Chapter 5
Serological and Molecular Investigations in Leprosy

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Abstract One of the most important and crucial aspects of leprosy control and elimination has been early diagnosis and treatment. Although we have been able to eradicate leprosy as a public health issue in many countries around the globe, its prevalence or new case diagnosis rate has not decreased significantly in the endemic countries over the last 15 years. The transmission of the disease and delayed detection of cases leading to deformities and even transmission are the major deterrents in our efforts to eradicate leprosy. Clinical criteria and slit skin smear (SSS) are the commonly used diagnostic modalities for leprosy. However, SSS is not practiced in most of the places due to increased risk of HIV/HBV/HCV and lack of expertise; hence, the diagnosis is based on clinical criteria only, which may miss some cases like polar lepromatous leprosy or pure neural leprosy. Extensive research has been carried out in the past to develop different serological and molecular assays for the diagnosis of leprosy. PGL-1 and LID-1 proteins are useful in serological testing; however, they have shown poor sensitivity in detection of paucibacillary and pure neuritic leprosy. The molecular-based approaches such as polymerase chain reaction (PCR) and real-time PCR are promising techniques for the diagnosis of leprosy because of higher sensitivity and specificity, but they are not feasible for use in the field settings due to requirement of equipment, setup, and expertise. We are still far from getting a rapid, easy point-of-care test for the diagnosis of leprosy as none of these diagnostic tests described are recommended by WHO for use in diagnosis of cases and contacts. Combination of both serological and molecular techniques will improve the leprosy diagnostics and will be helpful in diagnosis as well as monitoring the response to treatment as well.

Keywords Leprosy · Real-time PCR · qPCR, ELISA · Phenolic glycolipid-1 Leprosy · IDRI protein-1

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S. Pradhan, P. Kumar (eds.), *Clinical Cases in Leprosy*, Clinical Cases in Dermatology, https://doi.org/10.1007/978-3-031-08220-7_5

Introduction

Overview

Mycobacterium leprae (*M. leprae*) is the causative agent of leprosy, a chronic infectious disease with dermato-neurological and incapacitating symptoms. Despite various global initiatives to eradicate this disease, countries like India, Bangladesh, and Brazil still have high annual new case detection rates and it remains a public health issue. In this regard, WHO has designated the reduction of new cases as a priority in its global strategy, emphasising the importance of early detection, which seeks to minimise disease transmission in the population by early diagnosis of the suspicious case and contacts [1]. It is thought that a large number of people might have subclinical infection which self-heals with only minor symptoms. However, if untreated, leprosy may lead to a stage where it causes permanent nerve damage, including severe sensory and motor nerve loss, deformity, and blindness. It is already confirmed that sooner a leprosy patient is diagnosed and treated, the higher their chances of recovery. Identifying leprosy patients based on antigen-specific responses, ideally before the onset of symptoms, may seem to have a significant impact on clinical outcome [2].

Clinical criteria and slit skin smear (SSS) are the commonly used diagnostic modalities for leprosy. However, SSS is not being done in most of the places and in the field; hence, the diagnosis is based on clinical criteria only, which may miss some cases like polar lepromatous leprosy, histoid leprosy, and pure neural leprosy [3].

Early diagnosis and treatment have been the central tenets of leprosy control programmes. Early diagnosis is defined as diagnosis and start of treatment before onset of nerve impairment. Diagnosis of *M. leprae* at initial stages and early start of its treatment is essential not only for cure and prevention of deformities, but it may also prove useful in checking the transmission by earlier detection of multibacillary cases, but we need better diagnostic tests which could help in detecting leprosy cases that are missed by the clinical examination and slit skin smear examination.

Diagnosis of Leprosy

The detection of leprosy depends on existence of at least one of the three cardinal signs which include presence of skin lesions (that can vary widely in colour, appearance, and form) and existence of thickened or swollen peripheral nerves accompanied with varying degree of sensory loss, muscle weakness, and presence of acid-fast bacilli in the skin [4].

Detection of AFB in scraping of the skin is one of the cardinal signs for diagnosis of leprosy, and slit skin smear (SSS) has been one of the standard and most commonly used techniques, which has been discontinued over the last few years. It

needs to be reintroduced in the leprosy control programmes as it is not only helpful in early diagnosis of multibacillary cases of leprosy, but it also helps in assessing the response to treatment and stratification of cases as far as the risk of reactions and deformities is concerned.

Slit skin smears and histopathology of the skin are useful diagnostic modalities, but they also have their limitation for diagnosis as well as large-scale implementation in the leprosy control programmes. There is an urgent need for diagnostics (tests) that are inexpensive, specific, user-friendly, fast, accurate, and simple to deliver to end users. Broad surveys to determine the prevalence of leprosy in a given region would also benefit from such studies. Over the last few years, there has been a lot of research on the genomic structure and immune-pathogenesis of leprosy, and this has led to discovery of some useful antigens and techniques which are helpful in the diagnosis of leprosy.

The diagnostic tests used in leprosy can be broadly classified as bacterial tests which identify the bacillus in the samples like the SSS or the molecular tests like PCR or RT-PCR and the immunological tests which pick up the tissue or the immune response to the bacilli like serological tests against various leprosy antigens (PGL-1, LID-1).

Serological Investigations

Different mycobacterial antigens have been studied for the serological assays, and the basic principle is to study the antibodies directed against the antigen by using techniques like ELISA, agglutination, and lateral flow tests. Immunochromatographic lateral flow assay, detecting IgM antibodies against PGL-I and IgG antibodies to LID-1, is being developed as a point-of-care test for diagnosis of leprosy. We will be discussing some of the antigens which have been studied and have shown promise to be used for the diagnosis of leprosy (Table 5.1).

Phenolic Glycolipid-1 (PGL-1)

Phenolic glycolipid-1 (PGL-1) is an immunodominant antigen which induces a strong humoral immune response, mainly immunoglobulin M (IgM) which is measured using ELISA [5]. Brennan and Barrow in 1980 discovered PGL-1, and it was used by Payne et al. for the first time in serological studies in 1982. Identification of anti-PGL-1 antibody through ELISA is directly proportional to bacillary load which helps in classifying the severity of disease and also the response to treatment. During the treatment, decreased titre in anti-PGL-1 antibody is followed by antigen elimination and can correlate with BI [6]. However, PGL-1 antigen can persist in tissues for a long duration of time, even in the absence of live bacilli [7]. Therefore, positive anti PGL-1 titre is not always indicative of progressive disease [8, 9]. Although its

Table 5.1 Serological testing antigens and methods

Antigens	Presence in *M. leprae*	Efficacy	Drawbacks
Phenolic glycolipid-1	Cell wall protein of *M. leprae*	80–100% sensitivity in MB patients	1. Low titres in paucibacillary (PB) cases with sensitivity of 30–60% 2. No cut-off point for anti-PGL-1 titre to differentiate between disease and subclinical infection in leprosy patients and healthy individuals
35kD protein	Major membrane components of leprosy bacillus	98.5% sensitivity in MB patients	1. Only 46.7% sensitivity for PB patients 2. Poor performance with antibody levels near the cut-off value
LID 1 and NDO-LID	Protein	83.3% and 87%, respectively, sensitivity in MB patients	15.4% and 21.2%, respectively, in PB cases
IFN-γ	Pro-inflammatory marker against *M. leprae*	*M. Leprae* protein in combination with interferon gamma release assay (IGRA) provides better diagnosis	It can be detected in population who have developed sufficient immunity against *M. leprae*

performance in PB and pure neural leprosy cases is limited, serum anti-PGL-1 antibody response is a relatively reliable and simple method which is helpful to confirm diagnosis of MB leprosy and has even been used for prediction of type 2 lepra reactions [10–12].

35kD Protein

The epitope on the 35kD antigen of *M. leprae* reacts directly with MLO3-A1 monoclonal antibody [13]. Recent studies revealed that MLO3 shares 82% of its DNA and 90% of its amino acids with *M. avium also*, another species of mycobacterium [14]. Another specific sequence for 35kD, MLO4, is also used for serological tests. Initially developed as a radioimmunoassay based on competitive inhibition between patient's serum and I-125-labelled MLO4 [15], this assay eventually standardised as an ELISA using MLO4-labelled horse radish peroxidase [16, 17]. Despite the fact that this 35kD antigen shares certain genes with *M. avium*, *M. kansasii*, and *M. paratuberculosis*, the standardised serodiagnostic assay for leprosy diagnosis was found to be 97.5% precise and 90% sensitive [18]. Later, purified recombinant 35kD (r35kDa) protein was used and found 94.3% specific. The sensitivity for MB and PB cases was 83% and 17%, respectively. The presence of cross-reactive mycobacterial proteins of *M. smegmatis* in the cloned purified recombinant protein or the presence

of subclinical infection in the exposed contacts could explain the low sensitivity of the r35kD antigen assay [19].

M. Leprae *Recombinant Proteins and Development of LID-1 and NDO-LID Rapid Test*

The sequencing of *M. leprae* genome provided the opportunity for generation of protein diagnostic candidates, and a new fusion protein was developed by the Infectious Disease Research Institute, Seattle, USA, i.e., leprosy IDRI protein-1 (LID 1), which has expression of ML0405 and ML2331 antigens that have shown good immunogenicity in the serological assays and were considered appropriate alternatives for rapid diagnosis [20]. LID 1 can also be used as a carrier protein for the NDO to yield NDO-LID. Anti-natural octyl disaccharide-leprosy IDRI diagnostic (NDO-LID) is a ready-to-use kit for testing in the field and gives results within 20 min of charging of samples. Using this NDO-LID rapid diagnosis, the sensitivity and accuracy in detecting MB cases were found to be 87% and 96.1% [21].

IgA Antibody-Based Test

Salivary samples are used for the diagnosis of *M. leprae* using *M. leprae*-specific IgA antibodies in order to overcome the problem of invasive sampling. Different studies have used assays to measure salivary IgA/IgM antibodies against PGL-1 in patients and contacts and have found good correlation with serum IgM levels and recommend its use as a diagnostic tool for the contacts of leprosy patients [22]. Major problem with the serological assays in diagnosis of leprosy is their poor performance for detection of paucibacillary and pure neuritic leprosy.

Cytokines/Chemokines as Biomarker in Leprosy

During *M. leprae* infection, T cells get activated and secrete IFN-γ(interferon gamma) which is a pro-inflammatory marker against *M. leprae* and *M. tuberculosis* [23]. IFN-γ can be used as a marker for the diagnosis of *M. leprae*; however, we cannot differentiate between patients who have the disease and those who only have the infection or people who have been treated.

Moreover, immunopathogenicity induced by *M. leprae* infection activates host immune cells which secrete various effector and regulatory molecules. IL-1β, MIP-1, and MCP-1 can be used to differentiate pathogenic immunological responses existing in mycobacterial disease patients from those induced through asymptomatic *M. leprae* exposure.

Lastly, *M. leprae* protein such as ML-2478 in combination with interferon gamma release assay (IGRA) can be used as a novel method for anticipating the extent of *M. leprae* transmission in a given population and identifying people who are prone to contracting *M. leprae* infection and acquiring leprosy [24].

Gene-Based Assays

Molecular approaches like polymerase chain reaction (PCR) or real-time (RT)-PCR are routinely used for identification of specific *M. leprae* DNA sequence in clinical samples. These are highly sensitive assays which can be used for diagnosis of infection in doubtful/difficult cases, for assessing bacterial load, for detection of drug resistance, and for monitoring the response of treatment.

M. Leprae-*Specific PCR*

M. leprae-specific PCR could be carried out on routine basis in laboratory using DNA isolated from a wide range of biological specimens such as blood, skin smear, saliva, skin biopsy, oral or nasal swab, nerve section, and urine [25–28]. Detection range of *M. leprae* using PCR ranges between 10 and 30 fg which is equivalent to 2.8–8.3 bacilli [29]. Few *M. leprae*-specific PCR genes are RLEP, hsp85, 18 kDa, 36 kDa, 16S rRNA, and sodA (Table 5.2). Among these, the most sensitive and specific gene target-based PCR is *M. leprae*-specific repetitive element (RLEP) PCR [30]. The sensitivity of PCR is 100% in patients with a positive bacteriological index and lower in case of patients having low or negative bacteriological index.

Table 5.2 Comparative analysis of immunological and molecular markers in diagnosis of leprosy

Assay	Multibacillary patient's positivity (%)	Paucibacillary patient's positivity (%)
PGL-1 ELISA	80–100	30–60
35kD ELISA	98.5	46.7
r35kD ELISA	83	17
NDO-LID rapid test	87	21.2
PCR-using gene target RLEP	100	73
PCR-using 16S rRNA gene target	100	50
PCR-using 18 kDa gene target	99	74
PCR-using proline-rich antigen, 36 kDa	87–100	36–60

Multiplex PCR (M-PCR)

M-PCR is a better alternative and sensitive type of PCR technique in which two or more set of primers are used simultaneously for amplification of different target genes present in the same reaction (Table 5.3). However, selection of primers should be done carefully on the basis of these three parameters:

(a) The primers should have similar annealing temperature.
(b) The primers should not be complementary to each other.
(c) The size of the amplicon from each primer pair must be different so that they can be easily visualised as distinct bands by gel electrophoresis.

In *M. leprae* clinical diagnosis, M-PCR employs more than one specific gene to its DNA. This technique is used for the detection of paucibacillary forms or indeterminate leprosy by targeting pseudo genes of *M. leprae* such as ML1545, ML2180, and ML2179 with the positive detection range of 75.61% [31]. In case of PB patients, the positivity rate of M-PCR has been increased from 22.2% (conventional PCR) to 80.3% [32]. Different types of clinical samples can be used like blood, nasal swab, saliva, and SSS for the detection of PB and MB cases with the help of M-PCR using RLEP, 16S rRNA, and sodA targets [33] (Table 5.4).

After amplification of individual genes, products are electrophoresed using 2% agarose gel, whereas M-PCR-amplified gene products are electrophoresed using 4% agarose gel. The products are viewed using a gel documentation system. M-PCR using multiple gene targets improves the identification of *M. leprae* DNA with respect to sensitivity and specificity.

In Silico Molecular Techniques

In silico molecular techniques for drug resistance are used for the patients who are not responding to MDT. Resistance to anti-leprosy medicines like dapsone, rifampicin, and fluoroquinolones has been detected using molecular-based techniques to find mutation in drug resistance-determining regions (DRDR). Rifampicin resistance is associated with mutation in rpoB gene sequencing coding β-subunit of

Table 5.3 Sequences for commonly used primers in PCR

Gene	Sequence	Primer orientation	Amplicon size
16S rRNA	Forward	5′-CGGAAAGGTCTCTAAAAAATCTT-3′	171 bp
16S rRNA	Reverse	5′-CATCCTGCACCGCAAAAAGCTT-3′	
sodA	Forward	5′-CAGCTGTATGACCAACAGGC-3′	185 bp
sodA	Reverse	5′-TGCGTCTTAGATGTTGCAGC-3′	
RLEP	Forward	5′-TGCATGTCATGGCCTTGAGG-3′	129 bp
RLEP	Reverse	5′-CACCGATACCAGCGGCAGAA-3′	

Table 5.4 Sensitivity and specificity of different primers and different samples for diagnosis of leprosy

Clinical sample.	Target											
	M-PCR			RLEP			16S rRNA			sodA		
	Positivity(%)	Sensitivity	Specificity	Positivity (%)	Sensitivity	Specificity	Positivity (%)	Sensitivity	Specificity	Positivity (%)	Sensitivity	Specificity
SSS	93.33	0.93	1	51.66	0.51	1	31.66	0.31	1	21.66	0.21	1
Blood	86.66	0.86	1	46.66	0.46	1	53.33	0.53	1	53.33	0.53	1
Nasal swab	80	0.8	0.6	70	0.7	0.9	76.66	0.76	0.96	10	0.1	1
Saliva	54.48	0.548	1	45.16	0.45	1	35.48	0.35	1	6	0.06	1

RNA polymerase, dapsone resistance is associated with mutation within the folP1 sequence coding the dihydropteroate synthase (DDS), and ofloxacin resistance is associated with mutation within the gyrA sequence coding the subunit A of DNA gyrase [34]. To perform PCR for drug resistance, skin biopsy or SSS from the patient is preserved in 70% ethanol and sent to the laboratory to check mutation by gene sequencing in respective DRDR.

Loop-Mediated Isothermal Amplification (LAMP) Assay

It is a DNA amplification method that has been used to develop assays for various diseases like tuberculosis, nontuberculous mycobacteria, and COVID-19. Notomi et al. first devised this novel isothermal amplification method to amplify a limited amount of DNA copies into a million copies within an hour [35]. It utilises a set of four (or six) different primers which bind to six (or eight) different regions on the target gene making it highly specific. The end result or a positive test can be assessed easily by observing a change in turbidity or colour of the reaction with the naked eye or by using a turbidimeter or colorimeter or even a smartphone-based application for reading the colour or turbidity. It is an ideal assay for resource-constrained facilities due to minimal hardware requirements. The results can be read within an hour from the sample and the visualisation of the results is by seeing the change in colour of the analyte. Different primers have been used for the diagnosis of leprosy, a recent study by Jiang et.al developed a LAMP assay targeting the *M. leprae* RLEP gene and were of the opinion that the high sensitivity and rapidity of the LAMP assay, together with its ability to readily identify the *M. leprae* subspecies through naked eye evaluation, make it an attractive tool for routine diagnostics [36].

According to a recent meta-analysis of all leprosy diagnostic tests, agglutination tests had the highest sensitivity of the three serological tests studied (ELISA, agglutination test, and lateral flow), and all had comparable specificity. Among molecular analysis, qPCR had better sensitivity but lower specificity than traditional PCR. The PCR method was significantly more reliable than ELISA. However, the authors concluded that the findings among studies differed greatly, so they cannot suggest these tests for detection of leprosy patients due to heterogeneity in variation, thresholds, antigens targeted, and concerns about study aspect [37].

Conclusion

Early detection and management of *M. leprae* is the need of the hour if we want to check the transmission of leprosy and fulfil our dream of a leprosy-free world. We need more robust tests that can be used in the field to screen and diagnose leprosy patients and their contacts and maybe which can help us to classify the patients into paucibacillary and multibacillary so that adequate treatment can be given to those

diagnosed with leprosy. A combination of serological and molecular testing may prove to be useful and better and help to eradicate leprosy from society in order to have a *leprosy-free world*.

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