Mycobacterium tuberculosis is the single most deadly microorganism worldwide. A third of the world population is thought to have latent tuberculosis and approximately 2 million people die of the disease each year. Short and closely supervised treatment regimens are needed, but it is also essential to develop new strategies to ensure prompt diagnosis of the disease. In particular, cheap methods are needed to tackle tuberculosis from a population perspective. The present article reviews the advances in immunology and molecular strategies for epidemiological diagnosis and monitoring of tuberculosis patients.

Key words: Mycobacterium tuberculosis. Mimotopes. MIRU-VNTR. Hsp65. IS6110. Diagnosis.
8 million new cases of tuberculosis and 2 million people die of the disease each year. Although widespread availability of the bacillus Calmette–Guerin (BCG) tuberculosis vaccine, the incidence of the disease has increased. Moreover, the effectiveness of this vaccine has been questioned, although vaccinated individuals are less likely to develop extrapulmonary forms of the disease, and children in particular are afforded protection against meningeal tuberculosis.

In Mexico, according to official health ministry figures, tuberculosis is the 17th most common cause of death among the general working population. Nevertheless, as in the rest of the world, M. tuberculosis is the single most deadly microorganism. In Mexico, 7257 new cases of pulmonary tuberculosis and 76 new cases of meningeal tuberculosis had been reported through to week 28 of 2006, the World Health Organization reports a prevalence of 53 cases per 100 000 inhabitants throughout the Americas.

Traditionally, pulmonary tuberculosis is clinically suspected in individuals with chronic cough and radiological evidence of pulmonary lesions, which are most frequently located in the apical region of the right lung. Once all the clinical criteria have been assessed, diagnostic procedures can be performed to confirm the diagnosis. These laboratory tests consist of sputum smears to assess whether acid-fast bacilli are present. Definitive diagnosis is made by culturing and identifying the infectious species from bronchial secretions of the patient. A wide range of diagnostic strategies of varying diagnostic value are currently available (Table 1). Even without including the time necessary to test for sensitivity to antimicrobials, microbiological diagnosis of tuberculosis requires 6 to 8 weeks—a long delay before starting treatment with antituberculosis agents. On the other hand, prescription of drugs without waiting for the in vitro sensitivity results and lack of treatment compliance have favored the appearance of multidrug-resistant strains of M. tuberculosis. It is also important to note that factors other than diagnosis of infection, such as lack of access to health services and prompt medical care, also undermine attempts to control the pandemic.

Short and closely supervised treatment regimens are needed, but it is also essential to develop new strategies to ensure the disease is diagnosed promptly. In particular, cheap methods are needed to tackle tuberculosis from a population perspective. Below, we present the most promising strategies being developed for diagnosis and epidemiological surveillance of tuberculosis. These can be classified as: a) immunological methods that detect circulating antibodies in the patient (serological diagnosis), and b) molecular methods that aim to identify and genotype clinical isolates of M. tuberculosis.

**Immunological Methods**

The immunological methods most commonly studied for diagnosis of tuberculosis are based on detecting serum antibodies against mycobacterial antigens, which can be proteins, lipids, or polysaccharides. Alternatively, specific mycobacterial antigens can be identified in secretions of patients using ex vivo generated monoclonal antibodies.

**Tests Based on Antibody Detection**

Analyses based on detection of antibodies against M. tuberculosis are an important alternative to traditional methods for diagnosis of active tuberculosis because they can detect immune response induced during infection. This response is either not present or reduced in asymptomatic infected subjects (that is, those with latent tuberculosis). Furthermore, the type of immunoglobulin (Ig) detected (IgG or IgM) could indicate whether the infectious process is progressing or not. Serologic methods also have the advantage of being easy to do, cheap, and noninvasive, but they are limited in that there is a lack of highly specific and sensitive antigens, as discussed below.

In the enzyme-linked immunosorbent assay (ELISA), or a simplified form thereof (Dot-ELISA), M tuberculosis antigens are adsorbed onto a solid phase where they capture specific mycobacterial antibodies from serum samples. Antibody capture is detected with a second antibody conjugated to an enzyme (often peroxidase), which reacts with its substrate (hydrogen peroxide in the case of peroxidase) so indicating when antibody-antibody reaction has taken place. Unfortunately, this test cannot be used with whole protein extract from the bacillus in systematically vaccinated populations (such as in Mexico) because cross reactions occur with antigens in the vaccine strain (M bovis-BCG) and sometimes even with saprophytic mycobacteria found in abundance in the environment.

**Protein Antibodies**

Antigens purified from M tuberculosis have been used as an alternative to limit cross reactivity. However, the humoral response to most of the immunodominant antigens, although strong, varies greatly among tuberculosis patients, and so no antigen is systematically recognized by everyone. A sensitivity of around 80% in patients with positive sputum smears can be achieved using the 38-kDa protein, which is one of the most promising antigens for serodiagnosis. For patients with negative sputum smears, however, the sensitivity is as low as 15%. Thus, one of the main challenges is to design a strategy for rapid diagnosis of pulmonary tuberculosis in patients with...

**TABLE 1**

<table>
<thead>
<tr>
<th>Test</th>
<th>Time Required</th>
<th>Main Drawbacks</th>
<th>Diagnostic Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum smear</td>
<td>3 days (3 samples)</td>
<td>Low sensitivity and no specificity</td>
<td>No</td>
</tr>
<tr>
<td>Conventional culture</td>
<td>4-6 weeks</td>
<td>Needs time</td>
<td>Yes</td>
</tr>
<tr>
<td>Polymerase chain reaction</td>
<td>3-4 h</td>
<td>High cost</td>
<td>Yes</td>
</tr>
<tr>
<td>Immunodiagnosis</td>
<td>4-6 h</td>
<td>Lack of suitable antigens</td>
<td>No</td>
</tr>
</tbody>
</table>

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negative results for acid-fast bacilli and for diagnosis of extrapulmonary tuberculosis, for which sputum smears are not useful. Table 2 shows the main protein antigens used for diagnosis.16-25

To increase the sensitivity and specificity attainable with pure antigens, it has been proposed to use a mixture of different immunodominant antigens to cover the whole range of possible responses by all individuals in a population. A sensitivity of 90% was achieved with a battery of 9 antigens (ESAT6, 14 kDa, MPT63, 19 kDa, MPT64, MPT61, MTC28, 30 kDa, 38 kDa, and KatG).14

One of the advantages of using protein antigens for immunodiagnosis is that they can be prepared using recombinant DNA technology. Such methods of preparation allow large-scale expression and purification with the corresponding reduction in cost of the test. One of the most innovative recombinant antigens tested for immunodiagnosis of tuberculosis is a recombinant multiepitope polyprotein (TbF6), which is expressed in Escherichia coli as a fusion protein, and which contains antigenic regions of Mtb8, 38 kDa, Mtb11, and Mtb48. With this recombinant antigen, a sensitivity of 94% was achieved.25,26

However, protein antigens are not the only candidates for use in immunodiagnosis. The cell wall of M tuberculosis is extremely rich in lipids, glycolipids, and polysaccharides, and a humoral immune response has been demonstrated against all these components during infection.27,28

One of the advantages of using lipid antigens is that the humoral response is more uniform than with protein antigens.

**Polysaccharide Antibodies**

Lipoarabinomannan (LAM) and its biosynthetic precursors, phosphatidyl-myo-inositol mannosides, are also abundant in the cell wall of M tuberculosis. Likewise, it has been shown that IgM and IgG antibodies are raised against these glycoconjugates during active infection.34,35 It is particularly important to highlight that although LAM is widely distributed in different mycobacterial species, the type of capping differs for pathogenic and saprophytic species.36 The slow-growing pathogenic species (M tuberculosis, M bovis, M avium, Mleprae, etc) are capped with mannose residues (denoted manLAM), whereas fast-growing saprophytic species (Mycobacterium fortuitum, Mycobacterium smegmatis, etc) are either capped with arabinose residues (denoted araLAM) or are uncapped. It is therefore possible that the humoral response to LAM of the pathogenic mycobacteria may differ from that generated through contact with

### TABLE 2

<table>
<thead>
<tr>
<th>Antigen Sensitivity Specificity Application Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESAT6 27% Latent TB Silva et al 15</td>
</tr>
<tr>
<td>CFP10 25% 97% TB AFB- Dillon et al 17</td>
</tr>
<tr>
<td>16 kDa 57% Pulmonary TB Uma Devi et al 18</td>
</tr>
<tr>
<td>30 kDa 61% 95% Pulmonary TB Espitia et al 20</td>
</tr>
<tr>
<td>Mtb68 63% 90% TB HIV+ Ramalingam et al 21</td>
</tr>
<tr>
<td>TB16.3 66% 93% TB HIV+ Lodes et al 22</td>
</tr>
<tr>
<td>Mtb61 88% 98% TB HIV+ Welding et al 23</td>
</tr>
<tr>
<td>U1 (21 kDa) 70% 99% TB HIV+ Hendrickson et al 24</td>
</tr>
</tbody>
</table>

*AFB indicates acid-fast bacilli; TB, tuberculosis; HIV, human immunodeficiency virus.

### TABLE 3

<table>
<thead>
<tr>
<th>Antigen Sensitivity Specificity Application Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacyl trehalose 81%-88% 96%-99% Pulmonary and extrapulmonary TB Escamilla et al 25</td>
</tr>
<tr>
<td>Triacyl trehalose 91%-93% 96%-98% Pulmonary TB Escamilla et al 25</td>
</tr>
<tr>
<td>Trehalose dimycolate 69%-74% 95%-99% Pulmonary TB López-Marín et al 25</td>
</tr>
<tr>
<td>Sulfolipids (sulfolipid-1) 81% 77% Pulmonary TB Julió et al 25</td>
</tr>
</tbody>
</table>

*TB indicates tuberculosis.
has the drawback that lipids and polysaccharides as antigens for serodiagnosis specificity for diagnosis of tuberculosis.\(^3^9\)-\(^4^3\) A very on antigen capture can achieve higher sensitivity and As shown in Table 5, the immunological methods based LAM, followed by certain mycobacterial secretion proteins. the most widely used antigen in immunoassays is once again through use of highly specific monoclonal antibodies. The pathogen in serum and other body fluids, particularly urine, tuberculosis is detection of specific antigens for the important advantage of methods based on antigen detection is that they can be used for diagnosis of extrapulmonary tuberculosis.

### Peptide Mimotopes of Nonprotein Antigens

Glycolipids and polysaccharides in the cell wall of \(M\) \(tuberculosis\) are excellent alternative antigens for serodiagnosis. However, they are limited in that they cannot be produced by recombinant techniques because they are secondary gene products and, furthermore, the metabolic pathways that synthesize many of them have yet to be elucidated. Nevertheless, small peptides of \(7\) to \(15\) amino acid residues have long been known to mimic the 3-dimensional structure of some epitopes of polysaccharides.\(^4^4\) \(^4^5\) This means that such peptides, known as mimotopes because they mimic epitopes, can be used instead of complex lipids and polysaccharides, which cannot be synthesized chemically or generated by recombinant techniques. Currently, several systems are available commercially. These systems use a combinatorial peptide library expressed on filamentous phage displays to screen for sequences that are able to mimic the conformation of antigen determinants of complex molecules. Of the mimotopes that have been generated to mimic complex mycobacterial cell wall structures, the most noteworthy are those produced for antigen determinants of LAM for \(M\) \(tuberculosis\) and phenolic glycolipid-1 for \(M\) \(leprae\) (Table 4).\(^4^6\) \(^4^7\) Investigators have yet to explore the potential of any of these mimotopes for serodiagnosis.

### Other Tests

In many countries, particularly those where serodiagnosis is complicated by extensive cross reactions due to systematic vaccination programs or a high rate of infection with saprophytic mycobacteria found in the environment, methods based on the in vitro cell immune response to purified protein derivative (PPD) have started to be used. In these methods, the mononuclear cells of patients with a clinical diagnosis of tuberculosis are stimulated with PPD or with specific antigens (particularly \(ESAT6\) and \(CFP10\)), and cell proliferation and production of interferon-\(\gamma\) are assessed.\(^4^8\) \(^4^9\) The QuantiFERON-TB Test (Cellestis Ltd, Carnegie, Victoria, Australia) recently approved by the US Food and Drug Administration for the diagnosis of tuberculosis, is based on this system. A variation on quantification of in vitro production of interferon-\(\gamma\) is direct quantification of interferon-\(\gamma\)-producing cells with an enzyme-linked immunospot assay. Unfortunately, the need for in vitro culture of the cells of each patient pushes up the cost of diagnosis and limits its use in population studies.

### Molecular Methods

Although immunological methods, particularly serologic ones, are reasonably simple to use in population studies, they cannot provide information on the species or strain responsible for infection. Therefore, the preferred approach to epidemiological surveillance has been genotyping by

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Application</th>
<th>Sample</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>38 kDa</td>
<td>100%</td>
<td>100%</td>
<td>Meningeal TB</td>
<td>CSF</td>
<td>Sumi et al(^4^9)</td>
</tr>
<tr>
<td>43 kDa</td>
<td>96.7%</td>
<td>100%</td>
<td>Pulmonary TB</td>
<td>Pleural fluid</td>
<td>Wadler et al(^4^9)</td>
</tr>
<tr>
<td>LAM</td>
<td>96%</td>
<td>100%</td>
<td>Meningeal TB</td>
<td>CSF</td>
<td>Wadler et al(^4^9)</td>
</tr>
<tr>
<td>LAM</td>
<td>67%-88%</td>
<td>100%</td>
<td>Pulmonary TB</td>
<td>Urine</td>
<td>Tesema et al(^4^9)</td>
</tr>
<tr>
<td>LAM</td>
<td>94%</td>
<td>100%</td>
<td>Pulmonary TB</td>
<td>Serum</td>
<td>Sada et al(^4^9)</td>
</tr>
<tr>
<td>LAM</td>
<td>94%</td>
<td>100%</td>
<td>Pulmonary TB</td>
<td>Sputum</td>
<td>Perera et al(^4^9)</td>
</tr>
</tbody>
</table>

* LAM indicates lipoarabinomannan; CSF, cerebrospinal fluid; TB, tuberculosis.
isolating clinical cultures and applying molecular biology techniques. Recently, these techniques have started to be used diagnostically for direct identification of mycobacteria in body fluids of patients. However, M tuberculosis does not have a unique gene that allows the microorganism to be identified and diagnosed, and at least 3 genes or genomic regions have to be amplified to obtain a firm diagnosis.

Methods for Identifying Species

In the past, species corresponding to a clinical isolate were identified with a battery of sequential biochemical and physiological tests, and so an exact identification of the agent isolated from a patient’s secretions often took a long time. In recent years, DNA hybridization and polymerase chain reaction (PCR) techniques, and the availability of complete genomes of many microorganisms of medical interest have revolutionized how we approach microbiological diagnosis and the epidemiology of infectious diseases.

Regions of difference. Modern molecular biology methods, particularly those based on PCR, can accurately identify a wide range of microorganisms, including mycobacteria, through the use of PCR primers for genus- and species-specific genomic sequences. The sequencing of the genome of M tuberculosis and initial findings of comparative genomics have allowed the identification of large regions of missing genetic material (deletions) that characterize the different mycobacterial species. Some of these regions of difference are restricted to species of the M tuberculosis complex and so can help distinguish between pathogenic and nonpathogenic mycobacteria (nontuberculous mycobacteria).

The method described by Huard et al in 2003 used 6 PCR primer pairs for the Rv0577, Rv3349c, Rv1510, Rv1970, Rv3877/8, and Rv3120 loci. These primers generate amplicons of 400 to 1200 base pairs (Table 7), which allow the identification of M tuberculosis, M africanum subtype I, M africanum subtype II, M bovis, M bovis-BCG, Mycobacterium caprae, M microti, and M canetti. Furthermore, the mycobacterium corresponding to a clinical isolate can be classed as nontuberculous if amplification products from these genomic regions are not present.

Polymerase Chain Reaction–Restriction Fragment Length Polymorphism of hsp65

The hsp65 gene has been used to distinguish between mycobacteria that do not belong to the M tuberculosis complex. This gene codes for a heat shock protein that is present in all mycobacteria. The method originally described by Telenti et al in 1993, a 439-base pair region is amplified by PCR then digested with BstEII and HaeIII restriction enzymes. The restriction patterns of these enzymes for the amplified hsp65 sequence can be visually identified by eye or a computer program can be used to automate the analysis. It has been possible to identify as many as 62 species of nontuberculous Mycobacterium species through use of other restriction enzymes such as Sau96I and CfoI or AvaII, HpyII, and HpaII.

Methods for Genotyping Clinical Isolates of M tuberculosis

Genotyping of clinical isolates is done essentially with methods based on hybridization of DNA probes derived from repeat elements that characterize the M tuberculosis complex. The 2 most widely used techniques are Southern blot hybridization (restriction fragment length polymorphism [RFLP]-IS6110) and spoligotyping, although recently, the use of a variable number of tandem repeats (VNTR) has also been shown to be extremely useful.

RFLP-IS6110

Genotyping using RFLP takes advantage of discrete variations in the genetic material from different strains or clinical isolates. These variations can be detected by

<table>
<thead>
<tr>
<th>Target Region</th>
<th>Primers</th>
<th>Expected Product (Base Pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0577</td>
<td>5'-ATG CCC AAG AGA AGC GAA TAC AGG CAA-3'</td>
<td>786</td>
</tr>
<tr>
<td>Rv3349c</td>
<td>5'-CTT TGG TGG GCC GTG ACC GTG TGA ACT-3'</td>
<td>943</td>
</tr>
<tr>
<td>Rv1510</td>
<td>5'-CTT CTG ACC CAA AAA ATA GGA-3'</td>
<td>1.033</td>
</tr>
<tr>
<td>Rv1970</td>
<td>5'-GGG CGC CAG CTC CCT CAC TAC GTG AAC-3'</td>
<td>1.116</td>
</tr>
<tr>
<td>Rv3877/8</td>
<td>5'-GGG CAG GCC GCA ACC GCA-3'</td>
<td>999</td>
</tr>
<tr>
<td>Rv3120</td>
<td>5'-GGG CAG GCC GCA ACC GCA-3'</td>
<td>404</td>
</tr>
</tbody>
</table>
oligonucleotides covalently immobilized on a nitrocellulose
comparison with patterns produced by synthetic spacer
variability in the spacer regions is used to obtain different
used as a target for PCR amplification, and the extensive
spoligotyping, conserved sequences of direct repeats are
vary according to the number of direct repeats as well
by nonrepetitive sequences of 34 to 41 base pairs. Strains
locus contains direct repeats of 36 base pairs, interspaced

biotin or digoxigenin can be used. Given that
for IS6110, although currently a nonradioactive label with
IS6110); resolution of the digested DNA on agarose or
that cuts the insertion element (generally ProtIII for cutting
IS6110), resolution of the digested DNA on agarose or
culturing; digestion of the DNA with a restriction enzyme
contains 8 to 20 copies of IS6110 (according to the strain),
this method can detect 16 to 40 bands, which is enough
to differentiate and classify clinical isolates.62
Another of the insertion sequences used for
distinguishing between mycobacterial strains is IS1081
(1324 base pairs), which is found in strains of the
M tuberculosis complex but, due to the low number of
copies, is of limited use in epidemiological studies.
Techniques based on IS1081 also have the drawback of
not being able to differentiate between M bovis-BCG and
other members of the M tuberculosis complex.63

**Spoligotyping**

Spoligotyping takes advantage of the extensive
polymorphism of the DR locus of M tuberculosis.64 This
locus contains direct repeats of 36 base pairs, interspaced
by nonrepetitive sequences of 34 to 41 base pairs. Strains
vary according to the number of direct repeats as well as
the presence or absence of some spacers. With
spoligotyping, conserved sequences of direct repeats are
used as a target for PCR amplification, and the extensive
variability in the spacer regions is used to obtain different
hybridization patterns of the amplified product for
comparison with patterns produced by synthetic spacer
oligonucleotides covalently immobilized on nitrocellulose
membranes. The method described by Kamerbeek et al65
in 1997 makes use of 43 spacers which not only can be
used to type strains of M tuberculosis but also to distinguish
them from M bovis and M bovis-BCG.

In 2002, van der Zanden et al66 introduced 51 new spacers
thereby considerably increasing the capacity of this
technique for genotyping.

**VNTR**

Like the minisatellites described in eukaryotic genomes, 41
groups of 40 to 100 base pairs of repeat sequences
arranged in tandem have been found in M tuberculosis.
These sequences are known as mycobacterial interspersed
repetitive units (MIRU). Of these, 12 loci are sufficiently
polymorphic in terms of number of copies in clinical
isolates of M tuberculosis and have started to be used
successfully in epidemiological studies.67 In addition, the
use of MIRU-VNTR for genotyping clinical isolates has
the advantage that the process can be automated.

**Conclusions**

Molecular strategies are clearly an essential part of
the microbiological studies of tuberculosis because they
can accurately determine the species to which a given
clinical isolate belongs in a matter of hours. Furthermore,
acceptable epidemiological monitoring is possible with
spoligotyping or VNTR. Although molecular techniques are sensitive enough to detect DNA from just 2 mycobacteria, their application in tuberculosis
diagnosis has so far not been as successful as hoped,
mainly because of the presence of PCR inhibitors in
clinical samples. However, once the etiologic agent has
been isolated by culturing clinical samples in conventional
media, the use of PCR can reduce the time needed to
identify the species to a few hours. Another reason why
samples will continue to be cultured is that no other
technique can be used so successfully in drug sensitivity
studies, even though several molecular methods have
been described for the analysis of mycobacterial genes
associated with drug resistance.68

The characteristics that make serologic methods so
attractive (simplicity, low cost, etc) will allow these
techniques to replace reliance on sputum smears for
diagnosis, once the problem of availability of specific
antigens has been overcome. Population studies can
then be designed to investigate the prevalence of
tuberculosis.

The predictive value of the different methods has not
yet been fully determined, particularly in the case of
methods still in development. Sputum smears, for
example, have a low sensitivity (10%), and so a negative
value does not rule out the presence of the bacillus,
whereas a positive result is reliable evidence of the
presence of M tuberculosis in a patient with clinical
signs and symptoms of the disease. In turn, traditional
identifying methods have a higher predictive value (95%) and, along
with biochemical tests, is the benchmark technique for
identifying M tuberculosis, but of course the time
required to reach an accurate diagnosis is extremely
long.69 The sensitivity and specificity of innovative
immunological methods for antibody detection are
generally low, and although methods involving capture
and detection of mycobacterial antigens, particularly
glycolipids, appear promising, they are still not used
systematically, mainly because specific monoclonal
antibodies are lacking and/or are not commercially
available. Finally, of the molecular methods under
evaluation, PCR appears the best option for directly
identifying M tuberculosis although it can only detect
the presence of genetic material and does not provide
information on the viability of the bacillus. We are thus
drawn to conclude that we still lack a rapid diagnostic
strategy that meets all the requirements for implementation on a sufficiently large scale to deal with
the spread of the disease. Clinical judgment and
laboratory tests are therefore still required, and the
search for new strategies should continue.
PALMA-NICOLÁS JP ET AL. INNOVATIVE STRATEGIES TO DIAGNOSE AND MONITOR TUBERCULOSIS PATIENTS

Acknowledgments

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