A simple dipstick assay for leprosy: development, evaluation and application
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Citation for published version (APA):

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CHAPTER IV

THE USE OF ML DIPSTICK AS A TOOL TO CLASSIFY LEPROSY PATIENTS

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Int. J. Lepr. Other Mycobact. Dis. (accepted)
Chapter IV

4.1 Abstract

Leprosy control services face the problem of leprosy patients being misclassified by lack or poor quality of skin smear examination services. Misclassification increases the risk of relapse due to insufficient treatment if a multibacillary (MB) patient is classified as paucibacillary (PB), thereby also prolonging the time that the patient is infective.

The WHO recommends at present an alternative classification based on the number of skin lesions. Its reliability, however, has been questioned. Our investigation sought to determine the usefulness of the ML Dipstick, a simple field assay to detect IgM antibodies to PGL-I of *M. leprae*, for the classification of leprosy patients in addition to lesion count.

In this study 264 leprosy patients were investigated. Of 130 patients with a positive bacterial index (BI), 19 (14.6%) had less than six lesions and would have been classified as PB. Out of 134 patients with a negative BI, 26 (19.4%) had six or more lesions and would have been classified as MB patients if the lesion counting system would apply. Thus, the classification based on the number of lesions only was found to be 85% sensitive and 81% specific (using the BI as the gold standard) at detecting MB cases among the studied population.

Sensitivity would have increased if patients would have been classified according a combination of the number of lesions and the dipstick result. In that case patients are classified as MB when they are either dipstick positive (n=16), have more than 6 lesions (n=43), or both (n=94). Patients negative for both dipstick and number of lesions would have been classified as PB (n=111). The classification based on the number of lesions alone left 19 BI positive cases classified as PB, while the combination method of ML Dipstick and number of lesions left only 8 BI positive cases classified as PB (5 borderline, 2 borderline lepromatous and 1 tuberculoid), thus preventing undertreatment. The combination method of ML Dipstick and lesion counting was found to be 94% sensitive and 77% specific, which is an improvement of 9% (Chi square test, $p=0.025$) in the sensitivity compared to lesion counting only.

The results of this study indicate that testing with the dipstick all patients initially classified by lesion counting as PB (48% in our study population) can significantly contribute to improved classification of leprosy patients for treatment purposes.
4.2 Introduction

Classification of leprosy patients into PB and MB determines the duration of their treatment. MB patients are treated for a period of 24 months with a monthly-supervised combination therapy consisting of rifampicin, clofazamine and dapsone, whereas PB patients are treated for 6 months with rifampicin and dapsone. 24

The methods used for leprosy classification have changed significantly over the years. In 1982 the WHO defined PB patients as those with indeterminate (I), tuberculoid (TT) or borderline tuberculoid (BT) leprosy with a bacterial index (BI) of less than 2 at all sites, MB patients as those with a BI of 2 or higher at any site. 25 Later the definition was changed and patients with a positive BI at any site were classified as MB. 23 However, as the results of skin smears were often of poor quality or not available at all, 24 further simplification of the classification of patients has been introduced based on the number of body areas affected by skin and nerve lesions 22 and by an even simpler classification in some countries grouping patients with less than 6 skin lesions as PB patients and patients with 6 or more skin lesions as MB patients. 26 However, the reliability of classification on clinical criteria only has been questioned, since a classification system based on lesion count only is prone to-underestimation of the number of lesions, especially in areas of the world where cultural factors may influence the physical examination of the patient. Underestimation of the number of lesions can lead to misclassification of MB patients as PB 10 and thus undertreatment.

The changes over the years in leprosy patient classification systems illustrate the difficulty of defining the border between PB and MB. Misclassification increases the risk of relapse due to insufficient treatment of an MB patient classified as PB, thereby increasing the time that the patient is infective. Long before a relapse becomes manifest, leprosy patients may be highly infectious, transmitting the disease in the community.

The classification systems based either on bacteriological smear examination or on clinical findings have the need for well-trained leprosy workers in common, a possible limitation considering the integration of leprosy control into the general health service in many countries. In addition, both systems are liable to subjective interpretation. The currently practised classification system based on clinical findings would benefit from a simple and robust test, which gives results that are related to the bacterial load.

Several studies have shown that the presence of antibodies to the Mycobacterium leprae-specific phenolic glycolipid-I (PGL-I) correlates with the bacterial load of a leprosy patient. 21 The large majority of PB patients are seronegative whereas the large majority of MB patients are seropositive. 22 Studies monitoring the serum antibody levels to PGL-I during treatment further demonstrate that these levels correlate with the bacterial load: a decline during treatment corresponds with declining bacterial
indices. In addition, increasing levels of antibodies to PGL-I in patients have been associated with the development of relapses. Detection of anti-PGL-I antibodies may thus be a useful tool to confirm the diagnosis of MB leprosy.

Recently, we have developed a dipstick that detects IgM antibodies to PGL-I and which is suitable for field use. Studies demonstrate a high degree of agreement (97.2%) between the dipstick assay and the ELISA. The dipstick is a simple and rapid test, not dependent on specialized equipment and employs highly stable reagents that do not require refrigeration.

Here we investigated the potential usefulness of the dipstick as a tool in classifying leprosy patients into PB or MB for treatment purposes.

4.3 Materials and methods

4.3.1 Study population

The population studied included 264 untreated leprosy patients attending the leprosy clinic at Oswaldo Cruz Foundation, Rio de Janeiro, Brazil between January 1995 and May 1999. The Fiocruz Ethical Commission approved the study. All patients had given their consent to participate in the study.

Sera from patients were collected and kept frozen at -20°C. Patients were diagnosed based on clinical, bacteriological and histopathological findings according to Ridley and Jopling and the BI and the number of lesions were recorded. The BI was calculated as the mean BI of 6 skin smears. Clinical, bacteriological and histopathological findings were recorded. For our study, we use the BI results to divide the patients into PB and MB with a positive BI result at any site leading to classification as MB patient.

The study population was composed of 16 indeterminate (I), 4 tuberculoid (TT), 102 borderline tuberculoid (BT), 47 borderline (BB), 42 borderline lepromatous (BL), 43 lepromatous (LL) and 10 primary neuritic (PN) leprosy patients.

4.3.2 Dipstick assay

The dipstick assay for the detection of antibodies to PGL-I of *M.leprae* was prepared as described before. The dipsticks have two bands: an antigen band consisting of the *M.leprae*-specific and immunodominant disaccharide epitope of PGL-I linked to bovine serum albumin via an octyl linker arm (ND-O-BSA) and an internal control band consisting of anti-human IgM antibodies that bind IgM molecules from the serum. The IgM detection reagent consists of a lyophilised monoclonal anti-human IgM antibody linked with a colloidal dye. Briefly, dipsticks were wetted in distilled water for 15 sec and then incubated for 1 h in a reaction vial containing 200 μl of the reconstituted detection
The use of ML Dipstick as a tool to classify leprosy patients

reagent and 4 µl 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 indicates a positive reaction. The results were scored as positive when staining was observed; no coloring (but with a positive control band) was scored as negative.

4.3.3 ELISA

The ELISA for the detection of IgM antibodies to PGL-I of *M. leprae* was performed essentially as described previously \(^3\) using ND-O-BSA as the semi-synthetic analogue of PGL-I. ND-O-BSA (0.0023 µg of sugar/ml) was diluted in a volatile ammonium acetate carbonate buffer (pH 8.2). Nunc-Immunoplates-II (Life Technologies, Taastrup, Denmark) were coated with 50 µl/well and left to dry at room temperature. As a control 0.1 µg/ml bovine serum albumin (BSA) was used. Microtitre plates were blocked for 60 minutes with 100 µl of a 1% (w/v) BSA in PBS. After washing six times with PBS containing 0.1% (v/v) Tween-20 (PBST), the sera were diluted 1:300 in PBST containing 10% (v/v) normal goat serum (NGS) and 50 µl was added to each well. This was incubated at 37°C for 60 minutes and followed by another wash-step. Peroxidase conjugated anti-human IgM conjugate (Capple/Organon Teknika, Turnhout, Belgium) was added (50 µl/well) at a 1:2000 dilution in PBST-10% NGS to the microtitre plate. After incubation at 37°C for 60 minutes, the washing procedure was repeated and 50 µl of the Sigma 3,3',5,5'-tetramethyl-benzidine (TMB) liquid substrate system was added to each well. In order to control for plate-to-plate and day-to-day variation, a positive reference serum was included in quadruplicate on each plate. The color reactions of the entire plate were stopped with 50 µl 2.5N H2SO4 when the optical density (OD) at 450 nm from the reference control serum reached an OD value of 0.6. 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 only with BSA from the OD value of the test wells coated with ND-O-BSA. The cut-off value for positivity was OD=0.200. \(^4\)

4.3.4 Data analysis

Data were analyzed using Epi-info version 6. The agreement between the dipstick, number of lesions and bacterial index was determined by calculating kappa values with 95% confidence interval (CI). Kappa values express the agreement beyond chance. Generally, a kappa value of 0.60 to 0.80 represents a substantial agreement beyond chance and a kappa value of >0.80 represents almost perfect agreement beyond chance.
Chapter IV

4.4 Results

Of the 264 patients 134 were BI negative and classified as PB and 130 patients were BI positive and classified as MB.

4.4.1 Classification according to number of lesions

The columns in Table 1 show how patients would have been classified based on counting the number of lesions: 137 as MB and 127 as PB patients. Out of 130 patients with a positive BI, which are therefore by definition MB patients, 19 (14.6%) had less than six lesions and would have been classified as PB using the lesion counting system. Eighteen of them were treated with a MB regimen based on the histopathological results. The one treated with a PB regimen was a TT case with a negative dipstick result. Eleven were dipstick positive: 4 BB, 4 BL and 3 LL patients.

Out of 134 patients with a negative BI, therefore by definition PB patients, 26 (19.4%) had six or more lesions and would be classified as MB patients if the lesion counting system would have been applied. Five were dipstick positive: 4 BT and 1 PN. Twenty-three were treated as PB patients. The three patients treated as MB (1 BT, 1 BB, 1 BL) were all dipstick negative. The sensitivity of the WHO system of classification based on the number of lesions for detecting MB cases in our study was 85% (95%CI, 77.9-90.7) and the specificity 81%, for our study population the positive predictive value (PPV) was 81% and the negative predictive value (NPV) 85%. The agreement between the bacteriological classification and lesion counting classification was 83.0%, kappa 0.66 (95% CI, 0.54-0.78).

Table 1. Comparison between the BI and skin lesion counting systems of classification

<table>
<thead>
<tr>
<th>Number of lesions</th>
<th>&gt;6 (MB)</th>
<th>&lt;6 (PB)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI positive (MB)</td>
<td>111</td>
<td>19</td>
<td>130</td>
</tr>
<tr>
<td>BI negative (PB)</td>
<td>26</td>
<td>108</td>
<td>134</td>
</tr>
<tr>
<td>Total</td>
<td>137</td>
<td>127</td>
<td>264</td>
</tr>
</tbody>
</table>
4.4.2 Classification according to ML Dipstick

Table 2 shows how patients would have been classified based on the presence of antibodies to PGL-I using the results of ML Dipstick. One hundred and ten would have been classified as MB and 154 as PB patients. From 130 patients with a positive BI (MB patients by definition), 30 (23.1%) (1 TT, 15 BB, 8 BL, 6 LL) were dipstick negative and would have been classified as PB if only dipstick classification would have been applied.

Out of 134 patients with a negative BI, therefore by definition PB patients, 10 (7.5%) had antibodies to PGL-I and would have been classified as MB patients according to dipstick results. They were all treated as PB. The mean skin BI from the dipstick negative group was significantly lower than that of the dipstick positive group (0.336 [SD=0.994] versus 2.450 [SD=1.518]; p=0.00005), as was the mean ELISA value (0.117 [SD=0.250] versus 1.716 [SD=1.164]; p=0).

The sensitivity of the ML Dipstick was 77% (95% CI, 68.6-83.7) and the specificity 93%, with a PPV of 91% and an NPV of 81% in our study population. The agreement was 85%, kappa 0.7 (95% CI, 0.59-0.83).

Table 2. Comparison between the BI and ML Dipstick results

<table>
<thead>
<tr>
<th>ML Dipstick result</th>
<th>Positive (MB)</th>
<th>Negative (PB)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BI positive (MB)</td>
<td>100</td>
<td>30</td>
<td>130</td>
</tr>
<tr>
<td>BI negative (PB)</td>
<td>10</td>
<td>124</td>
<td>134</td>
</tr>
<tr>
<td>Total</td>
<td>110</td>
<td>154</td>
<td>264</td>
</tr>
</tbody>
</table>

4.4.3 Classification combining ML Dipstick and number of lesions

Table 3 gives a detailed overview of the correlation between BI classification on one hand and the results of ML Dipstick and lesion counting on the other. Table 4 shows how patient classification systems based on either BI or a combination of lesion counting and dipstick would compare.
**Table 3. Comparison between BI, skin lesion counting and dipstick results**

<table>
<thead>
<tr>
<th></th>
<th>Number of lesions and ML Dipstick</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N+/dip+</td>
<td>N+/dip-</td>
<td>N-/dip+</td>
<td>N-/dip-</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>BI positive (MB)</td>
<td>89</td>
<td>22</td>
<td>11</td>
<td>8</td>
<td>134</td>
<td></td>
</tr>
<tr>
<td>BI negative (PB)</td>
<td>5</td>
<td>21</td>
<td>5</td>
<td>103</td>
<td>130</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>43</td>
<td>16</td>
<td>111</td>
<td>264</td>
<td></td>
</tr>
</tbody>
</table>

N+ = number of lesions >6; N- = number of lesions <6; dip+ = ML Dipstick positive; dip- = ML Dipstick negative

**Table 4. Comparison between the BI classification system and the classification method based on a combination of lesion counting and dipstick result**

<table>
<thead>
<tr>
<th></th>
<th>Combination method result</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive(MB)(^a)</td>
<td>Negative(PB)(^b)</td>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BI positive (MB)</td>
<td>122</td>
<td>8</td>
<td>130</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BI negative (PB)</td>
<td>31</td>
<td>103</td>
<td>134</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>153</td>
<td>111</td>
<td>264</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Positive (MB) = (N+/dip+) + (N+/dip-) + (N-/dip+)
\(^b\) Negative (PB) = (N-/dip-)

N+ = number of lesions >6; N- = number of lesions <6; dip+ = ML Dipstick positive; dip- = ML Dipstick negative

Patients which scored positive in dipstick only (n = 16), in number of lesions only (n = 43) and in both methods (n = 94) would all be classified as MB. Patients which were negative for dipstick and had less than 6 lesions would be classified as PB (n = 111). Eight patients would be missed using the combined method (1 TT, 5 BB, 2 BL). All were treated with an MB regimen, except for the TT case.

The sensitivity of the combination method was 94% (95% CI, 87.8-97.1) and the specificity 77%. PPV and NPV were 80% and 93%, respectively. The observed agreement was 85%, kappa 0.71 (95% CI, 0.59-0.83).
4.5 Discussion

For operational use, in the great majority of leprosy endemic countries the classification of patients into PB and MB for treatment purposes is not based on the bacterial index but determined on the basis of the number of lesions of the patient. Here, we have explored the possibility of using the detection of antibodies to PGL-I through a simple dipstick assay as a marker for the bacterial load of a patient and consequently as an additional tool for the classification into pauci- and multibacillary leprosy. We showed that the system of clinical classification based on lesion counting in combination with the dipstick generated both a higher sensitivity for detecting MB patients and an increased ability to correctly identify PB cases.

Classification of patients is subjective and therefore it is difficult to get consistency under field conditions. Even in an ideal setting there will still be a small but significant number of BI positive, therefore MB, cases being treated with a PB regimen. Croft and co-workers \(^9\) reported a sensitivity of 89\% for the WHO system of classification based on counting lesions. In our study we found a somewhat lower sensitivity of 85\%. It means that 15\% of the MB patients would be incorrectly treated as PB. It is known that MB patients receiving PB treatment due to misclassification are susceptible to develop a relapse. \(^10\)

When comparing dipstick and BI, the 85\% agreement shows that the seropositivity found corresponded well with the BI of patients. Others have reported comparable results. \(^7;11;21\) Since antibodies to PGL-I are thought to reflect the bacterial load of the host, \(^1;2;11;13;15;17\) it is likely that the dipstick negative and BI positive patients have less bacteria in their bodies than the BI of the skin suggests, but it is impossible to assess whether these patients are being over-treated. We have seen in our previous study that BI positive patients, therefore MB, who did not present antibodies to PGL-I did not relapse although they had received a short course of treatment. In contrast, all relapsed MB cases were seropositive in the dipstick. \(^6\)

It was shown before that patients may have a low or negative BI in their skin, while bacteria may be found in the deeper tissue and in nerves. \(^18\) Since antibodies to PGL-I are a reflection of the total bacterial load in the body, again we could argue that the 10 PB patients with a positive dipstick result may have a higher bacterial load and therefore should have received longer treatment. False-positive classification into MB and consequent over-treatment, although slightly affecting cost-effectiveness of the control program, can nevertheless be favored over false-negativity and under-treatment, since that would result in a less effective control program due to the emergence of relapses. \(^22\)

As described above, we found a sensitivity of 85\% for the WHO system of classification according to the number of lesions. When combining the two methods, dipstick and lesion counting, we found a higher sensitivity (94\%) to detect true MB cases and the chance of
classifying a PB patient correctly was 93%. Still, 8 BI positive MB patients (6%) would be missed and treated as PB. Although they presented with low antibody levels it was not possible to assess if 6 months of therapy had been sufficient. For this, a trial should be performed in which treatment is co-determined by the dipstick result. Previous studies indicate that antibody detection can be used for classification of leprosy patients. The use of antibody detection as an additional tool for leprosy classification may be the key to decreasing the significant numbers of MB patients suspected to be under-treated by the lesion counting system. The results of this study suggest that testing all patients initially classified as PB (48% in our study population) with the dipstick assay would be very helpful in this respect.

A combination of both methods showed a significant decrease in misclassification compared to the lesion counting only. We report an improvement of 9% (Chi square test, $p= 0.025$) in the sensitivity, therefore the combined method is a useful tool for the classification of leprosy patients under field conditions. The dipstick method is simple, can be performed using either serum or finger prick blood, and is easy for health workers to use.

### 4.6 Acknowledgements

This study was financially supported by the Netherlands Leprosy Relief and the Scientific Research for the Tropics (WOTRO) fund of NWO (Nederlandse Organisatie voor Wetenschappelijk Onderzoek).

ND-O-BSA (Contract NO1 AI 55262, to CSU, PJB, PI) was kindly provided by Dr. D. Chatterjee, Colorado University, Denver, USA.

We thank Dr. Elisabeth Pereira Sampaio for her support and help during the research.
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