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

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Citation for published version (APA):
CHAPTER V

IDENTIFICATION OF LEPROSY PATIENTS WHO HAVE AN INCREASED RISK OF RELAPSE USING A SIMPLE DIPSTICK ASSAY

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(submitted)
5.1 Abstract

Classification of leprosy patients into paucibacillary (PB) and multibacillary (MB) determines the duration of treatment; misclassification increases the risk of relapse due to insufficient treatment if an MB patient is classified as PB. Due to the integration of leprosy control into the general health service, diagnosis and classification will be primarily in the hands of less experienced professionals. Therefore, a system which circumvents subjective interpretation and which requires only simple training is very much welcome.

Here, we explored the possibility of using a simple dipstick assay based on the detection of antibodies to the *Mycobacterium leprae*-specific phenolic glycolipid-I (PGL-I) as a tool for classification of patients into PB and MB for treatment purposes. The sensitivity of the dipstick test for detection of MB patients was 85.1%, the specificity 77.7%. We found that of the 71 dipstick negative PB patients 25 (35.2%) were clinically cured at the end of treatment, compared to only 2 (9.5%) of the 21 dipstick positive PB patients. Nine of 170 (5.3%) patients in the study population relapsed within the 5 year follow-up period. Seven were MB patients, all of which were dipstick positive. Two PB patients relapsed, one was dipstick negative and one was dipstick positive.

Here we show that dipstick positivity is a risk factor for the future development of relapses, especially in those groups of patients who had received a shorter-than-usual course of treatment and that the dipstick can be used as an additional, simple tool for classification of patients and for identification of those patients who have an increased risk of relapse.
Identification of leprosy patients who have an increased risk of relapse using a simple dipstick assay

5.2 Introduction

Leprosy is an infectious disease in which clinical signs and symptoms vary widely. There is no 'gold standard' for diagnosis and the diagnosis of leprosy is based generally on clinical symptoms. Classification of leprosy patients into paucibacillary (PB) and multibacillary (MB) determines the duration of treatment; misclassification increases the risk of relapse due to insufficient treatment if an MB patient is classified as PB. 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. The methods used for leprosy classification have changed significantly over the years. The classification criteria based on bacteriological findings have now been abandoned in most places, as the setting up and maintenance of reliable skin smear services was often difficult. Classification is now based on allocation of patients with less than 6 skin lesions to the PB group and patients with 6 or more lesions to the MB group. The reliability of classification on these criteria alone has been questioned since a classification system based on lesion counting only is prone to an underestimation of the number of lesions and will misclassify patients who have less than 6 lesions but should be classified as MB based on a positive bacterial index (BI).

Due to the integration of leprosy control into the general health service, diagnosis and classification will be primarily in the hands of less experienced professionals. Therefore, a system which is less liable to subjective interpretation and requires only simple training is very much welcome.

Several studies have shown that the presence of antibodies to the Mycobacterium leprae-specific phenolic glycolipid-I (PGL-I) correlate with the bacterial load of a leprosy patient. The majority of MB patients have high levels of antibodies to PGL-I, while in general PB patients are seronegative. Studies monitoring the serum antibody levels during treatment further demonstrate this correlation of serum antibodies to PGL-I as indicators of the bacterial load: declining antibody levels during treatment correspond with declining bacterial indices. The observation that increasing levels of antibodies to PGL-I in patients can be associated with the development of relapses also indicates a relation between PGL-I antibody levels and the presence of M. leprae.

Here, we explore the possibility of using a simple dipstick assay based on the detection of these antibodies as an easy tool for classification of patients into PB and MB for treatment purposes and for the prevention of relapse.
5.3 Materials and Methods

5.3.1 Study population

The samples used in this study were from leprosy patients that were included in a multi-center prospective study in Manaus, Brazil from 1992 until 1994. The patients were treated according to a randomized, double blind World Health Organization (WHO) protocol. MB patients were submitted to therapeutic regimens, as follows:

Regimen A - WHO/MDT/MB for 1 year: Rifampicin 600 mg + clofazimine 300 mg once every 4 weeks, supervised; clofazimine 50 mg daily + dapsone 100 mg daily, unsupervised.

Regimen B - The same as regimen A with the addition of ofloxacin 400 mg, daily for the first four weeks, supervised.

Regimen C - Rifampicin 600 mg + ofloxacin 400 mg daily, supervised, for four weeks, followed by appropriate placebo preparations.

Regimen D - The same as regimen A for two years (control group).

In PB cases the drug regimens tested were:

Regimen E - WHO/MDT/PB for 6 months: one monthly supervised dose of rifampicin 600 mg and dapsone 100 mg daily, unsupervised.

Regimen F - Rifampicin 600 mg and ofloxacin 400 mg in daily supervised doses for the first 4 weeks of the trial, followed by placebos for the remaining 5 months of the trial period.

The study population consisted of 67 untreated MB and 103 untreated PB leprosy patients. The inclusion criterion for MB patients in the trial was a positive BI of 2+ or more in at least one of five skin smears. MB patients with BI 1+ were not included in the trial. Based on clinical and histopathological findings, the MB group was found to be composed of 16 lepromatous (LL), 40 borderline lepromatous (BL) and 11 borderline patients (BB). The inclusion criterion for PB leprosy patients was a negative BI in all 5 skin smears. The PB group was composed of 6 indeterminate (I), 57 tuberculoid (TT), 40 borderline tuberculoid (BT). The number of skin lesions was only documented for the PB patients. Serum samples were collected from all 170 patients mentioned above.
5.3.2 Follow-up

Information on the clinical status after 6 months from the start of treatment was available for 92 of 103 PB patients. Clinical cure was defined as absence of skin and nerve lesions or a decrease in the number or clinical activity of lesions and nerves. All patients were followed for 5 years and relapses were recorded. In MB cases, the relapse was clinically confirmed if treated patients developed the following signs or symptoms: a) appearance of new skin lesion(s), not reactive lesions; b) increase in BI at least 2+ over previous value at any single site. In PB cases the relapse was clinically confirmed when new skin and/or nerve lesion(s) appeared and the patient did not respond to a therapeutic test with the following corticosteroid regimen: weeks 1 and 2 prednisone 10 mg four times per day; weeks 3 and 4 prednisone 10 mg three times per day.

5.3.3 Dipstick assay

The dipstick assay for the detection of antibodies to PGL-I of \textit{M.leprae} was described before.\textsuperscript{4} The dipsticks contain two bands: an antigen band consisting of the \textit{M.leprae}-specific and immunodominant disaccharide epitope of PGL-I linked to bovine serum albumin (BSA) (DBSA, kindly provided by WHO/IMMLEP through Dr. J. Colston, National Institute for Medical Research, London, UK)\textsuperscript{2} and an internal control band consisting of anti-human IgM antibodies that bind IgM molecules from the serum. The IgM detection reagent consisted of a lyophilized monoclonal anti-human IgM antibody linked with a colloidal dye.\textsuperscript{13}

Dipsticks were wetted in distilled water for 15 sec and then incubated for 3 h in a reaction vial containing 0.2 ml of the reconstituted detection 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. The results were scored as positive when staining was observed; no coloring (but with a positive control band) was scored as negative.

5.3.3 ELISA assay

The ELISA for the detection of IgM antibodies to PGL-I of \textit{M.leprae} was performed essentially as described previously\textsuperscript{2} using DBSA as the semi-synthetic analogue of PGL-I. DBSA (0.023μg of sugar per ml) was diluted in carbonate buffer (pH 9.6). Nunc-Immunoplates-II (Life Technologies, Taastrup, Denmark) were coated with 50 μl/well and incubated overnight at 37°C in a moist chamber. 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
Chapte rr V

(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 DBSA. The cut-off value for positivity was OD=0.250, which corresponded to the mean value of 108 local healthy blood donors plus two times the standard deviation.

5.3.4 Statistical evaluation

Data were analyzed using Epi-info version 6. The variation between test methods was determined by calculating kappa values with 95% confidence intervals (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.

5.4 Results

5.4.1 Comparison between dipstick and BI

Table 1 shows the comparison between the dipstick results and the classification according to BI of untreated leprosy patients. An agreement of 80.6%, kappa = 0.61 (95% CI, 0.46-0.76) was observed. The sensitivity of the dipstick test for detection of MB patients was 85.1%, the specificity 77.7%. The positive predictive value (PPV) for this study population was 71.3% and the negative predictive value (NVP) 88.9%.

The agreement between the ELISA results compared to dipstick results for the entire study population was 87.6%, kappa 0.75 (95% CI, 0.60-0.90).

Ten patients (14.9%) were classified as MB according their BI, which was by definition positive, but showed negative dipstick results. The mean BI of these ten MB patients was 1.4 (SD = 0.8), which was significantly lower than the mean BI (3.3, SD = 1.5) of the 57 MB patients with a positive dipstick (t test, p=0.023). There were 23 PB patients (22.3%) whose dipstick was positive and BI negative.
Identification of leprosy patients who have an increased risk of relapse using a simple dipstick assay

Table 1: Comparison between the dipstick results and the classification according to BI of untreated leprosy patients

<table>
<thead>
<tr>
<th></th>
<th>Dip pos (+)</th>
<th>Dip neg (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB patients BI (+)</td>
<td>57</td>
<td>10</td>
<td>67</td>
</tr>
<tr>
<td>PB patients BI (-)</td>
<td>23</td>
<td>80</td>
<td>103</td>
</tr>
<tr>
<td>Total</td>
<td>80</td>
<td>90</td>
<td>170</td>
</tr>
</tbody>
</table>

5.4.2 Comparison between dipstick result and number of lesions

For 95 patients classified as PB based on a negative BI, the number of lesions was recorded. Table 2 shows how these PB patients would have been classified (PB or MB) on the basis of lesion number and how on the basis of dipstick result.

Both methods of classification resulted in a similar number of patients being misclassified as MB (17/95 for lesion counting and 21/95 for dipstick, McNemar test, p=0.57). In this population dipstick classified 78% according to BI and the counting lesions method 82%. There was no agreement between dipstick and lesions counting (kappa 0.08 [95%CI, -0.12 - 0.28]).

Table 2: Comparison between lesion counting and dipstick results in 95 patients classified as PB on the basis of their negative BI

<table>
<thead>
<tr>
<th></th>
<th>Dip pos (+)</th>
<th>Dip neg (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions ≥ 6</td>
<td>5</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>Lesions &lt; 6</td>
<td>16</td>
<td>62</td>
<td>78</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>74</td>
<td>95</td>
</tr>
</tbody>
</table>
5.4.3 Follow-up of PB patients - at the end of treatment

Information on the clinical status after 6 months of treatment was available for 92 of 103 PB patients. Table 3 shows the clinical state of these patients in relation to dipstick results. It was observed that of the 71 dipstick negative PB patients 25 (35.2%) were already clinically cured, at the end of treatment, compared to only 2 (9.5%) of the 21 dipstick positive PB patients (Chi-square test with Yates correction, p = 0.04). In contrast, no significant difference in cure rate was found when these 92 PB patients were classified according to their number of lesions: of the 77 PB patients with less than 6 lesions, 22 (28.6%) were cured after 6 months compared to 5 (33.3%) of the 15 patients with 6 or more lesions (Chi square with Yates correction, p = 0.95).

Table 3: Follow up of PB patients after 6 months from the start of treatment

<table>
<thead>
<tr>
<th></th>
<th>Dip pos (+)</th>
<th>Dip neg (-)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cured</td>
<td>2</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>Not cured</td>
<td>19</td>
<td>46</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>71</td>
<td>92</td>
</tr>
</tbody>
</table>

5.4.4 Follow-up of all patients - after 5 years

Nine of 170 (PB-MB) patients relapsed within the follow-up period of 5 years. Seven were MB patients that were ELISA and dipstick positive at the start of their therapy. All of them were in the group that had received a shorter treatment than standard MDT. The mean ELISA OD of these 7 relapsed MB patients was 2.022 (SD = 1.016) (all strong dipstick-positive) in comparison to a mean OD of 1.382 (SD = 1.118) for the non-relapsed MB patients (Fisher exact test, p = 0.15). None of the 10 BI-positive (MB) patients that were dipstick negative and of which 8 had received a short course treatment (one year or shorter), relapsed within the five year follow-up period.

Two of the relapsed patients were initially classified as PB on the basis of their negative BI. One of them was dipstick and ELISA negative at the start of treatment (6 months MDT), the other dipstick and ELISA positive at the start of treatment (1 month daily rifampicin plus ofloxacin). The dipstick negative patient had 2 lesions and the dipstick positive patient had 8 lesions at the start of therapy. The dipstick positive patient was, upon recognition of his relapse (4 years after the release from treatment), retreated with a 6 months PB treatment (MDT) schedule and relapsed again one year later; he was then reclassified as MB and treated accordingly with good response. The dipstick negative patient responded well to the MDT regime for PB.
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5.5 Discussion

At present in many leprosy endemic countries the division of leprosy patients into PB and MB is determined solely on the basis of the number of lesions. This means that patients are classified without confirmation of the BI. 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 thus for the classification into PB or MB leprosy for treatment purposes. The results of this study indicate that patients with a high bacterial load as shown by their dipstick result, have an increased risk for relapse.

BI does not necessarily reflect the total bacterial load in the body. 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. 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. On the other hand, a positive BI patient may not have M. leprae heavily disseminated in the body. Antibodies are likely to be a better reflection of the total bacterial load in the body.

In our study dipstick positivity corresponded well with the BI of patients, as illustrated by an agreement of over 80%. Of the 67 patients which were classified as MB on the basis of their skin smear result, 10 (14.9%) were dipstick negative. It may be that in these patients the total bacterial load was located mainly in cutaneous lesions without dissemination. Their skin BI was significant lower than that of the dipstick positive MB patients. As the information on treatment received is still not available, it is at present impossible to assess whether these patients could have been treated for a shorter period of time.

Twenty-three of 103 (22.3%) patients with a negative BI had a positive dipstick result. The dipstick positive PB patients indeed showed a significantly lower cure-rate after 6 months compared to the dipstick negative and BI negative leprosy patients. This might be an indication that this group of patients indeed had a higher total bacterial load although their skin lesion BI was negative. The follow-up period of 5 years in which 22 of the 23 dipstick positive PB patients remained free of relapse support adequate treatment. However, it is not possible to give a definite answer because we are not aware of the treatment these patients have received. The low cure rate is at least an indication that the bacterial load of patients from the PB dipstick positive group may require the regular 6 months MDT.

Within the follow-up period of five years from the beginning of treatment, 1 of the 23 dipstick positive PB patients relapsed compared to 1 of the 80 dipstick negative PB patients. One can argue that the one PB patient who was dipstick-positive relapsed due to inadequate treatment of one month with rifampicin and ofloxacin. However, this patient was retreated with 6 months of MDT regimen for PB patients and then relapsed again, suggesting that this patient should have been classified as MB as was indicated initially by his dipstick result and this was confirmed in the end by his good clinical response.
when treated as an MB patient. The other dipstick negative patient who relapsed had received the regular MDT for PB and was negative by both dipstick and BI. This patient would neither have been identified by number of skin lesions nor by dipstick result as requiring a longer treatment course.

There was no relation between the number of lesions of the BI-negative PB patients and the dipstick result. The dipstick result correlated with the cure rate of these PB patients, but the lesion counting results did not. If these BI-negative patients had been classified only on the basis of dipstick result, 21/95 would have been classified as MB, while 17/95 would have been on the basis of the number of skin lesions. Over-classification of MB and over-treatment, which would likely occur when classification criteria would include dipstick result, is already general practice and fully accepted in leprosy control programs, which classify patients primarily on number of skin lesions. 7,23

The seven MB patients that relapsed within the five year follow-up period had all received one month of daily rifampicin treatment combined with ofloxacin instead of standard two years MDT. The mean anti-PGL-I antibody level at the start of treatment in these relapsed patients was higher than that of the non-relapsed patients. Strictly speaking, the difference was not significant (p = 0.15), but considering the small numbers involved, we believe this result at least illustrates the importance of the bacterial load in relation to the treatment given. The dipstick assay may be helpful in this respect as an indicator of the bacterial status of the patient and thus may help in identifying patients that could successfully receive a shorter treatment.

The WHO study on risk of relapse in leprosy judges it unnecessary to follow-up and carefully examine patients after MDT. 25 This is based on the observations that MDT is efficient and at most follow-up studies show only a low percentage of relapses. Nevertheless, when following up patients with high bacterial loads before treatment for longer periods the relapse rate increases twenty-fold. 7 Furthermore, in one study, the frequency of viable *M. leprae* extracted from nerves and skin of MB leprosy patients released from MDT was alarmingly high. 22 Based on the risk of relapse after MDT it is advised to give special attention to leprosy patients with high bacterial loads and that BI should be performed. 9

The risk of relapse is associated with both the sensitivity and the predictive value of a test or method to classifying a MB correctly (PPV). 23 In our study population (39.4% MB patients) we found a sensitivity of 85.1%, the chance of classifying a MB patient correctly was 71.3% (PPV). It should however be taken into account that our study population may not be representative for the total leprosy population in the study area since the MB group with BI <2 was not included. This group comprises usually 5-20% of the total number of patients depending on the leprosy control area. In a population predominantly formed by PB patients the ML Dipstick test would increase the number of PB patients falsely
classified as MB and further studies must be performed in order to assess the validity of the use of the test in such areas. However, 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.  \(^{23}\)

### 5.6 Conclusions

The long incubation period of leprosy and the risks of transmission associated with the difficulty to distinguish relapse from late reactions, are challenges for the leprosy control programs especially now that they are being absorbed by the primary care system. Studies on classification of patients with the help of a simple and robust tool maybe good to be considered. We show here that a simple serological assay, which can be used by health workers in the field, is capable of identifying those patients who have high antibody levels and thus in all probability have a high bacterial load. As these patients could be at an increased risk of developing relapses after treatment, special attention would be recommendable in the period after release from treatment, particularly in those groups of patients who had received a shorter than usual course of treatment.

The dipstick method is simple, can be performed using finger prick blood, \(^{3}\) and is easy for health workers to use. If it can be shown to provide a classification that fits better with response to treatment, then it could be an important additional tool for quick and accurate diagnosis of leprosy. The results of this study suggest that the dipstick test could play such a role, but further studies are needed to confirm whether the dipstick method to detect antibodies to PGL-I, alone or in combination with other criteria, would consistently produce more reliable patient classification.
5.7 Acknowledgments

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

We thank the Brazilian Government Department of Health Dermatology represented by Dr. Maria Leide Wan-Del-Rey de Oliveira for her suggestions and further assistance. To Dr. Euzenir Nunes Sarno and Dr. Pamela Wright a special thanks for their suggestions and constant stimulus.

We thank WHO/IMMLEP and Dr. Joe Colston (National Institute for Medical Research, London, UK) for provision of the DBSA antigen.
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5.8 Reference List


