonas aeruginosa* in six Athens hospitals. Eur. J. Clin. Microbiol. **6:**300–303.
24. **Levesque, C., L. Piche, C. Larose, and P. H. Roy.** 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. Antimicrob. Agents Chemother. **39:**185–191.
25. **Liebert, C. A., R. M. Hall, and A. O. Summers.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol. Mol. Biol. Rev. **63:**507–522.
26. **Liu, P. V., H. Matsumoto, H. Kusama, and T. Bergan.** 1983. Survey of heat-stable, major somatic antigens of *Pseudomonas aeruginosa*. Int. J. Syst. Bacteriol. **33:**256–264.
27. **Maruyama, F., T. Kenzaka, N. Yamaguchi, K. Tani, and M. Nasu.** 2003. Detection of bacteria carrying the *stx2* gene by in situ loop-mediated isothermal amplification. Appl. Environ. Microbiol. **69:**5023–5028.
28. **Mouneimne, H., J. Robert, V. Jarlier, and E. Cambau.** 1999. Type II topoisomerase mutations in ciprofloxacin-resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **43:**62–66.
29. **Naas, T., L. Poirel, and P. Nordmann.** 1999. Molecular characterisation of In51, a class 1 integron containing a novel aminoglycoside adenylyltransferase gene cassette, aadA6, in *Pseudomonas aeruginosa*. Biochim. Biophys. Acta **1489:**445–451.
30. **Nakamura, A., M. Hosoda, T. Kato, Y. Yamada, M. Itoh, K. Kanazawa, and H. Nouda.** 2000. Combined effects of meropenem and aminoglycosides on *Pseudomonas aeruginosa* in vitro. J. Antimicrob. Chemother. **46:**901–904.
31. **Notomi, T., H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase.** 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. **28:**E63.
32. **Ohtsuka, K., K. Yanagawa, K. Takatori, and Y. Hara-Kudo.** 2005. Detection of *Salmonella enterica* in naturally contaminated liquid eggs by loop-mediated isothermal amplification, and characterization of *Salmonella* isolates. Appl. Environ. Microbiol. **71:**6730–6735.
33. **Osano, E., Y. Arakawa, R. Wacharotayankun, M. Ohta, T. Horii, H. Ito, F. Yoshimura, and N. Kato.** 1994. Molecular characterization of an enterobacterial metallo-β-lactamase found in a clinical isolate of *Serratia marcescens* that shows imipenem resistance. Antimicrob. Agents Chemother. **38:**71–78.
34. **Pagani, L., C. Colinon, R. Migliavacca, M. Labonia, J. D. Docquier, E. Nucleo, M. Spalla, M. Li Bergoli, and G. M. Rossolini.** 2005. Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-beta-lactamase. J. Clin. Microbiol. **43:**3824–3828.
35. **Partridge, S. R., G. D. Recchia, C. Scaramuzzi, C. M. Collis, H. W. Stokes, and R. M. Hall.** 2000. Definition of the attI1 site of class 1 integrons. Microbiology **146:**2855–2864.
36. **Pellegrino, F. L. P. C., L. M. Teixeira, M. da Glória Siqueira Carvalho, S. Aranha Nouer, M. Pinto De Oliveira, J. L. Mello Sampaio, A. D'Ávila Freitas, A. L. P. Ferreira, E. de Lourdes Teixeira Amorim, L. W. Riley, and B. M. Moreira.** 2002. Occurrence of a multidrug-resistant *Pseudomonas aeruginosa* clone in different hospitals in Rio de Janeiro, Brazil. J. Clin. Microbiol. **40:**2420–2424.
37. **Pirnay, J. P., D. De Vos, C. Cochez, F. Bilocq, J. Pirson, M. Struelens, L. Duinslaeger, P. Cornelis, M. Zizi, and A. Vanderkelen.** 2003. Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. J. Clin. Microbiol. **41:**1192–1202.
38. **Pitt, T. L., D. M. Livermore, D. Pitcher, A. C. Vatopoulos, and N. J. Legakis.** 1989. Multiresistant serotype O 12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe. Epidemiol. Infect. **103:**565–576.
39. **Pournaras, S., M. Maniati, E. Petinaki, L. S. Tzouvelekis, A. Tsakris, N. J. Legakis, and A. N. Maniatis.** 2003. Hospital outbreak of multiple clones of

Pseudomonas aeruginosa carrying the unrelated metallo-beta-lactamase gene variants bla_{VIM-2} and bla_{VIM-4}. J. Antimicrob. Chemother. **51**:1409–1414.

40. **Ratnam, S., K. Hogan, S. B. March, and R. W. Butler.** 1986. Whirlpool-associated folliculitis caused by *Pseudomonas aeruginosa*: report of an outbreak and review. J. Clin. Microbiol. **23**:655–659.
41. **Richard, P., R. Le Floch, C. Chamoux, M. Pannier, E. Espaze, and H. Richet.** 1994. *Pseudomonas aeruginosa* outbreak in a burn unit: role of antimicrobials in the emergence of multiply resistant strains. J. Infect. Dis. **170**:377–383.
42. **Saiman, L., and J. Siegel.** 2004. Infection control in cystic fibrosis. Clin. Microbiol. Rev. **17**:57–71.
43. **Saito, R., Y. Misawa, K. Moriya, K. Koike, K. Ubukata, and N. Okamura.** 2005. Development and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Mycoplasma pneumoniae*. J. Med. Microbiol. **54**:1037–1041.
44. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York, NY.
45. **Seki, M., Y. Yamashita, H. Torigoe, H. Tsuda, S. Sato, and M. Maeno.** 2005. Loop-mediated isothermal amplification method targeting the *lytA* gene for detection of *Streptococcus pneumoniae*. J. Clin. Microbiol. **43**:1581–1586.
46. **Sekiguchi, J., T. Asagi, T. Miyoshi-Akiyama, T. Fujino, I. Kobayashi, K. Morita, Y. Kikuchi, T. Kuratsuji, and T. Kirikae.** 2005. Multidrug-resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its *aac(6')-Iae* gene cassette encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. **49**:3734–3742.
47. **Shibata, N., Y. Doi, K. Yamane, T. Yagi, H. Kurokawa, K. Shibayama, H. Kato, K. Kai, and Y. Arakawa.** 2003. PCR typing of genetic determinants for metallo-β-lactamases and integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. J. Clin. Microbiol. **41**: 5407–5413.
48. **Stanislavsky, E. S., and J. S. Lam.** 1997. *Pseudomonas aeruginosa* antigens as potential vaccines. FEMS Microbiol. Rev. **21**:243–277.
49. **Stokes, H. W., and R. M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. Mol. Microbiol. **3**:1669–1683.
50. **Tassios, P. T., V. Gennimata, A. N. Maniatis, C. Fock, N. J. Legakis, and The Greek *Pseudomonas aeruginosa* Study Group.** 1998. Emergence of multidrug resistance in ubiquitous and dominant *Pseudomonas aeruginosa* serogroup O:11. J. Clin. Microbiol. **36**:897–901.
51. **Tsuchizaki, N., K. Ishino, F. Saito, J. Ishikawa, M. Nakajima, and K. Hotta.** 2006. Trends of arbekacin-resistant MRSA strains in Japanese hospitals (1979 to 2000). J. Antibiot. **59**:229–233.
52. **Tsuji, A., I. Kobayashi, T. Oguri, M. Inoue, E. Yabuuchi, and S. Goto.** 2005. An epidemiological study of the susceptibility and frequency of multiple-drug-resistant strains of *Pseudomonas aeruginosa* isolated at medical institutes nationwide in Japan. J. Infect. Chemother. **11**:64–70.
53. **Walsh, T. R., M. A. Toleman, L. Poirel, and P. Nordmann.** 2005. Metallo-beta-lactamases: the quiet before the storm? Clin. Microbiol. Rev. **18**:306–325.
54. **Zimakoff, J., N. Hoiby, K. Rosendal, and J. P. Guilbert.** 1983. Epidemiology of *Pseudomonas aeruginosa* infection and the role of contamination of the environment in a cystic fibrosis clinic. J. Hosp. Infect. **4**:31–40.

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Evaluation of Antimicrobial Susceptibility For β-Lactams Using the Etest Method Against Clinical Isolates From 100 Medical Centers in Japan (2006)

Yoshikazu Ishii, Kazuhiro Tateda,
Keizo Yamaguchi, and the JARS