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

THE USE OF WHOLE BLOOD IN A DIPSTICK ASSAY FOR DETECTION OF ANTIBODIES TO *Mycobacterium leprae*: A FIELD EVALUATION

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3.1 Abstract

We describe a further simplification of a dipstick assay for the detection of antibodies to phenolic glycolipid I of *Mycobacterium leprae* by using whole blood and evaluated the assay performance in the leprosy endemic area of Amazonas in Brazil. The agreement with the “gold” standard ELISA was 94.9% (κ value = 0.87). This simple assay may be useful to identify those at risk of developing leprosy, for example among contacts of leprosy patients at lower levels in the health services.

3.2 Introduction

The introduction of relatively short treatment regimens for leprosy, an infection caused by *Mycobacterium leprae*, has resulted in a sharp decrease of the number of registered leprosy patients in the world. Notwithstanding the approximately constant number of new cases detected world-wide every year, leprosy seems thereby to have been reduced to a tangible health problem. Health reforms combined with the recent push to further eliminate leprosy as a public health problem will result in decentralization of leprosy control services and their integration into general health services. Health workers in non-specialized peripheral facilities will have to be able to diagnose the disease and monitor treatment results.

Elevated antibody levels to the phenolic glycolipid I (PGL-I) antigen of *M. leprae* indicate a risk for developing disease, as well as the type, transmission and extent of the disease. Detection of antibodies may assist in diagnosis of leprosy and mostly recognition of potential multibacillary (MB) cases among contacts.

An operational role for serology at a peripheral level in leprosy endemic areas would require a simple test system. To fulfil that requirement, we have recently developed a simple dipstick assay for the detection of antibodies to PGL-I, which does not require any specialized equipment and makes use of highly stable reagents, not depending on refrigeration. However, in this test serum was used.

Here, we describe a further simplification of the dipstick assay, using whole blood. In addition, we report on the performance of the test carried out by a leprosy field worker in an endemic area in Brazil.
3.3 Materials and Methods

3.3.1 Study population

The population studied included untreated and treated multibacillary (MB) \((n=123)\) and paucibacillary (PB) leprosy patients \((n=55)\), household contacts of leprosy patients \((n=42)\) and patients with skin diseases other than leprosy \((n=33)\) attending the Institute “Alfredo da Matta”, Manaus, Brazil. MB leprosy patients were those with more than five skin lesions or a positive bacterial index (BI) in any of 4 skin smears. The MB group was composed of 43 lepromatous (LL), 38 borderline lepromatous (BL), 24 borderline (BB) and 18 tuberculoid borderline patients (BT). PB leprosy patients were those with up to five skin lesions and negative BI in all 4 skin smears. The PB group was composed of 12 indeterminate (I), 31 tuberculoid (TT) and 12 borderline tuberculoid patients (BT). This classification follows the World Health Organization (WHO) recommendation. The contacts were persons living in the same household as the MB or PB cases.

Serum and blood samples were collected from all of the above mentioned study population \((n=253)\). Whole blood was collected by finger-prick in heparinized capillary tubes.

In addition, serum from 112 healthy blood donors residing in Manaus, Amazonas was used as negative controls in the ELISA (see below) to determine the cut-off value for positivity.

3.3.2 Dipstick assay

The dipstick assay for the detection of antibodies to PGL-I of \(M. \text{leprae}\) was used as described before. The dipsticks bear two bands: an antigen band consisting of the \(M. \text{leprae}\)-specific and immunodominant disaccharide epitope of phenolic glycolipid I (PGL-I) linked to BSA (disaccharide-BSA or DBSA) and an internal control band consisting of anti-human IgM antibodies that bind IgM molecules from the serum. The antigen was provided by WHO/IMMLEP through Dr. J. Colston (National Institute for Medical Research, London, UK). The IgM detection reagent consists of a lyophilized monoclonal anti-human IgM antibody linked with a colloidal dye. Briefly, dipsticks were wetted in distilled water for 15 s and then incubated for 3 h in a reaction vial containing 0.2 ml of the reconstituted detection reagent and 4 \(\mu\)l serum. At the end of the incubation period the dipsticks were rinsed with tap water and air-dried at ambient temperature. A reddish stained antigen band upon visual inspection indicates a positive reaction. The results were scored as positive when staining, irrespective of its intensity, was observed; no coloring was scored as negative.

When whole blood was used, the procedure followed was the same, except that 10 \(\mu\)l of the blood sample was diluted, immediately after collection, in 200 \(\mu\)l of the detection reagent.
The dipstick assay was performed in Manaus, Brazil by a fieldworker whose normal duty was to collect skin smears from leprosy patients and who had no experience with serological assays.

3.3.3 ELISA

The ELISA for the detection of IgM antibodies to PGL-I of *M. leprae* was performed essentially as described previously\(^2\) using DBSA as the semi-synthetic analogue of PGL-I. DBSA (0.1 μg ml\(^{-1}\)) was diluted in carbonate buffer (pH 9.6) and coated overnight at 37°C in a moist chamber, onto wells (50 μl well\(^{-1}\)) of Nunc-Immunoplates-II (Nunc, Denmark). As a control 0.1 μg ml\(^{-1}\) bovine serum albumin (BSA) was used. Microtitre plates were blocked for 60 min with 100μl of a 1% BSA in PBS containing 0.1% Tween 20 (PBST). After washing three times with PBST, the sera were diluted 1:300 in PBST containing 10% normal goat serum (NGS) and 50μl was added to each well. This was incubated at 37°C for 60 min and followed by another wash-step. Peroxidase conjugated anti-human IgM conjugate (Cappel/Organon Teknika, Turnhout, Belgium) was added (50 μl well\(^{-1}\)) at a 1:2000 dilution in PBST-10% NGS to the microtitre 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 0.4mg ml\(^{-1}\) O-phenylenediamine and 0.0066% hydrogen peroxide were added to each well. In order 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 H\(_2\)SO\(_4\) when the optical density at 492nm (OD\(_{492nm}\)) from a positive control serum reached an OD value of 0.6. ODs were measured in a spectrophotometer using a 492-nm filter. All sera were tested in duplo 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 only with BSA from the OD value of the test wells coated with DBSA. The cut-off value for positivity was OD=0.250, corresponding to the mean value of the presumably negative blood donors (n=112) plus two times the standard deviation.

3.3.4 Statistical evaluation

The variation between the dipstick assay and the IgM ELISA was determined by calculating values with 95% confidence intervals. \(\kappa\) values express the agreement beyond chance. Generally, a \(\kappa\) value of \(>0.80\) represents almost perfect agreement beyond chance. \(^1\)
3.4 Results and discussion

The agreement between ELISA (continuous measurement) and dipstick assay (discrete result) critically depends on the cut-off value of the ELISA. As can be seen in Fig. 1, the concordance, sensitivity and specificity of the dipstick assay performed on serum in relation to the ELISA are all consistently high above cut-off values of OD>0.25. At the cut-off value (OD=0.25) used in this study a high agreement (96.5%, κ=0.89) was observed between the ELISA and the dipstick assay both performed with sera (n=253). This cut-off value resulted in an ELISA-positivity rate among healthy blood donors of 1.8% (2/112) which was comparable to the one previously reported (1.7%; 2/116).

None of the blood donors was positive in the dipstick assay. These findings confirm previously reported results on sera from a geographically different population.

Figure 1. Performance characteristics of the dipstick assay using serum in relation to ELISA at different cut-off values
A similar high agreement was found comparing the dipstick assay performed on whole blood compared to the ELISA on the corresponding serum (Table 1). The concordance was 94.1% (κ=0.85). Table 2 shows the results of the dipstick assay performed on paired samples of whole blood and serum. The agreement was 94.9% with a κ value of 0.87. Together, these results confirm the good concordance between dipstick assay and ELISA results using sera, reported previously and in addition, show that the agreement is equally good when the dipstick assay for the detection of antibodies to PGL-I is performed on whole blood and under tropical field conditions.

**Table 1. Agreement between ELISA (serum) and dipstick assay (blood)**

<table>
<thead>
<tr>
<th>Category</th>
<th>ELISA+/Dipstick+</th>
<th>ELISA-/Dipstick-</th>
<th>ELISA+/Dipstick-</th>
<th>ELISA-/Dipstick+</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB leprosy</td>
<td>60</td>
<td>58</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>PB leprosy</td>
<td>5</td>
<td>44</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Household contacts</td>
<td>1</td>
<td>39</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Other skin diseases</td>
<td>1</td>
<td>30</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>67</strong></td>
<td><strong>171</strong></td>
<td><strong>8</strong></td>
<td><strong>7</strong></td>
</tr>
</tbody>
</table>

Agreement between ELISA and dipstick assay = 94.1% (kappa = 0.85)

MB = multibacillary

PB = paucibacillary

**Table 2. Agreement between dipstick assays performed with serum and with blood**

<table>
<thead>
<tr>
<th>Category</th>
<th>Dip S+/Dip B+</th>
<th>Dip S-/Dip B-</th>
<th>Dip S+/Dip B-</th>
<th>Dip S-/Dip B+</th>
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<tbody>
<tr>
<td>MB leprosy</td>
<td>60</td>
<td>56</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>PB leprosy</td>
<td>6</td>
<td>45</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Household contacts</td>
<td>1</td>
<td>39</td>
<td>1</td>
<td>1</td>
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<tr>
<td>Other skin diseases</td>
<td>1</td>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>68</strong></td>
<td><strong>172</strong></td>
<td><strong>7</strong></td>
<td><strong>6</strong></td>
</tr>
</tbody>
</table>

Dip S = dipstick performed on serum; Dip B = dipstick performed on blood

Agreement between dipstick assay performed with serum and dipstick assay performed with whole blood = 94.9% (kappa = 0.87)

MB = multibacillary PB = paucibacillary

The ELISA detected 39.3% (70/178), the dipstick 40.4% (72/178) of all leprosy patients using serum and 39.9% (71/178) using blood (Tables 1 and 2); these sensitivities were not significantly different from each other (χ² test; all P>0.8). It should be noted that the patient
population included paucibacillary patients and treated patients, both known to have a low bacillary load and presenting low antibody titers.  

Samples from 19 individuals (7.5%) showed discrepant results in any of the three tests. These same samples produced the same inconsistent results between the assays upon repeated testing in the Netherlands (results not shown). The OD values found in ELISA with most of these sera were close to the cut-off value for positivity (mean OD=0.322, SD=0.210). Several explanations can be put forward to explain discrepancies between the results of the assays. A positive dipstick in combination with a negative ELISA result may be due to the more stringent nature of the ELISA requiring a stronger affinity for the antibody, as ELISA uses multiple washing with detergent to ensure tight binding of the antigen/antibody complex. The differences in dilution of the sample, 1:50 in dipstick vs. 1:300 in ELISA, may also favor sensitivity of the dipstick. On the other hand, substances present in blood/serum that may inhibit antigen/antibody interaction would favor sensitivity of ELISA compared to the dipstick assay. The discrepancies between dipstick results obtained with blood and serum from the same patient may be due to the height of the hematocrit, which could effectively result in a different dilution of antibodies in whole blood compared to serum. The limited volume of the samples prevented further investigation of the precise nature of these few discrepant results.

In conclusion, the findings presented here show that whole blood can be used to perform the dipstick assay for the detection of antibodies to PGL-I of *M. leprae* and that this dipstick assay performs well under tropical field conditions. This facilitates the use of the test under field conditions by local health workers without the need of laboratory facilities. The next step will be to investigate the potential role of the dipstick test in the successful detection of leprosy in potential patients and its value as a tool to classify leprosy patients as PB or MB for treatment purposes.

3.5 Acknowledgement

The financial support by the Netherlands Leprosy Relief Association (NSL) and the Gastmann-Wichers Foundation is greatly appreciated. We thank the Brazilian Government’s Department of Health Dermatology represented by Dr. Maria Leide Wand-Del-Rey for constructive suggestions and assistance. We thank Dr. Dagmar Kiesslich, Fundação de Hematologia e Hemoterapia do Amazonas (HEMOAM), for her help in this study.
Chapter III

3.6 Reference List


