Review Article

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Molecular Diagnosis of Tuberculosis

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Tuberculosis (TB) is one of the leading causes of adult death in the Asia-Pacific Region, including Indonesia. As an infectious disease caused by *Mycobacterium tuberculosis* (MTB), TB remains a major public health issue especially in developing nations due to the lack of adequate diagnostic testing facilities. Diagnosis of TB has entered an era of molecular detection that provides faster and more cost-effective methods to diagnose and confirm drug resistance in TB cases, meanwhile, diagnosis by conventional culture systems requires several weeks. New advances in the molecular detection of TB, including the faster and simpler nucleic acid amplification test (NAAT) and whole-genome sequencing (WGS), have resulted in a shorter time for diagnosis and, therefore, faster TB treatments. In this review, we explored the current findings on molecular diagnosis of TB and drug-resistant TB to see how this advancement could be integrated into public health systems in order to control TB.

Key Words: *Tuberculosis; Nucleic Acid Amplification Techniques; Tuberculosis, Multidrug-Resistant*

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INTRODUCTION

In the Global Tuberculosis Report 2014 published by the World Health Organization (WHO), among the 249,866,000 Indonesian people, it was estimated that the epidemiological burden of tuberculosis (TB) per 100,000 populations was: 272 of prevalence (range 138-450); 183 of incidence (range 164-207); and 64 of mortality (range 14-37).[1] Recently, a genotyping study of *Mycobacterium tuberculosis* (MTB) was performed in Indonesia to reveal the TB molecular epidemiology and showed a high genetic diversity that varied by geographical aspects and found that the Beijing strain was the predominant strain in Indonesia.[2]

Fast and accurate detection of the pathogen and its drug susceptibility patterns is crucial for treatment initiation and disease control.[3] New techniques are available to optimize the culture medium including optimization of the culture system; early growth detection; strict control of oxygen tension; and the utilization of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) for identification.[4] Advancements in molecular methods for MTB detection has shortened the time to diagnosis to a few days, whereas diagnosis by conventional culture systems needs several weeks.[5,6] The majority of molecular tests have been aimed at the detection of MTB specific nucleic acids, both in DNA and RNA, by using amplification techniques such as polymerase chain reaction (PCR), and detection of genes mutation that are related with the resistance to anti-TB drugs by sequencing or nucleic acid hybridization.[7] Moreover, the WHO has announced the need for diagnostic options that are a sputum-based replacement test for smear microscopy, a non-sputum-based biomarker test that is resource-adjusted at facilities below microscopy laboratories, a simple initial test for first-contact care providers as a rule-out test, and a fast drug sensitivity test at the microscopy laboratory level.[8] In middle- and high-income countries, development continued with innovations in microscopy (for example, light emitting diode [LED] microscopes), MTB culture systems (for example, rapid automated liquid culture sys-

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tems, like the Becton Dickinson MGIT 960), nucleic acid amplification systems line probe assays and automated systems, such as the Cepheid Xpert® MTB/RIF system (Cepheid, Inc., Sunnyvale, CA, USA).[9] In the next section, we will discuss the role of the nucleic acid amplification test (NAAT) and whole-genome sequencing (WGS) in the diagnosis of TB, specimens transport medium technology, mycobacterial load detection assays, serological diagnosis of active TB, and molecular diagnostic of multi-drug resistant TB (MDR-TB).

NUCLEIC ACID AMPLIFICATION TEST (NAAT)

Because the conventional bacteriological diagnosis of TB has several limitations, the NAAT has emerged as a potential alternative.[10] The NAAT systems, with rapid turn-around times, facilitate testing and treatment initiation in the same visit and, therefore, loss to follow-up cases can be reduced.[11] Most NAAT assays detect the mycobacterial insertion element IS6110 for the identification of the MTB complex organisms.[12] NAAT detects MTB ribosomal RNA or DNA directly from sputum specimens, both the acid-fast bacilli (AFB) smear-positive and AFB smear-negative.[13] The NAAT showed very high sensitivity in sputum smear-positive patients and around 61 to 76% sensitivity in patients with smear-negative sputum.[11] Currently, the NAAT that is endorsed by the WHO is the Xpert/RIF MTB assay.[14] The other two NAATs, the Amplified Mycobacterium Tuberculosis Direct (MTD) Test (Gen-Probe, Inc),[15-17] and Amplicor Mycobacterium tuberculosis Test (Roche Molecular Systems, Inc),[18-20] have also been approved by the Food and Drug Administration (FDA) for testing respiratory AFB smear-positive specimens.[21] Other commercial NAATs are also available, including the loop-mediated isothermal amplification-based MTB detection system,[22-24] the cross-priming amplification-based TB diagnostic system,[25,26] and the Genedrive® *Mycobacterium tuberculosis* iD®.[27]

In multi-bacillary diseases with a high mycobacterial load, a positive AFB smear with a positive NAAT would indicate active tuberculosis whereas a positive AFB smear with a negative NAAT in the absence of inhibitors would indicate nontuberculous mycobacterial (NTM) disease.[28] If the culture was positive in the above case, the physician could consider the patient as a bacteriologically confirmed case of TB.[1] A NAAT could determine whether AFB smear-positive patients had TB or not.[29] Moreover, if the NAAT result is positive but the AFB smear result is negative, the decision to begin anti-TB treatment would rely on the clinical judgment while awaiting culture results.[30] According to Centers for Disease Control (CDC), if the sputum is smear-negative and the NAAT also negative, an additional specimen should be tested with NAAT.[31] However, if the culture results detected MTB bacteria growth, then the patient could also be classified as bacteriologically confirmed for having pulmonary TB. Finally, if the AFB smear, the NAAT, and the MTB culture showed negative results, the physician could classify the patients as a clinically diagnosed case of TB or consider another diagnosis. Fig. 1 described the algorithm of the NAAT in the diagnosis of TB that is in line with the recommendation of both the WHO and the CDC.

In locations where the rate of cultures positive for TB is low, it might be more efficient to limit the NAAT to cases with positive smear results; on the other hand, in locations where TB cases are high, a NAAT should be used in cases with negative smear results.[32] A meta-analysis that included 125 studies showed that a commercial NAAT alone should not replace conventional tests for diagnosing pulmonary TB.[33]

XPERT MTB/RIF ASSAY

The Xpert MTB/RIF assay is an nuclear acid amplification-based test using a cartridge based on the GeneXpert Instrument System.[34] The basis of the Xpert MTB/RIF as-

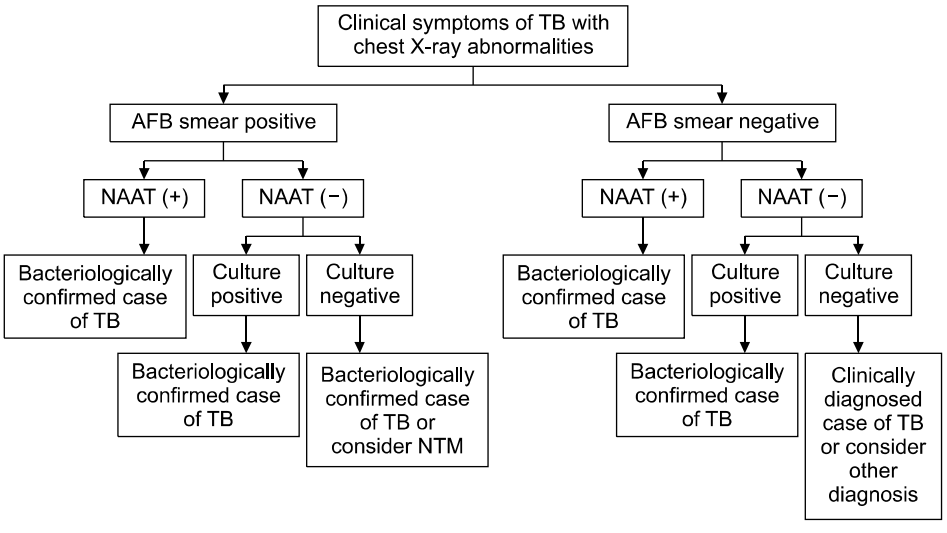

FIG. 1. The role of nucleic acid amplification test (NAAT) in the diagnosis algorithm of TB. TB: tuberculosis, NAAT: nucleic acid amplification test, NTM: nontuberculous mycobacterium.

say is a real-time PCR that can be used to detect DNA sequences specific to the MTB in sputum samples.[35] a ingle Xpert MTB/RIF test directly from sputum can detect 99% of smear-positive patients and more than 80% of smear-negative cases.[36] According to the WHO in 2013, a Xpert MTB/RIF assay could be used for: an add-on test following microscopic TB examination; a replacement examination for AFB smear microscopy; detection of MTB in both AFB smear-positive and smear-negative culture-positive cases; detection of MTB in pleural in pleural fluid; detection of MTB in lymph node in samples from biopsy or fine-needle aspiration; detection of MTB in gastric fluid; detection of MTB in samples of cerebrospinal fluid; and detection of MTB in tissue samples.[14] In 2014, the WHO stated that the Xpert MTB/RIF assay could be used as the initial diagnostic test in all subjects suspected on having pulmonary TB.[37]

The Xpert MTB/RIF assay detects rifampicin resistance by PCR amplification of the 81-bp fragment of the MTB *rpoB* gene and subsequent probing of this region for rifampicin resistant-associated mutations and the results can be obtained within 2 hours.[38,39] The WHO recommends subjects who are at high risk of MDR-TB should always have their sputum checked using the Xpert MTB/RIF test.[40] The Xpert MTB/RIF can be used as an initial test and as an add-on test after a negative AFB smear microscopy result.[41] The use of Xpert MTB/RIF test has shortened the median time to treatment for AFB smear-negative TB from 56 days (range 39-81 days) to 5 days (range 2-8 days).[42] However, Xpert MTB is more expensive than conventional sputum microscopy.[43] Nevertheless, in one analysis, if a rapid sputum-based test unit cost is of US$ 2-4, it would be lower to a similar cost of the conventional sputum smear microscopy.[44] In order to facilitate access, the Foundation for Innovative New Diagnostics (FIND) has negotiated significant price reductions.[45] Nevertheless, implementation of Xpert MTB/RIF would not be able to improve the control of drug-sensitive TB without improvements to the health system, especially as to reducing the initial loss to follow-up and reducing the time to treatment initiation.[46]

WHOLE-GENOME SEQUENCING (WGS)

Microbial genomics has allow us to investigate the organisms genetic markers that may impact treatment and infection prognosis.[47] Whole-genome sequencing (WGS) is becoming an affordable and accessible method that can identify microevolution within MTB lineages as they are transmitted between hosts.[48] Fig. 2 describes the workflow of WGS from specimen input until clinical diagnostic report. There are two classes of sequencers that exist: the first generation sequencer and the second generation (widely known as the next-generation sequencer [NGS]). The first generation sequencer is relatively slow, but has a high throughput and low cost (approximately $65 per bacterial genome). The second generation has a lower throughput, higher cost (approximately $150 per genome in the case of the Illumina® MiSeq) and is able to sequence multiple genomes in less than a day.[49] Table 1 describes the principles of the first generation sequencer and the next-generation sequencer.[50-53]

The WGS can detect various types of mutations better than the Xpert MTB assay. Moreover, WGS could avoid false positives when a polymorphism in the rifampicin-re-

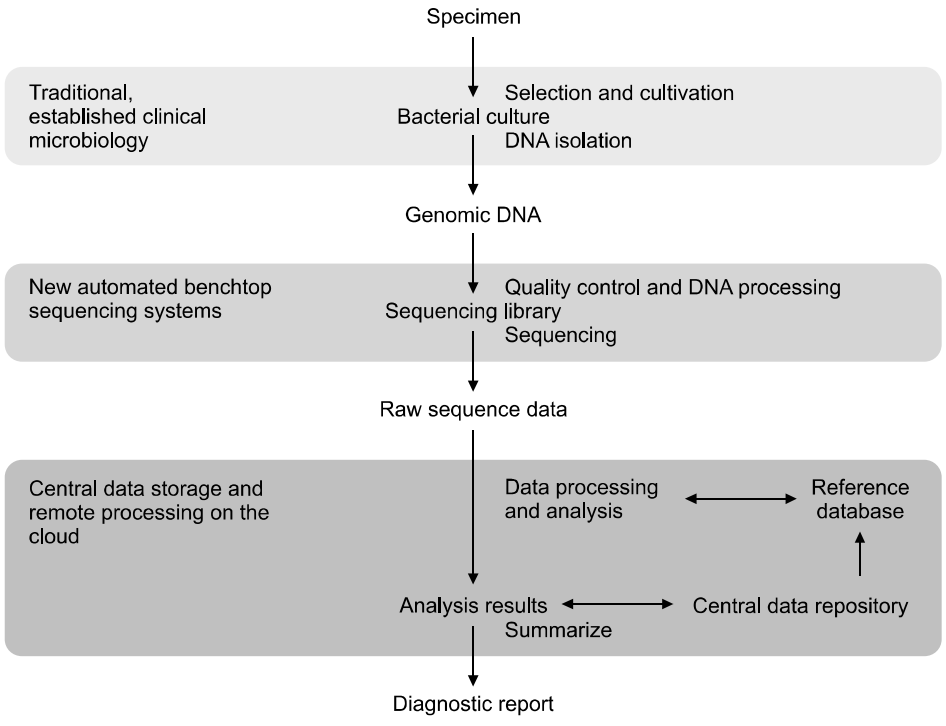

FIG. 2. Workflow of whole-genome sequencing from specimen processing until diagnostic report [Reprinted by permission from Macmillan Publishers Ltd: Nature Review Genetics 15: 49-55 copyright (2014)].

TABLE 1. Principles of first generation and next generation sequencer

	First generation sequencer	Next-generation sequencer
Principles and features	This first generation of DNA sequencers are essentially automated electrophoresis systems that detect the migration of labelled DNA fragments.[50]	Rapid generation of data by sequencing massive amounts of DNA in parallel using diverse methodologies.[52]
	Capable of genotyping of genetic markers where only the length of a DNA fragment(s) needs to be determined.[51]	Constitute various strategies that rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods.[53]

sistance determining region (RRDR) of *rpoB* is detected.[54] WGS has not been used as a routine diagnostic tool for TB, partly because of the need to culture MTB for several weeks until an adequate amount of DNA can be extracted.[55] Culturing slow-growing MTB before DNA extraction is a major time-consuming step in WGS , therefore, workflow to extract DNA from frozen isolates without re-culturing is important.[56] There is a reliable and low-cost DNA extraction method from 1 mL of early positive mycobacterial growth indicator tube (MGIT) cultures that is enough for the WGS to identify mycobacterial species and predict antibiotic resistance in clinical samples.[57] Recently, an important method that allows WGS without prior specimen culture has been discovered. The method utilizes MTB DNA-specific biotinylated RNA baits to capture full MTB genomes directly from non-cultured sputum samples.[58] WGS data can be obtained several weeks before the drug susceptibility test (DST) data is available.[59] DNA sequencing could also be used to confirm rifampin resistance from Xpert MTB/RIF.[60] However, with the price in the hundreds of dollars for this method, routine WGS for clinical specimens is limited to high-income countries.[61]

SPECIMENS TRANSPORT MEDIUM TECHNOLOGY

Sputum samples processing at a central diagnostic laboratory requires an efficient and safe system that is not compromised by potential prolonged transport times. Generally, a prolongation of sample storage results in reduced positivity of the target.[62] Therefore, transport medium technology plays an important role in the chain of TB diagnosis. The transport medium system consists of an MTB inactivation step and a DNA stabilization step with the goal to preserve the DNA quality and, at the same time, obtain high DNA output. Some of the known transport technologies are the PrimeStore®-Molecular Transport Medium (PS-MTM)[63,64] and cetylpyridinium chloride (CPC).[65] PS-MTM inactivates the MTB within the specimens and stabilizes both the DNA/RNA at an ambient temperature for further molecular processing and analysis.[64] As for the Xpert MTB assay, incubation of sputum with a sample reagent (SR) could maintain the sputum quality up to 72 h without a further decrease in MTB detection.[66]

MYCOBACTERIAL LOAD DETECTION ASSAY

Quantification of the bacillary load has an important prognostic role in TB patients.[67] There are several assays that can be used to quantify mycobacterial load, such as the BD ProbeTec system,[68] and AdvanSure TB/NTM real-time PCR kit.[69] Lee and colleagues evaluated the quantitative capability of the AdvanSure TB/NTM real-time PCR kit (LG Life Science, Korea) to determine the cycle threshold (Ct) for the mycobacterial burden.[70] A cut-off Ct value of <33.2 best-predicted stain positivity, with a sensitivity of 95.0% and a specificity of 32.0%. These findings suggest the potential use of AdvanSure TB/NTM real-time PCR kits for quantitatively determining mycobacterial burden. AdvanSure TB/NTM real-time PCR could be used to detect Mycobacterium and distinguish whether it is MTB or the NTM because it used the IS6110 primer for the detection of MTB complex and *rpoB* gene specific primer and probe for the detection of NTM.[71] However, this assay does not determine if there are viable Mycobacterium organisms that truly exist in the specimen.

SEROLOGICAL DIAGNOSIS OF ACTIVE TB

Serological tests for TB are widely used in developing countries.[72] In one study conducted in Indonesia, Senosaputra and colleagues found that plasma levels of anti-α-crystallin (ACR), anti-lipoarabinomannan, anti-trehalose 6,6'-dimycolate, and anti-tubercular-glycolipid antigen antibodies were higher in active TB patients compared to those in the latent TB infection (LTBI) and control subjects.[73] In another study, diverse IgG antibody responsiveness was demonstrated against five lipid antigens: cord factor (trehalose 6,6'-dimycolate) (TDM-T), monoacyl phosphatidylinositol dimannoside (Ac-PIM2), trehalose monomycolate isolated from *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) (TMM-T), trehalose monomycolate (TMM-M), and GPL-core from *Mycobacterium avium* complex (MAC).[74] However, a systematic reviews of the diagnostic accuracy of serological tests suggest that these tests are inaccurate and imprecise for both pulmonary and extra-pulmonary TB.[75] The serological assay of TB is limited in determining whether a person has had a previous TB infection, therefore it cannot inform whether the person has an active TB infection. Moreover, the WHO strongly recommends that serological commercial tests should not be used for the diagnosis of clinical pulmonary and extra-pulmonary TB.[76]

MOLECULAR DIAGNOSTIC OF MULTIDRUG RESISTANCE TUBERCULOSIS

The increasing incidence of drug-resistant, MDR (resistant to at least rifampicin and isoniazid), and extensively drug-resistant (XDR) (additionally resistant to a fluoroquinolone and kanamycin/amikacin/capreomycin) strains of MTB have been implicated as a major threat to TB control.[77] Detection of drug resistance to both first- and second-line anti-TB drugs is a key component of TB control programs.[78] Knowledge of the full drug susceptibility profile would enable customized treatment to increase efficacy and avoid exposure to ineffective toxic drugs.[79] Conventional phenotypic DST requires a minimum of 2 weeks: one week for the initial detection of microbial growth and another week to assess critical concentrations of first-line anti-TB drugs.[80] Obviously, there is an urgent demand for new, rapid, and accurate DST methods.[81] A large, multinational field trial, to compare the performance of the Line Probe Assay (LPA), Pyrosequencing® (PSQ), and Microscopic Observation of Drug Susceptibility (MODS) and compared to the BACTEC MGIT960 as reference standard to detect MDR/XDR-TB directly from patient sputum samples was performed, and the results indicated that mean time-to-result was 1.1 days for LPA and PSQ, 14.3 days for MODS, and 24.7 days for MGIT.[82]

LPAs are available in several methods, such as the AID LPA (AID Diagnostika, Germany),[83] the Hain LPA (Hain Lifescience, Germany),[84] the INNO LPA (Innogenetics, Belgium),[85] the GenoType MTBDR*plus*,[86,87] and the GenoType MTBDRsl.[88] The INNO LPA and GenoType MTBDR*plus* have been assessed and validated by the WHO in their Expert Group Report.[89] The AID LPA was designed to detect the most prevalent mutations that confer resistance to rifampin, isoniazid, streptomycin, capreomycin, amikacin, ethambutol, and fluoroquinolones.[90] Meanwhile, a comparison study suggested that MTBDR*plus* LPA was superior compared to Xpert MTB/RIF in the detection of rifampin-monoresistant MTB in terms of sensitivity.[91] Table 2 summarizes the molecular diagnostic of mono-resistant and MDR-TB detection.[77,92-103] Based on the list, different methods could explore different resistance-associated mutations, therefore variation of methods allows us to detect various resistances.

The PSQ method consists of seven PSQ assays for the mutations detection of the genes or promoter regions, which are commonly responsible for resistance to the first line and second line drugs.[104,105] In one study, the correlations between the PSQ results and the phenotypic DST results were 98.7% for rifampin, 94.3% for isoniazid, 99.2% for capreomycin, 99.2% for amikacin, 96.4% for kanamycin, and 97.6% for quinolones (levofloxacin, ofloxacin, or moxifloxacin).[106]

GenoType MTBDRsl (MTBDRsl) is the only molecular test that is commercially-available for detecting resistance to the fluoroquinolones (levofloxacin, ofloxacin, and moxifloxacin) and the second-line injectable drugs (kanamycin, capreomycin, and amikacin).[107] MTBDRsl detects *rrs* and *gyrA* genes mutations that confers resistance to injectable antibiotics and fluoroquinolones,, respectively.[108] Moreover, MTBDRsl also capable of detecting *embB* mutations that confer resistance to ethambuthol.[109] The sensitivity and specificity of the MTBDRsl test were as follows: 87% and 96%, respectively, for fluoroquinolones; 77% and 100%, respectively, for kanamycin; 80% and 98%, respectively, for capreomycin; 100% and 100%, respectively, for amikacin; and 57% and 92%, respectively, for ethambutol.[110] In one meta-analysis, it was reported that MTBDRsl showed good accuracy for detecting drug resistance to fluoroquinolones, capreomycin, and amikacin, but it may not be an appropriate choice for ethambutol and kanamycin.[111] However, despite the advances in the resistance detection tools, key challenges still exist including difficulties in documenting the impact on programmatic performance, long-term financial support, and a limited number of scientific publications.[112]

CONCLUSIONS

More rapid and appropriate TB detection and treatment

TABLE 2. Molecular diagnostic of mono-resistant and MDR-TB detection

Methods	Purpose	Reference
High-resolution melting curve analysis	Rifampicin resistance	92
Sloppy molecular beacon melting temperature coding	Rifampicin resistance	93
Nitrate reductase assay	Rifampicin and isoniazid resistance	94-96
Colorimetric redox-indicator methods	Rifampicin and isoniazid resistance	97, 98
PCR-single-strand conformational polymorphism (SSCP) method	Rifampicin resistance	77
Multi-PCR-SSCP method	Rifampicin and isoniazid resistance	99
VerePLEX Biosystem	Rifampicin and isoniazid resistance	100
Mycobacterial IDentification and Drug Resistance Screen (MID-DRS) assay	Rifampicin, isoniazid, and pyrazinamide resistance	101
Multiplex allele-specific PCR assay (MAS-PCR)	Rifampicin resistance	102
Direct DNA sequencing analysis	Rifampicin, isoniazid, pyrazinamide, and ethambutol	103

leads to reduced hospitalization and complications, as well as avoiding unnecessary treatment and isolation of false-positive cases.[113] The molecular diagnostic testing for active pulmonary TB is promising for the future and should be encouraged in a way that could improve population health.[114] Consideration is given to the requirements of future diagnostic tests and how these should be evaluated for their diagnostic accuracy and operational feasibility, and ultimately their clinical impact.[115]

CONFLICT OF INTEREST STATEMENT

None declared.

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