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Editorial

Current microbiological diagnosis of tuberculosis[☆]



Diagnóstico microbiológico actual de la tuberculosis

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Tuberculosis (TB) continues to be one of the most significant infectious diseases to devastate mankind.¹ According to the World Health Organization (WHO), in 2015 there were 10.4 million new cases of active TB, and 1.8 million people died from this disease.¹ Furthermore, it was estimated that 480,000 people developed multidrug-resistant tuberculosis (MDR-TB; resistance to at least rifampicin and isoniazid), and around a further 100,000 people had rifampicin-resistant TB in that year. Although TB mainly affects countries with limited economic resources, large movements of migrants are affecting the world's richest countries. Delayed diagnosis, both of the disease and of multidrug-resistant TB, is one of the biggest obstacles for controlling it globally.¹

From the beginning, the microbiological diagnosis of TB has been based on conventional methods, such as microscopy, culture and subsequent phenotypic identification. The quickest, simplest and cheapest method available is bacilloscopy, which is based on the direct observation of acid-alcohol resistant bacilli using different stains (Ziehl–Neelsen and Auramine).^{2,3} However, its limited overall sensitivity (22–80% of positive cultures) has restricted its diagnostic capacity, particularly in geographic areas where the incidence of TB is low and also in extrapulmonary (paucibacillary) forms of the disease.^{3,4} In addition, although bacilloscopy is used as a marker of the contagious capacity of the patient with tuberculosis, it has been observed that up to 17% of the transmission is due to patients with a negative bacilloscopy and a positive culture.⁵ Furthermore, despite being a test with good specificity ($\geq 90\%$), in those areas with the greatest incidence of clinical isolation of nontuberculous mycobacteria (NTM), bacilloscopy has a low positive predictive value ($\leq 70\%$).^{2–4} Therefore, bacilloscopy has limited usefulness in Spain, as Spain is currently included in the group of countries with a low incidence of tuberculosis (10.8 cases per 100,000 inhabitants per year). This is in contrast with

a growing rise in NTM infections.^{6,7} Unlike microscopy, culture remains the reference method due to its good sensitivity, and the fact that it enables the diagnosis of the majority of the 185 species of the *Mycobacterium* genus which are currently described. It also enables subsequent studies with the isolated mycobacteria (identification, sensitivity and epidemiological classification) to be carried out.^{2–4} However, the greatest drawback of culture is the slowness of the tubercle bacillus growth, which delays diagnosis of the disease. Although culture has greatly developed over the last two decades, through new resources and automated non-radiometric systems, such as MB/BacT ALERT[®] (bioMérieux, Marcy-l'Etoile, France), MGIT 960[®] (Becton Dickinson Diagnostics, Sparks, MD, USA) and VersaTREK[®] (Trek Diagnostic Systems, Westlake, USA), it still takes several weeks to reach the definitive microbiological confirmation, and even longer with phenotypic identification procedures.^{2,8} Therefore, in recent years, various strategies have been proposed to obtain a rapid diagnosis of active TB. These are quite varied and are based on the improvement of conventional techniques, the use of genotypic and proteomic methods, or even on mycobacteriophages. Furthermore, it is essential to differentiate the level of application, whether it is from a microorganism grown in a culture or grown directly from clinical samples. In the first case, the diagnosis is somewhat more delayed, but with a greater methodological variety and a better overall sensitivity. One of these possibilities incorporated in recent years is the immunochromatographic detection of the production of MPT64 antigen by the microorganism. It is a simple and very rapid technique, with hardly any manipulation, which is applied in cultures grown both in liquids and in solids, for the identification of the *Mycobacterium tuberculosis* complex.^{9,10} There are various commercial products (SD BIOLINE TB Ag MPT64 Rapid Test[®] kit, Standard Diagnostics Inc., Yongin, Korea; Capilia TB[®], TAUNS Laboratories Inc., Numazu, Japan; BD MGIT TBc identification test[®], Becton Dickinson Diagnostics) which have a specificity of greater than 99.5% and a sensitivity close to 98.5%. This has been determined because some strains of the complex (as has been seen in some *Mycobacterium bovis* BCG) do not manage to produce this protein due to mutations, deletions or insertions in the gene which codes for the MPT64 antigen.^{9,10} Molecular methods

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from grown cultures offer another technical possibility with significant potential. Chemiluminescent DNA probes were developed last century in the 1990s. These probes rapidly and specifically identify, by hybridisation with the mycobacterium's ribosomal RNA and, without previous amplification, the *M. tuberculosis* complex and that of some other relevant species.^{2,8} However, the probes are not available for all pathogenic species and require an initial orientation for the selection of the appropriate probe, they do not detect mixed cultures and, in some cases, there are minor specificity problems, such as those described for *M. tuberculosis* with *Mycobacterium terrae* and *Mycobacterium celatum*. Nevertheless, these probes have been a reference method in combination with the automated culture systems for a long time, obtaining a sensitivity and specificity of over 99% for the *M. tuberculosis* complex.^{2,8} Currently, the most used methods are based on the amplification of specific DNA sequences and a subsequent post-amplification analysis that may fundamentally consist of: (a) the sequencing of genes *hsp65*, *rpoB*, *gyrB* and/or 16S rDNA, which is usually reserved as a reference method for the identification of NTM when other routine methods have not been effective; and (b) solid-phase hybridisation, for which there are multiple commercial formats (MicroWell plates, nitrocellulose strips and DNA chips/arrays). Of these, the most widely used are the commercial products based on the amplification of a specific genetic area of the 16S-23S intergenic spacer region (INNO-LiPA[®], Innogenetics NV, Ghent, Belgium; Speed-Oligo[®]; Viracell, Granada, Spain) and the 23S rDNA (GenoType[®], Hain Lifescience, Nehren, Germany) and subsequent hybridisation of the amplified product on the different immobilised probes on a nitrocellulose strip. These are easy to read and interpret. It has been observed in multiple studies that these systems have a good sensitivity and specificity from both liquid and solid cultures, obtaining the results in one working day.^{2,8,11} By hybridising multiple areas simultaneously, these methods enable the detection of possible co-infections by diverse species in one single sample. In addition, a wide variety of strips have been developed, with the possibility of identifying the different species of the *M. tuberculosis* complex. This is of great epidemiological and, in particular, therapeutic usefulness, as not all species of this complex have the same sensitivity to anti-tuberculosis drugs, as in the case of *M. bovis* which is naturally resistant to pyrazinamide. Furthermore, cases of patients with superficial bladder cancer are noteworthy. Endocavity treatments are performed in these patients using instillations of *M. bovis*-BCG to reduce the number of recurrences and improve the disease-free interval. In these cases, it is necessary to monitor the presence of this mycobacterial species in urine.

Within the diagnosis of tuberculosis from a positive culture, the option of doing a proteomic study using MALDI-TOF MS has recently become possible. Although this system identifies the *M. tuberculosis* complex, it is still not capable of distinguishing between the different species that it contains, and requires a very well grown culture, especially when it is in a liquid medium. This has meant that its interest has focused on the identification of NTMs, although the possibility of it becoming a rapid, simple and cheap method for the diagnosis of tuberculosis from cultures, and even liquids, in the very near future is not being ruled out.¹²

In general, the most relevant aspect in the control of tuberculosis is the rapid diagnosis, thus managing to break the chain of transmission (by the isolation, treatment and study of contacts). This prevents multiple unsuccessful diagnostic tests, and even prolonged hospital stays in certain cases. For this purpose, it is necessary to work *directly on the clinical sample* without having to wait for the culture. One possibility is the application of bacteriophages with specific affinity for mycobacteria. Although they are simple and rapid (48 h) techniques which require little training and technical equipment, they have not shown sufficient sensitivity in

the majority of studies conducted for their implementation in clinical microbiology laboratories.^{8,11} The best alternative for detecting and identifying the *M. tuberculosis* complex, directly in the clinical sample, is to use molecular methods based on nucleic acid amplification (NAA). In the last two decades many molecular techniques have been developed, with commercial techniques being the most accepted and recommended, especially for issues of standardisation and quality control. In addition, it is recommended that these tests are performed strictly following the manufacturer's instructions, as well as the automated extraction of nucleic acids in order to prevent cross contamination, amongst other things. All these techniques are based on the amplification of specific genetic (DNA or RNA) sequences of the *M. tuberculosis* complex and its subsequent detection by hybridisation, which does not necessarily imply the viability of the mycobacteria. Despite the large quantity of evaluations performed, it has been difficult to obtain solid and conclusive scientific evidence on this issue over the years. This is due to the great heterogeneity of the studies and the lack of a good microbiological reference method (Gold Standard), as the method continues to be culture, which has a lower theoretical sensitivity than the NAA methods. However, it is known that these methods are very rapid, are becoming increasingly simpler and have a greater positive predictive value (PPV) than microscopy (>95%), and in particular in those geographic areas where there is a significant number of NTM isolations.¹³ Although there is a wide variety and quality of techniques, the current recommendations are confined to very few commercial methods. In fact, the CDC (Centers for Disease Control and Prevention) and the FDA (Food and Drug Administration), of the United States of America, recommended the AMTD[®] (Amplified *M. tuberculosis* Direct Test; Gen-Probe Inc., San Diego, CA, USA) and the Amplicor *M. tuberculosis* test[®] (Roche Diagnostic Systems Inc., Basel, Switzerland) in 1995 and 1996, respectively, for the rapid diagnosis of tuberculosis.^{11,13} The AMTD[®] is an isothermal method (42 °C) based on the amplification of 16S rRNA and a subsequent inverse transcription and hybridisation, while Amplicor is based on a conventional PCR of a specific fragment of 16S rRNA and subsequent hybridisation. Despite the fact that other good methods have been developed, including the BD Probe Tec ET Direct TB System[®] (Becton Dickinson Diagnostics), TB-LAMP[®] (Eiken Chemical Company Ltd, Japan), GenoQuick MTB[®] (Hain Lifescience), Fluorotype MTB[®] (Hain Lifescience), Abbott Real Time MTB[®] (Abbott, IL, USA) and the Anyplex plus MTB/NTM/DR-TB[®] (Seegene Inc., Seoul, Korea), amongst others, the GeneXpert MTB/RIF[®] (Cepheid, Sunnyvale, CA, USA and FIND Diagnostics, Geneva, Switzerland) was presented in 2010. It has become the most used method and is currently recommended by various international organisations.^{14–16} It is a semi-quantitative nested real-time PCR where the extraction of DNA, amplification of a specific region of the gene *rpoB* and simultaneous hybridisation with fluorogenic probes is performed in an automated manner, and is integrated into a cartridge. It is a rapid technique which detects, in less than 2 h and with hardly any manipulation, the presence of the *M. tuberculosis* complex and the mutations most frequently related to resistance to rifampicin in this microorganism (see below).^{14,16} Since the appearance of all these NAA methods for the diagnosis of TB, their indications for use have been evolving and expanding. It is currently recommended to use them (with low-quality evidence) in at least one respiratory sample (preferably the first one) of patients with signs and symptoms of pulmonary TB, but without a definitive diagnosis, and when the result of the test can influence the treatment and actions required to control the TB.^{4,13–16} When the bacilloscopy is positive and the NAA test is negative, the diagnosis of pulmonary TB is unlikely.^{4,13} However, when the bacilloscopy is negative and the clinical suspicion is high or intermediate, a positive NAA test presumptively suggests the diagnosis of pulmonary TB pending the culture.^{4,13} In the case of extrapulmonary TB, using NAA techniques (with very

low-quality evidence) is recommended, taking into account that if these are positive there is a high probability of suffering from extrapulmonary TB, as false positives are very rare.⁴ Furthermore, the indications for these techniques have been extended to the paediatric population and also for the detection of resistance to rifampicin and, therefore, of MDR-TB.^{4,14,16} In general, tuberculosis cannot be ruled out when these molecular tests are negative.^{4,13} Therefore, in the traditional way in the different guidelines and papers, it has been pointed out that these techniques should not be used routinely when the clinical suspicion of TB is low, as the PPV is less than 50%.^{4,13} As it is a methodology which does not replace other diagnostic techniques, but is instead complementary (with an important added value) to the standard methodology, the intrinsic cost of the NAA techniques has been a much-debated and controversial topic. However, in recent years, it has been observed that its routine application in patients with suspected TB enables an overall significant potential saving. It has been quite noticeable in areas with a high incidence of TB and even of MDR-TB.¹⁷ In this regard, the paper by Herráez et al. published in this issue of *Enfermedades Infecciosas y Microbiología Clínica* [Infectious Diseases and Clinical Microbiology], discusses an interesting study on cost-effectiveness in the diagnosis of TB using the GeneXpert MTB/RIF[®] system.¹⁸ This paper analyses the economic and health impact of three strategies of microbiological diagnosis of TB, comparing the classic standard method of the Centre in which it is carried out, with two alternative situations in which the bacilloscopy was negative, but in one situation patients with a high clinical suspicion of suffering from TB were selected, and in the other all cases were included. It was observed that the introduction of the GeneXpert was more cost-effective than the conventional procedure. The second alternative of application in all cases was particularly cost-effective with a 70% reduction in hospital stays and a 75% reduction in days without specific treatment. This is on top of an economic saving of 1.8 million euros annually, provided that TB patients are admitted to hospital under standard clinical practice, for 2–3 weeks depending on the case. The importance of this study lies in the fact that it was conducted in an area of low TB incidence, as in a previous study in Spain, in which it was observed that the introduction of the GeneXpert in patients with clinically suspected TB and negative bacilloscopy may reduce costs derived from hospitalisation and subsequent diagnostic tests. The time elapsed until tuberculosis treatment is started may also be reduced.¹⁹ Likewise in Germany, which has a low prevalence of TB, it was observed in a recent study that the routine introduction of the GeneXpert, complementing or replacing bacilloscopy, could significantly reduce the costs derived from TB diagnosis.²⁰

Other possibilities for the rapid diagnosis of TB would be the use of biomarkers at the site of patient care (point of care). This is a field which is still undergoing development where the T-cell activation marker (TAM-TB), volatile organic compounds or lipoarabinomannan (LAM) seem to be those of greatest interest.⁸ The latter has already been marketed for the detection of LAM in urine (LF-LAM, Alere DetermineTM TB LAM Ag[®], Alere Inc., Waltham, MA., USA), although at the moment its sensitivity is rather low for general implementation.

One of the most relevant and specific aspects in the rapid diagnosis of this disease is TB which is resistant to antimicrobials, and in particular MDR-TB due to its severe clinical and epidemiological implications. Molecular methods are based on the detection of mutations in the chromosomal targets most frequently related to the multidrug-resistance phenotype.^{11,21} There are currently several diverse commercial products based on PCR and hybridisation on nitrocellulose strips, such as the INNO-LiPA[®] (Innogenetics) and GenoType[®] (Hain Lifescience), which can detect multi-resistance from a culture or from a clinical sample with excellent sensitivity and specificity.^{11,21} Furthermore, the GenoType[®] system (Hain

Lifescience) has developed other strips for the genotypic study of resistance to other drugs, such as second-line injectables, fluoroquinolones and ethambutol. Although there is no totally unanimous consensus, since 2008 the WHO has endorsed the use of these commercial molecular systems in sputum samples with positive bacilloscopy or culture isolations in patients with a high risk of suffering from MDR-TB or XDR-TB.²¹ However, these methods do not replace conducting phenotypic sensitivity tests to anti-tuberculosis drugs.

In summary, molecular biology techniques have become a fundamental methodology in the rapid diagnosis of active pulmonary and extrapulmonary TB, and of the severe cases of multi-resistance with an excellent cost-effectiveness, even in geographic areas with a low prevalence of the disease.

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