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Selection of Antigens and Development of Prototype Tests for Point-of-Care Leprosy Diagnosis▽†

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Leprosy can be a devastating chronic infection that causes nerve function impairment and associated disfigurement. Despite the recent reduction in the number of registered worldwide leprosy cases as a result of the widespread use of multidrug therapy, the number of new cases detected each year remains relatively stable. The diagnosis of leprosy is currently based on the appearance of clinical signs and requires expert clinical, as well as labor-intensive and time-consuming laboratory or histological, evaluation. For the purpose of developing an effective, simple, rapid, and low-cost diagnostic alternative, we have analyzed the serologic antibody response to identify *Mycobacterium leprae* proteins that are recognized by leprosy patients. More than 100 recombinant antigens were analyzed in a protein array format to select those with discriminatory properties for leprosy diagnosis. As expected, multibacillary leprosy patients recognized more antigens with stronger antibody responses than paucibacillary leprosy patients. Our data indicate, however, that multibacillary patients can be distinguished from paucibacillary patients, and both of these groups can be segregated from endemic control groups. We went on to confirm the diagnostic properties of antigens ML0405 and ML2331 and the LID-1 fusion construct of these two proteins by enzyme-linked immunosorbent assay. We then demonstrated the performance of these antigens in rapid test formats with a goal of developing a point-of-care diagnostic test. A serological diagnostic test capable of identifying and allowing treatment of leprosy could reduce transmission, prevent functional disabilities and stigmatizing deformities, and facilitate leprosy eradication.

Leprosy is a chronic mycobacterial infection that can result in significant nerve function impairment if left untreated and is historically associated with social ostracism. Leprosy patients present with a spectrum of bacteriologic, clinical, immunologic, and pathologic indicators ranging from the extremes observed in paucibacillary (PB) to multibacillary (MB) patients (18, 22). PB leprosy patients have a low or absent bacterial index (BI; a measure of the number of acid-fast bacilli in the dermis expressed on a logarithmic scale), one or a few skin lesions, and low or absent titers of *Mycobacterium leprae*-specific antibodies. In marked contrast, MB leprosy patients have a high BI, multiple symmetric skin lesions, and high titers of anti-*M. leprae* antibodies (18). WHO experts have listed diagnostic criteria as one or more of the following: hypopigmented or reddish skin patches with definite loss of sensation, thickened peripheral nerves, and acid-fast bacilli on skin smears or biopsies (WHO Expert Committee on Leprosy, 1998). While there are field-based tests, these are not widely used to provide point-of-care leprosy diagnosis, and reductions in the number of trained leprologists has increased the likelihood that clinical diagnosis is delayed or even missed, especially in regions where leprosy has been "controlled" (1, 11, 14, 23).

The amount of serum immunoglobulin M (IgM) antibody that can bind *M. leprae*-specific phenolic glycolipid I (PGL-I) correlates with the BI in leprosy patients. Enzyme-linked immunosorbent assay (ELISA) and rapid lateral-flow (LF) test formats have been developed for the detection of anti-PGL-I antibody (3, 4, 8, 17, 19, 20, 24). In one study, an LF assay correctly diagnosed 97.4% of the MB leprosy patients tested, with a specificity of 86.2% (4). PB leprosy patients, however, have a low or no BI, and the majority of these patients are not identified by PGL-I-based tests (4, 7, 17). In addition, false-positivity rates in areas where leprosy is endemic are relatively high (>10%) (4, 7, 17). Studies have argued that the presence of anti-PGL-I antibodies is an indicator of leprosy development, but this is controversial (5, 6, 12, 13). Many contacts of leprosy patients have anti-PGL-I antibodies but do not develop the disease, limiting the capacity of PGL-I-based assays to predict disease development. Consequently, it recommended that PGL-I-based tests be used in support of clinical examination to direct treatment and none of these PGL-I-based tests have been widely implemented in field situations.

A simple, objective, and field-applicable diagnosis of leprosy

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TABLE 1. Sample group characteristics (Nepal)

Sample category (total no.)	Mean BI (range)	Male/female ratio	Median (range) age (yr)
MB (25)	2.44 (0–6)	1.8	52 (18–76)
PB (25)	0	1.5	34 (16–62)
EC (45)	NA[a]	1.4	25 (17–58)

[a] NA, not applicable.

would greatly benefit monitoring programs and alleviate the burden on skilled clinical diagnosis or laboratory testing. We sought to discover protein antigens that permit the identification of leprosy patients and can be used in simple tests to permit a clear, simple, and relatively cheap diagnosis of leprosy.

MATERIALS AND METHODS

Subjects and samples. Leprosy patient (MB and PB) and endemic control (EC) individual sera were obtained after blood was drawn. The MB and PB leprosy patient sera used in this study were derived from recently diagnosed and previously untreated individuals. Leprosy patients were recruited at Anandaban Hospital, Kathmandu, Nepal, in 2006. Ten MB leprosy patients attending the Leonard Wood Memorial Center, Cebu City, Philippines, were also recruited. Patients were categorized by BI, skin lesions, nerve involvement, and histopathology. EC individuals were healthy individuals who had never had tuberculosis, had no history of leprosy in the family, and were living in the area where leprosy is endemic. In all cases, drawing of blood was carried out with informed consent and the approval of the local ethics committee in the relevant country. The composition of the Nepalese study population is summarized in Table 1. All serum specimens were preserved with 40% glycerol and stored at −20°C prior to assay.

Cloning and purification of target antigens. DNA encoding selected *M. leprae* proteins was PCR amplified from *M. leprae* Thai-53 genomic DNA with *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA). Gene-specific PCR primers were designed to amplify the gene coding sequence with restriction enzyme sites at the 5′ and 3′ ends. The PCR product was restriction enzyme digested and directionally cloned into expression vector pET28a (Novagen, Madison, WI) with a six-His tag at the N terminus. Each sequence-verified expression construct was transformed into strain BL21(DE3) to produce recombinant protein. Recombinant proteins were purified with Ni-nitrilotriacetic acid resin (Qiagen, Gaithersburg, MD) and quantified with the bicinchoninic acid protein assay (Pierce, Rockford, IL), and quality was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. An additional 97 *M. leprae* genes were expressed with a rapid translation system (RTS; ImmPORT Therapeutics, Irvine, CA).

Determining patient reactivity by protein array. Glass-based chips were fabricated with duplicate sets of a total of 71 recombinant *M. leprae* proteins by Full Moon Biosystems, Sunnyvale, CA (see Table S1 in the supplemental material). Human IgG and Epstein-Barr virus nuclear antigen 1 were included as positive control proteins to verify array development, and buffer alone was included as a negative background control. Each protein was printed onto an array, and printing was repeated such that replicates were spatially separated. Identical print patterns were thereby applied to the top and bottom of each array. An additional 97 *M. leprae* genes were expressed with an RTS, and these proteins were fabricated in duplicate sets onto nitrocellulose-based chips by ImmPORT Therapeutics Inc., Irvine, CA (see Table S2 in the supplemental material). The expression system is a bacterial cell-free transcription-translation system with tolerance for toxic genes and transmembrane domain-containing genes. Sufficient material is generated from each reaction to enable array printing without the need for purifying the proteins. The proteins selected for inclusion in the RTS analysis included the PE and PPE proteins, cell wall and potentially outer membrane proteins, and conserved hypothetical proteins. In these arrays, human IgG1 and Epstein-Barr virus nuclear antigen 1 were included as positive control proteins to verify array development, and a no DNA reaction was included as a negative background control. Protein binding in both arrays was confirmed by probing with anti-His antibody. Slides were blocked with 1X Protein Array Blocking Buffer (Whatman S&S, Sanford, ME) at room temperature for 1 h. The slides were then washed and incubated with sera diluted at 1/100 with blocking buffer at room temperature for 2 h. After washing, slides were incubated with biotin-conjugated mouse anti-human IgG (heavy and light chains; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at 1:200 at room temperature for 1 h, washed, and then incubated with Cy5-conjugated streptavidin (Martek Biosciences, Columbia, MD) at 1:200 for another 1 h at room temperature. Slides were washed, dried, and scanned at 635 nm with GenePix Pro 6.0 (Molecular Devices, Sunnyvale, CA). The signal intensity of binding of each antigen for each individual serum was normalized versus the buffer-alone spots for each individual serum to derive a fold-over-control value. Data tables were statistically analyzed in MS Excel (Microsoft, Redmond, WA).

Determining patient reactivity by ELISA. Polysorp 96-well plates (Nunc, Rochester, NY) were coated with 2 μg/ml recombinant protein or 200 ng/ml NDO-BSA (the synthetically derived B-cell epitope of PGL-I conjugated to bovine serum albumin [BSA], provided by John Spencer, Colorado State University, Fort Collins, under NIH contract N01 AI-25469) in bicarbonate buffer overnight at 4°C and blocked for 2 h at room temperature with phosphate-buffered saline (PBS)–Tween 20 with 1% (wt/vol) BSA on a plate shaker. Wells were coated with a volume of 50 μl/well. 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 (Southern Biotech, Birmingham, AL), 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 (KPL, Gaithersburg, MD), and the reaction was quenched by the addition of 1 N H_2SO_4. The corrected optical density (OD) of each well at 450 nm was read with a VERSAmax microplate reader (Molecular Devices).

Selection of immune reactive antigens by multiantigen print immunoassay (MAPIA). We performed the MAPIA as described earlier (15). Briefly, purified antigens were immobilized on nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH) at protein concentrations of 0.2 and 0.05 mg/ml with a semiautomatic microaerosolization device (Linomat IV; Camag Scientific Inc., Wilmington, DE) to generate invisible parallel bands. After antigen printing, the membrane was cut into strips 3.5 mm wide, perpendicular to the antigen bands, so that each strip carried all of the antigens. Next, the strips were blocked for 1 h with 1% nonfat milk in PBS containing 0.05% Tween 20 (PBST) and then incubated with individual serum samples diluted 1:50 in blocking solution for 1 h at room temperature. After being washed five times with PBST, the strips were incubated for 1 h with alkaline phosphatase-conjugated anti-human IgG diluted 1:5,000 (Sigma, St. Louis, MO) and anti-human IgM diluted 1:3,000 (Sigma). The strips were washed with PBST as described above, and the human IgG and IgM antibodies bound to immobilized antigens were visualized with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium substrate (KPL). MAPIA results were scored by two independent operators who were unaware of the sample status. The appearance of any band of any intensity was read as a positive reaction.

Determining patient reactivity by a single LF test and a dual-path platform (DPP). Based on LF technology, the DPP antibody detection assay was developed by ChemBio Diagnostic Systems, Inc. Each ready-to-use disposable device consists of a plastic cassette containing a strip of nitrocellulose membrane impregnated with lines of test and control antigens and laminated with several pads made of glass fiber and cellulose. Serum and four drops of diluent buffer are added sequentially to the sample port. Driven by capillary forces, the test sample migrates to the conjugate pad, where antibody-antigen binding can occur. Development of the assay is achieved by adding four drops of diluent buffer containing protein A to the development port. Again, driven by capillary forces, the diluent buffer migrates to the conjugate pad, where it can bind any antibody/antigen complexes that have been formed and thereby allow color development of the test antigen line. Irrespective of the presence of a specific antibody in the test sample, the liquid continues to migrate along the membrane to produce a similar color band at the control line of the device, thereby demonstrating proper functioning of the test reagents. Any visible line in the test area, in addition to the control line, is considered an antibody-positive result. Results were read by at least two investigators (with at least one unaware of the sample status) after 10 to 15 min and, based on the strength of the test band, scored as negative, +, ++, or +++.

Statistical analyses. Individual *P* values between diagnostic groups were determined with Student's *t* test. For the larger protein array data sets, analysis of variance and discriminant analysis were performed in Excel with the statistiXL software package (version 1.8; StatistiXL, Broadway, Nedlands, Western Australia, Australia). Post-hoc analysis was performed by Tukey's method. *P* values resulting from the post-hoc analyses were then ranked by magnitude, and gene products giving the most significant differences were chosen for further analysis.

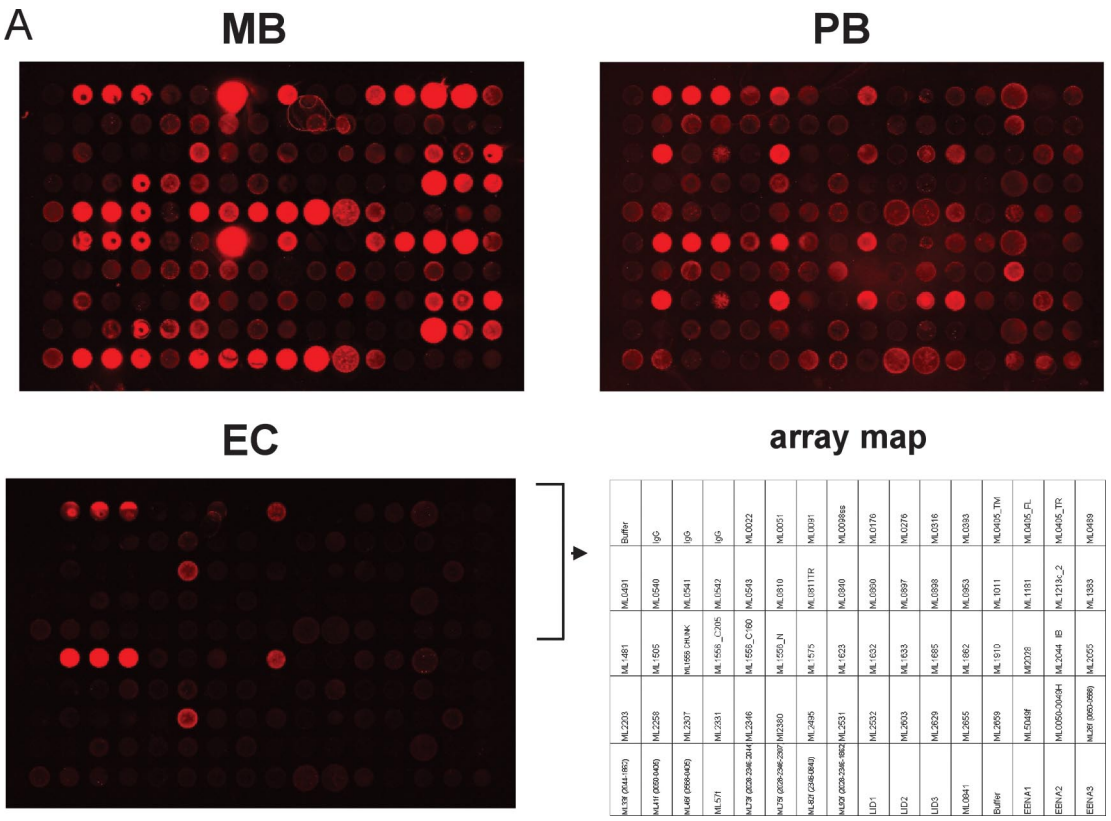

FIG. 1. Serum reactivity in *M. leprae* protein arrays. Protein arrays were incubated with MB or PB leprosy patient or EC individual sera, developed, and scanned. (A) Representative images of protein arrays from each group. Arrays were printed such that protein replicates were separated, and the identity of each spot is indicated by the array map. The protein pattern is duplicated at the top and bottom of each array. (B and C) Data sorted based upon the signal strength in arrays coated with recombinant proteins or RTS proteins, respectively. The *x* and *y* axes are scaled according to the OD at 635 nm. (D and E) Analysis of data from each individual patient to determine if they fall into distinct reactivity patterns by using data generated from all proteins or from a minimal protein set containing only the four genes with the most significant *P* values (ML0008, ML0308, ML1997, and ML2331). The axes represent the discriminant function score for each serum sample as evaluated with either the determined "MB discriminant function" (*x* axis) or the "PB discriminant function" (*y* axis).

RESULTS

Protein array screening for seroreactivity with *M. leprae* antigens. Following the recent publication of the *M. leprae* genome, our group and others have identified several antigens that demonstrate a potential to diagnose leprosy. We fabricated either glass- or nitrocellulose-based protein arrays with a total of 144 *M. leprae* proteins to comprehensively analyze the diagnostic potential of a multitude of antigens in a consistent and comparable fashion (see Tables S1 and S2 in the supplemental material).

In the initial testing, recombinant proteins, different variations of some proteins (e.g., ML0405FL, ML0405Tm, and ML0405Tr), and chimeric fusion proteins assembled from components of multiple proteins (e.g., LID-1, comprising ML0405 and ML2331) were expressed and immobilized in glass-based arrays. Arrays were tested with sera from untreated, clinically and histopathology-diagnosed leprosy patients (both MB and PB) or EC individuals from Nepal. Several proteins were recognized and bound by IgG within each serum and could be grouped as (i) nonspecific (i.e., binding all sera regardless of clinically diagnosed leprosy or not), (ii) leprosy sensitive but lacking specificity (i.e., binding leprosy patient sera but also binding some EC individual sera), and (iii) leprosy specific (i.e., binding leprosy patient sera but not EC individual sera) (Fig. 1A; see Table S1 in the supplemental material). Based on analysis of variance, the proteins that provided the best discrimination of MB leprosy patients from EC individuals were ML0405, ML2331, ML0050-0049 (a fusion construct of the *M. leprae* equivalent of ESAT-6 and CFP10), and a variety of fusion proteins containing either ML0405 or ML2331 (Fig. 1B). In Fig. 1B, only LID-1 is shown for clarity. Interestingly, two new proteins, ML2055 and ML0091, demonstrated robust discrimination between groups ($P < 0.001$) and are now being studied as diagnostic candidates. In this screen, only a few proteins showed a tendency to distinguish PB leprosy patients from EC individuals (Fig. 1B, $P > 0.01$).

In a second series of arrays, we used an RTS to express small amounts of proteins for immobilization on nitrocellulose-based membranes. Some proteins were duplicated across the two test formats to enable a fair comparison of the glass- and nitrocellulose-based arrays. In these arrays, only the control proteins (ML0405 and ML2331) and ML0411, a protein that had been previously described, demonstrated strong and re-

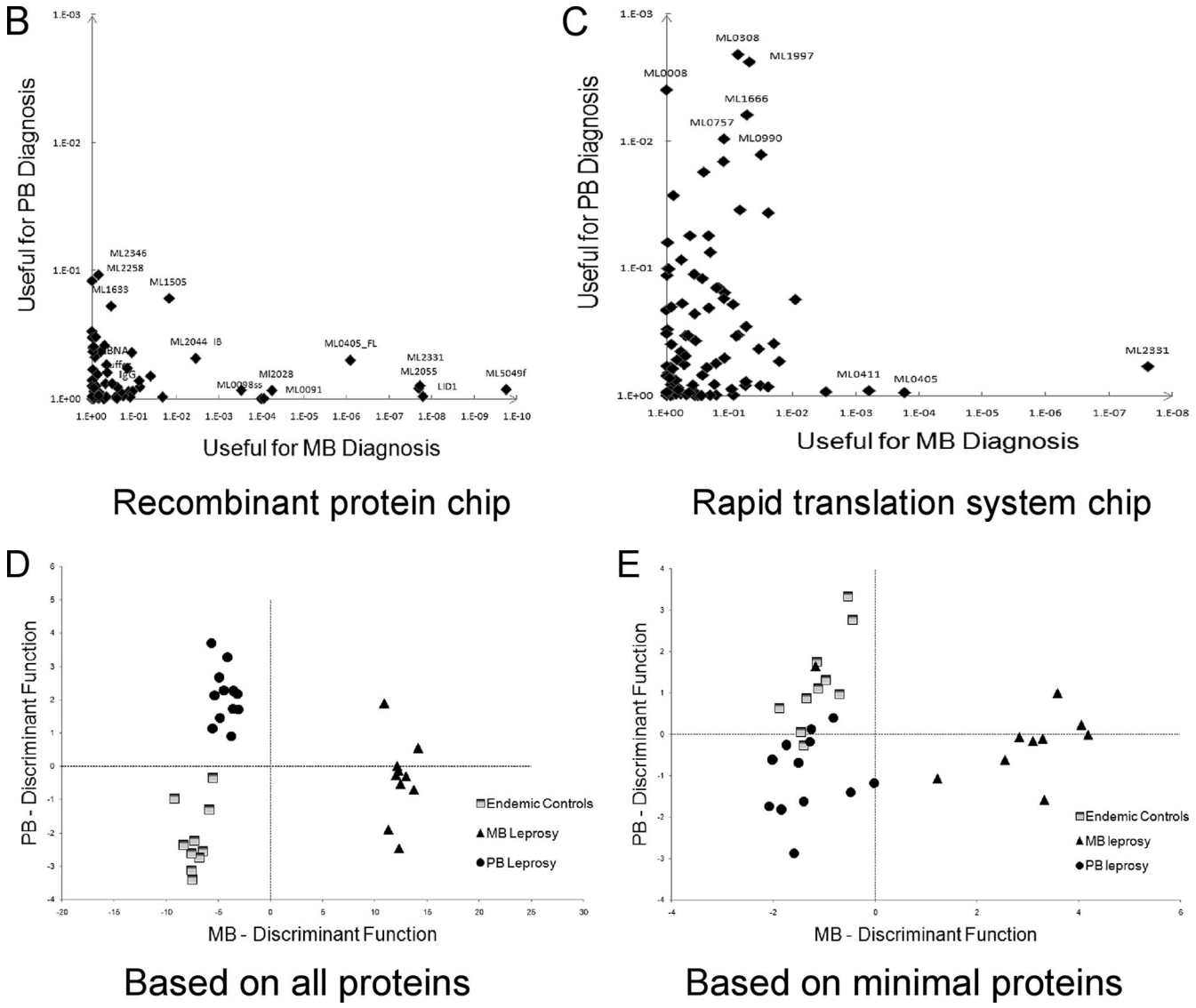

FIG. 1—*Continued.*

producible antibody binding when probed with leprosy patient sera (Fig. 1C; see Table S2 in the supplemental material). In this array, however, some candidate proteins had significant power to discriminate between the PB and EC groups ($P < 0.05$). Results from three proteins that provided significant PB discrimination (ML0008, ML0308, and ML1997) were combined with a protein that provided significant MB discrimination (ML2331) and used to perform a discriminant analysis. The discriminant functions explained 100% of the variance in the data set and had good statistical values (canonical correlations of 0.886 and 0.696, both $P < 0.001$). A holdout analysis to determine the predictive power of the model demonstrated that 90% of the MB leprosy patients were correctly classified, 83% of the PB leprosy patients were correctly classified, and 80% of the EC individuals were correctly classified.

Both protein arrays showed that of the 144 proteins investigated, ML0405 and ML2331 were robust markers of MB leprosy and are excellent candidates for an MB diagnostic test. Future studies of the other markers are now planned to add to the strength of MB leprosy diagnosis and to develop proteins for PB leprosy diagnosis.

Confirmation of seroreactivity by ELISA. To verify the results obtained by protein array analysis, we selected the antigens that provided the best sensitivity and specificity in protein array analyses and tested the same sera by ELISA. As expected, IgM in these sera bound NDO-BSA, the synthetic analogue of *M. leprae* PGL-I (Fig. 2). In agreement with data we recently generated with sera from Brazilian, Filipino, and Japanese leprosy patients, IgG in sera from MB leprosy patients in Nepal bound antigens ML0405Tr and ML2331, as well as LID-1 (the fusion construct of these two antigens; Fig. 2) (10). With positive values defined as two times the mean OD of the EC group, NDO-BSA detected slightly more MB leprosy patients than recombinant antigens did but was less specific (Table 2). When weakly positive responses (arbitrarily determined to be OD values less than five times the mean of the EC

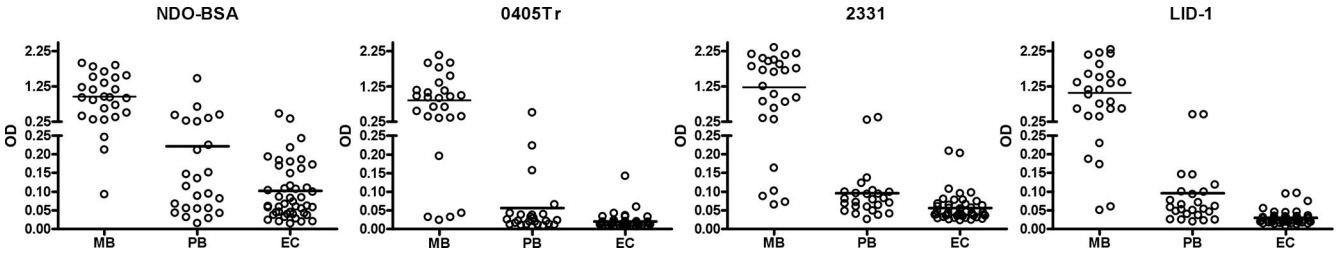

FIG. 2. *M. leprae* proteins react with leprosy patient sera. Antibody reactivity of sera from 25 clinically diagnosed MB leprosy patients, 25 clinically diagnosed PB leprosy patients, and 45 EC individuals was assessed against NDO-BSA, ML0405 (0405Tr), ML2331 (2331), and LID-1. NDO-BSA reactivity was assessed by IgM binding, and recombinant protein reactivity was assessed by IgG binding.

group) were excluded, however, we found that the recombinant protein antigens retained their diagnostic capacity for MB leprosy diagnosis but the ability of NDO-BSA waned (Table 2). Thus, the protein antigens provide the best discrimination between MB leprosy patients and EC individuals.

In comparison with MB leprosy patients, PB leprosy patients have low or absent anti-NDO-BSA responses and are more difficult to diagnose serologically. In agreement, although 32% of the PB leprosy patient sera tested positive with regard to NDO-BSA binding, only 8% of the sera yielded strongly positive results (Table 2). In contrast, 48% of the PB leprosy patient sera tested positive with regard to LID-1 binding but only 12% of the sera yielded strongly positive results.

Taken together, these results indicate that, similar to NDO-BSA, in Nepal, LID-1 may be useful for the diagnosis of MB leprosy but has a limited capacity for the diagnosis of PB leprosy.

MAPIA-based selection of antigens for rapid diagnosis platforms. MAPIA involves the immobilization of antigens on nitrocellulose membranes and constitutes a step in the development of a rapid point-of-care diagnostic test. Selected *M. leprae* antigens were painted onto nitrocellulose membranes to assess their performance in the MAPIA. Membranes were incubated with MB leprosy patient sera (Fig. 3A) or EC individual sera (Fig. 3B) and developed with either anti-IgG or anti-IgM antibodies. As expected, LID-1 (and its individual components) provided positive results when developed with anti-IgG antibody and NDO-BSA provided positive results when developed with anti-IgM antibody. The ability of these antigens to complement each other for leprosy diagnosis was also evident in the MAPIA. For example, sera 4 and 5 gave a weak or no response to NDO-BSA by anti-IgM detection but gave clear positive responses to LID-1 by anti-IgG detection. Serum 7 gave a weak or no response to LID-1 by anti-IgG detection but gave a clear positive response to NDO-BSA by anti-IgM detection. Also of note, several EC individual sera gave positive responses to NDO-BSA when detected by anti-IgM. This response was reduced when less NDO-BSA was painted onto the nitrocellulose membrane. This contrasted with the clean negative responses to LID-1 when detected by anti-IgG. These data indicate the utility of LID-1 in a simple test format for leprosy diagnosis.

Development of a rapid test for leprosy diagnosis. Previous reports have demonstrated that some simple PGL-1-based LF tests hold potential for the rapid point-of-care diagnosis of leprosy (3, 4). Most single LF tests, however, are limited in sensitivity. To test and assess rapid methods for leprosy diagnosis, we constructed both simple single (LF) and advanced DPP LF assays with LID-1 as the test antigen. When comparable quantities of protein (0.2 μg) were applied to these crude and unoptimized tests, the LF test was capable of detecting 5 of 10 MB leprosy patient sera and the DPP test detected 10 of 10 MB leprosy patient sera. In addition, stronger signals were obtained with the DPP test than with the LF test (Fig. 4, $P < 0.00001$). When serum was diluted to assess the sensitivity threshold of each assay, the DPP assay was approximately 100 times more sensitive than the LF assay (Fig. 4C). Taken together, these data demonstrate that the DPP test is an improved test platform for diagnosing leprosy.

TABLE 2. Comparison of antigen ELISA signal strengths

Sample response and category	% of samples reacting to:			
	NDO-BSA	ML0405Tr	ML2331	LID-1
Positive[a]				
MB	96	88	84	92
PB	32	20	16	48
EC	8.89	6.67	4.44	6.67
Strongly positive[b]				
MB	68	84	80	92
PB	8	12	8	12
EC	0	2.22	4.44	0

[a] A positive response was considered an OD more than two times the mean of EC samples.
[b] A strongly positive response was considered an OD more than five times the mean of EC samples.

DISCUSSION

The ultimate elimination of leprosy will be greatly aided by the development and availability of inexpensive, accessible, and accurate diagnostic tests capable of detecting early infection before symptoms of disease develop and transmission of infection occurs. To date, leprosy diagnosis has relied on invasive tests requiring trained clinicians (WHO Expert Committee on Leprosy, 1998). With the relatively successful implementation of multidrug therapy, however, the number of trained leprologists has been significantly reduced and fewer individuals can reliably diagnose leprosy. This is particularly true in regions where leprosy is not endemic or has been "controlled" and has increased the likelihood that diagnosis will be delayed or even missed (1, 11, 14, 23). This is particu-

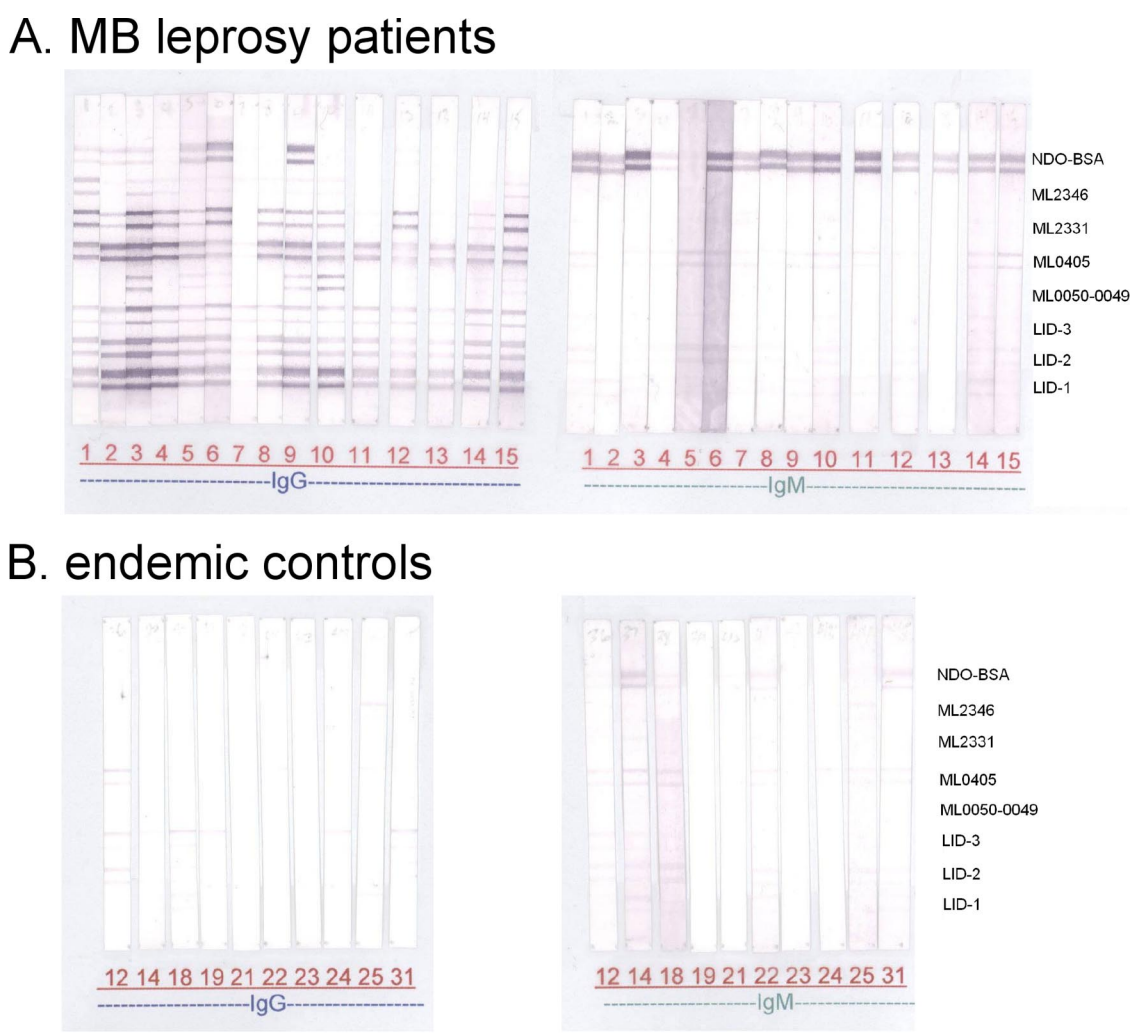

FIG. 3. Antigen reactivity in the MAPIA. Selected *M. leprae* antigens at 0.2 mg/ml (upper band) and 0.05 mg/ml (lower band) were used to coat nitrocellulose membranes, which were then incubated with sera from MB leprosy patients (A) or EC individuals (B). Following incubation with sera, membranes were developed with either anti-IgG (left panels) or anti-IgM (right panels). Results are representative of 25 MB leprosy patient sera and 20 EC individual sera.

larly troubling because the earlier a patient is identified and treated, the better the disease outcome (9). Additionally, it is well documented that close contact with leprosy patients, particularly MB leprosy patients, increases the risk of developing the disease (2, 16, 17, 21). Early detection and treatment will make it more likely that a patient's bacterial load will be limited and the potential to transmit viable *M. leprae* will be reduced.

To develop a simple and rapid diagnostic test for leprosy based on serologic responses, firstly, we evaluated the diagnostic potential of a large panel of *M. leprae* antigens by utilizing protein arrays to identify the antigens with the best diagnostic potential. Secondly, we confirmed and validated results obtained with protein arrays by ELISA. Thirdly, we selected the top candidate diagnostic protein and demonstrated the potential of the LID-1 antigen in a rapid diagnostic test format. The diagnostic capacity of several *M. leprae* proteins has been previously examined, providing a variety of results. To date, the presence of serum antibody to PGL-I has been used as an indicator of *M. leprae* infection or exposure, and anti-PGL-I levels correlate with BI in leprosy patients. MB leprosy patients with a high BI demonstrate high anti-PGL-I levels, whereas PB leprosy patients with a low or absent BI demonstrate low anti-PGL-I levels. ELISA and rapid LF test formats have been developed for the detection of anti-PGL-I antibody, and tests reliably detect MB leprosy patients (3, 4, 8, 17, 19, 20, 24). False-positive result levels in areas where leprosy is endemic, however, are relatively high, and many contacts of leprosy patients have anti-PGL-I antibodies despite never developing the disease (4, 7, 17).

We expressed a large panel of *M. leprae* antigens for screening in an array format against defined leprosy patient sera. As expected, many proteins were found not to react with patient sera (nonreactive) while others reacted with patient sera but also with control sera (nonspecific). These proteins were therefore incapable of diagnosing leprosy and were excluded from further analyses. Some antigens demonstrated various degrees of reactivity with leprosy patient sera but minimal reactivity

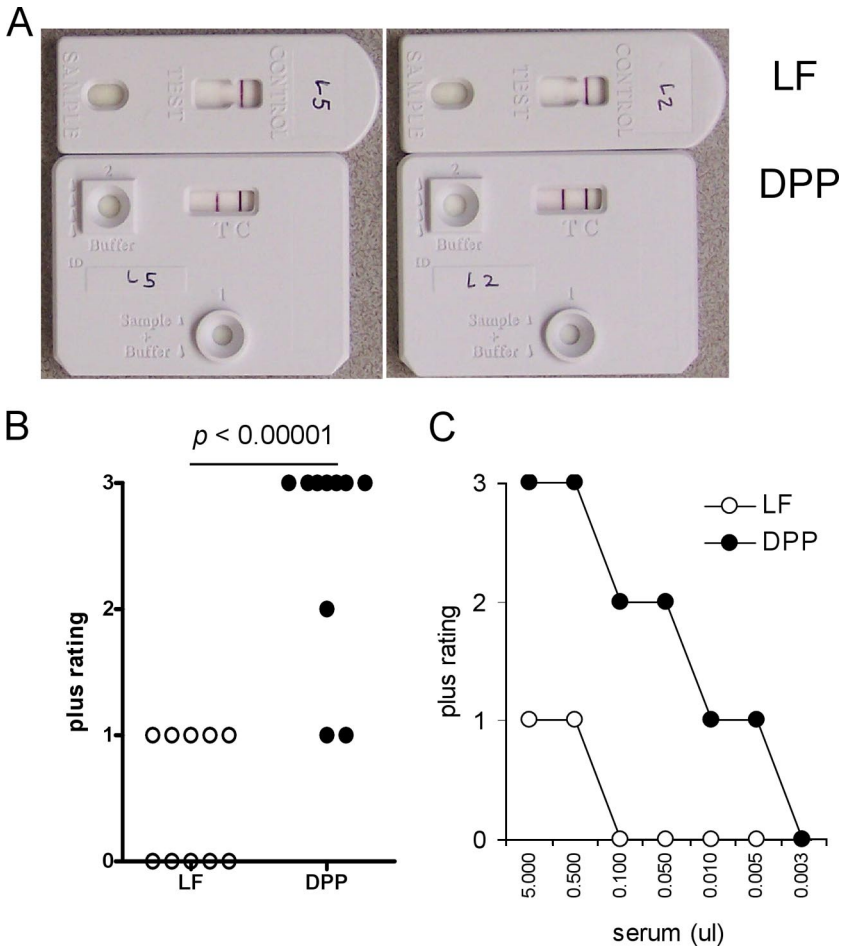

FIG. 4. Enhanced signal in DPP tests compared with single LF tests. LID-1 antigen was used to coat single LF and DPP tests, which were developed with sera from 10 Filipino MB leprosy patients. (A) Representative LF and DPP tests. The LID-1 (test) band develops in the left portion of the window, and the control band develops in the right portion of the window. (B) Undiluted sera were added to each test, and the test signal was scored as 0, +, ++, or +++. Each point describes the rank attributed to an individual sample. (C) A single serum sample that was positive by both the LF and DPP tests was serially diluted and retested in each format.

with control sera, indicating their potential for leprosy diagnosis. Analyses of antigens ML0405, ML2331, and LID-1 by ELISA allowed more accurate determination of patient serum reactivity. The antigens performed well when reacted with MB leprosy patient sera, returning a greater number of strongly positive responses than NDO-BSA. When reacted with PB leprosy patient sera, however, the antigens recognized only a subset of samples, similar to the pattern observed with NDO-BSA. We are currently further examining antigens that demonstrated reactivity with PB leprosy patient sera in the hope of identifying leprosy-specific B-cell epitopes. Using these epitopes as adjuncts to the LID-1 protein may improve sensitivity and signal strength without compromising specificity, thereby improving overall leprosy diagnosis.

Antigens were then assessed for retention of binding when immobilized on nitrocellulose membranes. The MAPIA format is a rapid test format that more closely resembles a final point-of-care test. In the MAPIA, the LID-1 antigen (and its components ML0405 and ML2331) showed good reactivity with MB leprosy patient sera when developed with anti-IgG antibody, with little or no binding of EC individual sera. Surprisingly, the fusion protein ML0050-0049 did not react with as many MB leprosy patient sera as the other antigens and was not selected for inclusion within a refined rapid test format. The reason for this discrepancy is unclear, but protein folding or charge may affect epitope availability and results.

In a preliminary evaluation of a potential final test format, we compared two variations of LF tests (LF and DPP). Recent advances in the design and construction of LF-based tests indicate that by allowing serum antibodies to interact with antigen as a first step and then detecting these complexes as a separate second step (as opposed to having antibodies interact with the detecting agent to form a complex prior to antigen binding, as occurs in a simple LF assay), the sensitivity of these assays can be markedly improved. We have confirmed the increased sensitivity of DPP test strips over similarly coated LF test strips, with our results indicating that the DPP test with LID-1-coated strips is 100 times more sensitive than the LF test with LID-1-coated strips. The study described here was conducted with stored sera, and this could result in higher sensitivity than in-field testing with whole blood. More viscous samples create larger clumps or aggregates with detector par-

ticles and result in poor performance of LF assays, as we have observed with several LF products. Viscous samples are less likely to compromise results in the DPP format, however, due to the independent migration of samples and detector particles. Regardless, whole blood may provide results inferior to those obtained with serum and extensive evaluation of the DPP leprosy assay with different test fluids is required.

Taken together, our data suggest that the DPP leprosy assay is a viable option to improve leprosy diagnosis when laboratory facilities are not available or when results are needed at the point of care. A simple test that can be performed within minutes and without expert technical skills such as those required for biopsy and histology will be significantly cheaper than current practice. Efforts are now focusing on wider evaluation of the DPP leprosy test in field trials.

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