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Review Article

Molecular and Biotechnological Approaches in the Diagnoses of Mycobacteriosis

Souza AG[1]*, Lima MIS[2], Bastos VAF[1], Marangoni K[1] and Goulart VA[1]

[1]Department of Genetics and Biochemistry, Federal University of Uberlândia, Brazil

[2]Department of Biology, Federal University of Maranhão, Brazil

***Corresponding author:** Aline Gomes de Souza, Laboratory of Nanobiotechnology, Department of Genetics and Biochemistry, Federal University of Uberlândia, 38400-902, Uberlândia-MG, Brazil

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Abstract

Mycobacteriosis such as tuberculosis and leprae are a worldwide health problem, which need the development of new and innovative strategies to be controlled. In some cases, the infection can be asymptomatic, wild animal can serve as reservoirs and opportunistic co-infections can occur. Those factors contribute to high rates of infection worldwide, and hamper the control and eradication of mycobacteriosis. Molecular biology and biotechnological methods have been used to assist in the diagnosis of those diseases, each with advantages and drawbacks. PCR diagnosis tuberculosis through the amplification of the IS6110 sequence, and it is a very reliable technique, but it is a time consuming and requires skilled professionals and well-prepared facilities. Enzyme-Linked Immunosorbent Assay (ELISA) is the most used method for tuberculosis diagnosis, it allows the detection of multiple infection related antigens and can be modified accordingly to the researcher needs, but it is also time consuming, and requires sample preparation and specialized laboratorial infrastructure. In other hand, Lateral Flow Assay (LFA) and biosensors are rapid and portable methods, capable of performing diagnosis in the field without sample preparation, however, these methods requires extensive standardization, and the number of antibodies used for detections are limited. This review presents such techniques their uses in the diagnosis and detection of *M. tuberculosis* and *M. leprae*, as well as the potential for the development of new techniques and strategies that can help to control and understanding mycobacteriosis.

Keywords: Mycobacteriosis; *M. tuberculosis*; *M. leprae*

Introduction

Singular characteristics such as: hydrophobicity in aqueous medium, slow growth, resistance to acids, disinfectants and antibodies are present in microorganisms of the genera *Mycobacterium spp* [1]. In these genera, we can highlight *M. tuberculosis* and *M. leprae* as they can cause Tuberculosis (TB) and leprosy respectively, two important infectious diseases [2,3].

Although those diseases were discovered more than a century ago, they still represent an important public health problem. In 2014, approximately 9 million new cases of tuberculosis and 1.5 million deaths were reported, according to World Health Organization (WHO). In the first quarter of the same year, the global prevalence of leprosy cases was 180 thousand worldwide, while the number of new cases reported in 2013 was 215.000 [4].

With the aim of a better, faster and reliable diagnose system for those diseases; molecular tools such as genetic sequencing, Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) and chromatography have been used [3-5]. Besides molecular techniques, the construction of biosensors has been proving to be a promising way to improve the actual diagnosis methods for *M. tuberculosis e M. leprae* [5,6].

Molecular Approaches

PCR

PCR utilizes the acid nuclei amplification to exponentially replicate specific target genes. Its simplicity, sensitivity and specificity, makes PCR one of the most used techniques in the diagnosis and study of many diseases, including tuberculosis and leprosy [7-9].

PCR can be used conventionally, to identify mycobacteria's genes presents in a sample, while real time PCR enables to verify the expression of infection related genes; and both can be used with others molecular techniques to improve mycobacteriosis diagnosis [10-12].

Chawla et al., (2009) evaluated the presence of the IS6110 insert in 104 different tissue samples using PCR and compared it to histopathological analysis, considered the gold standard for tuberculosis diagnosis. The results showed that PCR could achieve 74.1% sensitivity and 96.1% specificity. Thus, the authors suggest that PCR can be used for early diagnosis and treatment of tuberculosis in tissue samples, since histopathological diagnosis can take up to two weeks [7]. Ani et al., (2009) also presented PCR as a potent technique for the detection of *M. tuberculosis*. In his work, 141 patients (101 HIV-positive and 40 clinical specimens) were evaluated, all patients were suspected for pulmonary tuberculosis. PCR shown sensitivity and specificity above 70.0%, demonstrating that PCR is a rapid, sensitive and specific method in the investigation and detection of *M. tuberculosis* [13].

Lee et al., (2010) presented a novel multiplex PCR assay, were the amplification of the RD1 target gene can simultaneous identify and discriminate the presence of *M. tuberculosis* and *M. bovis*

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(BCG) in samples. In his work, reference strains were compared to nontuberculous mycobacteria, infected patients samples, vaccine extracts and nonmycobacterial specimens. The RD1 gene was used as target, since it is absent in all BCG strains and vaccines, but is present in the virulent *M. bovis* and *M. tuberculosis*. The authors suggest that multiplex PCR can be used in suspect of tuberculosis, as it is a fast, sensitive and reliable assay [14].

Malbruny et al., (2011) used a commercial real-time PCR assay kit to detect *M. tuberculosis*. In this work, 180 samples, 91 respiratory and 89 non-respiratory, from 132 patients with suspect of tuberculosis were submitted to real-time PCR and smear analysis. Only 17.2% of the analyzed samples yield *M. tuberculosis* on culture. Smear analysis had a sensitivity of 64.7% and 28.6% for respiratory and non-respiratory samples respectively, with >98% specificity, while the real-time PCR assay was able to achieve 100% of sensitivity and specificity on respiratory samples and 85.7% and 97.3% on non-respiratory samples. Accordingly to the authors, even though the test was validated on respiratory samples, it could also be used to diagnose extra-pulmonary tuberculosis [15].

Recently, Ushio et al., (2016) presented the use of digital PCR (dPCR) to detect *M. tuberculosis* in plasma samples of tuberculosis patients. The authors evaluated the copy number of IS6110 and gyrB genes, associated with tuberculosis infection. In both cases, significant differences were observed, and dPCR showed a sensitivity of 65% and 29% and specificity of 93% and 100% for IS6110 and gyrB respectively. Since sample collection is minimally invasive, the authors suggest dPCR as a potential diagnose option [16].

As well as in the detection of *M. tuberculosis*, PCR can also be used to detect *M. leprae*. Martinez et al., (2009) presented two methods for detection of *M. leprae* using RT-PCR targeting sodA mRNA and 16S rRNA. Both genes used showed high expression levels at pretreatment with rifampin and significant lower levels at 48 hours post treatment. Within one-week treatment, expression levels for both genes were considered background. Since viability of *M. leprae* is hard to predict as it is non cultivable on axenic media, the RT-PCR proposed by the authors could be useful for experimental studies and to predict the viability of *M. leprae* in biopsy samples [9].

In a case report, Edwards et al., (2014) showed that PCR can detect infections by *M. leprae*, with high sensitivity and specificity, helping clinical diagnosis and permitting timely treatment. The authors highlight the need for better and fast diagnosis both for tuberculosis and leprosy in Australia since both had high prevalence in some areas [17].

Considering those examples and the difficulties related to diagnosis and research of mycobacteriosis, the PCR technique has strongly contributed to the field as a versatile tool. The base technique can be improved and modified to attend the needs of better, faster and reliable detection.

ELISA (Enzyme-Linked Immunosorbent Assay)

Developed in the 70's, ELISA is a serological tool capable of determining quantitatively the presence of specific antigens in samples. The assay consists in the adsorption of a specific antibody (capture antibody) into a solid matrix; then the sample to be tested is added to the coated surface. The target molecule will bind to the capture antibody, while other molecules will be washed way. Another antibody (primary) is then added; this antibody will bind to the, already trapped, target molecule forming a sandwich-like structure. In the next step, the enzyme-linked antibody (secondary) is added to the mix. This conjugated antibody will emit a detectable light signal when the enzyme substrate is added. The signal detected is directly related to the amount of target molecules in the samples [18,19]. Thanks to its practicality, ELISA can be used to detect any number molecules and antigens, rendering it an amazing toll to diagnose of various diseases.

Some *M. leprae* antigens have been used in immune assays, amongst then, the Phenolic Glycolipid-1 (PGL-1). The evidence of his antigenicity and specificity has enable great innovation in the leprosy research, as his antigenic component is widely explored for serological diagnosis [20,21]. The use of native PGL-1 as antigen for ELISA is validated as a diagnosis method for new cases and subclinical infection of leprosy, since the antibodies production is a sign of antigenic stimuli [22,23]. Furthermore, there is a significant correlation between IgM anti-PGL-1 and the Baciloscopic Index level (BI), indicating that anti-PGL-1 levels are related to the bacillary load in leprosy patients, and thus, suggesting his use for monitoring the patient response to anti-leprosy chemotherapy [24-26].

In 2008, Aurtenetxe et al., (2008) reported the use of ELISA to detect *M. bovis* infection using PPD-B and protein G as the detection reagent. The authors tested 185 positive and negative serum samples of bovine tuberculosis. After standardization in diverse serum dilution, antigen and conjugate concentrations and ratios, the test yielded a specificity of 96.43%, and sensitivity of 72.60% [27].

Boadella et al., (2011) evaluated two types of ELISA assay, using *M. bovis* Purified Protein Derivative (bPPD) and Paratuberculosis Protoplasmatic Antigen 3 (PPA3) as antigens, and compared the assay with the Dual-Path Platform tuberculosis test (DPP). In his work, 200 serum samples (96 positive and 104 negative in culture) were analysed. DPP had sensitivity and specificity of 89.6% and 90.4% respectively, while ELISA presented sensitivity and specificity of 79.2% and 100.0% respectively. The wild boars are reservoirs for members of the *Mycobacterium tuberculosis* complex, especially for *Mycobacterium bovis*, and can contribute to the widespread of the disease. The development of rapid, cheap and reliable serological tests, as ELISA, can assist in the epidemiological research and in the monitoring of endemic regions [28].

Lateral-flow immunochromatographic assay

Lateral Flow Assay (LFA) is a rapid alternative to simple, but laborious serological tests. LFA's can provide qualitative and quantitative analyses within minutes, at low costs and eliminating the need of skilled professionals. Generally, the test is performed on a base strip, containing a sample application pad, conjugate pad, test line, control line and an absorbent pad, each part playing a specific and important role. The sample will flow through the strip until the conjugate pad, were it will bind to labeled conjugated antibodies. The complex (target molecule and labeled conjugate) will continue to flow until the test line. In the test line, only the complex will bind, giving off a colorimetric signal, just after the test line, is the control line composed of unspecific antibodies, and serving to validate the test [29,30].

The advantages of LFA's are many when compare to other diagnosis methods. However, LFA's are only recommended in cases where the relation antigen-antibody is well determined and error is unlikely to occur.

Various already characterized molecules, such as the leprosy antigen, PGL-1, Monosaccharide Octyl BSA (MD-O-BSA), Natural Disaccharide Octyl BSA (ND-O-BSA), Natural Disaccharide Octyl HSA (ND-O-HSA), Disaccharide BSA (D-BSA) and Natural Threesaccharide Phenol BSA (NT-P-BSA) have been used in LFA platforms [31]. Bührer-Sékula et al., (2008) demonstrated the use of a fast LFA method for detection of anti-PGL-1 in *M. leprae*. The test can be performed in whole blood and serum samples, takes only 10 minutes, and the apparatus and running buffer can be stored up to one year without affecting the results. The sensitivity and specificity were 97.4% and 90.2% respectively. In endemic regions, leprosy control can be difficult to achieve as asymptomatic patients can act as reservoirs with high bacillary loads. With rapid and inexpensive tests, the population can be monitored periodically, helping to control the disease spread [32].

Bobosha et al., (2014) developed new platforms and strategies for the detection and diagnosis of early stages infection by *M. leprae*. Aiming to detect antibodies and pro-/anti-inflammatory and regulatory cytokines against *M. leprae* in asymptomatic patients, the authors developed dry format LFA and multiplex LFA. Those platforms enable the detection of anti-PGL-1, IP10 and IL-10 with good correlation with ELISA assays [33].

In tuberculosis detection, LFA's have been suggested as primary diagnosis methods. Manabe et al., (2014) points the correlation between HIV immunosuppressed patients, co-infection by *M. tuberculosis, C. neoformans* and lethal outcome. HIV infected adults are prone to opportunistic infections, which can lead to death and potentiate an already major health problem. In this study, the authors analyzed sputum and urine samples of 351 HIV-TB positive patients. The mortality rate of HIV patients in Sub-Saharan Africa in six months after hospitalization is one third. The high mortality rate is associated with co-infections by *Mycobacteria cryptococcus*. The use of LFA to diagnose co-infections using urine samples, can help to start appropriate therapy and decrease the mortality rate [34].

Akyar et al., (2010) demonstrated the specificity of the mycobacterial antigen MPB64 for LFA. In his study, 94 reference strains of 34 *M. tuberculosis*, 97 nontuberculous bacilli, 7 *M. bovis* BCG substrains and 256 clinical mycobacterium isolates were tested. All samples were culture for 10 to 12 days prior the assay. The results were compared with PCR targeting the IS6110 sequence. The test had sensitivity and the specificity of 98.6% and 100% respectively [35].

New strategies can be incorporated aiming to detect specific or multiple antigens. Since LFA platforms are portable, cheap to produce, easy to use, dispense the need of lab professionals and equipment, and provide fast and reliable results, they are a good alternative for detecting and monitoring neglected infectious diseases in developing countries.

Biotechnological Methods

Biosensors

Biosensors have been developed aiming to easy the process of detection and quantification of various chemicals, bio products and contaminants. Their practical use, portability and low production costs, makes biosensors a formidable tool for a variety of applications [36,37].

Pathogen detection in most cases is laborious, time consuming and has the need of trained professionals and proper equipped test facilities. The majority of protocols implicated a culture or sample enrichment step [38,39]. Pathogen detection biosensors can ease the burden of routine test, since a portable biosensor can be operated by anyone and anywhere, giving fast results [36,38-40].

Most diagnosis methods for TB involves cell culture and pathogen enrichment, those protocols demand a great deal of time and work, since *M. tuberculosis* is a slow growth bacteria [38]. Aiming to provide fast and reliable diagnosis protocols, new techniques have been developed for the detection of *M. tuberculosis*, such as PCR, ELISA, flow cytometry and Xpert MTB/RIF assay, recommended by the WHO. However, those methods still demand high qualified professionals and specific instruments. In that context, the development of a portable, reliable, sensitive and easy to operate biosensors for the detection of *M. tuberculosis* and others mycobacteria is essential.

Shojaei et al., (2014) developed a method for rapid detection of *M. tuberculosis* using Fluorescence Resonance Energy Transfer (FRET) biosensor. In his work 50 clinical sputum samples of suspected tuberculosis patients were analyzed by FRET biosensor, PCR and nested PCR for comparison. The FRET biosensor system used gold nanoparticles and CdTe quantum dots conjugated with two complementary nucleotides probes aiming to detect the conserved region ESAT-6. When the target region is present in the sample a sandwich is formed and the Au NPs act as a fluorescence acceptor, emitting a detectable FRET signal. Compared to the PRC and nested PCR assay, the FRET system presented sensitivity and specificity of 94.2% and 86.6% respectively, against 74.2% and 73.3% for PCR and 82.8% and 80% for nested PCR. Although this system is more expensive than PCR related methods, it is a reliable alternative for the detection of *M. tuberculosis* when there is a high demand of tests [41,42].

Silva et al., (2011) presented a portable optoeletronic biosensor platform for the detection of *M. tuberculosis*. Using functionalized gold nanoparticles, a double color diode as light source and a silicon photodetector, the authors demonstrated the detection of *M. tuberculosis* sequences and their discrimination between *M. tuberculosis* and *M. bovis* in biological samples. This platform is portable and has low cost production; however, the sample has to be submitted to PCR for target sequence amplification and heat denaturation prior to detection [43].

Kaewphinit et al., (2012) used a quartz crystal microbalance DNA biosensor to detect the IS6110 sequence of *M. tuberculosis* in PCR products and in sputum samples. In his work, specific oligonucleotide sequences were immobilized on the gold electrode of a quartz crystal. The target sequence IS6110 was amplified by PCR, and the PCR product was incubated with the probes for signal detection observing the frequency changes in the biosensor. This method was also used to detect *M. tuberculosis* in sputum samples that tested positive and negative for TB in culture assay. The biosensor showed a sensitivity

and specificity of 100% and the authors claim that this method is able to detect as little as 5 pg of genomic DNA in the samples, characterizing this method as a possible assay for early TB diagnosis [44].

Xiang et al., (2015) developed a biosensor array using Surface Plasmon Resonance (SPR) and Rolling Circle Amplification (RCA) to detect *M. tuberculosis* and *M. avium*. This method combines the amplification and cleavage of target sequences as they are amplified, hybridization with immobilized probes, and detection in real-time by SPR, as the target sequences hybridize with the probes. All the steps occur simultaneously in the detection chamber, in that way there is no loss or degradation during the procedure. Thereof, the authors immobilized gold nanoparticles into the chip surface, aiming to boost the sensitivity of the biosensor and strengthen the detectable signal. When compared with conventional RCA or SPR methods, this approach showed sensitivity ten times higher. By this method, various samples can be analyzed faster, with low cost, low sample volume and less interference. Although the author focused on TB, this system can be used to diagnose any number of diseases and pathogens [45].

Aiming to reduce the costs of TB diagnosis in the clinic, Shin et al., (2015) developed an assay using Isothermal Solid-Phase Amplification and Detection (ISAD) and a Silicon Microring Resonator (SMR) for signal detection. His group tested 42 TB positive sputum samples, achieving sensitivity higher than 90%. The extracted DNA was mixed with primers targeting the sequence IS6110 and RPA solution (recombinase protease amplification), this solution was loaded into the MTB-ISAD device, and the temperature was maintained at 37°C. The reaction occurred for 20 minutes with the wavelength shift being monitored every five minutes. As the amplification progress, positive samples will produce IS6110 sequences that will hybridize with the complementary sequence immobilized at the SMR; the hybridization affects the wavelength in a detectable way [46].

Biosensors technology is in exponential expansion thanks to the rapid and portable mechanisms, low costs involved and the versatility of detection methods. Many approaches can be developed or improved, aiming to detect various pathogens and chemicals. In the case of mycobacteriosis, biosensor are especially attractive for the diagnosis of TB, since it is a prominent worldwide disease and the actual diagnosis methods require skilled professionals, great deal of time, and specialized facilities.

Conclusion

Advances in molecular biology favored more specific and selective research of diseases such as tuberculosis and leprosy. PCR, ELISA, LFA and the use of biosensors are only a few important examples of methods related to early diagnosis of specimens of the mycobacteria genera. Neglected infectious diseases present a permanent health risk, especially in endemic areas and in underdeveloped or poor countries. In this scenario, the development of faster and inexpensive, easy-to-use and reliable diagnosis methods are imperative, both to control the spread of the infection and to start early treatment, preventing worst outcomes.

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