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Chapter 4

Molecular and Biotechnological Approaches in the Diagnosis of Leprosy

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Abstract

Leprosy is a worldwide health problem, which needs the development of new and innovative strategies to be controlled. Early diagnosis of leprosy is an important contribution to reducing the incidence of the disease; thus, the development of biotechnology platforms, which include the mapping of antigens with potential to be used in immunodiagnostic and molecular methods for the detection of *Mycobacterium leprae*, is an important tool to confirm the clinical diagnostic. Molecular biology and biotechnological methods have been used to assist in the diagnosis of this disease, each one with its advantages and drawbacks. Enzyme-linked immunosorbent assay (ELISA) is the used method for leprosy diagnosis, and it allows the detection of infection-related antigens. Alternatively, due to their versatility to perform the same functions as the protein and non-protein natural antigens, mimetic peptides are considered an important tool. On the other hand, lateral flow assay (LFA) and optical and electrochemical biosensors are rapid and portable methods, capable of performing diagnosis in the field without sample preparation. This chapter presents such techniques, their uses in the diagnosis and detection of M. leprae, as well as the potential for the development of new techniques and strategies that can help to control and understand mycobacteriosis.

Keywords: *Mycobacterium leprae*, immunoassays, molecular tests, biomarkers

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1. Introduction

Leprosy is a chronic infectious disease, dermato neurological and incapacitating, which has *Mycobacterium leprae* as its causal agent [1]. Even with the worldwide effort to eliminate this disease as a public health problem [2], countries such as India and Brazil still present a higher number of cases than the World Health Organization (WHO) recommended [3]. In this sense, this organization defined as a global strategy the reduction in the incidence of new cases as a priority [4], highlighting the importance of early diagnosis, which aims to reduce the transmission of the disease in the community [5], which includes correctly diagnosing cases with suspicion of the disease and to identify subclinical infection.

The clinical and epidemiological diagnosis of leprosy represents the gold standard for confirmation of the disease [6]. Bacilloscopy and Mitsuda's reaction are important tests to identify the etiological agent [7, 8], but there is a need to use complementary tests that allow a more accurate diagnosis with high sensitivity and specificity. In this context, stand out the standardization of serological and molecular tests, important for the understanding of the epidemiological profile of the disease.

The use of *M. leprae* specific antigens in serological tests has been the subject of research. The use of phenol-glycolipid-1 (PGL-1) [9], lipoarabinomannan (LAM) [1] and heat shock proteins (GroES and GroEL) [10] as antigens for the enzyme-linked immunosorbent assay (ELISA) and in immunosensors can be validated as methods for detecting new cases of the disease and for early diagnosis [11]. In addition, molecular tests aid in the identification of specific *M. leprae* sequences in clinical samples, which can be amplified through the polymerase chain reaction (PCR) technique, allowing DNA detection of the infectious agent [12] and/or through the use of real-time PCR technology that allows the evaluation of bacterial load [13] and also the monitoring of drug resistance [14].

Thus, this chapter will present an overview of the laboratory diagnosis of leprosy in the world. Initially, we will present a review of the main tests traditionally used in clinical routine and regulated by the WHO, in addition to the complementary tests that have been focus of research as a future perspective for the early diagnosis of the disease. These diagnostic approaches may contribute to a reduction in the number of cases of the disease, since they allow the monitoring of populations and endemic and hyperendemic areas.

2. Leprosy diagnosis: traditional exams

The diagnosis of leprosy includes clinical observation of the patient, dermato-neurological clinical exams and complementary laboratory tests. Therefore, identify lesions and damaged nerves and analyze the life history of the patient are essential tools in the identification and detection of disease. These practices combined with other tools and methodologies are able to assist in epidemiological and disease control strategies, helping to map index cases and individuals who may develop leprosy, such as household contacts.

2.1. Dermato-neurological clinical exam, bacilloscopy, histopathology and Mitsuda test

For the diagnosis of leprosy, it is essential to perform clinical-dermatological exams that search for lesions in the epidermis, areas with changes in sensitivity (may be thermal, painful and tactile) and motor impairment-searching for thickened nerve trunks-which are classic signs of the disease [7]. In addition to clinical examination, there is a class of diagnostic exams and tests that are widely standardized and used in reference networks and centers [7, 15].

Bacilloscopy is the most commonly used exam in clinical practice and, together with the dermato-neurological clinical test, is the most useful methodology for diagnosis [16]. The test presents advantages such as reduced cost and aid in the confirmation of new cases and patients with relapse [15]. The methodology used to perform the exam is a dermal smear of sites, these being: ear lobes (LO), elbows (C) and active lesions, where it is possible to analyze the presence of the bacillus using a specific staining and optic microscopy [17, 18].

As a result, a Bacilloscopy Index (BI), proposed by Ridley and Joplin, is provided, where there is a logarithmic scale ranging from 0 to 6 [19–21]. Thus, after an average of the fields analyzed, the result may vary from BI = 0, associated with patients of the tuberculoid (TT) pole and BI = 3+ to 6+, associated with lepromatous patients (LL). It is still possible to analyze the morphological index (MI) of the bacilli arranged on the slide, describing aspects of their morphological integrity [20]. Thus, bacilli may present three aspects related to their structure, classified as integral, fragmented or granular [21, 22]. Integral bacilli are considered viable, that is, they are related to host susceptibility to the parasite. These bacilli exhibit cell structure with preserved ends and uniform staining and are seen in smears of patients who are either non-treated or have relapse. Both fragmented and granular bacilli present flaws in the cell wall structure, being considered unviable or dead and more observed in post-treatment patients [17–22].

Bacilloscopy is effective when associated with the results of clinical exams [7, 15]. However, it is an exam that presents low sensitivity, since 50% of the smears of the sick individuals are negative. In addition, the exam requires adequate laboratory infrastructure and trained professional apparatus, factors that are not always a reality in hyperendemic regions and where medical and financial resources are reduced [7].

Histopathology is commonly performed for the diagnosis of diseases caused by obligate intracellular parasites and has good indices of sensitivity and specificity in the detection of leprosy [23]. However, the method encounters issues related to cost, time of analysis and false-negative results, besides of being an invasive exam [24]. Exactly for these reasons, histopathology is only recommended for individuals where it is impossible to assess degrees of cutaneous sensitivity, such as in children, elderly and mentally handicapped people; when it is not possible to classify the dimorphic clinical form; or when there is uncertainty whether the diagnosis is leprosy or other diseases that cause local hypoesthesia [23]. The diagnosis using this exam also depends on the association with the clinical aspects and bacilloscopy. In addition, biopsies of peripheral nerve branches are not recommended and should only be performed in the last instance [23, 24].

The Mitsuda test or Mitsuda reaction does not present diagnostic value, but it is an alternative prognostic tool that assists in assessing susceptibility to lepromatous forms [27]. It is an exam based on the use of heat-killed bacilli (lepromin), derived from extract of the inactivated "leprosy bacillus" under the skin of LL patients. The test consists of inoculating the Mitsuda antigen intradermally and reading them between days 21 and 28 after the challenge in order to analyze the late cellular response of patients [8, 25].

Mitsuda's reaction has good agreement when related to bacilloscopy. Typically, individuals with a diameter reaction greater than 10 mm are considered resistant; they do not get sick or develop the TT shape, being Mitsuda positive [24]. While reactions with a diameter between 3 and 5 mm are indicative of dimorphic leprosy and, below this value, the test indicates anergy of the host's immune system to the bacillus, associated with patients in the LL pole, being Mitsuda negatives [8, 24, 26]

3. Complementary immunological tests

The discovery of the lipid apparatus present in the bacillus capsule and the characterization of a range of important lipidic and proteic components in the immunogenicity [30] allowed innovations in the leprosy serology [27].

Serological techniques are based on the detection of specific antibodies against the bacillus, since immunoglobulin production occurs in response to the antigenic signal of stimulation. These tests are important because they represent a class of complementary tests capable of detecting leprosy cases, besides the possibility of diagnosis recommendation, disease prevalence determination, infection evaluation in endemic and hyperendemic areas, and household contacts monitoring.

3.1. ELISA with native PGL-1 and its synthetic molecules

Many researches have used natural *M. leprae* antigens for the immunodiagnosis of leprosy [19, 28]. The elucidation of the structure of the PLG-1-the first *M. leprae* specific antigen to be isolated and the main antigenic glycolipid of the bacillus [29]-is a clear example of the widespread use of these molecules.

ELISA (enzyme-linked immunosorbent assay) has been widely used as a research tool for the detection of anti-PGL-1-native antibodies [29–32]. The technique consists of a quantitative test based on the IgM class antibodies response. In this scenario, important aspects in the biology and epidemiology of the disease, such as the determination and comparison of the positivity of patients and home contacts in several areas, besides the fluctuations in the reactivity profile in individuals from the hyperendemic area [33, 34] have been described from the studies with PGL-1.

Anti-PGL-1 antibodies are present in large numbers in untreated multibacillary patients, but paucibacillary patients naturally have low circulating antibody concentrations. For this reason, some of these patients present negative results against the diagnosis, even showing positive clinical signs [35, 36].

In addition, there's still a great limitation in obtaining the native molecule, restricted to the growth of *M. leprae* in armadillos. As an alternative, several synthetic analogous molecules associated with the tri or disaccharides of PGL-1 have been produced from the conjugation of these elements with BSA (Bovine serum albumin) and Phenol (P) or Octyl (O). The literature shows several semisynthetic analogues, among which the most well known are: monosaccharide-octyl-BSA (MO-BSA), disaccharide-BSA (D-BSA), natural disaccharide-octyl-BSA, natural octyl-HSA (ND-O -BSA and ND-O-HSA), natural trisaccharide-phenol-BSA (NT-P-BSA) which are used as antigens in immunodiagnosis [30, 36].

A study carried out in the hyperendemic region of the Brazilian Amazon points to the potential of synthetic molecules to identify new cases of leprosy and, similar to glycolipid, they have good detection rates in multibacillary patients. In addition, research shows that the molecules exhibit behavior related to the spectral immunology of the disease, where the LL pole has a higher antibody titer that decays at the boderline and tuberculoid poles [35]. When comparing two molecules derived from PGL-1 in the region, it was possible to observe that NT-P-BSA was very effective in the monitoring of home contacts and MB patients, whereas ND-O-BSA obtained better sensitivity and specificity indices in paucibacillary individuals [37].

ELISA requires skilled labor and specific equipment which is not always available. Therefore, the use of serological tests of both the native molecule and synthetic derivatives is important to validate increasingly adequate methods for the seroepidemiology of both endemic and hyperendemic regions, besides helping to standardize the positivity indices of the clinical forms that can vary intensely from one area to another.

3.2. ELISA with recombinant proteins

If the characterization of PGL-1 was important for the synthesis of several derived molecules with similar immunological aspects, the decoding of the *M. leprae* genome was essential for the identification of proteins and peptides with applicability in the laboratory detection of the disease [38]. Several advantages are associated with the use of these molecules, especially for reducing the cost of the assays and reflecting the spectral character of leprosy immunology. Thus, assays using recombinant proteins indicate high levels of antibodies in LL patients that decay in patients of the boderline and tuberculoid poles [38–43].Currently, there are a large number of *M. leprae* recombinant proteins, normally identified as ML, and several studies have evaluated the potential of these proteins [44]. In 2007, a survey conducted in Brazil, the Philippines and Japan evaluated the cellular and humoral response to 33 recombinant proteins across a broad population spectrum and identified three proteins (ML0405, ML2055 and ML2331) capable of inducing the humoral response in multibacillary (MB), production of IgG, as well as the cellular response in PB. Comparison between sites identified differential response patterns between populations, however, in all locations ML0405 and ML2331 showed similar results to PGL-1 serology in MB33 patients [40].

Due to the potential of these two proteins, the Leprosy IDRI Diagnostic protein (LID-1) was generated, resulting from the fusion between the two MLs. LID-1 was produced in order to maintain the reactivity profile of both proteins and was subsequently evaluated in several populations of Japan, Brazil, Venezuela, the Philippines and Nepal. The results pointed to the

potential of early detection of the disease using this protein, besides the possibility of its immobilization in different platforms [40].

Also in this scenario, in 2009 a chimeric protein with multiple epitopes (PADL), from the fusion of epitopes of recombinant proteins (ML0405, ML0049, ML0050, ML0091, ML0411, ML2055 and ML2311) was designed. The chimeric molecule was tested in serum from pauci and multibacillary patients living in Brazil and the endemic controls from the Philippines. The results demonstrated that all the portions that formed the protein have specific binding capacity to antibodies and the same showed great effectiveness in the diagnosis of MB patients and no specific response to the serum of the endemic controls, showing promise in the diagnosis of the disease [45].

3.3. Lateral flow tests

In addition to the ELISA immunoassays, leprosy serology may also be performed with the lateral flow test, known as ML-flow. The development of this immunochromatographic semiquantitative assay was due, in particular, to the possibility of field use. The test was developed by Burker-Sékula et al. [46], aiming the detection of IgM antibodies against several antigenic molecules such as PGL-1 and its synthetic derivatives, recombinant proteins and peptides [30].

The ML-flow test is not a diagnostic method, but assists in the classification of patients and presents low cost and easy execution, making its use possible in health services routine, especially in regions where laboratory resources are not available [30, 35, 46, 47]. Therefore, ML-flow is a methodology widely used in hyperendemic areas, especially in Brazil, where populations of Maranhão, São Paulo, Pará and Minas Gerais states were tested and reaffirm the importance of detection and control of disease cases through simple but reliable methodologies [32, 48–52].

In the search for increasingly fast and accurate tests, in 2012 the Brazilian Institute of Infectious Research together with the company OrangeLife developed a test capable of offering a diagnosis in only 10 minutes using only one drop of blood of the patient. The tool has received approval from the Brazilian National Sanitary Surveillance Agency (ANVISA) and has been field tested to evaluate the potential of the new platform. The assay is based on immunochromatography aspects, where recombinant proteins like LID-1, used as antigen, are immobilized on nitrocellulose membranes. Detection can be performed by the presence of IgM and IgG antibodies from various samples such as whole blood, plasma and serum.

Among the advantages of the test, it is mentioned the low cost of the tool, the possibility of early detection, agile and minimally invasive. In addition, it is associated with software stored on smartphones, further facilitating the interpretation of the exam and ensuring the availability of information in databases. Parallel analyzes of the rapid detection tool show the ability to diagnose, in most cases, the presence of the infection before clinical symptoms appear, contributing to the generation of accurate diagnoses and quality.

The interdisciplinary researches related to leprosy provided an amount of laboratory tools used as alternative methodologies for the more accurate and efficient diagnosis of the disease. Although there are still difficulties linked to the detection of paucibacillary forms, subclinical infections and contact monitoring, all research reinforces the importance of the search and use of efficient platforms and able to ally reduced cost and good indexes of sensitivity and specificity for the disease.

4. Complementary molecular tests

After the advent of the genome sequencing of the *M. leprae* bacterium [53], species-specific genetic sequences have been searched in order to standardize diagnostic tests based on DNA analysis. These sequences can be amplified through the PCR technique, which allows the detection of bacillus DNA from small amounts of *M. leprae* cells [12].

The first works using the PCR technique were performed a little over 20 years, but the data were not satisfactory for the identification of *M. leprae* DNA in paucibacillary (PB) patients [54]. Thus, the methodology of molecular biology began to be used as an alternative method to traditional diagnostic methods. These data stimulated the search for new specific sequences for the identification of the bacillus, as well as the use of several clinical samples [55].

Many studies have been carried out involving different sequences and target genes, with the aim of increasing sensitivity and specificity in the identification of bacillus, especially in patients with low bacillary load. The literature reports the use of sequences that amplify gene regions encoding the 36 kDa [56], 18-kDa [57] and 65-kDa antigens [58], complex 85 [59], 16S rDNA [60] as well as for repetitive sequences of *M. leprae* (RLEP) [61]. By comparing these sequences, RLEP has been shown to be more sensitive and more specific than the bacilloscopic index. This could be explained by the number of copies, estimated to be at least 28 units, of the RLEP sequence in the *M. leprae* genome. In addition, this sequence generates a 130pb amplicon, which is considerably small compared to the sequences mentioned above, that is an important factor in the best efficiency in conventional PCR. Having a specific sequence is of great importance, since the PCR technique may be useful in the differential dermatological diagnosis [62].

A significant advance in increasing bacillus identification occurred with the use of real-time PCR technology. This methodology has been used in the follow-up of leprosy patients undergoing treatment [63] evaluation of bacterial load [13] viable bacterial load [60] and determination of resistance to treatment [14].

In clinical practice, detection of *M. leprae* by PCR in patients with negative bacilloscopy or inconclusive histopathology is of great value to define the correct diagnosis and treatment scheme [64]. In the same way, the methodology can be useful, for those patients with the pure neural form (PNL), who usually do not have cutaneous lesions and because of this they have deficient treatment scheme [65, 66]. Further, the PCR technique may be useful in early identification, since a considerable number of studies have addressed the positivity of *M. leprae* DNA in contacts of leprosy patients [48, 67–69].

In a study carried out in a hyperendemic area in cases of leprosy, it was possible to identify DNA from the bacterium in buccal and nasal swab samples in individuals with subclinical infection with multibacillary or paucibacillary index cases [48]. The identification of DNA from the Hansen bacillus in buccal and nasal swab raises considerations about the participation of this risk group in the transmission chain, besides the route of infection of the bacillus [70]. Although PCR can be a useful tool for identification, few studies associate the presence of *M. leprae* DNA to the development of the disease [71, 72], highlighting the importance of the use of serological tools and the follow-up of patients with subclinical infection [73, 69]

5. New biotechnological tools in the diagnosis of leprosy

5.1. Mapping of new markers

Many studies have used post genomic procedures for the discovery of new antigens that can be used in the diagnosis of leprosy [71–75]. These studies have explored sequences of *M. leprae* for the identification of proteins or peptides that can be used in the serodiagnosis of the different clinical forms of leprosy [76].

The antigens ML0405, ML2331 and ML2055—the first two of previously unknown function and the latter a membrane protein - were used for serological tests in in multibacillary patients of the clinical forms boderline lepromatous (BL) and LL untreated [77]. The ML0308 and ML2498 proteins, a conserved hypothetical protein and an enoyl-CoA hydratase respectively, showed humoral and cellular immunogenicity and can be used in the diagnosis of tuberculoid and lepromatous forms [78]. These antigens were used in the production of fusion proteins, such as LID-1 (leprosy IDRI diagnostic-1) [42] and PADL [45, 79].

The tools of bioinformatics, genomic analysis and proteomics are also being used for mapping in silico of important antigenic targets of *M. leprae* [80]. This type of analysis was used to define a group of 50 potential antigens in mycobacteria, some being restricted to *M. leprae* [78].

Peptides derived from specific and immunogenic proteins of *M. leprae* have also been tested in patients with leprosy and controls [81]. Peptides obtained of proteins from *M. leprae* were promising as indicators of exposure [82].

The peptides are small in size and can be expressed on the surface of bacteriophage to select peptides that mimic different targets (pathogens, cellular receptors or antibodies) [83]. Mimetic peptides may have important applications in the diagnosis of leprosy, mimicking antigens such as PGL-1 [84] or other *M. leprae* antigens [85, 86]. Alternatively, due to their versatility to perform the same functions as the protein and non-protein natural antigens, mimetic peptides are considered an important tool in immunodiagnostic of infectious disease.

5.2. Biosensors as platforms for the diagnosis of leprosy.

The post-genomic, the identification and obtainment of hundreds of molecules with immunogenic potential have broadened the versatility of detection platforms and contributed to an optimal diagnostic test, especially for tropical diseases [87]. In recent decades, biosensors have been gaining more space in scientific research and diagnosis of various diseases [88].

Biosensors are analytical devices that have specific reactions and/or specific interactions mediated by a diversity of components (antigens, antibodies, enzymes, DNA fragments, organelles, receptors and even mimetic peptides) that, in contact with a transducer, have the conversion of a biological signal-a result of the interaction between specific components-in a measurable signal proportional to the analyte concentration [89]. These platforms can be electrochemical, piezoelectric, thermal, optical and based on surface plasmon resonance, depending on the

type of transducer used [90]. There are still specific classes of biosensors such as immunosensors [91], which evaluate interactions between antibodies and antigens, and genosensores, based on the hybridization of DNA-specific ribbons [87].

There is a wide range of studies showing the efficacy of biosensors for the detection of various diseases such as leishmaniasis, bacterial diseases, cystic fibrosis, dengue and leprosy itself [92–96]. In Brazil, a genosensor for *M. leprae* was constructed using the immobilization of a bacillus single-stranded DNA (ssDNA) on functionalized graphite electrodes. The interaction between the immobilized sequence and *M. leprae* double-stranded DNA (dsDNA) is measured electrochemically by reductions in the peak oxidation current and using ferrocenecarboxyaldehyde as the hybridization indicator. The result was very promising, showing efficient detection in only 3 minutes [87, 88, 96].

Currently, Brazilian research groups are betting on the use of electrochemical biosensors as an indispensable tool in the diagnosis and control of diseases. This innovation is mainly because these sensors are sensitive, reliable, fast response and operate in conditions that pretreat the samples. In addition, these techniques are capable of providing exceptionally low detection limits.

In this scenario, mimetic peptides of proteins and glycolipids present in the bacillus capsule have been validated for the immunogenic potential and immobilized in these electrochemical detection platforms. Thus, the proposals consist of using different biological fluids such as blood, secretion and saliva, ensuring a less invasive and more comfortable test to the patient and the manipulator. Subsequently, these platforms will be tested in hyperendemic areas, in order to evaluate their detection potential and help in the epidemiological control of the disease.

6. Conclusion

The early diagnosis of leprosy is one of the goals of the WHO for the control and reduction of new cases of the disease. This strategy will be implemented with the development of new diagnostic tools more sensitive and can be applied in large-scale monitoring. Molecular techniques and new biotechnological approaches can be used as complementary tests.The qualitative PCR, RLEP and real time PCR have been used for the detection of *M. leprae* in samples of different tissues of patients or of household contacts.

Immunodiagnosis can be done using different native *M. leprae* antigens such as PGL-1, LAM or their synthetic derivatives. Post-genomic technologies can be used for the production of recombinant chimeric proteins, peptides obtained in silico or mimetic peptides. Immunodiagnosis can be performed by ELISA, lateral flow tests and biological sensors.

Biotechnology and molecular biology have contributed to the development of research and improve the diagnosis of leprosy. Significant advances in laboratory diagnosis contribute to improving clinical practice.

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