Chapter 1

Paralleling of Diagnostic Endeavor for Control of Mycobacterial Infections and Tuberculosis

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Additional information is available at the end of the chapter

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Abstract

Mycobacterial infections and tuberculosis pose global public health threats. High tuberculosis morbidities and mortalities are due to the diagnosis problems among other causes. This chapter describes and compares diverse mycobacterial infections and tuberculosis diagnostic efforts and point-out the direction so as to inform areas of and motivate research toward early, rapid, and accurate diagnosis for effective TB control. We have grouped diagnostic approaches according to the type of sample taken for or organ targeted during diagnosis. The sputum-based methods include smear microscopy, culture, and rat sniffing. Interferon-γ (INF-γ) release assays, transcriptional blood signatures, and proteomic profiling use blood samples while colorimetric sensor array (CSA) and mass spectrometry use urine samples. Patho-physiological methods include tuberculin skin tests (TSTs) and radiography. Chromatography and acoustic wave detection can also be used to diagnose TB from breath. Comparative description of these methods is based on a time frame to diagnosis, accuracy, cost, and convenience. The trend shows that there is a move from time-consuming, slow and narrow-spectrum to quick and broad-spectrum TB diagnostic procedures. The sputum-based and patho-physiological approaches remain conformist while blood-based procedures lead research developments. Absence of single best approach calls for synergistic research combinations that form accurate, rapid, cheap, and convenient package at point-of-care centers.

Keywords: tuberculosis, zoonosis, Cricetomys gambianus, tuberculin, interferon, latent TB infection

1. Introduction

Tuberculosis caused by *M. tuberculosis* is the leading cause of human deaths from single infectious agent and fall in the top 10 causes of deaths worldwide. More than 10 million people suffered from TB in 2016 and the global case fatality and incident rates were 16% and 140/100,000 [1].

Inhalation of *M. tuberculosis* stimulates host cellular immune response with a consequence of either clearance of the organism or infection. Infection may result into latent mycobacteria infection (LTBI) (no clinical evidence) [2] or active tuberculosis (pulmonary or extra-pulmonary) [1].

The global LTBI prevalence is 23%, which is approximately 1.7 billion of the population [3]. About 5–15% of LTBI may progress to active TB [4]. LTBI is currently diagnosed by tuberculin skin test (TST) or gamma-interferon release assays (IGRA) [5]. On average the prevalence of active TB globally is 0.14% and the figures may vary according to regions [1].

The zoonotic potential of *M. bovis* constitutes a public health concern. Ingestion of *M. bovis* contaminated material is reported to be a primary cause of infection in humans and the resulting TB may take both pulmonary and extra-pulmonary form [6]. The burden of tuberculosis in humans due to *M. bovis* is variable in different countries, ranging from 5 to 28% with mortalities of 8.7% [6]. Therefore, this pathogen should be given due weight in TB diagnosis and consequently control plan.

According to WHO report, 53 million deaths have been averted from 2000 to 2016 following diagnosis and treatment. There is a big gap between diagnosis and treatment otherwise the number of deaths prevented could be even higher [1]. Every year the health systems fail to capture 3 million TB patients [7] some of them may be due to the nature of mycobacteria and diagnostic setup [6].

When prevention of pathogen-host contact is inadequate, early, rapid, and discriminative diagnosis become the primary factor for treatment success and reduction of further transmissions. Inappropriate diagnosis of symptomatic patients with active TB, LTBI, or other confounding diseases, for example, sarcoidosis, usually lead to delayed or wrong administration of full TB treatment or prophylactic dose [8]. This chapter, therefore, focuses on mycobacterial infections and tuberculosis diagnosis by assessing and comparing different procedures so as to suggest a best diagnostic method(s) and research direction.

The assessment and comparison of diagnostic procedures in this chapter are based on either single or combination of features such as the type of sample tested, pathological, or morphological changes or immunological reactions. Some of the known mycobacterial infections and tuberculosis diagnostic sample sources include sputum for smear, sniffing, and culture, blood for biomarkers such as interferon, inflammatory cells, proteins and transcriptomes, urine, and breath for volatile organic compounds (VOCs). Moreover, anatomical and physiological changes in tissues can allow diagnosis of tuberculosis through imaging techniques and skin reaction. Among these procedures, IGRA, TST and transcriptomic studies can detect mycobacterial infections while smear microscopy, culture and sniffing results of sputum,

radiography, and volatile organic compound determination can detect active tuberculosis. The latter group of procedures is assisted by the appearance of clinical symptoms. As the form of mycobacterial detection differs among these methods, sensitivity, and time frame to diagnosis vary as well. Nevertheless, the goal remains to end TB by 2035 [7].

2. Blood tests in TB diagnosis

Whole blood sample can be used to diagnose both mycobacterial infections and tuberculosis based on the host immune response. Characteristics of blood parameters such as interferon-induced genes, myeloid genes, inflammatory genes and B and T-cells regulation genes, and proteomic profiles are used as markers in the detection of mycobacterial infections and tuberculosis [9].

2.1. Interferon-γ release assays (IGRAs)

IGRAs are immune cell-mediated in vitro blood tests that measure mononuclear cell (lymphocytes: T and B cells) release of interferon-γ (INF-γ) after stimulation by antigens specific for Mycobacterium. Following infection, the body immune response is triggered and immune cells such as macrophages, T-cells, B-cells, and natural killer cell are involved. Macrophages, which form the first defense line, engulf, kill, and eliminate *Mycobacteria tuberculosis*. Some *M. tuberculosis* escapes the immune mechanism and survives and replicate in macrophages. T-cells, especially CD4 and CD8 T-cells, produce cytokine—interferon-gamma, which in turn activate infected macrophages to produce reactive nitric oxide and related reactive nitrogen intermediates to kill the *M. tuberculosis* and eliminate it through the actions of phagosomes and lysosomes [10]. The lymphocytes can produce interferon-gamma in vitro whenever stimulated by *M. tuberculosis* antigen. This feature is used to assist in the diagnosis of *Mycobacteria tuberculosis* infection. Principally, the lymphocytes of a person infected with *M. tuberculosis* release interferon-gamma when mixed with *M. tuberculosis* derived antigens. There are two enzyme-linked assays; enzyme-linked immunosorbent assay (ELISA), and enzyme-linked immunosorbent spot (ELISPOT) assay. The assays detect INF-γ release by lymphocytes of sample donor following exposure to antigens found on the *M. tuberculosis* complex (MTBC).

In ELISA based IGRA, fresh whole test blood sample is mixed with *M. tuberculosis* antigens and control. The antigen is made up of 6kDaA early secreted antigen target (ESAT-6), 10-kDa culture filtrate protein (CFP-10), and TB7.7 [11]. Normal saline is used as a control. The assay gives the concentration of INF-γ in international units per milliliter (IU/ml). The test result is considered positive when the difference in INF-γ response to the TB antigen between the test antigen value and the control value is greater than the cut-off of 0.35 IU [12].

Principally, the ELISPOT assay works on peripheral blood mononuclear cells (PBMCs) from a whole blood sample. The PBMCs are mixed with synthetic peptide antigens (ESAT-6 and CFP-10) and the control and incubated to stimulate interferon-γ secretion by the cells. Secreted interferon-γ is captured by specific antibodies and accumulated as spots. Thus, ELISPOT assay gives the number of interferon-γ producing cells (spots). The test result is

considered positive when the difference in a number of spots between the test sample and the negative control is greater than eight (8) [13].

Both IGRAs have the ability to diagnose latent mycobacterial infection in 24 h following the one-time patient visit and submission of the blood sample to a health center [11]. The sensitivity of the IGRAs in adults is 80–90% when mycobacterium standard culture is used as gold [13]. In contrast to tuberculin skin test, IGRAs do not cross-react with Bacille Calmette-Guérin (BCG) antigen hence prior BCG vaccination does not cause false positives [11]. IGRAs cannot discriminate active tuberculosis from LTBI and its sensitivity is low in immunocompromised patients and children under the age of 5 years [13]. The cost of mycobacterium infection diagnosis by interferon- γ release assays is more than 40 USD per sample, which is higher compared to 10.56–25.97 USD for tuberculin skin test [14, 15]. This factor may influence the availability of and accessibility to this diagnostic service, especially in low-income countries.

2.2. Mycobacterium global gene signature/transcriptional blood signatures

Whole blood transcriptional signature can assist in distinguishing latent from active tuberculosis by showing features, which are not present in asymptomatic patients. Moreover, the transition from latent infection to active TB can be predicted by assessing the blood parameter [9]. This approach is important because of the reported 10–20% of detectable tuberculosis in asymptomatic individuals diagnosed with latent *M. tuberculosis* infection [16].

TB risk signature or global gene expression based on whole blood RNA genotyping polymerase chain reaction (PCR and sequencing) can be used to predict the chance of progression from latent to active TB. It has been reported that the expression of signature genes (that is quantities of functional RNA) increases toward active TB development in contrast to non-TB progressor (latent mycobacterium infection). For instance, Zak and colleagues [17] have reported a 16-gene signature with a sensitivity of 71.2% and specificity of 80% (95% CI: 66·6–75·2) at a 6 month time before tuberculosis diagnosis. This approach shows promising future of TB diagnosis as early as possible when samples are submitted since PCR and sequencing can give diagnostic results within 24 h.

Transcriptional blood signatures studies have been conducted in many parts of the world including high-income countries (US, UK, and German), middle-income countries (Brazil, South Africa, and Indonesia) and low-income countries (Kenya, Gambia, and Malawi) [9]. However, the procedure is under research and there is hope that it will become a handy tool in TB diagnosis and treatment decision.

2.3. Blood proteomic profiling/fingerprinting

Proteome, the entire set of proteins produced or modified by a cell, system or organism, vary with time, requirement, stress or disease state that cell, and system or organism experiences. Studies on types and levels of plasma or serum proteins show variations between individuals with LTBI, active TB and those under different stages of treatment. These features can be used to differentiate LTBI from active TB and other perplexing diseases. Plasma or serum protein peaks are identified using high-performance liquid chromatography (HPLC)-tandem matrix-assisted laser desorption/ionization-TOF-MS (MALDI-TOF-MS) and the results are

statistically analyzed to define mass spectral patterns. Results show unique abundant spectra (up-regulation) in plasma from subjects with active TB, which are distinct from those of subjects with LTBI or controls.

Proteomic fingerprinting of plasma by surface-enhanced laser desorption/ionization—time of flight (SELDI-TOF) demonstrates that active TB can be discriminated from LTBI and other confounding diseases. Sandhu and colleagues [8] have reported that plasma from active TB patients had more distinct spectral peaks (at 5.8–11.5 kDa) than symptomatic LTBI and non-LTBI controls. The procedure discriminates the active TB patients from the undifferentiated controls with an accuracy of 87% (sensitivity 84% specificity 90%). Moreover, active TB can be differentiated from symptomatic controls with LTBI at 87% accuracy (sensitivity 89%, specificity 82%) and from symptomatic controls without LTBI at 90% accuracy (sensitivity 90%, specificity 92%). In this study Serum Amyloid A and transthyretin were reported as potential protein biomarkers for TB diagnosis. Similar procedure was also used by Agranoff and colleagues [18] on a serum to distinguish active TB patients from symptomatic controls, with an accuracy of 94% (sensitivity 93·5%, specificity 94·9%). Again, Serum Amyloid A and transthyretin were identified as potential biomarkers for TB diagnosis.

Liu and colleagues have also used SELDI-TOF MS to screen serum samples from TB patients and controls (lung cancer, pneumonia, chronic obstructive pulmonary disease, and healthy volunteers). Among different TB related peaks that were detected, fibrinogen with mass to charge ratio of 2554.6 Da was up-regulated in TB patients compared to controls. This discrimination of TB patients from controls had an accuracy of 83.8% (sensitivity of 83.3% and specificity of 84.2%) and suggests that fibrinogen could be a potential TB biomarker [19]. A similar procedure was performed by Zhang and colleagues [20], whereby TB patients were differentiated from controls (non-TB controls such as pulmonary cancer, pneumonia, chronic bronchitis, emphysema, asthma and flu, and healthy subjects) with a sensitivity of 96.9% and specificity of 97.8% (accuracy up to 97.3%). The most distinct protein peak at mass/charge ratio of 5643 Da was up-regulated in TB patients and identified as orosomucoid protein.

In most proteomic finger printings for TB diagnosis, comparison of active TB patients, LTBI, and controls (healthy subjects or patients with other non-TB diseases) has been done. The peaks of identified protein biomarkers in controls have served as references. However, the identified protein biomarkers are not TB specific.

According to expert opinions, blood-based mycobacterial diagnostic procedures for example, IGRAs can give supporting information in diagnosis in some situations like extra-pulmonary TB, testing of negative acid-fast bacilli (AFB) in sputum and/or culture negative for *M. tuberculosis*, TB diagnosis in children, or in the differential diagnosis of infection with non-tuberculous mycobacteria (NTM) [21]. IGRA can also support the diagnosis of *M. bovis* infection when single intradermal comparative tuberculin test (SICTT) is negative [22].

3. Sputum tests in TB diagnosis

Sputum of patients with pulmonary TB contains mycobacterium pathogens, which serves a role in transmission. The presence of the pathogens and their features such as viability,

staining, or release of odor compounds are useful in diagnosis. Staining and culture are two common procedures, which use sputum sample to diagnose tuberculosis, especially active form. An emerging third method, which also utilizes sputum, is sniffing by trained African giant pouched rats, the *Cricetomys gambianus*.

3.1. Sputum sample collection

In TB diagnosis, a collection of right samples is necessary. First morning samples are preferred as the overnight accumulation of secretions give better results. Sputum samples are normally collected from self-reporting presumptive TB cases at TB clinic. For samples collected far away from the diagnostic center, the samples are collected in transport medium, cetyl-pyridinium chloride (CPC) [23] for best subsequent results.

3.2. Ziehl Neelsen and fluorescent staining

A smear is made by even spreading of sputum over the glass slide to make a 20 mm by 10 mm eclipse shape. It may be dried and fixed by heat.

Smears from sputum samples are commonly stained using Ziehl Neelsen (ZN) or by fluorescence staining technique and examined microscopically for the presence of AFB. AFB testing is done as a screening procedure at the point of sample collection and on arrival at TB reference laboratory before culture and molecular confirmation. The waxy mycobacterial cell wall of mycobacterium is resistant to stain by conventional dyes and in one way the phenol-carbolfuchsin stain is forced to penetrate the cell wall by gentle heating underneath the slide flooded with carbolfuchsin. Then acid decolorizing solution is applied to remove the dye in cells, tissue, and any organisms in the smear except mycobacteria, which retain the dye. After decolorization, the smear is counterstained with either malachite green or methylene blue, which stains the background material green or blue, respectively. The contrasting background color enables the red Acid-Fast Bacilli to be visualized under a light microscope using the 100x oil immersion objective.

On the other hand, sputum smear can be stained with Auramine O or Auramine rhodamine dye and decolorized by a decolorizing agent (containing ethanol and hydrochloric acid). Counterstaining of the background by potassium permanganate enables visualization of orange-yellow acid-fast mycobacteria against a black back ground under a fluorescence microscope.

On comparison, fluorescent microscopy is faster in giving results (1 min) compared to ZN stain (4 min) and more sensitive (up to 97%) than ZN staining (up to 94%) and generally the accuracy of fluorescent microscopy is higher (Area Under the curve (AUC) = 96) compared to ZN staining (AUC = 94). However, due to resources constraint, ZN staining is more popular in low-income TB endemic areas [24].

3.3. Mycobacterial culture and molecular characterization

Culture isolation of mycobacteria is the gold standard method for TB diagnosis. Sputum smear stain positive samples are a good candidate for culture and molecular characterization.

However, manipulation of suspected mycobacterial samples should be handled in a containment facility to minimize the danger of exposure to personnel and the surrounding environment. If samples are collected far from the culture laboratory, and to prevent contamination, transport in CPC is recommended. Culturing of such samples should be within 7–8 days of collection and storage has given accurate results compared to culture from non-CPC stored samples [25]. Recent reports have shown good yield from CPC stored sputum samples for up to 21 days [26]. This is relevant in low-income countries where focal sample processing and culture centers are far from collection sites. Transport and storage times are worth noting as positivity from culture has been reported to be significantly affected beyond 2 weeks [27].

3.3.1. Sputum sample processing, culture, and identification

During sample processing, the sputum-CPC mixture is normally concentrated by centrifuging at 4000 rpm for 15 min, supernatants poured off into a splash proof container. The sediments can then be mixed with 20 ml of sterile distilled water, suspended by inverting the tubes several times, and then centrifuged at 3500 rpm for 15 min. The supernatant is then removed with the remaining pellets re-suspended in water for the inoculums ready for culture. Two Löwenstein-Jensen slants, one containing 0.75% glycerol and the other 0.6% pyruvate are used for inoculation of the sediments and incubated at 37°C. Growth of Mycobacterial inoculum is examined weekly for 8 weeks; at this juncture cultures with no growth after 8 weeks should be considered negative.

3.3.2. DNA extraction procedures

Several methods are available for extraction of mycobacterial DNA and follow pre-existing protocols depending on the purpose. However, boiling a loop full of bacteria in 100 μL H_2O at 80°C for 60 min can suffice to provide sufficient DNA for subsequent analysis. The crude DNA extracted as per protocol is ready for subsequent analyses or storage at −20°C until typing is done. The most used genotyping methods for mycobacterium DNA are both polymerase chain reaction (PCR)-based spoligotyping; and mycobacterial interspaced repetitive units-variable number tandem repeat (MIRU-VNTR) typing. These techniques are useful and can characterize mycobacterial DNA to strain level and later differentiate into sub-lineages that despite enabling the establishment of transmission chains [28] can assist the mapping of TB strains across regions. This can be done by matching strains in question to existing mycobacterium database at *Mtb* complex (http://www.MIRU-VNTRPlus.org/). Whole genome sequencing can be done to further characterize mycobacteria pathogens. Clinically, whole genome sequencing for *M. tuberculosis* has an advantage in the sense that it provides a more rapid and comprehensive view of the genotype of the pathogen, and with the timely and reliable prediction of drug susceptibility including detection of resistance mutations in clinical samples [29]. Despite the robust software and database tools, Whole genome sequencing needs to be developed for its full potential; it provides the highest resolution when investigating transmission events in possible outbreak scenarios [30]. All these methods are geared at improving treatment outcomes when integrated into routine diagnostic workflows, early reporting of drug resistance rather than dependence on phenotypic drug-susceptibility tests

[31]. In our context, we will discuss other characterization techniques, particularly spoligotyping, and MIRU-VNTR genotyping methods as our main focus rather than mycobacterium whole genome sequencing.

3.3.3. Spacer oligonucleotide typing (Spoligotyping)

In humans, TB infection is mostly due to MTBC, which comprises of a group of seven genetically similar species [32] namely *M. tuberculosis*, *M. bovis*, M. africanum, *M. microti*, *M. canettii*, *M. pinnipedii*, and *M. caprae*. To differentiate such genetically closely related species, special molecular techniques have been in place for quite some time now. Fortunately, despite the high genetic homogeneity among the members, they display divergent phenotypes, eliciting different pathologies, and while some show a degree of host specificities [33]. These qualities have made it possible to differentiate mycobacteria species to strain level and epidemiologically, determining their transmission chain using molecular techniques. Spoligotyping is among PCR-based technique that is used in combination with other molecular methods to establish strain variability in a given population. Using commercially available kits, spoligotyping can be done with reference to existing protocols described previously by Kamerbeek et al. [34]. This PCR-based fingerprinting method detects the presence or absence of 43 variable spacer sequences situated between short direct repeat (DR) sequences in the *M. tuberculosis* genome. The DNA from reference *M. tuberculosis* H37Rv and *M. bovis* BCG clones are commonly used as positive controls while autoclaved ultrapure water is used as a negative control. Visualization of presence (black squares) or absence (blank squares) of variable spacer sequences on film is achieved after incubation with streptavidin-peroxidase and detection of hybridized DNA using chemiluminescent detection liquid followed by exposure to radiography uses electromagnetic radiations (x-ray) film. Resulting spoligotypes are normally reported in octal and binary formats and compared to existing patterns in an international spoligotyping database profiles (SpolDB4.0) [35] available at http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). Spoligotype patterns can then be grouped as spoligotype international types (SITs) if they share identical spoligotype patterns with patterns present in the existing database. Such strain comparisons using SITs are used to identify TB strains circulating in the population as well as new strains and assign names for new strains, which cannot be found in previous studies where possible [35, 36]. By using spoligotyping, it is possible to map TB strains with their diversity within and between regions [37], not only revealing differences in circulating *M. tuberculosis* strains [38] but also identification of new strains [39] even within the country in endemic areas. Further, molecular characterization has yielded molecular type patterns suggestive of similar strains in humans and in wild ungulates [40].

3.3.4. Mycobacterial interspaced repetitive units-variable number tandem repeat (MIRU-VNTR) typing

This is a PCR-based method that exploits the presence of interspaced repetitive DNA in mycobacterium and other genomes to characterize mycobacteria strains and within strains circulating in a given population. In combination with spoligotyping, the method forms a large-scale, high-throughput genotyping of *M. tuberculosis* [28]. The method is based on the presence of more extensive loci that contain variable number tandem repeat (VNTR) of genetic elements named mycobacterial interspersed repetitive units (MIRUs) located

mainly in intergenic regions dispersed throughout the *M. tuberculosis* genome [28, 41]. The method combines the analysis of multiplex PCRs for the target loci on a fluorescence-based DNA analyzer with computerized automation of the genotyping [42]. Initially, the genotyping system used only 12 loci for epidemiological molecular studies in combination with spoligotyping, but now a more discriminatory 24 loci MIRU-VNTR is in place. This current genotyping system in combination with spoligotyping has been found to be 40% superior in a number of types among isolates from cosmopolitan origins, compared to those obtained with the original set of 12 loci [28]. Nevertheless, researchers still continue to optimize the 24-loci MIRU-VNTR genotyping in an attempt to reduce the turnaround time of typing and financial burden [33] although the MIRU-VNTR typing manual [43] remains primary and key guide to all MIRU-VNTR typing of MTBC strains.

The standardized 24 loci MIRU-VNTR typing protocol by Supply et al. [42] is performed using primers that amplify 24 polymorphic loci on the mycobacterial genome per DNA isolate. The number of tandem repeat units present at each locus is then calculated from the size of DNA fragments according to a standardized table (http://www.MIRU-VNTRplus.org). The results can be expressed in digital format where each number represents the number of repeat copies at a particular locus. Phylogenetic analysis and creation of dendrograms can then be done using MIRU-VNTR*plus* (http://www.MIRU-VNTRPlus.org/) to generate a categorical-based NJ-Tree dendrogram to enable comparison of strain genotypes [44]. Creation of dendrogram from phylogenetic analysis enables the establishment of transmission links.

3.4. Sputum sniffing by African giant pouched rats

In recent years diagnosis of pulmonary TB in humans has taken a turn to involve Trained African giant pouched rats (*Cricetomys gambianus*). The procedure takes advantage of the ability of the African giant pouched rats to detect and discriminate odor of volatile compounds produced by mycobacteria [45]. Weetjens and his colleges extended the dimension of research from landmine detection to TB diagnosis [46].

The rats are trained to sniff heat-inactivated sputum from presumptive TB patients for the purpose of discriminating positive samples from negatives. According to Weetjens and colleagues [46], the rats are domesticated, bred and that training is done to the young rats. At the age of 4 weeks, young rats are identified and assigned to a specific trainer. This is accompanied by socialization and habituation learning. Then the rats are trained to locate the sniffing hole in the cage and sniffing at the age of 8 weeks. Discrimination of odor is introduced and multiple sample evaluation follows, at the age of 8 months the rats start operational training. During training, the rats learn to associate odor of TB volatiles with reward (food: peanut or banana). The trained rats sniff holes with sputum samples in the cage. The negative samples are sniffed for less than a second and ignored while for the positive samples, the rat will fix their nose at the hole for at least 5 seconds. In this exercise, each of positive sample detection is accompanied by a reward in form of food [46].

The rats can detect *M. tuberculosis*-specific volatiles such as nicotinate, methyl para-anisate, and ortho-phenylanisole from sputum infected with MTBC, *M. avium*, *M. intracellulare* and other NTM [47]. The detection has been reported to be at 80.4% sensitivity, 72.4% specificity,

and 73.9% accuracy [47]. Sample evaluation results are potentiated by use of multiple rats (e.g. group of four) and positive indication by at least one rat criteria has been reported to be more sensitive than multiple positive indications [48].

Cricetomys gambianus are resistant to TB infection [48]. The rats can detect mycobacteria in sputum in as low concentration as a few bacteria in less than 10 sec [49]. In contrast to a trained microscopist who can examine less than 50 samples a day, the rats can detect hundreds of samples per day before exhaustion [50]. These features make sputum sniffing by the giant African rats a potential rapid screening test. Such technique has been used in Tanzania and Mozambique as a second line screening after ZN staining sputum smear microscopy where 44% increase in TB case detection rate has been reported [48]. However, sputum sniffing by the African giant rats does not guarantee 100% accurate TB diagnosis. Although this disqualifies it as a stand-alone TB diagnostic or first line screening test for presumptive test it can be useful in increasing TB cases among smear-negatives especially in low-income TB endemic countries where diagnostics capabilities are limited [51].

Moreover, TB diagnosis by sniffing rats works in active TB, it may not be handy in latent TB cases. Furthermore, the rats are living creature whose health status needs attention and any anatomical, physiological, or functional deformity may impair TB diagnosis.

4. Patho-physiological assessment in TB diagnosis

Pathological and or physiological changes in the body can be used to diagnose mycobacteria infections or TB. Two common in vivo methods, TST, and chest radiography are presented in this chapter.

4.1. Tuberculin skin test (TST)

The TST is the internationally recognized standard method to identify infection with *M. tuberculosis* and *M. bovis*. Intradermal tuberculin tests based on eliciting a delayed-type hypersensitivity response, which is mediated by a population of sensitized T-cells and takes some weeks to develop after infection [52]. The TST use the purified protein derivative (PPD) tuberculins that have been derived from *M. bovis* and *M. tuberculosis* for detection of latent TB in cattle and in human, respectively.

Techniques that are used for tuberculin skin testing in human include; Multiple puncture tests (tine test, heaf test, and MONO-VACC test) and intracutaneous injections either given by the jet injector or by the Mantoux test [53]. Multiple puncture tests introduce tuberculin into the skin through tuberculin-coated prongs while the Mantoux skin test involves the intracutaneous injection of tuberculin into the volar surface of the forearm [54]. The Mantoux test is recommended for clinical assessment or screening since it is more sensitive and specific than the other methods. The reported sensitivity and specificity of TST in human ranges from 59 to 100% and 70 to 100%, respectively [53].

There are several factors that are associated with false negative results of intradermal skin test in humans. These include recent or advanced TB, severe malnutrition, immuno deficiency

due to the HIV or immunosuppressive chemotherapy, co-infections with viral, bacterial and fungal diseases affecting lymphoid organs (lymphoma, chronic lymphoid leukemia sarcoidosis), metabolic derangement (chronic renal failure), stress (surgery, burns, and mental illness), and live vaccination (measles, mumps, and polio) [53, 54]. False positive reactions are more commonly attributed to co-infection or pre-exposure to other related NTM in cattle and human [55] and BCG vaccination in human [56].

Despite of the several major limitations, TST has been, until recently, a useful procedure for detecting LTBI [57] and most widely used test for control of Bovine TB due to the low-cost, low logistical demands, well-documented use, and its ability to screen the entire cattle populations [52]. Due to the limitations of the sensitivity and specificity of the skin tests, newer ancillary in vitro diagnostic assays for latent tuberculosis has been developed, which measures the production of interferons in whole blood upon stimulation with PPD [52].

4.2. Imaging in TB diagnosis

Radiography uses electromagnetic radiations (x-rays) to visualize internal body organs. Chest radiography is used for imaging lungs, airways, ribs, heart, and diaphragm. The rays are allowed to pass through tissues and captured by a film. Variation of absorption of x-rays by different tissues enables contrasting visualization. Hard tissues, for example, bones absorb more rays and appear dense while soft tissues allow more passage of rays and appear light. Knowledge of anatomy is important for diagnostic interpretation.

According to WHO [58] chest radiography is indicated as a pulmonary TB diagnostic tool for triaging, screening and as a diagnostic aid when clinical pulmonary TB cannot be confirmed bacteriologically. Chest radiography can also be used in the evaluation of TB treatment response. It is, therefore, part of an algorithm within health care system. It can be used to distinguish between active and inactive pulmonary TB based on a temporal evaluation of radiographs whereby the latter is characterized by stable radiographic pictures for 6 months [59].

Most common chest radiographic findings of TB include lymphadenopathy, parenchymal opacities, obstructive atelectasis, pleural effusion, cavitations, and tuberculomas [59]. Chest radiography has high-sensitivity (97%, 95% CI 0.90–1.00) and low-specificity (67%, 95% CI 0.64–0.70) [60]. It can detect any abnormality in the chest including those related to other non-TB pathological conditions. Other imaging techniques such as computed tomography (CT) and Magnetic resonance imaging (MRI) are superior to chest radiography and can be used for reference. It is important to note that besides the low specificity, chest radiography is expensive in terms of equipment and skilled labor, especially in low-income endemic areas.

5. Urine sample tests in TB diagnosis

Advocacy toward non-invasive, non-sputum simple TB diagnosis has pushed research into different directions including the use of urine sample from presumptive TB patients. There are a number of target TB biomarkers in urine including volatile compounds, proteins, and TB antigen.

In a study by Cannas and colleagues [61], traces of mycobacteria DNA were detected in the urine of TB patients at 79% while the controls were negative. In addition, proteins produced in lung lesions and excreted in urine were also recognized by immunoglobulin G (IgG) from active TB patients [62]. Other TB protein biomarkers in the urine of TB patients have been reported by Young and colleagues [63]. In a similar study, Lim and friends [64] tested urine for TB by using a colorimetric sensor array (CSA). Urine headspace analysis showed discrimination between TB and control patients with 85.5% sensitivity and 79.5% specificity. Analysis of volatile organic compounds using headspace gas chromatography/mass spectrometry (GC/MS) showed increased levels of o-xylene and isopropyl acetate and decreased levels of 3-pentanol, dimethylstyrene, and cymol in the urine of TB patients compared to controls with respiratory diseases other than TB [65].

Urine-based TB diagnostic procedures can only detect biomarkers related to active TB and not latent TB. However, the promising feature is that it can detect extra pulmonary TB. Nevertheless, more researches are required to unravel the practicability of the tests.

6. Breath tests in TB diagnosis

Volatile organic compounds (VOCs) in breath have shown to contain biomarkers of active pulmonary tuberculosis derived from the infectious organism (metabolites of *M. tuberculosis*) and from the infected host (products of oxidative stress).

6.1. Breath sample collection

According to the method described by Braden and colleague, [66], a portable breath collection apparatus (BCA) is used to capture the VOCs in 1.0 L breath and 1.0 L room air on to separate sorbent traps. The geometry of the breath reservoir of the BCA is set to ensure that the sample comprises >99% alveolar breath. Subjects wear a nose-clip and respire normally for 2.0 min through a disposable valve mouthpiece with a bacterial filter to prevent mycobacterial contamination of the instrument. The mouthpiece and filter present low-resistance to respiration ensuring that samples are collected without causing any discomfort to patients.

6.2. Breath sample analysis

The VOCs captured in the sorbent traps are then analyzed in the laboratory according to the method described by Phillips where an automated thermal desorption, gas chromatography, and mass spectroscopy (ATD/GC/MS) is employed. To quantify peak areas and control for drift in instrument performance, an internal standard is normally run with every chromatographic assay of breath and air (0.25 mL 2 ppm 1-bromo-4-fluorobenzene, Supelco, Bellefonte, and PA).

Chromatographic data collected are then converted into a series of data points by segmenting them into a series of time slices. The alveolar gradient of each time slice is then determined (i.e. abundance in alveolar breath minus abundance in ambient room air) [67, 68].

For each of the time slice, the alveolar gradient is calculated by taking: ¼ Vb /Ib Va/Ia.

Where, Vb is the integrated abundance of analytes detected by mass spectroscopy in a breath, and Ib is the area under the curve (AUC) of the chromatographic peak associated with the internal standard. Va and Ia denote corresponding values derived from the associated sample of room air.

6.3. Identification of biomarker time slices

The alveolar gradients are identified by comparing the patients positive or negative for active pulmonary TB and rank them as candidate biomarkers according to the value of the C-statistic that is, the AUC of the receiver operating characteristic (ROC) curve [69]. The Kovats Index windows for active TB are clearly distinct from controls in a test that takes 6 min. The detection of TB biomarkers is 80% accurate with 71.2% sensitivity and 72% specificity [70].

TB testing using breath provides hope for a future non-invasive diagnostic procedure. Phillips et al. have put effort to assess the presence of volatile organic compounds in the breath of active TB patients. A special device collected and concentrated breath from TB patients and controls at point-of-care centers. However, the procedure requires special set up and equipment and may not be suitable in low-income endemic settings.

7. Challenges in diagnosis of tuberculosis

In the diagnosis of tuberculosis in both humans and animals, the challenges have always been the availability of better and affordable diagnostic methods. While conventional tests such as ZN test for sputum has been in place and are the gold standard, it can miss some cases due to low-sensitivity. Mycobacterial cultures can improve detection but takes longer to get results. Molecular characterization to nucleotide level that is, through sequencing is perfect but usually unaffordable in low-income countries diagnostic settings. In addition, DNA isolation, species identification, and obtaining cultures from a sophisticated system may face limitations as well. All these challenges necessitate for advocacy focusing on innovations that deliver better tools to confidently diagnose TB and at affordable costs [71]. Although, international and national laboratory partnerships are encouraged particularly to boost diagnostic services in resource-poor countries, the need for diagnostic tests that allow rapid testing at point-of-care is necessary [72]. However, all these need acceptance by health authorities in respective countries for incorporation into countries diagnostic algorithms while ensuring inherent and adequate quality assurance programs in dedicated laboratories. In marginalized communities, refusal of diagnosis, high indirect costs, and anticipated treatment side-effects have posed barriers to TB diagnosis [73]. All these need to be considered if we want to perform right diagnosis and management of tuberculosis across endemic regions. Screening programs for both human and animal tuberculosis could cut the costs of modern molecular diagnostics and characterization but these programs are costly and might currently not be of primary priority by responsible authorities. Otherwise, screening programs despite potentially expected high initial capital investment, their value on cutting down diagnostic costs for TB cannot be overemphasized.

8. Features of desired TB diagnostic procedure

Currently, the procedure includes medical history, physical examination, chest radiography, TST, serologic tests (e.g. IGRA), microbiologic smear (e.g. ZN), and culture [59].

The distinction between and transition from LTBI to active TB as well as differentiation of stages of active TB toward recovery are attributes of a nearly perfect TB diagnostic procedure. This should be coupled with the ability to detect early stages of the TB related health status and a short time to diagnosis. Applicability and convenience of diagnostic (set of) procedures in all life settings are factors to consider when planning a TB diagnostic package. It is not easy to find the majority of these merits in one procedure but a combination of both conventional and molecular tools starting from screening to diagnosis can facilitate the mission of fighting TB.

9. Diagnostic research direction

TB diagnostic research course moves toward blood parameters such as immune response components, protein, and pathological parameters or other tissues (cerebral-spinal fluid). Such parameters shed more light for future informed diagnosis including status and stage of infection or treatment. Converging different research routes may provide an outcome, which shows effects of interactions. For sure there may be antagonistic but also synergistic outcome. For instance, an approach that will detect the presence of the pathogen and also inform about immune status, pathological, or prognostic prediction is required. All in all, instituting programs that focus on screening tests for early detection of the disease can perfectly fit in the strategy of disease control and management. In so doing, disease elimination can be made possible.

10. Conclusion

There is no single best diagnostic approach that can suit all settings including low-income endemic areas. Some procedures are weak in some aspects and can be complemented by others synergistically. This calls for research focus on combinations of procedures and programs, which give out quick and discriminatory results. For instance, a diagnostic procedure should be able to detect LTBI, active TB and different stages of recovery during treatment. Nevertheless, having programs that focus on screening of the disease in our population will definitely reduce diagnostic as well as treatment costs. This can be done particularly through active case finding (ACF) that will enable screening of TB by systematically searching individuals who would otherwise spontaneously not nock the door and present for care at health facility. In so doing early case detection will be increased particularly in marginalized populations [73–76]. Such areas are devoid of reliable and dedicated diagnostic facility under normal settings.

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References

[1] WHO. Global Tuberculosis Report 2017. Geneva: World Health Organization; 2017

[2] Mack U, Migliori GB, Sester M, Rieder HL, Ehlers S, Goletti D, et al. LTBI: Latent tuberculosis infection or lasting immune responses to *M. tuberculosis*? A TBNET consensus statement for the TBNET. European Respiratory Journal. 2009;**33**:956-973. DOI: 10.1183/09031936.00120908

[3] Houben RMGJ, Dodd PJ. The global burden of latent tuberculosis infection: A re-estimation using mathematical modelling. PLoS Medicine. 2016;**13**:e1002152. DOI: 10.1371/journal.pmed.1002152

[4] Getahun H, Matteelli A, Chaisson RE, Raviglione M. Latent *Mycobacterium tuberculosis* infection. The New England Journal of Medicine. 2015;**372**:2127-2135. DOI: 10.1056/NEJMra1405427

[5] Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. Clinical Microbiology Reviews. 2014;**27**:3-20. DOI: 10.1128/CMR.00034-13

[6] Olea-Popelka F, Muwonge A, Perera A, Dean AS, Mumford E, Erlacher-Vindel E, et al. Zoonotic tuberculosis in human beings caused by *Mycobacterium bovis*—A call for action. The Lancet Infectious Diseases. 2017;**17**:e21-e25. DOI: 10.1016/S1473-3099(16)30139-6

[7] WHO. Gear up to End TB: Introducing the End TB Strategy. Geneva: World Health Organization; 2017

[8] Sandhu G, Battaglia F, Ely BK, Athanasakis D, Montoya R, Valencia T, et al. Discriminating active from latent tuberculosis in patients presenting to community clinics. PLoS One. 2012;**7**:e38080. DOI: 10.1371/journal.pone.0038080

[9] Cliff JM, Kaufmann SHE, McShane H, van Helden P, O'Garra A. The human immune response to tuberculosis and its treatment: A view from the blood. Immunological Reviews. 2015;**264**:88-102. DOI: 10.1111/imr.12269

[10] Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: Mechanisms of host and bacillus that contribute to persistent infection. The Lancet Infectious Diseases. 2003;**3**:578-590. DOI: 10.1016/S1473-3099(03)00741-2

[11] CDC. TB Elimination. Interferon-Gamma Release Assays (IGRAs) – Blood Tests for TB Infection. Natl Cent HIV/AIDS, Viral Hepatitis, STD, TB Prev Div Tuberc Elimin; 2011. pp. 1-3. https://www.cdc.gov/tb/publications/factsheets/testing/IGRA.pdf [Accessed March 21, 2018]

[12] Seyhan EC, Gunluoglu M, Tural S, Sokucu S, Gunluoglu G. Predictive value of the tuberculin skin test and QuantiFERON-tuberculosis gold in-tube test for development of active tuberculosis in hemodialysis patients. Annals of Thoracic Medicine. 2016;**11**: 114-120. DOI: 10.4103/1817-1737.180023

[13] Starke JR. Committee on infectious diseases. Interferon-γ release assays for diagnosis of tuberculosis infection and disease in children. Pediatrics. 2014;**134**:e1763-e1773. DOI: 10.1542/peds.2014-2983

[14] Steffen RE, Caetano R, Pinto M, Chaves D, Ferrari R, Bastos M, et al. Cost-effectiveness of quantiferon®-tb gold-in-tube versus tuberculin skin testing for contact screening and treatment of latent tuberculosis infection in Brazil. PLoS One. 2013;**8**:e59546. DOI: 10.1371/journal.pone.0059546

[15] Nijhawan AE, Iroh PA, Brown LS, Winetsky D, Porsa E. Cost analysis of tuberculin skin test and the QuantiFERON-TB gold in-tube test for tuberculosis screening in a correctional setting in Dallas, Texas, USA. BMC Infectious Diseases. 2016;**16**:564. DOI: 10.1186/s12879-016-1901-8

[16] Berry MPR, Graham CM, McNab FW, Xu Z, Bloch SAA, Oni T, et al. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature. 2010;**466**:973-977. DOI: 10.1038/nature09247

[17] Zak DE, Penn-Nicholson A, Scriba TJ, Thompson E, Suliman S, Amon LM, et al. A blood RNA signature for tuberculosis disease risk: A prospective cohort study. Lancet. 2016;**387**:2312-2322. DOI: 10.1016/S0140-6736(15)01316-1

[18] Agranoff D, Fernandez-Reyes D, Papadopoulos MC, Rojas SA, Herbster M, Loosemore A, et al. Identification of diagnostic markers for tuberculosis by proteomic fingerprinting of serum. Lancet. 2006;**368**:1012-1021. DOI: 10.1016/S0140-6736(06)69342-2

[19] Liu J, Jiang T, Wei L, Yang X, Wang C, Zhang X, et al. The discovery and identification of a candidate proteomic biomarker of active tuberculosis. BMC Infectious Diseases. 2013;**13**:506. DOI: 10.1186/1471-2334-13-506

[20] Zhang J, Wu X, Shi L, Liang Y, Xie Z, Yang Y, et al. Diagnostic serum proteomic analysis in patients with active tuberculosis. Clinica Chimica Acta. 2012;**413**:883-887. DOI: 10.1016/J.CCA.2012.01.036

[21] ECDC. Use of Interferon-Gamma Release Assays in Support of TB Diagnosis. Stockholm: European Centre for Disease Prevention and Control; 2011. DOI: 10.2900/38588

[22] Clegg TA, Good M, Doyle M, Duignan A, More SJ, Gormley E. The performance of the interferon gamma assay when used as a diagnostic or quality assurance test in Mycobacterium bovis infected herds. Preventive Veterinary Medicine. 2017;**140**:116-121. DOI: 10.1016/J.PREVETMED.2017.03.007

[23] Pardini M, Varaine F, Iona E, Arzumanian E, Checchi F, Oggioni MR, et al. Cetylpyridinium chloride is useful for isolation of *Mycobacterium tuberculosis* from sputa subjected to long-term storage. Journal of Clinical Microbiology. 2005;**43**:442-444. DOI: 10.1128/JCM.43.1.442-444.2005

[24] Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: A systematic review. The Lancet Infectious Diseases. 2006;**6**:570-581. DOI: 10.1016/S1473-3099(06)70578-3

[25] Pal N, Sharma B, Malhotra B, Rishi S. Transport and storage of sputum specimen by using cetylpyridinium chloride for isolation of mycobacteria. Indian Journal of Pathology & Microbiology. 2009;**52**:59-61. DOI: 10.4103/0377-4929.44966

[26] Hiza H, Doulla B, Sasamalo M, Hella J, Kamwela L, Mhimbira F, et al. Preservation of sputum samples with cetylpyridinium chloride (CPC) for tuberculosis cultures and Xpert MTB/RIF in a low-income country. BMC Infectious Diseases. 2017;**17**:542. DOI: 10.1186/s12879-017-2642-z

[27] Das D, Dwibedi B, Kar SK. Transportation of sputum samples in cetylpyridinium chloride for drug resistance studies from remote areas of Odisha, India. Journal of Infection in Developing Countries. 2014;**8**:1059-1062. DOI: 10.3855/jidc.4384

[28] Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology. 2006;**44**:4498-4510. DOI: 10.1128/JCM.01392-06

[29] Brown AC, Bryant JM, Einer-Jensen K, Holdstock J, Houniet DT, Chan JZM, et al. Rapid whole-genome sequencing of *Mycobacterium tuberculosis* isolates directly from clinical samples. Journal of Clinical Microbiology. 2015;**53**:2230-2237. DOI: 10.1128/JCM.00486-15

[30] Witney AA, Cosgrove CA, Arnold A, Hinds J, Stoker NG, Butcher PD. Clinical use of whole genome sequencing for *Mycobacterium tuberculosis*. BMC Medicine. 2016;**14**:46. DOI: 10.1186/s12916-016-0598-2

[31] Walker TM, Kohl TA, Omar SV, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: A retrospective cohort study. The Lancet Infectious Diseases. 2015;**15**:1193-1202. DOI: 10.1016/S1473-3099(15)00062-6

[32] Imaeda T. Deoxyribonucleic acid relatedness among selected strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. International Journal of Systematic Bacteriology. 1985;**35**: 147-150. DOI: 10.1099/00207713-35-2-147

[33] de Beer JL, Akkerman OW, Schürch AC, Mulder A, van der Werf TS, van der Zanden AGM, et al. Optimization of standard in-house 24-locus variable-number tandem-repeat typing for *Mycobacterium tuberculosis* and its direct application to clinical material. Journal of Clinical Microbiology. 2014;**52**:1338-1342. DOI: 10.1128/JCM.03436-13

[34] Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. Journal of Clinical Microbiology. 1997;**35**:907-914

[35] Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: Mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiology. 2006;**6**:23. DOI: 10.1186/1471-2180-6-23

[36] Vitol I, Driscoll J, Kreiswirth B, Kurepina N, Bennett KP. Identifying *Mycobacterium tuberculosis* complex strain families using spoligotypes. Infection, Genetics and Evolution. 2006;**6**:491-504. DOI: 10.1016/J.MEEGID.2006.03.003

[37] Mbugi EV, Katale BZ, Streicher EM, Keyyu JD, Kendall SL, Dockrell HM, et al. Mapping of *Mycobacterium tuberculosis* complex genetic diversity profiles in Tanzania and other African countries. PLoS One. 2016;**11**:e0154571. DOI: 10.1371/journal.pone.0154571

[38] Gulich GA, Tulu KT, Worku A, Zewude A, Chimidi GA. Molecular characterization of *Mycobacterium tuberculosis* complex in Gambella region, south West Ethiopia. Journal of Medical Diagnostic Methods. 2015;**4**. DOI: 10.4172/2168-9784.1000175

[39] Debebe T, Admassu A, Mamo G, Ameni G. Molecular characterization of *Mycobacterium tuberculosis* isolated from pulmonary tuberculosis patients in Felege Hiwot referral hospital, Northwest Ethiopia. Journal of Microbiology, Immunology, and Infection. 2014;**47**:333-338. DOI: 10.1016/j.jmii.2013.03.012

[40] Gortazar C, Vicente J, Samper S, Garrido JM, Fernández-De-Mera IG, Gavín P, et al. Molecular characterization of *Mycobacterium tuberculosis* complex isolates from wild ungulates in south-Central Spain. Veterinary Research. 2005;**36**:43-52. DOI: 10.1051/vetres: 2004051

[41] Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Molecular Microbiology. 2002;**36**:762-771. DOI: 10.1046/j.1365-2958.2000.01905.x

[42] Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. Journal of Clinical Microbiology. 2001;**39**:3563-3571. DOI: 10.1128/JCM.39.10.3563-3571.2001

[43] Supply P, Gaudin C, Raze D. Optimization of standard 24-locus variable-number tandem-repeat typing of *Mycobacterium tuberculosis* isolates: A multicenter perspective. Journal of Clinical Microbiology. 2014;**52**:3518-3519. DOI: 10.1128/JCM.01790-14

[44] Weniger T, Krawczyk J, Supply P, Niemann S, Harmsen D. MIRU-VNTRplus: A web tool for polyphasic genotyping of *Mycobacterium tuberculosis* complex bacteria. Nucleic Acids Research. 2010;**38**:W326-W331. DOI: 10.1093/nar/gkq351

[45] Mgode GF, Weetjens BJ, Nawrath T, Lazar D, Cox C, Jubitana M, et al. *Mycobacterium tuberculosis* volatiles for diagnosis of tuberculosis by Cricetomys rats. Tuberculosis (Edinburgh, Scotland). 2012;**92**:535-542. DOI: 10.1016/j.tube.2012.07.006

[46] Weetjens BJ, Mgode GF, Machang'u RS, Kazwala R, Mfinanga G, Lwilla F, et al. African pouched rats for the detection of pulmonary tuberculosis in sputum samples. The International Journal of Tuberculosis and Lung Disease. 2009;**13**(6);737-743

[47] Mgode GF, Weetjens BJ, Nawrath T, Cox C, Jubitana M, Machang'u RS, et al. Diagnosis of tuberculosis by trained African giant pouched rats and confounding impact of pathogens and microflora of the respiratory tract. Journal of Clinical Microbiology. 2012;**50**:274-280. DOI: 10.1128/JCM.01199-11

[48] Weetjens BJ, Mgode GF, Machang'u RS, Kazwala R, Mfinanga G, Lwilla F, et al. African pouched rats for the detection of pulmonary tuberculosis in sputum samples. The International Journal of Tuberculosis and Lung Disease. 2009;**13**:737-743

[49] Mahoney A, Edwards TL, Weetjens BJ, Cox C, Beyene N, Jubitana M, et al. Giant African pouched rats (*Cricetomys gambianus*) as detectors of tuberculosis in human sputum: Two operational improvements. Psychological Record. 2013;**63**:583-594. DOI: 10.11133/j.tpr.2013.63.3.012

[50] Mahoney A, Weetjens BJ, Cox C, Jubitana M, Kazwala R, Mfinanga GS, et al. Giant African pouched rats as detectors of tuberculosis in human sputum: Comparison of two techniques for sputum presentation. Psychological Record. 2013;**63**:21-26. DOI: 10.11133/j.tpr.2013.63.1.002

[51] Mulder C, Mgode GF, Ellis H, Valverde E, Beyene N, Cox C, et al. Accuracy of giant African pouched rats for diagnosing tuberculosis: Comparison with culture and Xpert ® MTB/RIF. The International Journal of Tuberculosis and Lung Disease. 2017;**21**:1127-1133. DOI: 10.5588/ijtld.17.0139

[52] Bezos J, Marqués S, Álvarez J, Casal C, Romero B, Grau A, et al. Evaluation of single and comparative intradermal tuberculin tests for tuberculosis eradication in caprine flocks in Castilla y León (Spain). Research in Veterinary Science. 2014;**96**:39-46. DOI: 10.1016/J.RVSC.2013.10.007

[53] Goldstein EJC, Lee E, Holzman RS. Evolution and current use of the tuberculin test. Clinical Infectious Diseases. 2002;**34**:365-370. DOI: 10.1086/338149

[54] Huebner RE, Bass MFS, JB J. The tuberculin skin test. Clinical Infectious Diseases. 1993;**17**:968-975. DOI: 10.2307/4457498

[55] Harboe M. Antigens of PPD, old tuberculin, and autoclaved *Mycobacterium bovis* BCG studied by crossed immunoelectrophoresis. The American Review of Respiratory Disease. 1981;**124**:80-87. DOI: 10.1164/ARRD.1981.124.1.80

[56] Daniel TM, Janicki BW. Mycobacterial antigens: A review of their isolation, chemistry, and immunological properties. Microbiological Reviews. 1978;**42**:84-113

[57] Jasmer RM, Nahid P, Hopewell PC. Latent tuberculosis infection. The New England Journal of Medicine. 2002;**347**:1860-1866. DOI: 10.1056/NEJMcp021045

[58] WHO. Chest Radiography in Tuberculosis Detection: Summary of Current WHO Recommendations and Guidance on Programmatic Approaches. Geneva: WHO; 2016

[59] Piccazzo R, Paparo F, Garlaschi G. Diagnostic accuracy of chest radiography for the diagnosis of tuberculosis (TB) and its role in the detection of latent TB infection: A systematic review. The Journal of Rheumatology. Supplement. 2014;**91**:32-40. DOI: 10.3899/jrheum.140100

[60] den Boon S, White NW, van Lill SWP, Borgdorff MW, Verver S, Lombard CJ, et al. An evaluation of symptom and chest radiographic screening in tuberculosis prevalence surveys. The International Journal of Tuberculosis and Lung Disease. 2006;**10**:876-882

[61] Cannas A, Goletti D, Girardi E, Chiacchio T, Calvo L, Cuzzi G, et al. *Mycobacterium tuberculosis* DNA detection in soluble fraction of urine from pulmonary tuberculosis patients. The International Journal of Tuberculosis and Lung Disease. 2008;**12**:146-151

[62] Kashino SS, Pollock N, Napolitano DR, Rodrigues V Jr, Campos-Neto A. Identification and characterization of *Mycobacterium tuberculosis* antigens in urine of patients with active pulmonary tuberculosis: An innovative and alternative approach of antigen discovery of useful microbial molecules. Clinical and Experimental Immunology. 2008;**153**:56-62. DOI: 10.1111/j.1365-2249.2008.03672.x

[63] Young BL, Mlamla Z, Gqamana PP, Smit S, Roberts T, Peter J, et al. The identification of tuberculosis biomarkers in human urine samples. The European Respiratory Journal. 2014;**43**:1719-1729. DOI: 10.1183/09031936.00175113

[64] Lim SH, Martino R, Anikst V, Xu Z, Mix S, Benjamin R, et al. Rapid diagnosis of tuberculosis from analysis of urine volatile organic compounds. ACS Sensors. 2016;**1**:852-856. DOI: 10.1021/acssensors.6b00309

[65] Banday KM, Pasikanti KK, Chan ECY, Singla R, Rao KVS, Chauhan VS, et al. Use of urine volatile organic compounds to discriminate tuberculosis patients from healthy subjects. Analytical Chemistry. 2011;**83**:5526-5534. DOI: 10.1021/ac200265g

[66] Braden B, Lembcke B, Kuker W, Caspary WF. 13C-breath tests: Current state of the art and future directions. Digestive and Liver Disease. 2007;**39**:795-805. DOI: 10.1016/j.dld.2007.06.012

[67] Phillips M. Method for the collection and assay of volatile organic compounds in breath. Analytical Biochemistry. 1997;**247**:272-278. DOI: 10.1006/ABIO.1997.2069

[68] Phillips M, Greenberg J, Awad J. Metabolic and environmental origins of volatile organic compounds in breath. Journal of Clinical Pathology. 1994;**47**:1052-1053. DOI: 10.1136/JCP.47.11.1052

[69] Cook NR. Statistical evaluation of prognostic versus diagnostic models: Beyond the ROC curve. Clinical Chemistry. 2008;**54**:17-23. DOI: 10.1373/clinchem.2007.096529

[70] Phillips M, Basa-Dalay V, Blais J, Bothamley G, Chaturvedi A, Modi KD, et al. Point-of-care breath test for biomarkers of active pulmonary tuberculosis. Tuberculosis (Edinburgh, Scotland). 2012;**92**:314-320. DOI: 10.1016/j.tube.2012.04.002

[71] Nema V. Tuberculosis diagnostics: Challenges and opportunities. Lung India. 2012;**29**: 259-266. DOI: 10.4103/0970-2113.99112

[72] Parsons LM, Somosközi A, Gutierrez C, Lee E, Paramasivan CN, Abimiku A, et al. Laboratory diagnosis of tuberculosis in resource-poor countries: Challenges and opportunities. Clinical Microbiology Reviews. 2011;**24**:314-350. DOI: 10.1128/CMR.00059-10

[73] Lorent N, Choun K, Malhotra S, Koeut P, Thai S, Khun KE, et al. Challenges from tuberculosis diagnosis to care in community-based active case finding among the urban poor in Cambodia: A mixed-methods study. PLoS One. 2015;**10**:e0130179. DOI: 10.1371/journal.pone.0130179

[74] Corbett EL, Bandason T, Duong T, Dauya E, Makamure B, Churchyard GJ, et al. Comparison of two active case-finding strategies for community-based diagnosis of symptomatic smear-positive tuberculosis and control of infectious tuberculosis in Harare, Zimbabwe (DETECTB): A cluster-randomised trial. Lancet. 2010;**376**:1244-1253. DOI: 10.1016/S0140-6736(10)61425-0

[75] Lorent N, Choun K, Thai S, Kim T, Huy S, Pe R, et al. Community-based active tuberculosis case finding in poor urban settlements of Phnom Penh, Cambodia: A feasible and effective strategy. PLoS One. 2014;**9**:e92754. DOI: 10.1371/journal.pone.0092754

[76] Sekandi JN, Neuhauser D, Smyth K, Whalen CC. Active case finding of undetected tuberculosis among chronic coughers in a slum setting in Kampala, Uganda. The International Journal of Tuberculosis and Lung Disease. 2009;**13**:508-513

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