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Bacteriologic Diagnosis of Tuberculosis. Current State of Knowledge

First part

Diagnóstico bacteriológico de la tuberculosis. Estado actual del conocimiento

Primera parte

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TB Diagnostic Study Group

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DIAGNOSIS OF TUBERCULOSIS

Claudio D. González

One of the main challenge for Tuberculosis Control Programs (TCPs) is early detection of open forms of the disease. The World Health Organization (WHO) has estimated that the development of a tuberculosis (TB) diagnostic method that offers 85% sensitivity and 95% specificity in sputum samples would allow saving around 400,000 lives per year.[1] Under ideal conditions, it would also be necessary to have an affordable and precise method applicable to the most vulnerable groups which contributes to the identification of the species and its resistance profile, especially in cases that imply a higher risk of therapeutic failure.[1] In the last decade, the development of the GeneXpert MTB/RIF diagnostic system has been a major breakthrough in that regard. At a cost of USD 9.98 per determination (in the 145 subsidized countries), the method helped get closer to the mentioned objectives, that is to say, early detection of TB and detection of resistance to rifampicin, usually considered an indicator of therapeutic failure.[2-4]

Unfortunately, the emergency of the SARS-CoV-2 pandemic impacted negatively on the achievements. Two aspects of TB patients´ care were affected: one, the regular and complete provision of supplies for the diagnosis and treatment of the disease; the other aspect was related to delays and postponement of consultations caused by lockdown measures and social

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distancing, adopted by most countries. Some studies have calculated the direct and indirect impact of the pandemic on the performance of the TCPs through mathematical models.[5]

The purpose of this work was to review the current state of knowledge of valid TB diagnostic methods. To make reading easier, this updating document has been divided into three publications. This first publication includes the diagnostic methods aimed at identifying the causative agent and its sensitivity profile, that is to say, the *bacteriologic or certainty diagnosis*. The second publication will address the methods that evaluate the host response to the bacillus, in other words, the *non-bacteriologic or presumptive diagnosis,* which includes some methods that are still under investigation. The third publication will refer to the diagnosis of TB in children.

BACTERIOLOGIC DIAGNOSIS OF TUBERCULOSIS.

Norberto Símboli

The National TB Diagnostic Laboratory Network is a pyramid organizational structure where each level has specific infrastructure and biosafety requirements defined by the activities and diagnostic methods performed in each laboratory. As the laboratory level increases (1 to 3), the technologies get more innovative; as a result, the personnel need to have more abilities and higher competence, and training requirements increase.[6]

Diagnostic methods are classified based on the three laboratory levels, according to the risk level associated with each procedure, the epidemiological situation of the disease and the available resources .[6] Following such classification, this document has the purpose of reviewing the current state of knowledge about available techniques and offering an initial approach to methods of bacteriologic diagnosis which are still under development. The complexity levels mentioned before are:

1. FIRST LEVEL OF COMPLEXITY

It includes peripheral laboratories located, in some cases, in health centers offering direct test by sputum (DT) through the Ziehl Neelsen (ZN) technique and Kudoh-Ogawa (KO) culture medium.

At present, the GeneXpert MTB-RIF, TB-LAMP (loop-mediated isothermal amplification) and LF-LAM (lateral flow lipoarabinomannan assay) diagnostic methods are being included.[6]

2. SECOND LEVEL OF COMPLEXITY

This level includes laboratories of local or regional hospitals with the capacity to do all level 1 activities plus solid- or liquid-medium cultures, identification of the *Mycobacterium tuberculosis* (*M. tuberculosis*) complex and sensitivity tests (STs) for first-line drugs (isoniazid and rifampicin), plus those with the capacity to perform FL-LPA (*line probe assay* for first-line drugs) and SL-LPA (*line probe assay* for second-line drugs), always from sputum samples with positive bacilloscopy.[6]

3. THIRD LEVEL OF COMPLEXITY

It consists of national or provincial reference laboratories, or specialized laboratories that have the resources to carry out all the studies of the two previous levels plus STs for second-line drugs and complex molecular techniques.

1. FIRST LEVEL OF COMPLEXITY

Direct test (DT)

For some time, countries with limited resources have used the microscopy as the main method to detect *M. tuberculosis*. Although the DT is low-cost and requires minimum biosafety conditions, it has limited sensitivity, especially in patients living with HIV/AIDS and children under 5 years, and it doesn't provide information about the drug resistance profile of bacilli.[7] Despite the fact that the microscopy is not able to differentiate *M. tuberculosis* from other mycobacteria, in countries with TB endemic, the positive bacilloscopy of a respiratory sample from an immunocompetent patient has very high predictive value for TB diagnosis.[7]

ZN staining has been the most widely used technique for TB diagnosis in Latin American countries.[7] Compared to fluorescence microscopy (FM), the conventional microscopy has the advantage that it requires less training, because it is easier to acquire the capacity to identify the bacillus through this methodology. Also, the DT through ZN staining is still a useful resource in our country for TB screening in patients with respiratory

symptoms (RSs), that is to say, people with cough and expectoration for more than two weeks.[7]

In 2011, the WHO (World Health Organization) recommended the use of the FM with LED light. The FM is at least 20% more sensitive than conventional microscopy through ZN.[8] Given that it reduces the time necessary for the reading and requires trained personnel, it is especially recommended for laboratories with heavy workloads. In comparison with conventional FM (with a mercury lamp), the FM with LED light offers considerable operational advantages because it has a long-life span, it doesn't generate heat and doesn't involve environmental pollution risks if it breaks. If a center uses this method instead of ZN staining, it must meet the technical requirements demanded by the WHO and the corresponding external quality monitoring.[8] For the past few years, rapid and sensitive tests have been available; such tests are based on molecular methods to replace or complement the microscopy.

Kudoh-Ogawa culture method

Laboratories without the necessary conditions for culturing through methods that require centrifugation, which are located far away from a reference laboratory or don't have a regular sample transportation system can inoculate the samples with the Kudoh-Ogawa method and send the inoculated tubes to the reference laboratory.[9]

GeneXpert MTB/RIF- MTB/ULTRA- Xpert XDR methods

The development of the Xpert® MTB/RIF assay for the GeneXpert platform was completed in 2009, and is considered an important breakthrough in the fight against TB. For the first time, a molecular test was simple and robust enough to be introduced and used outside the conventional laboratories' environment.[10]

It detects the *Mycobacterium tuberculosis* complex (MTBC) and also the most common mutations that confer resistance to rifampicin using three specific primers and five unique molecular probes to ensure a high degree of specificity. It is a closed automated system of real-time extraction and amplification. It allows the detection of the MTBC in a great amount of clinical samples in 2 hours, with a detection limit of 114 ufc/mL, and reasonable conditions of accessibility, cost and security.[2, 3, 10]

It is a rapid, simple test that can be used in laboratories with minimum infrastructure, and allows for an increased number of detected TB cases, compared to the microscopy. This is favorable in terms of reducing investment in infrastructure and equipment for health services. There are also other benefits for public health, such as the potential reduction in the secondary transmission of resistant strains, an aspect that assumes especial importance within the context of a multidrug-resistant tuberculosis epidemiology (MDRTB) in our country, where the greater MDRTB-generating impulse is associated with strain transmission in the community.[11]

When different studies evaluated the overall pooled sensitivity-specificity of lung samples compared to the DT, it was shown that their performance reached 88% and 99%, respectively.[2-4] In positive sputum DT/culture samples, sensitivity was 98%, whereas in negative DT/positive culture samples, pooled sensitivity was 80%. This performance, replacing the DT as the initial test or in comparison with negative DT samples, would allow 30% detection improvement through bacilloscopy, compared to the ZN technique, and would considerably reduce the time since the beginning of treatment.[12] This performance also includes the group of patients living with HIV, in which it doubles the TB detection rate and reaches a global performance of 79%.[2, 3]

With the same cartridge, the system offers a second use, which is to do the sensitivity test (ST) for rifampicin, reaching a pooled sensitivity-specificity of 95% and 99%, respectively.[2, 3]

On the other hand, in extrapulmonary samples of adults and children, the highest pooled sensitivity-specificity obtained with Gene-Xpert, compared to the culture samples, was found in ganglion samples (84.9% and 94.2%, respectively), followed by gastric lavage and aspirate samples (83.8% and 98.1%, respectively), cerebrospinal fluid (79.5% and 98.6%), and, at last, pleural liquid (43.7% and 98.1% pooled sensitivity and specificity, respectively).[2, 3]

The Xpert® MTB/RIF Ultra has been developed as a new generation assay to overcome the limitations of the Xpert MTB/RIF, and uses the same GeneXpert®platform.[13]

In order to improve sensitivity for detecting the MTBC, the Xpert Ultra incorporates two different multicopy amplification targets (IS6110 and IS1081) and one DNA reaction chamber bigger than Xpert MTB/RIF (PCR [polymerase chain

reaction] of 50 μL in the Ultra versus 25 μL in Xpert MTB/RIF).[13]

It also incorporates nested-like nucleic acid amplification, faster thermal cycling and improved fluids and enzymes. This resulted in the Xpert Ultra having a detection limit of 16 ufc/mL (compared to 114 ufc/mL for the Xpert MTB/RIF). In order to improve the accuracy of rifampicin resistance detection, the Ultra incorporates an analysis based on the melting temperature instead of a real-time PCR. Specifically, four probes identify rifampicin resistance mutations in the determining region of the rpoB gene and move the melting temperature away from the reference value of the wild type.[13]

Investigation of TB with MTB/RIF cartridges has consistently proven to be more sensitive than bacilloscopy. The Ultra version of the Xpert MTB/RIF cartridges is even more sensitive, especially in negative DT samples, positive cultures, and samples of HIV patients, but less specific than the previous version, mostly among patients with history of TB treatment.[9]

Patients with TB and rifampicin resistant TB (RRTB) should promptly do additional tests for detection of resistance at least to isoniazid and fluoroquinolones, respectively, so as to guide treatment decisions.

In 2020, the WHO requested a systematic review of published and unpublished data regarding three classes of nucleic acid amplification tests that hadn't been previously reviewed by that organization.[14] One of them was the new MTB/XDR cartridge, which showed excellent sensitivity and specificity to rapidly detect resistance to isoniazid, fluoroquinolones and aminoglycosides.

Recently, the WHO recommended the use of the cartridge for the rapid detection of mutations that confer resistance to these drugs.[14] This recommendation was based on the analysis of three studies including sputum samples of 1605 participants. This analysis showed that the overall combined sensitivity of this cartridge (95% CI [confidence interval]) for the detection of resistance to isoniazid was 94.2% (89.3% to 97.0%), and the specificity was 98.0% (95.2% to 99.2%). The overall combined sensitivity (95% CI) for the detection of resistance to fluoroquinolones was 93.1% (88.0% to 96.1%), and the specificity was 98.3% (94.5% to 99.5%). The global combined sensitivity (95% CI) for the detection of resistance to amikacin was 89.1% (80.9% to 94.1%), and the specificity was 99.5% (96.9% to 99.9%). The phenotypic ST was used as standard of reference for the three estimations mentioned before. The overall sensitivity (95% CI) for the detection of resistance to ethionamide was 96.4% (92.2% to 98.3%), and the specificity was 100.0% (82.5% to 100.0%). The gene sequencing of the *inhA* promoter region was used as standard of reference for the detection of resistance to ethionamide.[14]

The Xpert system, in all its forms of presentation, can be used in low-complexity laboratories, under the same conditions required to perform a bacilloscopy.

Recommendations for the use of Xpert MTB/Rif and ULTRA:

The WHO recommends the use of the Xpert MTB/RIF and Xpert Ultra as initial tests in adults and children with signs and symptoms of pulmonary and extrapulmonary TB basing on current scientific evidence.[15]

Given the fact that when this consensus was achieved our country had limited access to rapid molecular tests, due to the restricted availability of the Gene-Xpert equipment, this test is *recommended* mainly for the following groups:

I. Adult or pediatric patients with high clinical and epidemiological suspicion of TB and risk of multiresistant TB (strong recommendation).
II. Adult or pediatric patients with high clinical and epidemiological suspicion of TB or MRTB living with HIV (strong recommendation).
III. Adult or pediatric patients with high clinical and epidemiological suspicion of meningeal TB (strong recommendation).
IV. Adult or pediatric patients with high clinical suspicion of extrapulmonary TB (conditional recommendation).[15]

Its use is indicated in the following cases:

a) Respiratory samples of adult or pediatric patients who show signs and symptoms compatible with TB and higher risk of suffering resistant TB: people living with HIV, immunosuppressed patients, healthcare personnel, contacts of patients with RRTB or MRTB, and patients who completed treatment with antituberculous drugs more than 1 year before.
b) Cerebrospinal fluid samples, lymph node aspiration, synovial fluid, pleural liquid, peritoneal fluid, pericardial fluid, urine and biopsies from adult or pediatric patients with high clinical or epidemiological suspicion of extrapulmonary TB.[15]

These recommendations are periodically reviewed, basing on technological development and the existence of scientific evidence that justifies such review.

The use of the Xpert MTB/XDR cartridge is recommended in sputum samples of patents with RRTB.

Truenat MTB, MTB Plus and MTB-Rif DX

These are new molecular methods, developed in India, which may be used on the same laboratory level as the Gene-Xpert. They are based on a real-time micro-PCR that allows for the detection of the MTC and its resistance to rifampicin from a sputum sample in less than 1 hour. The Truenat MTB and the MTB Plus can be used as initial diagnostic tests in adults and children with signs and symptoms of pulmonary TB, whereas the MTB-RIF Dx is used to detect resistance to rifampicin in samples that had positive results in the initial test.[16]

TB-LAMP (loop-mediated isothermal amplification)

TB-LAMP is a commercial molecular assay based on loop-mediated isothermal amplification which requires minimum laboratory infrastructure and biosafety requirements.[17] It has been evaluated as a rapid test (<2 h) at the point of care testing as an alternative to the sputum DT, which is still the primary diagnostic test for pulmonary TB in limited resource environments.

In January 2016, the WHO organized a meeting with the Guideline Development Group (GDG), to review the evidence published from 2012 to that moment.[17] The review included all prospective studies evaluating the TB-LAMP assay in sputum samples of adults with signs and symptoms compatible with pulmonary TB that were carried out in environments with high or intermediate TB burden. In this review, which included twenty studies (4760 adult patients), the TB-LAMP showed a combined sensitivity 15% higher than the DT to detect pulmonary TB in adults (78% versus 63%), though the combined specificity was 2% lower (98% versus 100%). This can partly be explained by the identification of TB cases wrongly classified as negative TB through the use of reference cultures. The evaluation of TB-LAMP accuracy in adults living with HIV with signs and symptoms of pulmonary TB showed sensitivity and specificity percentages similar to those of sputum DTs (64% and 62%) and (99% and 99%), respectively.[17]

In accordance with this evidence evaluation, and taking into account the costs and benefits associated with the use of TB-LAMP, the WHO recommends it for use only in sputum samples in one of these ways:

a) As an alternative to the bacilloscopy for the diagnosis of pulmonary TB in adults with signs and symptoms compatible with TB (conditional recommendation, very low- quality evidence).
b) As additional test, apart from the DT, in adults with signs and symptoms compatible with pulmonary TB, especially in cases of negative sputum DTs (conditional recommendation, very low-quality evidence).

In our country, this method isn't available yet.

LF-LAM (lateral flow lipoarabinomannan assay)

Tests based on the detection of the mycobacterial lipoarabinomannan (LAM) antigen in urine have emerged as potential rapid tests at the point of care testing for the diagnosis of TB.[18] The LAM antigen is a lipopolysaccharide present in the cell walls of mycobacteria, which is released from metabolically active or degenerating bacterial cells and seems to be present only in patients with active TB. This test would be better than sputum-based tests because urine is easy to collect and store and doesn't entail the infection control risks associated with sputum collection.[18]

LAM detection assay in urine through lateral flow immunochromatography is commercially available. The test is done manually, applying 60 μL of urine to the reagent strip and incubating at room temperature for 25 min. Then the strip is inspected visually. The intensity of any of the bands visible in the reagent strip is classified, comparing it with the band intensities of a reference card provided by the manufacturer.[18]

Several studies and meta-analyses of a previous generation test (LAM-ELISA) have shown good sensitivity for detecting urinary LAM in cases of HIV-TB coinfection, and sensitivity increases even more with lower LTCD4$^+$ counts. This finding contrasts with traditional diagnostic methos for TB in patients with HIV. Several hypotheses can explain the higher sensitivity of LAM detection in urine in patients with HIV-associated immunosuppression: higher bacillary and antigen burden, higher probability of having TB in the urogenital tract and higher glomerular

permeability to allow increased levels of antigen in the urine.[18]

Some published studies reported much higher mortality rates in patients with HIV with low LTCD4$^+$ counts who have detectable urinary LAM, compared to individuals with negative LF-LAM results.[18] Given the potential of the assay to help reduce mortality in patients living with HIV and the fact that the test is easy to do and requires minimum biosafety infrastructure, the WHO requested a systematic review of the use of the LF-LAM assay for the diagnosis and detection of active TB in people living with HIV. After that review, the organization made the following *recommendations on the use* of this assay:[15, 18]

a) Except for people with HIV infection who are seriously ill or have low LTCD4$^+$ counts, the LF-LAM SHALL NOT be used for the diagnosis of TB (strong recommendation, low quality evidence).
b) LF-LAM can be used to help diagnose TB in HIV patients with signs and symptoms of TB (pulmonary or extrapulmonary) with a LTCD4$^+$ cell count lower than, equal to or higher than 100 cells/μL, or HIV-positive patients who are seriously ill regardless of their LTCD4$^+$ count, or with an unknown count (conditional recommendation, low quality evidence).

Table 1 summarizes the diagnostic methods related to this level.

2. SECOND LEVEL OF COMPLEXITY

Culture

As we mentioned before, the DT is still the primary diagnostic test for pulmonary TB in limited resource environments.

The culture complements the DT in that it allows us to show viable bacilli present in low amounts in a lesion sample, to characterize them and know whether they are sensitive or resistant to antituberculous drugs. The role of the culture is more important in a context of medium or low incidence of TB, with a high incidence of TB bacillus/HIV coinfection, and medium or high MRTB burden.[19]

Through the culture, it is possible to increase the number of cases with confirmed diagnosis of TB in approximately 15%-20% of the total number of cases, and 20%-30% of the cases with pulmonary TB. If we take into account the total number of cases with a bacteriologically confirmed diagnosis of pulmonary TB, the bacilloscopy detects 70%-80% of the cases, and the culture detects the remaining 20%-30%.[19]

TABLE 1. Diagnostic methods related to the first level of complexity

Method	Indications	Advantages	Limitations
DT through ZN	Patients with respiratory symptoms	Accessibility and low cost. Fast results	Low sensitivity.
DT through FM with LED	Patients with respiratory symptoms Centers with high burden of samples	Higher sensitivity than the ZN technique. Fast results	Requires highly trained staff.
Kudoh-Ogawa culture	Laboratories without suitable equipment	Accessibility and low cost	Recommended only for sputum samples.
XPERT-TB	TB and RRTB diagnosis as initial test	Fast results. High sensitivity to detect MTBC in samples from different origins High sensitivity to detect RRTB	Relative cost. Requires uninterruptible power supply system (UPS) and controlled room temperature
XPERT-TB Ultra	Diagnosis of TB and RRTB as initial test	Higher sensitivity in both functions	Equal limitations
XPERT- MTB/XDR	Diagnosis of MRTB and XDRTB	Provides resistance profile to H, FQ, aminoglycosides and ethionamide	Equal limitations
TB-LAMP	Diagnosis of pulmonary TB as alternative to DT	Fast results. Higher sensitivity than the DT	Not available in our country yet. Recommended only for sputum samples
LF-LAM	Diagnostic complement only in HIV patients with low LTCD4+ counts or severe forms of the disease	Simple and safe	Restricted to immunocompromised patients

DT: direct test. **ZN:** Ziehl-Neelsen technique. **FM:** fluorescence technique. **RRTB:** rifampicin resistant tuberculosis **MTBC:** Mycobacterium tuberculosis complex. UPS: Uninterruptable Power Supply. **MRTB:** multi-drug resistant tuberculosis. **TBXDR:** extensively drug-resistant tuberculosis. **H:** isoniazid. **FQ:** fluoroquinolone. **TB-LAMP:** loop-mediated isothermal amplification technique. **LF-LAM:** lateral flow lipoarabinomannan assay. **HIV:** human immunodeficiency virus. **LTCD4+:** CD4+ lymphocyte count.

The solid-medium culture is still being used as a reference point for the more modern, automated liquid media, and continues to be the reference method compared to other diagnostic systems. The solid medium has the advantage of being low-cost, but takes more time to detect bacterial growth.

Liquid-medium culture methods

The main advantage of these systems, compared to the traditional culture, has to do with their fast results. They use a colorimetric system to inform about bacterial growth (MB Bact Alert) or the detection of consumed oxygen by fluorescence (MGIT 960). For blood and bone marrow samples, the lysis-centrifugation technique for blood cultures is applied.[7]

The biosafety conditions required by methods are different from those of solid media; they can be used on this level of complexity only if such conditions are observed.

Culture indications

a) Given the fact that lesions in children are usually paucibacillary, there is a strong recommendation that all pediatric samples should be cultured, because they increase the DT performance by 20%.[3] The following respiratory samples are indicated, in order of preference: sputum, gastric aspirate in RS children with pathological chest X-ray (Rx), induced sputum, bronchial aspirate and bronchoalveolar lavage; among non-respiratory samples, the content of serous cavities and biopsies.[3]

b) Samples of symptomatic patients with clinical signs, Rx or other images compatible with TB and one of the following characteristics:

- Negative bacilloscopy of three respiratory samples.
- Extrapulmonary localization of the disease.
- Immunosuppressed patients, particularly HIV positive individuals.
- Positive bacilloscopy in gastric lavage, bronchial lavage or swabs.
- History of anti-tuberculous treatment, especially in cases of loss to follow-up or treatment failure.
- Exposure to infection by drug-resistant bacilli (contact with cases of resistant TB, hospitalized patients or workers of health institutions or prisons with registered cases of MRTB).[7, 19]
- To complement rapid diagnostic tests when they are used as the initial diagnostic test.

First-line drugs sensitivity testing (H and R)

Phenotypic tests.

On this level of complexity, the Löwenstein-Jensen medium proportion method (method of Canetti, Rist and Grosset) still provides the well-known simplicity and reliability for which it has been considered the method of reference, compared to molecular-based genotypic methods. It is an economical method, but it has the disadvantage of taking 30 to 40 days to obtain a sensitivity result.

An economical alternative to accelerate results is the nitrate reductase assay (NRA). Ideally, the method shall be used directly with positive DT samples collected at the moment or as soon as the primoculture is developed. This test is supported by the WHO, for being considered an accessible and effective ST for determining resistance to isoniazid and rifampicin.[19]

A more expensive alternative is the use of liquid-medium cultures (MGIT) that accelerate results because they use semi-automated equipment that detects bacterial growth before it is visible.[19]

ST indications

Ideally, all cases with bacteriologically confirmed diagnosis of TB must have access to the ST, at least for drugs that are crucial to treatment success (H and R). Universal access to recommended rapid tests shall be guaranteed (Xpert5, LPAS, etc.). In the process of achieving this objective, the ST should be the priority in cases with the following characteristics, which increase the risk of drug resistance.[7]

a) Treatment failure.
b) History of previous treatment, irregularity in treatment compliance or prescription of an incomplete or inadequate regimen.
c) Exposure to infection by drug-resistant TB.
d) Children.
e) Immunosuppressed patients (people living with HIV and/or diabetes, etc.).
f) Previous residence in countries with a high burden of drug-resistance
(Ecuador, Peru, some Asian and East European countries).
g) Drug or alcohol abuse.

New platforms

New technologies for rapid detection of TB and resistance to rifampicin are becoming more and more available and are being adopted by several countries. Several manufacturers have developed

automated platforms for detecting TB and resistance to rifampicin and isoniazid (Abbott, Becton Dickinson, Roche, Hain Lifescience/Bruker) basing on nucleic acid amplification.[20]

These tests are faster and less complex than the phenotypic drug-sensitivity tests based on cultures and line probe assays (LPA). They have the advantage of being mostly automated, and may be used as the initial test to detect TB and resistance to both first-line drugs simultaneously (rifampicin and isoniazid). They offer fast and accurate results and process a large number of samples; thus, they are adequate for laboratories of medium and high burden of sensitivity tests. So, these technologies are suitable for high-density population areas and fast sample reference systems.[20]

Table 2 summarizes the diagnostic methods related to this level.

3. THIRD LEVEL OF COMPLEXITY

Species identification. First- and second-line drugs sensitivity testing

Phenotypic tests for species identification

Apart from the traditional system used for species identification in solid-medium cultures, there is a lateral flow immunochromatography that identifies the *M. tuberculosis* complex qualitatively from positive liquid-medium cultures. This system detects a protein (MPT64) that is secreted by the bacteria into the culture medium. It is a simple, fast, low-cost, high sensitivity-specificity technique. The disadvantage of this technique is that it can't differentiate the species of that complex, and the results must be contextualized with the clinical information.[19] It can also be used for the identification of MTBC in positive solid-medium cultures.

First- and second-line drugs phenotypic sensitivity testing

Indirect ST through the proportion method using solid media is the most common method to show the sensitivity of *M. tuberculosis* isolates.

The BACTEC-MGIT system is the preferred method for the ST of many antibacillary agents, given the standardization of MGIT media and instruments. This system of automated reading is the most widely used on this level of complexity, because it considerably reduces the time of detection of rifampicin and isoniazid resistance, with 95%-98% sensitivity.[2] The disadvantage regarding detection of resistance to ethambutol, pirazinamide and streptomycin (this drug is no longer used for the treatment, but sometimes it's necessary to include it) lies in its low reproducibility; so, it is

TABLE 2. Diagnostic methods related to the second level of complexity

Method	Indications	Advantages	Limitations
Solid-medium culture	Identification of mycobacteria in areas of medium-low incidence of TB and medium incidence of MRTB. Samples of paucibacillary or negative pulmonary samples by DT. Children. Immunocompromised patients	High-sensitivity. Low-cost	Takes 4-8 weeks to obtain results. Requires strict biosafety measures
Liquid-medium culture	Patients who underwent previous treatment, who have been exposed to cases of MRTB, or those who come from a country with high prevalence of MRTB	Faster and more sensitive	More expensive. Increased risk of pollution; requires strict biosafety measures
Phenotypic STs for HR			
LJ proportions	Therapeutic failure or previous treatment. Children. Immunocompromised patients. Exposure to foci of confirmed MRTB or patients from countries with high-incidence	Low-cost	Takes 3-4 weeks since isolation. Requires strict biosafety measures
NTA	Study of bacterial resistance to RH on DT samples	Simple. Fast results	Requires strict biosafety measures Relative cost
MGIT	Study of bacterial resistance to RH in culture samples	High-sensitivity. Fast results	Requires strict biosafety measures

MRTB: multi-drug resistant tuberculosis; **DT:** direct test; PS: sensitivity tests; **R:** rifampicin; **H:** isoniazid; **LJ:** Löwenstein-Jensen medium; **NTA:** nitrilotriacetate assay; **MGIT:** mycobacteria growth indicator tube.

used for national reference laboratories that have other methods to confirm results related to these drugs. Indications were already described before.

Genotypic sensitivity testing (line probe assay, LPA)

The amplification and detection of the nucleic acids of the *M. tuberculosis* complex is a technology that has proven to be very sensitive and specific. Some amplification techniques have the advantage of being able to detect resistance to certain antituberculous drugs.[21]

Real-time PCR applied to some tools is the most widely used technique at present. These tools detect the DNA of the *Mycobacterium tuberculosis* complex and distinguish gene mutations related to drug resistance. Generally speaking, they have a considerable cost and require staff training in order to meet the requirements of international standards and external audits.[19, 21]

The LPAs are a group of tests based on multiplex PCR and strip-based reverse hybridization. They amplify gene segments where the most frequent mutations that originate resistance are produced.[21]

They also amplify a specific segment of the *M. tuberculosis* complex, so it is also possible to detect the complex. The resistance of *M. tuberculosis* rifampicin and isoniazid-resistant isolates, which is 5% and 15% , respectively, may not be detected by these systems because they have genetic alterations in regions that are not covered by them.

The LPAs are technically more complex than the Xpert MTB/RIF-ULTRA or XDR assays; however, they can also detect resistance to a variety of first- and second-line agents (for example, isoniazid, fluoroquinolones and injectable drugs), and their results can be obtained in 24 hours.

There are two large groups of assays:[21]

- Those which detect MTC and resistance to first-line antituberculous agents (known as first-line LPA [FL-LPA]), as for example, GenoType MTBDRplus v1 and v2, Genoscholar NTM + MDRTB II.
- Those which detect resistance to second-line antituberculous agents (known as second-line LPA [SL-LPA]), as for example, GenoType MTBDRsl.

The use of the LPA to detect resistance doesn't eliminate the need to do a conventional culture and the phenotypic ST, since they have a critical role in the follow-up of treatment response and the detection of additional resistance to other drugs.[7]

In general, the LPAs can't be used as the initial diagnostic test as a replacement for the DT because they have limited sensitivity and have to be done in laboratories with a specific level of complexity.[21]

Given the increasing incidence of MRTB, the LPA system has been evaluated to detect or discard MRTB and extensively-drug resistant tuberculosis (XDRTB).

Other molecular techniques for the identification of species or their clonality

The next-generation sequencing (NGS) has great potential as a method for the rapid diagnosis of drug-resistant tuberculosis (DRTB) in various environments of clinical reference laboratories throughout the world.[22]

The NGS approach overcomes many of the important challenges associated with conventional phenotypic tests, as well as the limitations of other less complete molecular tests, by providing fast and detailed information of sequences for multiple gene regions or complete relevant genomes. However, the use of these technologies for the diagnosis of DRTB has been obstructed due to elevated costs, integration in the laboratory workflow, technical training requirements necessary to use the technology, and the need of expert guidance for clinical data management and interpretation.[22]

Other complex diagnostic methods aim at knowing about the transmission of the disease within the community; this can be achieved by identifying the clonality of species through molecular techniques, such as RFLP (restriction fragment length polymorphism) or by whole genome sequencing techniques (WGS). In all cases, its use is restricted to central reference laboratories.[22]

Table 3 summarizes the diagnostic methods related to this level of complexity.

CONCLUSIONS

The strategy and goals for the prevention, care, and control of tuberculosis after 2015, briefly called "An end to TB", approved by the 67th World Health Assembly in May 2014 through resolution WHA 67.1, and launched by the WHO, proposes a TB control approach that goes beyond the health-care sector. It takes into account biological factors and socio-economic conditions that define which are the populations with higher risk of suffering

TABLE 3. Diagnostic methods related to the third level of complexity

Method	Indications	Advantages	Limitations
Phenotypic tests for species identification. LF	Positive liquid or solid cultures.	Fast results. Simple. High sensitivity and specificity	They don't identify species in the MTC
First-line drugs phenotypic STs	The same indications as those of the second level of complexity. The MGIT is the most widely used system for the detection of resistance to R and H	Fast results. Simple. High sensitivity and specificity. Gold standard methodology	Low reproducibility for certain drugs such as E, Z and S
First- and second-line drugs phenotypic STs GenoType MTBDRplus and GenoType MTBDRsl	. Need to rapidly detect drug resistance	Fast results. Detects MRTB and RRTB.	Elevated cost. Staff training. They don't eliminate the need to do cultures and conventional STs
Other genotypic tests for species identification NGS	Next-generation sequencing for the identification of mutations	Fast results. Very detailed information about sequences	Elevated cost. Staff training. Interpretation of results
Genotypic clonality tests RFLP and WGS	Study of strain transmission within a community	Fast results. Very detailed information about sequences	Elevated cost. Staff training. Restricted to reference centers

MTC: *Mycobacterium tuberculosis* complex; **LF:** lateral flow immunochromatography; **MGIT:** mycobacteria growth indicator tube; **R:** rifampicin; **H:** isoniazid; **E:** ethambutol; **Z:** pyrazinamide; **S:** streptomycin; **MDRTB:** multi-drug resistant tuberculosis; **TBXDR:** extensively drug-resistant tuberculosis; **ST:** sensitivity test; **NGS:** next-generation sequence; **RFLP:** restriction fragment length polymorphism; **WGS:** whole genome sequencing.

TB, as well as the strengths of the research on new vaccines, diagnostic methods, and drugs that will make way for the elimination of this disease.

Recent discoveries about the diagnosis of TB provide an opportunity to improve the capacity of the laboratories to reach an early and accurate diagnosis of sensitive and resistant TB. One of the most important elements for adopting these new technologies is the existence of diagnostic policies that incorporate these techniques in the diagnostic algorithms and establish training plans and external evaluations of the quality of said techniques.

Conflict of interest

The authors of this work have no conflicts of interest to declare**.**

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