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

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CHAPTER 1

INTRODUCTION
Chapter 1

1.1 Leprosy, agent and infection

*Mycobacterium leprae* is an acid-fast, slow growing, obligate intracellular bacillus that preferably invades the nerve Schwann cells and macrophages. The rate at which leprosy spreads in a community depends on the proportion of susceptible individuals in the population, the opportunity for contact with an infected person and the force of infection in the community. The outcome of infection is based on the capacity of the host to mount an effective immune response. In the great majority of infected persons the cellular immune response is efficient and clinical symptoms never appear (subclinical infection). Leprosy has a long incubation period and it generally takes two to five years for the disease to appear.

Van Beers et al. showed in an elegant and clear way that leprosy is clustered and that it spreads in concentric circles around a patient similar to the "stone in the pond" model as described for tuberculosis. Therefore, definition of "contacts" is crucial to understand the transmission of leprosy. Fine has also shown that large proportion of new leprosy patients arises among individuals with temporary or indirect contact with known cases. Household contacts of leprosy patients have a risk of developing leprosy around 30 times higher than non-contacts. In addition, other factors like socioeconomic status, sanitary conditions and genetic background also play a role in infection and development of disease.

1.2 Incidence and prevalence

The latest available information on leprosy by the World Health Organization (WHO) indicates that around 800,000 new cases were detected in 1998. The number of new cases detected (rate per 100,000 as per 1998) per WHO region is as follows: South-East Asia 689,069 (46.5), Africa 53,096 (8.3), Americas 47,218 (5.8), Eastern Mediterranean 5,923 (1.2), Western Pacific 11,446 (0.7) and Europe 92 (-). This clearly shows that the majority of the new cases are concentrated in certain limited geographic areas. The worldwide number of newly diagnosed patients has remained more or less stable or even increased over the past decade, going from 571,792 in 1990, through 552,416 in 1995, to 755,305 in 1998.

Although the detection rate remains constant or is in some countries even increasing, the prevalence rate at the global level has dropped from 21.1 per 10,000 in 1985, through 13.9 in 1990 and 4.0 in 1995, to 1.4 cases per 10,000 people in 1999. However, this decrease is due to administrative actions rather than to a true decline in leprosy incidence. With the introduction of multi drug therapy (MDT), patients who were previously life long registered as leprosy cases are now removed from the register at the end of treatment, which is after two years at most. With even shorter treatment regimens which are being
introduced (and more can be expected in future) this “artificial decline” in leprosy prevalence may continue even though it is not corroborated by a decline in incidence of leprosy.

In order of prevalence the 12 most endemic countries are: Madagascar (8.0 per 10,000), India (5.9), Brazil (4.3), Nepal (3.6), Mozambique (3.3), Guinea (3.3), Nigeria (2.9), Myanmar (2.4), Cambodia (1.7), Ethiopia (1.3), Indonesia (1.1) and the Democratic Republic of Congo (1.0). Although the prevalence has dropped considerably, this is still higher than the aimed target of the WHO by the year 2000, which is one case of leprosy per 10,000 people.

1.3 Clinical signs and symptoms in leprosy

Physically and psychologically, leprosy is a striking disease. Initially symptoms may be hardly noticed. During the course of the disease skin lesions become more visible and nerves may be damaged irreversibly, resulting in deformities and handicaps. The consequent stigma generates an extensive fear in both patients and the general public.

Lesions are frequently noted on exposed surfaces of the skin but can be present all over the body. They may be single or numerous, varying widely in form, appearance, and color. The margins of lesions range from well delineated to rather vague, or may fade imperceptibly into the surrounding normal skin. Peripheral and cutaneous nerves may be palpably thickened.

Sensory loss can be localized to the lesions but also total anesthesia involving hands and feet may be present. Once anesthesia has occurred, wounds are easily caused by trauma or burning. Motor loss may result in muscle function loss.

A special type of leprosy is primary neuritic leprosy. The patient presents clinically with peripheral neuropathy with functional impairment of one or more nerves but without skin lesions, making diagnosis extremely difficult.

1.4 Diagnosis of leprosy

The diagnosis is based on clinical signs and symptoms that can be recognized by a health worker after a period of training. In endemic countries the diagnosis is usually made clinically on the presence of skin lesions, anesthesia of the skin lesions, thickened nerves, and the presence of acid-fast bacilli in slit skin smears.
1.4.1 Clinical examination

The whole body surface should be examined for skin lesions, and one or few of them are tested for loss of sensation. The main peripheral nerves are palpated to ascertain any thickening, or tenderness. Eyes, hands and feet are examined for any disability.

1.4.2 Microscopy

*M. leprae* bacteria are found by microscopy using acid-fast staining of skin smears or biopsies. *M. leprae* presents as slightly curved, rod-shaped bacteria, around 0.3 μm wide and about 1-8 μm long, lying either singly or in larger bundles, or in characteristic compact masses known as globi. The bacillary load is expressed as the bacterial index (BI). The BI reports the number of bacteria in one microscope field under an oil-immersion objective, on a logarithmic scale, from 1+ (1 bacterium in 100 fields) to 6+ (with more than 1000 in an average field). Smears should be taken from a minimum of three sites, including at least one earlobe.

1.5 Classification of leprosy

1.5.1 General

Leprosy classification used to be based on clinical, bacteriological and histopathological criteria. Upon the introduction of multiple drug therapy (MDT), the WHO, as assistance to the assignment of patients, has introduced a simplified classification system in which patients are categorized as either paucibacillary (PB) or multibacillary (MB). In PB patients *M. leprae* is not found in skin smears or biopsies. This group includes tuberculoid leprosy (TT), borderline tuberculoid leprosy (BT), and indeterminate leprosy (I). MB patients are lepromatous leprosy (LL), borderline lepromatous leprosy (BL) and mid-borderline leprosy (BB), in which bacteria are found by microscopy on acid-fast staining of skin smears or biopsies.

Facilities for bacteriological examination of skin smears and histopathological examination of skin and/or nerve biopsies are often not available, and when available the results may be subject to biased interpretation. Therefore, there is a serious risk of poor performance: low densities of acid-fast bacilli are not detected, with the outcome that patients are misclassified. Discrepancies in histopathological diagnosis of skin biopsies, even when performed by experienced pathologists, have been reported, illustrating the difficulty in correctly diagnosing the disease.

Classification of leprosy based on clinical findings only is now a routine procedure in most places. With the attempt to overcome the problem of the absence of diagnostic facilities, WHO adopted an even more simplified method of classification based on
Introduction

counting the number of lesions. Leprosy patients with less than 6 lesions will receive a PB treatment regimen and patients with 6 or more lesions will be treated as MB.

Until 1982, MB patients referred to those who had a BI of at least 2 at any site in the initial skin smear. From 1988, MB leprosy includes all smear-positive patients, as well as patients with more than five lesions.

1.5.2 Classification according to immune response

Ridley and Jopling proposed a classification based on the level of cellular immunity that a patient mounts against the bacterium. The specific cellular immune response can be vigorous as in tuberculoid leprosy or deficient or absent as in lepromatous leprosy.

Tuberculoid leprosy represents the highly resistant form of the disease with no or few bacteria and few cutaneous lesions and extensive local nerve damage. The histological findings include granulomata, which consist of multinucleated giant cells, epitheloid cells, macrophages and T-lymphocytes. Low or absent anti-\textit{M. leprae} antibody levels are seen in tuberculoid leprosy.

Lepromatous leprosy represents extreme susceptibility to \textit{M. leprae} as manifested by a large number of skin lesions containing undifferentiated macrophages in which large numbers of bacilli are present. Few lymphocytes can be detected, mainly of the suppressor CD8 subset. Such cells stimulate humoral responses while suppressing cell-mediated response. LL patients generally have high anti-\textit{M. leprae} antibody levels as opposed to TT patients.

In addition to TT and LL, there are three intermediate forms of leprosy namely borderline lepromatous (BL), mid-borderline (BB) and borderline tuberculoid (BT). Indeterminate (I) leprosy is often the beginning of the disease and can be self-limiting, but may progress to other forms of leprosy.

A visual presentation of the cellular and humoral immune response in leprosy is given in Figure 1.
1.5.3 Clinical features

1.5.3.1 Indeterminate leprosy (I)
The skin lesions consist of a single flat macule, or a few macules in some cases, usually slightly hypopigmented or slightly erythematous, roughly oval or rounded in shape. Surface is flat and may be smooth and the texture of the skin is usually not affected. Margins may be definite but are usually rather vague. The lesions are frequently localized on exposed surfaces of the skin. Sensory loss over the macules is generally minimal, and may involve only sections of the macule. Bacteriologic smears are usually negative, occasionally slightly positive.

1.5.3.2 Tuberculoid leprosy (TT)
In TT leprosy, skin lesions are single or few, asymmetrical and well demarcated from the normal surrounding skin. Margins may be thinly elevated throughout or only in segments. The surface is dry and may show central healing and slight atrophy. Definite sensory loss is demonstrable. Loss of hair, and anhydrosis may be present. Bacteriologic smears are negative.

1.5.3.3 Borderline leprosy
This comprises the Borderline Tuberculoid (BT), Borderline (BB) and Borderline Lepromatous (BL) types of leprosy.

**Borderline Tuberculoid (BT)**
The skin lesions are few or numerous and hypopigmented. The individual lesions may be elevated throughout or may have central clearing; margins are well delineated from the normal surrounding skin. The surface may be smooth, but usually rough and may be scaly. Peripheral and cutaneous nerves may be palpably thickened. Bacteriologic smears can be positive.
**Introduction**

**Mid-Borderline (BB)**
The skin lesions vary in number from few to numerous, rounded or oval in shape. Lesions are infiltrated throughout or with a central clear area or areas producing a punched out appearance, with vague peripheral edges. Sensory loss is generally mild with the exception of the central areas of the lesions. Bacteriologic smears are usually positive.

**Borderline Lepromatous (BL)**
Skin lesions vary in size, are thickened or infiltrated, often numerous and widespread. They may be bilateral in distribution, but not symmetric. The surface of the lesions is usually smooth and shiny with ill-defined edges. Patchy areas of diffuse infiltration may be noted. Sensory loss is more generalized with usually asymmetric sensory loss involving hands and feet. Bacteriologic smears are positive.

1.5.3.4 Lepromatous leprosy (LL)
Skin lesions are widespread, generalized and symmetric, in the form of diffuse thickening and appear small, ill defined, slightly hypopigmented or faintly erythematous. Papular or nodular lesions are seen in advanced disease, commonly over areas already infiltrated. Sensory loss occurs later in the disease, is bilateral and symmetric resulting in "glove and stocking" type of anesthesia.

1.5.3.5 Primary neuritic leprosy
Primary neuritic leprosy is a form of leprosy without history or evidence of skin lesions. This type of leprosy is characterized by neuritic manifestations caused by the asymmetrical involvement of usually one or, at times, several peripheral nerves. The ulnar nerve is most commonly affected. In the majority of cases the underlying changes in the affected nerves in primary neuritic leprosy are due to tuberculoid or borderline leprosy. Bacteriologic skin smears are negative but nerve biopsies near the site of the neurological deficit may be positive.

1.6 Leprosy treatment
The treatment has changed over the years. In the 1940s dapsone was developed and introduced in leprosy therapy. The treatment regimen was 100 mg daily for a minimum period of 5 years to treat paucibacillary (PB) leprosy, and lifelong to treat multibacillary (MB) leprosy. The treatment of leprosy based on dapsone monotherapy was in use until recently in some areas.

In the sixties *M. leprae* started to develop resistance to dapsone, and in 1981 the WHO recommended the introduction of a multidrug therapy (MDT). PB patients are treated for 6 months with 600 mg rifampicin supervised once a month and 100 mg dapsone
Chapter I

daily. The recommended standard treatment for MB leprosy consisted until recently of 24 months of treatment with rifampicin (600 mg once monthly, supervised), dapsone (100 mg daily), and clofazimine (300 mg once monthly, supervised and 50 mg daily). The duration of the above MDT regimen for MB leprosy is currently recommended to be 12 months.

For the treatment of single skin lesion PB leprosy WHO recommended, as an acceptable and cost-effective alternative regimen, a single dose of rifampicin 600 mg plus ofloxacin 400 mg and minocycline 100 mg.

1.7 Reactions in leprosy

The two major complications in leprosy are reversal reactions (RR) (also known as type I reaction) and erythema nodosum leprosum (ENL) (also known as type II reaction). These reaction episodes may occur during and/or after treatment and often cause permanent disabilities. Figure 2 represents the occurrence of reactions in relation to the leprosy spectrum.

1.7.1 Reversal reaction (RR)

RR appears to be associated with a sudden change in the cell-mediated immunity (CMI) against *M. leprae* antigens and occurs mainly in borderline (BT, BB and BL) patients. It is considered to be delayed type hypersensitivity (DTH) response to bacillary antigens and is associated with clinical and histological changes towards the tuberculoid pole of the leprosy spectrum. The bacilli are eliminated but the tissue is destroyed and extensive nerve damage occurs frequently. As an increase in the CMI is witnessed, RR is also called "upgrading reaction".

In RR, the expanding granulomata, seen on histopathological examination, are the cause of the nerve damage. The nerves, especially the small nerve branches, are compressed and may become dysfunctional due to lack of nutrients. This lack of nutrients happens also in subcutaneous nerves and in the nerve trunks but these are mainly destroyed indirectly by an inflammatory edema in the non-elastic impermeable perineurium that causes an increased intraneural pressure, which compresses the axon.
1.7.2 Erythema nodosum leprosum (ENL)

ENL is a type of reaction that can occur in BL and LL patients. A viral infection, vaccination, pregnancy or a period of acute stress may trigger an ENL episode. During ENL episodes painful, tender, erythema nodosum-like nodules are present. In severe cases these papules may even pustulate or result in necrosis. ENL episodes are often accompanied by fever and malaise and may be complicated by neuritis, orchitis, lymphadenopathy, iridocyclitis, arthritis and proteinuria. Nerve damage is less severe than in RR, but a relapsing long-standing course of ENL can result in irreversible nerve damage.

The ENL reaction is associated with a migration of newly recruited monocytes and T cells into the site of the lesion, with predominance of T helper cells in the inflammatory infiltrate. Although a role for immune complex-mediated injury has been suggested in the past, convincing data are still lacking. More recent studies suggest TNF-α to be a key mediator in the immunopathology of tissue damage in both the RR and the ENL.

1.8 Relapse of leprosy

Relapse is the consequence of renewed multiplication and spread of surviving leprosy bacilli after treatment. Diagnosis of a relapse in an MB patient is based on the increase of the BI, and the presence of viable bacilli may be confirmed by inoculation in the footpads of mice. A PB relapse is more difficult to diagnose since the signs, including the histological picture, strongly resemble those of reversal reaction.
It was observed that new activity in lesions during the first 3-4 years after MDT is mostly due to a late reaction and that the longer the period after MDT, the higher the chance that signs of new clinical activity signify a relapse. Still, there is no general rule and since therapy differs - a reaction is suppressed by corticosteroids and a relapse is treated with MDT - there is a need for easy tools to differentiate between reaction and relapse.

1.9 Leprosy control

In the majority of endemic countries, vertical leprosy control programs have existed for a long time. At this moment, discussions are ongoing to integrate these leprosy control programs into the general health services. In a number of countries this integration is already being implemented. The future implications of this action are not known, especially taking into account that leprosy elimination as a public health problem is more an operational feature than a scientific fact. The long incubation period of leprosy and the risks of transmission and relapse will pose challenges to leprosy control when merged into the normal health care system. Integration implies that less experienced professionals are expected to diagnose and classify leprosy. In communities where leprosy has been eliminated as a public health problem hardly any case will be diagnosed. Most health professionals would never encounter a leprosy patient and may not include leprosy in the differential diagnosis if they believe it to have been eliminated. Furthermore, the social stigma of leprosy would not necessarily be eliminated, and stigma would continue to discourage patients from self-reporting. In addition, if the socioeconomic situation remains the same in high endemic areas, the susceptibility in the community will remain high.

Early diagnosis is a key to the interruption of leprosy transmission. Silent transmission is facilitated by the slow growth characteristic of the bacilli, the long incubation period of leprosy, the slow progress of the irreversible nerve damage and the stigma of leprosy. In leprosy endemic areas, the number of undetected leprosy cases may increase and silent transmission over the years could finally reverse the achieved status of leprosy elimination to one of even high leprosy endemicity.

Health workers in the field lack tools which could assist in establishing an early diagnosis, monitoring chemotherapy and the emergence of relapse, and identifying patients with a risk of developing leprosy reactions during and after therapy.

Therefore, the main challenges in the field of leprosy control are: (i) to develop a better understanding of the mechanisms of transmission and protective immune response to further boost the effectiveness of the control measures; and (ii) to develop efficient diagnostic tests to help the public health systems in developing countries to overcome the problems related to diagnosis of the various forms of leprosy and its complications.
1.10 Leprosy serology

As indicated above, there is an urgent need for simple diagnostic tests for leprosy. For a number of other diseases, simple tests are based on serology, i.e. the detection of specific antibodies in the body fluids from suspected patients.

1.10.1 Development of leprosy serological techniques

Serological techniques for leprosy are aimed at the detection of specific anti-\textit{M. leprae} antibodies which reflect current infection and are useful for follow-up during therapy and for the assessment of the prevalence of the disease and the spread of infection in the community. Many serological techniques have been developed.

The first generation of serological tests described for leprosy consisted of a radioimmunoassay (RIA) using antigen 7, the fluorescent leprosy antibody absorption test (FLA-ABS) using crude \textit{M. leprae} and a passive haemagglutination test (PHA). Even though lacking specificity, these tests made it possible to study subclinical leprosy infection.

Next, indirect enzyme-linked immunosorbent assays (ELISA) using crude antigens or different proteins from \textit{M. leprae} and other mycobacteria were described. In addition, monoclonal antibody inhibition ELISA using the 35kDa, 36kDa and 65kDa proteins were developed.

The low sensitivity, high cross reactivity and limited availability of the antigens restricted the use of most of these tests to small-scale epidemiological studies of clinical and subclinical infection, prevalence of infection and transmission of the disease.

With the advent of \textit{M. leprae}-specific phenolic glycolipid (PGL-I) and the subsequent reproducible production of semi-synthetic neoglycolipids, large scale studies of the humoral response in leprosy became easier and many studies were performed. Still, incorporation of serology into the leprosy control services required a simpler test system than ELISA. "Simple assays" using the same neoglycolipids were developed. These include the passive haemagglutination test (PHA) and a particle agglutination gelatin test (MLPA). Sensitivities and specificities comparable to those obtained with ELISA were found. These assays, however, are still dependent on a cold chain and require serum for testing.

1.10.2 Detection of antibodies to PGL-I

Most mycobacteria have specific, highly antigenic glycolipids that can be used as tools for the serodiagnosis of distinct mycobacterial infections. As the mycobacterium enters
the human body, the cell wall is the first to be encountered by the immune system. The cell envelope of \textit{M. leprae} mainly consists of lipids including a considerable glycolipid fraction with a unique carbohydrate composition.

One of the first \textit{M. leprae}-specific antigens to be isolated and characterized was phenolic glycolipid-I (PGL-I), the major antigenic glycolipid in the bacterium. The PGL-I molecule is composed of a unique trisaccharide, the 3,6-di-\textit{O}-methyl-\textit{\beta}\text{-D}-glucopyranosyl-(1→4)-2,3-di-\textit{O}-methyl-\textit{\alpha}\text{-L}-rhamnopyranosyl-(1→2)-3-\textit{O}-methyl-\textit{\alpha}\text{-L}-rhamnopyranose, see figure 3.\textsuperscript{18,37,21,45,105} The principal antigenic determinant of PGL-I is the ultimate disaccharide part of the molecule.\textsuperscript{10,37} Monoclonal antibodies were used to further analyze this antigenic determinant of PGL-I. It was shown that the removal of the terminal sugar residue resulted in loss of binding of most antibodies, while removal of the long chain fatty acids of the molecule had no effect on antibody binding.\textsuperscript{105} These and other results suggested that chemical synthesis of the ultimate disaccharide part would provide an antigenic epitope that would be specific enough to be applied in the serology of leprosy.

\textbf{Figure 3 - Chemical structure of PGL-I}

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\textbf{Figure 3 - Chemical structure of PGL-I}
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The main obstacle in obtaining larger quantities of the PGL-I molecule was the difficulty of cultivating \textit{M. leprae} \textit{in vivo}. In addition, the PGL-I molecule is quite polar and in order to use it as an antigen in ELISA, deacylation, sonication or detergent treatment was required. Therefore the option to produce the antigen synthetically was explored. The sugar part of the PGL-I was synthesized and conjugated to bovine serum albumin (BSA) either directly or through different linkers, namely an octyl (O) or phenyl (P) linker arm.

The following neoglycolipids were produced: monosaccharide-octyl-BSA (M-O-BSA),\textsuperscript{36} disaccharide-BSA (D-BSA),\textsuperscript{40} natural disaccharide-octyl-BSA (ND-O-BSA)\textsuperscript{18} and natural trisaccharide-phenyl-BSA (NT-P-BSA).\textsuperscript{36}
Based on native PGL-I and its semi-synthetic derivatives, numerous serological assays have been developed to detect the presence of antibodies of the immunoglobulin IgM, IgG and IgA classes. As the semi-synthetic antigens could be produced in larger quantities and they could be applied easier than native PGL-I, a wide variety of applications ranging from clinical diagnosis to large epidemiological studies became feasible. It soon became evident that the antibody response mainly consists of the production of IgM antibodies in a T cell-independent fashion.\(^6\)

### 1.10.3 Serological studies using PGL-I

The vast majority of studies published so far have used the ELISA technique. In the next paragraphs, the results of these studies will be presented and discussed. However, for a proper understanding, it is essential to first discuss some of the advantages and potential pitfalls that may occur when using this technique.

#### 1.10.3.1 ELISA: advantages and pitfalls

The advantages of the ELISA lie in (i) the wide availability of the necessary equipment as the technique is almost universally applicable; (ii) the easy separation of solid and liquid phases, and (iii) the lack of a requirement for labeling the \textit{M. leprae}-specific components of the test (either antigen or antibody). Still, ELISA is a laborious technique and pitfalls due to differences in antigen preparations, antigen concentrations, buffers, washing procedures, serum dilution and type of substrate used in the technique must be taken into consideration.

**Non-specific binding**

ELISA for PGL-I derivatives involves the immobilization of the protein part of the molecule (BSA) to a plastic surface via passive interactions. The ability of the plastic surface to interact with proteins is an essential feature. However, non-specific binding of other proteins or biomolecules to non-occupied spaces on the surface during the performance of the assay can be detrimental to the specificity and sensitivity of the assay results. Blocking is therefore essential.

The use of non-sugar conjugated BSA coated wells is crucial to enable to correct for any (non-specific) binding that may occur to BSA when using the semi-synthetic antigens. Similarly, one should correct for non-specific binding to uncoated wells (but treated with the same buffers) when using native PGL-I.

**Variations in the antigen preparation**

The ratio of sugar molecules to the protein (BSA) will vary in every antigen preparation. Thus, it is important to report the sugar/protein ratio of the antigen, to allow comparison between studies.
Chapter 1

**Precision and variation**
In order to maximize assay precision and sensitivity, complete removal of loosely or not bound fractions is required. In addition, daily variation in assay performance will also influence the results and is mainly related to the enzyme-substrate reaction. Several requirements, such as timing and development conditions, need to be optimized to result in a precise, accurate and reproducible assay. Since enzyme-substrate reactions are kinetic, the time elapsed from the start of the reaction to the end of the reaction as well as the temperature can and will affect the final concentration of product developed. In order to obtain comparable results there is a need to include an internal standard serum in the assay and to stop the reaction when the standard serum has achieved a certain optical density (OD) value.

**Serum dilution**
The reported serum dilutions in different studies ranged from 1:20 to 1:500. The serum dilution highly influences the end-result of an ELISA. For example, a low dilution usually results in a lot of non-specific binding. On the other hand, it allows binding of low affinity specific antibodies. Thus, serum dilutions may affect both specificity and sensitivity.

**Standard ELISA procedure for PGL-I**
A standardized ELISA protocol was published as a result of a workshop in June 1985, but still studies were and are being performed using a variety of alternative procedures. Differences in the study design and methodology of the ELISA techniques used in the studies will directly affect the sensitivity and specificity of the test.

1.10.3.2 Evaluation of serological studies using PGL-I

**Demographic trends in serology**
The percentage of seropositivity in the general population was found to be consistently higher in females at all ages. Also, a consistent age trend in seropositivity is observed in both sexes: the seropositivity rates being higher in younger groups and highest in the group between 10 and 20 years of age. It is well known that females and young people have higher IgM levels. Adding the fact that leprosy does not manifest preferentially in women or children, these high levels are more likely to be just a common feature of the immune system and do not specifically reflect differences in anti-PGL-I antibody levels in these groups.

**Before treatment**
One very important finding is that there is a significant correlation between antibody levels and the BI. This is important because the classification of patients for treatment is essentially based on the number of bacteria present in the
Introduction

body. Therefore, these findings are a first indication that serological results can be used as an alternative technique for the classification of patients.

Another consistent finding is that antibody levels increase from TT to LL, which is in agreement with the correlation between BI and antibody levels. The percentages of seropositivity in leprosy patients vary upon the classification criteria used. It was reported that between 85% and 100% of the MB patients and between 5% and 34% of the PB patients are seropositive. This is again an indication that serology may be used as an (additional) tool for classification of leprosy patients. Few studies were performed with PN patients, but in one study 30% of PN patients were found to be seropositive.

During treatment

During treatment there is a decline in antibody levels which correlates with the decline of BI. The initial load of bacteria in the body determines the slope of decline.

After treatment

In patients with a high bacterial load before treatment, chronic elevated levels of antibodies may persist for many years after therapy. An abnormal increased antibody response after therapy could be seen prior to relapse.

Serology and disabilities

Although few studies compared serology and disability it is important to mention that there is a positive correlation between seropositivity and the degree of disability in leprosy patients: patients with established disability at the time of diagnosis are more likely to be seropositive. However, as the PB group presents low antibody levels the relation between neuropathy and serology may be weak. Roche and coworkers, in a selected PN leprosy group, found that when the neuropathy involved more than 2 regions there was also concomitant rise in seropositivity.

Serology among contacts of leprosy patients

Many studies found a significant difference between seropositivity rates when comparing contacts and non-contacts. The fact that some studies found no significant difference may be due to one or more of the following reasons:

(i) definition of the study population, such as inclusion of contacts from patients who had been treated for leprosy in the (distant) past;
(ii) comparison between contacts of leprosy patients and a group which had not been matched for age and/or sex;
(iii) a small sample size not representing properly the population of the study area or not giving significantly different results, and/or

(iv) the high incidence of leprosy in the studied area.

A study performed in Korea and in the Philippines showed differences between contacts and non-contacts only in the Korean situation (low incidence), where a statistically significant higher seropositivity was seen in the contact group in comparison to the non-contact population. This could not be repeated in the Philippines (high incidence). The high seropositivity in non-contacts in high endemic areas may indicate that leprosy is widespread in these communities. Repeated testing of seropositive contacts suggested that an increase in antibody levels was an indication of the development of clinical disease. The risk of developing leprosy was shown to be around 30 times higher for in seropositive contacts of MB patients. Contacts of MB patients exhibit a significantly higher seropositivity rate and higher mean OD value than contacts of PB patients.

The results indicate that testing close contacts of newly identified MB cases could help to identify those most probably infected who may be incubating leprosy and as a consequence may function as a potential source of further transmission. The contact population is an important target for interrupting leprosy transmission. The earlier a new case is identified, the shorter will be the time for transmission.

Serology in the general population
It was found that in the general population the seropositivity has a unimodal distribution. This means that it is not possible to make a clear-cut division of the general population into two groups, namely infected and not infected. It also means that it is impossible to distinguish between subclinical infection and disease. These findings imply that serological tests based on detection of IgM antibodies to PGL-1 cannot be used as a sole diagnostic tool for screening the whole population for the detection of leprosy patients.

The seropositivity rate in the population varies from high to low depending on the incidence of leprosy in the community.

Serology in relation to Mitsuda reaction and BCG
No relation was found between seropositivity and Mitsuda reaction positivity. This is not surprising, as Mitsuda positivity is an indication of cell-mediated immunity as opposed to serology, which is a measurement of the humoral response.

No difference was observed in seropositivity between individuals with or without BCG vaccination.
1.10.4 Conclusion

Detection of IgM antibodies to PGL-I can be used for the diagnosis of leprosy when the results are considered together with other diagnostic and clinical data. Antibody detection is especially useful for the diagnosis of MB leprosy; antibody levels may be low or undetectable in PB patients.

During treatment of MB patients, repeated testing provides an additional technique to measure the effect of therapy. Furthermore, increasing antibody levels may indicate a relapse. However, serological results must always be interpreted in combination with other diagnostic information.

Clearly, PGL-I antibodies are reflective of bacterial load in an individual, reflecting subclinical infection or disease. Serological screening and follow-up of contacts of leprosy patients is a useful tool for early detection of new cases. In addition, it may be useful to measure the extent of the leprosy problem in a population.
Chapter I

1.11 Outline of this thesis

Although many studies were performed using ELISA for the detection of IgM antibody to PGL-I, this assay is too complicated to be applied in most areas where leprosy remains a public health problem. In order to simplify the use of serology in leprosy control a simple assay is urgently required. This thesis describes the development, evaluation and application of a simple dipstick assay (ML Dipstick).

Chapter II describes the development of the ML Dipstick for the detection of IgM antibodies to PGL-I of *M. leprae* in serum. The test does not require any specialized equipment and the highly stable reagents make the test robust and suitable for use in tropical countries.

Chapter III describes a further simplification of the ML Dipstick assay by using whole blood and an evaluation of the assay performance in the leprosy endemic area of Amazonas in Brazil.

Chapter IV shows how ML Dipstick could contribute to improved classification of leprosy patients for treatment purposes. In this chapter the results of ML Dipstick were combined with clinical classification by counting the number of lesions. Results were compared with the classification based on the bacteriological index.

Chapter V investigates whether ML Dipstick is capable of identifying patients with a higher risk of relapse after treatment. With the integration of leprosy control into the general health system, diagnosis and classification will be primarily in the hands of less experienced professionals. Misclassification leads to a higher risk of relapse. Identifying those patients who have high antibody levels would in all probability recognize patients that have a high bacterial load and consequently should receive longer treatment.

Chapter VI shows the results of an epidemiological study performed in Brazil using ML Dipstick for the detection of seropositivity among 7073 school children in three different leprosy endemic states. It was examined whether seropositivity rates could be related to leprosy detection rates and thus to be used as an indicator of the magnitude of leprosy problem in the community. As such it would be useful to evaluate the effect of control measures.

Chapter VII gives a summary and the main conclusions of the study.
1.2 Reference List


Chapter I


Chapter I


32


