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Diagnostic Evaluation of Tuberculosis

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1. Introduction

Tuberculosis is still one of the leading causes of death by infectious diseases with 2 million deaths per year and 9.2 million new cases of tuberculosis disease annually [1-3]. Besides, more than 2 milliard people are infected with latent tuberculosis infection (LTBI) [1-3]. Despite continuous effort in the prevention, monitoring and treatment of tuberculosis, the disease remains a major health problem in many countries [4-6], particularly in developing countries like Indonesia [7]. National tuberculosis programs and other programs conducted by foreign organizations still fail to eliminate the transmission and incidence of tuberculosis. Transmission is even on the rise in developing countries despite the availability of effective therapies for tuberculosis, whereas the spread and the incidence of tuberculosis in Europe and North America are under control. Several reasons may be responsible for this failure, such us the difficulty of providing adequate anti-tuberculosis medication in many developing countries due to cost issues, the emergence of multi-drug resistant (MDR) strains of *M. tuberculosis,* and the dramatically high co-incidence of tuberculosis in HIV-infected patients [2, 7]. Another important issue is delay of diagnosis due to the lack of a proper method to identify tuberculosis agents [1, 8].

Smear is the cheapest and most widely available detection method for *M. tuberculosis*. In this technique, the diagnosis of tuberculosis is based on identification of acid-fast bacilli (AFB) in a patient`s sputum [9, 10]. Many staining techniques are available for AFB smear, the most common one of which is the modified Ziehl-Neelsen stain. Unfortunately, the sensitivity and the specificity of those techniques are low due to difficulty in the identification and differentiation of the various species of *M. tuberculosis* [10]. Two studies found that the AFB smear was positive in only half of patients with subsequent culture positive for *M. tuberculosis* [9, 10]. Another worldwide available detection method is the conventional culture method on

Lowenstein-Jensen (LJ) medium. This method is the gold standard in the identification of *M. tuberculosis* and still serves as the reference method due to its high sensitivity (89%) and specificity (98%) [4, 7, 9, 10]. However, this technique requires equipments or materials that are often unavailable in resource-poor settings. In addition, this technique is time consuming; the results only can be obtained after 6–12 weeks. In addition, the incidence of other bacterial contamination on culture tends to be high [7, 11]. Even a modern culture method such as the BACTEC MGIT 960 culture system, which uses the modified Middlebrook 7H9 broth and a fluorescent signaling system, allows for earlier detection of growth, but still takes at least 10 days to give any result [9].

The goal of tuberculosis control programs is to identify and to cure as many cases as possible; therefore the critical role of early diagnosis is obvious [11]. Under-diagnosis may lead to further spread of the disease because undiagnosed patients can spread the disease unnoticeably [11]. Accurate and early diagnosis is the first important step to effective management. Several new methods for the identification of tuberculosis are available, which including serologic tests and also various molecular methods developed as a result of major advances in understanding the genetic aspects of tuberculosis [8, 9, 11]. Those detection methods can be grouped into two types First, by detection of mycobacteria or its components directly; second by measurement of immunologic responses to mycobacterium infection [9]. In this chapter we present a short review of some these promising detection methods used in the laboratory to identify tuberculosis.

2. Direct detection methods

The genus mycobacterium consists of almost 100 different species, which all appear similar on AFB staining and culture [7, 10, 12]. Many of these can be isolated from humans, although many also can be found in the environment including in animals. It is not easy, however, to distinguish between pathogen and saprophyte species. Each mycobacterium isolate must be evaluated individually regarding its potential to cause a disease; therefore identification of mycobactera is a lengthy and tedious effort. Since the introduction of nucleic acid amplification assays as diagnostic tool for mycobacteria identification, several probes/gene amplification systems for tuberculosis have been developed for rapid and specific identification of *M. tuberculosis* and other mycobacteria [12, 13]. These techniques allow for the confirmation of identity of isolates, direct detection of gene sequences from the clinical specimens and also for molecular detection of drug resistance [12]. Many previous publications have shown the sensitivity and specificity of several molecular detection assays such as BDProbeTec ET, (Becton Dickinson), COBAS AMPLICOR (Roche), Amplified *M. tuberculosis* Direct Test AMTDT (Gen Probe, USA) for identification of mycobacteria[9].

The use of nucleic-acid probe identification systems was a one step ahead in the rapid identification of mycobacterium species of *M. tuberculosis* complex, *M. avium* complex, *M.*

avium, M. intracellulare, M. kansasii, and *M. gordonae* and also other nontuberculous mycobacteria (NTM) in culture because the result can be obtained after 2 hours [10, 12]. But the sensitivity and specificity of this probe technology will only approximate 100% if there are more than 100 *mycobacteria* present in the sample, except for *M. kansasii* (87%) [12]. Thus, these probes are not sensitive enough to be used directly in clinical specimens like sputum. Also, it still needs to be confirmed by other conventional detection methods such as biochemical test and molecular tests to able to identify the species identity within the *M. tuberculosis* complex, such as for *M. microti, M. bovis, M. bovis of* BCG,, *M. canettii,* and *M. africanum* [10]. There has been extensive research to design an identification system for ribosomal RNA/DNA fingerprinting and for development of probes that targeting specific rRNA, ribosomal DNA, spacer and flanking sequences of various types of mycobacterium species including *M. tuberculosis, M. leprae, M. avium, M. gardonae,* etc [12, 13]. Those rRNA targeting probes are 10-100 fold more sensitive than DNA targeting. However, since the lowest detection limit is still around 100 organisms. it still needs more evaluation before it can be applied to clinical specimens [12].

Several techniques based on polymerase chain reaction (PCR) and isothermal amplification assay have been developed [7-10, 12]. Various researchers have described the rapid detection of *M. tuberculosis* by PCR, and many have reported a high sensitivity in detecting *M. tuberculosis* in clinical samples by means of DNA amplifications [7, 14]. Such techniques involve amplification of specific gene regions followed by hybridization with species specific primers, and also frequently followed by sequencing and or restriction fragment length polymorphism (RFLP) analysis [12]. RFLP is still most widely used in clinical microbiology laboratories due to its simplicity and lower costs than PCR Sequencing [12]. Multiplex PCR has been used to detect *M. tuberculosis* complex bacteria and other mycobacterium. This technique is based on the amplification of the most widely used specific insertion sequences IS6110 and 16S [7-9]. Based on our experience, multiplex PCR has sensitivity up to 81.62% with negative predictive value up to 79.51% [7]. Nevertheless, taking into account the "simple and economical" issue this technique is probably not suited for most of the countries with a high tuberculosis burden [11]. Other rapid molecular amplification detection method which is being used in our laboratory is multiplex PCR-reverse cross blot hybridization, which can be modified to identify multiple species of mycobacteria at one time by using a specific probe for each species. Compared to the culture and microscopic method, this technique had a sensitivity of 86.03%, negative predictive value of 82.41% and it can be applied to detect NTM [7]. The multiplex PCR reverse cross blot hybridization technique is more complicated than conventional multiplex PCR; but it can detect considerably more NTM species such as *M. avium, M. intracellulare, M. kansasii, M. fortuitum, M. chelonae, M. genavense and M. smegmatis* (Fig. 1) [7].

In term of accuracy and duration time that it needs to get a result, Raman spectroscopy is one of the most promising techniques. This vibrational spectroscopy-based detection method can detect and differentiate various molecular compositions of microorganism [15-18] and therefore is suitable to identify the species and strains of microorganism. Buijtels et al., demonstrated that Raman spectroscopy differentiated between *M. tuberculosis* with NTM with accuracy up to 100% and with 92.5% correct species identification. This

technique is also are much faster; results can be obtained within 3 hours since positive automated cultured system is obtained [18]. In view of the importance of early diagnosis to prevent further spread of tuberculosis in the community, this time efficiency is the most significant contribution of Raman spectroscopy.
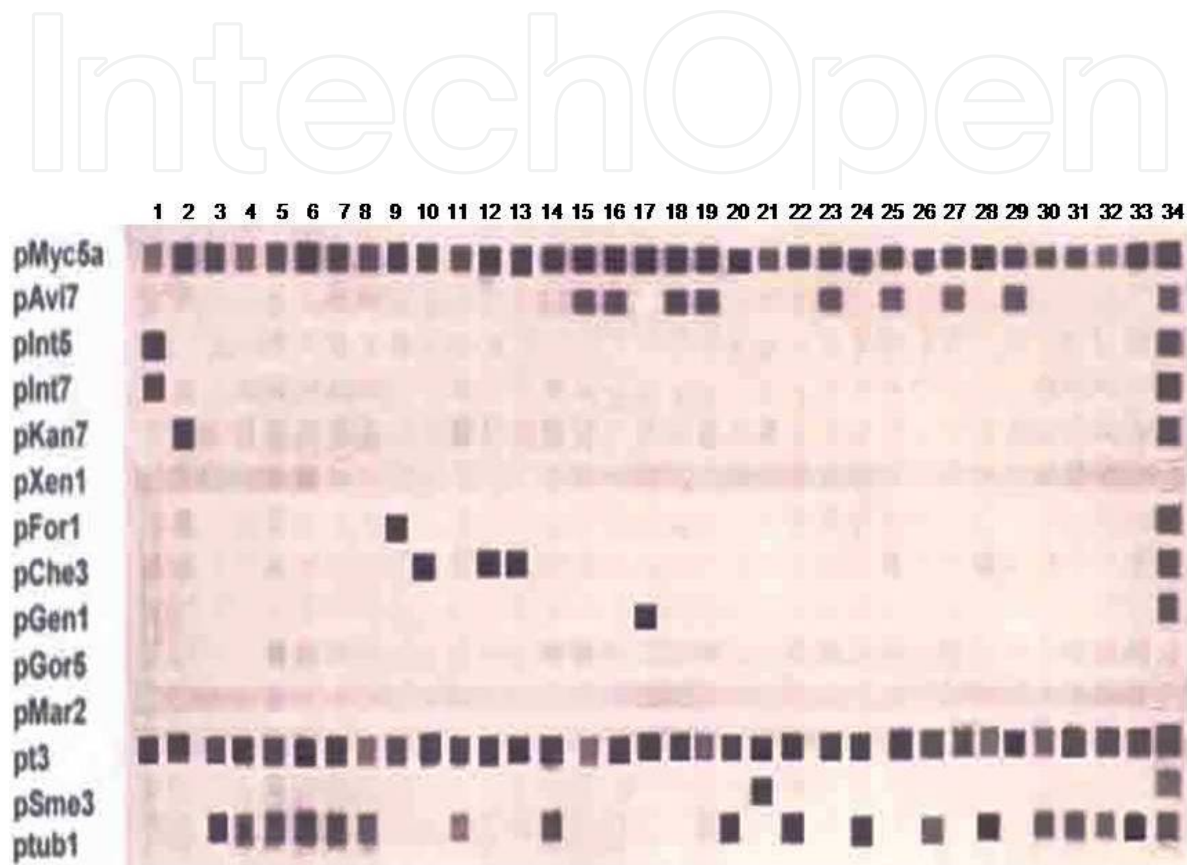

Figure 1. Multiplex PCR reverse cross blot hybridization assay is able to detect various species of mycobacteria simultaneously. Each column (Col) represents certain species of mycobacteria; Col 1, *M. intracellulare*; Col 2, *M. kansasii* ; Col 3-8, 11, 14, 20, 22, 24, 26, 28, 30-33, *M. tuberculosis*; Col 9, *M. fortuitum*; Col 10, 12, 13, *M. chelonae*; Col 15, 16, 18, 19, 23, 25, 27, 29, *M. avium*; Col 17, *M. genavense*; Col 21, *M. smegmatis* ; 34, pool PCR product of mycobacteria. [7]

3. Indirect detection methods

Even those remarkable molecular detection methods are not yet up to the mark when it comes to in the identification of tuberculosis, particularly latent tuberculosis infection (LTBI). Approximately 2 milliard people are silent tuberculosis patients, i.e. they have been infected by *M. tuberculosis* but show no tuberculosis symptoms [1, 2]. LTBI has been defined by evidence of a cellular immune response to *M. tuberculosis* derived antigens. It may be the result of incomplete elimination of *M. tuberculosis* by the host's adaptive immune system, resulting in asymptomatic infection with almost undetectable bacilli [2]. Thus, the diagnosis of LTBI currently depends on detecting the host's immune response to the infection [2]. Affected individuals have little risk

of progression from LTBI to active tuberculosis, but any disruption of their cellular immunity – such as in HIV co-infection cases – can considerably increase this risk [2]. Currently, the diagnosis of LTBI is commonly made with the tuberculosis skin test (TST), which is based on the delayed hypersensitivity to purified protein derivative (PPD). Unfortunately, patients sensitized to environmental nontuberculous mycobacteria or patients vaccinated with the bacillus Calmette–Guérin (BCG) vaccine may have a false positive result. On the other hand, a false negative result may occur in immunosuppressed patients and also in children [2]. This immunologic response is often not conclusive as antibodies and delayed type hypersensitivity response persist long after infection or after the diseases has disappeared [12]

Interferon Gamma Release Assays (IGRAs) have been introduced in the clinical setting for the diagnosis of LTBI [19-21]. These more specific whole-blood tests are based on the principle of measuring host interferon-y (IFN-y) released by T-cells specific to *M. tuberculosis* as a marker. IFN-y is stimulated by *early secretory antigen target 6* (ESAT-6) and *culture filtrate protein 10* (CFP-10). These are not present in the BCG or in the most of the NTM [2]. There are two types of IGRAs: The enzyme-linked immunospot assay (ELISpot)-based IGRA, where individual IFN-y producing T-cells responding to *M. tuberculosis* antigens stimulation are counted [22], and the QuantiFERON-TB Gold In-Tube test, an ELISA-based IGRA where the IFN-y produced by those T-cells is measured after stimulation with *M. tuberculosis* antigens [2]. Pai et al. showed that the sensitivity of the ELISpot and ELISA-based approach was around 90% and 70%, respectively, and that the specificity of both was 93% [2, 20]. As there is still no gold standard for the diagnosis of LTBI, these assays potentially may serve as routine diagnosis test other than TST to identify people with LTBI [2].

Cytokine-based detection methods could be useful not only in the detection of LTBI cases but also of active tuberculosis cases. However, considering the high number of LTBI in the community, a single cytokine identification method such as IGRAs is not sufficient to detect active tuberculosis. For this reason the identification of multiple tuberculosis biomarkers-cytokines seems to be a promising strategy. Several studies have shown the potential usefulness of TNA-a, IL-2, IP-10, MIG along with IF-g simultaneously [23-26]. Using a multiplex microbead-based assay, Wang et al. showed significant differences in expression of these cytokines/chemokines between active tuberculosis patients and healthy controls. Regarding active pulmonary tuberculosis the sensitivity of IFN-y, IP-10 and MIG was 75.3% and the specificity was 89.7%. They also demonstrated the potential usefulness of this multiplex microbead-based assay for the detection of new tuberculosis cases by documenting a sensitivity of 96.3% [23].

Untill now, smear and culture methods are still the gold standard to detect mycobacteria. Based on our experience, combination of conventional and advanced detection methods would greatly improve the sensitivity and specificity of the assays. Detection of the *mycobacteria* species are quite difficult with culture, therefore we using multiplex PCR as the first confirmation assay to detect the species while it also as confirmation test for negative results from either smear or culture assay. Hence, to overcome the limitation of multiplex PCR in species detection, multiplex PCR- reverse cross blot hybridization assay would further expand the range of *mycobacteria* species detection (Fig. 2).
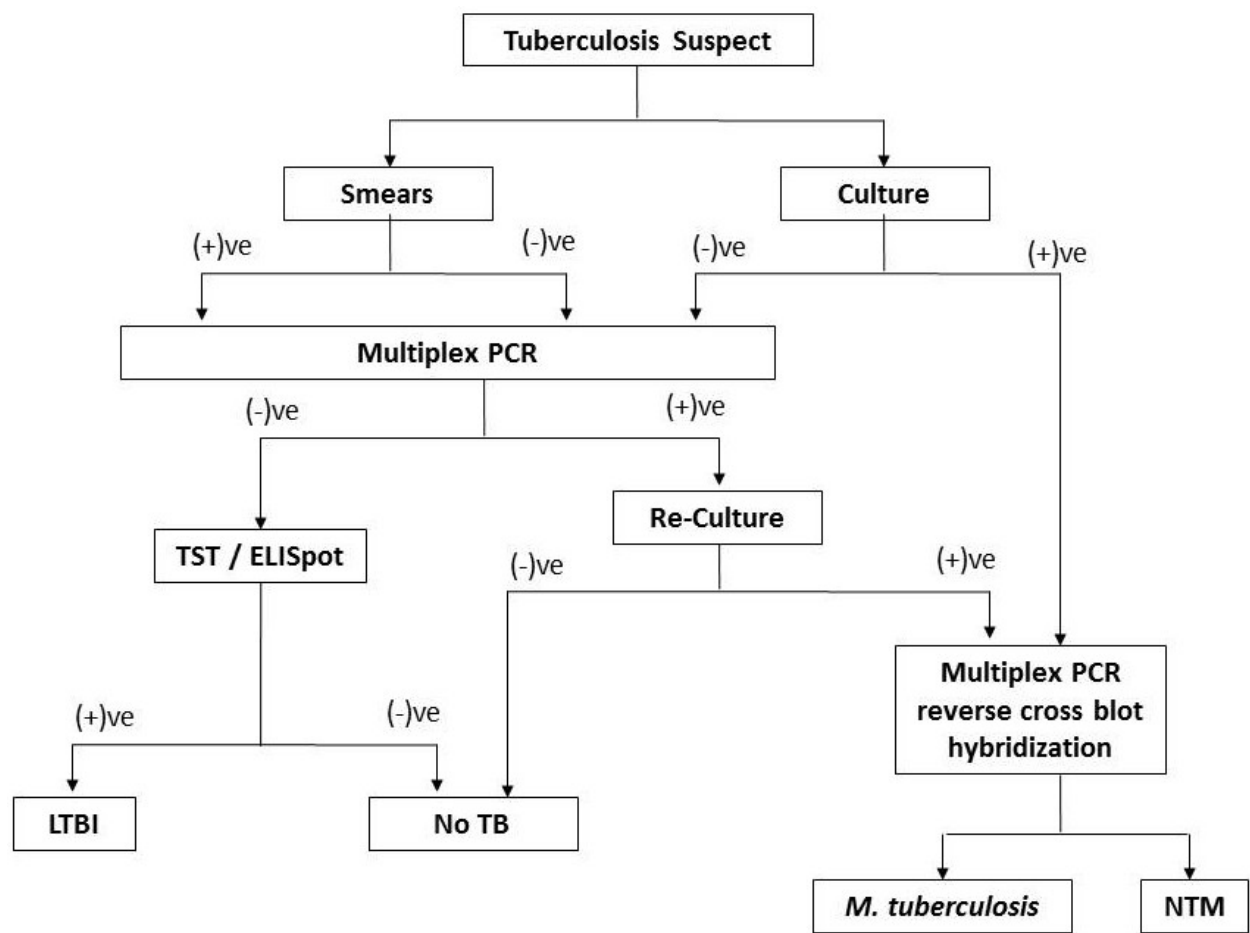
Figure 2. Microbiologic diagnosis of tuberculosis. Multiplex PCR are used to confirm smears results and negative result of culture assay. Patients with negative multiplex PCR result would be proceed for ELISpot or Tuberculin skin test (TST) to detect latent tuberculosis (LTBI), while specimen from patient with positive culture result would get final confirmation by Multiplex PCR reverse cross blot hybridization assay to further detect the mycobacterium species

4. Conclusion

Conventional methods for the diagnosis of tuberculosis, such as the smear and culture methods have some limitations, particularly the low specificity and sensitivity as well as the time-consuming nature. Now these limitations have been overcome in some novel and rapid detection methods. Various gene amplification techniques have demonstrated their usefulness in the identification of mycobacteria and its various species. The rapid detection of *M. tuberculosis* by probes, PCR or other molecular techniques and some newest serologic assays offer good opportunities to improve the diagnosis and therapy of tuberculosis [2, 7-9, 12, 13].

However despite the availability of diagnostic tools for laboratory identification of tuberculosis at high sensitivity and specificity, the "simple and economically" aspect of those new methods is still a matter of consideration. The question is whether they can be used in simple

clinical settings and whether they are economically affordable for developing countries, in most of which tuberculosis is still rampant [11].

Summary

Tuberculosis still remains a major health problem in many developing countries, despite continuous long-standing vaccination and surveillance programs, and worldwide availability of effective anti-tuberculosis drugs. Early detection is of major importance in the control of tuberculosis. The emergence of multidrug resistant *Mycobacterium tuberculosis* and the association of HIV with tuberculosis outbreaks in community both illustrate that rapid diagnosis is essential. Therefore, a fast and reliable diagnosis of tuberculosis would greatly improve the control of the tuberculosis. Regrettably, current conventional laboratory diagnostic methods of tuberculosis are still time-consuming. The rapid development of novel diagnostic methods for the identification of mycobacteria and its species bring new hope, however, for the diagnosis and management this infectious disease. Meanwhile those techniques still seem to clash with simplicity and economically affordable issues.

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