

Review Article

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Current tuberculosis diagnostic tools & role of urease breath test

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Tuberculosis (TB) remains a significant public health issue worldwide especially in developing countries, where the disease is endemic, and effective TB diagnostic as well as treatment-monitoring tools are serious barriers to defeating the disease. Detection of pathogen-specific metabolic pathways offers a potential alternative to current methods, which focus on bacterial growth, bacterial nucleic acid amplification, or detection of host immune response to the pathogen. Metabolic pathway detection may provide rapid and effective new tools for TB that can improve TB diagnostics for children and HIV infected patients. Metabolic breath tests are attractive because these are safe, and provide an opportunity for rapid point of care diagnostics and tool for drug efficacy evaluation during clinical trials. Our group has developed a rabbit urease breath test model to evaluate the sensitivity and the specificity of urease based detection of *Mycobacterium tuberculosis*. TB infected rabbits were given stable isotopically labelled urea as the substrate. The urea tracer was metabolized to $^{13}\text{C-CO}_2$ and detected in exhaled breaths using portable infrared spectrometers. The signal correlated with bacterial load both for primary diagnostics and treatment monitoring. Clinical trials are currently ongoing to evaluate the value of the test in clinical management settings. Urea breath testing may provide a useful diagnostic and biomarker assay for tuberculosis and treatment response.

Key words Biomarker - TB diagnostic and treatment monitoring - UBT

Introduction

About two billion people (one-third of the world's population) carry latent tuberculosis (TB) infection and more than nine million of them become sick each year with active TB, which can be spread to others. It disproportionately affects people in resource-poor settings, particularly those in Asia and Africa^{1,2}. More than 90 per cent of new TB cases and deaths occur in those parts of the world, posing significant challenges to the livelihood of individuals and developing economies, as TB primarily affects people during their most productive years^{1,2}. Poor health systems, limited laboratory capacity for case detection, treatment barriers

and complications (unreliable drug supply, patients not completing treatment, or prescribing errors), TB and HIV co-infection, and the emergence of drug-resistance^{3,4} make TB a major challenge facing public health programmes, particularly in the 22 countries with the highest TB burden (Fig. 1). In addition, TB is difficult to diagnose and its diagnosis is more difficult in individuals with TB/HIV co-infection. The current diagnostic techniques are either inadequate to detect TB cases with precision, or are time consuming, expensive, and require highly equipped laboratories which are not available in developing countries, where the disease is endemic. In this paper, we reviewed the current available diagnostic tools for TB and the potential role

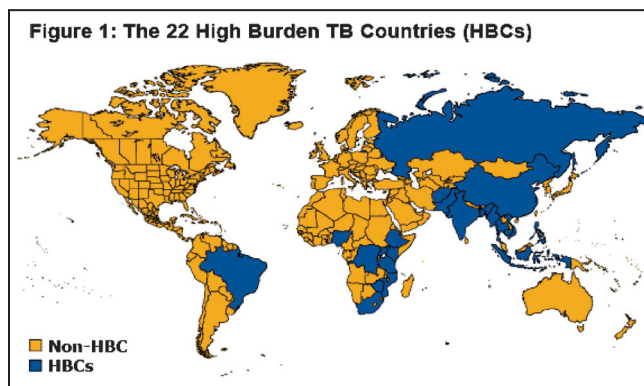


Fig. 1. The 22 high burden TB countries in the world (Source: Kaiser Family Foundation, www.GlobalHealthFacts.org, based on WHO, *Global tuberculosis control 2010*, Slide Date: March 04, 2011; reprinted with permission from the Henry J. Kaiser Family Foundation, California, USA).

of urease breath test (UBT) to both diagnose TB and monitor treatment response.

Current TB diagnostic tools

Sputum smear is used to detect acid fast bacilli (AFB) in clinical specimens by Ziehl-Neelsen (Z-N) or fluorescent staining. It is a cost-effective tool for diagnosing patients with TB and to monitor the progress of treatment especially in developing countries. However, there are many drawbacks such as the difficulty of obtaining the sputum sample especially in children and the low sensitivity especially in HIV-infected individuals with AFB smear positivity ranging from 31 to 90 per cent⁵.

Sputum culture is the gold standard for TB diagnosis with an excellent sensitivity and specificity. The traditional method of inoculating solid medium such as Lowerstein-Jensen (L-J) or 7H10/7H11 media is slow and takes 6-8 wk of incubation to diagnose the infection and further more time to determine the susceptibility patterns. That results in delay in initiation of appropriate therapy.

The introduction of the BACTEC[®] TB 460 radiometric system (Becton Dickinson, Sparks, MD, USA) in the 1980s allowed the detection of *Mycobacterium tuberculosis* in a few days as compared with the conventional culture media. The introduction of these rapid and automated systems have increased the sensitivity of isolation of *Mycobacterium* from clinical samples and has brought down the time required for positive culture substantially (9-10 days). Faster culture results in *M. tuberculosis* infected patients can result in evidence-based therapy and faster implementation

of the planned treatment⁶. Although a combination of traditional solid and liquid media is recommended and helpful for the primary isolation of *Mycobacterium*, the process still requires several days and expensive infrastructure.

The microscopic observation drug susceptibility (MODS) assay relies on light microscopy to visualize the characteristic cording morphology of *M. tuberculosis* in liquid culture. MODS has shorter time to culture positivity (average 8 days) compared with LJ medium (average ~26 days). The cost-effectiveness of MODS (~US \$2/₹102/- per sample) is favourable compared with L-J culture (~ US \$6/₹307/- per sample)⁷. Hence, MODS appears to be a promising, novel and inexpensive tool that can rapidly detect TB and drug resistance directly from sputum samples. However, the effectiveness of MODS in HIV/TB case detection, which is a very common co-infection in developing countries, is not yet clearly established. Also the test is only validated for isoniazid and rifampicin susceptibility testing, excluding the other first lines and second lines TB therapy.

The Gen-Probe assay specific for *M. tuberculosis* complex is a rapid detection, nucleic acid amplification (NAA) test, where results can be obtained as fast as in two hours (provided if a positive culture is present); it also has a high sensitivity of 99 per cent and specificity of 99.2 per cent⁸. It holds the disadvantage of needing of positive culture that can take several days.

Spoligotyping is a technique for concurrent detection and typing of *M. tuberculosis* complex bacteria⁹. This method relies upon amplification of a highly polymorphic direct repeat locus in the *M. tuberculosis* genome and results can be obtained within one day. Thus, the clinical usefulness of spoligotyping is determined by its rapidity, both in identifying causative bacteria and in providing molecular epidemiologic information on strains. Spoligotyping holds drawbacks of being expensive, and requiring well-equipped laboratory and skilled laboratory personnel, which are not always available in endemic areas.

Line-probe assays (INNO-LiPA[®] Rif. TB and HAIN Genotype MTBDR plus) and the microfluidic PCR device (GenExpert[®]/Cepheid) allow both rapid detection of TB and also drugs resistance patterns^{10,11}. The INNO-LiPA[®] Rif. TB can simultaneously detect *M. tuberculosis* and the presence of a mutation in the *rpoB* gene, which confers resistance to rifampicin with a sensitivity of 95 per cent and specificity nearly 100

per cent when isolates from culture were used^{12,13}. However, INNO-LiPA® Rif. TB is less accurate when directly applied on clinical sputum specimens¹². The new Hain “GenoType® MTBDR plus” test can be used both on culture-based isolates and directly on smear positive sputum samples from patients with pulmonary TB. The MTBDR assay has the advantage of identifying mutations in the *rpoB* gene (rifampicin resistance) as well as mutations in the *katG* gene (high-level of isoniazid resistance) and *inhA* gene (low-level of isoniazid resistance). The MTBDR assay can detect at least 90 per cent of MDR-TB cases in a few hours¹³. GeneXpert® MTB/ RIF uses heminested real-time polymerase chain reaction (PCR) assay to amplify *M. tuberculosis* specific sequence of the *rpoB* gene, which is probed with molecular beacons for mutations within the rifampin-resistance determining region^{14,15}. The sensitivity of a single direct MTB/RIF test for culture-confirmed tuberculosis was 92.2 per cent and rose to 96.0 per cent with the additional testing of a pelleted sample¹⁶. The MTB/RIF test provided sensitive detection of tuberculosis and rifampicin resistance directly from untreated sputum in less than 2 h with minimal hands-on time¹⁶.

Such novel technologies have been criticized for being expensive and excluding many drugs for the resistance patterns profile.

Urease activity of *M. tuberculosis* and applications

The *M. tuberculosis* urease enzyme encoded by *ureA*, *ureB* and *ureC* (*Rv1848*, *Rv1849* and *Rv1850*) hydrolyzes urea into carbon dioxide and ammonia¹⁷. The *M. tuberculosis* urease is a likely bacterial virulence factor, which may subvert the host acidification of the phagosome microenvironment. Urease activity has been used in the past as a key test in the identification of microorganisms¹⁸. Sohngen reported urease activity in mycobacteria in 1913 (as noted by Urabe and Saito)¹⁹. With the classification of mycobacteria in the 1950s by Timpe²⁰ and Runyon²¹, medical and taxonomic interest in mycobacteriology increased. During the following decade, biochemical and morphological findings established mycobacteriology as a distinct area for development²²⁻²⁴ and tests based on urease activity have been consistently utilized for the identification of mycobacterial species²⁵⁻²⁸. Despite the role of urease testing for mycobacterial species identification, little has been done to employ urease-based tests as point-of-care primary TB diagnostics until 2009, when our group studied the usefulness of rabbit urease breath test for TB diagnosis and treatment monitoring²⁹. An

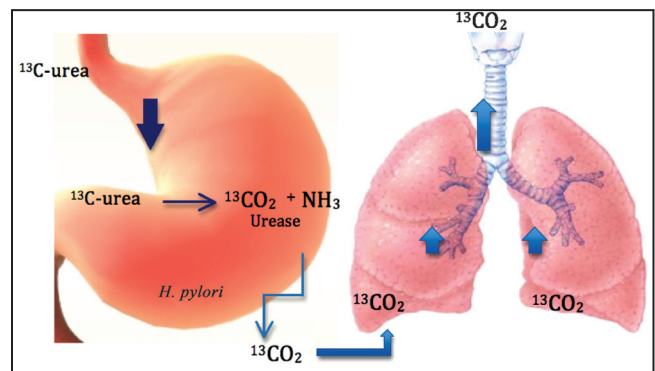


Fig. 2. Labelled urea is degraded by *H. pylori* in the stomach whereby the labelled CO₂ produced is then exhaled and detected in the lungs. The detection of the labelled CO₂ implies infection with *H. pylori*.

attractive aspect of urease-based diagnostics is that humans lack urease enzymes. Hence, the detection of urease enzyme activity in humans signifies the presence of microbial pathogen or commensal which is urease positive.

Metabolomic application of *Helicobacter pylori* urease activity

As *M. tuberculosis*, *H. pylori* also possesses urease activity to promote its survival. Gastric urease detection has been classically utilized for rapid and non-invasive diagnostics of *H. pylori* infection³⁰. This assay introduced during 1980s, uses an oral dose of labelled ¹³C-urea with sampling of the ratio of ¹³CO₂ to ¹²CO₂ in exhaled air after the urea is hydrolyzed in the stomach by the *H. pylori* urease (Fig. 2). This ratio is readily measured using small portable infrared spectrometers, and the entire procedure can be completed within 30 min³¹. The test called *H. pylori* urease breath test (UBT-HP) received United States FDA approval in 1993 and is currently popular for point-of-care diagnosis of *H. pylori* active infection and its post-treatment eradication. This success of the *H. pylori* urease breath test comprises the basis of developing a similar breath test for TB.

Rabbit model of TB-urease breath test (UBT-TB)

Our approach has been to develop a breath test for use in TB similar to the rapid diagnostic for *H. pylori* infection and as a biomarker of its eradication by drug treatment³⁰. We delivered ¹³C-urea into lungs of rabbits infected with *M. bovis* or *M. tuberculosis* (both of which are urease producing mycobacteria) via bronchoscopic infection, and estimated lung burdens of disease and

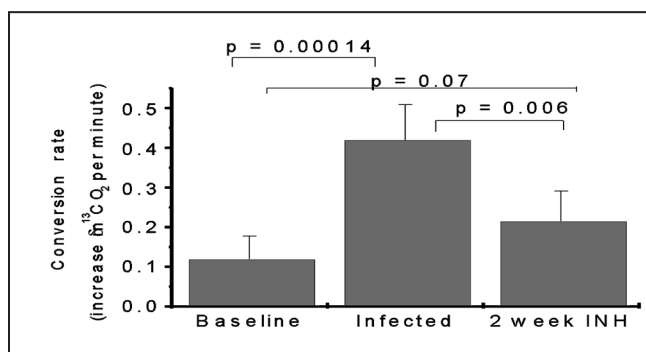


Fig. 3. UBT signal increased in rabbits infected by *M. tuberculosis* and declined significantly in response to partial TB therapy. Source: Ref 29 (Reprinted with permission).

response to therapy by comparing the UBT results over time²⁹. These rabbits converted ¹³C-labelled urea into ¹³CO₂ in a matter of minutes (Fig. 3). The ¹³CO₂ in exhaled breath was rapidly detectable with an isotopic ratio mass spectrometer or a small infrared absorbance spectrometer. The UBT signal in rabbits declines as the rabbits are treated with antimicrobial and increases again after therapy is halted²⁹. In contrast to oral UBT-HP, our experiments with rabbit UBT implemented direct instillation of ¹³C-urea substrate into the lungs or by intravenous injection to increase the specificity for *M. tuberculosis*. The rabbit UBT model has been limited by the fact that exhaled air is difficult to obtain and animals need to be anesthetized (Fig. 4).

Potential utilities of UBT-TB for patient management

UBT provides the possibility of using exhaled breath samples to diagnose TB. It is attractive because it offers a path towards a novel point-of-care diagnostic for TB and TB treatment response. Point-of-care diagnostics are needed for TB especially in high-burden settings in order to reduce the disease impact by providing the possibility to quickly prescribe the appropriate treatment. Thus the UBT may be able to serve as a rapid indicator of treatment failure as occurs in patients with MDR-TB. Resistance to TB drugs is genotypic and new nucleic acid amplification tests (such as GeneXpert[®]) offer significant steps forward in rapidly detecting certain forms of resistant clinical isolates. However, a proportion of resistant infections will not be identified molecularly and some molecular resistant genotypes may not follow the phenotype expected³². UBT will have the advantage to follow the *in vivo* real time impact of the treatment on bacterial death. Furthermore, the conversion rate per minute showed that the test may rapidly and reliably detect

pulmonary tuberculosis associated with a variety of pathologic presentations²⁹.

Isotopic gas ratio analysis can be conducted with compact infrared spectrophotometers such as POcone[™] or UBiT[®] (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan). The exhaled breath sample is easily obtained through one-way valve bags and analyzed by such devices. These devices can be modified and filters incorporated for use for TB infected patients (to avoid contaminations).

The UBT-TB test may also provide a novel biomarker for TB drug therapy trials to improve therapeutic strategies and validate novel TB drug and vaccine candidates. If validated, it may provide valuable early information on whether bacterial burdens are responding to antimicrobial therapy. As opposed to currently utilized sputum microbiology analyses being used for drug susceptibility, urea breath testing for *M. tuberculosis* may allow a rapid assessment of treatment efficacy and the need for treatment modifications for drug resistant TB^{33,34}.

UBT, as a metabolomic based test, works on live bacteria. A large cell population of *M. tuberculosis* during the disease and even during TB treatment is non-replicating and non culturable in normal conditions³⁵. However, UBT has the potential to detect this population and monitor its response to chemotherapy, although the relative expression of urease activity by dormant cells requires investigations.

Specificity of UBT-TB

UBT-TB specificity may be limited by other urease-producing microbial species, such as *H. pylori*,

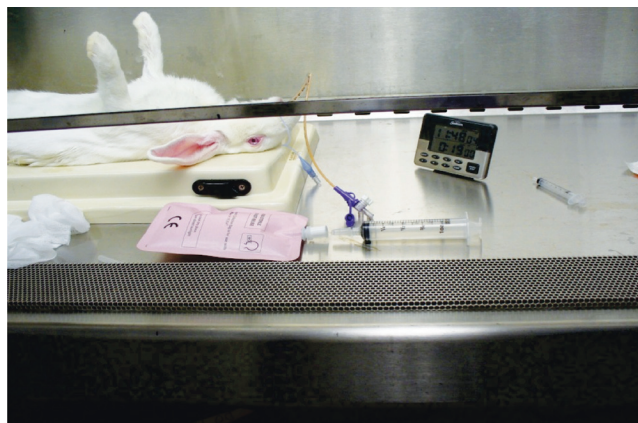


Fig. 4. Materials to get exhaled air breath from anaesthetized rabbits and transfer into Breathek[™] air bag.

which is common in high TB incidence areas reaching 70 to 90 per cent of the population³⁶. Interestingly, the specificity may be ameliorated by oral non-absorbable urease inhibitors such as bismuth subsalicylate (commonly used to treat dyspepsia) or proton pump inhibitors (PPIs) by allowing selective suppression of gastrointestinal *H. pylori*³⁷. TB therapy known to have the potential to suppress *H. pylori* in the first two weeks also increases the specificity for TB treatment monitoring³⁸.

In addition, specificity may be gained by introducing the ¹³C-urea tracer via the respiratory tract or intravenously, thereby avoid gastric *Helicobacter* species.

An additional approach would be to co-administer an unlabelled urea drink not enriched with ¹³C, but rather has this at normal abundance. This approach would saturate gut urease producers with “cold” tracer and, therefore, any CO₂ produced by gut organisms would not be enriched in ¹³CO₂ and thus not confound the signal from the lungs.

Ongoing clinical trials on UBT-TB

In collaboration with partners, our group is investigating the sensitivity and the specificity of oral UBT both for TB diagnostic and treatment response in Mali and South Africa³⁹. Pranactin®-Citric (Breathtek™), an oral granulated powder of ¹³C-urea approved by United States Food and Drug Administration (FDA) for UBT-HP will be used with different time points to assess the best moment to test for pulmonary TB. While the test may be confounded by co-existing gastric *H. pylori* infection, bismuth subsalicylate will be also evaluated in certain arms of the studies to specifically reduce false-positive signal from *H. pylori*. The studies will compare UBT-TB to TB standard tests and will include both HIV co-infected and non-HIV TB patients. Tolerability studies upon the nebulized delivery of ¹³C-urea solution directly to the lungs are also in planning stages.

Conclusion

Despite progress in the last decade in TB diagnostic tool discovery, effort is still needed to have impact on the time and the accuracy of the results. The urea breath test may fulfill the criteria for service as an effective point-of-care diagnostic which requires an easily instituted, cost-effective and rapidly applied technology. UBT may also be valuable for response to therapy and thereby offer early information on the possibility of drugs resistant infections monitoring.

Further clinical studies are needed to evaluate the benefits of metabolic breath tests compared to the currently available ones.

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