Improving weak links in the diagnosis and treatment of tuberculosis

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GENERAL INTRODUCTION

Tuberculosis (TB) is one of the oldest infectious diseases but remains a worldwide problem. TB spreads easily from person-to-person by air. The only relevant reservoir of the causative organism of TB, *Mycobacterium tuberculosis*, is the human population; patients with pulmonary TB are the source of transmission [1]. By coughing and sneezing, they excrete TB bacilli that become air-born in tiny droplets or aerosols. Tubercle bacilli can survive in these droplets that in turn can be inhaled by individuals in close contact to the index TB patient. Subsequent infection may either result in self-healing or latent infection, depending on virulence of the *M. tuberculosis* strain, inoculum size, and host genetic and acquired immune factors, such as coinfection with HIV or diabetes mellitus [2,3]. By definition, latent infection reflects a state in which the immune system of the host controls the infection; live tubercle bacilli persist and survive in organelles of host immune cells [4]. Macrophages activated by cytokines like tumor necrosis-factor alpha, interleukin-12 and gamma-interferon, prevent both the metabolic activity and the multiplication rate of bacilli; their numbers are very low during latent infection, below the threshold to detect these organisms by microbial diagnostic assays like culture and DNA amplification techniques. Current techniques to test for latent infection are based on immune recognition by the host. They are unable to make the distinction among individuals that were self-healed, with no viable bacilli left in their system, those with latent infection, carrying live intra-cellular tubercle bacilli in a quiescent, metabolically inactive and very slowly replicating dormant state, or those with active TB. However, approximately 23% of the world's population is believed to be latently infected with TB bacteria, and 5-15% of them will progress at any point in time to develop the disease [5–7]. When these active TB cases are not promptly diagnosed, greater transmission happens in the community. The following symptoms, in decreasing order of frequency, usually occur in someone with active pulmonary TB disease: cough with production of sputum (with or without blood), fever, unintentional weight loss, night sweats, dyspnea, and chest pain [8].

In 2017, there was a global diagnostic gap of 3.6 million between notifications of new cases and the estimated number of incident cases [7], indicating an underreporting and under-diagnosis of TB cases. The top three countries accounting for almost half of this gap were: India (26%), Indonesia (11%), and Nigeria (9%) [7]. Therefore, the World Health Organization (WHO) launched an initiative called: 'Find. Treat. All', with a target to detect and treat 40 million people with TB in 2018-2022 [7].

Even though conceptually TB is almost always curable, it is currently the world's single leading infectious killer, indicating that the treatment of TB is not yet optimal [7]. Besides the challenge of treating drug-sensitive TB cases, the incidence of Multi Drug Resistance (MDR)-TB (caused by *M. tuberculosis* which is not sensitive to Isoniazid and Rifampicin, the two most powerful first line anti-TB drugs) is increasing. MDR-TB invariably results from spontaneous mutations in the genome of *M. tuberculosis* changing the structure of the target of TB drugs; there is no horizontal gene transfer [9] and selective drug pressure facilitates resistant clones to multiply. Inadvertent monotherapy is the main driver of drug resistance that was first identified soon after the discovery of streptomycin, the first TB drug that was first used as monotherapy [10]. Besides this factor, a history of previous TB

treatment, inadequate drug exposure, either because of inadequate dosing, or because of the presence of conditions that change resorption or distribution of TB medications, e.g., diabetes mellitus[11–15], are also associated with the development of MDR-TB. Indeed, inadequate dosing rather than violations of National TB treatment guidelines have recently emerged as an important driver of the MDR-TB epidemic [11,16]. Initial drug resistance, acquired by transmission from patients with MDR-TB, has now become even more important than acquired resistance emerging during TB treatment in many areas around the world [7].

DIAGNOSIS OF PULMONARY TUBERCULOSIS

According to the WHO criteria, pulmonary TB (PTB) is diagnosed by clinical symptoms and isolation of *M. tuberculosis* from sputum by culture or by a newer method such as molecular line probe assays (LPAs), or isolation of acid-fast bacilli by sputum smear microscopy (SSM) if culture or LPAs is unavailable, or smear-negative PTB patients with chest radiography (CXR) showing abnormalities consistent with active PTB[17]. CXR has low specificity and expose patients to radiation[8], while SSM lacks sensitivity because of high threshold of TB detection (5,000 bacilli/ml of sputum) thus it can only detect TB in patients in progressive stage [18]. Its sensitivity is even lower in HIV-coinfected individuals, and it cannot differentiate *M. tuberculosis* from non-tuberculosis mycobacteria (NTM)[19,20].

Culture is the diagnostic modality that is currently considered the reference standard [17]. Unlike molecular genetic tests and SSM, it is the only technique that allows identification of the presence of live TB bacilli. It needs only 10-100 *M. tuberculosis* bacilli to establish a diagnosis, however, it is relatively expensive and needs 2 to 8 weeks to obtain results [7]. Immune-based tests, such as IGRAs or serological antibody assays, are not useful for the diagnosis of active TB because it cannot differentiate active TB from latent TB [8]. Other sputum-dependent tests have been developed more recently, *i.e.* nucleic acid amplification techniques which enable fast identification of *M. tuberculosis* (such as TB LAMP (Eiken, Japan)), as well as rapid assessment of rifampicin susceptibility (such as GeneXpert® MTB/RIF (Cepheid, USA) and Truenat MTB assays® (Molbio Diagnostics, Bangalore, India)), or rapid assessment of susceptibility to first and second line anti-TB drugs (such as LPAs (Hain Lifescience, Germany and Nipro, Japan)).[7] However, most of these techniques are not widely available in many countries, and although Xpert MTB/RIF is available with a concessional price for low-middle income countries[7], it requires cartridges which are more expensive than microscopy, and it is not suitable for using in peripheral health centres because it needs stable electricity.

With a population of over 265 million, Indonesia is the third most burdened country with TB around the world [9]. Many patients in Indonesia live in remote rural areas with difficult access to health care facilities and human resources [21]. The case finding efforts in the country were predominantly based on passive case finding and contact tracing. In line with the national guidelines of Indonesia, culture examinations are only conducted for particular cases, such as extra pulmonary TB or in patients suspected to have drug-resistant and MDR-TB [22].

Sensitivity and specificity of routine diagnostic work-up for pulmonary tuberculosis

For the reliability of the case definition for active pulmonary TB, it is important to assess the reliability of the diagnostic work-up under service conditions in the study area. There was no study regarding the sensitivity and specificity of routine diagnostic work-up for TB in lung clinics in Indonesia, while such data are crucial to inform the stakeholders regarding the performance of routine diagnostic work-up as well as to identify opportunities to improve current diagnostic practice. It has been estimated that there is an average loss of 1 to 3 months delay between the first day of visit to a health facility and the correct diagnosis [23,24]. Clearly, delay to reach the correct diagnosis of TB has major implications for ongoing transmission, as well as the development of clinically extensive and advanced TB with the inherent risk of poor outcome. Meanwhile, the non-TB patients who are misdiagnosed as TB will suffer from unnecessary treatment with loss of resources, and unjustified adverse drug effects. Nontuberculous mycobacteria (NTM) lung disease and lung cancer may mimic TB in chest radiographic imaging, resulting in misdiagnosis of TB [25]. In Taiwan, patients who had negative sputum smear results were more likely to have an incorrect TB diagnosis [26]. A recent study in Surakarta, a small city on Java island, Indonesia, showed that in 2014-2015, 28.7% lung cancer patients were misdiagnosed with pulmonary TB and 73.4% of those patients received anti TB drugs for more than one month [27].

Breath test to diagnose pulmonary tuberculosis

One third of TB cases had difficulty to collect an adequate and good quality sputum sample, especially children or people living with HIV [28]. Therefore, non-sputum-based tests would be a tremendous asset. Currently several non-sputum based tests are in development, such as urinary lipoarabinomannan (LAM), paediatric stool processing prior to Xpert [29], computer-aided detection systems[7], immune-based tests (such as blood host markers), skin patches, and breath tests[30].

A breath test has several advantages; being non-invasive, conceptually applicable as a point-of-care test, that is easy-to-perform, fast, and convenient for children and mechanically ventilated patients[31]. The concept of breath test is to recognize volatile organic compounds (VOCs) that are produced by the host metabolism due to infections, which are different from standard conditions [32], or VOCs that are produced by *M. tuberculosis*[33–35].

There are two techniques used to analyse breath in diagnosing TB, *i.e.* a chemical or a physical technique. A chemical technique is based on chemical interactions between VOCs and the devices, such as gas chromatography combined with mass spectrometry (GC/MS), electronic noses, and immunosensor and bio-optical technology[36–41], while the physical technique measures a physical property of the molecule, such as Field Asymmetric Ion Mobility Spectrometry (FAIMS)[42]. GC/MS is used to find specific VOCs of *M. tuberculosis*[33–35,43,44], but this method requires complex equipment and operation skills, and different studies reported different VOCs[35,43–45]. Meanwhile, electronic nose is an easy-to-use tool based on an

array of sensors that can learn and diagnose a disease from the pattern of VOCs contained in any biological materials, such as breath, urine, or feces.

Electronic-noses have been used for diagnosis of various pulmonary and non-pulmonary diseases, for example asthma[46], chronic obstructive pulmonary disease[46,47], urinary tract infection[48], or cancer[49,50]. More recently, electronic-nose has been investigated as a diagnostic tool for TB[37,39–41,51]. The sensor array in electronic nose comprises non-specific sensors. An odour stimulates the sensor array to produce a specific fingerprint. Patterns or fingerprints from known odours are used to build a model and train a pattern recognition system to classify unknown odours based on this model. A hardware part of the device collects and transports odours to the sensor array, while the electronic circuitry digitizes and stores the responses of sensor for signal processing[52].

TREATMENT OF PULMONARY TUBERCULOSIS

In 1944, Schatz and Waksman reported that streptomycin was active against TB bacilli, although drug resistance emerged soon with monotherapy. This marked the start of discovery of several other TB drugs such as isoniazid, pyrazinamide, and para-amino salicylic acid [17], and in 1952, the concept of multi-drug treatment was established [53–55]. With the discovery of rifampicin in 1968, and the different randomized studies conducted by the British Medical Research Council that followed, a standardized short-course regimen with first-line drugs was adopted by the WHO [56]. The current recommendation is to use isoniazid, rifampicin, pyrazinamide and ethambutol as first line treatment during the first two months, called the intensive phase, and isoniazid and rifampicin for the next four months, referred to as the continuation phase [7]. These anti-TB drugs work with the following mechanisms: 1) bactericidal action, explained as the capability of the drugs to kill the actively growing and multiplying bacilli, a role that is conducted by isoniazid and rifampicin; 2) sterilising action, defined as the capability of the drugs to kill the semi-dormant bacilli. Rifampicin and pyrazinamide fall under this category; 3) prevention from bacillary resistance to happen, a role that is carried out most by isoniazid and rifampicin, and to a lesser extent by ethambutol and pyrazinamide [57].

Treatment success rates reach between 60 to 87%, [7,58]. The success of TB treatment results from many factors including: adherence, comorbidity, type of TB, residence, income, and drug exposure, measured by multiple measurements of drug concentrations over time in the bloodstream [59–61]. Poor treatment outcome has increasingly been associated with low TB drug exposure. Patients with low plasma drug concentrations over time (the area under the plasma-concentration-time curve, or AUC) and low peak concentration of drugs in the blood (C_{max}) of rifampicin and isoniazid may result in selection pressure facilitating repopulation of organisms with reduced drug susceptibility, eventually resulting in acquired drug resistance [61].

In addition, recent studies showed that anti-TB drugs do not penetrate *M. tuberculosis* niches, such as the caseating granulomas, which explains poor correlation between

pharmacokinetic-pharmacodynamics profiles and efficacy [62,63]. Therefore, the efficacy of available treatment strategies is questionable and TB drug discovery and delivery strategies need innovation [64].

Pharmacokinetics of anti-tuberculosis drugs

Pharmacokinetics describes how the body processes the drugs, through absorption, distribution, metabolism, and excretion. The most widely used and most useful pharmacokinetic parameters for TB drugs are AUC and C_{max}. Higher AUC values indicate higher drug exposure and increased efficacy, while low C_{max} values are associated with the occurrence of drug resistance [61].

Several factors influence the pharmacokinetic of TB drugs, which are divided in inter-individual variability factors caused by comorbidities such as HIV and diabetes mellitus [65], or pharmacogenetics of N-acetyltransferase 2 (NAT2) [66], and intra-individual variability, which includes the auto-inducing activity of rifampicin [67], variability of MICs, and/or concomitant food intake along with the ingestion of TB drugs [68].

In healthy volunteers, administration of drugs with meals results in lower AUC and C_{max} values of isoniazid [69], and lower C_{max} of rifampicin and ethambutol [70,71]. Meanwhile, in TB patients, one study that was conducted after two weeks of TB treatment showed that a high carbohydrate diet decreased $AUC_{0\text{-}8h}$ and C_{max} of isoniazid [72], and another study that was conducted at least after four days of TB treatment found that food reduced C_{max} and $AUC_{0\text{-}10h}$ of all first-line anti-TB drugs [73].

There may be distinct pharmacokinetics of first-line drugs in treatment-naïve patients compared to TB patients who are already on treatment because of discrepancy in severity of disease, malnutrition and hypoalbuminemia [74,75]. Furthermore, in treatment-naïve patients, there is a higher number of bacilli and higher risk of acquired drug resistance when the drug level is insufficient [61].

Optimal sampling strategy of blood samples to measure exposure to anti-tuberculosis drugs

There are many factors that could influence the concentration of TB drugs in the blood, thus Therapeutic Drug Monitoring (TDM) may be useful, especially for patients who show slow clinical response to treatment [76], a poor clinical condition during treatment [76], comorbidities that could interfere with the TB drug exposure [14,77], or a condition that puts the patient at risk of adverse TB drug reactions [78].

TDM uses information of plasma drug concentrations (the area under the concentration-time curve, or AUC) to determine the appropriate dose for the patient [76]. However, at least six or seven samples are needed to estimate the AUC [76]. Therefore, some alternative approaches were developed to assess drug exposure and to estimate AUC values in a particular patient.

Optimal sampling strategy (OSS) is a strategy that includes a limited number of blood samples from a patient to estimate $AUC_{0\text{-}24h}$ -the AUC over 24 h- instead of using a full

concentration-time curve that requires frequent sampling time points [76]. Thus, burden for the patient, cost of examination, and time needed to approximate the AUC_{0-24h} of the investigated drug could be reduced[79].

For anti-TB drugs, OSS has been done for some individual drugs such as moxifloxacin or rifampicin, but only one study described an OSS for multiple anti-TB drugs, while this study only assessed OSS of anti-TBs drugs in fasting condition [80].

Early bactericidal activity of inhalation drugs for multi drug-resistant tuberculosis

Treatment for MDR-TB needs long duration (9-12 months for short regimen, and 18-20 months for long regimen), and uses many drugs with many side effects [81]. Therefore, efforts have been put to find a more effective and safer MDR-TB treatment. In developing treatment for MDR-TB, besides searching new anti MDR-TB drugs, exploring other routes of administration for existing drugs is another option.

Since most of TB cases are pulmonary TB, a pulmonary route of drug administration would be an asset. This administration route may use drugs with a different mechanism of action against *M. tuberculosis* than the standard 1st or 2nd line drugs in current use. We were interested in the concept to use an efflux pump inhibitor that causes lesions in the cell wall or cell membrane of tubercle bacilli. In this manner other drugs may act more potently against *M. tuberculosis*.

Colistin sulphomethate sodium pokes holes in cell membranes, causes cell wall damage, deformation and bulging, and may have a synergistic effect with other anti-TB drugs [82]. This mechanism was shown by scanning electron micrographs of cultured isolates of extremely drug resistant *M. tuberculosis*, which were treated with 12,5 mcg/mL colistin [83]. Recently, Lee *et al.* showed a synergistic effect of colistin and rifampicin in *A. baumannii* [84]. Meanwhile, Bax *et al.* and van Breda *et al.* indicated that colistin could potentiate the anti-TB drug activity [85,86].

Inhalation of colistin sulphomethate sodium might therefore be an interesting candidate as additional drug for MDR-TB treatment. For a successful inhalation route, suitable particle properties and appropriate delivery device should be considered. There are several forms of drugs available, such as solutions, emulsions, suspensions or dry powders [64]. Because of the higher sterility, storage stability, and easier handling, a dry powder form is preferred [64]. Currently, the most advanced technologies developed for inhalation drugs are pressurized metered-dose inhalers (pMDIs) and dry powder inhalers (DPIs) [64]. Colistin sulphomethate sodium DPI has already been tested in healthy volunteers [87], patients with cystic fibrosis [88], and TB patients in South Africa (unpublished data), showed that the Colistin sulphomethate sodium DPI was well tolerated by the subjects.

An early bactericidal activity (EBA) is defined as "The fall in counts/mL sputum/day during the first 2 days of treatment" [89]. It is used to measure rates of sterilization of an anti-bacterial drug and is the best method to investigate the efficacy of a drug candidate in a pipeline [90]. To investigate the added value of a drug in an EBA study, another drug in the current regimen which has low EBA should be used as it will not mask the effect of the investigated drug.

Aminoglycosides were used as the second-line injectable agents in the MDR-TB regimen [81]. The EBA for amikacin, one of the aminoglycosides, is low [91]. Meanwhile, the bactericidal activity of amikacin is very similar with kanamycin [92], thus intravenous kanamycin could be used to investigate the added value of Colistin sulphomethate sodium DPI in treating TB.

AIMS AND OUTLINE OF THE THESIS

The aim of Chapter 2 is to investigate the sensitivity and specificity of the routine diagnostic work-up for tuberculosis in lung clinics in Yogyakarta, Indonesia, and explore possible ways to improve current diagnostic standards.

In Chapter 3, we present a study evaluating the diagnostic accuracy of breath test using an electronic nose for PTB. We investigated the sensitivity and specificity of this diagnostic tool using a standard as described in Chapter 2 – the reference was based on clinical symptoms, culture, sputum smear examination, chest X-ray results, and clinical follow-up among patients presenting with complaints warranting a diagnostic work-up for PTB in Yogyakarta, Indonesia.

In Chapter 4, we present a systematic review and meta-analysis of breath test in diagnosing TB to investigate the diagnostic accuracy of breath test with electronic-nose and other devices using culture or other tests for TB (sputum smear microscopy, chest radiography, Gene Xpert, pleural biopsy, or combination of these tests) as a reference for comparison.

As explained above, adequate drug exposure is critical to prevent the emergence of drug-resistant mutants. The objective of Chapter 5 is to quantify the influence of food on the pharmacokinetics of isoniazid, rifampicin, ethambutol, and pyrazinamide in treatment-naïve TB patients. For this purpose, we carried out a prospective randomized crossover pharmacokinetic study in Yogyakarta, Indonesia.

In Chapter 6, we developed an optimal sampling procedure with best-subset multiple linear regression to predict AUC_{0-24h} of first-line anti-TB drugs, which were administered on an empty stomach, fed condition, with intravenous administration as a comparison.

The aim of Chapter 7 is to investigate the early bactericidal activity of Colistin sulphomethate sodium inhalation and intravenous Kanamycin in patients with pulmonary TB.

In Chapter 8, we discuss outcomes of these studies and future perspectives.

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