A simple dipstick assay for leprosy: development, evaluation and application
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CHAPTER II

A SIMPLE DIPSTICK ASSAY FOR THE DETECTION OF ANTIBODIES TO PHENOLIC GLYCOLIPID-I OF MYCOBACTERIUM LEPRAE

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2.1 Abstract

Among the many reported applications of the detection of antibodies to phenolic glycolipid - I (PGL-I) of *Mycobacterium leprae*, in particular, the use of seroprevalence as an indicator of the magnitude of the leprosy problem may turn out to be very useful in leprosy control programs. An operational function of serology within the leprosy control services requires a simple test system. We have developed a simple dipstick assay for the detection of antibodies to PGL-I and we compared its performance with that of ELISA. A high degree of agreement (97.2%) was observed between ELISA and the dipstick assay when tested on 435 sera; the agreement beyond chance (Kappa value) was 0.92. No significant difference was found between the dipstick assay and ELISA when seropositivity rates obtained in groups of leprosy patients, household contacts, and controls were compared. The interpretation of the dipstick results as positive or negative was unequivocal, as illustrated by the high agreement between different persons reading the test (Kappa values >0.88). Storage of the only reagents required, the dipsticks and the stabilized detection reagent, up to three weeks under tropical conditions of high temperatures, high humidity, and exposure to light did not influence the results of the assay. The dipstick assay described here is an easy-to-perform method for the detection of IgM antibodies to PGL-I of *M. leprae*; it does not require any special equipment and the highly stable reagents make the test robust and suitable for use in tropical countries. An internal control validates the performance of the assay. This dipstick assay may be the method of choice for epidemiologic mapping of leprosy.
2.2 Introduction

Leprosy, caused by *Mycobacterium leprae*, is a disease that at present has a registered prevalence of 1.3 million people in the world currently on anti-leprosy chemotherapy, but the estimated number of cases in the world reaches about 1.8 million. Because of the severe handicaps, as a result of permanent and extensive deformities of the skin and the peripheral nerves, leprosy is associated with social stigma and economic loss. Thus, the problem of leprosy is greater than the number of cases would suggest. Although leprosy is likely to decrease to less than one case per 10,000 by the year 2000, many more leprosy patients can be expected to emerge in several leprosy-endemic countries beyond that year.

Serologic tests for detection of antibodies to *M. leprae* have a clear, though limited value. Notably, serologic tests based on the detection of IgM class antibodies against the immunodominant 3,6-di-O-methyl-glucopyranosyl residue of the trisaccharide component of the phenolic glycolipid-I (PGL-I) antigen of *M. leprae* have been widely studied. These serologic tests have been shown to be a useful addition to the classic methods of diagnosis. Furthermore, they may be valuable in monitoring the effectiveness of chemotherapy, the emergence of relapse, and in identifying patients with a high risk of presenting a sudden inflammatory phase of exacerbation (lepra reactions) during therapy. In addition, serologic testing of populations has been shown to be of value in monitoring changes in the magnitude of *M. leprae* infection and as such may provide a rapid method to establish the effect of control measures.

An operational function of serology within the leprosy control services would certainly require a simpler test system than the ELISA. Agglutination tests are simpler than the ELISA, but these are still too elaborate for widespread use under field conditions.

Here, a simple dipstick for the detection of antibodies to PGL-I is described and its performance is compared with that of an ELISA. This dipstick test does not depend on any specialized equipment and makes use of highly stable reagents that do not require cold storage.
2.3 Materials and Methods

2.3.1 Study population

The population studied included multibacillary (MB) and paucibacillary (PB) patients classified according to the load of bacteria in the skin, household contacts, and controls from the Philippines. The MB leprosy patients included borderline, borderline lepromatous (BL), and lepromatous patients with a bacterial index (BI) of at least 2+ at any one site. The PB leprosy patients included indeterminate, tuberculoid, and borderline tuberculoid (BT) with BIs < 2+ at any one site. These parameters follow the World Health Organization (WHO) recommendation. The contacts were persons living in the same household as the MB or PB cases in the last three years. The control population was composed of persons living in the same community as the patients (presenting other forms of skin diseases) but free of clinical signs of leprosy and with no case of leprosy in their households. In addition, sera were tested from patients residing in The Netherlands with syphilis (n=5), hepatitis A infection (n=5), hepatitis B infection (n=5), toxoplasmosis (n=5), human immunodeficiency virus (HIV) infection (n=5), and autoimmune disease (n=5).

The collection of blood from the study subjects was approved by the Leonard Wood Memorial Institutional Review Board (Human Rights Committee) (Cebu, The Philippines) and the Ministry of Health (Manila) Ethical Committee. Written informed consent was obtained from all subjects. For collection of sera from patients residing in The Netherlands, approval was obtained from the Medical Ethical Committee of the Academic Medical Center (Amsterdam, The Netherlands).

2.3.2 Preparation of dipsticks

The dipsticks were prepared exactly as previously described, except that disaccharide-bovine serum albumin (DBSA), a semi-synthetic analog of the PGL-I antigen of M. leprae, was used at a concentration of 2 μg/ml to coat the nitrocellulose strips. The antigen was provided by WHO/Steering Committee on Immunology of Leprosy through Dr. J. Colston (National Institute for Medical Research, London, United Kingdom). The nitrocellulose strips containing the antigen were then adhered to a plastic support with double-sided tape and cut in 2.5 mm-wide sticks. To obtain an internal control, anti-human IgM antibodies were coated onto the nitrocellulose as a separate band.

2.3.3 Preparation of the detection reagent

A monoclonal anti-human IgM antibody was conjugated to palanil red, a colloidal dye, according to a patented method. The stained antibody was suspended in a special freeze-drying solution as previously described and lyophilized for preservation.
2.3.4 Execution of the dipstick assay

Serum dilutions (1:50) were made directly into the reconstituted detection reagent. Dipsticks were wetted in distilled water for 15 sec to prevent adsorption of the detection reagent to the solid support and then incubated for 3 hr at room temperature in a reaction vial containing 0.2 ml of the detection reagent and 4 μl of serum. At the end of the incubation period, the dipsticks were rinsed with tap water and after removal of excess of liquid with a paper towel, air-dried at ambient temperature. A reddish-stained antigen band indicated a positive reaction. Staining of varying intensities was scored as positive; no color was scored as negative.

2.3.5 Internal control

The internal control consisted of anti-human IgM antibodies coated to nitrocellulose. These antibodies bind IgM molecules from the serum that are stained by the detection reagent. The internal control facilitates interpretation of the assay results and checks the integrity of the ingredients.

2.3.6 Serological assay

The ELISA for the detection of IgM antibodies to PGL-I of *M. leprae* was performed essentially as described previously using the semi-synthethic analog DBSA. The DBSA (0.1 μg/ml) was diluted in carbonate buffer (pH 9.6) and coated overnight at 37°C in a moist chamber onto wells (50 μl/well) of microtiter plates (Immunoplates-II (Nunc, Roskilde, Denmark). As a control 0.1 μg/ml bovine serum albumin (BSA) was used. The microtiter plates were blocked for 60 min with 100 μl of 1% BSA in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). After the plates were washed three times with PBST, the sera were diluted 1:300 in PBST containing 10% normal goat serum (NGS) and 50 μl was added to each well. The plates were then incubated at 37°C for 60 min and another wash step was performed. Peroxidase-conjugated anti-human IgM conjugate (Cappel/Organon Teknika, Turnhout, Belgium) was added (50 μl/well) at a 1:2,000 dilution in PBST-10% NGS to the microtiter plate. After incubation at 37°C for 60 min, the washing procedure was repeated and 50 μl of a 0.1 M citrate-phosphate buffer containing 0.4 mg/ml of o-phenylenediamine and 0.0066% hydrogen peroxide were added to each well. To control for plate-to-plate and day-to-day variation, a positive reference serum was included in triplicate on each plate. The color reactions of the entire plate were stopped with 50 μl 2 N H₂SO₄ when the optical density at 492 nm (OD₄₉₂ₙ㎜) from a positive control serum reached a value of 0.6. The ODs were measured in a spectrophotometer using a 492 nm filter. All sera were tested in duplicate and the ELISA results were expressed as mean absorbance of the duplicates. The final OD value of each serum sample was calculated by subtracting the OD value of wells coated with BSA from the OD value of the test wells coated with DBSA. The cut-off value for positivity was an OD of 0.200.
2.3.7 Statistical analysis

The variation between the dipstick assay and the IgM ELISA was determined by calculating Kappa values with 95% confidence intervals. Kappa values express the agreement beyond chance. Generally, a Kappa value $> 0.80$ represents almost perfect agreement beyond chance.
2.4 Results

2.4.1 Reading the results of the dipstick assay

Figure 1 shows representative results of the dipstick assay obtained with serum from an endemic control, a BT leprosy patient, and a BL leprosy patient. The internal control band is clearly visible in all three cases (Figure 1), as was true for every tested serum.

![Figure 1. Representative results of the dipstick assay. Dipstick A shows serum from an ELISA-negative (OD$_{492\text{nm}}$ = 0.001) endemic control, dipstick B shows serum from a borderline tuberculoid leprosy patient (OD$_{492\text{nm}}$ = 0.250) and dipstick C shows serum from a borderline lepromatous leprosy patient (OD$_{492\text{nm}}$ = 0.929). Both the antigen band (Ag) and the internal control band (IC) are indicated. OD = optical density.](image)

2.4.2 Comparison between ELISA and dipstick assay

A high degree of agreement (97.2%) was observed between the ELISA and the dipstick assay (Table 1). The agreement beyond chance (Kappa value) was 0.92. A similar number of sera was found positive for the study group with both tests (Table 2). In the group of MB patients, 74.1% and 79.6% seropositivity was observed for the ELISA and the dipstick assay, respectively. In the PB group, we found 13.6% seropositivity for both the ELISA and dipstick assay. Seropositivity for household contacts was 3.7% for the ELISA and 1.9% for the dipstick assay. In the group of controls from the same endemic area, 1.7% seropositivity was observed in both tests. No significant difference was found between dipstick assay and ELISA seropositivity in any of the groups (Table 2). When only endemic controls and household contacts were considered (n=224), agreement between the two tests was 99.1 %, with a Kappa value of 0.79 (standard error of Kappa: 0.06). All of the sera (n=30) from the patients with syphilis, hepatitis A or B, toxoplasmosis, HIV infection or autoimmune disease were negative in both the dipstick assay and ELISA.
Table 1. Comparison between ELISA and dipstick assay for the detection of antibodies to *Mycobacterium leprae* phenolic glycolipid I*

<table>
<thead>
<tr>
<th></th>
<th>ELISA</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ (N)</td>
<td>- (N)</td>
<td></td>
</tr>
<tr>
<td>Dipstick</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ (N)</td>
<td>96</td>
<td>8</td>
<td>104</td>
</tr>
<tr>
<td>- (N)</td>
<td>4</td>
<td>327</td>
<td>331</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>335</td>
<td>435</td>
</tr>
</tbody>
</table>

*The agreement was 97.2% with a kappa value of 0.92 (SE 0.04, p<0.0001)

Table 2. Comparison between ELISA and dipstick assay according to the classification of the study group

<table>
<thead>
<tr>
<th>Class</th>
<th>N^0</th>
<th>Dipstick Positivity (%)</th>
<th>ELISA Positivity (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endemic controls</td>
<td>116</td>
<td>1.7</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Household contacts</td>
<td>108</td>
<td>1.9</td>
<td>3.7</td>
<td>0.68</td>
</tr>
<tr>
<td>PB patients</td>
<td>103</td>
<td>13.6</td>
<td>13.6</td>
<td>1.00</td>
</tr>
<tr>
<td>MB patients</td>
<td>108</td>
<td>79.6</td>
<td>74.1</td>
<td>0.41</td>
</tr>
<tr>
<td>Total</td>
<td>435</td>
<td>23.9</td>
<td>23.0</td>
<td></td>
</tr>
</tbody>
</table>

*No significant difference was found between ELISA and dipstick assay seropositivity in any of the groups (Chi square test)
2.4.3 Discrepancy analysis of the dipstick assay and ELISA results

Seven MB leprosy patients and one PB patient were found to be ELISA negative (mean OD = 0.165; SD = 0.037; minimum value = 0.037; maximum value = 0.200) and dipstick positive. Conversely, two patients (1 MB and 1 PB) and two household contacts were ELISA positive and dipstick negative (mean OD 0.260; SD = 0.044, minimum value = 0.210; maximum value = 0.310). Figure 2 illustrates graphically the relationship between the dipstick and the ELISA results.

Figure 2. Graphic presentation of ELISA and dipstick results of 435 sera
The horizontal line indicates the cut-off value of the ELISA (optical density [OD] = 0.2).
2.4.4 Reproducibility

The results of 344 dipstick assays were independently read by two other observers and their results were compared with ours. The agreement was 97.1% (Kappa = 0.91, SE = 0.05) and 96.4% (Kappa = 0.89, SE = 0.05). The differences in the readings were due to interpretation of very weak positive results.

2.4.5 Test conditions

In all experiments the dipsticks were incubated for 3 hr at room temperature (20-24°C). In addition, we performed the dipstick assay with 10 of the samples at 37°C. Performance of the assay at this temperature, while reducing the incubation time to 2 hr, did not affect the results. Incubation at 37°C for 2 hr caused background staining.

2.4.6 Storage

The lyophilized detection reagent was stored for one year at various temperatures (i.e. 4°C, 20°C, 37°C and 56°C) without loss of activity. The dipsticks were stored at temperatures of 4°C, 20°C, 37°C, 40°C and 56°C, and exposed to light (SON-T, 400 W, 380-780 nm; Philips, Eindhoven, The Netherlands) and high humidity. When a sample of the sera (n=5) were tested with the dipsticks stored under the various conditions, we observed that exposure to light and humidity longer than 28 days interfered with the reading of the dipstick results: the staining of both the antigen band and the internal control band became weaker compared with the unexposed dipsticks. The dipsticks stored at 56°C showed an increased background staining after 14 days of storage, but the results could still be properly interpreted up to 21 days. The other conditions had no influence on the readability of the test results up to three months of storage.
2.5 Discussion

Among the many reported applications of the detection of antibodies to PGL-I of *M. leprae* is its use in leprosy control programs with seroprevalence as an indicator of the magnitude of the leprosy problem.

Several studies have documented a relationship between the leprosy burden in a population and seroprevalence in different parts of the world. In addition, Baumgart and others found that a decrease in leprosy prevalence upon introduction of multi-drug therapy in a confined community in Papua New Guinea was accompanied by a reduction in the seropositivity rate among children.

Mandatory to further evaluation of serology as a tool for epidemiologic mapping of leprosy would be the availability of a simple assay. We have developed a simple dipstick assay for the detection of antibodies to PGL-I of *M. leprae* with a performance comparable with an ELISA, but which does not require specialized equipment or refrigeration.

We found high agreement between the dipstick assay and the ELISA on an individual specimen basis. Seropositivity rates obtained with the dipstick assay in different groups of patients and controls did not significantly differ from the ELISA and were in agreement with ELISA seropositivity rates reported previously in this same population. Disagreement between the results of the dipstick assay and the ELISA were found in only 2.8% of the sera tested. All these sera had values in the ELISA close to the cut-off value. A high agreement (99.1%) was also found between the two tests when only the endemic controls and the household contact population were considered for analysis.

The specificity of the detection of antibodies to PGL-I in the ELISA has been previously shown to be high. The dipstick assay is also specific, as illustrated by the absence of reactivity with 30 sera from patients with common diseases other than leprosy.

Although the dipsticks results are assessed by the human eye as positive or negative, in contrast with the ELISA results, which are determined by a spectrophotometer, the interpretation of the dipstick results was unequivocal, as illustrated by the high agreement between different test observers.

To mimic tropical conditions, we examined the influence of high temperatures, light and high humidity on the performance of the dipstick assay. Storage of the dipsticks and the lyophilized detection reagent up to three weeks under such conditions did not influence the results, while the lyophilized detection reagent was stable for at least one year under these conditions. Performance of the dipstick assay at a temperature of 37°C did not influence the result of the assay, provided that the incubation period was reduced to prevent background staining.
In conclusion, the dipstick assay described here is an easy-to-perform method for the detection of IgM antibodies to PGL-I of *M. leprae*. An internal control validates the performance of the assay. Due to its robustness and simplicity the dipstick assay seems to be highly suitable for application under field conditions, and may prove to be suitable to quickly and easily assess seroprevalence and thus indicate the magnitude of the leprosy problem in a specific area.

2.6 Acknowledgement

We are grateful for the financial support received from the Netherlands Leprosy Relief Association.
2.6 Reference List


Chapter II
