

New developments in the laboratory diagnosis of tuberculosis

New and faster ways of diagnosing tuberculosis are needed urgently.

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Although the incidence of tuberculosis (TB) has declined in many regions of the world over the past decade, absolute numbers of cases continue to increase, with 9.27 million new cases diagnosed in 2007.¹ Africa is particularly affected, with the incidence and mortality both rising steadily, owing, at least in part, to the deadly synergy between TB and HIV.

TB control programmes still rely primarily on smear microscopy for diagnosis – a technique that has remained essentially unchanged for the past 100 years, although the introduction of fluorescent acid-fast staining for smear microscopy has reduced operator time and resulted in a modest increase in sensitivity. Incremental advances in culture technology have also been made in the past decades, including the introduction of liquid culture and automated systems for detection of mycobacterial growth. This has significantly reduced time to detection – from an average of 20 - 24 days to 10 - 12 days. However, culture results are still frequently received too late to have an impact on clinical management. Moreover, culture remains cumbersome and costly, involving decontamination and concentration of sputum, which requires bio-safety level 3 facilities, expensive equipment and highly trained personnel. These facilities are often not available in high-burden regions. There is therefore an urgent need for more rapid and sensitive diagnostic tests for TB.

The rise of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB has drawn attention to the need for rapid diagnosis of drug-resistant TB. Delay in diagnosis is associated with unfavourable outcomes and an increased opportunity for transmission of resistant strains, both within the community and in the health care setting, as demonstrated by the Tugela Ferry outbreak of XDR TB.

The past decade has seen significant advances in diagnostics for TB, most notably the introduction of interferon-gamma release assays (IGRAs) for the diagnosis of latent TB infection (LTBI) and the development of rapid genotypic tests for drug resistance. There is much optimism that these tests, and novel fully automated systems for real-time PCR-based detection, will finally enable clinicians to diagnose TB accurately and rapidly.

Interferon-gamma release assays

For over a century, the tuberculin skin test (TST) has remained the established screening method to identify persons with LTBI. In children, TST is also frequently used as an adjunctive test to support a diagnosis of active TB disease. TST is, however, an imperfect marker for TB exposure as the test reagent, purified protein derivative (PPD), is cross reactive and contains antigens present in bacille Calmette-Guérin (BCG) and numerous non-tuberculous mycobacteria, diminishing test specificity. TST is also prone to errors in placement and interpretation. Most importantly,

the sensitivity of TST is reduced in individuals with advanced TB disease, malnutrition or HIV infection, leading to false-negative results.

An important advance has been the discovery of *Mycobacterium tuberculosis*-specific antigens, including early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), encoded by the region of difference 1 (RD1) of *M. tuberculosis* and absent from all BCG vaccine strains and most environmental mycobacteria. IGRA (such as QuantiFERON-TB Gold assay, Cellestis Limited, Victoria, Australia) and T-SPOT.TB (Oxford Immunotec, Oxford, UK) detect the presence of circulating T cells sensitised to TB-specific antigens by the release of IFN- γ . A growing body of evidence supports the use of IGRAs for the diagnosis of LTBI in adults.² Studies have shown that IGRAs have a reasonably high sensitivity for TB disease and correlate better with *M. tuberculosis* exposure than TST.

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IGRAs for the diagnosis of active TB in South Africa

The major drawback of IGRAs is their inability to distinguish between active and latent TB infection. In South Africa, where the majority of adults have been exposed to TB (and will therefore have a positive IGRA), these assays have no place in the diagnosis of active pulmonary TB. Their role in the diagnosis of extra-pulmonary TB in adults, when performed on site-of-disease samples, such as cerebrospinal fluid, is currently under investigation.

In children, however, even in high-prevalence regions of South Africa, rates of TB infection are approximately 3% per annum. A positive IGRA may therefore provide useful supportive evidence of recent exposure or infection with *M. tuberculosis* in a young child with suspected TB. There is no clear evidence, however, that IGRAs perform substantially better than TST in HIV-uninfected children in our context.³ In contrast, IGRAs are more sensitive than TSTs in HIV-infected children (64% v. 29%);⁴ however, false-negative

IGRA tests are not uncommon, both in HIV-infected and -uninfected children. These tests are costly and require access to sophisticated laboratory facilities and highly trained personnel.

IGRAs for the diagnosis of latent TB infection in South Africa

HIV-infected adults in South Africa more frequently have positive IGRAs than TST.⁵ This may reflect increased sensitivity of IGRA for the diagnosis of LTBI in HIV-infected individuals (or, alternatively, decreased specificity). Since a positive TST is frequently used as a marker for the potential beneficial effect of isoniazid (INH) prophylaxis in this context, it has been suggested that adults with a positive IGRA, but negative TST, may also benefit from INH prophylaxis, although this has not been proven. There are limited data suggesting that a positive IGRA is predictive of the later development of active TB in adults.⁶

Asymptomatic young children (<5 years of age) with a positive IGRA should be assumed to be infected with TB and should be offered INH prophylaxis, although South African guidelines advocate the use of INH for all young children in contact with an adult source case, irrespective of TST (or IGRA) result.

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A new look at some old tools

Recent reviews have confirmed the importance of specimen processing and fluorescence microscopy in achieving optimal sensitivity for smear microscopy. Concentration of sputum (by centrifugation) increases the sensitivity of smear microscopy by 18%,⁷ while fluorescence microscopy is on average 10% more sensitive than standard microscopy.⁸

Clinicians should ensure that sputum from their patients is processed using these techniques.

A drawback to the widespread implementation of fluorescence microscopy has been the cost and limited lifespan of fluorescent light sources. The recent development of high-powered, cheap and durable light-emitting diode (LED) light sources for fluorescent microscopes is likely to improve access to fluorescence microscopy within the near future.

Nucleic acid amplification (NAA) assays

In the best of hands smear microscopy is able to detect approximately 80% of culture-proven cases (in HIV-uninfected adults). However, in field settings, the real performance of smear microscopy is typically 50 - 60% compared with culture. HIV infection has further reduced the sensitivity of smear microscopy, with up to 60% of culture-proven cases from HIV-infected patients having a negative acid-fast smear.⁹ This further highlights the need for a rapid test with improved sensitivity over smear microscopy.

NAA assays rely on the specific detection of *M. tuberculosis* DNA or RNA in patient samples. These tests, while theoretically highly sensitive and specific, have been reported to produce variable results and to be of limited use in children and smear-negative cases. Tests fall into two categories, i.e. 'in-house' (developed for own use by a laboratory) and commercial assays. The performance of in-house assays varies widely, probably because they depend heavily on the expertise of the operator performing the test.¹⁰ Many in-house assays have not been rigorously validated by a clinical evaluation study. Caution should therefore be taken in interpreting the results of such tests. The performance of commercial assays for smear-positive disease is excellent; however, there is no clear benefit for performing NAA assays on smear-positive samples in South Africa where acid-fast bacilli in respiratory samples are virtually pathognomonic for TB. In smear-negative disease, the sensitivity of commercial assays is significantly lower (66%) while specificity is high (98%).¹¹

Commercial NAA assays are typically performed using decontaminated, concentrated sputum sediment and therefore require a fully equipped TB laboratory. This drawback, the need for laboratory expertise and scrupulous laboratory technique and the significant expense involved, have further limited

the use of NAA assays. However, NAA tests are attractive as they amplify regions specific to *M. tuberculosis*, are potentially highly sensitive and may allow for rapid detection of mutations associated with drug resistance.

A major recent advance has been the development of a fully automated sputum-processing, DNA extraction and real-time PCR-based detection platform for TB. The GeneXpert MTB/RIF system is able to simultaneously detect the presence of TB in a sample and determine susceptibility to rifampicin. A published pilot study showed promise, with detection of 100% of smear-positive and 72% of smear-negative cases and high specificity.¹² The results of a large multicentre evaluation study are awaited. The major advantage of this system is the need for minimal operator training, suitability for decentralised models of TB diagnosis (this test can easily be performed at a microscopy centre) and ability to rapidly detect both the presence of TB and rifampicin resistance.

Other NAA technologies in the pipeline include the loop-mediated isothermal amplification assay (LAMP), which does not require costly equipment and relies on simple visual inspection of the assay tube to detect amplification product (and hence the presence of TB).

Genotypic detection of drug resistance

The GeneXpert system relies on the detection of mutations in a small region of the TB chromosome associated with 95% of rifampicin resistance. The same principle underlies the now widely used Hain MTBDR_{plus} line probe assay (LPA). LPA involves a PCR-hybridisation technique to identify the most common mutations giving rise to resistance to rifampicin or INH. The major advantage of this technique is that the test can be directly applied to smear-positive sputum samples, giving rapid drug susceptibility results without the need for culture. Many laboratories now also use LPA as the primary method for drug susceptibility testing on cultured isolates of *M. tuberculosis* in the place of phenotypic (growth-dependent) drug susceptibility testing. A large evaluation study performed in South Africa showed excellent concordance between LPA performed on smear-positive sputum and conventional culture-based (phenotypic) drug susceptibility testing.¹³ The World Health Organization (WHO) subsequently issued a statement supporting the use of MTBDR_{plus} directly on smear-positive samples for rapid detection of drug resistance. Limitations of the assay include

reduced sensitivity for detection of INH resistance compared with rifampicin resistance (89% and 98%, respectively)¹⁴ and relatively poor performance on smear-negative samples. The assay is labour intensive and requires highly trained personnel and dedicated laboratory space.

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Antigen detection assays

A major drawback of NAA assays in their current format is the requirement for costly and fragile equipment. They are therefore unsuitable for true point-of-care testing for TB. In contrast, antigen detection assays offer the promise of simple lateral flow-type assays (such as the home pregnancy test) that can be performed with minimal resources. Unfortunately, to date there has been no candidate target antigen identified that can be reliably detected in samples from TB-infected patients. Mycobacterial lipoarabinomannan (LAM) can be detected in the urine of a subset of TB patients by an enzyme-linked immunosorbent assay (ELISA) technique; however the sensitivity of this assay is poor in HIV-uninfected adults (14 - 21%). The sensitivity is higher in HIV-infected patients (38 - 67%), particularly in those with more advanced immunosuppression (and therefore presumably a higher TB bacterial burden) but remains suboptimal.

HIV-infected adults in South Africa more frequently have a positive IGRA than TST.

Serological tests

The major challenge facing serological (antibody) testing is the difficulty in distinguishing between TB exposure, latent infection and active disease. It seems likely that no single antigen will provide acceptable sensitivity and specificity; however, combinations of antigens may

improve performance. A WHO-sponsored evaluation of commercial serological tests using well-characterised serum specimens concluded that these tests vary widely in performance but in general perform poorly.¹⁵ Their use in clinical practice cannot be supported at present.

Conclusion

While there has been significant progress in the past decade, the goal of a rapid, inexpensive, sensitive, point-of-care test for TB remains elusive. It is likely that in the near future novel integrated NAA assays will provide clinicians with rapid results with improved sensitivity compared with smear microscopy. It is important that clinicians are aware of the strengths and limitations of novel diagnostic test methods.

Mark Nicol's research work is supported by the Wellcome Trust, European and Developing Countries Clinical Trials Partnership (EDCTP), National Institutes of Health (NIH), Medical Research Council (MRC), National Research Foundation (NRF) and PATH.

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In a nutshell

- Sputum concentration and fluorescence microscopy improve the sensitivity of smear microscopy for TB.
- Interferon-gamma release assays are not helpful for the diagnosis of adult pulmonary TB in South Africa.
- Interferon-gamma release assays may be useful adjunctive tests for the diagnosis of HIV-associated TB in children.
- The performance of 'in-house' nucleic acid amplification assays is highly variable and results should be interpreted with caution.
- Commercial nucleic acid amplification assays have high specificity but relatively poor sensitivity for smear-negative disease.
- Novel automated sputum processing and real-time PCR systems may offer substantial improvements over current technology.
- Currently available serological tests for TB diagnosis perform poorly and should not be used.