Revista Argentina de Microbiología 57 (2025) 49–53

asociación argentina de microbiología

REVISTA ARGENTINA DE MICROBIOLOGÍA

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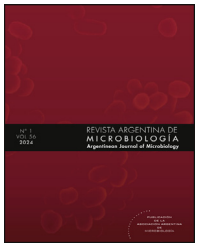

BRIEF REPORT

Update of diagnostic methods in tuberculosis (TB)

Mario José Matteo[a], **María Cecilia Latini**[b], **Davor Nicolás Martinovic**[c], **Marina Bottiglieri**[d,*]

[a] *Laboratorio de TB Dr Abel Cetrangolo, Hospital F. J. Muñiz, Ciudad Autónoma de Buenos Aires (CABA), Argentina*
[b] *Instituto de Tisioneumonología Dr Raúl Vaccarezza, Universidad de Buenos Aires, CABA, Argentina*
[c] *División Microbiología, Servicio de Laboratorio, Hospital Rawson, Córdoba, Argentina*
[d] *Servicio de Microbiología, Universidad Católica de Córdoba, Clínica Universitaria Reina Fabiola, Córdoba, Argentina*

Received 10 April 2024; accepted 26 December 2024
Available online 18 January 2025

KEYWORDS
Tuberculosis;
Diagnosis;
Traditional methods;
Current diagnosis methods

Abstract The WHO aims to reduce the number of deaths from TB by 95% and decrease its incidence rate by 90% between 2015 and 2035. The recommended rapid diagnostic tests are accurate and cost-effective, allow for a prompt start to treatment, and influence other outcomes that are important to the patient. To detect latent infection, the tuberculin skin test and interferon γ release (IGRA) tests are used. Although IGRA is an expensive test, it has greater specificity and is not affected by previous exposure to the BCG vaccine, among other advantages. For the diagnosis of active TB, smear microscopy is commonly employed. Culture is a more sensitive, but also more complex method. It constitutes the definitive diagnosis and allows phenotypic sensitivity tests to be performed. TB-LAM has limited sensitivity; however, unlike other methodologies, it has shown promising results in individuals living with HIV and CD4 T-cell counts below 200/mm^3. Finally, among the molecular biology-based tests, commercial methods using real-time PCR allow mass diagnosis and sensitivity testing to first- and second-line drugs to be conducted within a few hours of receiving the sample. These are highly sensitive and specific tests, and their use is recommended as the initial diagnostic test in both pulmonary and extrapulmonary TB cases.

* Corresponding author.
E-mail address: marinabottiglieri@gmail.com (M. Bottiglieri).

https://doi.org/10.1016/j.ram.2024.12.008

PALABRAS CLAVE
Tuberculosis;
Diagnóstico;
Métodos tradicionales;
Métodos de diagnóstico actuales

Actualización de los métodos de diagnóstico en tuberculosis (TB)

Resumen La OMS propuso lograr la reducción en el número de muertes por tuberculosis (TB) en un 95%, y en la tasa de incidencia de esta enfermedad en un 90%, entre 2015 y 2035. Las pruebas de diagnóstico rápido recomendadas son precisas y costo-efectivas, permiten iniciar el tratamiento rápidamente e influyen en otros resultados importantes para el paciente. Para la detección de la infección latente se utilizan las pruebas de la tuberculina y de liberación del interferón γ (IGRA, por sus siglas en inglés). Si bien el IGRA es una prueba costosa, presenta mayor especificidad y no se ve afectada por la exposición previa a la vacuna BCG, entre otras ventajas. En casos de TB activa, la baciloscopia es una forma habitual de diagnóstico. El cultivo es más sensible, pero de mayor complejidad. Constituye el diagnóstico de certeza y permite realizar pruebas de sensibilidad fenotípica a drogas antituberculosas. El kit TB-LAM tiene una sensibilidad limitada; sin embargo, a diferencia de otras metodologías, ha mostrado resultados prometedores en personas que viven con el virus de la inmunodeficiencia humana (VIH) y tienen recuentos de linfocitos T CD4 por debajo de 200/mm^3. Por último, dentro de las pruebas basadas en biología molecular, los métodos comerciales fundamentados en PCR en tiempo real permiten realizar un diagnóstico masivo y pruebas de sensibilidad a drogas de primera y segunda línea en el plazo de pocas horas desde la recepción de la muestra. Son altamente sensibles y específicos; su utilización está recomendada como primera prueba diagnóstica en casos de TB tanto pulmonar como extrapulmonar.

According to the Global Tuberculosis Report (published by the World Health Organization (WHO)) in 2023, 7.5 million people were reported to be newly diagnosed with TB in 2022, with a total estimated number of more than 10 million cases. Moreover, an estimated 410 000 individuals developed multidrug-resistant or rifampicin-resistant TB. In light of these conditions, WHO calls for urgent actions required to end the global TB epidemic[13]. The recommended rapid diagnostic tests (RDTs) recommended by WHO are highly accurate, shorten the time to treatment initiation, influence important patient outcomes and are cost-effective. Although the target for 2025 is for all notified patients to initially have an RDT, by 2022 only 47% had it and its use varied substantially among countries[13].

This paper provides a review of traditional and new diagnostic techniques that accurately and rapidly detect infection, disease and emerging resistance to *Mycobacterium tuberculosis*.

Certain conditions favor the development of the disease, and patients living with HIV, immunosuppressed hosts, diabetics, cohabitants of patients with pulmonary TB, among others, constitute populations at high risk of contracting TB. The detection of TB infection in these groups represent an important step forward in the implementation of a tuberculosis preventive treatment (TPT)[3,4].

M. tuberculosis infection is a state of persistent immune response to antigens of the bacterium. Infected individuals have no symptoms or signs of TB. The tuberculin skin test (TST) and/or the interferon γ release assay (IGRA) can be used[1] to determine this condition.

Both TST and IGRA do not differentiate latent infection (LTB) from active disease (ATB) and are not recommended for patients who already have a positive test, for treatment monitoring, for individuals who have received live virus vaccines, such as measles or mumps, who have or have had a viral infection within the previous 30 days, or those who are allergic to the purified protein derivative (PPD) used for the test[3].

The TST consists of an intradermal injection of a small amount of PPD derived from a nonspecific mixture of antigens from *M. tuberculosis* bacteria. In an individual who has previously been infected and developed cell-mediated immunity to these tuberculin antigens, a delayed hypersensitivity reaction will occur within 48–72 h. The reaction will cause localized swelling and will manifest as induration of the skin at the injection site. It cross-reacts with other mycobacteria of the TB complex, especially *Mycobacterium bovis* and other non-TB mycobacteria (NTM), which reduces its specificity[5].

A return visit required to read the reaction and the low specificity in immunocompromised patients are disadvantages; the low price is an advantage[3].

IGRA (interferon-gamma release assay) tests measure the interferon-gamma released by circulating lymphocytes in whole blood upon exposure to *M. tuberculosis*-specific antigens, ESAT6 and CFP-10 proteins. These antigens are also present in *Mycobacterium kansasii*, *Mycobacterium szulgai* and *Mycobacterium marinum*, but not in *M. bovis*[3]. In 2011, WHO recommended the use of QIAGEN QuantiFERON®-TB Gold and QuantiFERON-TB Gold In-Tube, and Oxford Immunotec T-SPOT®TB assays; in 2021, the recommendation from WHO was extended to include other technologies: Beijing Wantai's TB-IGRA and QuantiFERON-Plus. The sample to be tested requires whole blood[17]. Advantages include their higher specificity, no BCG vaccine involvement and a single patient visit to the laboratory. They are expensive

and require trained personnel. Indeterminate results may occur in immunocompromised hosts, HIV and advanced TB and immunosuppressive treatments[4]. Future development of TB vaccines that include the antigens present in IGRA will render this assay useless. For this reason, other antigens are being tested to allow differentiation between LTB, ATB and vaccinated individuals.

Currently in Argentina, a new IGRA assay that uses a specific antigen called Rv2626c is being developed, which would allow differentiation between ATB, LTB, recently exposed individuals and healthy people[1].

The bacteriological diagnosis of certainty of tuberculosis is made by the detection of the microorganism in the patient's sample. Direct microscopy or smear microscopy is the most common form of diagnosis. The Ziehl–Neelsen technique is a stain that evidences the presence of acid-fast bacilli of the genus *Mycobacterium*. Although its specificity is limited to the genus level, it has a sensitivity close to 70% in respiratory samples (5000–10 000 bacilli per ml of sample), allowing for the diagnosis of most pulmonary forms, which are those that maintain transmission in the community[9,10].

Alternatively, bacilloscopy can be performed using fluorescence microscopy and auramine dyes. In this case, reading with the 20× and 40× objectives enables the observation of a larger surface of the smear in less time, favoring the work in laboratories with high sample load and increasing sensitivity in paucibacillary samples. Both methods require trained operators[9,10].

Culture is a more sensitive yet more complex method. It can detect up to 10 bacilli/ml of sample and determine their viability, but requires laboratories with a high level of biosafety. Results are obtained between 20 and 60 days and its cost is markedly higher than bacilloscopy. In non-sterile samples it is necessary to perform a decontamination technique prior to culturing[10]. There are two groups of culture media: solid and liquid. Among the solid media, the most frequently used are based on coagulated eggs, such as Löwenstein–Jensen and Stonebrink (the latter promotes the growth of *M. bovis*). Although mycobacteria grow slowly on these media, the colony characteristics can be observed, and semiquantification can be performed. Semi-automated methods have the advantage of detecting development in a shorter time (≈8 days). The most currently used is the Bactec MGIT 320 fluorometric method using Middlebrook 7H9 liquid medium supplemented with oleic acid, albumin, dextrose and catalase and a mixture of antibiotics[10].

Culture continues to be an important methodology in many aspects, including[10]:

- It is the gold standard.
- Due to its higher sensitivity, it is essential in the diagnosis of paucibacillary patients (low-grade pulmonary tuberculosis, extrapulmonary tuberculosis, children) where molecular tests may be negative.
- It is essential for bacterial isolation, typing, drug resistance studies, and strain conservation and transport.
- Diagnosis of active TB in recently pretreated patients.
- Identification of mixed infections with mycobacteria of the *M. tuberculosis* complex and NTM, which usually occur in immunocompromised patients.
- Detection of NTMs which cannot be identified by smear microscopy or detected by molecular biology methods.
- Semiquantification in treatment controls, especially in cases with high numbers of non-viable mycobacteria that result in persistent positive smears.
- Follow-up of patients with multidrug-resistant tuberculosis.
- Suspicion and subsequent detection of bovine tuberculosis.
- Diagnosis of resistance not detected by molecular biology methods, for example, other mutations for isoniazid outside the *kat*G 315 and *inh*A promoter region −15 or outside the rifampicin resistance determinant region.
- Confirmation of active TB in samples with positive results from molecular methods with minimal quantification (traces). In these cases, culture is a complementary tool to consider in the diagnosis. In some cases, although culture can be negative, they are still considered true TB.

Although in the first quarter of the 21st century, molecular methods offered great advantages in terms of speed and sensitivity, culture continues to be a tool that complements the diagnosis and in some cases is still irreplaceable.

One of the new RDTs is the technique for the determination of mycobacteril by lateral flow immunochromatography (LF-LAM), since it yields promising results, especially in individuals living with HIV (PLHIV).

The first recommendation for its use as a support in the diagnosis of TB in HIV-positive patients was issued in 2015, as it is an inexpensive, simple, and quick methodology to perform, which makes it a good point-of-care strategy[15]. No extensive training is required to perform the test, although it is necessary to evaluate the result in the clinical context and in support of other diagnostic methods, especially an Xpert-Ultra test.

The sensitivity of LF-LAM is moderate and varies between studies (57–64%), being higher in PLHIV with low CD4 T-lymphocyte counts. Specificity is high (85–95%), although it may be compromised in regions with high endemicity of infections caused by NTMs and other Actinomycetales. Despite the simplicity of the technique, variability in interpretation among different operators, caused by the low clarity in the appearance of the band, can lead to errors or delays in diagnosis[7].

Currently the only commercial kit validated and recommended by WHO is Alere Determine TB LAM Ag (TB-LAM, Abbott, United States), although the Fujifilm SILVAMP TB LAM kit (FujiLAM, Fujifilm, Japan) is in development and undergoing a validation process. Comparative studies show increases in sensitivity up to 15–20% with respect to TB-LAM[6].

In 2019, WHO updated its guidance on the use of this methodology, issuing a strong recommendation for its use in inpatient settings. This recommendation applies to adults, adolescents, and children living with HIV who present with signs and symptoms of TB, have advanced disease, are seriously ill, or have CD4 T-lymphocyte counts below 200/mm^3, regardless of the presence of symptoms. The recommendation is conditional in those adults, adolescents and children living with HIV who do not require hospitalization, but have

signs or symptoms of TB, are severely ill or have CD4 counts below 100/mm^3[15].

As has been the case for many infectious diseases in the last 20 years, methods based on molecular biology began to be used for the diagnosis of TB. These methods are highly accurate, cost-effective and rapid, generating results within a few hours from the start of the test.

As a result, large laboratories began to produce these tests at a large scale, ensuring the massiveness of their use. The WHO recommended several of these tests, among which we can mention: (a) tests with linear probes (LPA)[14], (b) isothermal amplification-mediated looping (TB-LAMP)[17] and (c) real-time polymerase chain reaction (rtPCR), which are the most widely used[16,17]. These tests were proposed as first-line options for TB diagnosis in replacement of bacilloscopy and set a target for the year 2030 for the diagnosis of 90% of the patients with clinical suspicion of TB with a molecular test[12,16,17].

LPA tests were validated by WHO in 2008[14]. They enable the diagnosis and detection of resistance to first- and second-line drugs. They involve, briefly, the extraction of nucleic acids from the sample, subsequent multiplex PCR amplification of the target genes and finally, reverse hybridization of the amplified products against specific DNA probes immobilized on a nitrocellulose strip. The result is obtained through colorimetric reaction based on the profile of colored bands on the strip. These are highly complex assays, as several steps are required (extraction-PCR-hybridization), and are only recommended in smear positive respiratory samples or from respiratory or extrapulmonary culture isolates. The most frequently used commercial methods are Genotype®MDRTBplus and Genotype MTBDR[14]. Genoscholar PZA-TB II is a LPA test useful to detect pyrazinamide resistance[17].

The loop-mediated isothermal amplification (TB-LAMP) assay is a low-cost, low-complexity test that requires virtually no equipment. It was recommended by the WHO in 2016 as a diagnostic test in first-line care laboratories. It is based on an amplification technique which requires four primers to perform the amplification of gene replication forks, which subsequently leads to concatemer formation and massive amplification. Its simplicity lies in the fact that the entire process is carried out in a single amplification tube and the final reading is colorimetric or fluorometric. Its disadvantages include its unsuitability for drug resistance screening (only recommended in areas with low prevalence of resistance) and its lower sensitivity in PLHIV[17].

rtPCR assays are used to test for the presence of *M. tuberculosis* complex genes and are the most frequently used worldwide. Among the first technologies to be massively applied was Xpert MTB/RIF[17], capable of simultaneously detecting the presence of the *M. tuberculosis* complex and rifampicin resistance within 2 h. The test is simple, it does not require laboratories with high biosafety conditions, and is made entirely in a closed cartridge that prevents contamination[2]. It had a subsequent evolution that was named Xpert MTB/RIF-Ultra that improved the sensitivity of the method, has been validated by WHO since 2016, and can be used in both respiratory and extrapulmonary samples. The test is based on the amplification of the IS-6110 and IS-1081 insertion sequences, as well as of a specific 81-base pair region of the gene for the *rpo*B polymerase called the *rpoB* resistance determinant region, where a very large majority of the mutations that are found confer resistance to the drug. At the same time, the amplified products are detected through a system of DNA probes linked to fluorophores which emit fluorescence at different wavelengths when there is binding specific to amplicons[17]. Interpretation of resistance in Xpert-Ultra is made by the analysis of melting curves. The sensitivity and specificity of the method exceeds average values of 90% for both the diagnosis and detection of rifampicin resistance in respiratory specimens (including specimens with negative bacilloscopy where sensitivity reaches a value close to 80%)[17]. In extrapulmonary samples, sensitivity is also high and depends on the sample involved.

In 2020, the technology underwent a further evolution with Xpert MTB/XDR, which detects resistance to isoniazid and other second-line drugs such as ethionamide, aminoglycosides and quinolones, but requires equipment that allows fluorescence readings at 10 different wavelengths[17].

Alternatively, WHO subsequently recommended other tests based on rtPCR reaction for respiratory samples only: (a) the Truenat system consisting of a micro-PCR running inside a chip and (b) methods provided by multi-platform teams that developed their version of the TB diagnostics kits, such as Real Time MTB RIF/INH (Abbott), BD MAX/MDR-TB (Becton Dickinson) and Cobas MTB RIF/INH (Roche). All of them have sensitivity and specificity similar to those of Xpert. These assays are somewhat more complex but have the advantage that they perform a greater number of tests simultaneously (important in laboratories with a high workload) and detect resistance to isoniazid and rifampicin simultaneously in the initial test[11,17].

Another test to be mentioned is FluoroType MTBDR, a new molecular assay for diagnosis and detection of multidrug-resistant tuberculosis, which uses a nonsymmetric PCR, together with sets of lights-on/lights-off probes and allows to detect resistance to rifampin and isoniazid[17].

Finally, the sequencing of the complete genome of *M. tuberculosis* using state-of-the-art sequencers is becoming increasingly relevant. It quickly provides extensive information, such as the analysis of many specific genes (e.g. all those involved in the production of the mechanisms involved in drug resistance) and precise molecular typing to ensure the comparative analysis of strains for in-hospital outbreak descriptions, differentiation between relapses and reinfections, and characterization of endemic strains to a geographic region, among other uses[8].

Targeted next generation sequencing (NGS) was recommended by WHO in 2024. These tests couple amplification of selected genes with NGS technology to detect resistance to many drugs with a single test, promising to be an important tool for the future[17].

In summary, molecular methods have brought about a revolution in TB diagnosis and detection of antimicrobial resistance. Training to learn the rationale behind them to use them rationally is essential. Moreover, social policies should be implemented to assist and follow-up vulnerable patients. Governments need to invest in equipment, infrastructure and trained human resources to ensure that these technological advances are effectively translated into improvements in the fight against TB, and thus move us closer to the ''End TB'' goal proposed by the WHO[12].

Funding

The authors declare that no funding was received for this article.

Conflict of interest

The authors declare that they have no conflict of interest.

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