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# Introduction

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*A priori*, it seems surprising that in 2011 a medical journal in a developed country such as Enfermedades Infecciosas y Microbiología Clínica (EIMC) should publish a special issue entitled "Update in Tuberculosis (TB)". However, as this issue stresses, the disease remains a major public health problem and one of the most important infectious diseases affecting mankind today.

According to estimates by the World Health Organization (WHO; fifteenth annual report)[1], about a third of the world's population (2 bn people) is infected with *Mycobacterium tuberculosis*; in 2009 there were 9.4 million new TB patients, with 1.3 million deaths among HIV-negative subjects and 0.38 million deaths among HIV-positives. The situation is particularly dramatic in the poorest areas of the world: 85% of these cases are concentrated in South-East Asia, Africa and the Western Pacific (35%, 30% and 20% respectively).[1] In developed nations, including Spain, because of the phenomenon of mass immigration from countries with high endemic rates of TB the incidence of the disease has not fallen as much as had been hoped.

TB control is based on complicated programs that focus on adherence to treatment (some of which date from as long ago as 1977), contact tracing, early diagnosis and surveillance. Unfortunately, few of the world's countries are able to implement these programs effectively. To make matters worse, the BCG vaccine has demonstrated very low efficiency in tuberculosis control. Since 2003, the Global Fund to fight AIDS, Tuberculosis and Malaria has made great efforts to promote the expansion of better control programs in countries with limited economic resources. As a result, important health benefits have been observed – for example, in the case of TB, the large number of lives saved after the implementation of directly observed treatment (DOT). It has been estimated that by the end of 2007, DOT would have saved about 1.63 million lives compared with no TB treatment, and more than 400,000 lives compared with non-DOT TB treatment.[2]

Identification of multidrug-resistant (resistance to at least rifampin and isoniazid) *Mycobacterium tuberculosis* (MDR-TB) is one of the cornerstones for global TB control as it allows early epidemiological and therapeutic interventions. In 2008, the WHO estimated that almost 500,000 new cases of MDR-TB occur globally every year, and placed special emphasis on the new cases of extensively drug-resistant TB (XDR-TB; MDR-TB plus resistance to any fluoroquinolone and any of the second-line anti-TB injectable drugs) which have been reported in 46 countries in all five continents.[3] However, trend data from different countries suggest that adequate treatment could reverse the spread of MDR-TB cases.[4]

Although TB has been relatively neglected in recent years, several organizations have set up product development partnerships (PDPs) which have made significant joint efforts to push TB research forward. Substantial resources from public, private, academic, and philanthropic sectors have been allocated to the development of new diagnosis tools (FIND), drugs (TB Alliance) and vaccines (AERAS), as is the case in other neglected diseases. In addition to these and other global institutions such as the WHO, IUATLD, TB-NET and CDC, several small publicly and privately-funded groups around the world are also heavily involved in TB research. As a result, the scientific community currently has a growing body of information on TB at its disposal.

This special issue of the journal EIMC presents an update on the most relevant and innovative aspects of TB control, with contributions from international scientific experts. Multidisciplinary approaches that include studies of TB epidemiology, comparative genomics, evolution and host-pathogen interaction are essential to the development of better tools and strategies to control and eliminate TB. This issue is divided into nine chapters, each of which focuses on the most important features of the disease —epidemiology, immunology and pathogenesis, diagnostics, new drugs and vaccines— and discusses the most promising recent results. It is our hope that the insights presented here will contribute to the control of this disease, and to its eventual elimination.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References

1. World Health Organization. Global tuberculosis control: epidemiology, strategy and financing. WHO report 2009. WHO/HTM/TB/2009.411. WHO Press, 2009 [consulted 3 December 2010]. Available at: http://www.who.int/tb/publications/global_report/2009/en/index.html
2. Komatsu R, Korenromp EL, Low-Beer D, Watt C, Dye C, Steketee RW, et al. Lives saved by Global Fund-supported HIV/AIDS, tuberculosis and malaria programs: estimation approach and results between 2003 and end-2007. BMC Infect Dis. 2010;10:109.
3. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. World Health Organization, WHO Press, 2010. WHO/HTM/TB/2010.3. [consulted 10 Juanuary 2011]. Available at: http://www.who.int/tb/features_archive/m_xdrtb_facts/en/index.html.
4. Dye C, Williams BG. Slow elimination of multidrug-resistant tuberculosis. Sci Transl Med. 2009;1:3ra8.

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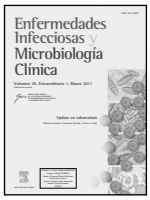

# Present epidemiology of tuberculosis. Prevention and control programs

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*Keywords:*
Tuberculosis
Surveillance
Control
Immigration

A B S T R A C T

Tuberculosis (TB) has affected humanity since the beginning of the recorded time and is associated with poverty, malnutrition, overcrowding, and immunosuppression. Since Koch discovered the infectious nature of the disease in 1882, knowledge about its history and physiopathology has advanced, but it continues to be a global public health problem.

More than 9 million new cases occurred in 2008 worldwide (with an incidence of 139/100,000 inhabitants), of whom more than one million died. Over half million of the cases presented with multidrug resistant-TB. Africa represents the continent with the highest incidence and the most HIV co-infection. The situation in Eastern Europe is also worrisome because of the high incidence and frequency drug resistance.

In developed countries, TB has been localized in more vulnerable populations, such as immigrants and persons with social contention. There is an increase of extra-pulmonary presentation in this context, related to non-European ethnicity, HIV infection, and younger age. In Spain, the increasing immigrant population has presented a need to improve coordination between territories and strengthen surveillance.

The global control plan is based on the DOTS strategy, although the objectives and activities were redefined in 2006 to incorporate the measurement of global development, and community and healthcare strengthening. Adequate control measures in a more local context and continual activity evaluation are necessary to decrease the burden of suffering and economic loss that causes this ancient disease.



## Epidemiología actual de la tuberculosis. Programas de prevención y control

*Palabras clave:*
Tuberculosis
Vigilancia
Control
Inmigración

R E S U M E N

La tuberculosis (TB) afecta a la humanidad desde tiempos inmemoriales y se asocia a la pobreza, mala alimentación, hacinamiento e inmunodepresión. Desde que Koch descubrió su naturaleza infecciosa en 1882, se ha avanzado mucho en el conocimiento de la historia natural y la fisiopatología de la infección, pero, sin embargo, continúa siendo un problema global de salud pública.

En 2008 ocurrieron más de 9 millones de nuevos casos en el mundo (incidencia de 139/100.000 habitantes) de los que más de 1 millón falleció. También más de medio millón de casos presentaron TB multirresistente. África es el continente con mayor incidencia y el más afectado por la coinfección por el virus de la inmunodeficiencia humana (VIH). Es preocupante la situación epidemiológica del este de Europa por su elevada incidencia y resistencia a los fármacos.

En los países desarrollados, la enfermedad se está concentrando en poblaciones vulnerables, como inmigrantes y personas con exclusión social. En este contexto aumentan las localizaciones extrapulmonares, relacionadas con etnias no europeas, infección por VIH y menor edad. En España, el aumento de la proporción de inmigrantes pone de manifiesto la necesidad de mejorar la coordinación territorial y fortalecer la vigilancia.

La estrategia global de control se ha basado en la estrategia DOTS, aunque en 2006 se redefinieron los objetivos y actividades incorporando medidas globales de desarrollo y fortalecimiento de las comunidades y de los sistemas sanitarios. La adecuación de las medidas de control al contexto específico y la evaluación continuada de las actividades son imprescindibles para conseguir disminuir la carga de sufrimiento y las pérdidas económicas que causa esta vieja enfermedad.



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## Introduction

Tuberculosis (TB) has affected humanity since the beginning of time:[1] earlier well-described as consumption and historically causing high morbidity and mortality as the white plague. During the industrial revolution, urban living can be identified as a source of health and nutrition deficiencies and long work hours which facilitated TB transmission. Later progressive socio-economic improvement resulted in a decline in morbidity and mortality.

In 1882, Robert Koch's *Die Aetiologie der Tuberculose*[2] caused a great revolution, clarifying the infectious etiology of the disease which had been in question for so long. The introduction of modern anti-TB drugs in 1944 also contributed to a decrease in mortality in industrialized countries; however, as we will see in this chapter, TB has continued to be an important public health issue on a global scale.

The objective of this chapter is to review the present-day epidemiologic TB situation, and control programs, as well as additional contributing factors such as HIV infection, immigration, and the global economic recession.

### *Causal agent*

*Mycobacterium* is a immobile, aerobic, acid-fast bacteria which is 0.8-4 microns in size, sensitive to solar and ultraviolet light, heat, and disinfectants, but resistant to drying. TB is caused by *Mycobacterium tuberculosis* complex, mainly by *M. tuberculosis*, *M. bovis, M. caprae and M. africanum.* Other mycobacteria, (known as non-TB, atypical or environmental) can also more rarely cause pulmonary or extra-pulmonary pathology.

### *The host*

Host susceptibility is universal, but the risk of infection is directly and mainly related to the degree of exposure. The highest probability of progression to active disease occurs during the first 12-24 months after infection, especially among children, adolescents, the elderly, or immunosuppressed individuals.[3] In countries of high or intermediate TB incidence, the disease more frequently affects children under 4 years of age, followed by young adults. Young adults many times can be smear-positive and transmit the disease to children, who are more susceptible because of their immature immune system. It is also important to note that after 20 years of age, TB tends to affect more males due to higher exposure to infection and higher prevalence of risk factors. In countries where good control programs are in place, incidence among any age group is low and increases slightly with age; in this context, TB is caused by endogenous re-activation, affecting more males and the elderly.

### *Reservoir and source of infection*

An infected person is the reservoir and can be considered a source of infection if active disease occurs. Patients who produce smear-positive sputum are the most contagious, followed by those with positive culture results. It has been documented that even cases with smear and culture negative sputum might also transmit the disease. A correctly treated patient is unlikely to be contagious after 2-4 weeks of treatment. Another source of infection which is of epidemiological interest is the bovine livestock. Other reservoirs also exist but are only of anecdotal interest.

### *Transmission mechanism*

Aerosol transmission plays an important and relevant role in public health. A patient with pulmonary or laryngeal TB produces aerosol contaminants and expulses bacteria which others could inhale, when he or she coughs talks or sings. In industrialized countries, milk pasteurization has made digestive transmission of *M. bovis* anecdotal. Nonetheless, it is important to remember that bovine TB is far from eradicated in cattle, even in some developed countries, and as a consequence, the risk of infection in poor regions continues to be significant. Other transmission mechanisms include cutaneous-mucosal, urogenital, transplacentarial and percutanial inoculation.

### *Natural history of Mycobacterium tuberculosis infection*

Using the "iceberg" epidemiological model, *M. tuberculosis* infection can be separated into infected patients, sick patients and deceased. The sick and deceased patients, or tip of the iceberg, are easily detected, whereas it is difficult to identify the infected patients, which would require testing the entire population. These infected patients represent the hidden and larger part of the iceberg.

A physician can identify TB infection, for example, using tuberculin skin test conversion in the context of contact tracing. Infected patients have the highest probability (about 5%) of developing TB during the first few years after infection (exogenous TB infection), and another 5% could develop TB during their lifetime (endogenous TB reactivation). Therefore, more TB infected individuals means more future TB cases, some with severe clinical forms, such as the TB meningitis. During childhood, a pediatrician plays an important role in the early diagnosis of TB infection and disease, as well as ensuring the compliance of an adequate TB regimen to avoid further complications which could accompany the child throughout his or her life or even result in premature death. In our setting, TB lethality among sick patients must always be under 1%.

### *Epidemiological indicators to evaluate tuberculosis in a community*

The most relevant indicators include: TB incidence, the decrease of this incidence over time, and TB meningitis incidence among children between 0 and 4 years of age. It is also important to use TB infection indicators such as infection prevalence at a specific age and annual infection incidence (AII). In a cohort of tuberculin skin test negative children, a second test must be performed after one year to determine AII. However, this measurement can be difficult to perform and a bias exists for those who are BCG vaccinated, which produces more positive results and hence an inflated AII. To avoid these complications, Styblo defines annual risk of infection (ARI) as the prevalence of infection in two cohorts of the same age during consecutive years. ARI is determined by calculating the decline and prevalence of the last year, which should be the same as the AII. Styblo also estimated that 8-12 infected cases would arise from one smear-positive TB case, even though a recent study reduced this approximation to 2.6-5.8 because of improved TB control.[4]

### *Disease burden in the world and control plans*

Despite the wealth of knowledge about the disease's natural history and the availability of adequate drugs to cure the majority of patients, TB continues to be a global public health problem and the second most common cause of death by an infectious agent in the world, following HIV.[5] The last WHO report estimated that more than 9 million cases occurred worldwide in 2008, with an incidence of 139 cases per 100,000 inhabitants (Fig. 1), just under that of 2007. The majority of these cases are reported in Asia (55%) and Africa (31%) and one half of the total cases occur in 5 countries: India, China, Indonesia, Nigeria and South Africa. Of the total newly diagnosed cases, four million are smear-positive (incidence of 61/100,000) and almost one and a half million are infected with HIV (80% live in Africa).[6,7] Even though the absolute number of cases is increasing with the rising total population, the global incidence rate began to decrease in 2004, with an annual decline of less than 1%.

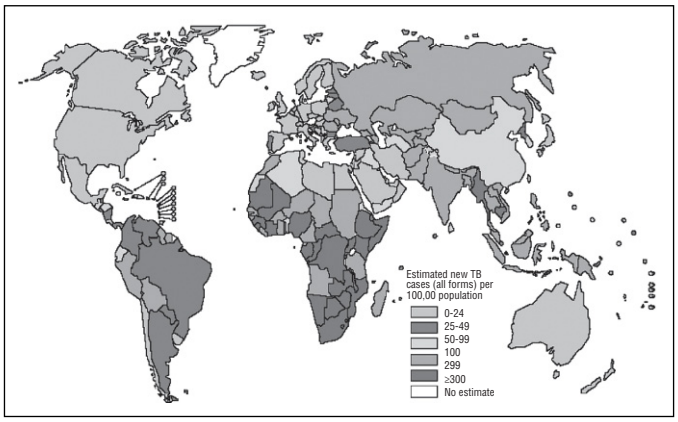


**Figure 1.** Estimated tuberculosis (TB) incidence rates, 2008.

This pattern was also observed with HIV prevalence in Africa. Of the 6 WHO regions, TB incidence is decreasing in five, with the exception of the European region, where it remains stable. There are more than a half million estimated cases of multi-drug resistant (MDR) TB, 56% of which are newly diagnosed cases and 70% are smear-positive. The 5 countries with the highest number of MDR TB cases are India, China, the Russian Federation, South Africa and Bangla Desh.[8,9] In 2007, TB mortality was estimated at 19/100,000 habitants, with 1.3 million non-HIV deaths and almost 1.5 million HIV infected deaths.

In the WHO European region, almost one half million cases were reported (incidence 52/100,000) in 2008, comprising 6% of the reported cases in the world. Incidence was increasing until 2004 but decreased by 2.5% last year and it is still early to predict whether this tendency will continue. Notification rates and mortality increase following a west to east gradient. Eighteen countries have been defined as High Priority Countries (HPC)*. These countries all present incidences over 100 cases/ 100,000 inhabitants, represent more than 80% of the TB cases in the region and report rates more than 6 times higher than those of the European Union. The percentages of MDR TB cases among new diagnoses and previously treated cases are 14% and 50%, respectively, in HPC. Extensively multi-drug resistant (XDR) TB prevalence is around 1.4%. The number of HIV infected TB patients has doubled since 2006 in HPC. This trend has only been observed in the HPC and it is assumed to be associated with improved detection of co-infected TB.[10] Treatment outcome among new cases is also not good, with a success rate of 70%. Low treatment adherence, an elevated proportion of MDR TB and an increasing proportion of HIV co-infected patients all contribute to this poor result. Surveillance data in the region demonstrate diverse epidemiological trends, with the disease burden concentrated in the 18 HPC, which is truly worrisome.[10]

In European Union and European Economical Area countries, more than 80,000 cases were detected in 2008. Incidence was 16.7 cases/100,000 inhabitants, which was 1% lower than in 2007. In the EU, incidence has been steadily declining in the last 5 years. Rates less than 20/100,000 have been reported in 21 countries, but high rates remain in Romania (115), the Baltic countries (between 33 and 67), Bulgaria (41), Portugal (28) and Poland (21). Twenty three percent of the reported cases were immigrants, ranging from 20-88%. Six percent of the reported cases with available drug susceptibility results present a MDR resistance pattern and 90 cases of XDR TB were reported.[11]

Spain reported one of the highest numbers of cases of EU in 2008 (third after Romania and Great Britain), with an incidence of 18.4/100,000 and 30% immigrants.[12] Knowledge of TB epidemiology in our country is far from optimal, especially regarding important factors such as HIV co-infection and drug resistance.[10,12] Program evaluation indicators are also not available on the national level. Two years ago in Spain, a national TB control program was created to improve program coordination between each autonomous community and to enforce disease surveillance and control. However, it does not appear to have reached the initial expectations.[13]

Only 3 countries have achieved the successful treatment rate defined by the WHO objective and many countries report a rate under 85%, usually because of lack of information. Some countries, such as Spain, do not even report it. Even though an unknown treatment result does not necessarily represent a negative result, the absence of such important information complicates TB control planning.[14] The TB epidemiological situation in the EU is heterogeneous; the poor evolution of TB incidence in high or intermediate burden countries requires their close follow-up. The quality of reported information, with respect to important indicators such as absolute number of cases, drug resistance and treatment outcome, is poor and complicates the control of the disease. It is also important to note that the disease is affecting vulnerable populations within low incidence countries, such as immigrants and the poor.[15,16]

## Have there been changes in the site of tuberculosis? Epidemiological significance of extrapulmonary tuberculosis

Pulmonary TB is always the most frequent, even though extrapulmonary (EP) forms have increased its frequency in recent years among HIV infected patients and even more recently among distinct groups of immigrants. EP TB is a clinical problem rather than a public health problem, since isolated non-pulmonary disease is not contagious. However, between 6% and 20% of patients with EP TB also have active pulmonary infection.[17-20] In addition, about 20% of patients with EP disease may have a positive sputum culture and 40% of them a positive sputum smear, even in the presence of normal or non-suspicious chest X-ray findings.

Several studies from European, North American and Asian countries in the last decade indicate that between 12% and 53% of TB patients present with a major form of EP TB. The variability of EP TB depends not only on the prevalence of well-characterized risk factors among the population, but also on the definitions. For example, patients with concomitant pulmonary involvement, intrathoracic lymphadenopathy, pleurisy or miliary disease may or may not be included.[21-29] Longitudinal studies performed in industrialized countries have shown that the proportion of EP cases has remained stable or increased over time due to a disproportionally slower decrease in EP rates as compared to pulmonary TB[18,24,25] This may be explained in part by the growing number of individuals of a non-European ethnic background living in industrialized countries. However, a different dynamic of disease reactivation, in which EP forms are favored over time since infection, may also be involved. Although TB control is improving, clinicians should be aware of this trend in order to accurately diagnose EP TB among both specific high-risk groups and the native population.

Characterized risk factors for EP TB include a young age, female gender, non-European ethnic background and immunosuppression from HIV infection, end-stage renal disease, liver cirrhosis,[17-20,22-29] and probably anti-TNF therapy[30] and solid organ transplantation.[31] Conversely, classic TB risk factors, such as diabetes mellitus, alcoholism, homelessness, incarceration, smoking, previous TB history and previous contact with a TB patient, are associated with pulmonary presentation rather than EP disease.[18,19] Although literature is limited, strains isolated from EP TB cases seem to belong

* Armenia, Azerbaijan, Bielorrusia, Bulgaria, Estonia, Georgia, Kazakhastan, Kyrgyzstan, Letonia, Lituania, Moldavia, Rumania, Rusia, Tajikistan, Turquia, Turkmenistan, Ucrania, Uzbekistan

less frequently to cluster[18] and MDR strains than those from pulmonary cases.[19] One study demonstrated that the time from migration to TB diagnosis was almost double for EP cases compared to pulmonary cases (45 vs 24 months)[25] and within an endemic region, the time elapsed since contact with a contagious case to diagnosis was significantly longer for EP cases (<5 years for 23% of cases) than pulmonary cases (<5 years for 73% of cases).[17] Several polymorphisms in some genes have also been associated with EP TB.[32-36] However, the number of EP TB cases that are explained by these polymorphisms or their relationship to predisposing ethnic factors is unknown. Finally, some strain characteristics, such as belonging to the W-Beijing family[37] or certain phosfolipase-C gene D *(plcD)* mutations are associated with EP disease.

The most common sites of EP TB are the lymph nodes and pleura, although significant differences of frequency are observed by age, gender, ethnic background and immunosuppressant conditions. Nonetheless, some classic associations still hold true, such as predominance of intrathoracic lymphatic disease, higher frequency of meningitis in children under 15 years of age, absence of genitourinary TB before 35 years of age and the predilection of pleurisy for older teenagers and young men. Lymphatic TB has been repeatedly associated with ethnic backgrounds other than white European, osteoarticular TB appears to be more frequent in sub-Saharan Africa and peritonitis is more frequent in North Africa and Asia.[25-27]

## Tuberculosis prevention and control programs

The present global TB control strategy was started during the 90's, when TB incidence and mortality continued to increase. The WHO created the "Directly Observed Treatment, Short Course" (DOTS) strategy, which requires each country to detect smear-positive TB cases and offer standardized DOT , with the objective of curing over 85% of TB patients.[7] DOTS has been implemented in 180 countries and it has cured an estimated 25 million patients (Table 1).[6,38]

**Table 1**
Components of "Directly Observed Treatment, Short Course" (DOTS) strategy

| |
|---|
| Political commitment with increased and sustained financing |
| Case detection through quality-assured bacteriology |
| Standardized treatment with supervision and patient support |
| An effective drug supply and management system |
| Monitoring and evaluation system and impact measurement |

Despite these advances, TB has continued presenting new challenges on a global scale. In 2006, the TB Alliance created the Global Plan to Stop TB during 2006-2015. This strategy is the WHO-recommended approach to reduce TB burden. The principle components of the strategy include:

- Pursue high-quality DOTS expansion and enhancement.
- Address TB/HIV, MDR-TB and the needs of poor and vulnerable populations.
- Contribute to health system strengthening based on primary healthcare.
- Engage all care providers.
- Empower people with TB and communities through partnership.
- Enable and promote research.[39]

Therefore, the global TB control objectives have been re-defined to detect 70% of new smear-positive cases and to cure at least 85% of them. TB incidence should start to decrease by 2015 and TB prevalence and mortality should decrease by half by 2015 as compared to figures from 1990. Various activities centered around DOTS were outlined to achieve the Stop TB Global Plan objectives. The 70% detection and 85% cure rates will decrease the number of infectious TB cases and TB-infected contacts, thus decreasing disease burden and mortality. According to estimations from epidemiological parameters obtained in developed countries (average ARI and risk of disease development by primary infection, re-infection or reactivation), the goal of the proposed objectives is to decrease TB incidence to at least 5-10% per year, if no other event associated to greater risk of disease progression, such as HIV infection, occurs.

Nonetheless, interventions directed at increasing the quality of health and life in general, such as better housing, better nutrition or reducing smoking, will also influence infection transmission and the risk of disease progression, independent of DOTS implementation. In fact, in an analysis of the impact of specific activities for TB control compared to general country development factors (improved healthcare, economic growth, etc), better TB evolution was explained mainly by improved socio-economic development in most coutries.[40,41]

Though the Global Plan calls for the coalition of various organizations to present diverse interventions which can be implemented, much criticism has been made about its difficulty.[42] Some challenges include to sustain the political commitment, the competition with other priorities, the threat of HIV, the quality of patient management to prevent drug resistance, to build human resources capacity, to improve the quality of diagnostic and to foster operative research.[43] Furthermore, the adaptation of field activities and quality of information must also be taken into account. The use of the appropriate strategy requires full knowledge of the local TB situation, an adequate evaluation system of control activities and a functioning surveillance system.[44] In high TB burden countries, considerable uncertainty exists about the indicators used to measure progress towards Global Plan objectives.[45]

In low burden countries, TB disproportionately affects vulnerable populations (immigrants, homeless, etc), which complicates case management (poor treatment adherence, increased drug resistance), requires additional professional training, and can increase diagnostic delays and disease advancement.[46] In Spain, the decrease in TB incidence has been accompanied by a change in the epidemiological profile, especially in large cities such as Barcelona, where the proportion of immigrant cases has reached up to 50% (Fig. 2).[47] Similarly, highest incidence rates among native patients are found among the elderly, demonstrating improved disease control (Fig. 3). The city control program has incorporated the use of Community Health Workers to support immigrants in treatment adherence and contact tracing, and to act as facilitators during communication with the healthcare system.[47,48] However, program development with respect to surveillance and TB control is uneven in each Autonomous Community in Spain, even though organizational models do exist, such as in Galicia, which produce positive results and considerable decreases in TB incidence.[49,50]

Another aspect to consider in a low burden setting is the absence of clinical suspicion in the presence of compatible symptoms. From a public health perspective, this increases diagnostic delays, time for transmission and the possibility of epidemic outbreaks especially affecting children.[51] One study performed on 1,000 pediatric active TB cases in Barcelona from 1987 to 2007, the index case was identified in almost half (478) of the cases. The same study describes the outbreaks reported from 2000 to 2007 and states that 75 of the 219 outbreaks (34%) involved children. The 98 secondary cases under 15 years of age represented half of the pediatric TB cases in that period.[52]

As demonstrated, there is much knowledge about this old disease in the world and about the complications that exist to decrease its impact. The Global Plan to control TB should serve as a stimulant to

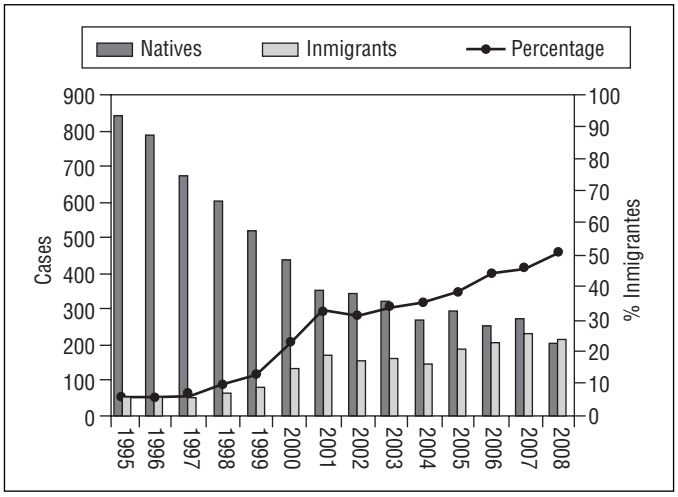


**Figure 2.** Evolution of tuberculosis by origin country. Barcelona, 1995-2008.

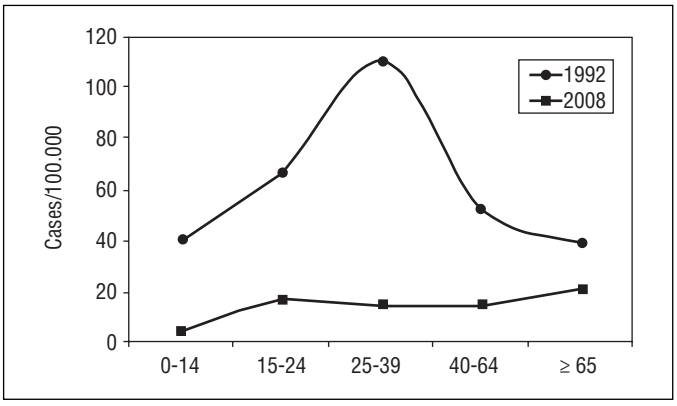


**Figure 3.** Incidence by age-groups in natives. Barcelona, 1992-2008.

decrease the burden of suffering and economical loss that is represented by TB, and also to direct us to the elimination of TB as a public health problem by 2050.[39] It can also contribute to other initiatives, such as the Lancet TB Observatory, which will assess and monitor the progress in TB control and research, assess domestic and global financing, regularly disseminate information and advocate for intensified efforts with stakeholders at all levels.[53]

## Conflict of interest

The authors declare they have not any conflict of interest.

## References


1. Gutiérrez MC, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, et al. Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. PLoS Pathog. 2005;1:e5.
2. Koch R. Die Aetiologie der Tuberculose. Berl Klin Wochenschr. 1882;15:221-30.
3. Buff A, Raviglione M. Tuberculosis. In: Heymann DL, editor. Control of Communicable Diseases Manual. Washington: American Public Health Association; 2008. p. 639-58.
4. Van Leth F, Van der Werf MJ, Borgdorff MW. Prevalence of tuberculous infection and incidence of tuberculosis; a re-assessment of the Styblo rule. Bull World Health Organ. 2008;86:20-6.
5. Frieden TR, Sterling TR, Munsiff SS, Watt CJ, Dye C. Tuberculosis. Lancet. 2003; 362:887-99.
6. Jassal MS, Bishai WR. Epidemiology and challenges to the elimination of global tuberculosis. Clin Infect Dis. 2010;50 Suppl 3:S156-64.
7. World Health Organization. Tuberculosis surveillance and monitoring: report of a WHO workshop, Geneva, 20-22 March 1991.
8. World Health Organization. Global tuberculosis control 2009: surveillance, planning, financing. Available at: www://who.int/tb/publications/global_report/es
9. Glaziou P, Floyd K, Raviglione M. Global burden and epidemiology of tuberculosis. Clin Chest Med. 2009;30:621-36.
10. OMS-ECDC Editor. Tuberculosis surveillance in Europe 2008. Available at: www.edcd.europa.eu/en/publications/Publications/1003_SUR_tuberculosis_surveillance_in_europe_2008.pdf
11. Ködmön C, Hollo V, Huitric E, Amato-Gauci A, Manissero D. Multidrug-and extensively drug-resistant tuberculosis: a persistent problem in the European Union European Union and European Economic Area. Euro Surveill. 2010;15. pii: 19519.
12. Rodríguez E, Hernández G, Villarrubia S, Díaz O, Tello O. Casos de tuberculosis declarados a la Red Nacional de Vigilancia Epidemiológica, España, 2008. Bol Epidemiol Semanal. 2009;17:121-32.
13. Ministerio de Sanidad y Consumo. Plan para la Prevención y Control de la Tuberculosis en España. Available at: www.msc.es/profesionales/saludPublica/prevPromocion/docs/planTuberculosis.pdf
14. Manissero D, Hollo V, Huitric E, Kodmon C, Amato-Gauci A. Analysis of tuberculosis treatment outcomes in the European Union and European Economic Area: efforts needed towards optimal case management and control. Euro Surveill. 2010;15. pii: 19514.
15. Hollo V, Amato-Gauci A, Kodmon C, Manissero D. Tuberculosis in the EU and EEA/EFTA countries: what is the latest data telling us? Euro Surveill. 2009;14. pii: 19151.
16. D'Ambrosio L, Centis R, Spanevello A, Migliori GB. Improving tuberculosis surveillance in Europe is key to controlling the disease. Euro Surveill. 2010;15. pii: 19513.
17. Musellim B, Erturan S, Sonmez Duman E, Ongen G. Comparison of extra-pulmonary and pulmonary tuberculosis cases: factors influencing the site of reactivation. Int J Tuberc Lung Dis. 2005;9:1220-3.
18. Sreeramareddy CT, Panduru KV, Verma SC, Joshi HS, Bates MN. Comparison of pulmonary and extrapulmonary tuberculosis in Nepal- a hospital-based retrospective study. BMC Infect Dis. 2008;8:8.
19. Peto HM, Pratt RH, Harrington TA, LoBue PA, Armstrong LR. Epidemiology of extrapulmonary tuberculosis in the United States, 1993-2006. Clin Infect Dis. 2009;49:1350-7.
20. Lin JN, Lai CH, Chen YH, Lee SS, Tsai SS, Huang CK, et al. Risk factors for extra-pulmonary tuberculosis compared to pulmonary tuberculosis. Int J Tuberc Lung Dis. 2009;13:620-5.
21. Noertjojo K, Tam CM, Chan SL, Chan-Yeung MM. Extra-pulmonary and pulmonary tuberculosis in Hong Kong. Int J Tuberc Lung Dis. 2002;6:879-86.
22. González OY, Adams G, Teeter LD, Bui TT, Musser JM, Graviss AE. Extra-pulmonary manifestations in a large metropolitan area with a low incidence of tuberculosis. Int J Tuberc Lung Dis. 2003;7:1178-85.
23. Yang Z, Kong Y, Wilson F, Foxman B, Fowler AH, Marrs CF, et al. Identification of risk factors for extrapulmonary tuberculosis. Clin Infect Dis. 2004;38:199-205.
24. Cailhol J, Decludt B, Che D. Sociodemographic factors that contribute to the development of extrapulmonary tuberculosis were identified. J Clin Epidemiol. 2005;58:1066-71.
25. Te Beek LA, Van der Werf MJ, Richter C, Borgdorff MW. Extrapulmonary tuberculosis by nationality, The Netherlands, 1993-2001. Emerg Infect Dis. 2006;12:1375-82.
26. Asghar RJ, Pratt RH, Kammerer JS, Navin TR. Tuberculosis in South Asians living in the United States, 1993-2004. Arch Intern Med. 2008;168:936-42.
27. Forssbohm M, Zwahlen M, Loddenkemper R, Rieder HL. Demographic characteristics of patients with extrapulmonary tuberculosis in Germany. Eur Respir J. 2008;31:99-105.
28. Kruijshaar ME, Abubakar I. Increase in extrapulmonary tuberculosis in England and Wales 1999-2006. Increase in extrapulmonary tuberculosis in England and Wales 1999-2006. Thorax. 2009;64:1090-5.
29. Fiske CT, Griffin MR, Erin H, Warkentin J, Lisa K, Arbogast PG, et al. Black race, sex, and extrapulmonary tuberculosis risk: an observational study. BMC Infect Dis. 2010;10:16.
30. Raval A, Akhavan-Toyserkani G, Brinker A, Avigan M. Brief communication: characteristics of spontaneous cases of tuberculosis associated with infliximab. Ann Intern Med. 2007;147:699-702.
31. García-Gómez JF, Linares L, Benito N, Cervera C, Cofán F, Ricart MJ, et al. Tuberculosis in solid organ transplant recipients at a tertiary hospital in the last 20 years in Barcelona, Spain. Transplant Proc. 2009;41:2268-70.
32. Wilkinson RJ, Patel P, Llewelyn M, Hirsch CS, Pasvol G, Snounou G, et al. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1beta on tuberculosis. J Exp Med. 1999;189:1863-74.
33. Wilkinson RJ, Llewelyn M, Toossi Z, Patel P, Pasvol G, Lalvani A, et al. Influence of vitamin D deficiency and vitamin D receptor polymorphisms on tuberculosis among Gujarati Asians in west London: a case-control study. Lancet. 2000;355:618-21.
34. Kim JH, Lee SY, Lee SH, Sin C, Shim JJ, In KH, et al. NRAMP1 genetic polymorphisms as a risk factor of tuberculosis pleurisy. Int J Tuberc Lung Dis. 2003;7:370-5.
35. Thuong NT, Hawn TR, Thwaites GE, Chau TT, Lan NT, Quy HT, et al. A polymorphism in human TLR2 is associated with increased susceptibility to tuberculous meningitis. Genes Immun. 2007;8:422-8.
36. Fernando SL, Saunders BM, Sluyter R, Skarratt KK, Goldberg H, Marks GB, et al. A polymorphism in the P2X7 gene increases susceptibility to extrapulmonary tuberculosis. Am J Respir Crit Care Med. 2007;175:360-6.
37. Nicol MP, Wilkinson RJ. The clinical consequences of strain diversity in Mycobacterium tuberculosis. Trans R Soc Trop Med Hyg. 2008;102:955-65.
38. WHO Editor. Global Tuberculosis Control: a short update to 2009 report. Available at: http://www.who.int/tb/publications/global_report/2009/update/en/index.html

39. Stop TB partnership. The Global Plan to Stop TB 2006-2015. Geneva: World Health Organization; 2006.
40. Dye C, Lönnroth K, Jaramillo E, Williams BG, Raviglione M. Trends in tuberculosis incidence and their determinants in 134 countries. Bull World Health Organ. 200987:683-91.
41. Suk JE, Manissero D, Büscher G, Semenza JC. Wealth inequality and tuberculosis elimination in Europe. Emerg Infect Dis. 2009;15:1812-4.
42. Maher D, Dye C, Floyd K, Pantoja A, Lonnroth K, Reid A, et al. Planning to improve global health: the next decade of tuberculosis control. Bull World Health Organ. 2007;85:341-7.
43. Enarson DA, Billo NE. Critical evaluation of the Global DOTS Expansion Plan. Bull World Health Organ. 2007;85:395-8; discussion 399-403.
44. Seita A. The critical challenge in tuberculosis programmes: are we thinking critically? Int J Tuberc Lung Dis. 2009;13:1444-6.
45. Van der Werf MJ, Borgdorff MW. Targets for tuberculosis control: how confident can we be about the data? Bull World Health Organ. 2007;85:370-6.
46. Migliori GB, Weis S. Searching for the tuberculosis "needle in the haystack": do we need a new approach to find tuberculosis in countries with a low burden of tuberculosis? Am J Respir Crit Care Med. 2009;15;180:916-7.
47. Agència de Salut Pública de Barcelona. Servei d'Epidemiologia. La Tuberculosi a Barcelona, 2008. Available at: www.aspb.es/quefem/docs/TBC_2008.pdf
48. Grupo de trabajo para el control de las tuberculosis importadas. Prevención y Control de la tuberculosis importada. Available at: www.aspb.es/uitb/docs/DocConsTBimport.pdf
49. Rodrigo T, Caylà JA y Grupo de Trabajo para Evaluar Programas de Control de Tuberculosis. Med Clin (Barc). 2003;121:375-8.
50. Dirección General de Salud Pública y Planificación. Informe de tuberculosis en Galicia: Características de los casos de tuberculosis de Galicia de los años 2007 y 2008. Evolución en el período 1996-2008. Available at: http://www.sergas.es/MostrarContidos_N3_T02.aspx?IdPaxina=61317&uri=http://www.sergas.es/cas/DocumentacionTecnica/docs/SaudePublica/Tuberculose/informe_TUBERCULOSIS_castellano_WEB.pdf&hifr=1250&seccion=0
51. Gillman A, Berggren I, Bergström SE, Wahlgren H, Bennet R. Primary tuberculosis infection in 35 children at a Swedish day care center. Pediatr Infect Dis J. 2008;27:1078-82.
52. Nelson JL, Moreno A, Orcau A, Altet N, Martínez-Roig A, Caylà J, et al. Transmission of childhood tuberculosis: risk factors associated with an unidentified index case and outbreak evolution in Barcelona (1987-2007). Pediatr Infect Dis J. 2010; 29:876-9.
53. Marais BJ, Raviglione MC, Donald PR, Harries AD, Kritski AL, Graham SM, et al. Scale-up of services and research priorities for diagnosis, management, and control of tuberculosis: a call to action. Lancet. 2010;375:2179-91.

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# Innovations in the molecular epidemiology of tuberculosis

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ABSTRACT

The application of genotyping tools to the analysis of tuberculosis (TB) has allowed us to identify clinical isolates of *Mycobacterium tuberculosis* to strain level. *M. tuberculosis* fingerprinting has been applied at different levels: *a)* in the laboratory, to optimize identification of cross-contamination events which can lead to a false diagnosis; *b)* in the patient, to determine whether recurrences are due to reactivations or exogenous reinfections or to identify cases coinfected by more than one strain; *c)* at the micropopulation level, to identify clusters of cases infected by the same strains (recent transmission) and to differentiate them from orphan cases that are most probably due to reactivations; and *d)* at the macropopulation level, to define the global distribution of *M. tuberculosis* lineages, to monitor the international spread of high-risk strains, and to explore the evolutionary features of *M. tuberculosis*. In recent years, important methodological and strategic advances have been applied at these different levels of analysis. Rather than provide an exhaustive review, the present study focuses on specific advances in micropopulation and macropopulation analysis.



## Innovaciones en la epidemiología molecular de la tuberculosis

*Palabras clave:*
Tuberculosis
Epidemiología molecular
Innovaciones
Transmisión reciente
Evolución

RESUMEN

La aplicación de estrategias de genotipado al análisis de la tuberculosis (TB) ha permitido discriminar los aislados de *Mycobacterium tuberculosis* a nivel de cepa en distintos contextos: *a)* en el laboratorio, para optimizar eventos de contaminación cruzada; *b)* en el paciente, para discriminar recurrencias debidas a reactivaciones o reinfecciones e identificar casos con infecciones mixtas; *c)* en el contexto "micropoblacional", para identificar casos infectados por una misma cepa (transmisión reciente), y *d)* en el contexto "macropoblacional", para definir la distribución internacional de linajes de *M. tuberculosis*, de cepas de alto riesgo o analizar aspectos evolutivos. En los últimos años hemos asistido a avances metodológicos y analíticos en cada uno de los contextos mencionados. Esta revisión no pretende ofrecer un análisis exhaustivo de éstos, sino destacar algunos avances de especial interés en el contexto del análisis micro y macropoblacional.



## Genotyping of *Mycobacterium tuberculosis* at the micropopulation level: analysis of epidemiologically-related populations

An understanding of how *Mycobacterium tuberculosis* is transmitted requires the analysis of well-defined epidemiologically consistent populations based on universal genotyping of *M. tuberculosis* isolates and long-term surveillance.

The way in which *M. tuberculosis* genotyping data are used for epidemiological purposes has changed. Most molecular epidemiology studies of TB have been performed using IS6110-RFLP (restriction fragment length polymorphism), due to its reproducibility, discriminatory power, and low cost. Initially, *M. tuberculosis* genotyping patterns provided a picture of the features of recent transmission in a population. In the present review, this approach is termed descriptive molecular epidemiology, which defines cases in the population infected by the same strain in order to calculate the percentage of clustered tuberculosis (TB) cases (recent transmission events). The traditional application of molecular epidemiology techniques is moving toward real-time *M. tuberculosis* genotyping, which will make it possible to take advantage of cluster data for epidemiological research. We call this approach interventionist epidemiology, namely, an attempt to exercise control over TB transmission.

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## Descriptive molecular epidemiology

Molecular epidemiology allows us to identify recent transmission events, analyze the risk factors associated with recent transmission, detect non-conventional transmission contexts, identify weaknesses in the assignment of microepidemics, and measure the impact of TB control programs.

In recent years, changes in the socioepidemiological scenarios generated by immigration have revealed new challenges in molecular epidemiology. The key questions include comparison of the role of recent transmission with that of reactivation/importation in immigrant TB cases, the impact of potential importation of previously unidentified *M. tuberculosis* strains, and cross-transmission between cases from different nationalities. Without the support of molecular epidemiology, these questions would prove difficult to answer.

Scenarios in recent transmission and immigration are country-specific. Several studies have found that recent transmission plays a minor role in TB in immigrants,[1,2] thus suggesting that it is mainly due to reactivation/importation. However, recent transmission involving immigrants has been observed, and some authors have identified transmission between cases from different origins and between immigrant and autochthonous populations.[3-5] One recent study applied questionnaires to calculate an "integration-index," and revealed higher values for cases in multinational clusters.[6]

One concern in molecular epidemiology studies is the low frequency with which clusters are confirmed by epidemiological data. Some authors[7] consider that both, standard and molecular epidemiology, analyze independent subsets of cases, and discrepancies between their findings are therefore expected. Alternatively, a more frequent-than-expected role has been proposed for casual contacts, which are not identified by contact tracing. In fact, the difficulties in performing contact tracing in immigrant populations are usually considered responsible for the poor correlation between epidemiological data and molecular data.

Some authors are using novel, innovative approaches to resolve this lack of correlation. One strategy has been to refine the quality and amount of epidemiological data obtained from the cases. Questionnaires compiling detailed information about cases and their social networks[8] increase the limited data obtained using standard contact tracing. The clear improvements brought about by the application of these questionnaires[9] have led to a high correlation between standard and molecular epidemiology. Another approach involves the use of photographs of cases as a tool to establish epidemiological links through visual recognition.[10] When this strategy has been applied, clustered patients recognized more photographs of individuals in their cluster than from outside their cluster. These findings validate the strategy, and its application has made it possible to identify sites of TB transmission that could be open to intervention. Some authors have integrated the use of photographs with the application of detailed questionnaires, which have made it possible to identify transmission contexts that had not been identified by standard epidemiological approaches, thus increasing the percentage of clusters with demonstrated links.[11]

The descriptive application of molecular epidemiology has shown us that a certain proportion of transmission is expected to occur outside households, and this has generated interest in identifying transmission hot spots. In this sense, geographic information systems (GIS) to identify areas where transmission is likely to be occurring seem particularly useful. Cluster data obtained by genotyping have been integrated with those obtained by GIS to determine the spatial distribution of clustered cases in order to identify discrete geographic areas where ongoing transmission is actively occurring. Once hot spots were identified, targeted screening was applied and the number of cases of undiagnosed TB and latent TB infection identified were shown to exceed the number of cases normally revealed by standard screening programs.[12]

## Interventionist molecular epidemiology

The refinements developed in descriptive studies have been followed by attempts to improve the speed with which genotyping data are made available, so that researchers can switch from retrospective descriptive designs to prospective real-time interventions. This approach makes it possible to identify ongoing transmission events in real time, to help epidemiologists identify sites of transmission —thus influencing their choices in contact tracing— and, to design efficient intervention strategies to improve TB control.

IS6110-RFLP-based fingerprinting has consolidated the role of molecular epidemiology. However, this technique is labor-intensive and requires well-grown cultures, thus leading to delays in obtaining genotypes. Alternative polymerase chain reaction (PCR)-based strategies can facilitate the switch between descriptive studies and challenging interventional approaches, and several PCR-based genotyping alternatives have been developed in recent years. The most representative techniques were compared several years ago in a multicenter study,[13] which concluded that variable number tandem repeat (VNTR)-based typing was the most robust approach. In parallel, more traditional approaches have also been improved, and amplified fragment length polymorphism (AFLP) including fluorescence targeting has also been used to analyze IS6110 locations.[14] Spoligotyping has evolved towards microbead-based designs and covers a higher number of targets.[15] Application of the multilocus VNTR-based methods mentioned above has been studied, and mycobacterial interspersed repetitive unit (MIRU)-VNTR[16] has been shown to meet new requirements.

Initially, MIRU-VNTR involved a 12-loci set which was considered[17] efficient for epidemiological purposes.[18] However, some authors found it to have limitations,[19,20] although it did have good discriminatory power when applied together with a second-line genotyping method.[20,21] A new improved set of 24 targets with enhanced discriminatory power has been recommended, and a 15-loci subset has been shown to ensure reasonable discrimination.[22,23] In addition, the initial design, which was based on simplex PCR, has been converted to a high-throughput format. Some authors apply high-performance liquid chromatography (HPLC),[24] although most use multiplex fluorescence-labelled PCR and capillary electrophoresis.[17] MIRU-15 and MIRU-24 have been considered an alternative to RFLP, and several studies have found a good correlation with RFLP-based typing data using different population-based approaches and independent settings.[22,25-27] Nevertheless, contradictory data have emerged.[19,28] Analysis of homogeneous lineages, such as the Beijing family, offers poorer results, and some authors have proposed suitable VNTR markers,[29,30] although MIRU-24 recently provided acceptable results in settings where the Beijing family is prevalent.[31] Inclusion of other hypervariable loci in standardized panels has recently been recommended in order to increase the quality of genotyping data.[32] The speed of MIRU-VNTR typing makes the technique suitable for intervention schemes.[25,33]

In the context of intervention, epidemiological resources are generally not sufficient to monitor all recent transmission chains. After identifying the more actively spread strain in a setting, some authors have developed targeted PCR-based genotypic strategies for specific monitoring.[34] Epidemiological control of TB can also be optimized by identifying clusters that are expected to be more robust indicators of recent transmission, as some clusters are never supported by epidemiological data, even in innovative approaches that refine the epidemiological survey. The lack of epidemiological support behind some clusters may be due to the fact that the genotyping tool applied to define clusters has insufficient resolution to determine genetic heterogeneity between the isolates. Alternatively, some epidemiologically unrelated cases could be independently infected by strains that are highly prevalent or endemic, and therefore appear as clustered cases. In this context,

several authors favor second-line genotyping methods to target epidemiological resources at clusters that are confirmed to be genotypically homogeneous by two genotyping tools (and therefore expected to be epidemiologically robust). IS6110-fAFLP has been applied on a selection of RFLP-defined clusters with various degrees of epidemiological support; it split mainly clusters with weak support, thus confirming those that had already been identified.[35] Similarly, MIRU-VNTR splits RFLP-defined clusters lacking epidemiological links and confirms homogeneity in clusters that are epidemiologically robust.[22,25,36,37] In addition to second-line genotyping, other analytical innovations have been applied to evaluate the robustness of RFLP-defined clusters and thus direct epidemiological resources more efficiently towards more epidemiologically significant clusters. Some research lines have determined whether the genotypes defining clusters in a population can also be involved in clusters in unrelated populations, thus minimizing their usefulness as markers of recent transmission. Strains belonging to the Haarlem lineage, which is prevalent in many settings, are preferentially split by MIRU,[22,37] and some RFLP patterns belonging to this lineage are prevalent in unrelated populations.[37]

*Future challenges*

A novel interventional molecular approach is necessary to reduce genotyping response times and thus enable ongoing transmission events to be analyzed in real time. Research will be aimed at direct genotyping of bacilli in clinical specimens. Innovative approaches in the characterization of resistance mutations and species identification by direct analysis of bacilli in clinical specimens now offer good specificity and sensitivity.[54] Therefore, future approaches should attempt to obtain fingerprints from bacilli in clinical samples before culture.

## Application of molecular markers at the macropopulation level

The standard typing methods applied at the micropopulation level —IS6110 RFLP (based on mobile DNA elements), spoligotyping, and MIRU-VNTR (based on repetitive DNA elements)— have high discriminatory power. However, they are not completely suitable at the macropopulation level, because identical fingerprints can emerge in unrelated lineages (homoplasy) as a result of convergent evolution.[38-40] The slower molecular clock of large-sequence polymorphisms (LSPs) and single-nucleotide polymorphisms (SNPs) makes these markers stable enough to fulfill the conditions necessary to define phylogenetic associations unambiguously in *M. tuberculosis*. LSPs in mycobacteria can be detected by comparative whole-genome hybridization using DNA microarrays,[41] whereas SNPs can be identified using currently available in silico comparisons of the multiple whole-genome sequences of *M. tuberculosis*.[42] In view of the high clonality of *M. tuberculosis* species, it is not surprising that the use of different markers produces highly congruent phylogenetic trees consisting of a limited number of large phylogenetic/phylogeographic lineages.[43]

*Evolutionary framework of M. tuberculosis*

The genetically homogeneous *M. tuberculosis* complex is ecologically very diverse and includes *M. tuberculosis*, *Mycobacterium africanum* and *Mycobacterium canetti* (exclusively human pathogens), *Mycobacterium microti* (a rodent pathogen), and *Mycobacterium bovis* (with a wide host range), as well as *Mycobacterium pinnipedii* (seals) and *Mycobacterium caprae* (goats).

Analysis based on large deletions and other markers has made it possible to propose a new evolutionary scenario for *M. tuberculosis* complex, while a series of deletions have sequentially differentiated *M. africanum*, *M. microti*, and the various subspecies of *M. bovis*.[44,45] The RD1 region that contains the highly immunogenic ESAT 6 family of antigens has been found in both *M. tuberculosis* complex and other mycobacteria, but not in *M. microti* or *M. bovis*.[45,46] In particular, *M. bovis* has undergone many deletions of sequences that are present in *M. tuberculosis*; therefore, the hypothesis that *M. tuberculosis* evolved from *M. bovis* following the domestication of cattle is incorrect. Analysis based on large deletions also helps to elucidate the position of *M. africanum* and subdivide it into *africanum* I (an independent lineage within *M. tuberculosis* complex) and *M. africanum* II (a part of *M. tuberculosis sensu stricto*).

Horizontal gene transfer is virtually absent in *M. tuberculosis*, thus implying that its clonal population structure is presented by genetic families, namely, monophyletic clusters of genetically related strains. These families, or genotypes, originated in well-delimited geographic areas and were usually named according to the geographic, historical, or cultural name of the region/country where they were first isolated.

The low levels of sequence variation in *M. tuberculosis* have long precluded the use of multilocus sequence typing. However, recent advances in mycobacterial genomics show more substantial genetic variation at the whole-genome level. A recent study analyzing polymorphisms in DNA repair, recombination, and replication (3R) genes of a worldwide collection of tubercle bacilli[42] revealed a surprisingly high level of polymorphism for the 3R genes as compared to housekeeping genes. The study also underlined the usefulness of 3R-based trees for future discrimination between *M. tuberculosis* complex phylogenetic groups when more microbial genomes are sequenced. Indeed, suboptimal activity of the 3R genes (reportedly caused by a general negative/purifying selection) is reflected by their relaxed fidelity, which may in turn lead to adaptive variants, some of which will be able to survive. Niemann et al[47] compared the complete genomes of Beijing representatives in a high-incidence region (Karakalpakstan, Uzbekistan). One was drug-susceptible (isolated in 2001) and the other multidrug-resistant (MDR) isolated in 2004. Both isolates shared the same IS6110-RFLP pattern and the same allele at 23 out of 24 MIRU-VNTR loci, although they differed by 130 SNPs and one large deletion. The susceptible isolate had 55 specific SNPs, while the MDR variant had 75 specific SNPs, including resistance-conferring mutations. This finding underlines that an identical genotypic pattern may not denote clonality *sensu stricto*, even when multiple (and independent) genetic markers are used. Differences in genetic diversity using additional markers reveal remote links during earlier transmission events. Additionally, some of the strain-specific SNPs in the MDR isolate might represent mutations compensating for putative fitness effects of resistance-conferring mutations.

*Human demography-influenced macropopulation structure of M. tuberculosis*

The genetic diversity of *M. tuberculosis* may be linked to human demographic and migratory events; differences in the occurrence of given lineages and sublineages, as well as their local gradients, could be strongly influenced by the historical events affecting the human host.[48-51] Recent estimates[49] based on the application of Bayesian statistics to VNTR allelic diversity suggested that *M. tuberculosis* complex might be 40,000 years old, a figure which coincides with the expansion of "modern" human populations out of Africa.[52] Additionally, the strong and recent demographic spread of nearly all *M. tuberculosis* complex lineages, which coincided with the increase in the world's population over the last two centuries, has been corroborated by a coalescence analysis.[49]

Principal components analysis and its variant, multidimensional scaling (MDS), are widely used in human population genetics to visualize interpopulation relationships based on complex genetic data. The first application of MDS to *M. tuberculosis* VNTR data

highlighted strong geographic specificities of the local clonal variants of *M. tuberculosis* Beijing genotype.[53] The strong affinity observed for Russian strains, even among geographically distant *M. tuberculosis* complex populations, suggests relatively recent propagation of the Beijing strains presumably exacerbated by massive human migrations in 20th century Russia. Nonetheless, some weak and less expected affinities observed for Beijing strains in distant *M. tuberculosis* complex populations (northern Vietnam, South Africa, Beijing, and Hong Kong) are fascinating and should be closely analyzed to elucidate concealed patterns of human migration or as yet unfamiliar epidemiological links between distant regions. It has been suggested that dissemination of the *M. tuberculosis* Beijing genotype to other regions of the world was driven by population movements to Russia during the Middle Ages, or, more recently, to South Africa (since the 17th century) and to Australia (since the 19th century). Their differential dissemination within these areas, on the other hand, has been influenced by climatic factors in addition to demographic factors.[48,53]

*Local M. tuberculosis clones and human-microbial co-adaptation*

Interplay between human host genetics and microbial burden have led to co-adaptation. In Vietnam, individuals with the T597C allele of the human *TLR-2* gene were more likely to have tuberculosis caused by the East-Asian/Beijing genotype than other individuals.[54] In the Russian Slavic population in Siberia, the -336G allele of *CD209*, the gene encoding DC-SIGN was more common among patients infected with TB caused by Beijing strains than in those infected with non-Beijing strains.[55]

The acquisition of differential pathogenic characteristics by different *M. tuberculosis* complex lineages may lead to locally prevalent clones of the tubercle bacillus, some of which are better adapted to local human populations, such as a specific Beijing subtype in South Africa;[56] others may have evolved in response to selection factors, such as long-term mass BCG vaccination in Vietnam[57] and Tunisia.[58] At the same time, some clones may develop a stable association with a given population leading to non-competitive local circulation. In 2008, Namouchi et al[58] showed that >60% of TB cases were caused by a single genotype in each of the prevalent clades, in contrast to the more clustered ST50/Haarlem, which is predominant in northern Tunisia. The more widespread ST42/LAM (Latin-American-Mediterranean), with a low transmission rate and weak clustering, suggests its stable association with the Tunisian population.[58]

Local specificity of clones can be explained by recent importation and fast dissemination (due to specific pathogenic properties), outbreak conditions, or long-term historical presence in an area. The Beijing genotype is the best known, although it is not exceptional. The heterogeneous genetic family of *M. tuberculosis* LAM has remarkable pathogenic features in settings as geographically distant as Brazil, Russia and Cameroon.[59-61] Examples of the locally predominant but drug-susceptible clonal groups come from geographically diverse areas, both island and continental settings.[30,62]

*New technologies and algorithms*

*Spoligotyping*

A new microsphere (bead)-based laser technology (Luminex, Austin, Texas, USA) permitting the identification and quantification of each PCR product was applied to spoligotyping as an alternative to reverse line blot hybridization.[63] This method makes it possible to analyze a sample in less than 15 seconds and has recently been re-evaluated in France by Zhang et al[15], who found perfect agreement with the results of the membrane-based technique.

A novel alternative has recently been developed:[64] automated MALDI-TOF mass spectrometry (MALDI-TOF MS) was adopted for spoligotype detection and replaced the hybridization step with a multiplexed primer extension assay. A homogeneous assay format of PCR and multiplexed primer extension assay followed by MALDI-TOF MS detection on the MassARRAY® system (Sequenom, Inc.) streamlines sample processing by avoiding extensive washing steps and microsphere conjugation.

*New algorithms*

Sequence evolution models are not appropriate for many non-sequenced-based markers. In particular, adequate treatment of binary spoligotyping data is especially challenging, since the mode of evolution of the DR locus in *M. tuberculosis* is not completely clear. Numerical taxonomy is a simple approach that makes it possible to infer spoligotype-based phylogenies, although it is not very reliable. An interesting method has recently been proposed to visualize relationships between spoligotypes as a "spoligoforest" graph (http://www.emi.unsw.edu.au/spolTools/). This method is based on a statistically tested model showing that changes in the DR locus more frequently involve the loss of a single or a low number of adjacent spacers.

Bionumerics and PAUP (Phylogenetic Analysis Using Parsimony) packages are the most frequently used approaches to analyze VNTR data. More recently, the BURST algorithm (http://eburst.mlst.net), initially implemented for MLST data, has also been applied to infer VNTR-based phylogenies. This algorithm identifies mutually exclusive groups of related genotypes in the population and attempts to identify the founding genotype of each group. It then predicts the pattern of descent from the predicted founding genotype to the other genotypes in the group and displays the output as a radial diagram centered on the predicted founding genotype.

There is still a certain degree of controversy surrounding the treatment of VNTR data. First, mathematical modeling suggested that VNTR loci in *M. tuberculosis* more likely evolve via a single locus change (loss rather than gain); therefore, VNTR alleles should be treated as quantitative variables. Second, currently used programs still consider VNTR alleles as categorical variables, that is, any change is assumed to be equally likely.

*Databasing in molecular tuberculosis control*

Monitoring and timely reporting of circulating *M. tuberculosis* complex clones are essential in anti-TB strategies in order to pinpoint the subpopulations that are susceptible to a targeted, high-priority response by TB control programs. A suitable molecular marker must be chosen for macropopulation studies; thus LSPs and SNPs are well suited for phylogeographical classification of strains, but not for purely epidemiological purposes, as opposed to IS6110-RFLP and 24-loci MIRUs, which are well adapted for molecular epidemiology. Combined use of spoligotyping and MIRU typing is probably the best available compromise that enables a relatively good insight into the major genotypic lineages of *M. tuberculosis* complex, and makes it possible to efficiently investigate clustered cases to monitor ongoing TB transmission in a given setting.[65] Publicly available databases to compare the ever-increasing amount of genotyping data are necessary.

One of the first databases used for inter-laboratory comparison of IS6110-RFLP patterns was developed, maintained, and housed at the National Institute of Public Health and the Environment (Dutch: Rijksinstituut voor Volksgezondheid en Milieu or simply RIVM), Bilthoven, The Netherlands. This database was widely used under the auspices of a project on new-generation genetic markers for the study of the epidemiology of TB (EU project Q2K2-CT-2000-630). However, for reasons of security and confidentiality, this database was not made publicly available.

Although public MIRU-VNTR databases focusing on a global collection of *M. tuberculosis* complex strains did not become available until 2008, MIRU-VNTR patterns could be compared with those

included in an MLVA database at http://minisatellites.u-psud.fr/MLVAnet. This option has recently been complemented by a freely accessible web-based server (http://www.MIRU-VNTRplus.org) that provides information on geographical origin, drug susceptibility profile, corresponding genetic lineage, IS6110-RFLP, 24-locus MIRU-VNTR, spoligotyping, and SNP and LSP profiles for 186 reference strains.[66] However, it cannot compare genotyping results at a global level, since the information it provides serves to predict lineages for classification purposes rather than to describe the worldwide diversity of *M. tuberculosis* complex genotypes.

Spoligotyping is the backbone of the largest publicly available database, SpolDB4, which, on its release in 2006, described a total of 1939 clustered patterns (shared-types) representing 39,295 strains from 122 countries.[67] It tentatively classified *M. tuberculosis* complex into 62 clades/lineages using a mixed expert-based and bioinformatics approach (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo). Developed and housed at Institut Pasteur de Guadeloupe, SpolDB4 has recently evolved to a proprietary multimarker database (SITVIT2) that contains genotyping information on nearly 75,000 *M. tuberculosis* complex isolates from 160 countries of isolation (including MIRU-VNTRs on about 15,000 isolates). A smaller version of this proprietary database will be released online in 2010 and will contain information on about 62,500 clinical isolates (105 countries of isolation and 153 countries of patient origin) and 3 markers (Spoligotyping, 5-loci ETR, and 12-loci MIRU-VNTR; personal communication from N. Rastogi).

Other databases that are not publicly available include: the database of the University of Zaragoza, Spain (5,694 IS6110-RFLP entries, of which 4,637 are from Spanish isolates);[68] the database of the Public Health Research Institute Tuberculosis Center (http://www.phri.org/programs/program_tbcenter.asp) that gives information on over 17,000 clinical isolates, as well as the Houston and the Centers for Disease Control and Prevention databases.

### *Future challenges*

Recent advances in mycobacterial genomics have shown a more substantial genetic variation at the whole-genome level. Accordingly, whole-genome sequencing may become a tool for routine molecular epidemiology studies if its cost becomes comparable to that of traditional typing techniques.

Whereas several global TB databases collectively contain a large amount of genotypic information, discrepancies between them make information sharing difficult, if not impossible. Thus, a synchronization mechanism must be set up, in order to check similar genotypic patterns across databases and establish a common nomenclature.

Accumulation of new data on the global diversity of different molecular markers, together with application of the refined statistical approaches, should better define a time scale for the evolution of *M. tuberculosis* and its families. A truly quantitative approach to the co-evolution of *M. tuberculosis* and humans that takes into account the host-pathogen relationship has yet to be developed.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References


1. Dahle UR, Sandven P, Heldal E, Caugant DA. Continued low rates of transmission of *Mycobacterium tuberculosis* in Norway. J Clin Microbiol. 2003;41:2968-73.
2. El Sahly HM, Adams GJ, Soini H, Teeter L, Musser JM, Graviss EA. Epidemiologic differences between United States- and foreign-born tuberculosis patients in Houston, Texas. J Infect Dis. 2001;183:461-8.
3. Diel R, Rusch-Gerdes S, Niemann S. Molecular epidemiology of tuberculosis among immigrants in Hamburg, Germany. J Clin Microbiol. 2004;42:2952-60.
4. Alonso Rodríguez N, Chaves F, Iñigo J, Bouza E, García de Viedma D, Andrés S, et al. Transmission permeability of tuberculosis involving immigrants, revealed by a multicentre analysis of clusters. Clin Microbiol Infect. 2009;15:435-42.
5. Borrell S, Español M, Orcau A, Tudó G, March F, Caylà JA, et al. Tuberculosis transmission patterns among Spanish-born and foreign-born populations in the city of Barcelona. Clin Microbiol Infect.2010;16:568-74.
6. Barniol J, Niemann S, Louis VR, Brodhun B, Dreweck C, Richter E, et al. Transmission dynamics of pulmonary tuberculosis between autochthonous and immigrant subpopulations. BMC Infect Dis. 2009;9:197.
7. Borrell S, Español M, Orcau A, Tudó G, March F, Caylà JA, et al. Factors associated with differences between conventional contact tracing and molecular epidemiology in study of tuberculosis transmission and analysis in the city of Barcelona, Spain. J Clin Microbiol. 2009;47:198-204.
8. Fitzpatrick LK, Hardacker JA, Heirendt W, Agerton T, Streicher A, Melnyk H, et al. A preventable outbreak of tuberculosis investigated through an intricate social network. Clin Infect Dis. 2001;33:1801-6.
9. Van Deutekom H, Hoijng SP, De Haas PE, Langendam MW, Horsman A, Van Soolingen D, et al. Clustered tuberculosis cases: do they represent recent transmission and can they be detected earlier? Am J Respir Crit Care Med. 2004;169:806-10.
10. Weis SE, Pogoda JM, Yang Z, Cave MD, Wallace C, Kelley M, et al. Transmission dynamics of tuberculosis in Tarrant county, Texas. Am J Respir Crit Care Med. 2002;166:36-42.
11. Martínez-Lirola M, Alonso-Rodríguez N, Sánchez ML, Herranz M, Andrés S, Peñafiel T, et al. Advanced survey of tuberculosis transmission in a complex socioepidemiologic scenario with a high proportion of cases in immigrants. Clin Infect Dis. 2008;47:8-14.
12. Moonan PK, Oppong J, Sahbazian B, Singh KP, Sandhu R, Drewyer G, et al. What is the outcome of targeted tuberculosis screening based on universal genotyping and location? Am J Respir Crit Care Med. 2006;174:599-604.
13. Kremer K, Van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martin C, et al. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol. 1999;37:2607-18.
14. Thorne N, Evans JT, Smith EG, Hawkey PM, Gharbia S, Arnold C. An IS6110-targeting fluorescent amplified fragment length polymorphism alternative to IS6110 restriction fragment length polymorphism analysis for *Mycobacterium tuberculosis* DNA fingerprinting. Clin Microbiol Infect. 2007;13:964-70.
15. Zhang J, Abadía E, Refregier G, Tafaj S, Boschiroli ML, Guillard B, et al. *Mycobacterium tuberculosis* complex CRISPR genotyping: improving efficiency, throughput and discriminative power of 'spoligotyping' with new spacers and a microbead-based hybridization assay. J Med Microbiol. 2010;59:285-94.
16. Mazars E, Lesjean S, Banuls AL, Gilbert M, Vincent V, Gicquel B, et al. High-resolution minisatellite-based typing as a portable approach to global analysis of *Mycobacterium tuberculosis* molecular epidemiology. Proc Natl Acad Sci USA. 2001;98:1901-6.
17. Supply P, Lesjean S, Savine E, Kremer K, Van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J Clin Microbiol. 2001;39:3563-71.
18. Sun YJ, Bellamy R, Lee AS, Ng ST, Ravindran S, Wong SY, et al. Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. J Clin Microbiol. 2004;42:1986-93.
19. Scott AN, Menzies D, Tannenbaum TN, Thibert L, Kozak R, Joseph L, et al. Sensitivities and specificities of spoligotyping and mycobacterial interspersed repetitive unit-variable-number tandem repeat typing methods for studying molecular epidemiology of tuberculosis. J Clin Microbiol. 2005;43:89-94.
20. García de Viedma D, Alonso Rodríguez N, Andrés S, Martínez-Lirola M, Ruiz Serrano MJ, Bouza E. Evaluation of alternatives to RFLP for the analysis of clustered cases of tuberculosis. Int J Tuberc Lung Dis. 2006;10:454-9.
21. Cowan LS, Diem L, Monson T, Wand P, Temporado D, Oemig TV, et al. Evaluation of a two-step approach for large-scale, prospective genotyping of *Mycobacterium tuberculosis* isolates in the United States. J Clin Microbiol. 2005;43:688-95.
22. Oelemann MC, Diel R, Vatin V, Haas W, Rusch-Gerdes S, Locht C, et al. Assessment of an optimized mycobacterial interspersed repetitive- unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. J Clin Microbiol. 2007;45:691-7.
23. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006;44:4498-510.
24. Evans JT, Hawkey PM, Smith EG, Boese KA, Warren RE, Hong G. Automated high-throughput mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* strains by a combination of PCR and nondenaturing high-performance liquid chromatography. J Clin Microbiol. 2004;42:4175-80.
25. Alonso-Rodríguez N, Martínez-Lirola M, Sánchez ML, Herranz M, Peñafiel T, Bonillo MC, et al. Prospective universal application of mycobacterial interspersed repetitive-unit-variable-number tandem-repeat genotyping to characterize *Mycobacterium tuberculosis* isolates for fast identification of clustered and orphan cases. J Clin Microbiol. 2009;47:2026-32.
26. Allix-Beguec C, Fauville-Dufaux M, Supply P. Three-year population-based evaluation of standardized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing of Mycobacterium tuberculosis. J Clin Microbiol. 2008;46:1398-406.
27. Valcheva V, Mokrousov I, Narvskaya O, Rastogi N, Markova N. Utility of new 24-locus variable-number tandem-repeat typing for discriminating *Mycobacterium tuberculosis* clinical isolates collected in Bulgaria. J Clin Microbiol. 2008;46:3005-11.

28. Blackwood KS, Wolfe JN, Kabani AM. Application of mycobacterial interspersed repetitive unit typing to Manitoba tuberculosis cases: can restriction fragment length polymorphism be forgotten? J Clin Microbiol. 2004;42:5001-6.
29. Mokrousov I, Narvskaya O, Vyazovaya A, Millet J, Otten T, Vishnevsky B, et al. *Mycobacterium tuberculosis* Beijing genotype in Russia: in search of informative variable-number tandem-repeat loci. J Clin Microbiol. 2008;46:3576-84.
30. Millet J, Miyagi-Shiohira C, Yamane N, Sola C, Rastogi N. Assessment of mycobacterial interspersed repetitive unit-QUB markers to further discriminate the Beijing genotype in a population-based study of the genetic diversity of *Mycobacterium tuberculosis* clinical isolates from Okinawa, Ryukyu Islands, Japan. J Clin Microbiol. 2007;45:3606-15.
31. Shamputa IC, Lee J, Allix-Beguec C, Cho EJ, Lee JI, Rajan V, et al. Genetic diversity of *Mycobacterium tuberculosis* isolates from a tertiary care tuberculosis hospital in South Korea. J Clin Microbiol. 2010;48:387-94.
32. Velji P, Nikolayevskyy V, Brown T, Drobniewski F. Discriminatory ability of hypervariable variable number tandem repeat loci in population-based analysis of *Mycobacterium tuberculosis* strains, London, UK. Emerg Infect Dis. 2009;15:1609-16.
33. Hawkey PM, Smith EG, Evans JT, Monk P, Bryan G, Mohamed HH, et al. Mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* compared to IS6110-based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. J Clin Microbiol. 2003;41:3514-20.
34. Ashworth M, Horan KL, Freeman R, Oren E, Narita M, Cangelosi GA. Use of PCR-based *Mycobacterium tuberculosis* genotyping to prioritize tuberculosis outbreak control activities. J Clin Microbiol. 2008;46:856-62.
35. Borrell S, Thorne N, Español M, Mortimer C, Orcau A, Coll P, et al. Comparison of four-colour IS6110-fAFLP with the classic IS6110-RFLP on the ability to detect recent transmission in the city of Barcelona, Spain. Tuberculosis (Edinb). 2009; 89:233-7.
36. Van Deutekom H, Supply P, De Haas PE, Willery E, Hoijng SP, Locht C, et al. Molecular typing of *Mycobacterium tuberculosis* by mycobacterial interspersed repetitive unit-variable-number tandem repeat analysis, a more accurate method for identifying epidemiological links between patients with tuberculosis. J Clin Microbiol. 2005;43:4473-9.
37. Alonso Rodríguez N, Martínez-Lirola M, Chaves F, Iñigo J, Herranz M, Ritacco V, et al. Differences in the robustness of clusters involving the *Mycobacterium tuberculosis* strains most frequently isolated from immigrant cases in Madrid. Clin Microbiol Infect. 2010;16:1544-54.
38. Gutacker MM, Smoot JC, Migliaccio CA, Ricklefs SM, Hua S, Cousins DV, et al. Genome-wide analysis of synonymous single nucleotide polymorphisms in *M. tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. Genetics. 2002;162:1533-43.
39. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM. Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. Proc Natl Acad Sci USA. 2004;101:4871-6.
40. Filliol I, Motiwala AS, Cavatore M, Qi W, Hazbón MH, Bobadilla del Valle M, et al. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. J Bacteriol. 2006;188:759-72.
41. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host-pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA. 2006;103:2869-73.
42. Dos Vultos T, Mestre O, Rauzier J, Golec M, Rastogi N, Rasolofo V, et al. Evolution and diversity of clonal bacteria: the paradigm of *Mycobacterium tuberculosis*. PLoS One. 2008;3:e1538.
43. Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. Lancet Infect Dis. 2007;7:328-37.
44. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, et al. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci USA. 2002;99:3684-9.
45. Marmiesse M, Brodin P, Buchrieser C, Gutiérrez C, Simoes N, Vincent V, et al. Macro-array and bioinformatic analyses reveal mycobacterial 'core' genes, variation in the ESAT-6 gene family and new phylogenetic markers for the *Mycobacterium tuberculosis* complex. Microbiology. 2004;150:483-96.
46. Mahairas, GG, Sabo PJ, Hickey MJ, Singh DC, Stover CK. 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J Bacteriol. 1996;178:1274-82.
47. Niemann S, Köser CU, Gagneux S, Plinke C, Homolka S, Bignell H, et al. Genomic diversity among drug sensitive and multidrug resistant isolates of *Mycobacterium tuberculosis* with identical DNA fingerprints. PLoS One. 2009;4:e7407.
48. Mokrousov I, Ly HM, Otten T, Lan NN, Vyshnevskyi B, Hoffner S, et al. Origin and primary dispersal of the *Mycobacterium tuberculosis* Beijing genotype: clues from human phylogeography. Genome Res. 2005;15:1357-64.
49. Wirth T, Hildebrand F, Allix-Béguec C, Wölbeling F, Kubica T, Kremer K, et al. Origin, spread and demography of the *Mycobacterium tuberculosis* complex. PLoS Pathog. 2008;4:e1000160.
50 Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, et al. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol. 2008;6:e311.
51. Mokrousov I, Valcheva V, Sovhozova N, Aldashev A, Rastogi N, Isakova J. Penitentiary population of Mycobacterium tuberculosis in Kyrgyzstan: Exceptionally high prevalence of the Beijing genotype and its Russia-specific subtype. Infect Genet Evol. 2009;9:1400-5.
52. Cavalli-Sforza LL, Menozzi P, Piazza A. The History and Geography of Human Genes. Princeton: Princeton University Press; 1996.
53. Mokrousov I. Genetic geography of *Mycobacterium tuberculosis* Beijing genotype: a multifacet mirror of human history. Infect Genet Evol. 2008;8:777-85.
54. Caws M, Thwaites G, Dunstan S, Hawn TR, Lan NT, Thuong NT, et al. The influence of host and bacterial genotype on the development of disseminated disease with *Mycobacterium tuberculosis*. PLoS Pathog. 2008;4:e1000034.
55. Ogarkov OB, Medvedeva TV, Nekipelov OM, Antipina SL, Men'shikov ML. Study of DC-SIGN gene polymorphism in patients infected with *Mycobacterium Tuberculosis* strains of different genotypes in the Irkutsk Region [article in Russian]. Probl Tuberk Bolezn Legk. 2007;(11):37-42.
56. Hanekom M, Van der Spuy GD, Gey Van Pittius NC, McEvoy CR, Ndabambi SL, Victor TC, et al. Evidence that the spread of *Mycobacterium tuberculosis* strains with the Beijing genotype is human population dependent. J Clin Microbiol. 2007;45:2263-6.
57. Kremer K, Van-der-Werf MJ, Au BK, Anh DD, Kam KM, Van-Doorn HR, et al. Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. Emerg Infect Dis. 2009;15:335-9.
58. Namouchi A, Karboul A, Mhenni B, Khabouchi N, Haltiti R, Ben Hassine R, et al. Genetic profiling of *Mycobacterium tuberculosis* in Tunisia: predominance and evidence for the establishment of a few genotypes. J Med Microbiol. 2008;57:864-72.
59. Lazzarini LC, Spindola SM, Bang H, Gibson AL, Weisenberg S, Da Silva Carvalho W, et al. RDRio *Mycobacterium tuberculosis* infection is associated with a higher frequency of cavitary pulmonary disease. J Clin Microbiol. 2008;46:2175-83.
60. Dubiley S, Ignatova A, Mukhina T, Nizova A, Blagodatskikh S, Stepanshina V, et al. Molecular epidemiology of tuberculosis in the Tula area, central Russia, before the introduction of the Directly Observed Therapy Strategy. Clin Microbiol Infect. 2010;16:1421-6.
61. Niobe-Eyangoh SN, Kuaban C, Sorlin P, Thonnon J, Vincent V, Gutiérrez MC. Molecular characteristics of strains of the Cameroon family, the major group of Mycobacterium tuberculosis in a country with a high prevalence of tuberculosis. J Clin Microbiol. 2004;42:5029-35.
62. Valcheva V, Mokrousov I, Panaiotov S, Bachiiska E, Zozio T, Sola C, et al. Bulgarian specificity and controversial phylogeography of *Mycobacterium tuberculosis* spoligotype ST125_BGR. FEMS Immunol Med Microbiol. 2010;59:90-9.
63. Cowan LS, Diem L, Brake MC, Crawford JT. Transfer of a *Mycobacterium tuberculosis* genotyping method, Spoligotyping, from a reverse line-blot hybridization, membrane-based assay to the Luminex multianalyte profiling system. J Clin Microbiol. 2004;42:474-7.
64. Honisch C, Mosko M, Arnold C, Gharbia SE, Diel R, Niemann S. Replacing reverse line-blot hybridization spoligotyping of the *Mycobacterium tuberculosis* complex. J Clin Microbiol. 2010;48:1520-6.
65. Rastogi N, Sola C. Molecular Evolution of the *Mycobacterium tuberculosis* Complex In: Palomino JC, Cardoso Leão S, Ritacco V, editors. Tuberculosis 2007. Available at: http://www.tuberculosistextbook.com/index.htm
66. Allix-Béguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2008;46:2692-9.
67. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6:23.
68. López-Calleja AI, Gavín P, Lezcano MA, Vitoria MA, Iglesias MJ, Guimbao J, et al. Unsuspected and extensive transmission of a drug-susceptible *Mycobacterium tuberculosis* strain. BMC Pulm Med. 2009;9:3

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# The secret trumps, impelling the pathogenicity of tubercle bacilli

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ABSTRACT

Confrontation between invading microbial pathogens and host defense systems involves intricate cellular and molecular interactions. Here we discuss the virulence factors as trumps, overriding the contest in favor of the tubercle bacillus *(Mycobacterium tuberculosis)*. It evolved a number of molecular constituents, which can interfere with antigen presentation and Toll receptor function, thus impairing immune defenses. It also evolved stress responses, which can drive its cell cycle into a non-replicating, low metabolic mode. Although the low counts of latent bacilli prevent their direct detection, we contend that they retain a capacity to survive for long periods in foamy macrophages and within the necrotic parts of lung granulomas. We attributed significance to drainage of *M. tuberculosis* by the alveolar fluid: while out-flow is responsible for the clearance, the reverse-flow has an important capacity to re-infect the lungs and to transmit the infection to new recipients. We consider the cycling between replicating and latent organisms to be a continuous process, which is a departure from the concept of long-lived dormant organisms, with a capacity to resuscitate. These aspects impinge also on the actions of isoniazid (INH) chemotherapy and on the topography of human lung lesions. Eventually, fibrosis of the connective tissue of the lungs is known to encapsulate lung lesions, thus limiting the impact of both outward and reverse drainage. In conclusion, the novelty of our views on *M. tuberculosis*-host interactions rests in the dynamic perception of *M. tuberculosis* latency and its evolutionary importance for the pathogenesis of tuberculosis.



## Los triunfos secretos que dan fuerza a la patogenicidad del bacilo tuberculoso

*Palabras clave:*
Tuberculosis
Inmunología
Patogénesis
Latencia bacteriana
Macrófagos

RESUMEN

El enfrentamiento entre los patógenos invasivos y los sistemas defensivos del huésped implica interacciones celulares y moleculares. En el presente artículo se discuten los factores de virulencia como triunfos, favoreciendo el éxito de la contienda a favor del bacilo tuberculoso *(Mycobacterium tuberculosis)*. Éste desarrolla un número de constituyentes moleculares que pueden interferir con la presentación antigénica y la función Toll receptor, deteriorando las defensas inmunes del huésped, así como respuestas al estrés que enlentecen su ciclo celular hasta convertirlo en no replicante. Aunque el recuento bajo de bacilos latentes previene su detección directa, postulamos que retienen cierta capacidad de sobrevivir dentro de macrófagos espumosos y en las partes necróticas de los granulomas pulmonares. Mientras que el circuito natural del fluido alveolar hacia las vías respiratorias superiores es el responsable de la eliminación de bacilos, su retorno para generar aerosoles de forma fisiológica también implica la posibilidad de que con él ciertos bacilos puedan reinfectar de forma endógena los pulmones y transmitir la infección a nuevos individuos. Consideramos, pues, la tuberculosis latente como un proceso continuo, en contraposición al concepto de la existencia de bacilos largamente durmientes y con capacidad de resucitar. Creemos, además, que la fibrosis del tejido conectivo de los pulmones, capaz en ocasiones de encapsular lesiones pulmonares, es la responsable de frenar el drenaje y la diseminación de bacilos, limitando el ciclo reinfectivo. En conclusión, la novedad de nuestra visión radica en la percepción dinámica de la latencia de *M. tuberculosis* y sus consecuencias sobre la patogénesis de la tuberculosis.



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*"Behold, I tell you a mystery: we shall all be changed, in the twinkling of an eye.*

*For our earthly bodies that can die, must be transformed into heavenly bodies that cannot perish but will live forever."*

*1 Corinthians, 15:51-53.*

## Evolution of virulence by adapting the bacterial life cycle

The long history of co-evolution between *Mycobacterium tuberculosis* and our human ancestors is pertinent to the recent 200th birthday anniversary of Charles Darwin. It has been deduced that an ancestor of *M. tuberculosis* was able to infect our predecessors, the *Australopithecus.*[1] *M. tuberculosis* adapted itself efficiently to the physiology of man and took advantage of it, for evolving its host pathogenicity and transmittance. The intricate outcome of this co-evolution made the life cycle of the bacillus highly sophisticated to an extent that still frustrates current attempts for the global eradication of tuberculosis (TB). The menacing epidemiological figures suggest that one third of mankind carry latent *M. tuberculosis* infection (latent TB infection [LTBI]). There are 9 million cases of active TB and 1.8 million TB-related deaths per year.[2] Unlike other microbial pathogens, the virulence of *M. tuberculosis* is not caused by any overtly cytopathic constituents, but rather by the intricate action of immunomodulatory cytokines. It has previously been suggested, that they act as "decoys" to provoke adaptive macrophage and immune reactions, pretending to be protective, but in effect causing host pathology.[3,4] Here, we postulate, that the "secret trump" of *M. tuberculosis* for winning over the infected host involves an adaptation of its life cycle. We further discuss how the unraveling of the underlying mechanisms could be mandatory for turning the tables in favor of the host.

## *Mycobacterium tuberculosis* constituents involved in pathogenicity

*M. tuberculosis* infects the host by the inhalation of small diameter infected aerosols of "droplet nuclei" into alveoli.[5] After being phagocytosed by the alveolar "resident macrophages", several constituents of *M. tuberculosis* cell walls can mediate a number of different strategies, by which the bacilli can avoid their destruction inside a phagolysosome.[6-8] *M. tuberculosis* can inhibit the phagolysosome fusion by: increasing the pH,[9] disturbing the ATPase pump,[10] secreting the ESAT-6/CFP-10 (early secreted antigenic target 6/culture filtered protein 10) complex[11] or by the autophagy mechanism.[12] Then, bacilli can grow until the death of the macrophage itself. This outcome is manifested by necrosis, which prevents the bacilli being killed by macrophage apoptosis.[13-19] However, the burst of bacilli, released from the infected macrophages into the stressful extracellular milieu, instantly curtails their growth.[20-22] Alternatively, a number of constituents of intracellular *M. tuberculosis* can interfere with the host defense mechanisms.[23] Thus, Man-lipoarabinomannan (Man-LAM) and 19 kDa lipoglycoprotein can inhibit the antigen presenting functions and TLR-mediated inflammatory responses of infected cells.[24,25]

## Granuloma and tumour necrosis factor-alpha functions

Blood derived monocytes and neutrophils are attracted to infected sites by the continuous production of tumour necrosis factor-alpha (TNF-α) by the infected resident macrophages (Fig. 1). The most prominent cellular response is represented by the phagocytosis of *M. tuberculosis* by macrophages. The induced accumulation of macrophages leads to granulomatous lesions (Fig. 1). While preventing the dissemination of bacilli, they are also an ideal milieu for the bacilli to grow. This has been called the "Citadel paradox"[26], in analogy to the Citadel of 18th century (Barcelona), which had an

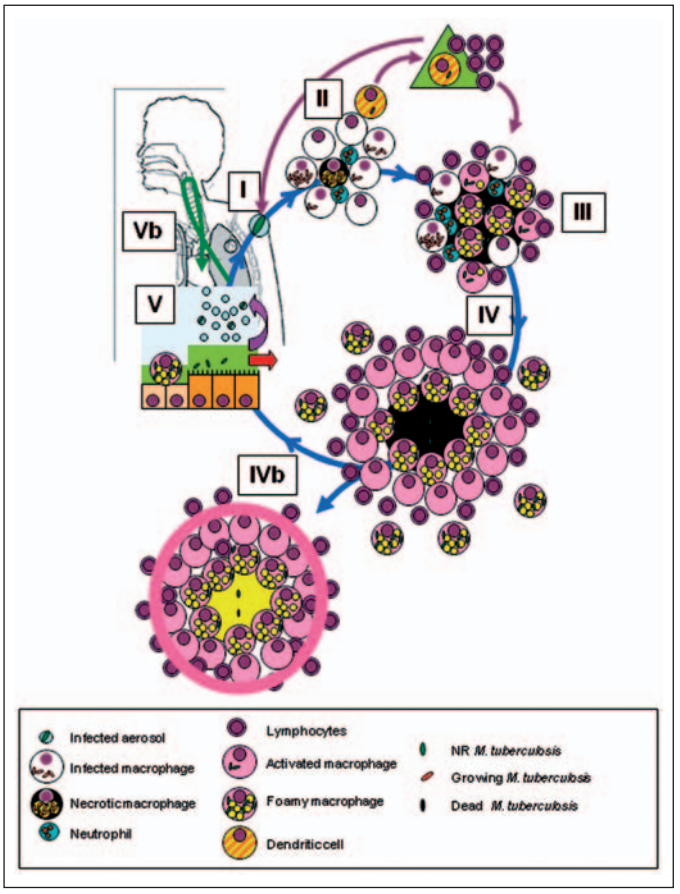


**Figure 1.** Lung pathology changing the life cycle of *Mycobacterium tuberculosis* in the lungs. (I) *M. tuberculosis* transmitted by aerosol settles in the alveoli. (II) *M. tuberculosis* growing inside macrophages, causing their necrosis. Infected monocytes become dendritic cells that are drained to the lymph nodes (green triangle) for antigen presentation. (III) Neutrophils, natural killer cells, lymphocytes and new macrophages are attracted to the granuloma; infected macrophages, bactericidal or bacteriostatic develop into foamy macrophages. *M. tuberculosis* changed to non-replicating (NR) *M. tuberculosis* in necrotic tissue are drained by foamy macrophages towards alveoli. (IVb) Encapsulated necrotic granuloma, starting to mineralize; NR *M. tuberculosis* cannot drain. (V) NR *M. tuberculosis* infected alveolar fluid generates aerosols with the inhaled air or is swallowed and killed/drained in the gastrointestinal tract (Vb). Drainage of bacilli from infected lymph nodes through the thoracic ducts to the right atrium to be pumped back to the lung across the pulmonary artery also contributes to the re-infection process. Symbols: black, necrotic tissue; yellow, mineralized tissue.

architecture pretending to defend against foreign invaders, but in fact serving against the potential rioting of its own residents. Sustained TNF-α signaling is required to maintain the local chemokine gradients for holding the cells in close apposition, which favors the activation of infected macrophages.[27-30] Although *M. tuberculosis* infected TNF-knock-out mice also develop granulomas,[31] this requires a high bacillary concentration and results in larger and more necrotic structures. TNF-α apparently can induce the granulomatous response even with a lower bacillary concentration, thus helping to save the host integrity.

## The role of neutrophils, natural killer cells and dendritic cells, before specific immune response onset

Neutrophils have both mycobactericidal[32] and regulatory anti-inflammatory activities[33] but their role is not fully understood. However, interferon-gamma (IFN-γ) producing natural killer cells can activate or lyse macrophages, containing other intracellular microbial infections,[34] but do not curtail the TB infection. Monocytes

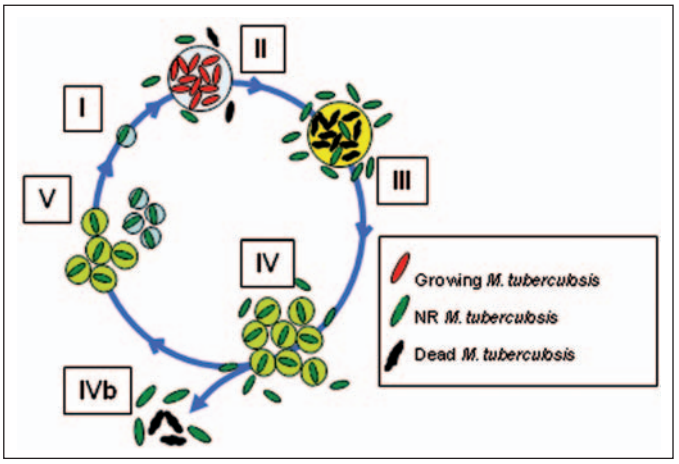


**Figure 2.** Changing life styles of *Mycobacterium tuberculosis* within and outside granulomas. (I) Non-replicating (NR) *M. tuberculosis* in aerosol. (II) Growing *M. tuberculosis*. (III) NR *M. tuberculosis* developing within and outside activated macrophages. (IV) NR *M. tuberculosis* being either killed or persisting inside the phagolysosome or (IVb) inside the mineralized tissue or (V) spreading out by alveolar fluid aerosols.

in the vicinity of infection develop rapidly into dendritic cells (DCs)[35] and emigrate to regional lymph nodes, thus favorable to the host by presenting antigen to T cells. However, infected DCs could also be the "Trojan horse" spreading the bacilli haematogenically from lymph nodes to distant organs. Gradually, antigen stimulated CD4 Th1 cells will proliferate[36] and will be attracted to the lung granulomas to activate infected macrophages through the secretion of IFN-γ.[37] When immune responses to *M. tuberculosis* infection had been established and tubercle bacilli become trapped in granulomas, there is notable bacillary killing.[38] However, not all bacilli get killed and some will enter a stationary, non-replicating, latent phase and will become "hidden" and extremely resistant to host immunity generated stress (Fig. 2), conditions[39,40] which are bactericidal toward replicating *M. tuberculosis.*[41]

## Closing the ring by foamy macrophages

Foamy macrophages (FMs) develop as a consequence of macrophage necrosis and the accumulation of apoptotic short-lived neutrophils.[42-44] FMs accumulate the cellular debris and generate lipid bodies in their cytoplasm. They can phagocytose from the extracellular milieu tubercle bacilli which had already become non-replicating (or latent) under the influence of the stressing inflammatory process; this is represented by acidic pH and intermediate oxygen and nitrogen radicals.[45-47] Oxidation of extravasal LDLs[48] accumulate in macrophages and develop into FMs.[49] In mice, these cells are drained into lung alveoli, building an external ring around the granulomas,[42] because of limited alveolar space.[50] However, FMs can be drained into upper bronchi by the alveolar fluid in guinea pigs[20] or minipigs[21] which have larger alveolar spaces.

## Reverse passage of alveolar fluid

Particles are drained out from the parenchyma of lungs in humans, towards the upper bronchial tree and then into the gastrointestinal tract. This process could be protecting the host by draining replicating *M. tuberculosis* and latent *M. tuberculosis*, organisms out of the body, in actively and latently infected individuals. However, there is also a reverse flow, as induction of aerosols with the inhaled air could lead to re-infection of the lungs (Figs. 1 and 2).[22] This process could not be prevented by T cell immunity, because that develops only after the emigration of the infected DCs to the draining lymph nodes.[51]

## "Continuous re-infection" origin of latent *Mycobacterium tuberculosis*

The continuous re-infection of lungs through the reverse flow of alveolar fluids has been integral to the previously formulated "dynamic hypothesis" of LTBI pathogenesis.[52] Drained bacilli from infected hilar lymph nodes through the thoracic ducts to the right atrium are pumped back to the lung across the pulmonary artery. The mandatory role for a cycle between replicating and latent organisms in LTBI is supported also by the knowledge that T cells in LTBI recognize predominantly antigens expressed by replicating *M. tuberculosis* bacilli. Hence, the "dynamic hypothesis" differs from the traditional concept, which assumes that latent *M. tuberculosis* remain dormant for many years, while retaining a potential to resuscitate into active TB.

Chronic production of new granulomas has been demonstrated by the production of small new lesions (0.25 to 2.5 mm of diameter) in minipigs (with pulmonary structure similar to human's).[21] Furthermore, lesions of about 2 mm of diameter, which are too small to be detected by a routine chest X-ray, have been identified in human lungs, using High resolution CT (data not published). However, direct evidence for the "hidden trump" of *M. tuberculosis* bacilli, represented by their ability for continuous low-grade infection of aerosols would require more sensitive detection techniques.

Continuous re-infection of macrophages apparently proceeds despite pronounced T cell immunity, which accompanies LTBI. As existence of an extracellular stage seems integral for the re-infection concept, one can speculate, that antibodies against surface expressed antigens might have a protective potential for LTBI.[52]

## Fibrotic processes in the lungs

The fibrotic process is important for stabilizing cellular accumulation in granulomas. With an early onset, TNF-α and chemokines attract new macrophages and neutrophils to alveoli. Here, new macrophages take up apoptotic bodies containing bacilli, or (in larger proportion) the extracellular bacilli released from necrotized macrophages. Epithelial and endothelial cells and fibroblasts also participate in this process. These cells build a cellular architecture involving fibrin,[53] proliferation of transforming growth factor-beta (TGF-β) stimulated fibroblasts[54] and production of mainly type III collagen.[21,55] The TGF-β anti-inflammatory response may counterbalance excessive local Th1 reactions. In guinea pigs and minipigs the structure of granulomas containing necrosis is also stabilized by collagen.[56] In these animals, TGF-β transforms fibroblasts to myofibroblasts[21] which organize collagen fibers, leading to sphere-like structures that help to control the mechanical stress, induced by lung respiration. The necrotic tissue of granulomatous lesions in guinea-pigs[57] and minipigs[21] is linked to the accumulation of the apoptotic cells and phosphatidylserine-rich lipid bodies[58] from destroyed FMs.[49] Phosphatidylserine accumulation, retaining calcium and phosphate from the local blood transudate leads to calcification, particularly when the inflammatory response is reduced.[59] The mineralization process at alkaline pH is an important starvation, hypoxic and osmotic stress inducer, trapping latent *M. tuberculosis* extracellular bacilli in necrotic tissues.[60]

A different type of fibrotic process, encapsulating the granulomas using a net of intralobular septa, takes places in larger mammals, including humans.[21] Fibroblasts producing type I collagen in these septa proliferate around the granuloma, when stimulated by TGF-β. This process named as the "double patron" of fibrosis, was observed by Canetti[61] in necropsies of *M. tuberculosis*-infected subjects without active TB, whom he classified as a "benign" progression of TB infection (Fig. 1).

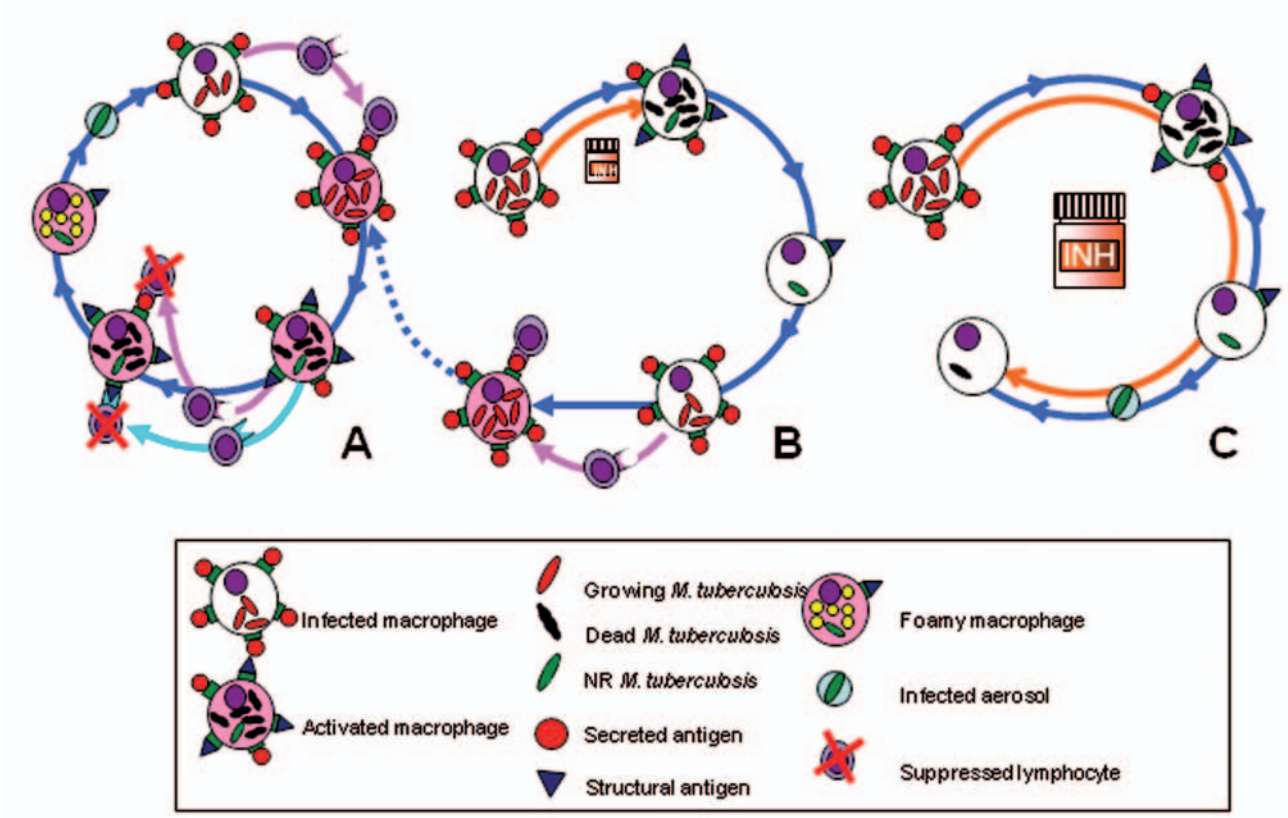


**Figure 3.** Impact of T cell immunity and isoniazid (INH) treatment on the life style of *Mycobacterium tuberculosis*. (A) Activated macrophages present secreted antigens from live *M. tuberculosis*. Negative feedback of responses against structural antigens allows the "escape and hiding" of non-replicating (NR) *M. tuberculosis*.[41] (B) Short term treatment with INH kills the growing bacilli, but leave the NR *M. tuberculosis*. Once the treatment ceases, these NR *M. tuberculosis* can reactivate. (C) Long term INH treatment kills the growing bacilli; drained NR *M. tuberculosis* avoid re-growth risk.

A strong fibrotic process also takes place around the granulomas in the hilar lymph nodes to curtail further drainage to the right atrium.

## Isoniazid-chemotherapy needs to be long-term

Slow metabolism is one of the most important, fundamental features of *M. tuberculosis* bacilli. It leads to a slow cell division cycle and thus favors bacterial persistence. Since INH chemotherapy is bactericidal only at a certain stage of the cycle, it has to be delivered for a long period (Fig. 3). However, INH by its bactericidal action and by inducing a stress response[62] in bacilli also reduces the occurrence of infected DCs and of the induction of T cell mediated surveillance of new infected cells. Nevertheless, *M. tuberculosis* has a possibility of returning and re-growing in the lungs, if INH is discontinued. The longer the INH treatment period, the lower the probability of bacillary re-growth: a 6 months treatment has about 60% efficacy and 9 months 90% efficacy.[63] During that period, latent *M. tuberculosis* would be removed, returned to the parenchyma, but could not re-grow in the presence of INH. Drainage of macrophages through the alveolar fluids and their removal through the gastrointestinal tract would hinder the return of viable bacteria to the lung parenchyma.

## Association of tuberculosis with the topography of the lungs

The upper lobes of the lung are known to be a predilected site for cavitary lesions, harbouring extracellular bacilli in adult patients with reactivated TB.[64] Reactivation of latent *M. tuberculosis* bacilli when reaching an upper lobe, could involve a number of different mechanisms, none of which is fully understood. These could be related to increased speed of bacillary growth, caused by: *a)* high oxygen pressure;[65-67] *b)* the more discrete net of capillaries; and *c)* less acidity in upper lobes; all these factors may be somehow unfavourable to immune surveillance. A burst of inflammation with high IFN-γ/TNF-α ratio[53] in response to bacterial replication would then interfere with the development of fibrosis. Lung lesions would develop into cavities, under influence of increased levels of plasmin, generated from plasminogen, trapped at the bacillary cell wall.[68,69] The stronger mechanical ventilation of the upper lobes, leading to liquefaction, will impair the fibrotic structure of granulomas.[70]

According to the "damage framework" concept of infectious diseases,[71] the development of TB could be favoured by either a too strong or too weak host responses to the infection. In the first instance, an excessive IFN-γ level could interfere with the production of fibrin, thus inducing liquefaction. This can lead to extracellular growth of the *M. tuberculosis* bacilli, enhancing the local inflammatory response and tissue destruction. Eventually, large cavities eroding the bronchi would drain out massive numbers of bacilli, thus generating highly contagious aerosols (Fig. 4).

In immunosuppressed patients however, bacilli grow diffusely, without predilection to the upper lobe, inducing weak inflammation and less liquefaction and the aerosols are less contagious.[72] Malnutrition interferes with the development of both innate and acquired immunity, because they require a large supply of nutrients;[73] consequently, this is a significant factor that favors TB incidence, particularly in poor-resource countries.

It has previously been proposed, that the evolutionary advantage of tubercle bacilli rests in efficient transmission, rather than in killing of the infected host.[74] From this angle, infection of immunocompetent individuals appears to be to the best advantage of the tubercle bacilli.

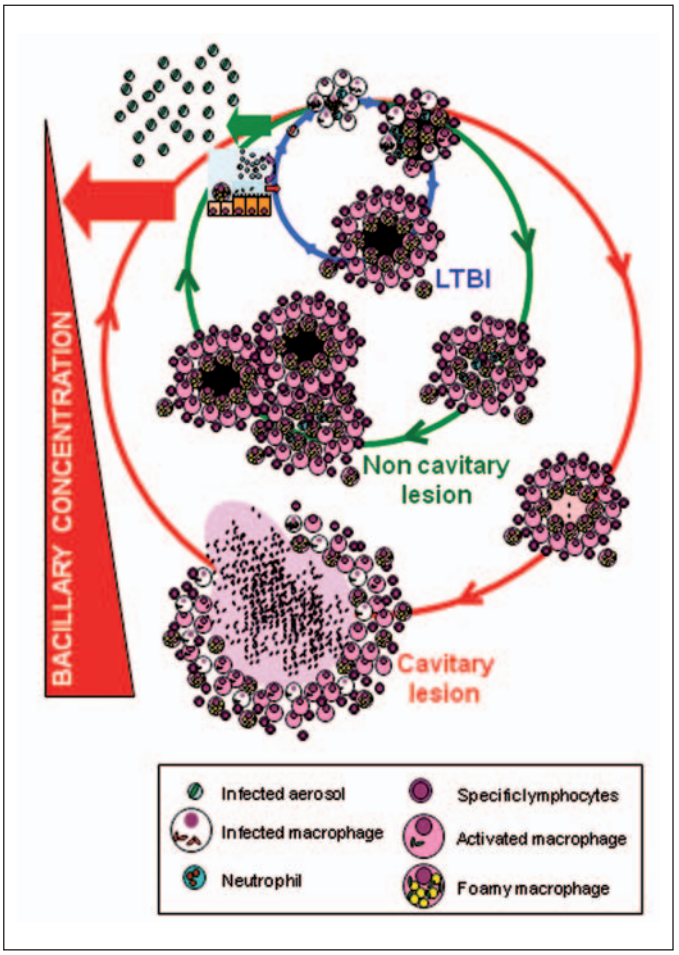


**Figure 4.** Transmission of *Mycobacterium tuberculosis* infection. Latent tuberculosis infection (LTBI) (green circles) results from protracted endogenous re-infection of macrophages from drained non-replicating (NR) *M. tuberculosis*. Aerosol spread of drained NR *M. tuberculosis* to susceptible hosts occurs from cavitary (red circles), and less frequently from non-cavitary (e.g. immunocompromised patients) (green circles) granuloma lesions. Symbols: black, necrotic tissue; pink, liquefacted tissue.

Moreover, the predilection to the lungs seems mandatory for transmission, while extrapulmonary localization of lesions (about 10% of active TB) is a wasted option for the pathogen's expansion as a microbial species

## Conclusions

Persistance of infection with *M. tuberculosis* leading to tuberculosis symbolically resembles the "mysteries", eluded to in the 1 Corinthians (see motto). We discussed here the trumps played by the bacillus and the tenacity of the infected host's resistance. The bacterial response to the stress, encountered with the infected host cells drives the *M. tuberculosis* cell cycle into a non-replicating (latent, dormant) mode and a low metabolic rate. Latent bacilli retain a capacity to induce necrotic granulomas in the lungs and to survive embedded in the necrotic tissue for long periods. The flow of draining fluids in cases of active TB re-infects the original host and also transmits the infection to new susceptible hosts. Though the host immune reactions are not capable to destroy *M. tuberculosis* bacilli, the host tries at least draining them out by the flow of alveolar fluids. However, this reverse flow is harnessed in favor of the pathogen through continuous re-infection of the lungs. Eventually, a mineralization process, leading to fibrosis of the connective tissue of the lungs counters the pathogen. This encapsulates the lesions and a limits both outward and reverse drainage. However, continuous re-infection by the surviving *M. tuberculosis* persists. In conclusion, the trumps used in the intricate contest between *M. tuberculosis* and humans have been perfected over millions of years of evolution. Better understanding of the formidable natural resilience of the *M. tuberculosis* bacillus in relation to humans gives at least some clue, why it evaded so far elimination by the various used strategies. Better understanding of these factors is mandatory for developing more effective means of control for the many, still vulnerable populations around the world.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References

1. Gutiérrez M, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, et al. Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. PLoS Pathog. 2005;1:e5.
2. WHO. Global tuberculosis control: epidemiology, strategy, financing: WHO report 2009. Geneva: World Health Organization; 2009.
3. Ivanyi J. Pathogenic and protective interactions in mycobacterial infections. In: Britten V, Hughes HPA, editors. Immunological recognition of altered cell surfaces in infection and disease. London: WB Saunders; 1986. p. 127-57.
4. Ivanyi J. Local immune responses in tuberculosis. In: Mestecky J, editor. Mucosal Immunology. Amsterdam, London: Elsevier Academic Press; 2005. p. 1465-74.
5. Mastorides SM, Oehler R, Greene J, Sinnott JT, Kranik M, Sandin RL, et al. The detection of airborne *Mycobacterium tuberculosis* using micropore membrane air sampling and polymerase chain reaction. Chest. 1999;115:19-25.
6. Gatfield J, Pieters J. Essential role for cholesterol in entry of mycobacteria into macrophages. Science. 2000;288:1647-50.
7. Kang PB, Azad AK, Torrelles JB, Kaufman TM, Beharka A, Tibesar E, et al. The human macrophage mannose receptor directs *Mycobacterium tuberculosis* lipoarabinomannan-mediated phagosome biogenesis. J Exp Med. 2005;202:987-99.
8. Torrelles J, Knaup R, Kolareth A, Slepushkina T, Kaufman T, Kanf P, et al. Identification of *Mycobacterium tuberculosis* clinical isolates with altered phagocytosis by human macrophages due to a truncated lipoarabinomannan. J Biol Chem. 2008;283:31417-28.
9. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, et al. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guérin mutants that secrete listeriolysin. J Clin Invest. 2005;115:2472-9.
10. Kusner D, Barton J. ATP stimulates human macrophages to kill intracellular virulent *Mycobacterium tuberculosis* via calcium-dependent phagosome-lysosome fusion. J Immunol. 2001;167:3308-15.
11. Xu J, Laine O, Masciocchi M, Manoranjan J, Smith J, Du SJ, et al. A unique *Mycobacterium* ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. Mol Microbiol. 2007;66:787-800.
12. Liu PT, Modlin RL. Human macrophage host defense against *Mycobacterium tuberculosis*. Curr Opin Immunol. 2008;20:371-6.
13. Chen M, Gan H, Remold HG. A mechanism of virulence: virulent *Mycobacterium tuberculosis* strain H37Rv, but not attenuated H37Ra, causes significant mitochondrial inner membrane disruption in macrophages leading to necrosis. J Immunol. 2006;176:3707-16.
14. Loeuillet C, Martinon F, Pérez C, Muñoz M, Thome M, Meylan PR. *Mycobacterium tuberculosis* subverts innate immunity to evade specific effectors. J Immunol, 2006;177:6245-55.
15. Mustafa T, Wiker HG, Morkve O, Sviland L. Reduced apoptosis and increased inflammatory cytokines in granulomas caused by tuberculous compared to non-tuberculous mycobacteria: role of MPT64 antigen in apoptosis and immune response. Clin Exp Immunol. 2007;150:105-13.
16. Park JS, Tamayo MH, González-Juarrero M, Orme IM, Ordway DJ. Virulent clinical isolates of *Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. J Leukoc Biol. 2006;79:80-6.
17. Ríos-Barrera VA, Campos-Peña V, Aguilar-León D, Lascurain LR, Meraz-Ríos MA, Moreno J, et al. Macrophage and T lymphocyte apoptosis during experimental pulmonary tuberculosis: their relationship to mycobacterial virulence. Eur J Immunol. 2006;36:345-53.
18. Gan H, Lee J, Ren F, Chen M, Kornfeld H, Remold HG. *Mycobacterium tuberculosis* blocks crosslinking of annexin-1 and apoptotic envelope formation on infected macrophages to maintain virulence. Nat Immunol. 2008;9:1189-97.
19. Lee J, Remold H, Ieong M, Kornfeld H. Macrophage apoptosis in response to high intracellular burden of *Mycobacterium tuberculosis* is mediated by a novel caspase-independent pathway. J Immunol. 2006;176:4267-74.
20. Cáceres N, Tapia G, Ojanguren I, Altare F, Gil O, Pinto S, et al. Evolution of foamy macrophages in the pulmonary granulomas of experimental tuberculosis models. Tuberculosis (Edinb). 2009;89:175-82.
21. Gil O, Díaz I, Vilaplana C, Tapia G, Díaz J, Fort M, et al. Granuloma encapsulation is a key factor for controlling latent tuberculosis infection in mini-pigs. PLoS One. 2010;5:e10030.
22. Bui T, Dabdub D, George S. Modeling bronchial circulation with application to soluble gas exchange: description and sensitivity analysis. J Appl Physiol. 1998;84:2070-88.

23. Ramachandra L, Smialek J, Shank S, Convery M, Boom WH, Harding CV. Phagosomal processing of *Mycobacterium tuberculosis* antigen 85B is modulated independently of mycobacterial viability and phagosome maturation. Infect Immun. 2005;73:1097-105.
24. Riedel D, Kaufmann S. Chemokine secretion by human polymorphonuclear granulocytes after stimulation with *Mycobacterium tuberculosis* and lipoarabinomannan. Infect Immun. 1997;65:4620-3.
25. Tobian A, Potter N, Ramachandra L, Pai R, Convery M, Boom WH, et al. Alternate class I MHC antigen processing is inhibited by Toll-like receptor signaling pathogen-associated molecular patterns: *Mycobacterium tuberculosis* 19-kDa lipoprotein, CpG DNA, and lipopolysaccharide. J Immunol. 2003;171:1413-22.
26. Cardona PJ. The granuloma in tuberculosis: friend or foe? (towards the resolution of the Citadel paradox). 11th International Symposium of KU Leuven. Leuven, Belgium, March 2010.
27. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P. The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell. 1989; 56:731-40.
28. Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ. TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol. 2002;168:4620-7.
29. Algood HM, Lin PL, Flynn JL. Tumor necrosis factor and chemokine interactions in the formation and maintenance of granulomas in tuberculosis. Clin Infect Dis. 2005;41 Suppl 3:S189-93.
30. Saunders BM, Britton WJ. Life and death in the granuloma: immunopathology of tuberculosis. Immunol Cell Biol. 2007;85:103-11.
31. Gil O, Guirado E, Gordillo S, Díaz J, Tapia G, Vilaplana C, et al. Intragranulomatous necrosis in lungs of mice infected by aerosol with *Mycobacterium tuberculosis* is related to bacterial load rather than to any one cytokine or T cell type. Microbes Infect. 2006;8:628-36.
32. Martineau AR, Newton SM, Wilkinson KA, Kampmann B, Hall BM, Nawroly N, et al. Neutrophil-mediated innate immune resistance to mycobacteria. J Clin Invest. 2007;117:1988-94.
33. Zhang X, Majlessi L, Deriaud E, Leclerc C, Lo-Man R. Coactivation of Syk kinase and MyD88 adaptor protein pathways by bacteria promotes regulatory properties of neutrophils. Immunity. 2009;31:761-71.
34. Feng CG, Kaviratne M, Rothfuchs AG, Cheever A, Hieny S, Young HA, et al. NK cell-derived IFN-γ differentially regulates innate resistance and neutrophil response in T cell-deficient hosts infected with *Mycobacterium tuberculosis*. J Immunol. 2006;177:7086-93.
35. Serbina N, Jia T, Hohl T, Pamer E. Monocyte-mediated defense against microbial pathogens. Annu Rev Immunol. 2008;26:421-52.
36. Wolf AJ, Desvignes L, Linas B, Banaiee N, Tamura T, Takatsu K, et al. Initiation of the adaptive immune response to *Mycobacterium tuberculosis* depends on antigen production in the local lymph node, not the lungs. J Exp Med. 2008;205:105-15.
37. North RJ, Jung YJ. Immunity to tuberculosis. Annu Rev Immunol. 2004;22:599-623.
38. Gill WP, Harik NS, Whiddon MR, Liao RP, Mittler JE, Sherman DE. A replication clock for *Mycobacterium tuberculosis*. Nat Med. 2009;15:211-14.
39. Wallace JG. The heat resistance of tubercle bacilli in the lungs of infected mice. Am Rev Respir Dis. 1961;83:866-71.
40. Muñoz-Elías E, Timm J, Botha T, Chan W, Gómez J, McKinney JD. Replication dynamics of *Mycobacterium tuberculosis* in chronically infected mice. Infect Immun. 2005;73:546-51.
41. Andersen P. Host responses and antigens involved in protective immunity to *Mycobacterium tuberculosis*. Scand J Immunol. 1997;45:115-31.
42. Cardona PJ, Gordillo S, Díaz J, Tapia G, Amat I, Pallarés A, et al. Widespread bronchogenic dissemination makes DBA/2 mice more susceptible than C57BL/6 mice to experimental aerosol infection with *Mycobacterium tuberculosis*. Infect Immun. 2003;71:5845-54.
43. Cardona PJ, Llatjos R, Gordillo S, Díaz J, Ojanguren I, Ariza A, et al. Evolution of granulomas in lungs of mice infected aerogenically with *Mycobacterium tuberculosis*. Scand J Immunol. 2000;52:156-63.
44. D'Avila H, Roque NR, Cardoso RM, Castro-Faria-Neto HC, Melo RC, Bozza PR. Neutrophils recruited to the site of *Mycobacterium* bovis BCG infection undergo apoptosis and modulate lipid body biogenesis and prostaglandin E production by macrophages. Cell Microbiol. 2008;10:2589-604.
45. Hemsworth GR, Kochan I. Secretion of antimycobacterial fatty acids by normal and activated macrophages. Infect Immun. 1978;19:170-7.
46. Leemans JC, Thepen T, Weijer S, Florquin S, Van Rooijen N, Van de Winkel JG, et al. Macrophages play a dual role during pulmonary tuberculosis in mice. J Infect Dis. 2005;191:65-74.
47. Martínez D, Vermeulen M, Von Euw E, Sabatté J, Maggíni J, Ceballos A, et al. Extracellular acidosis triggers the maturation of human dendritic cells and the production of IL-12. J Immunol. 2007;179:1950-9.
48. Chisolm GM 3rd, Hazen SL, Fox PL, Cathcart MK. The oxidation of lipoproteins by monocytes-macrophages. Biochemical and biological mechanisms. J Biol Chem. 1999;274:25959-62.
49. Russell DG, Cardona PJ, Kim MJ, Allain S, Altare F. Foamy macrophages and the progression of the human tuberculosis granuloma. Nat Immunol. 2009;10: 943-8.
50. Sapoval B, Filoche M, Weibel ER. Smaller is better—but not too small: a physical scale for the design of the mammalian pulmonary acinus. Proc Natl Acad Sci USA. 2002; 99:10411-6.
51. Jung Y, Ryan L, LaCourse R, North R. Properties and protective value of the secondary versus primary T helper type 1 response to airborne *Mycobacterium tuberculosis* infection in mice. J Exp Med. 2005;201:1915-24.
52. Cardona PJ. A dynamic reinfection hypothesis of latent tuberculosis infection. Infection. 2009;37:80-6.
53. Mullarky IK, Szaba FM, Berggren KN, Kummer LW, Wilhelm LB, Parent MA, et al. Tumor necrosis factor alpha and gamma interferon, but not hemorrhage or pathogen burden, dictate levels of protective fibrin deposition during infection. Infect Immun. 2006;74:1181-8.
54. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM, et al. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest. 1998;101:890-8.
55. Fraser RS. Histology and gross anatomy of the respiratory tract. In: Hamid Q, Shannon J, Martin J, editors. Physiologic basis of respiratory disease. Hamilton: B.C. Decker Inc; 2005.
56. Plopper CG, Harkema JR. The respiratory system and its use in research. The laboratory primate. 2005;30:503-26.
57. Lenaerts AJ, Hoff D, Aly S, Ehlers S, Andries K, Cantarero L, et al. Location of persisting mycobacteria in a Guinea pig model of tuberculosis revealed by r207910. Antimicrob Agents Chemother. 2007;51:3338-45.
58. Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest. 2002;109:41-50.
59. Torday JS, Rehan VK. The evolutionary continuum from lung development to homeostasis and repair. Am J Physiol Lung Cell Mol Physiol. 2007;292:L608-11.
60. Hsu HH, Abbo BG. Role of bicarbonate/CO2 buffer in the initiation of vesicle-mediated calcification: mechanisms of aortic calcification related to atherosclerosis. Biochim Biophys Acta. 2004;1690:118-23.
61. Canetti G. The tubercle bacillus in the pulmonary lesion of man. Histobacteriology and its bearing on the therapy of pulmonary tuberculosis. New York: Springer Publishing Company; 1955.
62. Rieder R, Zhao Z, Zavizion B. New approach for drug susceptibility testing: monitoring the stress response of mycobacteria. Antimicrob Agents Chemother. 2009;53:4598-603.
63. Comstock G. How much isoniazid is needed for prevention of tuberculosis among immunocompetent adults? Int J Tuberc Lung Dis. 1999;3:847-50.
64. Balasubramanian V, Wiegeshaus E, Taylor B, Smith D. Pathogenesis of tuberculosis: pathway to apical localization. Tuber Lung Dis. 1994;75:168-78.
65. Vargas M, Furuya M, Pérez-Guzmán C. Effect of altitude on the frequency of pulmonary tuberculosis. Int J Tuberc Lung Dis. 2004;8:1321-4.
66. Park MK, Myers RA, Marzella L. Oxygen tensions and infections: modulation of microbial growth, activity of antimicrobial agents, and immunologic responses. Clin Infect Dis. 1992;14:720-40.
67. Milic-Emili J. Ventilation distribution. In: Hamid Q, Shannon J, Martin JG, editors. Physiologic basis of respiratory disease. Hamilton, ON, Canada: BC Decker; 2005. p. 133-41.
68. Monroy V, Amador A, Ruiz B, Espinoza-Cueto P, Xolalpa W, Mancilla R, et al. Binding and activation of human plasminogen by *Mycobacterium tuberculosis*. Infect Immun. 2000;68:4327-30.
69. Xolalpa W, Vallecillo A, Lara M, Mendoza-Hernández G, Comini M, Spallek R, et al. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. Proteomics. 2007;7:3332-41.
70. West J, Matthews F. Stresses, strains, and surface pressures in the lung caused by its weight. J Appl Physiol. 1972;32:332-45.
71. Casadevall A, Pirofski LA. The damage-response framework of microbial pathogenesis. Nat Rev Microbiol. 2003;1:17-24.
72. Pepper T, Joseph P, Mwenya C, McKee G, Haushalter A, Carter A, et al. Normal chest radiography in pulmonary tuberculosis: implications for obtaining respiratory specimen cultures. Int J Tuberc Lung Dis. 2008;12:397-403.
73. Schwenk A, Macallan D. Tuberculosis, malnutrition and wasting. Curr Opin Clin Nutr Metab Care. 2000;3:285-91.
74. Doenhoff MJ. Granulomatous inflammation and the transmission of infection: schistosomiasis--and TB too? Immunol Today. 1998;19:462-7.

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# Tuberculosis in special populations

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ABSTRACT

The susceptibility to infection, the pathogenesis and the clinical manifestations of tuberculosis (TB) depend on the immunological status of the host. Immunological status is largely determined by age and comorbidities, but is also affected by other less well known factors. In Spain, most incidental cases of TB arise from the reactivation of remotely acquired latent infections and are favored by the aging of the population and the use of aggressive immunosuppressive therapies. The diagnosis and management of TB in these circumstances is often challenging. On the one hand, the atypical presentation with extrapulmonary involvement may delay diagnosis, and on the other, the toxicity and interactions of the antituberculous drugs frequently make treatment difficult. Immigration from resource-poor, high incidence TB countries, where the social and economic conditions are often suboptimal, adds a new challenge to the control of the disease in Spain. This chapter summarizes our current knowledge of epidemiological, clinical and treatment aspects of TB in particularly susceptible populations.



*Palabras clave:*
Tuberculosis
Inmunosupresión
Comorbilidad
Inmigración

## Tuberculosis en poblaciones especiales

RESUMEN

La susceptibilidad a la infección, patogenia y manifestaciones clínicas de la tuberculosis (TB) dependen de la situación inmunológica del hospedador, lo cual, a su vez, está determinado en gran medida por la edad y las comorbilidades, pero también por otros factores no bien conocidos. La mayor parte de casos nuevos de TB en España tiene su origen en la reactivación de una infección remota latente, y es favorecida por el envejecimiento y las terapias inmunosupresoras agresivas. A menudo, el diagnóstico y tratamiento de la TB en este contexto representan un reto. Las presentaciones atípicas, con afectación extrapulmonar, pueden retrasar el diagnóstico, pero además la toxicidad y las interacciones de los fármacos antituberculosos, a menudo, dificultan el tratamiento. La inmigración de países en vías de desarrollo y alta incidencia de TB, frecuentemente con condiciones sociales y económicas desfavorables, añade un nuevo reto al control de la enfermedad en España. En este capítulo se resume el conocimiento actual acerca de los aspectos epidemiológicos, clínicos y terapéuticos de la TB en poblaciones especialmente susceptibles.



## Introduction

The pathogenesis of tuberculosis is closely related to immunity. The host's immune response plays a key role in the proliferation and spread of the tubercle bacilli, and in the tissue damage that is responsible for the clinical manifestations of the disease. Therefore, both the susceptibility of progression to tuberculosis after infection and the degree of dissemination of the primary infection are directly determined by the age and comorbidity of the patient affected[1] (Table 1).

In developed countries, immigrants and refugees born in countries with a high endemicity of tuberculosis make up a significant proportion of incident cases of the disease. As a result, the implementation of adequate policies for rapid detection of active cases and prophylaxis for high risk immigrants is essential in tuberculosis prevention and control.

## Tuberculosis in immunosuppressed people

### *Tuberculosis and human immunodeficiency virus infection*

The convergence of the human immunodeficiency virus (HIV) epidemic and tuberculosis has had a considerable impact on morbidity and mortality worldwide. In some areas of sub-Saharan

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**Table 1**
Risk for developing active tuberculosis relative to a control population[1]

| Condition | Relative risk |
|---|---|
| Silicosis | 30 |
| Diabetes mellitus | 2-4 |
| Chronic renal failure-hemodialysis | 10-25 |
| Jejunoileal by-pass | 27-63 |
| Renal transplantation | 37 |
| Heart transplantation | 20-74 |
| Head and neck cancer | 16 |
| HIV infection | 20-100 |

HIV: human immunodeficiency virus.

Africa, annual notification rates of tuberculosis are above 400 cases per 100,000 population, and more than two-thirds of patients are co-infected with HIV.[2]

HIV infection produces cellular immunodeficiency by depleting $CD4^+$ lymphocyte cells and impairing macrophage function, which increases the risk of infection with *M. tuberculosis*, progression to active disease, and reactivation of latent infection (LTBI). The occurrence of multidrug-resistant epidemics among HIV groups reflects the extreme vulnerability of these patients to tuberculosis infection and disease.[3,4] The clinical presentation of tuberculosis in HIV-infected patients varies with the immunological status of the host. Patients with early HIV infection frequently present a "classic" pattern of pulmonary tuberculosis. Conversely, in patients with low CD4 cell counts, extrapulmonary involvement and disseminated disease are frequent even though chest radiographs may be normal.[5,6]

HIV-related tuberculosis should be treated with a regimen including rifamycin for the full course of therapy. If a rifamycin cannot be given, treatment should be extended to 18-24 months. The rifamycins induce the cytochrome P450-3A (CYP3A) system, increasing the metabolism of most antiretroviral drugs. Rifabutin is a less potent inhibitor than rifampin and may be a reasonable alternative.[7] Table 2 shows the interactions between rifamycins and antiretrovirals and recommended dose adjustments. Although increased rates of recurrence have been reported with the standard regimen of six months in HIV-infected patients, there is insufficient evidence to recommend more prolonged therapy in this setting.[8,9] Regardless of HIV status, patients with cavitation and positive cultures after the first two months of therapy should be treated for nine months.[9] Intermittent treatment with rifampin twice a week or rifapentine weekly is not recommended in HIV patients because of the development of resistance to rifampin, especially in patients with CD4 cell counts less than 100 cells/$mm^3$.[10,11]

The ideal time to start antiretroviral therapy in HIV-infected patients with tuberculosis when both are diagnosed simultaneously has not been definitively established. Deferring antiretroviral therapy until treatment of tuberculosis has been completed avoids potential drug interactions, overlapping drug toxicities, and paradoxical reactions. However, this strategy is not safe in patients with low CD4 cell counts, in whom delaying treatment may increase mortality due to opportunistic infections and AIDS progression.[12,13] The current ATS/CDC/IDSA guidelines suggest an individualized decision on timing of antiretroviral therapy in patients with CD4 counts below 350 cells/$mm^3$ and advise delaying antiretroviral therapy for 4–8 weeks if possible.[9]

The paradoxical worsening of symptoms known as the "immune reconstitution inflammatory syndrome" (IRIS) is a frequent complication when antiretroviral therapy is started in patients being treated for active tuberculosis.[14] Immune reconstitution symptoms typically appear in the first days or weeks of treatment and frequently affect patients with low CD4 cell counts.[15] A short course of steroids may be of benefit in these cases. IRIS may also explain the development of tuberculosis in patients with subclinical or latent infection and low CD4 cell counts presenting with clinical symptoms of the disease shortly after initiating antiretroviral therapy.[16]

**Table 2**
Interactions between rifampin-rifabutin and antiretroviral drugs

| Antiretroviral | Rifampin (RIF) | Rifabutin (RFB) |
|---|---|---|
| *Protease inhibitors (PI)* | | |
| Atazanavir | | RFB AUC ↑ 2-3 fold |
| Ritonavir-atazanavir | PI concentrations markedly decreased (>75%): co-administration contraindicated | Decrease RFB to 150 mg/48 h or 3 times a week |
| Ritonavir-lopinavir | | No change in PI dose |
| Ritonavir-saquinavir | | |
| Ritonavir-fosamprenavir | | |
| Ritonavir-darunavir | | |
| *Non-nucleoside reverse transcriptase inhibitors (NNRTI)* | | |
| Nevirapine (NVP) | NVP AUC ↓ 37%; NVP dose unchanged; RIF dose unchanged[a] | NVP $C_{min}$ ↓ 16%; RFB AUC ↑ 17%; NVP dose unchanged; RFB unchanged[b] |
| Efavirenz (EFV) | EFV AUC ↓ 26%; EFV dose unchanged; consider 800 mg/d; RIF dose unchanged | RFB ↓ 38%; EFV dose unchanged; RFB 450-600 mg/d if EFV is not coadministered with a PI |
| Etravirine (ETR) | ETR concentration markedly decreased: do not coadminister | ETR AUC ↓ 37%; RFB ↓ 17%; no dose adjustment unless PI co-administration |
| *Entry inhibitors* | | |
| Enfuvirtide (T20) | No significant interaction: dose unchanged | No significant interaction: dose unchanged |
| Maraviroc (MVC) | MVC ↓ 78%: Increase MRV dose 600 mg/12 h; RIF dose unchanged | No clinical experience; a significant interaction is unlikely |
| *Integrasa inhibitors* | | |
| Raltegravir (RAL) | RAL AUC ↓ 40% and $C_{min}$ ↓ 61% with RAL 400 mg Rifampin with RAL 800 mg BID compared with RAL 400 mg BID alone: RAL AUC ↑ 27% and $C_{min}$ ↓ 53% | Dose: RAL 800 mg BID |

[a]Recent issued guidelines recommend do not coadminister. If coadministration is needed, use 600 mg BID. Available at: http://aidsinfo.nih.gov
[b]Standard rifabutin dose: 300 mg/day or 300 mg/48 h.

HIV-infected people should be screened for LTBI with the TST. Reactors to TST must be thoroughly evaluated, and once active disease has been ruled out preventive chemotherapy must be offered. Although early studies suggested that HIV-seropositive patients with cutaneous anergy had a risk of tuberculosis similar to that of patients with positive TST, nowadays the evidence argues against treating anergic HIV-infected patients.[17] The role of interferon–gamma release assays (IGRA) either in addition to TST or instead of it is still controversial.[18] Daily isoniazid for 9 months remains the treatment of choice for LTBI in HIV-infected patients. Multidrug short-course regimens (rifampin plus pyrazinamide for 2 months, and rifampin plus isoniazid for 3 months) have proved as effective as standard isoniazid treatment in HIV-infected patients.[1,19,20] However, the combination of rifampin and pyrazinamide is no longer recommended due to the unacceptable rate of severe liver toxicity.[21] Rifampin or rifabutin for four months are also good alternatives.

*Tuberculosis in transplant recipients*

The incidence of tuberculosis in either solid organ or hematopoietic progenitor cell recipients is closely related to the background of tuberculosis infection in the area considered, and it is estimated to be 30 to 100-fold higher than in the general population.[22] A prospective cohort study[23] in Spain found an incidence was 0.48% and an estimated relative risk in relation to the general population of 26.6.

Most cases of tuberculosis in transplant recipients arise from a reactivation of remote infection. Rarely, tuberculosis is transmitted by the transplanted graft. Most cases of tuberculosis develop in the first year after transplantation. Late infections may be related to the treatment of chronic rejection or graft versus host disease in allogenic bone marrow recipients.[23,24]

Treatment of tuberculosis in transplant recipients may be complicated by the potential of drug interactions and toxicity. Rifampin interacts with most immunosuppressive drugs, potentially leading to graft rejection. The experience in renal transplants has been favorable provided that levels of immunosuppressive drugs are carefully monitored.[25] There is no consensus on other solid organ or hematopoietic progenitor cell transplants. Experience with rifabutin, a weaker *cytochrome* P-450 inducer, is scarce. Regimens not containing a rifamycin must be prolonged up to 18-24 months. The Spanish network for transplant related infections (RESITRA) recommends the use of a rifamycin-based regimen in solid organ transplants which develop a severe or disseminated form of tuberculosis or if isoniazid resistance is suspected.[26]

Pre-transplant evaluation of candidates for transplantation should include screening for tuberculosis, and treatment of patients with positive TST and those with evidence of inappropriately treated or untreated past tuberculosis. Treatment of latent infection in candidates for liver transplantation is challenging as the risk of severe liver toxicity is very high in patients with end stage liver disease. Some authors recommend postponing chemoprophylaxis until after transplantation.[26]

*Tuberculosis in anti-TNF treated patients*

Anti-tumor necrosis factor (TNF) drugs are used in a growing number of immune-mediated inflammatory diseases (IMID). The main drawback of suppressing TNF activity is the blockage of the immune response to infection. Not surprisingly, several opportunistic infections have been reported in association with the use of these agents, such as systemic mycosis and mycobacterial infections.[27] The first descriptions of tuberculosis associated with anti-TNF agents were in patients treated with infliximab, and showed that more than half had extrapulmonary and disseminated infections.[28] Later series found similar features of tuberculosis in other anti-TNF treated patients.[29] After recommendations of screening and chemoprophylaxis for candidates to anti-TNF treatment were issued, the incidence of tuberculosis fell notably.[30] Candidates for anti-TNF therapy and diagnosed with LTBI should receive either isoniazid for nine months or rifampin for four months.[31] There is also some experience with IGRA in patients with IMID for the screening of tuberculosis infection before anti-TNF therapy. The currently available data suggest that these tests perform better than TST in individuals receiving immunosuppressive treatment, but false-negative and indeterminate results also occur.[32,33]

*Tuberculosis in the pediatric patients*

The burden of childhood tuberculosis has been somewhat neglected in the past. However, of the estimated 8.3 million new tuberculosis cases diagnosed in 2000, 884,019 (11%) were children.[34]

Most cases of pediatric tuberculosis are acquired through inhalation of bacilli from a person with active tuberculosis. Rarely, transplacental infection may occur after acute primary or miliary tuberculosis during pregnancy. Neonatal infection may very occasionally be acquired by fetal aspiration or ingestion of infected amniotic fluid. The risk of developing active tuberculosis in children varies with age: infants in the first year of life have the highest risk (40-50%), and also the most severe forms (10 to 20% miliary tuberculosis or meningitis). The risk of progression is still high in children infected up to the fifth year of life, and then falls between 5 and 10 years of age.

Primary lung disease may be asymptomatic and may resolve spontaneously. The classic form of primary lung disease is a parenchymal infiltrate with hilar lymphadenopathy. Progression of primary tuberculosis leads to lung consolidation, lymph node enlargement with bronchial obstruction, and pleural disease.[35] Common symptoms of pulmonary tuberculosis in children include chronic cough, prolonged fever and weight loss or failure to thrive.[36] Complications related to massive lymph node enlargement are more frequent in children under five years of age. Lung cavitation is rare in the under-tens. Peripheral lymphatic tuberculosis and meningitis are the most common forms of extrapulmonary disease. Microbiologic confirmation of tuberculosis in children is hampered by the difficulty of obtaining appropriate respiratory secretions.[35] Sputum smears are positive in less than 20% of cases, and culture confirmation is achieved in less than half of these. The yield of 3 consecutive early morning gastric aspirates is higher than bronchoalveolar lavage in the diagnosis of pulmonary pediatric tuberculosis.[37] Nasopharyngeal aspiration and induced sputum with hypertonic saline aerosol are good alternatives.[38]

A six-month regimen of isoniazid and rifampin with pyrazinamide during the initial phase is effective in most forms of pulmonary or extrapulmonary disease. In meningeal and disseminated tuberculosis, as well as in HIV-infected children, treatment should be extended to 9-12 months.[9] Ethambutol is contraindicated in children less than 13 years, in whom ophthalmological monitoring may not be sufficient to exclude optical neuropathy. However, in the experience reported with 15-20 mg/kg daily doses in young children, optical toxicity is exceptional.[39]

Children are high priority targets of preventive chemotherapy after contact with active tuberculosis. Household exposure must be treated as infection from the first moment onwards, as tuberculin response may be delayed for up to 3 months. Treatment may be stopped if the second TST remains negative. In immunosuppressed children TST is not sensitive enough to rule out infection, and therefore chemotherapy must be completed for the whole nine-month course despite the lack of conversion. Isoniazid is well tolerated, and severe liver toxicity is extremely rare. As in adults, rifampin is the alternative of choice in intolerant children or after contact with isoniazid resistant strains.[40]

*Tuberculosis in pregnancy and breastfeeding*

The stress and the physiological changes experienced in pregnancy create a state of cellular immunodeficiency that may facilitate the development of tuberculosis.[41] In the early 20th century, after a publication that reported a less favorable course of tuberculosis in pregnant than in non-pregnant women, therapeutic interruption of the pregnancy was recommended. Nowadays, it is clear that if anti-tuberculosis treatment is started early, the outcome is the same as in non-pregnant women.[42,43]

Diagnosis may be delayed because certain manifestations of pregnancy, such as asthenia, tachycardia and anemia, and manifestations of tuberculosis may confuse clinicians. Diagnosis of tuberculosis infection relies on TST, which is valid in pregnancy and is safe for the woman and fetus. New diagnostic blood tests (IGRA) can be used, but they have not been evaluated in pregnant women. Chest x-ray can be performed safely by shielding the abdomen, as fetal exposure is below 0.3 mrads.[44]

Treatment for active tuberculosis in pregnant women should be started promptly since its delay has been associated with hazard for mother and fetus. The preferred regimen includes rifampin, isoniazid and ethambutol for two months, followed by rifampin and isoniazid for seven additional months. Pirazynamide is not recommended because its effect on the fetus is unknown.[9] However, no theratogenic effects have been reported; the WHO recommends it, and in fact its use is widespread in many countries. Streptomycin should not be used because of its interference with the aural development of the fetus. Due to the potential harmful effects on the fetus and the unknown risks of second-line drugs, terminating pregnancy or suspending treatment during pregnancy is frequently advised in pregnant women who require treatment with these drugs.[42] A recent retrospective study of pregnant women treated with second-line drugs for multidrug-resistant tuberculosis (MDR-TB) showed similar birth outcomes to those of healthy women and no congenital defects in newborns.[45] This experience indicates that the benefits of treating MDR-TB probably outweigh the risk for mother and fetus. Therefore, after receiving counselling concerning the potential risks, women should have the option to continue treatment without termination of pregnancy.[45]

Breastfeeding is not contraindicated in women treated with first-line antituberculous drugs. Isoniazid, rifampin, ethambutol, and pyrazinamide are considered safe for breastfeeding because the levels of these drugs achieved in breast milk are too low to produce toxicity in the baby.[46,47] Fluoroquinolones are not recommended during breastfeeding.[9]

## Tuberculosis in the frail population

*Tuberculosis in the elderly*

Incidence of tuberculosis is higher in the elderly, due to the increased prevalence of the infection and the higher rates of reactivation as a result of impairment of T-cell mediated immune response with aging. Other age-associated factors, such as malignancy and chronic diseases, also contribute to the greater risk of reactivation of latent infection.[48] In addition, nursing home residents have a two to four-fold higher incidence of tuberculosis, due not only to reactivation but also to an increased transmission in this clustering environment.[49,50]

Around 75% of elderly persons with tuberculosis present pulmonary involvement, and the clinical features are similar to those found in younger persons.[51] However, tuberculosis may present atypically: unexplained low-grade fever, fatigue, reduction in daily living activities, anemia and liver function test abnormalities being the predominant manifestations.[52] Respiratory sample collection may be limited by the difficulty of obtaining sputum from old people. In addition, the sensitivity of TST wanes with aging. Radiographic findings do not differ significantly from those in younger people, except for the lower proportion of cavitation.[52]

Current standard treatment for tuberculosis is also recommended for older people. While also applicable to young patients, two main issues are of particular relevance in the elderly: the greater risk of drug toxicities, and the risk of interactions with other drugs, since these patients are more likely to be taking other medications.[53,54] Particularly, an increased risk of hepatic toxicity from isoniazid with aging has been found.[48,55] Preventive treatment may also be indicated in elderly people.[1] Prevention of tuberculosis in long-term care facilities requires each unit to apply an appropriate prevention and control program in order to protect residents and staff.[56]

*Tuberculosis and end-stage renal disease*

Patients with end-stage renal disease (ESRD) undergoing hemodialysis are at increased risk of developing tuberculosis.[57] Diagnosis may be challenging due to false-negative TST results, more frequent extrapulmonary involvement and on occasion the non-specificity of symptoms, which may be attributable to uremia or other infections.

Since some antituberculous medications are cleared by the kidney, drug-dosing adjustment is required, which complicates treatment of tuberculosis in these patients.[9] Furthermore, some antituberculosis drugs are removed by haemodialysis.[58] Rifampin and isoniazid are metabolized by the liver, so dosing adjustment is not necessary in ESRD.[9] Pyrazinamide, which is metabolized by the liver but whose metabolites are cleared by the kidneys, and ethambutol which is 80% cleared by the kidneys, may accumulate in case of renal insufficiency and require adjustment.[9] Although isoniazid, pyrazinamide and ethambutol are removed by haemodialysis, only in the case of pyrazinamide is the dialysis significant. So, if pyrazinamide is given after dialysis, a supplementary dose is not necessary. Rifampin is not dialyzable and does not require dosing-adjustment.[9] Renal clearance of fluoroquinolones varies from drug to drug. Of the two most commonly used for tuberculosis treatment, levofloxacin is mainly cleared by kidneys; moxifloxacin undergoes hepatic metabolism, and the urinary excretion of the unchanged drug only accounts for 19-22% of the given dose.[59] Antituberculosis aminoglycosides (streptomycin, kanamycin and amikacin) and capreomycin are excreted by kidneys, and require dosing-adjustment in patients with renal insufficiency.[9] Table 3 shows the dosing adjustment for the commonest antituberculosis agents in patients with renal function impairment.

*Tuberculosis and liver disease*

Treatment of tuberculosis in patients with chronic liver disease is complicated by the increased hepatotoxicity of antituberculosis drugs, the potentially harmful consequences of toxicity in patients with marginal liver function reserve and, finally, the difficulty of monitoring drug-related alterations in liver tests.[9] In addition, the high incidence of alcohol use may lead to poor compliance and treatment failure. The first line antituberculosis agents rifampin, isoniazid and pyrazinamide may produce hepatotoxicity, but they should be used whenever possible. Careful assessment of hepatic disease and expert consultation is advisable in treating these patients with drugs or regimens other than the standard ones.[9]

*Tuberculosis and immigration*

The increase in immigration during the last decade has slowed the steady fall in the incidence of tuberculosis in Spain. The proportion of foreign-born subjects among tuberculosis patients has increased consistently since the late 1990s, reaching figures as high as 67% in some areas.[60,61]

**Table 3**
Recommended dosing adjustment of antituberculosis agents in adult patients with impaired renal function (creatinine clearance <30 ml/min) or receiving hemodialysis[a]

| Drug | Dosing adjustment |
|---|---|
| Rifampin | Not required |
| Isoniazid | Not required |
| Pyrazinamide | 25-35 mg/Kg 3 times per week |
| Ethambutol | 15-25 mg/Kg 3 times per week |
| Streptomycin | 12-15 mg/Kg two or 3 times per week[b] |
| Kanamycin | 12-15 mg/Kg two or 3 times per week[b] |
| Amikacin | 12-15 mg/Kg two or 3 times per week[b] |
| Capreomycin | 12-15 mg/Kg two or 3 times per week[b] |
| Levofloxacin | 750 mg 3 times a week |
| Moxifloxacin | Not required |
| Ethionamide | Not required |
| *p*-Aminosalicylic acid | Not required |
| Cycloserine | 500 mg 3 times per week |
| Linezolid | Not required[c] |

[a]Treatment should be taken after the dialysis session.
[b]Dose and interval according to the American Thoracic Society Guidelines.
[c]Recommended dose: 600 mg per dose once daily. Two primary metabolites may accumulate in patients with renal insufficiency, whose clinical significance is largely unknown.

It has commonly been assumed that tuberculosis in immigrants results from the reactivation of latent infection acquired in their country of origin.[62-64] However, data from two molecular epidemiology studies in Spain, in which 29% and 33% respectively of isolates from immigrant patients were clustered, suggest that recent transmission also plays a significant role in the development of tuberculosis in immigrants in our country.[60,65] Most cases of tuberculosis in immigrants occur within two or 3 years of arrival.[66,67] However, the risk not only persists beyond this point but its incidence remains higher than in the native-born population for more than five years.[68,69] Clinical and radiographic characteristics do not differ substantially from those of Spanish-born patients, except for a younger age at diagnosis.[67,70] Some authors reported a higher proportion of extrapulmonary involvement than in native-born patients.[70]

Most immigrants to Spain come from Latin American and African countries with higher rates of resistant tuberculosis.[71] Although the resistance of *M. tuberculosis* in Spain has not increased substantially in recent years, rates of resistance in immigrants are higher than in Spanish-born people.[3,66,70,72,73] In a study conducted in Madrid the rate of resistance to any first-line antituberculous drug was 33% in immigrants who had no prior treatment, compared with 10.7% among all patients with tuberculosis.[72]

Treatment for tuberculosis in immigrants should not differ from that of native-born patients, and all isolates should be tested for susceptibility to first-line antituberculous drugs. Early diagnosis and treatment are the two best preventive and control measures. Diagnosis of tuberculosis should be coupled with detection and treatment of secondarily infected people. Treatment of active disease and infection in the immigrant population presents significant challenges to tuberculosis control measures: several studies have found lower rates of treatment completion, including preventive treatment, among immigrants than in native-born people.[74,75]

How to screen people from areas with high tuberculosis rates who migrate to developed countries is a matter of debate. Strategies including chest radiograph screening and TST to detect cases of active tuberculosis have little (if any) public health impact and are not cost-effective.[76] On the other hand, detection of LTBI using the TST, and universal treatment of those with positive results is limited by the low positive predictive value of the test, low rates of treatment completion, and toxicity. Nowadays, early diagnosis and treatment of active tuberculosis cases and detection of subjects with latent infection at risk of progression seems to be the most reasonable strategy for tuberculosis control.

## Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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## References

1. Targeted tuberculin testing and treatment of latent tuberculosis infection. American Thoracic Society. MMWR Recomm Rep. 2000;49(RR-6):1-51.
2. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC, et al. The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Arch Intern Med. 2003;163:1009-21.
3. Guerrero A, Cobo J, Fortún J, Navas E, Quereda C, Asensio A, et al. Nosocomial transmission of Mycobacterium bovis resistant to 11 drugs in people with advanced HIV-1 infection. Lancet. 1997;350:1738-42.
4. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006;368:1575-80.
5. Keiper MD, Beumont M, Elshami A, Langlotz CP, Miller WT Jr. CD4 T lymphocyte count and the radiographic presentation of pulmonary tuberculosis. A study of the relationship between these factors in patients with human immunodeficiency virus infection. Chest. 1995;107:74-80.
6. Perlman DC, El-Sadr WM, Nelson ET, Matts JP, Telzak EE, Salomon N, et al. Variation of chest radiographic patterns in pulmonary tuberculosis by degree of human immunodeficiency virus-related immunosuppression. The Terry Beirn Community Programs for Clinical Research on AIDS (CPCRA). The AIDS Clinical Trials Group (ACTG). Clin Infect Dis. 1997;25:242-6.
7. Baciewicz AM, Chrisman CR, Finch CK, Self TH. Update on rifampin and rifabutin drug interactions. Am J Med Sci. 2008;335:126-36.
8. El-Sadr WM, Tsiouris SJ. HIV-associated tuberculosis: diagnostic and treatment challenges. Semin Respir Crit Care Med. 2008;29:525-31.
9. Blumberg HM, Burman WJ, Chaisson RE, Daley CL, Etkind SC, Friedman LN, et al. American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med. 2003;167:603-62.
10. Burman W, Benator D, Vernon A, Khan A, Jones B, Silva C, et al. Acquired rifamycin resistance with twice-weekly treatment of HIV-related tuberculosis. Am J Respir Crit Care Med. 2006;173:350-6.
11. Updated guidelines for the use of rifabutin or rifampin for the treatment and prevention of tuberculosis among HIV-infected patients taking protease inhibitors or nonnucleoside reverse transcriptase inhibitors. MMWR Morb Mortal Wkly Rep. 2000;49:185-9.
12. McIlleron H, Meintjes G, Burman WJ, Maartens G. Complications of antiretroviral therapy in patients with tuberculosis: drug interactions, toxicity, and immune reconstitution inflammatory syndrome. J Infect Dis. 2007;196 Suppl 1:S63-75.
13. Abdool Karim SS, Naidoo K, Grobler A, Padayatchi N, Baxter C, Gray A, et al. Timing of initiation of antiretroviral drugs during tuberculosis therapy. N Engl J Med. 2010;362:697-706.
14. Meintjes G, Lawn SD, Scano F, Maartens G, French MA, Worodria W, et al. Tuberculosis-associated immune reconstitution inflammatory syndrome: case definitions for use in resource-limited settings. Lancet Infect Dis. 2008;8:516-23.
15. Müller M, Wandel S, Colebunders R, Attia S, Furrer H, Egger M, et al. Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection: a systematic review and meta-analysis. Lancet Infect Dis. 2010;10:251-61.
16. Park WB, Choe PG, Jo JH, Kim SH, Bang JH, Kim HB, et al. Tuberculosis manifested by immune reconstitution inflammatory syndrome during HAART. AIDS. 2007;21:875-7.
17. Prevention and treatment of tuberculosis among patients infected with human immunodeficiency virus: principles of therapy and revised recommendations. Centers for Disease Control and Prevention. MMWR Recomm Rep. 1998;47(RR-20):1-58.
18. Centers for Disease Control and Prevention. Guidelines for prevention and treatment of opportunistic infections in HIV-infected adults and adolescents. MMWR Early Release. 2009;58:1-206.

19. Rose DN. Short-course prophylaxis against tuberculosis in HIV-infected persons. A decision and cost-effectiveness analysis. Ann Intern Med. 1998;129:779-86.
20. Gordin F, Chaisson RE, Matts JP, Miller C, De Lourdes García M, et al. Rifampin and pyrazinamide vs isoniazid for prevention of tuberculosis in HIV-infected persons: an international randomized trial. Terry Beirn Community Programs for Clinical Research on AIDS, the Adult AIDS Clinical Trials Group, the Pan American Health Organization, and the Centers for Disease Control and Prevention Study Group. JAMA. 2000;283:1445-50.
21. Update: adverse event data and revised American Thoracic Society/CDC recommendations against the use of rifampin and pyrazinamide for treatment of latent tuberculosis infection--United States; 2003. MMWR Morb Mortal Wkly Rep. 2003;52:735-9.
22. Muñoz P, Rodríguez C, Bouza E. *Mycobacterium tuberculosis* infection in recipients of solid organ transplants. Clin Infect Dis. 2005;40:581-7.
23. Torre-Cisneros J, Doblas A, Aguado JM, San Juan R, Blanes M, Montejo M, et al. Tuberculosis after solid-organ transplant: incidence, risk factors, and clinical characteristics in the RESITRA (Spanish Network of Infection in Transplantation) cohort. Clin Infect Dis. 2009;48:1657-65.
24. Muñoz P, Palomo J, Muñoz R, Rodríguez-Creixéms M, Peláez T, Bouza E. Tuberculosis in heart transplant recipients. Clin Infect Dis. 1995;21:398-402.
25. European best practice guidelines for renal transplantation. Section IV: Long-term management of the transplant recipient. IV.7.2. Late infections. Tuberculosis. Nephrol Dial Transplant. 2002;17 Suppl 4:39-43.
26. Aguado JM, Torre-Cisneros J, Fortún J, Benito N, Meije Y, Doblas A, et al. Tuberculosis in solid-organ transplant recipients: consensus statement of the group for the study of infection in transplant recipients (GESITRA) of the Spanish Society of Infectious Diseases and Clinical Microbiology. Clin Infect Dis. 2009;48:1276-84.
27. Dunlop H. Infliximab (Remicade) and etanercept (Enbrel): serious infections and tuberculosis. CMAJ. 2004;171:992-3.
28. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med. 2001;345:1098-104.
29. Winthrop KL, Siegel JN. Tuberculosis cases associated with infliximab and etanercept. Clin Infect Dis. 2004;39:1256-7.
30. Carmona L, Gómez-Reino JJ, Rodríguez-Valverde V, Montero D, Pascual-Gómez E, Mola EM, et al; BIOBADASER Group. Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. Arthritis Rheum. 2005;52:1766-72.
31. Rodríguez-Valverde V, Cáliz R, Álvaro-Gracia JM, Marenco de la Fuente JL, Mulero J, Tornero J, et al. III Actualización del Consenso de la Sociedad Española de Reumatología sobre terapia biológica en la artritis reumatoide. Reumatol Clin. 2006;2 Supl 2:S52-9.
32. Bartalesi F, Vicidomini S, Goletti D. QuantiFERON-TB Gold and the TST are both useful for latent tuberculosis infection screening in autoimmune diseases. Eur Respir J. 2009;33:586-93.
33. Lalvani A, Millington KA. Screening for tuberculosis infection prior to initiation of anti-TNF therapy. Autoimmun Rev. 2008;8:147-52.
34. Marais BJ, Gie RP, Schaaf HS, Beyers N, Donald PR, Starke JR. Childhood pulmonary tuberculosis: old wisdom and new challenges. Am J Respir Crit Care Med. 2006;173:1078-90.
35. Schaaf HS, Marais BJ, Whitelaw A, Hesseling AC, Eley B, Hussey GD, et al. Culture-confirmed childhood tuberculosis in Cape Town, South Africa: a review of 596 cases. BMC Infect Dis. 2007;7:140.
36. Marais BJ, Obihara CC, Gie RP, Schaaf HS, Hesseling AC, Lombard C, et al. The prevalence of symptoms associated with pulmonary tuberculosis in randomly selected children from a high burden community. Arch Dis Child. 2005;90:1166-70.
37. Rigouts L. Clinical practice: diagnosis of childhood tuberculosis. Eur J Pediatr. 2009;168:1285-90.
38. Hatherill M, Hawkridge T, Zar HJ, Whitelaw A, Tameris M, Workman L, et al. Induced sputum or gastric lavage for community-based diagnosis of childhood pulmonary tuberculosis? Arch Dis Child. 2009;94:195-201.
39. Graham SM, Daley HM, Banerjee A, Salaniponi FM, Harries AD. Ethambutol in tuberculosis: time to reconsider? Arch Dis Child. 1998;79:274-8.
40. Finnell SM, Christenson JC, Downs SM. Latent tuberculosis infection in children: a call for revised treatment guidelines. Pediatrics. 2009;123:816-22.
41. Bindu R, Schluger NW. Tuberculosis and pregnancy. Semin Respir Crit Care Med. 1998;19:295-306.
42. Arora VK, Gupta R. Tuberculosis and pregnancy. Ind J Tub. 2003;50:13-6.
43. Figueroa-Damien R, Arredondo-García JL. Pregnancy and tuberculosis: influence of treatment on perinatal outcome. Am J Perinatol. 1998;15:303-6.
44. Miller K, Miller JM Jr. Tuberculosis in pregnancy: interactions, diagnosis, and management. Clin Obstet Ginecol. 1996;39:120-42.
45. Palacios E, Dallman R, Muñoz M, Hurtado R, Chalco K, Guerra D, et al. Drug-resistant tuberculosis and pregnancy: treatment outcomes of 38 cases in Lima, Peru. Clin Infect Dis. 2009;48:1413-9.
46. Brost BC, Newman RB. The maternal and fetal effects of tuberculosis therapy. Obstet Gynecol Clin North Am. 1997;24:659-73.
47. Snider DE, Powell KE. Should women taking antituberculosis drugs breast-feed? Arch Intern Med. 1984;144:589-90.
48. Yoshikawa TT. Tuberculosis and aging: a global health problem. Clin Infect Dis. 2001;33:1034-9.
49. Stead WW. Special problems in tuberculosis: tuberculosis in the elderly and in residential homes, correctional facilities, long-term care hospitals, mental hospitals, shelters for the homeless, and jails. Clin Chest Med. 1989;10:397-405.
50. Stead W, Lofgren J, Warren E, Thomas C. Tuberculosis as an endemic and nosocomial infection among the elderly in nursing homes. N Engl J Med. 1985;312:1483-7.
51. Pérez-Guzmán C, Vargas MH, Torres-Cruz A, Villarreal-Velarde H. Does aging midify pulmonary tuberculosis? A meta-analytical review. Chest. 1999;116: 961-7.
52. Vasankari T, Liippo K, Tala E. Overt and cryptic miliary tuberculosis misdiagnosed until autopsy. Scand J Infect Dis. 2003;35:794-6.
53. Byrd RB, Horn BR, Solomon DA. Toxic effects of isoniazid in tuberculosis chemoprophylaxis: role of biochemical monitoring in 1,000 patients. JAMA. 1978;241:1239-41.
54. Chmura K, Chan E. Tuberculosis in the elderly: keep a high index of suspicion. J Respir Dis. 2006;27:307-15.
55. Pande JN, Singh SP, Khilnani GC, Khilnani S, Tandon RK. Risk factors for hepatotoxicity from antituberculosis drugs: a case-control study. Thorax. 1996; 51:132-6.
56. CDC. Prevention and control of tuberculosis in facilities providing long-term care to the elderly. Recommendations of the advisory committee for elimination of tuberculosis. MMWR. 1990;39(RR-10):7-20.
57. Chia S, Karim M, Elwood RK, Fitzgerald JM. Risk of tuberculosis in dialysis patients: a population-based study. Int J Tuberc Lung Dis. 1998;2:989-91.
58. Malone RS, Fish DN, Spiegel DM, Childs JM, Peloquin CA. The effect of hemodialysis on isoniazid, rifampin, pyrazinamide, and ethambutol. Am J Respir Crit Care Med. 1999;159:1580-4.
59. Stass H, Kubitza D. Pharmacokinetics and elimination of moxifloxacin after oral and intravenous administration in man. J Antimicrob Chemother. 1999;43:83-90.
60. Martínez-Lirola M, Alonso-Rodríguez N, Sánchez ML, Herranz M, Andrés S, Peñafiel T, et al. Advanced survey of tuberculosis transmission in a complex socioepidemiologic scenario with a high proportion of cases in immigrants. Clin Infect Dis. 2008;47:8-14.
61. Sanz-Peláez O, Caminero-Luna J, Pérez-Arellano L. Tuberculosis e inmigración en España. Evidencias y controversias. Med Clin (Barc). 2006;126:259-69.
62. Dahle UR, Sandven P, Heldal E, Caugant DA. Continued low rates of transmission of *Mycobacterium tuberculosis* in Norway. J Clin Microbiol. 2003;41:2968-73.
63. Geng E, Kreiswirth B, Driver C, Li J, Bruzynski J, DellaLatta P, et al. Changes in the transmission of tuberculosis in New York City from 1900 to 1999. N Engl J Med. 2002;346:1453-8.
64. Ravins M, Bercovier H, Chemtob D, Fishman Y, Rahav G. Molecular epidemiology of *Mycobacterium tuberculosis* infection in Israel. J Clin Microbiol. 2001;39: 1175-7.
65. Borrell S, Español M, Orcau A, Tudó G, March F, Caylà JA, et al. Tuberculosis transmission patterns among Spanish-born and foreign-born populations in the city of Barcelona. Clin Microbiol Infect. 2009 Epub ahead of print.
66. Diz S, López-Vélez R, Moreno A, Fortún J, Moreno L, Gómez-Manpaso E, et al. Epidemiology and clinical features of tuberculosis in immigrants at an infectious diseases department in Madrid. Int J Tuberc Lung Dis. 2007;11:769-74.
67. Ramos JM, Masiá M, Rodríguez JC, Padilla I, Soler MJ, Gutiérrez F. Tuberculosis en inmigrantes: diferencias clínico-epidemiológicas con la población autóctona/1999-2002). Enferm Infecc Microbiol Clin. 2004;22:315-8.
68. Zuber PL, McKenna MT, Bibkin NJ, Onorato IM, Castro KG. Long-term risk of tuberculosis among foreign-born persons in the United States. JAMA. 1997; 278:304-7.
69. Cain KP, Haley CA, Armstrong LR, Garman KN, Wells CD, Iademarco MF, et al. Tuberculosis among foreign-born persons in the United States. Am J Respir Crit Care Med. 2007;175:75-9.
70. Arce Arnáez A, Iñigo Martínez J, Cabello Ballesteros L, Burgoa Arenales M. Tuberculosis e inmigración en un área sanitaria de Madrid. Situación epidemiológica y evolución en la década 1994-2003. Med Clin (Barc). 2005;125:210-2.
71. The WHO/IUATLD Global Project on anti-tuberculosis drug resistance surveillance. Four Global Report. Available at: http://www.who.int/tb/publications/
72. Huerga H, López-Vélez R, Navas E, Gómez-Manpaso E. Clinicoepidemiological features of immigrants with tuberculosis living in Madrid, Spain. Eur J Clin Microbiol Infect Dis. 2000;19:236-7.
73. Tirado M, Moreno R, Marín M, González F, Pardo F, García del Busto A, et al. Impact of immigration on drug resistance to *Mycobacterium tuberculosis* in Castellon (Spain): 1995-2003. Med Clin (Barc). 2006;126:761-4.
74. Caylà JA, Rodrigo T, Ruiz-Manzano J, Caminero JA, Vidal R, García JM, et al, and the Working Group on Completion of Tuberculosis Treatment in Spain (Study ECUTTE). Tuberculosis treatment adherence and fatality in Spain. Respir Res. 2009;10:121.
75. Anibarro L, Casas S, Paz-Esquete J, González L, Pena A, Guerra M, et al, and the Mycobacteria Study Group (GEIM) of Spanish Society of Clinical Microbiology and Infectious Diseases (SEIMC).Treatment completion in latent tuberculosis infection at specialist tuberculosis units in Spain. Int J Tuberc Lung Dis. 2010;14:701-7.
76. Dasgupta K, Menzies D. Cost-effectiveness of tuberculosis control strategies among immigrants and refugees. Eur Respir J. 2005;25:1107-16.

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# Diagnosis of tuberculosis infection using interferon-γ-based assays

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A B S T R A C T

Interferon-γ-based assays, collectively known as IFN-γ release assays (IGRAs), have emerged as a reliable alternative to the old tuberculin skin test (TST) for the immunodiagnosis of tuberculosis (TB) infection. The 2 commercially available tests, the enzyme-linked immunosorbent assay (ELISA), QuantiFERON-TB Gold In-tube (QFT-IT), and the enzyme-linked immunospot assay (ELISPOT), T-SPOT.TB, are more accurate than TST for the diagnosis of TB, since they are highly specific and correlate better with the existence of risk factors for the infection. According to the available data, T-SPOT.TB obtains a higher number of positive results than QFT-IT, while its specificity seems to be lower. Although the sensitivity of the IFN-γ -based assays may be impaired to some extent by cellular immunosuppression and extreme ages of life, they perform better than TST in these situations. Data from longitudinal studies suggest that IFN-γ-based tests are better predictors of subsequent development of active TB than TST; however this prognostic value has not been consistently demonstrated. This review focuses on the clinical use of the IFN-γ -based tests in different risk TB groups, and notes the main limitations and areas for future development.



## Diagnóstico de la tuberculosis mediante las técnicas basadas en la detección de interferón-γ

*Palabras clave:*
Tuberculosis
Infección tuberculosa
Determinación de liberación de interferón-γ
QuantiFERON-TB Gold In-Tube
T-SPOT.TB

R E S U M E N

Las técnicas de detección de la liberación de interferón-γ conocidas como IFN-γ *release assays* (IGRA), constituyen una alternativa fiable a la clásica prueba de la tuberculina (PT) para el inmunodiagnóstico de la infección tuberculosa. Las 2 pruebas comerciales disponibles, QuantiFERON-TB Gold In-tube (QFT-IT) y T-SPOT.TB, son más precisas que la PT para el diagnóstico de tuberculosis (TB), ya que son muy específicas y presentan una mejor correlación con la existencia de factores de riesgo para la infección tuberculosa. Con los datos disponibles, T-SPOT.TB detecta mayor número de positivos que QFT-IT, pero parece ser menos específica. Aunque pueden verse afectadas en determinadas situaciones de inmunosupresión celular y en las edades extremas de la vida, estas técnicas siguen siendo superiores a la PT en estas situaciones. Estudios longitudinales sugieren que las pruebas de liberación de IFN-γ son mejores predictores de la progresión a enfermedad tuberculosa; sin embargo, este hecho no ha sido demostrado completamente. Esta revisión trata el uso de los test de liberación de IFN-γ en diferentes grupos de riesgo de TB. Asimismo, remarca sus principales limitaciones y las áreas de desarrollo futuro.



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## From the tuberculin skin test to the interferon-γ-based assays

The tuberculin skin test (TST), that recalls the delayed-type hypersensitivity response to the intradermal inoculation of purified protein derivate (PPD),[1] has been used to diagnose TB infection for the last hundred years. The PPD contains a mixture of more than 200 antigens that are widely shared by mycobacteria other than *Mycobacterium tuberculosis,* including the vaccinal strain of *Mycobacterium bovis* bacilli Calmette-Guérin (BCG) and many non-tuberculous mycobacteria (NTM). As a result, individuals sensitized by previous exposure to NTM or BCG vaccine may respond immunologically to PPD. The other main limitation of the TST is its low sensitivity in certain groups of individuals, such as immunosuppressed patients and young children.[2]

Immunodiagnostic methods have been developed based on the *in vitro* quantification of the cellular immune response, by detecting interferon-gamma (IFN-γ) released by sensitized T-cells stimulated with specific *M. tuberculosis* antigens. The two main antigens used are the 6-kD *M. tuberculosis* early-secreted antigenic target protein (ESAT-6) and the 10-kD culture filtrate protein (CFP-10), encoded in the region of difference 1 (RD1), which is present in *M. tuberculosis* but not in BCG or in most NTM.[3] This new *in vitro* technology has been rapidly adapted from initial *in-house* methods to the two commercially available techniques: QuantiFERON-TB Gold assays (QFT-G) (Cellestis Limited, Carnegie, Victoria, Australia) and T-SPOT.TB assay (Oxford Immunotec, Oxford, UK). Both tests, collectively known as IGRAs (Interferon-Gamma Release Assays), have been approved for sale in Europe and have received final approval from the U.S. Food and Drug Administration (FDA) as an aid for diagnosing *M. tuberculosis* infection. T-SPOT.TB detects the number of IFN-γ producing T-cells after stimulating a definite number of isolated peripheral blood mononuclear cells (PBMCs) with ESAT-6 and CFP-10 separately by means of enzyme-linked immunospot assay (ELISPOT). QFT-G tests are whole blood assays that use an enzyme-linked immunosorbent assay (ELISA) to detect IFN-γ produced in supernatants by stimulated T-cells. The QFT-G In Tube version (QFT-IT), includes a third antigen, TB7.7. This new antigen is encoded in RD11 and is missing from the BCG strains as well as most common environmental mycobacteria.[4] In the QFT-IT assay, the three specific *M. tuberculosis* antigens are already incorporated into the same tube (Fig. 1). Both *in vitro* tests include a positive control that detects the capacity of T cells to produce IFN-γ upon stimulation with a mitogen (phytohemagglutinin), in order to distinguish false-negatives from indeterminate results.

## Interferon-γ-based assays for detecting latent infection in high-risk populations

This section will discuss the potential value of the IFN-γ-based tests in diagnosing latent TB infection (LTBI) in people at high risk of progression to active disease.

### *Contact tracing study*

Between 5% and 10% of recently infected contacts will develop active TB within 2-5 years after exposure. The identification and treatment of these individuals constitutes an essential component of the TB control strategy in low-prevalence countries. Numerous studies have explored the utility of the IFN-γ-based tests in contact investigations.[5-9] In the absence of a gold standard test for the diagnosis of LTBI, the best approach to compare IFN-γ-based tests and TST consists of correlating their results with the degree of exposure to an infectious case. Positive results of IFN-γ-based tests were found to be more strongly associated with greater recent exposure than TST;[5,8] however, this association could not be demonstrated by others.[9] Besides, IFN-γ-based tests offer the advantage of high specificity, since they are not affected by prior BCG vaccination or by infection with most NTM.

### *Health care workers*

Due to the risk of infection with *M. tuberculosis* through occupational exposure, periodical testing is recommended for all health care workers (HCWs). Serial TST testing may induce a boosting phenomenon, compromising its interpretation.[10] In a study performed in Barcelona (Spain),[11] prevalence of LTBI in HCWs without a previous positive TST was higher according to T-SPOT.TB (23.1%), and QFT-IT (17.3%) than according to TST (15.4%). Positive IFN-γ tests were associated with age and degree of occupational exposure, but not with BCG vaccination, a finding consistent with previous studies with QFT-G tests.[12,13] Although IFN-γ-based tests have became a good alternative to TST for serial testing of HCWs, factors such as reversions and conversions should be taken into account. Choi et al[14] described conversions of QFT-IT in HCWs 2-4 weeks after performing a TST test among TST reactors, but not among non-reactive individuals. Similarly, van Zyl-Smit et al[15] reported conversions a week after TST administration. However, when using a two-step screening strategy, IFN-γ test results were not influenced if TST was performed within three days.

### *Immunocompromised patients*

The performance of the IFN-γ tests in immunocompromised patients and the effect of immunosuppression on these tests remains unclear.[16] Previous studies including different groups of immunocompromised patients found impaired performance of IFN-γ-based tests related to malfunction of cellular immune system, but they performed better than TST nonetheless.[17,18] In a prospective study including 369 immunosuppressed participants, Richeldi et al[19] found that IFN-γ tests detected more patients as being infected with *M. tuberculosis* than did TST.

### *HIV infected patients*

Patients co-infected with HIV and *M. tuberculosis* are particularly prone to a reactivation of LTBI and development of disseminated disease. In studies evaluating T-SPOT.TB and its ELISPOT pre-commercial version[20,21] or the QFT-G tests,[22,23] *in vitro* tests obtained higher rates of positive results than TST in diagnosing LTBI,[23,24] and a better association between positive results and presence of risk factors for LTBI.[22,23] In recent years, some studies in HIV-infected populations reported similar sensitivities for both IFN-γ tests.[21-25] As regards indeterminate results, a correlation between low $CD4^+$ cell counts and a low control positive response was found with QFT-IT,[22] while T-SPOT.TB and *in-house* ELISPOT appeared to be relatively unimpaired by low $CD4^+$ cell counts.[24,26,27] However, higher rates of indeterminate results with T-SPOT.TB and *in-house* ELISPOT have also been reported.[24,28]

### *Chronic immune-mediated inflammatory disease*

Tumor necrosis factor (TNF)-α antagonists provide reliable treatment in patients with immune-mediated inflammatory diseases (IMID).[29] TNF-α is one of the key molecules involved in granuloma formation and containment of TB infection. Due to the increased risk of TB in patients receiving anti-TNF-α agents,[30] exclusion of active TB and screening for latent infection is mandatory before starting anti-TNF-α therapy.[31] However, cellular-mediated response to PPD is compromised by the corticosteroids and/or immunosuppressive drugs that most patients with IMID are already taking.[29,31] Experience with the IFN-γ-based tests in this population, although promising, is still limited.[32,33] Overall, agreement with the TST seems to be poor.[34-36] The discordant positive TST and negative IFN-γ-based test results have been attributed to false-positive TST results,[37,38] whereas the

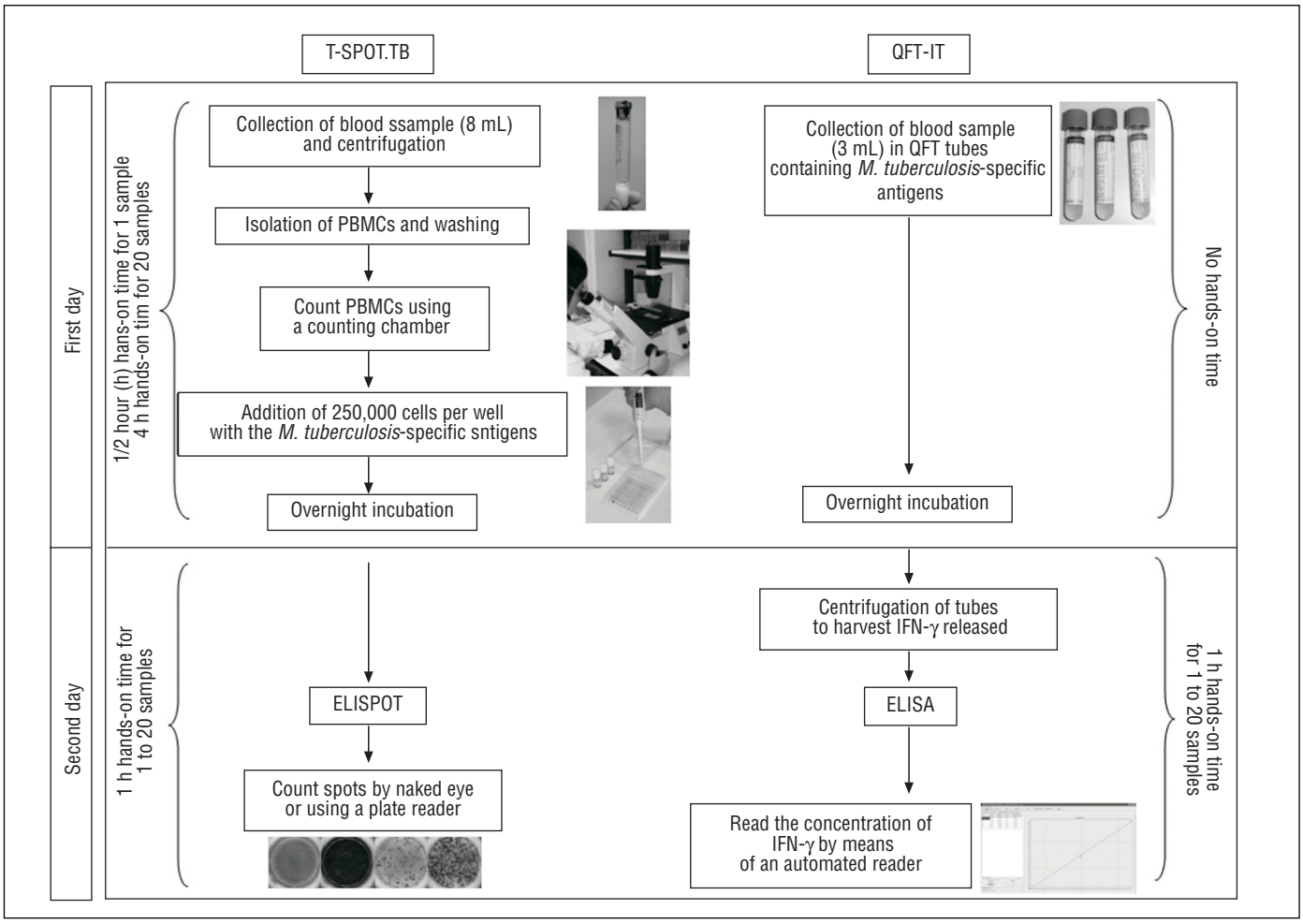


**Figure 1.** Comparison of T-SPOT.TB and QuantiFERON-TB Gold In Tube (QFT-IT) methodology. ELISA: enzyme-linked immunosorbent assay; ELISPOT: enzyme-linked immunospot assay; IFN: interferon; PBMCs: peripheral blood mononuclear cells.

discordant negative TST and positive IFN-γ-based test results have been considered false-negative TST results due to the immunosuppressive therapy being taken by these patients.[35,36,39] The available data show that the IFN-γ-based tests detect more cases of LTBI than TST does,[36,40] and a closer association with the presence of risk factors for TB infection.[34,35] The effect of IMID-associated immunosuppression on the performance of the IFN-γ-based tests has not been completely clarified. In a study involving 398 consecutive subjects, Bartalesi et al[41] did not find an association between results of the TST or QFT-IT and the use of conventional disease-modifying antirheumatic drugs, but reported an association of steroids with a lower likelihood of a positive result. In view of the high risk of TB in IMID patients receiving anti-TNF-α therapy, a strategy based on a simultaneous TST and one of the IFN-γ tests might maximize diagnostic sensitivity for the detection of LTBI.

*Paediatric population*

Children have a high risk of progression to active TB, especially infants under the age of two.[42] Early, specific diagnosis of LTBI is therefore crucial to prevent active disease. The sensitivity of TST in young children is unknown, but the existence of immaturity certainly induces a lower cutaneous response. In addition, BCG-vaccination, especially in TB endemic areas, reduces the test's specificity. Overall, T-SPOT.TB provides higher rates of positive results for LTBI than TST or the QFT-G tests. Recently, Davies et al[43] found that, in contrast to TST, ELISPOT results were not affected by young age or severe immunosuppression. Furthermore, a high correlation with the degree of exposure to *M. tuberculosis* with both IFN-γ-based tests has been demonstrated.[44] Results of both IFN-γ-based tests are unrelated to the BCG-vaccination status, which contributes to their high specificity.[4,7] Discordant results between these tests and TST are frequently found.[7,45-48] In a study conducted in Barcelona (Spain),[7] among BCG-unvaccinated children, 60% and 57% of those with positive TST had negative QF-IT and T-SPOT.TB respectively. Latorre et al[49] reported that 48% of children with TST positive and negative T-SPOT.TB had sensitized T cells against *Mycobacterium avium* sensitins. As regards the indeterminate results, a significantly lower IFN-γ release in response to mitogen (positive control) has been described in young children tested with QFT-IT, suggesting an age-dependent response.[47,50] As for T-SPOT.TB, it does not seem to be related to age,[17] except in the first weeks of life.[51] However, Nicol et al[48] reported a decline in positive T-SPOT.TB results in children less than 1 year of age, whereas TST results were unaffected.

## Interferon-γ-based assays for diagnosing active tuberculosis

Although the IFN-γ-based tests are widely used together with or in place of TST, their role in the diagnosis of active disease is still undefined. According to the results of two recent meta-analyses,[52,53] both commercial IFN-γ-based tests, performed in blood, have better sensitivity than TST for active TB. As expected, specificity for active disease was low, ranging from 59% for T-SPOT.TB to 79% for QFT-IT and 75% for TST.[53]

While a positive result of an IFN-γ-based assay does not distinguish between active and latent infection, in combination with the TST result it may help to exclude active TB.[54-56] A recent multicentre study[55] showed a very low likelihood of TB with a negative result on both TST and IFN-γ-based tests. Unfortunately, only 4% of patients were immunosuppressed, which precludes its generalization to the whole risk population. The usefulness of levels of IFN-γ, measured by QFT-IT, to predict clinical outcome was evaluated in two studies.[56,57] Although active TB was associated with higher IFN-γ levels, the benefit was presumed to be marginal in highly experienced centres.

Cellular immunosuppression and age, among other factors, may impair performance of IFN-γ-based tests.[58] In 4 studies that compared performance of QFT-IT in HIV-infected and non-infected adults with active TB, mean sensitivity was 64% in HIV-infected patients and 79% in non-HIV-infected patients.[59-62] Although the overall sensitivity of T-SPOT.TB is higher than that of QFT-IT in otherwise healthy people, data from head-to-head comparisons in HIV-infected patients are scarce.[60,63,64] In three studies, covering a total of 39 patients with culture-confirmed TB, QFT-IT and T-SPOT.TB detected 74% and 82% of cases respectively.[60,64] Despite impaired sensitivity, IFN-γ-based tests are clearly superior to TST for the diagnosis of active TB in these patients.[61,65-67]

IFN-γ-based tests may be of value in diagnosing TB in childhood, due to the absence of microbiological confirmation in a high proportion of cases. In a large prospective study, the sensitivity of T-SPOT.TB was 83%, and was not affected by HIV status.[20] A hospital-based study reported sensitivity rates of 100% for TST and 73% for T-SPOT.TB and QFT.IT, and specificity rates of 58%, 98% and 100% for TST, T-SPOT.TB and QFT-IT respectively.[68] A multicentre study comparing both IFN-γ-based tests with TST in 333 children aged 2 months to 16 years found that in 49 TB-confirmed cases, sensitivity was 82% for TST, 78% for QFT-IT and 66% for T-SPOT.TB, increasing to 96% and 91% when TST was combined with T-SPOT and QFT-IT respectively.[69]

As regards aged patients, the data available show that QFT-G is more sensitive than TST in patients older than 80 years with active TB.[70,71] Although the sensitivity of QFT-G decreased with age, it remained better than that of TST.[71,72]

*Interferon-γ-based assays in fluids other than blood*

IFN-gamma is predominantly produced by effector T-cells. The recruitment of specific T cells during active TB and the process via which antigen-specific cells clonally expand and migrate to the site of infection have been described.[73] Therefore, during active TB, it makes sense to apply IFN-based assays in samples collected directly from the site of infection. Data from a recent metanalysis[53] indicate that the T-SPOT.TB assay in extrasanguineous fluids is a promising tool for the diagnosis of active TB.

*Pulmonary tuberculosis*

Rapid diagnosis of pulmonary TB relies on the detection of acid-fast bacilli (AFB). However, this can be difficult due to the low sensitivity of the sputum smear. In addition, a significant proportion of cases cannot be confirmed by culture. In a prospective study by Jafari et al,[74] all 12 patients with smear-negative pulmonary TB, but none of the 25 controls, had positive T-SPOT.TB test from the bronchoalveolar lavage (BAL). TB-specific T cells were more concentrated in BAL than in peripheral blood, indicating a highly selective compartmentalization at the site of infection.[75] A recent large study carried out by the TBNET confirmed the high sensitivity, specificity and predictive values of T-SPOT.TB from the BAL.[76]

*Pleural tuberculosis*

The diagnosis of pleural TB is often difficult due to the limitations of conventional tests.[77] Wilkinson et al[73] found a 15-fold greater concentration of ESAT-6-specific spot-forming T cells in pleural fluid than of PBMCs in 10 patients with pleural TB. These cells were not found in the pleural fluid of 8 patients with nontuberculous pleuritis.[73] In a TBNET study,[78] T-SPOT.TB was performed on mononuclear cells from blood and pleural fluid in 20 patients with pleural TB and in 21 with pleural effusion of other causes. T-SPOT.TB was positive in 90% of cases on blood samples and in 95% of cases pleural fluid. Specificity was 67% for blood and 76% for pleural fluid. In another study of 28 patients with pleural TB, results in pleural fluid were inconclusive in 52% of cases, due to high background IFN-γ production.[79] Commercial IFN-γ tests, T-SPOT.TB and QFT-IT in pleural fluid were compared to unstimulated IFN-γ for the diagnosis of pleural TB in 74 patients.[80] In 11 (15%) cases, the cell counts were not large enough to perform the tests. In the 63 remaining patients, sensitivity, specificity, positive predictive value and negative predictive value were: for T-SPOT.TB, 86, 60, 84 and 64% respectively; for QFT-IT, 57, 80, 87 and 44% respectively, and for unstimulated IFN-γ, 97, 100, 100 and 94% respectively. The authors concluded that the IFN-γ-based assays had suboptimal accuracy for the diagnosis of pleural TB.

*Tuberculous meningitis*

Tuberculous meningitis (TBM) is a challenge for clinicians because of the frequent absence of microbiological confirmation and high mortality if not promptly treated. In one study including 10 patients with a diagnosis of TBM, T-SPOT.TB detected *M. tuberculosis* antigen-specific IFN-γ in CSF from nine patients (90%), but in none of the seven controls (specificity 100%).[81] In a study with 12 patients with TB of the central nervous system and 25 without TB, T-SPOT's sensitivity and specificity in CSF were 75%.[82] Recently, in a prospective observational study of 31 patients with confirmed or probable TBM, the same group of investigators[83] reported a sensitivity of 59% and a specificity of 89% for T-SPOT.TB in CSF mononuclear cells. However, since the diagnosis was not confirmed microbiologically in 21 of these patients, the sensitivity may have been underestimated. Similarly, in a study of 140 patients with meningitis (81% HIV-infected), using ≥46 spot-forming cells as cut-off point and after excluding bacterial and cryptococcal meningitis, the positive and negative predictive values of T-SPOT.TB in CSF were 100% and 68% respectively.[84]

## Interferon-γ-based assays for predicting subsequent active tuberculosis

The ability to predict subsequent active TB among latently infected people is essential in order to select those who would benefit from chemoprophylaxis and to avoid unnecessary treatment for low-risk persons. Doherty et al[85] demonstrated a strong association of reactivity to ESAT-6 and progression to active TB in twenty-four household contacts of smear-positive TB patients. In a study involving 601 close contacts of sputum smear-positive TB, Diel et al[86] found that while 14.6% of contacts with positive QFT-IT who declined treatment developed TB within the 2-year follow-up, only 2.3% of those with positive TST did. This difference between TST and QFT-IT disappeared when only unvaccinated contacts were considered.[86] More recently, the same group of investigators[88] extended the original study and reported progression to active TB for up to four years for a cohort of 954 close contacts of smear-positive index cases. Of 147 untreated contacts with a positive QFT-IT test 19 (12.9%) developed active TB, whereas only 17 of 555 (3.1%) with TST >5 mm did. The progression rate was higher among children (28.6%). In addition, none of 824 untreated contacts with negative QFT-IT developed active TB, confirming the high negative predictive value of the test.[87] In a cohort of 308 silicosis patients, Leung et al[88] found that a positive T-SPOT.TB significantly predicted development of active TB during a follow-up of more than 2 years (RR 7.80; 95%CI 1.02-59.6). Unexpectedly, TST was not predictive of TB, regardless of the cut-off point used. In the study by Kik et al[89] of 339 close contacts of sputum

**Table 1**
Summary of 9 studies evaluating the predictive value of the interferon-γ based tests for the development of tuberculosis

| Study (reference) | Country | Study population | Period of follow-up (years) | Test | Positive test n/N (%) | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|---|---|---|
| Doherty et al[85] | Ethiopia | Household contact adults | 2 | ELISA (PPD) | 21/24 (88) | 100 | 18 | 33 | 100 |
| | | | | ELISA (ESAT-6) | 9/24 (38) | 86 | 83 | 67 | 93 |
| Diel et al[87] | Germany | Close contact adults | 4 | TST | 555/903 (60) | 89 | 61 | 3.1 | 99 |
| | | | | QFT-IT | 147/903 (16) | 100 | 86 | 12.9 | 100 |
| Leung et al[88] | Hong Kong | Silicosis patients | 2.5[a] | TST (10 mm) | 203/308 (66) | 77 | 35 | 6.4 | 96 |
| | | | | T-SPOT.TB | 204/308 (66) | 88 | 36 | 7.4 | 98 |
| Kik et al[89] | Netherlands | Contact immigrant adults | 2 | TST | 339[b] | 100 | 16 | 3.1 | 100 |
| | | | | QFT-IT | 178/324 (55)[b] | 63 | 45 | 2.8 | 98 |
| | | | | T-SPOT.TB | 181/299 (61)[b] | 75 | 40 | 3.3 | 98 |
| Hill et al[90] | Gambia | Household contact childre & adults | 2 | TST | 843/2230 (38) | 54 | 62 | 1.7 | 99 |
| | | (HIV +ve and -ve) | | T-SPOT.TB | 649/1736 (37) | 42 | 63 | 1.7 | 99 |
| Aichelburg et al[91] | Austria | HIV +ve adults | 1.6 | QFT-IT | 44/783 (5.6) | 100 | 95 | 8.1 | 100 |
| Santín et al[92] | Spain | HIV –ve adults | 1.6[a] | TST | 7/120 (6) | – | 94[c] | – | 100[c] |
| | | | | QFT-IT | 13/120 (11) | – | 89[c] | – | 100[c] |
| Bakir et al[93] | Turkey | Contact children | 1.3 | TST | 550/908 (61) | 80 | 40 | 2 | 99 |
| | | | | T-SPOT.TB | 381/908 (42) | 73 | 59 | 3 | 99 |
| Higuchi et al[94] | Japan | Contact adolescents | 3.5 | TST | 95 | – | 73 | – | 100 |
| | | | | QFT-G | 4/88[d] | – | 96[e] | – | 100[e] |

NPV: negative predictive value; PPV: positive predictive value.
[a]Mean follow-up.
[b]Only subjects with positive TST were tested with QFT-IT and T-SPOT.TB.
[c]Calculated with 120 non-treated patients (those with negative TST and negative/indeterminate QFT-IT).
[d]Only subjects with positive TST were tested with QFT-G.
[e]Calculated with 84 non-treated subjects (those with negative QFT-G).

smear-positive TB, TST, QFT-IT and T-SPOT.TB were comparable in predicting development of TB during a two-year period. Positive predictive values were 3.1% for TST ≥10 mm, 3.8% for TST ≥15 mm, 2.8% for positive QFT-IT and 3.3% for T-SPOT.TB. In a study with 2348 household contacts in Gambia, Hill et al[90] found that neither the TST nor the T-SPOT.TB predicted development of TB. The lack of predictive value was attributed to the high-burden of TB and recent transmission.

Two studies assessed QFT-IT and progression to active disease in HIV-seropositive individuals.[91,92] In a large study in a low-prevalence country, 8.1% of HIV-seropositive patients with a positive QFT-IT result at baseline, and left untreated, developed TB during a median follow-up of 19 months. None of the 738 patients with negative results had TB.[91] In a study in Spain of 135 HIV-infected individuals without active disease, none of the patients who had a negative or indeterminate QFT-IT result at baseline had TB after a median follow-up of 20 months.[92]

Development of TB was also assessed in child and adolescent contacts. Bakir et al[93] studied 908 children with recent household exposure to TB, most of whom received preventive therapy. During a follow-up of 1.3 years, children with positive T-SPOT.TB had a 3- to 4-fold higher risk of developing active TB than those with negative T-SPOT.TB. However, rates of progression were similar in children with positive T-SPOT.TB and TST reactors. Since a high proportion of children were treated, the true incidence rates may have been underestimated. In the study by Higuchi et al,[94] 349 students underwent QFT-G and TST simultaneously, but only those with positive QFT-G were given chemoprophylaxis. Follow-up of the 91 students with positive TST but negative QFT-G showed no cases of active TB.

Although IFN-γ-based tests seem to predict subsequent active TB better than TST, the majority of high-risk people with positive tests will not develop active TB. Conversely, subsequent active TB in the next two to three years seems to be extremely low among people with a negative result.[85,87,89-94] Table 1 summarizes the nine studies assessing development of active TB with IFN-γ-based tests.

## Final remarks and areas of future development

IFN-γ-based assays have become a reliable alternative to the old TST for the diagnosis of TB infection. Both commercial tests, QFT-IT and T-SPOT.TB, have a higher specificity than TST, and a better correlation with risk factors for TB and the degree of contact with an infectious case. Although their sensitivity may be affected to some extent by immunosuppression and extreme ages of life, they perform better than TST in these situations. Besides, IFN-γ-based tests do not induce boosting, and no additional visits are required for reading.

A great deal is now known about IFN-γ-based assays, and their use has expanded considerably. However, the prognostic value of a positive/negative result for the development of active TB, the significance of discordant results, the cut-off points to use in immunosuppressed people, the conversion/reversion phenomenon, and their role in the diagnosis of paucibacillary forms of TB, are some of the important questions that remain unresolved.

The actual prognostic value of a positive IFN-γ result needs to be clarified. Although the available data suggest that IFN-γ tests predict progression to active disease better than TST, most people with a positive result will not develop TB. Large prospective studies are urgently needed. Furthermore, the question of whether quantification of IFN-γ release may be of help in this situation, as has been previously suggested,[85,86] should also be addressed. Because of the discordance between IFN-γ tests and TST results, practitioners are reluctant to use them in everyday clinical practice. Trials focusing specifically on understanding the discordant results between IFN-γ tests and the TST, and between IFN-γ tests themselves, are required. This issue is especially relevant in childhood, where the effect of NTM infection may play an important role.[49] Since *in vitro* assays rely on the secretion of IFN-γ, which is largely produced by $CD4^+$ T cells, determining the CD4 threshold at which the performance of these assays declines is of particular importance. In addition, studies exploring the effect of the different immunosuppressor drugs on the response, as well as the accuracy of new cut-offs for diagnosing LTBI in immunosuppressed patients, are needed. Detection of *M. tuberculosis* specific T cells in samples other than blood with ELISPOT is a promising tool for the diagnosis of smear-negative pulmonary TB[76] and other paucibacillary forms of TB.[78,81] The methodological procedures and appropriate cut-offs should be established.

While awaiting answers to these questions, the use of the IFN-γ-based tests in clinical practice should be guided by clinical judgement and evidence-based guidelines for different groups of patients must be developed.

Finally, technical modifications of IFN-γ-based tests are being explored.[95] The attempts to improve IFN-γ-based tests include the study of alternative readouts to measure IFN-γ release.[95,96] the use of alternative *M. tuberculosis* specific antigens,[4,97] and the simultaneous measurement of chemokines[98] and interleukins.[99] The next generation of IFN-γ-based tests will significantly enhance diagnostic sensitivity without diminishing specificity, and will also reduce the rate of indeterminate results, especially in immunosuppressed patients and children.

## Conflict of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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## References

1. Jasmer RM, Nahid P, Hopewell PC. Clinical practice. Latent tuberculosis infection. N Engl J Med. 2002;347:1860-6.
2. Horsburgh CR Jr. Priorities for the treatment of latent tuberculosis infection in the United States. N Engl J Med. 2004;350:2060-7.
3. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet. 2000;356:1099-104.
4. Brock I, Weldingh K, Leyten EM, Arend SM, Ravn P, Andersen P. Specific T-cell epitopes for immunoassay-based diagnosis of *Mycobacterium tuberculosis* infection. J Clin Microbiol. 2004;42:2379-87.
5. Arend SM, Thijsen SF, Leyten EM, Bouwman JJ, Franken WP, Koster BF, et al. Comparison of two interferon-gamma assays and tuberculin skin test for tracing TB contacts. Am J Respir Crit Care Med. 2007;175:618-27.
6. Diel R, Loddenkemper R, Meywald-Walter K, Gottschalk R, Nienhaus A. Comparative performance of tuberculin skin test, QuantiFERON-TB-Gold In Tube assay, and T-Spot.TB test in contact investigations for tuberculosis. Chest. 2009;135:1010-8.
7. Domínguez J, Ruiz-Manzano J, De Souza-Galvao M, Latorre I, Milà C, Blanco S, et al. Comparison of two commercially available gamma interferon blood tests for immunodiagnosis of tuberculosis. Clin Vaccine Immunol. 2008;15:168-71.
8. Lalvani A, Pathan AA, McShane H, Wilkinson RJ, Latif M, Conlon CP, et al. Rapid detection of *Mycobacterium tuberculosis* infection by enumeration of antigen-specific T cells. Am J Respir Crit Care Med. 2001;163:824-8.
9. Janssens JP, Roux-Lombard P, Perneger T, Metzger M, Vivien R, Rochat T. Contribution of a IFN-gamma assay in contact tracing for tuberculosis in a low-incidence, high immigration area. Swiss Med Wkly. 2008;138:585-93.
10. Menzies R, Vissandjee B, Rocher I, St Germain Y. The booster effect in two-step tuberculin testing among young adults in Montreal. Ann Intern Med. 1994; 120:190-8.
11. Casas I, Latorre I, Esteve M, Ruiz-Manzano J, Rodríguez D, Prat C, et al. Evaluation of interferon-gamma release assays in the diagnosis of recent tuberculosis infection in health care workers. PLoS One. 2009;4:e6686.
12. Nienhaus A, Schablon A, Bacle CL, Siano B, Diel R. Evaluation of the interferon-gamma release assay in healthcare workers. Int Arch Occup Environ Health. 2008;81:295-300.
13. Pai M, Gokhale K, Joshi R, Dogra S, Kalantri S, Mendiratta DK, et al. *Mycobacterium tuberculosis* infection in health care workers in rural India: comparison of a whole-blood interferon gamma assay with tuberculin skin testing. JAMA. 2005;293:2746-55.
14. Choi JC, Shin JW, Kim JY, Park IW, Choi BW, Lee MK. The effect of previous tuberculin skin test on the follow-up examination on whole-blood interferon-gamma assay in the screening for latent tuberculosis infection. Chest. 2008;133:1415-20.
15. Van Zyl-Smit RN, Pai M, Peprah K, Meldau R, Kieck J, Juritz J, et al. Within-subject variability and boosting of T-cell interferon-gamma responses after tuberculin skin testing. Am J Respir Crit Care Med. 2009;180:49-58.
16. Menzies D, Pai M, Comstock G. Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research. Ann Intern Med. 2007;146:340-54.
17. Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, et al. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: a prospective study. Lancet. 2006;367:1328-34.
18. Kobashi Y, Mouri K, Obase Y, Fukuda M, Miyashita N, Oka M. Clinical evaluation of QuantiFERON TB-2G test for immunocompromised patients. Eur Respir J. 2007; 30:945-50.
19. Richeldi L, Losi M, D'Amico R, Luppi M, Ferrari A, Mussini C, et al. Performance of tests for latent tuberculosis in different groups of immunocompromised patients. Chest. 2009;136:198-204.
20. Liebeschuetz S, Bamber S, Ewer K, Deeks J, Pathan AA, Lalvani A. Diagnosis of tuberculosis in South African children with a T-cell-based assay: a prospective cohort study. Lancet. 2004;364:2196-203.
21. Mandalakas AM, Hesseling AC, Chegou NN, Kirchner HL, Zhu X, Marais BJ, et al. High level of discordant IGRA results in HIV-infected adults and children. Int J Tuberc Lung Dis. 2008;12:417-23.
22. Brock I, Ruhwald M, Lundgren B, Westh H, Mathiesen LR, Ravn P. Latent tuberculosis in HIV positive, diagnosed by the *M. tuberculosis* specific interferon-gamma test. Respir Res. 2006;7:56.
23. Jones S, De Gijsel D, Wallach FR, Gurtman AC, Shi Q, Sacks H. Utility of QuantiFERON-TB Gold in-tube testing for latent TB infection in HIV-infected individuals. Int J Tuberc Lung Dis. 2007;11:1190-5.
24. Stephan C, Wolf T, Goetsch U, Bellinger O, Nisius G, Oremek G, et al. Comparing QuantiFERON-tuberculosis gold, T-SPOT tuberculosis and tuberculin skin test in HIV-infected individuals from a low prevalence tuberculosis country. AIDS. 2008;22:2471-9.
25. Rangaka MX, Wilkinson KA, Seldon R, Van Cutsem G, Meintjes GA, Morroni C, et al. Effect of HIV-1 infection on T-Cell-based and skin test detection of tuberculosis infection. Am J Respir Crit Care Med. 2007;175:514-20.
26. Clark SA, Martin SL, Pozniak A, Steel A, Ward B, Dunning J, et al. Tuberculosis antigen-specific immune responses can be detected using enzyme-linked immunospot technology in human immunodeficiency virus (HIV)-1 patients with advanced disease. Clin Exp Immunol. 2007;150:238-44.
27. Rivas I, Latorre I, Sanvisens A, Domínguez J, Tor J, Prat C, et al. Prospective evaluation of latent tuberculosis with interferon-gamma release assays in drug and alcohol abusers. Epidemiol Infect. 2009;137:1342-7.
28. Karam F, Mbow F, Fletcher H, Senghor CS, Coulibaly KD, LeFevre AM, et al. Sensitivity of IFN-gamma release assay to detect latent tuberculosis infection is retained in HIV-infected patients but dependent on HIV/AIDS progression. PLoS ONE. 2008;3:e1441.
29. Panes J, Gomollón F, Taxonera C, Hinojosa J, Clofent J, Nos P. Crohn's disease: a review of current treatment with a focus on biologics. Drugs. 2007;67:2511-37.
30. Gardam MA, Keystone EC, Menzies R, Manners S, Skamene E, Long R, et al. Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis. 2003;3:148-55.
31. Carmona L, Gómez-Reino JJ, Rodríguez-Valverde V, Montero D, Pascual-Gómez E, Mola EM, et al. Effectiveness of recommendations to prevent reactivation of latent tuberculosis infection in patients treated with tumor necrosis factor antagonists. Arthritis Rheum. 2005;52:1766-72.
32. Domínguez J, Latorre I. Role of the T-cell interferon-gamma release assays in preventing reactivation of latent tuberculosis infection in immunosuppressed patients in treatment with anti-TNF agents. J Crohn's & Colitis. 2008;2:250-4.
33. Lalvani A, Millington KA. Screening for tuberculosis infection prior to initiation of anti-TNF therapy. Autoimmun Rev. 2008;8:147-52.
34. Bocchino M, Matarese A, Bellofiore B, Giacomelli P, Santoro G, Balato N, et al. Performance of two commercial blood IFN-gamma release assays for the detection

of *Mycobacterium tuberculosis* infection in patient candidates for anti-TNF-alpha treatment. Eur J Clin Microbiol Infect Dis. 2008;27:907-13.
35. Matulis G, Juni P, Villiger PM, Gadola SD. Detection of latent tuberculosis in immunosuppressed patients with autoimmune diseases: performance of a *Mycobacterium tuberculosis* antigen-specific interferon gamma assay. Ann Rheum Dis. 2008;67:84-90.
36. Ponce de León D, Acevedo-Vásquez E, Alvizuri S, Gutiérrez C, Cucho M, Alfaro J, et al. Comparison of an interferon-gamma assay with tuberculin skin testing for detection of tuberculosis (TB) infection in patients with rheumatoid arthritis in a TB-endemic population. J Rheumatol. 2008;35:776-81.
37. Cobanoglu N, Ozcelik U, Kalyoncu U, Ozen S, Kiraz S, Gurcan N, et al. Interferon-gamma assays for the diagnosis of tuberculosis infection before using tumour necrosis factor-alpha blockers. Int J Tuberc Lung Dis. 2007;11:1177-82.
38. Martin J, Walsh C, Gibbs A, McDonnell T, Fearon U, Keane J, et al. Comparison of interferon-{gamma}-release assays and conventional screening tests before tumour necrosis factor-{alpha} blockade in patients with inflammatory arthritis. Ann Rheum Dis. 2010;69:181-5.
39. Vassilopoulos D, Stamoulis N, Hadziyannis E, Archimandritis AJ. Usefulness of enzyme-linked immunosorbent assay (Elispot) compared to tuberculin skin testing for latent tuberculosis screening in rheumatic patients scheduled for anti-tumor necrosis factor treatment. Addendum. J Rheumatol. 2008;35:1464.
40. Takahashi H, Shigehara K, Yamamoto M, Suzuki C, Naishiro Y, Tamura Y, et al. Interferon gamma assay for detecting latent tuberculosis infection in rheumatoid arthritis patients during infliximab administration. Rheumatol Int. 2007;27:1143-8.
41. Bartalesi F, Vicidomini S, Goletti D, Fiorelli C, Fiori G, Melchiorri D, et al. QuantiFERON-TB Gold and the TST are both useful for latent tuberculosis infection screening in autoimmune diseases. Eur Respir J. 2009;33:586-93.
42. Lalvani A, Millington KA. T cell-based diagnosis of childhood tuberculosis infection. Curr Opin Infect Dis. 2007;20:264-71.
43. Davies MA, Connell T, Johannisen C, Wood K, Pienaar S, Wilkinson KA, et al. Detection of tuberculosis in HIV-infected children using an enzyme-linked immunospot assay. AIDS. 2009;23:961-9.
44. Altet-Gómez N, De Souza-Galvao M, Latorre I, Milá C, Jiménez MA, Solsona J, et al. Diagnosing TB infection in children: analysis of discordances using in vitro tests and tuberculin skin test. Eur Respir J. 2010;doi:10.1183/09031936.00022710.
45. Connell TG, Ritz N, Paxton GA, Buttery JP, Curtis N, Ranganathan SC. A three-way comparison of tuberculin skin testing, QuantiFERON-TB gold and T-SPOT.TB in children. PLoS ONE. 2008;3:e2624.
46. Kampmann B, Whittaker E, Williams A, Walters S, Gordon A, Martínez-Alier N, et al. Interferon-gamma release assays do not identify more children with active TB than TST. Eur Respir J. 2009;33:1374-82.
47. Lighter J, Rigaud M, Eduardo R, Peng CH, Pollack H. Latent tuberculosis diagnosis in children by using the QuantiFERON-TB Gold In-Tube test. Pediatrics. 2009; 123:30-7.
48. Nicol MP, Davies MA, Wood K, Hatherill M, Workman L, Hawkridge A, et al. Comparison of T-SPOT.TB assay and tuberculin skin test for the evaluation of young children at high risk for tuberculosis in a community setting. Pediatrics. 2009;123:38-43.
49. Latorre I, De Souza-Galvao M, Ruiz-Manzano J, Lacoma A, Prat C, Altet N, et al. Evaluating the non-tuberculous mycobacteria effect in the tuberculosis infection diagnosis. Eur Respir J. 2010;35:338-42.
50. Connell TG, Curtis N, Ranganathan SC, Buttery JP. Performance of a whole blood interferon gamma assay for detecting latent infection with *Mycobacterium tuberculosis* in children. Thorax. 2006;61:616-20.
51. Richeldi L, Ewer K, Losi M, Bergamini BM, Roversi P, Deeks J, et al. T cell-based tracking of multidrug resistant tuberculosis infection after brief exposure. Am J Respir Crit Care Med. 2004;170:288-95.
52. Diel R, Loddenkemper R, Nienhaus A. Evidence-based comparison of commercial interferon-gamma release assays for detecting active TB: a metaanalysis. Chest. 2010;137:952-68.
53. Sester M, Sotgiu G, Lange C, Giehl C, Girardi E, Migliori GB, et al. Interferon-{gamma} release assays for the diagnosis of active tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2011;37:100-11.
54. Domínguez J, De Souza-Galvao M, Ruiz-Manzano J, Latorre I, Prat C, Lacoma A, et al. T-cell responses to the *Mycobacterium tuberculosis*-specific antigens in active tuberculosis patients at the beginning, during, and after antituberculosis treatment. Diagn Microbiol Infect Dis. 2009;63:43-51.
55. Goletti D, Stefania C, Butera O, Amicosante M, Ernst M, Sauzullo I, et al. Accuracy of immunodiagnostic tests for active tuberculosis using single and combined results: a multicenter TBNET-Study. PLoS ONE. 2008;3:e3417.
56. Metcalfe JZ, Cattamanchi A, Vittinghoff E, Ho C, Grinsdale J, Hopewell C, et al. Evaluation of quantitative IFN-gamma response for risk stratification of active tuberculosis suspects. Am J Respir Crit Care Med. 2010;181:87-93.
57. Latorre I, De Souza-Galvao M, Ruiz-Manzano J, Lacoma A, Prat C, Fuenzalida L, et al. Quantitative evaluation of T-cell response after specific antigen stimulation in active and latent tuberculosis infection in adults and children. Diagn Microbiol Infect Dis. 2009;65:236-46.
58. Pai M, Menzies D. Interferon-gamma release assays: what is their role in the diagnosis of active tuberculosis? Clin Infect Dis. 2007;44:74-7.
59. Aabye MG, Ravn P, PrayGod G, Jeremiah K, Mugomela A, Jepsen M, et al. The impact of HIV infection and CD4 cell count on the performance of an interferon gamma release assay in patients with pulmonary tuberculosis. PLoS ONE. 2009;4:e4220.
60. Chee CB, Gan SH, Khinmar KW, Barkham TM, Koh CK, Liang S, et al. Comparison of sensitivities of two commercial gamma interferon release assays for pulmonary tuberculosis. J Clin Microbiol. 2008;46:1935-40.
61. Raby E, Moyo M, Devendra A, Banda J, De Haas P, Ayles H, et al. The effects of HIV on the sensitivity of a whole blood IFN-gamma release assay in Zambian adults with active tuberculosis. PLoS ONE. 2008;3:e2489.
62. Tsiouris SJ, Coetzee D, Toro PL, Austin J, Stein Z, El-Sadr W. Sensitivity analysis and potential uses of a novel gamma interferon release assay for diagnosis of tuberculosis. J Clin Microbiol. 2006;44:2844-50.
63. Markova R, Todorova Y, Drenska R, Elenkov I, Yankova M, Stefanova D. Usefulness of interferon-gamma release assays in the diagnosis of tuberculosis infection in HIV-infected patients in Bulgaria. Biotechnol & Biotechnol Eq. 2009;23:1103-8.
64. Leidl L, Mayanja-Kizza H, Sotgiu G, Baseke J, Ernst M, Hirsch C, et al. Relationship of immunodiagnostic assays for tuberculosis and numbers of circulating CD4+ T-cells in HIV infection. Eur Respir J. 2010;35:619-26.
65. Vincenti D, Carrara S, Butera O, Bizzoni F, Casetti R, Girardi E, et al. Response to region of difference 1 (RD1) epitopes in human immunodeficiency virus (HIV)-infected individuals enrolled with suspected active tuberculosis: a pilot study. Clin Exp Immunol. 2007;150:91-8.
66. García-Gasalla M, Fernández-Baca V, Mir-Viladrich I, Cifuentes-Luna C, Campins-Rosselló A, Payeras-Cifre A, et al. Quantiferon-TB-Gold In Tube test in the diagnosis of pulmonary and extra-pulmonary tuberculosis. Enferm Infecc Microbiol Clin. 2010;28:685-9.
67. Syed Ahamed Kabeer B, Sikhamani R, Swaminathan S, Peromal V, Paramasivam P, Raja A. Role of interferon gamma release assay in active TB diagnosis among HIV infected individuals. PLoS One. 2009;4:e5718.
68. Detjen AK, Keil T, Roll S, Hauer B, Mauch H, Wahn U, et al. Interferon-gamma release assays improve the diagnosis of tuberculosis and nontuberculous mycobacterial disease in children in a country with a low incidence of tuberculosis. Clin Infect Dis. 2007;45:322-8.
69. Bamford AR, Crook AM, Clark JE, Nademi Z, Dixon G, Paton JY, et al. Comparison of interferon-{gamma} release assays and tuberculin skin test in predicting active tuberculosis (TB) in children in the UK: a paediatric TB network study. Arch Dis Child. 2010;95:180-6.
70. Kobashi Y, Mouri K, Yagi S, Obase Y, Miyashita N, Okimoto N, et al. Clinical utility of the QuantiFERON TB-2G test for elderly patients with active tuberculosis. Chest. 2008;133:1196-202.
71. Mori T, Sakatani M, Yamagishi F, Takashima T, Kawabe Y, Nagao K, et al. Specific detection of tuberculosis infection: an interferon-gamma-based assay using new antigens. Am J Respir Crit Care Med. 2004;170:59-64.
72. Kobashi Y, Mouri K, Miyashita N, Okimoto N, Matsushima T, Kageoka T, et al. QuantiFERON TB-2G test for patients with active tuberculosis stratified by age groups. Scand J Infect Dis. 2009;41:841-6.
73. Wilkinson KA, Wilkinson RJ, Pathan A, Ewer K, Prakash M, Klenerman P, et al. Ex vivo characterization of early secretory antigenic target 6-specific T cells at sites of active disease in pleural tuberculosis. Clin Infect Dis. 2005;40:184-7.
74. Jafari C, Ernst M, Kalsdorf B, Greinert U, Diel R, Kirsten D, et al. Rapid diagnosis of smear-negative tuberculosis by bronchoalveolar lavage enzyme-linked immunospot. Am J Respir Crit Care Med. 2006;174:1048-54.
75. Jafari C, Ernst M, Strassburg A, Greinert U, Kalsdorf B, Kirsten D, et al. Local immunodiagnosis of pulmonary tuberculosis by enzyme-linked immunospot. Eur Respir J. 2008;31:261-5.
76. Jafari C, Thijsen S, Sotgiu G, Goletti D, Domínguez-Benítez JA, Losi M, et al. Bronchoalveolar lavage enzyme-linked immunospot for a rapid diagnosis of tuberculosis: a Tuberculosis Network European Trialsgroup study. Am J Respir Crit Care Med. 2009;180:666-73.
77. Trajman A, Pai M, Dheda K, Van Zyl Smit R, Zwerling AA, Joshi R, et al. Novel tests for diagnosing tuberculous pleural effusion: what works and what does not? Eur Respir J. 2008;31:1098-106.
78. Losi M, Bossink A, Codecasa L, Jafari C, Ernst M, Thijsen S, et al. Use of a T-cell interferon-{gamma} release assay for the diagnosis of tuberculous pleurisy. Eur Respir J. 2007;30:1173-9.
79. Baba K, Sornes S, Hoosen AA, Lekabe JM, Mpe MJ, Langeland M, et al. Evaluation of immune responses in HIV infected patients with pleural tuberculosis by the QuantiFERON TB-Gold interferon-gamma assay. BMC Infect Dis. 2008;8:35.
80. Dheda K, Van Zyl-Smit RN, Sechi LA, Badri M, Meldau R, Meldau S, et al. Utility of quantitative T-cell responses versus unstimulated interferon-{gamma} for the diagnosis of pleural tuberculosis. Eur Respir J. 2009;34:1118-26.
81. Thomas MM, Hinks TS, Raghuraman S, Ramalingam N, Ernst M, Nau R, et al. Rapid diagnosis of *Mycobacterium tuberculosis* meningitis by enumeration of cerebrospinal fluid antigen-specific T-cells. Int J Tuberc Lung Dis. 2008;12:651-7.
82. Kim SH, Chu K, Choi SJ, Song KH, Kim HB, Kim NJ, et al. Diagnosis of Central Nervous System Tuberculosis by T-cell-based Assays on Peripheral Blood and Cerebrospinal Fluid Mononuclear cells. Clin Vaccine Immunol. 2008;15:1356-62.
83. Kim SH, Cho OH, Park SJ, Lee EM, Kim MN, Lee SO, et al. Rapid diagnosis of tuberculous meningitis by T cell-based assays on peripheral blood and cerebrospinal fluid mononuclear cells. Clin Infect Dis. 2010;50:1349-58.
84. Patel VB, Singh R, Connolly C, Coovadia Y, Peer AK, Parag P, et al. Cerebrospinal T-cell responses aid in the diagnosis of tuberculous meningitis in a human immunodeficiency virus- and tuberculosis-endemic population. Am J Respir Crit Care Med. 2010;182:569-77.
85. Doherty TM, Demissie A, Olobo J, Wolday D, Britton S, Eguale T, et al. Immune responses to the *Mycobacterium tuberculosis*-specific antigen ESAT-6 signal subclinical infection among contacts of tuberculosis patients. J Clin Microbiol. 2002;40:704-6.
86. Diel R, Loddenkemper R, Meywald-Walter K, Niemann S, Nienhaus A. Predictive value of a whole-blood IFN-gamma assay for the development of active TB disease. Am J Respir Crit Care Med. 2008;177:1164-70.

87. Diel R, Loddenkemper R, Niemann S, Meywald-Walter K, Nienhaus A. Negative and Positive Predictive Value of a Whole-Blood IGRA for Developing Active TB - An Update. Am J Respir Crit Care Med. 2011;183:88-95.
88. Leung CC, Chang KC, Chau CH. Is the whole-blood gamma interferon assay better than the tuberculin skin test in predicting active tuberculosis? Am J Respir Crit Care Med. 2008;178:210-11; author reply 211.
89. Kik SV, Franken WP, Mensen M, Cobelens FG, Kamphorst M, Arend SM, et al. Predictive value for progression to tuberculosis by IGRA and TST in immigrant contacts. Eur Respir J. 2010;35:1346-53.
90. Hill PC, Jackson-Sillah D, Fox A, Brookes RH, De Jong BC, Lugos MD, et al. Incidence of tuberculosis and the predictive value of ELISPOT and Mantoux tests in Gambian case contacts. PLoS ONE. 2008;3:e.1379.
91. Aichelburg MC, Rieger A, Breitenecker F, Pfistershammer K, Tittes J, Eltz S, et al. Detection and prediction of active tuberculosis disease by a whole-blood interferon-gamma release assay in HIV-1-infected individuals. Clin Infect Dis. 2009;48:954-62.
92. Santín M, Casas S, Saumoy M, Andreu A, Moure R, Alcaide F, et al. Detection of latent tuberculosis by the tuberculin skin test and a whole-blood interferon-gamma release assay, and the development of active tuberculosis in HIV-seropositive persons. Diagn Microbiol Infect Dis. 2011;69:59-65.
93. Bakir M, Millington KA, Soysal A, Deeks JJ, Efee S, Aslan Y, et al. Prognostic value of a T-cell-based, interferon-gamma biomarker in children with tuberculosis contact. Ann Intern Med. 2008;149:777-87.
94. Higuchi K, Harada N, Mori T, Sekiya Y. Use of QuantiFERON-TB Gold to investigate tuberculosis contacts in a high school. Respirology. 2007;12:88-92.
95. Lalvani A, Millington KA. T-cell interferon-gamma release assays: can we do better? Eur Respir J. 2008;32:1428-30.
96. Lalvani A, Pareek M. Interferon gamma release assays: principles and practice. Enferm Infecc Microbiol Clin. 2010;28:245-2.
97. Liu XQ, Dosanjh D, Varia H, Ewer K, Cockle P, Pasvol G, et al. Evaluation of T-cell responses to novel RD1- and RD2-encoded *Mycobacterium tuberculosis* gene products for specific detection of human tuberculosis infection. Infect Immun. 2004;72:2574-81.
98. Ruhwald M, Bodmer T, Maier C, Jepsen M, Haaland MB, Eugen-Olsen J, et al. Evaluating the potential of IP-10 and MCP-2 as biomarkers for the diagnosis of tuberculosis. Eur Respir J. 2008;32:1607-15.
99. Millington KA, Innes JA, Hackforth S, Hinks TS, Deeks JJ, Dosanjh DP, et al. Dynamic relationship between IFN-gamma and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. J Immunol. 2007;178:5217-26.

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# Advances in rapid diagnosis of tuberculosis disease and anti-tuberculous drug resistance

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A B S T R A C T

Rapid diagnosis of tuberculosis (TB) and multidrug-resistant (resistance to at least rifampin and isoniazid) *Mycobacterium tuberculosis* (MDR-TB) is one of the cornerstones for global TB control as it allows early epidemiological and therapeutic interventions. The slow growth of the tubercle bacillus is the greatest obstacle to rapid diagnosis of the disease. However, considerable progress has recently been made in developing novel diagnostic tools, especially molecular methods (commercial and 'in-house'), for direct detection in clinical specimens. These methods, based on nucleic acid amplification (NAA) of different targets, aim to identify the *M. tuberculosis* complex and detect the specific chromosome mutations that are most frequently associated with phenotypic resistance to multiple drugs. In general, commercial methods are recommended since they have a better level of standardization, reproducibility and automation. Although some aspects such as cost-efficiency and the appropriate setting for the implementation of these techniques are not yet well established, organizations such as the WHO are strongly supporting the implementation and universal use of these new molecular methods. This chapter summarizes current knowledge and the available molecular methods for rapid diagnosis of TB and anti-tuberculous drug resistance in clinical microbiology laboratories.



## Avances en el diagnóstico rápido de la enfermedad tuberculosa y de la resistencia a los fármacos antituberculosos

*Palabras clave:*
Tuberculosis
Diagnóstico molecular
Detección rápida de la resistencia

R E S U M E N

El diagnóstico rápido de la enfermedad tuberculosa y la resistencia múltiple a los fármacos antituberculosos (al menos isoniazida y rifampicina) en *Mycobacterium tuberculosis* complex (MDR-TB) es una de las piedras angulares en el control de esta enfermedad, ya que permite una acción epidemiológica y terapéutica precoz. El crecimiento lento del bacilo tuberculoso es uno de los mayores impedimentos para un diagnóstico rápido. En los últimos años ha existido un importante avance en el desarrollo de nuevas herramientas diagnósticas, sobre todo moleculares (comerciales y caseras), para el diagnóstico directo de muestra clínica. Estos métodos se basan en la amplificación de diversas dianas de ácidos nucleicos (AAN), para la identificación de *M. tuberculosis* complex y la detección de las mutaciones cromosómicas más frecuentemente relacionadas con la resistencia fenotípica a diversos fármacos. En general, entre las múltiples técnicas existentes, se recomiendan los métodos comerciales por su mayor estandarización, reproducibilidad y automatización. A pesar de que aspectos como el coste-efectividad y las indicaciones para la adecuada implementación de estas técnicas no están del todo bien establecidos, organizaciones como la OMS están apoyando de forma firme la aplicación y utilización universal de estos nuevos métodos moleculares. Este capítulo resume el conocimiento actual y los métodos moleculares disponibles para el diagnóstico rápido de la TB y la resistencia a los fármacos en los laboratorios de microbiología clínica.



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## Introduction

Delayed diagnosis of tuberculosis (TB) and multi-drug resistant forms of the disease constitute one of the biggest obstacles to effective control of TB worldwide.[1] Recently, the Stop TB Partnership, a network of concerned governments, organizations and donors led by the World Health Organization (WHO) (http://www.stoptb.org/stop_tb_initiative/), outlined a global plan to halve TB prevalence and mortality by 2015 and eliminate the disease as a public health problem by 2050. Multidisciplinary approaches, including studies of TB epidemiology, comparative genomics, evolution and host-pathogen interaction, will be necessary in order to develop better tools and strategies to control and eliminate TB.[2] In this context, several rapid and accurate diagnostic methods have recently appeared, and they will be briefly discussed in this chapter.

## Rapid detection of *Mycobacterium tuberculosis*

Although the presumptive diagnosis of TB is often based on clinical suspicion and radiological data, a definitive diagnosis of disease and drug resistance requires microbiological assays. Laboratory diagnosis of TB has traditionally been based on smear microscopy, culture and phenotypic identification. While the quickest, easiest and cheapest method available is acid-fast staining, its low sensitivity (45%-80% of positive cultures) has limited its usefulness, especially in geographical areas of lower incidence, in extrapulmonary forms (paucibacillary) of TB, and in HIV-infected patients. It should also be noted that a significant percentage (17%) of transmission occurs from smear-negative pulmonary tuberculosis patients.[3] A further point is that despite having good overall specificity the smear has a low positive predictive value (50%-80%) in areas of higher incidence of non-tuberculous mycobacteria (NTM) clinical isolates.[4-6]

By contrast, the culture technique is still regarded as the reference method due to its sensitivity and the fact that further studies can be conducted with the isolated mycobacteria (identification, sensitivity and epidemiological typing).[5,6] However, the slow growth of the tubercle bacillus is a major obstacle to rapid disease diagnosis. Indeed, while the last two decades have witnessed spectacular improvements to the culture method through the use of new media and automated systems such as Bactec 460TB (Becton Dickinson Diagnostics, Sparks, USA), MB/BacT ALERT (bioMérieux, Marcy-l'Etoile, France), MGIT 960 (Becton Dickinson Diagnostics) and VersaTREK (Trek Diagnostic System, Westlake, USA), several weeks are still required to obtain the final laboratory confirmation, and even longer in the case of conventional phenotypic identification procedures.[4-7] Therefore, in recent years new methods have been developed for the rapid diagnosis of active TB, the best alternative being the molecular or genotypic techniques.

### *Chromatographic methods*

Direct *M. tuberculosis* identification from clinical samples has been attempted by using different chromatography methods to detect tuberculostearic acid (TBSA) alone or in combination with other structural components of the mycobacterial cell wall.[8,9] Several fairly fast and sensitive methods have been developed so far,[9] one of the most interesting of which is fast gas chromatography mass spectrometry (GC-MS).[10,11] However, because TBSA is not specific to species and its detection requires a differential diagnosis between *Mycobacterium* and *Nocardia* species and other Gram-positive bacilli, which also contain the same acid, other lipids have been studied and proposed. Among these, hexacosanoic acid in combination with TBSA appears to be quite specific for the presence of *M. tuberculosis.*[11] However, although chromatographic methods may have some utility for mycobacterial identification from positive cultures, such as in the case of the new immunochromatographic assays based on the MPT-64 antigen,[12] they do not yet represent a significant alternative for the rapid diagnosis of tuberculosis from clinical specimens.

### *Phagotypic methods*

During the last decade a number of bacteriophages with specific affinity for mycobacteria have appeared for the rapid diagnosis of tuberculosis. Since 1947, over 250 different types of mycobacteriophages have been isolated and described, and they have constituted important tools for the genetic manipulation of mycobacteria. However, a degree of clinical utility has only been shown by two of the phage-based approaches developed to date, namely the Luciferase Reporter Phage Assay (LRP) and the Phage Amplified Assay (PhaB or MAB).[13-15] The most important difference between these methods concerns the detection of phage-infected mycobacterial cells. LRP relies on the emitted light that is encoded by the gene for luciferase (*fflux*), which is inserted into the phage genome. By contrast, PhaB or MAB is based on the presence of viable *M. tuberculosis* complex cells after phage amplification (Mycobacteriophage D29) in *Mycobacterium smegmatis*.

LRP has proven useful in differentiating *M. tuberculosis* and NTM from culture and, especially, in susceptibility tests to isoniazid and rifampin.[13] PhaB or MAB has been commercialized (FASTPlaque-TB or the variant PhageTeK MB, Biotec Laboratories Ltd, Ipswich, Suffolk, UK) for diagnosing tuberculosis in respiratory specimens,[14] and has also been studied for antimicrobial susceptibility testing in *M. tuberculosis.*[16] Both techniques are generally quick and simple, requiring little training and technical equipment, and they are relatively inexpensive. However, although they have demonstrated good specificity, several problems of sensitivity have been encountered in most studies.[14,15,17] Their routine application has therefore been somewhat delayed, and it remains to be seen what their real usefulness will be in the diagnosis of tuberculosis or the detection of resistance to anti-tuberculosis drugs

### *Genotypic methods*

Numerous molecular techniques (commercial and 'in-house') and various applications of them are now available for the microbiological diagnosis of mycobacterial infections.[7,18-20] Although DNA probes were the first major innovation in the molecular diagnosis of tuberculosis, the direct detection from clinical samples of *M. tuberculosis* and specific mutations correlating with resistance (see below) requires methods based on amplifying specific sequences of nucleic acids (NAA). These techniques have several advantages, such as a fast turnaround time and feasibility for automation. However, a number of disadvantages emerge when applying these methods directly to clinical specimens, for example, problems with inhibitors, sensitivity in smear-negative samples and DNA extraction.

Although the clinical utility of these methods has been widely discussed, solid evidence and a global consensus regarding their implementation has yet to be definitively achieved.[21] This is due in part to the wide variety of techniques available, as well as to the lack of standardization between studies, most of which use culture as the *gold standard*, which theoretically has a lower sensitivity than nucleic acid amplification (NAA) tests. Furthermore, the lack of assessment of clinical aspects in most studies has led to some confusion regarding how, with whom and when to use this technology. Nevertheless, the current findings regarding the use of NAA tests to diagnose tuberculosis suggest that: *a)* they can quickly detect the presence of *M. tuberculosis* in 50%-85% of acid-fast bacillus (AFB) smear-negative and culture-positive specimens; *b)* the positive predictive value in AFB smear-positive specimens is higher (>95%) than that of microscopy in geographical areas with a large number of NTM isolates; and *c)* in general, these molecular methods can diagnose TB

**Table 1**
Comparison of different commercial nucleic acid amplification (NAA) tests for direct detection of *Mycobacterium tuberculosis* complex from clinical samples

| Assay | Amplification method | Target | Detection | Sample vol (µl) | Turnaround Time (h) | Automation | IAC |
|---|---|---|---|---|---|---|---|
| Cobas Amplicor | PCR | 16S rRNA | Colorimetric | 100 | 6-7 | Yes | Yes |
| AMTD | TMA | 16S rRNA | Chemiluminiscent | 450 | 2.5 | No | No |
| LCx | LCR | PAB | Fluorimetric | 500 | 6 | Yes | No |
| BD Probe Tec | SDA | IS*6110* – 16S rRNA | Fluorimetric | 500 | 3.5-4 | Yes | Yes |
| Inno-Lipa | Nested-PCR | *rpoB* gene | Colorimetric | 500 | 12 | Yes | No |
| GenoType MD | NASBA | 23S RNA | Colorimetric | 500 | 5.5 | Yes | Yes |
| RT-PCR* | Real-time PCR | 16S rRNA | Fluorimetric | 10-100 | 2-3 | Yes | Yes |
| GeneXpert | Real-time PCR | *rpoB* gene | Fluorimetric | 1,000 | 2 | Yes | Yes |
| GenoQuick | PCR | IS*6110* | Colorimetric | 500 | 2.5 | No | Yes |

IAC: internal amplification control; LCR: ligase chain reaction; NASBA: nucleic acid sequence-based amplification; PCR: polymerase chain reaction; SDA: strand displacement amplification; TMA: transcription-mediated amplification.
*RT-PCR: several commercial techniques of real-time PCR.

several weeks earlier than culture in 80%-90% of patients with a high level of TB suspicion.[21-23]

NAA tests include a wide variety of 'in house' methods with multiple protocols of nucleic acid extraction and amplification (PCR) of different genetic targets (IS*6110*, *rpo*B, *hsp*65, 16S rDNA or MBP64). However, although these 'in house' amplification tests have generally improved in recent years, the recommendation is to use commercial tests that have a greater level of standardization and reproducibility.[20,23,24]

All NAA methods require further post-amplification analysis by electrophoretic observation of the amplified fragment or hybridization, restriction or sequencing.[18,20,25] The analysis based on sequencing (16S rDNA) or restriction (such as PCR-RFLP of the *hsp*65 gene or 16S-23S spacer region) could theoretically be used on clinical specimens. However, for the diagnosis of tuberculosis the most developed and commercialized methods are based on hybridization assays, and these are briefly described below, especially the newly marketed systems (Table 1).

*Conventional DNA amplification by PCR.* The Amplicor *Mycobacterium tuberculosis* test (Roche Diagnostic System Inc., Basel, Switzerland) is one of the oldest marketed techniques to rely on standard PCR. It is a DNA-based test that amplifies a specific segment of the 16S rRNA gene, followed by hybridization and colorimetric detection. This method may be automated (Cobas Amplicor) and was approved in 1996 by the US Food and Drug Administration (FDA) for use in respiratory samples that have positive AFB smears.[23] Numerous studies have reported high sensitivity in smear-positive respiratory specimens (87%-100%), the figure being lower in smear-negative cases (40%-73%) and extrapulmonary samples (27%-98%). The specificity of this method ranges from 91% to 100%.[20,24,26,27]

*Transcription-mediated amplification (TMA).* The commercial Amplified *M. tuberculosis* Direct Test (AMTD; Gen-Probe Inc., San Diego, CA, USA) is a rapid isothermal (42 ºC) method based on the amplification of 16S rRNA. Reverse transcriptase is used to copy rRNA to a cDNA-RNA hybrid, and the chemiluminiscent method is then applied to detect the *M. tuberculosis* complex by specific DNA probes. The AMTD was the first test to be approved by the FDA (1995) for smear-positive respiratory specimens, and in 2000 the FDA recommendation was extended to smear-negative samples.[23] There is now evidence that AMTD shows high specificity (95%-100%) and high sensitivity (91%-100%) for smear-positive respiratory samples, although the latter is lower for smear-negative (65%-93%) and extrapulmonary samples (63%-100%). The most important disadvantages are the lack of internal amplification control (IAC) and no possibility of automation.[20,24,26,27]

*Ligase chain reaction (LCR).* The LCX *M. tuberculosis* assay (Abbot Laboratories, Chicago, IL, USA) is a semi-automated DNA amplification method using LCR for direct detection, from clinical samples, of chromosomal gene encoding the *M. tuberculosis* protein antigen b. However, although good specificity (90%-100%) and sensitivity (65%-90%) were reported in several studies from respiratory specimens, this product was withdrawn from the European market in 2002.[20,26,27,28]

*Strand displacement amplification (SDA).* The BD ProbeTec ET Direct TB System (DTB; Becton Dickinson) was introduced in 1998 as a semi-automated technique for rapid detection of MTBC in respiratory samples. It is an isothermal (52.5 ºC) enzymatic amplification process for generating multiple copies of target sequences of the IS*6110* and 16S rRNA genes, whose amplification product is detected by the fluorescent method. Evaluations in respiratory samples have shown a sensitivity of 90%-100% in smear-positive samples and 30%-85% in smear-negative ones, with high specificity (90%-100%).[20,24,26,27]

*Solid-phase hybridization assays.* Three line probe assays are commercially available: the INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium), the GenoType MTBDR*plus* assay and the GenoType Mycobacterium Direct (MD) assay (Hain Lifescience, Nehren, Germany). Although the first two systems can detect and identify *M. tuberculosis* complex from clinical samples their major use is with positive cultures and for the detection of rifampin and isoniazid (by GenoType MTBDR*plus* only) resistance (see below). However, the GenoType MD assay is specific for the direct detection of RNA in clinical specimens of *M. tuberculosis* complex and other common NTM (*Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium kansasii* and *Mycobacterium malmoense*) by the NASBA amplification method. The limited data available to date indicate good sensitivity and specificity in respiratory specimens, with the potential advantage that this assay can detect five clinically common mycobacterial species.[29-31]

*Real-time PCR (RT-PCR).* These techniques are based on simultaneous amplification of different DNA targets and fluorimetric detection by labelled probes (for example, TaqMan, molecular beacons, bioprobes or FRET). These tests have a number of important advantages, especially their rapidity and fewer cross-contamination problems; this is because the processes, after DNA extraction, occur in a single tube. In recent years, numerous commercial techniques, such as the Cobas TaqMan MTB test (Roche Diagnostic System), have been developed with high overall sensitivity and specificity, especially in smear-positive respiratory samples (Table 1).[19,20,24,32] Among these, the GeneXpert MTB/RIF (Cepheid, Sunnyvale, CA, USA and FIND Diagnostics, Geneva, Switzerland) has recently been introduced as a semi-quantitative nested RT-PCR *in vitro*

diagnostic test, one that integrates and automates sample processing (DNA extraction) and offers simultaneous detection of *M. tuberculosis* complex and rifampin resistance (see below) within single-use disposable cartridges. The time to result is less than two hours and minimal training is required to use the test. Preliminary studies suggest good sensitivity and specificity in pulmonary samples.[33-36] Although further research is needed, the WHO has recently supported the use of this system as an initial diagnostic test in respiratory specimens of patients with high clinical suspicion of having tuberculosis or who could be multidrug resistant (see below).[37]

*Other new methods*. The loop mediated isothermal amplification (LAMP; Eiken Chemical Co. Japan and FIND Diagnostics, Geneva, Switzerland) is a relatively new isothermal (64-65 °C) amplification DNA technique.[38] The LAMP assay can synthesize large numbers of DNA targets (*gyrB* or IS*6110*) in a single tube, and the amplification product may be detected by turbidity or colorimetric and fluorimetric methods. Despite limited testing in the context of tuberculosis the early data are promising and the assay has the advantage of being rapid (2 hours) and relatively inexpensive, which could be useful in resource-limited settings.[24,39] Another new commercial NAA assay for rapid diagnosis of tuberculosis in respiratory samples is the GenoQuick MTB test (Hain Lifescience), which is based on PCR and subsequent hybridization. The complex obtained binds selectively to a dipstick and is detected by a colorimetric method (gold labelling). No studies have been published to date, but the preliminary data are promising.

## Rapid diagnosis of anti-tuberculous drug resistance

The rapid emergence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB: resistance to at least rifampin and isoniazid) and extensively drug-resistant tuberculosis (XDR-TB: MDR plus resistance to fluoroquinolone and one of the three injectable second-line drugs, amikacin, kanamycin and capreomycin) poses a serious threat to the treatment of tuberculosis. The World Health Organization estimates that 500,000 new cases of MDR-TB occur globally every year and more than 45 countries have reported XDR cases.

Because genetic resistance to an anti-tuberculosis drug is due to spontaneous chromosomal mutations the MDR/XDR phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance. This drug resistance may be attributable to direct transmission of drug-resistant strains (primary resistance) or to *de novo* acquisition of resistance during individual patient treatment (secondary resistance), i.e. due to inappropriate treatment or poor adherence to treatment.

A delay in diagnosing MDR-TB associated with standard drug susceptibility testing methods is likely to contribute to the acquisition of further drug resistance, as well as to the dissemination of drug-resistant strains through person-to-person transmission. By contrast, the rapid detection of drug-resistant strains facilitates early access to the appropriate therapy, reduces transmission rates and improves treatment outcomes. The long turnaround time and laboriousness of drug susceptibility testing methods has therefore stimulated the search for alternative and faster techniques. New genotypic methods search for the genetic determinants of resistance rather than the resistance phenotype. In this regard, the WHO has recommended the worldwide use of rapid genotypic assays for the rapid diagnosis of MDR-TB. Those genotypic assays should be able to detect the mutations responsible for isoniazide (INH) and rifampin (RMP) resistance. Moreover, if XDR-TB is to be ruled out, then the mutations responsible for resistance to streptomycin (STR), amikacin (AMK), kanamycin (KAN), capreomycin (CM) and fluoroquinolones should also be screened for.

Mutations confined to a short 81bp DNA region of the *rpoB* gene, encoding the β-subunit of RNA polymerase and spanning codons 507-533, have been found in ~95% of RMP-resistant strains. Mutations in rpoB generally result in high-level resistance to RMP and cross resistance to all rifamycins. However, specific mutations in codons 511, 516, 518 and 522 result in a phenotype of lower-level resistance to RMP and rifapentin, and retained susceptibility to rifabutin and rifalazil.[40] As RMP monoresistance is relatively rare, molecular detection of mutations in this region (RRDR, rifampin resistance determining region) is a good indicator of MDR-TB. It should be noted that a recent paper by Zaczek et al[41] reported that direct molecular identification for RMP-resistant *M. tuberculosis* clinical isolates is only possible for strains carrying selected mutations in RpoB. The identification of other mutations suggests that investigated strains might be resistant to this drug, in other words, these mutations require a specific genetic background to develop resistance.

The molecular mechanisms of resistance to INH are more complex. They have been associated with a variety of mutations which affect one or several genes involved in mycolic acid biosynthesis or that are overexpressed as a response to the build-up or cellular toxicity of INH. Mutations in the *katG* gene are responsible for 60%-70% of INH-resistant strains, with the most frequent mutation occurring at codon 315 (S315T, serine-to-threonine substitution). The S315T alteration located within the active site of *katG* prevents KatG-mediated activation of INH and results in a high level of resistance. Mutations in the *mabA-inhA* regulatory region that exhibit both low-level INH resistance and ethionamide resistance account for 8%-20% of INH resistance. A C-to-T substitution at nucleotide -15 results in the overexpression of InhA, an NADH-dependent enoyl-acyl reductase involved in mycolic acid synthesis, and INH resistance arises as a result of drug titration.[42] In our experience, a rapid genotypic assay including the 315-*katG* codon and -15 nt of the *mabA-inA* regulatory region would cover 62% of isoniazid-resistant strains in Barcelona.[43]

STR acts on the ribosome, inhibiting the translation of mRNA and, therefore, disrupting protein synthesis. Mutations associated with STR resistance in *M. tuberculosis* have been identified in the *rpsL* and *rrs* genes, which encode the ribosomal proteins S12 and 16S rRNA, respectively. More than half the STR-resistant clinical isolates present mutations associated with these genes.[44] The most common mutations in the *rpsl* gene have been detected in codons 43 and 48, and in two specific regions (the 530 loop and 912 regions) of the *rrs* gene.[45] In our experience, mutations in the *rpsL* and *rrs* genes are detected in 37.7% of STR-resistant *M. tuberculosis* isolates[46]. There is also a strong correlation between the level of resistance and the type and position of mutations.[46,47] High-level resistance has mainly been associated with *rpsL* gene alterations, whereas intermediate and low levels of resistance have been linked with mutations in the *rrs* gene and wild-type patterns.[46,47]

Only a few studies have investigated the genetic background of AMK, KAN and CM resistance.[48-50] Resistance to AMK and CM is associated with mutations in the *rrs* gene, especially in the region between nucleotides 1.400 and 1.500, each of which are responsible for a specific resistance pattern. Mutations G1484T and A1401G were found to cause high-level resistance to all drugs, whereas C1402T only causes resistance to CM and KAN. Resistance to CM is thought to be additionally mediated by mutations located anywhere in the *tlyA* gene, which encodes a 2'-O-methyltransferase.

Resistance to fluoroquinolones is mediated mainly by mutations in *gyrA* (around 85%) and less frequently by those in *gyrB* (around 10%),[51] which are genes that encode the respective subunits of the DNA topoisomerase gyrase.[52] Most mutations accumulate in a short discrete region known as the quinolone resistance-determining region (QRDR). It has been observed that certain isolates show a mixture of wild-type and multiple mutant alleles of *gyrA* (heteroresistant isolates).[53] Heteroresistance is considered a preliminary stage of full resistance.

In summary, a genotypic method to detect INH resistance should be based on the analysis of the 315-*katG* codon and -15 nt of the *mabA-inA* regulatory region. For RMP resistance the method should explore the short 81bp DNA region of the *rpoB* gene. In this context, it has to be stressed that RMP resistance is a surrogate marker for

**Table 2**
Description of studies that have evaluated line probe assays*

| Author (year) | Country | Reference test | Sample | Susceptible/resistant | Sensitivity | Specificity |
|---|---|---|---|---|---|---|
| *Genotype MTBDRplus assay (Hain Lifescience, Nehren, Germany)* | | | | | | |
| Isoniazid | | | | | | |
| Lacoma (2008) | Spain | Bactec460TB | Isolate | 14/48 | 73 | 100 |
| Mohito (2008) | Italy | Bactec460TB | Isolate | 0/173 | 79 | 100 |
| Hillemann (2007) | Germany | Bactec460TB | Isolate | 50/75 | 92 | 100 |
| Evans (2009) | South Africa | DNA sequencing | Isolate | 90/123 | 83.8 | 98.9 |
| Lacoma (2008) | Spain | Bactec460TB | Clinical specimen | 21/30 | 93 | 100 |
| Causse (2008) | Spain | MGIT | Clinical specimen | 22/37 | 94.6 | |
| Hillemann (2007) | Germany | Bactec460TB | Clinical specimen | 31/41 | 90.2 | 100 |
| Rifampin | | | | | | |
| Lacoma (2008) | Spain | Bactec460TB | Isolate | 50/12 | 91.7 | 100 |
| Hillemann (2007) | Germany | Bactec460TB | Isolate | 50/75 | 98.7 | 100 |
| Evans (2009) | South Africa | DNA sequencing | Isolate | 131/92 | 90.8 | 100 |
| Lacoma (2008) | Spain | Bactec460TB | Clinical specimen | 22/29 | 100 | 95.4 |
| Causse (2008) | Spain | MGIT | Clinical specimen | 23/36 | 100 | |
| Hillemann (2007) | Germany | Bactec460TB | Clinical specimen | 41/31 | 96.8 | 100 |
| *INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium)* | | | | | | |
| Ahmad (2002) | Kuwait | Bactec460TB | Isolate | 29/12 | 97 | 100 |
| De Oliveira (1998) | Brazil | Proportion | Isolate | 113/15 | 97 | 100 |
| Gamboa (1998) | Spain | Bactec460TB | Isolate | 46/13 | 100 | 100 |
| Hirano (1999) | Japan | Proportion | Isolate | 90/26 | 92 | 100 |
| Johansen (2003) | Denmark | Bactec460TB | Isolate | 35/24 | 97 | 100 |
| Jureen (2004) | Sweden | Bactec460TB | Isolate | 27/26 | 100 | 92 |
| Lemus (2004) | Belgium | Bactec460TB | Isolate | 10/10 | 100 | 100 |
| Rossau (1997) | Belgium | Proportion | Isolate | 203/61 | 98 | 100 |
| Sintchenko (1999) | Australia | Bactec460TB | Isolate | 22/11 | 96 | 100 |
| Somoskovi (2003) | USA | Proportion | Isolate | 64/37 | 95 | 100 |
| Srivastava (2004) | India | MIC | Isolate | 45/10 | 82 | 100 |
| Tracevska (2002) | Latvia | Bactec460TB | Isolate | 34/19 | 100 | 100 |
| Traore (2000) | Belgium | Proportion | Isolate | 266/145 | 99 | 100 |
| Watterson (1998) | England | Bactec460TB | Isolate | 16/16 | 100 | 94 |
| De Beenhouwer (1995) | Belgium | Proportion | Clinical specimen | 21/46 | 91 | 100 |
| Gamboa (1998) | Spain | Bactec460TB | Clinical specimen | 46/13 | 98 | 100 |
| Johansen (2003) | Denmark | Bactec460TB | Clinical specimen | 26/21 | 100 | 100 |
| Watterson (1998) | England | Bactec460TB | Clinical specimen | 10/24 | 80 | 100 |

*Modified from references 56 and 57.

MDR-TB. Finally, detection of XDR-TB would be based on the study of *rpsL*, *rrs* and *tlyA*, for resistance to STR, AMK, KAN and CM, and of *gyrA* and *gyrB*, for fluoroquinolone resistance.

Several molecular methods have been proposed to detect the specific mutations correlating with resistance in the amplified products: DNA sequencing, PCR-single-strand conformation polymorphism, PCR-heteroduplex formation, RT-PCR or solid-phase hybridization assays.[20,54] As solid-phase hybridization assays and RT-PCR have been commercialized and are widely used in clinical laboratories, they will be reviewed in some detail.

*Solid-phase hybridization assays*

*Line probe assays*. Line probe assays are a family of novel DNA strip tests that use PCR and reverse hybridization methods. Results are determined by colorimetric development. They have been designed to identify *M. tuberculosis* complex and simultaneously detect genetic mutations related to drug resistance. Amplified DNA can be obtained from cultured strains or clinical samples. Commercially available kits include the INNO-LiPA Rif. TB kit (Innogenetics, Gent, Belgium), the GenoType MTBDR*plus* assay and the GenoType MTBDR*sl* assay (Hain Lifescience).[54] The INNO-LiPA Rif. TB kit hybridizes the amplified DNA to ten oligonucleotide probes (one specific for the *M. tuberculosis* complex, and nine encompassing the core region of the *rpoB* gene: five overlapping wild-type S probes and four R probes for detecting specific mutations) that are immobilized on a nitrocellulose strip.[55] A number of studies have evaluated the diagnostic accuracy of LiPA for detecting resistance in several settings (Table 2). A recent metaanalysis[56] suggests that the LiPA assay is highly sensitive and specific for detecting rifampin-resistant *M. tuberculosis* in culture

and, to a slightly lesser degree, in clinical specimens. The Genotype MTBDR*plus* assay detects mutations in the *rpoB* gene for rifampin resistance, in the *katG* gene (S315T) for high-level INH resistance and in the promoter region of the *inhA* gene (nucleotides -8, -15, and -16) for low-level INH resistance. Various studies on the kit's accuracy have been performed and summarized in a recent metaanalysis.[57] Sensitivities for the detection of rifampin resistance range from 91% to 100%, whereas in the case of INH they range from 73% to 94%. The main limitation for the detection of INH resistance is that the molecular mechanisms behind some INH-resistant *M. tuberculosis* isolates are not known. Differences in the observed sensitivity could be due to the distribution of resistance-associated mutations in the different studies.[58] The GenoType *Mycobacterium tuberculosis* second line (MTBDR*sl*) assay was developed with a specific focus on the most prevalent *gyrA*, *rrs* and *embB* mutations. Although few studies have been published to date, this new assay may represent a reliable tool for the detection of fluoroquinolone and amikacin/capreomycin resistance, and, to a lesser extent, ethambutol resistance. In combination with a molecular test for the detection of RMP and INH resistance, the potential to detect XDR-TB can also be postulated.[53]

*LCD microarrays.* A low cost and density microarray (LCD) to detect RMP and INH resistance has been developed by Chipron GmbH (Berlin, Germany). Owing to high costs, complex protocols and the need for substantial additional laboratory equipment, microarrays have yet to become part of routine molecular diagnostics. However, LCD arrays do not need special equipment and the working protocols are similar to those used with line probe assays. Moreover, the LCD array offers increased throughput (eight samples per chip). The LCD array has been tested with *M. tuberculosis* clinical isolates[59] and a good correlation with sequencing data was obtained for *katG* S315T and S315N, for -8, -15, -17 nt of the *mabA-inA* regulatory region, and for *rpoB* core region mutations. Additional studies based on clinical samples are now needed.

*Real-time PCR (RT-PCR)*

As mentioned above, the GeneXpert MTB/RIF system (Cepheid) has recently been introduced. In addition to the high sensitivity and specificity obtained for the detection of *M. tuberculosis*, the few studies performed to date have also observed a good response as regards resistance to rifampin.[33-36] Although these results are promising, they obviously require further validation.

It is widely accepted that the extent of any future MDR or XDR tuberculosis epidemic will largely depend on the transmission efficiency or relative fitness of drug-resistant *M. tuberculosis* compared to drug-susceptible strains. For infectious pathogens, fitness is a composite measure of an organism's ability to survive, reproduce and be transmitted. However, the fitness cost associated with drug-resistance, in terms of reduced virulence and transmissibility, remains largely unknown.[60] Although INH-resistant strains were, in general, less often transmitted between humans in recent years, several studies have shown that the *katG* S315T mutation is associated with INH resistance without diminishing the virulence or transmissibility of *M. tuberculosis* strains.[61] This lack of attenuation, its high frequency among INH-resistant clinical isolates and the association between *katG* S315T and the Haarlem strain family (which may partly explain the successful spread of Haarlem strains in South America) suggests that the majority of these isolates will be virulent. In other words, it can therefore be considered a 'no-cost' mutation. Similarly, several studies have shown that different mutations conferring resistance to RMP varied in their effects on bacterial fitness.[62] It is important to highlight that a strain's genetic background could also influence the fitness effects of particular mutations. In light of these data, it may become necessary to provide information not only about the molecular mechanisms of resistance, but also about the particular clone that harbours them.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References


1. World Health Organization. Global tuberculosis control: epidemiology, strategy and financing. WHO report 2009. WHO/HTM/TB/2009.411. WHO Press, 2009 [consulted 3 December 2010]. Available at: http://www.who.int/tb/publications/global_report/2009/en/index.html
2. Young DB, Perkins MD, Duncan K, CE Barry CE 3rd. Confronting the scientific obstacles to global control of tuberculosis. J Clin Invest. 2008;118:1255-65.
3. Behr MA, Warren SA, Salamon H, Hopewell PC, Ponce de León A, Daley CL, et al. Transmission of *Mycobacterium tuberculosis* from patients smear-negative for acid-fast bacilli. Lancet. 1999;353:444-9.
4. American Thoracic Society; Centers for Disease Control and Prevention; Council of the Infectious Disease Society of America. Diagnostic standards and classification of tuberculosis in adults and children. Am J Respir Crit Care Med. 2000;161:1376-95.
5. Alcaide F, Esteban J, González J, Palacios JJ. Micobacterias. In: Cercenado E, Cantón R, editores. Procedimientos en Microbiología Clínica. Recomendaciones de la Sociedad de Enfermedades Infecciosas y Microbiología Clínica [9a]. SEIMC; 2005. Available at: http://www.seimc.org/documentos/protocolos/microbiologia/cap9a.pdf
6. Pfyffer GE. *Mycobacterium*: general characteristics, laboratory detection, and staining procedures. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of Clinical Microbiology. 9th ed. Washington DC: ASM Press; 2007. p. 543-72.
7. Vincent V, Gutiérrez MC. *Mycobacterium*: Laboratory characteristics of slowly growing mycobacteria. In: Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA, editors. Manual of Clinical Microbiology. 9th ed. Washington DC: American Society for Microbiology; 2007. p. 573-88.
8. Salfinger M, Pfyffer GE. The new diagnostic mycobacteriology laboratory. Eur J Clin Microbiol Infect Dis. 1994;13:961-79.
9. Jost KC Jr, Dunbar DF, Barth SS, Headley VL, Elliott LB. Identification of *Mycobacterium tuberculosis* and *M. avium* complex directly from smear-positive sputum specimens and BACTEC 12B cultures by high-performance liquid chromatography with fluorescence detection and computer-driven pattern recognition models. J Clin Microbiol. 1995;33:1270-7.
10. Cha D, Cheng D, Liu M, Zeng Z, Hu X, Guan W. Analysis of fatty acids in sputum from patients with pulmonary tuberculosis using gas chromatography-mass spectrometry preceded by solid-phase microextraction and post-derivatization on the fiber. J Chromatogr A. 2009;1216:1450-7.
11. Kaal E, Kolk AH, Kuijper S, Janssen HG. A fast method for the identification of Mycobacterium tuberculosis in sputum and cultures based on thermally assisted hydrolysis and methylation followed by gas chromatography-mass spectrometry. J Chromatogr A. 2009;1216:6319-25.
12. Park MY, Kim YJ, Hwang SH, Kim HH, Lee EY, Jeong SH, et al. Evaluation of an immunochromatographic assay kit for rapid identification of *Mycobacterium tuberculosis* complex in clinical isolates. J Clin Microbiol. 2009;47:481-4.
13. Jacobs WRJ, Barletta RG, Udani R, Chan J, Kalkut G, Sosne G, et al. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science. 1993;260:819-22.
14. Alcaide F, Galí N, Domínguez J, Berlanga P, Blanco S, Orus P, et al. Usefulness of a new mycobacteriophage-based technique for rapid diagnosis of pulmonary tuberculosis. J Clin Microbiol. 2003;41:2867-71.
15. Kalantri S, Pai M, Pascopella L, Riley L, Reingold A. Bacteriophage- based tests for the detection of *Mycobacterium tuberculosis* in clinical specimens: a systematic review and meta- analysis. BMC Infect Dis. 2005;5:59.
16. Galí N, Domínguez J, Blanco S, Prat C, Alcaide F, Coll P, et al. Use of a mycobacteriophage-based assay for rapid assessment of susceptibilities of *Mycobacterium tuberculosis* isolates to isoniazid and influence of resistance level on assay performance. J Clin Microbiol. 2006;44:201-5.
17. McNerney R, Kambashi BS, Kinkese J, Tembwe R, Godfrey-Faussett P. Development of a bacteriophage phage replication assay for diagnosis of pulmonary tuberculosis. J Clin Microbiol. 2004;42:2115-20.
18. Alcaide F. New methods for mycobacteria identification. Enferm Infecc Microbiol Clin. 2006;24 Suppl 1:53-7.
19. Domínguez J, Blanco S, Lacota A, García-Sierra N, Prat C, Ausina V. Utility of molecular biology in the microbiological diagnosis of mycobacterial infections. Enferm Infecc Microbiol Clin. 2008;26 Suppl 9:33-41.
20. Palomino JC. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. Eur Respir. 2005;26:339-50.
21. Dinnes J, Deeks J, Kunst H, Gibson A, Cummins E, Waugh N, et al. A systematic review of rapid diagnostic tests for the detection of tuberculosis infection. Health Technol Assess. 2007;11:1-96.
22. Catanzaro A, Perry S, Clarridge JE, Dunbar S, Goodnight-White S, LoBue PA, et al. The role of clinical suspicion in evaluating a new diagnostic test for active tuberculosis. Results of a multicenter prospective trial. JAMA. 2000;283:639-45.
23. CDC. Update Guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. Morb Mortal Wkly Rep. 2009;58:7-10.
24. Palomino JC. Molecular detection, identification and drug resistance detection in Mycobacterium tuberculosis. FEMS Immunol Med Microbiol. 2009;56:103-11.
25. Alcaide F. What does molecular biology contribute to the diagnosis of tuberculosis? Enferm Infecc Microbiol Clin. 2009;27:493-5.
26. Piersimoni C, Scarparo C. Relevance of commercial amplification methods for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J Clin Microbiol. 2003;41:5355-65.

27. Neonakis IK, Gitti Z, Krambovitis E, Spandidos DA. Molecular diagnostic tools in mycobacteriology. J Microbiol Methods. 2008;75:1-11.
28. Ausina V, Gamboa F, Gazapo E, Manterola JM, Lonca J, Matas L, et al. Evaluation of the semiautomated Abbott LCx *Mycobacterium tuberculosis* assay for direct detection of *Mycobacterium tuberculosis* in respiratory specimens. J Clin Microbiol. 1997;35:1996-2002.
29. Neonakis IK, Gitti Z, Baritaki S, Petinaki E, Baritaki M, Spandidos DA. Evaluation of GenoType mycobacteria direct assay in comparison with Gen-Probe *Mycobacterium tuberculosis* amplified direct test and GenoType MTBDRplus for direct detection of *Mycobacterium tuberculosis* complex in clinical samples. J Clin Microbiol. 2009;47:2601-3.
30. Syre H, Myneedu VP, Arora VK, Grewal HM. Direct detection of mycobacterial species in pulmonary specimens by two rapid amplification tests, the gen-probe amplified *Mycobacterium tuberculosis* direct test and the genotype mycobacteria direct test. J Clin Microbiol. 2009;47:3635-9.
31. Kiraz N, Saglik I, Kiremitci A, Kasifoglu N, Akgun Y. Evaluation of the GenoType Mycobacteria Direct assay for direct detection of the *Mycobacterium tuberculosis* complex obtained from sputum samples. J Med Microbiol. 2010;59:930-4.
32. Kim JH, Kim YJ, Ki CS, Kim JY, Lee NY. Evaluation of COBAS TaqMan® MTB PCR for the detection of *Mycobacterium tuberculosis*. J Clin Microbiol. 2010;49:173-6. Epub 2010 Nov 3.
33. Blakemore R, Story E, Helb D, Kop J, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol. 2010;48:2495-501.
34. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010;363:1005-15.
35. Helb D, Jones M, Story E, Boehme C, Wallace E, Ho K, et al. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. J Clin Microbiol. 2010;48:229-37.
36. Moure R, Muñoz L, Torres M, Santín M, Martín R, Alcaide F. Rapid detection of *Mycobacterium tuberculosis* complex and rifampin resistance in smear-negative clinical samples using an integrated real time PCR method. J Clin Microbiol. 2011;49(3). Doi: 10.1128/JCM. 01831-10.
37. World Health Organization. Roadmap for rolling out Xpert MTB/RIF for rapid diagnosis of TB and MDR-TB. 2010 [consulted 7 December 2010]. Available at: http://www.who.int/entity/tb/laboratory/roadmap_xpert_mtb-rif.pdf
38. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun. 2001;289:150-4.
39. Lee MF, Chen YH, Peng CF. Evaluation of reverse transcription loop-mediated isothermal amplification in conjunction with ELISA-hybridization assay for molecular detection of *Mycobacterium tuberculosis*. J Microbiol Methods. 2009; 76:174-80.
40. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PR, et al. Contribution of rpoB mutations to development of rifamycin cross-resistance in Mycobacterium tuberculosis. Antimicrob Agents Chemother. 1998;42:1853-7.47.
41. Zaczek A, Brzostek A, Augustynowicz-Kopec E, Zwolska Z, Dziadek J. Genetic evaluation of relationship between mutations in rpoB and resistance of Mycobacterium tuberculosis to rifampin. BMC Microbiol. 2009;9:10.
42. Hazbon MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis.* Antimicrob Agents Chemother. 2006;50:2640-9.
43. Coll PL, Aragón M, Alcaide F, Espasa M, Garrigó M, González J, et al. Molecular analysis of isoniazid and rifampin resistance in *Mycobacterium tuberculosis* isolates recovered from Barcelona. Microb Drug Resist. 2005;11:107-14.
44. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med. 2009;6:e2.
45. Meier A, Kirschner P, Bange FC, Vogel U, Bottger EC. Genetic alterations in streptomycin-resistant *Mycobacterium tuberculosis*: mapping of mutations conferring resistance. Antimicrob Agents Chemother. 1994;38:228-33.
46. Tudó G, Rey E, Borrell S, Alcaide F, Codina G, Coll P, et al. Characterization of mutations in streptomycin-resistant *Mycobacterium tuberculosis* clinical isolates in the area of Barcelona. J Antimicrob Chemother. 2010;65:2341-6.
47. Meier A, Sander P, Schaper KJ, Scholz M, Bottger EC. Correlation of molecular resistance mechanisms and phenotypic resistance levels in streptomycin-resistant *Mycobacterium tuberculosis.* Antimicrob Agents Chemother. 1996;40: 2452-4.
48. Alangaden GJ, Kreiswirth BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL, et al. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis.* Antimicrob Agents Chemother. 1998;42:1295-7.
49. Maus CE, Plikaytis BB, Shinnick TM. Molecular analysis of cross-resistance to capreomycin, kanamycin, amikacin, and viomycin in *Mycobacterium tuberculosis.* Antimicrob Agents Chemother. 2005;49:3192-7.
50. Maus CE, Plikaytis BB, Shinnick TM. Mutation of tlyA confers capreomycin resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2005;49:571-7.
51. Feuerriegel S, Cox HS, Zarkua N, Karimovich HA, Braker K, Rüsch-Gerdes S, et al. Sequence analyses of just four genes to detect extensively drug-resistant *Mycobacterium tuberculosis* strains in multidrug-resistant tuberculosis patients undergoing treatment. Antimicrob Agents Chemother. 2009;53:3353-6.
52. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, et al. Cloning and nucleotide sequence of *Mycobacterium tuberculosis* gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994;38:773-80.
53. Hillemann D, Rüsch-Gerdes S, Richter E. Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol. 2009;47:1767-72.
54. Nahid P, Pai M, Hopewell PC. Advances in the diagnosis and treatment of tuberculosis. Proc Am Thorac Soc. 2006;3:103-10.
55. De Beenhouwer H, Lhiang Z, Jannes G, Mijs W, Machtelinckx L, Rossau R, et al. Rapid detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by PCR and line probe assay. Tuber Lung Dis. 1995;76:425-30.
56. Morgan M, Kalantri S, Flores L, Pai M. A commercial line probe assay for the rapid detection of rifampicin resistance in *Mycobacterium tuberculosis:* a systematic review and meta-analysis. BMC Infect Dis. 2005;5:62.
57. Bwanga F, Hoffner S, Haile M, Joloba ML. Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. BMC Infect Dis. 2009;9:67.
58. Lacoma A, García-Sierra N, Prat C, Ruiz-Manzano J, Haba L, Rosés S, et al. GenoType MTBDRplus assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* strains and clinical samples. J Clin Microbiol. 2008;46:3660-7.
59. Aragón LM, Navarro F, Heiser V, Garrigó M, Español M, Coll P. Rapid detection of specific gene mutations associated with isoniazid or rifampicin resistance in *Mycobacterium tuberculosis* clinical isolates using non-fluorescent low-density DNA microarrays. J Antimicrob Chemother. 2006;57:825-31.
60. Borrell S, Gagneux S. Infectiousness, reproductive fitness and evolution of drug-resistant *Mycobacterium tuberculosis.* Int J Tuberc Lung Dis. 2009;13:1456-66.
61. Van Doorn HR, De Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, Van Soolingen D. Public health impact of isoniazid-resistant Mycobacterium tuberculosis strains with a mutation at amino-acid position 315 of katG: a decade of experience in The Netherlands. Clin Microbiol Infect. 2006;12:769-75.
62. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJ. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis.* Science. 2006; 312:1944-6.

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# Present-day treatment of tuberculosis and latent tuberculosis infection

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*Keywords:*
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Multi-drug-resistant tuberculosis
Antituberculosis treatment

ABSTRACT

The major objectives of tuberculosis (TB) control are to reduce morbidity and mortality via an early and appropriate treatment of the disease, to prevent carriers of the *Mycobacterium tuberculosis* bacillus from transmitting it to others, and to prevent latent tuberculosis infection (LTB) sufferers from progressing to the disease. To achieve these objectives, it is imperative to start an appropriate, effective antituberculosis treatment as early as possible, as well as identify contacts of the infected TB patient and others at risk of LTB progressing to TB, in order to establish an appropriate treatment for them. Here we review the bases for treating TB and LTB infections, including those produced by strains resistant to anti-TB drugs.



## Tratamiento actual de la infección y la enfermedad tuberculosas

*Palabras clave:*
Tuberculosis
Infección latente tuberculosa
Tuberculosis extensamente resistente
Tuberculosis multirresistente
Tratamiento antituberculoso

RESUMEN

El objetivo del control de la tuberculosis (TB) es reducir su morbilidad y mortalidad mediante el tratamiento precoz y adecuado de la enfermedad, la prevención de la transmisión de *Mycobacterium tuberculosis* desde personas bacilíferas y la prevención de la progresión a enfermedad de personas con infección latente tuberculosa (ILT). Para alcanzar estos objetivos se requieren el inicio precoz y la correcta cumplimentación de un tratamiento antituberculoso efectivo, y la identificación de contactos de pacientes con TB infectante y de otras personas con ILT con riesgo de progresar a enfermedad tuberculosa para establecer el tratamiento adecuado de estas personas. Revisamos las bases del tratamiento de la TB y de la ILT, incluyendo las producidas por cepas resistentes a los fármacos antituberculosos.



## Introduction

Early treatment of tuberculosis (TB) and preventing latent tuberculosis infection (LTB) from progressing to disease are two fundamental strategies of the objective of TB control. In this monograph chapter, we deal with both aspects which are essential for reducing morbidity and mortality due to TB.

## Antituberculosis drugs

Antituberculosis drugs (ATD) have traditionally been classified in terms of first-line, second-line and third-line drugs. The objective of this classification is to identify which ATDs —on the basis of their effectiveness, strength, toxicity or tolerance— should be used preferentially in the initial treatment of TB, and to distinguish them from others that should only be used in situations of intolerance or resistance to the previous drugs.[1-3] In addition, this classification is of particular interest currently in the treatment of in multi-drug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), since it provides a framework for taking decisions when designing a treatment regimen.[1-3]

## General principles of antituberculosis treatment

The major objectives of anti-TB therapy are: to eliminate the TB bacillus as rapidly as possible; to prevent the emergence of ATD-resistance; and to avoid relapses by eliminating persistent bacilli from their reservoirs[2] To achieve these objectives, an ATD combination should be employed for an extended period of time.[1,2] *Mycobacterium tuberculosis* has a long generation time and the ability to enter periods of dormancy with slow metabolic activity when antimicrobial penetration is difficult.[4] In TB patients, most of the bacillary population is made up of rapidly multiplying extracellular bacilli, located fundamentally in the tuberculous cavitary walls and the caseum, where the number of microorganisms may reach $10^9$ and $10^5$/mL, respectively. This population, because of its size, is the one

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which most often gives rise to the appearance of ATD-resistant mutations. The second of these populations is made up of slow growing bacilli, located at the periphery of the cavities in necrotic tissue in an acid-fast medium, and the third would consist of sporadically replicating dormant bacilli. The two latter populations are responsible for the appearance of TB relapse. In order to eradicate them properly, extended treatment is fundamental, using ATDs with a sterilizing effect.[1,2,4]

*M. tuberculosis* has the ability to spontaneously mutate, and this may give rise to the development of ATD-resistance. The bacillary population necessary to generate an ATD-resistant mutant varies according to the drug: $10^6$ for isoniazid (H), $10^8$ for rifampicin (R), $10^5$ for ethambutol (E), $10^6$ for streptomycin (S) and $10^3$ for pyrazinamide (Z). The bacillary population necessary to develop a mutation resistant to more than one ATD is calculated by adding together the exponents of the bacillary population needed to develop resistance to each individual drug; so, a bacillary population of $10^{21}$ would be needed to acquire resistance to a regimen containing H, R and Z. In the absence of pre-existing mutations, an appropriate three drug combination therapy would make resistance development impossible.[1,2,4,5]

Data arising from experimental observations and randomized clinical trials enable us to assert that of all the combinations tried, the one with greatest bactericidal and sterilizing activity is H plus R, and when administered for 9 months, the relapse rate is less than 3%. Reducing the period of this combination to 6 months gives rise to an increase in the incidence of relapses, although adding Z to RH during the first two months is successful in sterilizing the cultures more rapidly without increasing the relapse rate.[1,3,6-9]

## The treatment of tuberculosis disease

### *Indications for start of treatment*

The decision to start anti-TB treatment should be based on epidemiological, clinical, radiological and microbiological data. In the case of clinical forms following a serious, potentially fatal course and where TB is highly suspected, the priority objective should be to initiate empirical anti-TB therapy, even without microbiological confirmation.[1,3,6-8] In such cases it is important, before commencing treatment, to obtain the biological samples needed for the subsequent confirmation of the diagnosis and an ATD sensitivity analysis of the isolated strain. For suspected TB patients in a stable clinical condition, the priority should be to arrive at a microbiological diagnosis before starting treatment, since there is no clinical manifestation or X-ray image —however suggestive it may appear— specific to TB.

### *Recommended therapeutic regimens*

Currently recommended schedules for treating TB last for 6 months. This period is made up of an initial phase of 2 months —during which the basic objective is to eliminate the rapid growth bacilli and reduce the period of infection and contagion— and a maintenance phase of 4 months, during which the objective is to eliminate all intracellular bacilli and prevent a relapse.[1,3,6-8] An extension of the continuation phase to 7 months is indicated in cases where cultures remain positive beyond the first two months of initial therapy, and in special situations.[1,3,6-8] At present, courses of treatment lasting less than 6 months are not recommended, although trial schedules using new drugs are being tested which will enable this possibility to be explored.[9,10]

#### *Guidelines for the initial phase of treatment*

The prescription of choice for the initial phase of TB treatment consists of a daily administration of combinations of H, R and Z for 2 months.[1,3,6-8] Fixed dose preparations of these drug combinations are available, and the use of these formulations confers undoubted benefits, simplifying the complexity of the treatment and making it easier to follow correctly, which, in turn, minimizes the risk of monotherapy and the consequent development of resistance. The replacement of H by moxafloxacin (M) in the initial phase of TB treatment in one randomized clinical trial did not demonstrate greater effectiveness or safety.[11] The addition of a fourth drug to the selected regimen does not increase the bactericidal or sterilizing activities of the regimen but it does supply extra protection against developing resistant mutations. Its use is indicated in patients with suspected baseline resistance to one of the drugs included in the initial treatment plan, or when the primary resistance rate to H exceeds 4% in any particular population, or is unknown.[1,3,6-8] The recommendation for an associated fourth drug has traditionally been one of the first-line drugs (E or S), although it should be borne in mind that in one double-blind randomized clinical trial, the use of M instead of E as the fourth drug was associated with more rapid sterilization of the sputum,[9] and another non-randomized study also demonstrated that adding M to the standard TB treatment with 4 ATDs (RHEZ) was associated with more rapid negative sputum culture conversion.[10] Once the results of the laboratory ATD sensitivity tests are known, treatment can be adjusted accordingly, with the fourth drug in the regimen being suspended in cases where the bacilli are susceptible to all the drugs. There is no evidence arising from randomized clinical trials that enables firm recommendations to be made about the best pattern of treatment in situations where either R or H cannot be used in the initial treatment schedule because of intolerance or because it has been contraindicated. It is however recommended to replace the drug concerned with another first-line ATD and to increase the treatment period to 12-18 months.[3] Nor is there evidence to enable recommendations to be laid down concerning the best treatment option when neither R nor H can be used in the initial treatment because of intolerance, although replacing both drugs by first-line ATDs or M and increasing the treatment period to a minimum of 18-24 months, are recommended.[3]

#### *Guidelines for continuation phase treatment*

After the initial treatment phase, H and R may be continued for 4 months. There is no evidence which permits firm recommendations to be made about the therapeutic strategy or its duration in the maintenance phase when it is impossible to use R or H, either separately or together. However, it seems reasonable to recommend increasing the treatment time in accordance with the indications made in the previous point, and to lay down a sequence for the drugs to be used on an individual basis, depending on the toxicity profile of the drugs used.[1,3,6-8]

### *Treating antituberculosis drugs-resistant tuberculosis*

#### *Concepts*

ATD-resistant TB is a worldwide problem of the first order which makes the disease difficult to control and entails an increase in morbidity and mortality rates.[12] The presence of resistance to a single drug is referred to as monoresistance, and resistance to more than one drug, not including the H and R combination, polyresistance. The term MDR-TB is reserved for the presence of resistance to at least H and R,[13] and that of XDR defines resistance to H, R, fluoroquinolones and at least one of the following parenterally-administered drugs: amikacin, kanamycin and capreomycin.[14]

#### *Basic principles for treating resistant tuberculosis cases.*[1,3,6,12-14]

- When it comes to designing a new therapeutic regimen in cases of TB resistance, the possibility should always be considered that the patient may have followed covert monotherapy or bitherapy during the initial treatment phase which may have given rise to the development of resistance to

one of the initially susceptible ATDs that the patient received. This situation is especially likely in scenarios of MDR-TB and XDR-TB.
- In patients with therapeutic failure, the addition of a new ATD to the failing regimen may give rise to covert monotherapy and the acquisition of resistance to the new ATDs introduced, thus compromising the success of future therapy.
- A laboratory report for ATD sensitivity that includes at least R and H should be carried out for every patient diagnosed with TB. The diagnosis of MDR-TB or XDR-TB requires sensitivity testing that extends to second- and third-line ADTs.
- Changing treatment due to therapeutic failure should always be guided by the results of the baseline sensitivity analysis to ATDs, while taking into account the existence of cross-resistances between different ATDs.
- It is not advisable to reduce the number of ATDs in the initial regimen of TB treatment before the baseline sensitivity test is available, especially in patients who do not respond to treatment.
- MDR-TB and XDR-TB treatments should be carried out in consultation with and on the advice of a TB specialist.
- Appropriate therapy for MDR-TB and XDR-TB requires the use of at least 4 active drugs not used previously, and always including as far as possible a drug for parenteral administration.
- There is insufficient evidence of the kind necessary to be able to make firm recommendations concerning the optimal duration of MDR-TB and XDR-TB treatments. Given these limitations, we consider that the minimum period of treatment should be 12-18 months after the sterilization of cultures, with an injectable agent being maintained, whenever possible, for up to 6 months after their negative conversion, and continuing with a minimum of 3 active oral drugs until the end of treatment.

*Treatment of monoresistant and polyresistant tuberculosis*

The existence of baseline *M. tuberculosis* resistance to one or two ATDs requires a change in the initially established therapeutic regimen to be evaluated. How the new regimen should be drawn up will depend on factors such as: the number of ATDs the patient received during the initial regimen, the number of ATDs received to which the infectious strain is resistant, the presence or absence of a response to treatment, and the estimated initial bacillary population in relation to the clinical form of TB. We can distinguish various scenarios:

- The patient is treated initially with 4 ATDs (R, H, Z and E), with baseline resistance to one of them. In this scenario, once the sensitivity test results are known, the drug to which there is resistance will be suspended, while the others are maintained until the initial phase of treatment is complete. During the continuation phase, where the M. tuberculosis strain is susceptible to R and H, treatment with R and H will be maintained until 6 months are up, given that the patient will have undergone an initial treatment phase with 3 active ATDs that include R and H. If the strain is resistant to H or R, the initial phase of treatment can be completed with the remaining 3 active ATDs; in the continuation phase, the drug to which the strain is resistant will be replaced with Z or E and treatment prolonged until 9 months are completed.
- The patient is treated initially with 3 ATDs (R, H and Z), with baseline Z resistance. In this case, Z will be replaced by a new first-line drug, and R and H maintained until the initial phase of treatment is complete. Subsequently, treatment with R and H will continue for a total of 9 months.
- The patient is initially treated with 3 ATDs (R, H and Z) with baseline resistance to R or H. In this scenario, account should be taken of the time that the patient has received treatment with only 2 active drugs and the bacillary population required to develop resistant mutations which, in the cases of resistance to R or H, would be 109 and 1011 respectively. It should be remembered that this bacillary population may be present in cases of cavitary TB, and that there is, therefore, a possibility that new drug resistances may have developed, especially if the treatment time has been prolonged. In this situation the most conservative approach would be to assume that the patient may have developed new ATD resistances. We should therefore suspend the drug to which the strain is resistant, maintain the other ATDs to which the strain is susceptible (in view of the possibility that they might still be active), add at least 3 new active ATDs including first-line drugs wherever possible, and carry out a new ATD sensitivity test when the treatment is changed. After the results are known, the therapeutic regimen and its duration can be optimized.
- The patient is treated initially with 3 ATDs, with baseline resistance to two of them. In this scenario, it should be considered that the patient may have received covert monotherapy during the anti-TB medication and that the probability of developing resistance to this drug is consequently very high. In this case, the drugs to which the strain is resistant should be suspended, the drug to which the strain was susceptible be maintained, and at least 4 active drugs added, always including first-line ATDs wherever possible. A new sensitivity test should be performed at the time the treatment is changed. After the laboratory results are known, the therapeutic regimen and its duration can be optimized.
- The patient is treated initially with 4 ATDs with baseline resistance to two of them. If the isolate is not resistant to H or R, the drugs to which it is resistant should be replaced by a new first-line ATD, and the others maintained until the initial phase of treatment has been completed. Treatment with R and H will be maintained subsequently until a total of 9 months of treatment has been completed. In cases where R or H is one of the drugs to which the strain is resistant, the period during which the patient has received treatment with only 2 active drugs, and the bacillary population necessary for the development of mutations resistant towards them, should be considered (see scenario C, above)

*Treatment of multi-drug-resistant tuberculosis*

There are many possible combinations of ATD resistance. On a practical level, however, we can distinguish 2 scenarios, based on the number of ATDs used in the initial regimen:

- Patients treated initially with a regimen including 3 ATDs (R, H and Z or E). In this situation, the patient should be considered as having received monotherapy with Z or E, and has most probably developed a resistance to the drug. In this situation, the whole failing regime should be suspended and replaced by a combination of 4 active ATDs, selected in the following order of priority (Table 1): *a)* include, wherever possible, the first-line ATD not used previously (E or Z), because of its profile of baseline resistance; *b)* include a fluoroquinolone (preferably M); *c)* include a parenterally-administered ATD (S, kanamycin, capreomycin or amikacin); *d)* include a second-line ATD (cycloserine, para-aminosalicylic acid [PAS] or ethionamide) until 4 active drugs have been incorporated; and *e)* if it has not been possible to select the desired number of active ATDs in the 4 previous steps, include third-line drugs until this has been achieved (linezolid, clofazimine, amoxicillin-clavulanate, clarithromycin, imipenem).
- Patients treated initially with a regimen that included 4 ATDs (R, H, Z and E). In this situation, the patient should be considered

**Table 1**
Selection of drugs for multi-drug-resistant tuberculosis treatment: order of priority

| 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|
| Pyrazinamide | Moxifloxacin | Amikacin | Cycloserine | Clofazimine |
| Ethambutol | Levofloxacin | Streptomycin | PAS | Clarithromycin |
| | | Kanamycin | Ethionamide | Amoxicillin-Clavulanate |
| | | Capreomycin | | Linezolid |
| | | | | Imipenem |

PAS: para-aminosalicylic acid.

as having undergone bitherapy with Z plus E. Since the bacillary population necessary to develop mutations resistant to these drugs is $10^8$, it may have triggered off resistance to both, especially if the period of treatment was prolonged and the patient presented with cavitary TB. In this situation, R and H should be suspended and a new regimen designed with 6 active drugs, selected in the following order of priority: if possible, include E and Z because of their profile of baseline resistance and regardless of the risk, already mentioned, of secondary resistance; then, steps 2, 3, 4 and 5 outlined in the previous case should be followed.

*Treatment for extensively drug-resistant-tuberculosis*

The new regimen should consist of a combination of 6 active ATDs, selected in the following order (Table 2): *a)* include E or Z wherever possible because of their profile of baseline resistance; *b)* if the resistance profile permits, include an ATD for parenteral administration (S, kanamycin, capreomycin or amikacin); *c)* include a second-line ATD (cycloserine, PAS or ethionamide); and *d)* include third-line ATDs (linezolid, clofazimine, amoxicillin-clavulanate, clarithromycin, imipenem) until there are 6 active ATDs. The future treatment of patients with MDR and XDR-TB may change substantially as a result of the development of new ATDs, as is explained in a separate chapter of this monograph.

## Treatment of latent tuberculosis infection

After the detection and treatment of active TB cases, the second most pressing measure in the control of TB is the diagnosis and treatment of people with a high risk of developing the disease.

*General principles for treating latent tuberculosis infection*

The therapeutic objective is to avoid progression from latent to active TB. Only 10% of LTB patients are at risk of developing tuberculosis disease. For this reason, and also because treating it may provoke serious adverse and potentially fatal side effects, LTB treatment is only recommended in those individuals who are at greatest risk of progressing to TB.[3] On the other hand it should be remembered that TB may also develop because of the rapid progression of a recent infection, especially in immunocompromised patients (ICP). This possibility should be borne in mind when considering starting prophylactic treatment in IPC who have been in contact with active TB. Some ICP may have a limited ability to respond to the tuberculosis antigen and show negative to the tuberculin test, despite being infected with *M. tuberculosis*; for this reason, starting prophylaxis ought to be considered in ICP following obvious exposure, despite the negative tuberculin test.[12] Finally, the bacillary population in LTB patients is considerably lower than that found in patients with TB, so that the use of combination drug therapy is not necessary to avoid the development of resistant mutations.[3]

**Table 2**
Selection of drugs for treating extensively drug-resistant tuberculosis: order of priority

| 1 | 2 | 3 | 4 |
|---|---|---|---|
| Pyrazinamide | Amikacin | Cycloserine | Clofazimine |
| Ethambutol | Streptomycin | PAS | Clarithromycin |
| | Kanamycin | Ethionamide | Amoxicillin-Clavulanate |
| | Capreomycin | | Linezolid |
| | | | Imipenem |

PAS: para-aminosalicylic acid.

*Who should receive preventive therapy?*

Ideally, LTB treatment should be recommended exclusively for those with a significant risk of developing TB and a low risk of toxicity. LTB treatment is recommended for those who have had a recent tuberculin skin test conversion, for people with a positive reaction to tuberculin who may have had significant contact with TB carriers, and for patients with a high risk of developing TB.[3,15]

*Guidelines for treating latent tuberculosis infection*

*Isoniazid*

H has been considered the therapy of choice for LTB since 1965. The earliest randomized clinical trials that evaluated the effectiveness of H in LTB therapy were carried out between 1950 and 1970. Most of these compared H with a placebo for 12 months (12H), and demonstrated 90% effectiveness in reducing TB development.[16] In a clinical trial designed to evaluate the optimum treatment time for LTB using H,[17] a placebo was compared with H regimens for 3 (3H), 6 (6H) and 12 months (12H). In this study, the incidence of TB at 5 years was 1.4% for the placebo and 1.1%, 0.5% and 0.3% for 3H, 6H, and 12H, respectively. Patients in receipt of 6H had a 40% higher risk of TB than those in receipt of 12H. For this reason, 12H is considered the standard for LTB treatment. In a study was observed that the TB rate dropped the longer that H treatment continued, although beyond 9 months, it did not manage to bring down the incidence of TB any further. The study concluded that 9 months treatment could be enough for LTB therapy.[16] Intermittent administration of 9H in LTB treatment has not been studied comparatively, but by analogy with what was demonstrated in the continuation phase of TB treatment —where a twice-weekly dose was equivalent to daily administration— the twice-weekly administration of 9H is an alternative choice for LTB treatment.[15] Likewise, 6H administered daily or intermittently is also recommended as an alternative option in LTB treatment.15 In short, 9H is the preferred regimen recommended at the present time for treating LTB.15), on the grounds that this length of treatment offers maximum benefits, and that an extension to 12 months would bring only minimal extra benefit.

*Rifampicin*

The evidence concerning the effectiveness and safety of R administered daily for 4 months (4R) in LTB treatment is limited. In a placebo-controlled double-blind clinical trial, the effectiveness of 3 regimens of LTB treatment —R for 3 months (3R), 6H, and RH for 3 months (3RH)— was compared with the placebo.[18] After 5 years of follow-up, there was a higher incidence of TB in the placebo group than in the 3 therapeutic regimens, with no differences of effectiveness and safety being found between the 3 regimens evaluated. Available evidence, therefore, suggests that a 4R in LTB treatment achieves higher rates for completion of treatment, with a lower incidence of serious adverse outcomes and at less cost than the standard 9H regimen.[19-22] However, due to the absence of solid evidence concerning its effectiveness, 4R should be regarded as an alternative regimen for treating LTB, although given its low cost and short duration, 4R may be of some therapeutic use for LTB in certain special situations: in areas with a high incidence of H-resistance and in populations where a short treatment program may be indicated.

*Rifampicin with isoniazid*

The first clinical trial in which the effectiveness and safety of this program was evaluated was mentioned earlier.[18] In another randomized open clinical trial including 196 patients, the effectiveness of 3RH turned out to be similar to that of 9H, although the size of the population sample in the study did not permit demonstrate the comparability across the two regimens.[23] The 3RH regimen has been evaluated in various clinical trials in HIV-infected LTB patients. So, in a randomized clinical trial carried out on patients with a positive tuberculin skin test and infected with HIV, 3RH gave a similar level of protection to 6H and reduced the risk of TB compared to the placebo by 60%.[24] In another randomized open trial, 3RH demonstrated a similar effectiveness to 12H; however, the study included both tuberculin reactor and anergic patients, and lacked sufficient power to demonstrate the comparability of the two regimens. In another randomized open clinical trial which evaluated the safety and adherence to 3 short courses of LTB treatment, the TB rate and safety profiles of the 3RH and 6H schedules were similar, although the population sample size did not enable the equivalence of the two regimens to be evaluated.[25] Finally, in a clinical trial carried out on HIV-infected patients with cutaneous anergy, the safety of 3RH was comparable to 6H.[26] In a meta-analysis of randomized clinical trials which included the data of 1926 patients, the effectiveness and incidence of serious adverse outcomes and mortality were equivalent in 3RH and 6-12H.[27]

Therefore, the available data suggest that the safety of 3RH in treating LTB is equivalent to that of 6-12H. However, given that there is currently no solid evidence of its effectiveness, 3RH should be considered an alternative regimen for treating LTB.

*Rifampicin with pyrazinamide*

In a randomized clinical trial performed among HIV-infected patients, it was demonstrated that the effectiveness and safety of 2RZ and 12H were equivalent, although there was a significantly higher rate of compliance with treatment in the former.[28] On the basis of these results, the 2RZ and 12H regimens were regarded as preferential for treating LTB in HIV-infected patients,[29] with 2RZ as an alternative therapeutic course in patients not infected with HIV.[15] However, the description of a higher incidence of hepatotoxicity with 2RZ,[30] the communication of cases of severe hepatitis during LTB therapy in patients not infected with HIV in receipt of 2RZ,[31] and the observation that there was a higher incidence of hepatotoxicity with 2RZ than 6H in a randomized clinical trial carried out on patients not infected by HIV.[32] all served to dissuade use of 2RZ in HIV-negative patients and sowed doubts about the advisability of administering it to HIV-positive ones.[31] The greater risk of hepatotoxicity has been corroborated by other observational studies.[33] In a meta-study comparing the safety and effectiveness of 2RZ with 6-12H, which included clinical trials carried out on HIV-infected and non-infected patients, it was affirmed that 2RZ was equivalent to H for treating LTB in terms of effectiveness and mortality, but that it increased the risk of a serious negative outcome in patients not infected with HIV.[34]

Nevertheless, it is worth mentioning that none of the randomized clinical trials carried out among HIV-infected patients verified a significant increase in hepatotoxicity due to 2RZ.[25,26,28,35,36] In a meta-analysis of randomized clinical trials, designed to compare rates of severe hepatotoxicity in HIV-infected patients using 2RZ against 6H-12H, an increased risk of severe hepatotoxicity due to 2RZ was not demonstrated.[37] However, it should be remembered that the population represented in the clinical trials carried out among HIV-infected patients may not be representative of present-day HIV-infected populations, since all the studies were carried out before powerful antiretroviral treatment was available.

In summary, LTB treatment with 2RZ provokes a higher incidence of adverse outcomes and serious hepatotoxicity than a regimen using H in patients not infected by HIV, so that its use is not advised. Despite the fact that this has not been verified in HIV-infected patients, for reasons already remarked upon, it seems reasonable to advise against its use in these patients too.

*Treatment of latent tuberculosis infection due to antituberculosis drugs-resistant M. tuberculosis strains*

The preferential regimen for LTB treatment in subjects in contact with TB patients brought about by H-resistant strains should be 4R. The treatment of LTB in subjects in contact with MDR-TB has not been evaluated in randomized clinical trials, so that there is insufficient evidence to make firm recommendations about it. In such cases, a careful evaluation should be made of the risk of the LTB patient developing TB. In cases where the risk is high, it seems reasonable to use a combination of two drugs, adjusting the choice of these to the resistance profile of the infecting strain, with a preference —if the resistance profile of the infectious strain permits— for first-line drugs or fluoroquinolones. In cases of XDR-TB, the use of two drugs to which the infecting strain is susceptible is recommended, basing choice on the order set out in Table 2.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References

1. American Thoracic Society Documents. ATS/CDC/IDSA: Treatment of tuberculosis. Am J Respir Crit Care Med. 2003;167:603-62.
2. Coll P. Active drugs against *Mycobacterium tuberculosis*. Enferm Infecc Microbiol Clin. 2009;27:474-80.
3. González-Martín J, García-García JM, Anibarro L, Vidal R, Esteban J, Blanquer R, et al. Documento de consenso sobre diagnóstico, tratamiento y prevención de la tuberculosis. Enf Infecc Microbiol Clin. 2010;28:297.e1-20.
4. Wayne L. Dormancy of *Mycobacterium tuberculosis* and latency of disease. EurJ Clin Microbiol Infect Dis. 1994;13:908-14.
5. David HL. Probability distribution of drug-resistant mutants in unselected populations of *Mycobacterium tuberculosis*. Appl Microbiol. 1970;20:810-4.
6. Domínguez-Castellano A, Del Arco A, Canueto-Quintero J, Rivero-Román A, Kindelán JM, Creagh R, et al. Guía de práctica clínica de la Sociedad Andaluza de Enfermedades Infecciosas (SAEI) sobre el tratamiento de la tuberculosis. Enferm Infecc Microbiol Clin. 2007;25:519-34.
7. Consenso Nacional para el Control de la Tuberculosis en España. Grupo de Trabajo sobre Tuberculosis. Med Clin (Barc). 1992;98:24-31.
8. World Health Organisation. Global Tuberculosis Programmes. Treatment of tuberculosis: guidelines for national programmes. (WHO/CDS/TB/2003.13). Genève: World Health Organisation; 2003.
9. Wang JY, Wang JT, Tsai TH, Hsu CL, Yu CJ, Hsueh PR, et al. Adding moxifloxacin is associated with a shorter time to culture conversion in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2010;14:65-71.

10. Conde MB, Efron A, Loredo C, De Souza GR, Graça NP, Cezar MC, et al. Moxifloxacin versus ethambutol in the initial treatment of tuberculosis: a double-blind, randomised, controlled phase II trial. Lancet. 2009;373:1183-9.
11. Dorman SE, Johnson JL, Goldberg S, Muzanye G, Padayatchi N, Bozeman L, et al. Substitution of moxifloxacin for isoniazid during intensive phase treatment of pulmonary tuberculosis. Am J Respir Crit Care Med. 2009;180:273-80.
12. Rivero A, Márquez M, Santos J, Pinedo A, Sánchez MA, Esteve A, et al. High rate of tuberculosis reinfection during a nosocomial outbreak of multidrug-resistant tuberculosis caused by *Mycobacterium bovis* strain B. Clin Infect Dis. 2001;32:159-61.
13. Mukherjee JS, Rich ML, Socci AR, Joseph JK, Virú FA, Shin SS, et al. Programmes and principles in treatment of multidrug-resistant tuberculosis. Lancet. 2004;363:474-81.
14. Revised definition of extensively drug resistant tuberculosis. MMWR Morb Mortal Wkly Rep. 2006;55:1176.
15. CDC. Targeted Tuberculin Testing and Treatment of Latent Tuberculosis Infection. MMWR. 2000;49 RR6:1-71.
16. Ferebee SH. Controlled chemoprophylaxis trials in tuberculosis: a general review. Adv Tuberc Res. 1970;17:28-106.
17. International Union Against Tuberculosis Committee on Prophylaxis. Efficacy of various durations of isoniazid preventive therapy for tuberculosis: five years of follow-up in the IUAT trial. Bull WHO. 1982;60:555-64.
18. Hong Kong Chest Service/Tuberculosis Research Centre, Madras/British Medical Research Council. A double-blind placebo-controlled clinical trial of three antituberculosis chemoprophylaxis regimens in patients with silicosis in Hong Kong. Am Rev Respir Dis. 1992;145:36-41.
19. Page KR, Sifakis F, Montes de Oca R, Cronin WA, Doherty MC, Federline L, et al. Improved adherence and less toxicity with rifampin vs isoniazid for treatment of latent tuberculosis: a retrospective study. Arch Intern Med. 2006;166:1863-70.
20. Lardizabal A, Passannante M, Kojakali F, Hayden C, Reichman LB. Enhancement of treatment completion for latent tuberculosis infection with 4 months of rifampin. Chest. 2006;130:1712-7.
21. Menzies D, Long R, Trajman A, Dion MJ, Yang J, Al Jahdali, et al. Adverse events with 4 months of rifampin therapy or 9 months of isoniazid therapy for latent tuberculosis infection: a randomized trial. Ann Intern Med. 2008;149:689-97.
22. Ziakas PD, Mylonakis E. 4 months of rifampin compared with 9 months of isoniazid for the management of latent tuberculosis infection: a meta-analysis and cost-effectiveness study that focuses on compliance and liver toxicity. Clinical Infectious Diseases. 2009;49:1883-9.
23. Martínez Alfaro E, Solera J, Serna E, Cuenca D, Castillejos ML, Espinosa A, et al. Compliance, tolerance and effectiveness of a short chemoprophylaxis regimen for the treatment of tuberculosis [in Spanish]. Med Clin (Barc). 1998;111:401-4.
24. Whalen CC, Johnson JL, Okwera A, Hom DL, Huebner R, Mugyenyi P, et al. A trial of three regimens to prevent tuberculosis in Ugandan adults infected with the human immunodeficiency virus. N Engl J Med. 1997;337:801-8.
25. Rivero A, López-Cortés L, Castillo R, Verdejo J, García MA, Martínez-Marcos FJ, et al. Ensayo clínico aleatorizado para evaluar tres pautas cortas de tratamiento de la infección latente tuberculosa en pacientes infectados por el VIH. Enferm Infecc Microbiol Clin. 2007;25:305-10.
26. Rivero A, López-Cortés L, Castillo R, Lozano F, García MA, Díez F, et al. Ensayo clínico aleatorizado de tres pautas de quimioprofilaxis para prevenir la tuberculosis en pacientes infectados por el VIH con anergia cutánea. Enferm Infecc Microbiol Clin. 2003;21:287-92.
27. Ena J, Valls V. Short-course therapy with rifampin plus isoniazid, compared with standard therapy with isoniazid, for latent tuberculosis infection: a meta-analysis. Clinical Infectious Diseases. 2005;40:670-6.
28. Gordin F, Chaisson RE, Matts JP, Miller C, De Lourdes García M, Hafner R, et al. Rifampin and pyrazinamide vs. isoniazid for prevention of tuberculosis in HIV-infected persons: an international randomized trial. Terry Beirn Community Programs for Clinical Research on AIDS, the Adult AIDS Clinical Trials Group, the Pan American Health Organization, and the Centers for Disease Control and Prevention Study Group. JAMA. 2000;283:1445-50.
29. CDC. Prevention and treatment of tuberculosis among patients infected with human immunodeficiency virus: Principles of therapy and revised recommendations. MMWR. 1998;47:1-58.
30. Cook PP, Maldonado RA, Yarnell CT, Holbert D. Safety and completion rate of short-course therapy for treatment of latent tuberculosis infection. Clin Infect Dis. 2006;43:271-5.
31. CDC. Fatal and severe liver injuries associated with rifampin and pyrazinamide for latent tuberculosis infection, and revisions in American Thoracic Society/CDC Recommendations. MMWR. 2001;50:733-5.
32. Jasmer RM, Saukkonen JJ, Blumberg HM, Daley CL, Bernardo J, Vittinghoff E, et al. Short-course rifampin and pyrazinamide compared with isoniazid for latent tuberculosis infection: a multicenter clinical trial. Ann Intern Med. 2002;137:640-7.
33. McNeill L, Allen M, Estrada C, Cook P. Pyrazinamide and rifampin vs isoniazid for the treatment of latent tuberculosis: improved completion rates but more hepatotoxicity. Chest. 2003;123:102-6.
34. Gao XF, Wang L, Liu GJ, Wen J, Sun X, Xie Y, et al. Rifampicin plus pyrazinamide versus isoniazid for treating latent tuberculosis infection: a meta-analysis. Int J Tuberc Lung Dis. 2006;10:1080-90.
35. Halsey NA, Coberly JS, Desormeaux J, Losikoff P, Atkinson J, Moulton LH, et al. Randomised trial of isoniazid versus rifampicin and pyrazinamide for prevention of tuberculosis in HIV-1 infection. Lancet. 1998;351:786-92.
36. Mwinga A, Hosp M, Godfrey-Faussett P, Quigley M, Mwaba P, Mugala BN, et al. Twice weekly tuberculosis preventive therapy in HIV infection in Zambia. Aids. 1998;12:2447-57.
37. Camacho A, Pérez-Camacho I, Rivero A, Natera C, García-Lázaro M, Castón JJ, et al. Usar rifampicina más pirazinamida en profilaxis antituberculosa no incrementa el riesgo de hepatotoxicidad grave en pacientes infectados por virus de la inmunodeficiencia humana: metaanálisis de ensayos clínicos aleatorizados y controlados. Enferm Infecc Microbiol Clin. 2010;28:239-44.

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# New drugs for tuberculosis treatment

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ABSTRACT

Available data on anti-tuberculosis drug research reveal different properties of the agents and provoke speculation about future directions. Higher doses of the rifamycins are promising and are currently being evaluated in regimens of shorter duration that the isoniazid plus rifampin-based, six-to-nine month-course therapy. Moxifloxacin and gatifloxacin might shorten tuberculosis treatment as well, possibly in combination with rifapentine, while SQ109 could enhance the activity of rifampin-containing regimens. On the other hand, co-administration of moxifloxacin and PA-824 could be active against latent tuberculosis, whereas linezolid, PA-824 and TMC207 are candidates for a rifampin-free regimen in multidrug-resistant and extensively-resistant tuberculosis. Unfortunately, shorter than existent treatment regimens based on the new agents discussed here are likely to take at least another decade to be fully developed and implemented in clinical practice.



## Nuevos fármacos para el tratamiento de la tuberculosis

*Palabras clave:*
Altas dosis de rifamicinas
Nuevas combinaciones de fármacos

RESUMEN

Los datos disponibles en el proceso de investigación de nuevos fármacos antituberculosos han descubierto diferentes propiedades de los compuestos que permiten crear expectativas acerca de sus futuras indicaciones. Modelos terapéuticos que incluyan altas dosis de rifamicinas y pautas que asocien rifapentina con moxifloxacino o gatifloxacino podrían acortar el tratamiento de la tuberculosis, mientras que SQ109 incrementaría la actividad de las combinaciones basadas en esta rifamicina. Por otra parte, la tuberculosis latente podría tratarse adecuadamente con la asociación de moxifloxacino y PA-824, y la tuberculosis multirresistente y extensamente resistente con linezolid, PA-824 y TMC207, en pautas sin rifampicina. Desgraciadamente, tratamientos más cortos que los existentes, basados en asociaciones de los fármacos que se comentan en este trabajo, llevarán al menos otra década para ser completamente desarrollados e introducidos en la práctica clínica.



*In every bit of honest writing in the world there is a base theme: Try to understand men.*[1] But for every bit of research, to be properly done, there should be a base theme as well: Try to comprehend Nature.

### From mice to men I. Old drugs, higher doses, and new combinations

Required properties of tuberculosis drugs are summarized in Table 1.

#### *Rifampin*

Rifampin is considered to be the cornerstone in the current treatment of tuberculosis (TB).[2] Results from studies with mice and early bactericidal activity (EBA), in which the fall in colony forming units (CFU) during the first 2 days of therapy is measured, suggest that the standard dose of rifampin in TB treatment is at the lower end of the concentration-response curve.[3] Rifampin inhibits the β-subunit of the RNA polymerase, a multisubunit enzyme that transcribes bacterial RNA.[2] Mycobacterial resistance to the rifamycins results from mutations in the *rpoB* gene that codes for that β-subunit,[4] and its increasing prevalence concurrently with isoniazid resistance (multidrug-resistant tuberculosis [MDR-TB]) is a serious concern.[5] The pharmacokinetics (PK) and pharmacodynamics (PD) of rifampin

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**Table 1**
Required properties of new anti-tuberculosis (TB) drugs

| What a new drug should do? | Characteristics required |
|---|---|
| Simplify treatment or reduce treatment duration | Strong early bactericidal and sterilizing activity |
| | Low pill count, fixed-dose combination |
| | Allow for intermittent therapy |
| Have an acceptable toxicity profile | Low incidence of treatment-limiting adverse events |
| | No overlapping toxicity profile with other TB drugs |
| Be active against MDR-TB/XDR-TB | No cross resistance with first-line drugs |
| Be useful in HIV-infected patients with TB | Minimal interactions with antiretroviral drugs |
| | No overlapping toxicity profile with antiretrovirals |
| Be active against latent TB | Active against dormant bacilli |
| | Favorable toxicity profile |

HIV: human immunodeficiency virus; MDR-TB: multi-drug resistant tuberculosis; XDR-TB: extensively drug-resistant tuberculosis.

**Table 2**
Summary of pharmacokinetics/pharmacodynamics and activity of rifampin and rifapentine*

| Drug dose | Bio availability (%) | AUC (mg·h/l) | Cmax (mg/L) | $t_{1/2}$ (h) | Plasma protein binding (%) | MIC (mg/L) | AUC/MIC ratio |
|---|---|---|---|---|---|---|---|
| Rifampin (10 mg/Kg) | 68 | 21.5 | 8-20 | 2-5 | 85 | 0.15 | 70.8 |
| Rifapentine (600 mg) | Unknown | 319-394 | 10-18 | 13-20 | 97 | 0.02-0.125 | 76.6 |

AUC: area under the curve; Cmax: maximum concentration; MIC: minimal inhibitory concentration.
*Drug concentrations estimated for single compounds, not in combination.

in adults treated with the licensed dose (10 mg/Kg of body weight) are summarized in Table 2. In the 1960s and 1970s, PK of higher single doses (up to 30 mg/Kg) and repeated doses (up to 16 mg/Kg) of rifampin in adults were assessed, showing nonlinear increases in exposure.[6] More recently, PK of daily rifampin at 13 mg/Kg have been compared with 10 mg/Kg in 50 Indonesian patients with pulmonary TB who were treated with the standard regimen (2 months of RHZE followed by 4 months of rifampin and isoniazid [2RHZE/4RH]).[7] Increasing the dose by 30%, the maximum concentration (Cmax) increased by 49%, and the area under the curve (AUC) increased by 65%. AUC is an important parameter for concentration-dependent killers such as the rifamycins. It indicates total exposure to the drug over a certain time period. A higher dose of rifampin is not likely to affect the PK of other anti-TB drugs and antiretroviral drugs more strongly than the standard dose, as rifampin's inductive effect on the cytochrome P450 (CYP450) enzyme system appears to be maximal at a daily dose of 300 mg.[8] The minimal inhibitory concentration (MIC) of rifampin is 0.15 mg/L in broth culture.[9] Rifampin exhibits concentration-dependent activity that correlates best with the AUC/MIC ratio, as was shown in the mouse model. Results from an efficacy study in mice predicted a one-third reduction in TB treatment duration when the rifampin dose was increased by 50%.[10] Only a few data are available on the efficacy of regimens based on a higher dose of rifampin in humans. A short regimen of a high dose of rifampin (1,200 mg daily or every other day) with a high dose of isoniazid (900 mg) and streptomycin (1,000 mg) daily yielded almost 100% sputum culture conversion after 3 months.[11] All patients remained culture negative for up to 1 year, but sixteen percent of patients relapsed after 12 to 24 months. Another study in TB patients did not show a difference in efficacy between 600 mg or 750 mg rifampin daily combined with 300 mg isoniazid for 20 weeks,[12] however, the EBA of 1,200 mg rifampin daily was studied in 14 patients with pulmonary TB in another study and the mean 2-day EBA was almost twice as high as that of 600 mg rifampin.[3]

Little is known about the tolerability of higher than standard doses of rifampin. Past attempts to use large intermittent rather than daily doses of rifampin were met with a high incidence of the flu-like syndrome. This was ascribed to the intermittency of dosing rather than the size of the dose.[2] Daily rifampin at 13 mg/Kg was tolerated well by Indonesian patients.[7] Grade 1 and 2 hepatotoxicity was more common in the higher-dose group (46% versus 20%; $P = 0.054$), but none of the patients developed serious hepatotoxicity. Higher doses of rifampin did not cause tolerability problems in patients with brucellosis (900 mg, 45 days) or cutaneous leishmaniasis (1,200 mg, divided in two doses, 28 days).[13,14] The tolerability of rifampin in other diseases was reviewed in 650 patients; it was good with doses up to 1,200 mg but less favorable with doses of 1,800 mg.[15]

Available data suggest that higher daily doses of rifampin can shorten TB treatment. The maximum tolerable dose of rifampin and the EBA of a range of higher doses of rifampin given alone and in combination are planned to be investigated more extensively in phase II and III clinical trials in different multidrug regimens. Drawbacks of rifampin are its inductive effect on the CYP450 enzyme system, which is involved in the metabolism of many other drugs, and the increasing rate of mycobacterial resistance to rifampin.

*Rifapentine*

Rifapentine is a cyclopentyl rifamycin, whose activity and resistance mechanisms are the same as rifampin and other rifamycins.[16] PK of rifapentine in the standard dose is shown in Table 2.[17] When the dose of rifapentine was increased from 600 to 900 or 1,200 mg in 35 TB patients, the AUC increased by 39% or 61%, respectively.[18] Rifapentine induces the CYP450 enzyme system to a lesser extent than rifampin.[2] Rifapentine autoinduction —the phenomenon that induction of the CYP450 enzyme system also increases metabolism of the drug itself— was shown in a phase I study: rifapentine AUC decreased by 20% after 7 days of thrice-weekly rifapentine at 900 mg in 13 healthy volunteers.[19] Rifapentine (900 mg thrice weekly) reduced the AUC of moxifloxacin (400 mg daily) by 17% in the same study. The MIC of rifapentine ranges from 0.02 to 0.125 mg/L, i.e., two to four times lower than that of rifampin.[20] When adjusted for protein binding, the AUC/MIC ratio for rifapentine in standard dose is 76.6 (AUC/MIC ratio for rifampin, 70.8).[21]

**Table 3**
Overview of anti-tuberculosis (TB) drugs in the clinical pipeline

| Drug | Trial phase | Potential to shorten treatment | Acceptable toxicity profile | Active against MDR-TB | Useful in HIV-infected patients with TB | Active against latent TB | Interaction with rifampin |
|---|---|---|---|---|---|---|---|
| High-dose rifampin | II | Yes | To be established | Limited | Yes but not with protease inhibitors | Yes, but not first choice | – |
| High-dose rifapentine | II | Yes | To be established | Limited | To be established | Yes | – |
| Moxifloxacin | III | Yes | Yes | Yes | Yes | Yes | Reduced AUC of moxifloxacin by 30% |
| Gatifloxacin | III | Yes | Caution in elderly | Yes | Yes | Unknown | Possible |
| TMC207 | II | Yes | To be established | Yes | Unknown | Unknown | Reduced serum TMC207 concentration by 30% |
| PA-824 | II | Doubtful | Moderate increase in creatinine observed | Yes | Unknown | Yes | No |
| OPC-67683 | I/II | Yes | To be established | Yes | Unknown | Unknown | No |
| SQ 109 | I/II | Yes | To be established | Yes | Unknown | Unknown | Synergism in vitro |
| LL3858 | I | Yes | Unknown | Yes | Unknown | Unknown | Synergism in vitro |

AUC: area under the curve; HIV: human immunodeficiency virus; MDR-TB: multi-drug resistant tuberculosis.

Rifapentine (10 mg/Kg) was approved for the treatment of pulmonary TB by the U.S. Food and Drug Administration (FDA) in 1998 (Priftin, Hoechst Marion Roussel, Kansas City, MO), but it has not been approved by the European Medicines Agency (EMEA). Based upon its long half-life, rifapentine allows for intermittent dosing at wider intervals, which facilitates observed treatment. However, regimens with rifapentine and isoniazid once weekly in the continuation phase of treatment are slightly inferior to regimens with rifampin and isoniazid twice weekly, especially in patients with cavitary TB.[22] A high rate of mycobacterial monoresistance to rifamycins was seen in human immunodeficiency virus (HIV)-infected patients treated with rifapentine and isoniazid.[23] The use of rifapentine once weekly has therefore been restricted to HIV-negative pulmonary TB patients without cavitation and with a negative sputum culture after the intensive phase of treatment.[24] Higher than standard doses of rifapentine have shown the potency to shorten TB treatment in mice, especially when combined with moxifloxacin.[25] Rifapentine is being evaluated with moxifloxacin as a companion drug. A higher dose of rifapentine (15 mg/Kg) with moxifloxacin (100 mg/Kg twice per day) in a once-weekly continuation-phase regimen in mice showed better sterilizing activity than once-weekly rifapentine (15 mg/Kg) and isoniazid (75 mg/Kg) or twice-weekly rifampin (10 mg/Kg) and isoniazid (75 mg/Kg).[26] A twice-weekly regimen in mice containing rifapentine (15 or 20 mg/Kg), pyrazinamide (300 mg/Kg), and moxifloxacin (100 mg/Kg), preceded by 2 weeks of daily rifampin (10 mg/Kg), pyrazinamide (150 mg/Kg), and moxifloxacin (100 mg/Kg), resulted in stable cure after 4 months of treatment. Substitutions of rifampin (10 mg/Kg) by rifapentine (10 mg/Kg) and of isoniazid (25 mg/Kg) by moxifloxacin (100 mg/Kg twice per day) in a daily standard regimen in mice lead to bacillus eradication rates twice as fast as the standard regimen.[27] A recent study in mice showed that the main sterilizing component in regimens containing rifapentine, moxifloxacin, and pyrazinamide is rifapentine, rather than moxifloxacin.[28] An experiment in mice revealed a dramatic increase of bactericidal activity with increased rifapentine dose up to 80 mg/Kg in a regimen of rifapentine, moxifloxacin (100 or 400 mg/Kg), and pyrazinamide (150 or 600 mg/Kg), indicating the potential of higher doses of rifapentine to shorten TB treatment.[29] The optimum higher dose of rifapentine has not yet been defined, but it is assumed that rifapentine will cause fewer problems of drug-drug interactions than rifampin.

A study in 150 HIV-negative TB patients treated with either 600, 900, or 1,200 mg rifapentine plus isoniazid at 15 mg/Kg once weekly in the continuation phase showed good tolerability of the 900-mg dose and an insignificant trend towards more adverse events in the 1,200-mg arm.[30] In another study (35 patients), no association between the occurrence of adverse events and a higher dose of rifapentine (up to 1,200 mg) was found.[31] Two of 14 healthy volunteers developed adverse events (grade 2 hepatitis and a flu-like syndrome with rash) after treatment with daily moxifloxacin (400 mg) and thrice-weekly rifapentine (900 mg).[19]

There is a renewed rifapentine registration trial, conducted by the Tuberculosis Trials Consortium (TBTC), in which rifapentine 600 mg, daily dose, substitutes rifampin in the standard intensive phase, four-drug regimen, for treating smear-positive, pulmonary TB (TBTC study 29). On the other hand, the International Consortium for Trials of Chemotherapeutic Agents in Tuberculosis (INTERTB) is currently conducting the RIFAQUIN trial with moxifloxacin (400 mg) instead of isoniazid (300 mg) in the standard regimen and with rifapentine once weekly (20 mg/Kg for 4 months) or twice weekly (15 mg/Kg for 2 months) in the continuation phase.

Rifapentine is also a candidate drug for latent TB. A once weekly, 3-month regimen of rifapentine (15 mg/Kg) plus either moxifloxacin (100 mg/Kg) or isoniazid (75 mg/Kg) was as active as 6 months of daily isoniazid (25 mg/Kg) in monotherapy in a mouse model for latent TB.[32] A regimen of rifapentine (900 mg) plus isoniazid (900 mg) once weekly for 12 weeks was tolerated better than daily rifampin (450 to 600 mg) plus pyrazinamide (750 to 1,500 mg) for 8 weeks by patients with latent TB. The regimen protected well against active TB.[33] TBTC study 26 is also a clinical trial comparing rifapentine plus isoniazid (900 mg each), administered weekly for 12 weeks, to the standard regiment of daily isoniazid (300 mg) during 9 months. Summarizing, increasing the dose of rifapentine could shorten TB treatment, especially in combination with moxifloxacin, and may be useful against latent TB as well.

## From mice to men II. New agents under clinical investigation

Table 3 provides an overview of new anti-TB drugs in the clinical pipeline, and new indications and combinations of anti-TB drugs currently assessed in clinical trials are summarized in Table 4.

**Table 4**
Summary of clinical trials on going as 31st of May, 2010

| Study Title | Characteristics | Sponsor |
|---|---|---|
| TBTC Study 26 | Phase III randomized, open-label clinical trial of ultra short-course treatment of latent TB infection among contacts of active cases, using a 3-month once-weekly regimen of isoniazid and rifapentine, compared to standard 9-month daily therapy with isoniazid. Enrolment of main populations is completed (more than 8,100 participants have been enrolled and are in follow-up). Extended enrollment for HIV+ and children closed as December 15th, 2010 | CDC |
| TBTC Study 29 | Phase II randomized, open-label clinical trial assessing the antimicrobial activity and safety of substituting rifampin for rifapentine in standard intensive phase TB treatment regimen | CDC |
| TBTC Study 29PK | Substudy to characterize rifapentine PK parameters in patients with TB | CDC |
| TBTC Study 30 | Phase I/II randomized, placebo-controlled, double-blind clinical trial assessing the safety and microbiological activity of linezolid added to OBT for MDR-TB or XDR-TB | CDC |
| TBTC Study 30PK | Substudy to characterize to characterize linezolid PK and linezolid time over the MIC in patients with MDR-TB and XDR-TB | CDC |
| Rifaquin Study | Phase II, open-label 3-arm clinical trial powered to demonstrate non-inferiority of the two trial arms compared with the standard 6-month TB regimen. Trial regimens are: 2 months of daily ethambutol, moxifloxacin (400mg/qd), rifampin and pyrazinamide, followed by *a)* 2 months of twice weekly moxifloxacin (400 mg) and rifapentine (900mg) (2EMRZ/2P2M2) or *b)* 4 months of once weekly moxifloxacin (400mg) and high dose rifapentine (1,200mg) (2EMRZ/4P1M1) | INTERTB Biomedical Research and Training Institute, Zimbabwe |
| Linezolid for XDR-TB | Phase 2a, randomized, 2-arm, open-label, clinical trial of the efficacy of linezolid combined with anti-TB therapy in subjects with XDR-TB | NIAID |
| 4-month quinolone for treating pulmonary TB | Phase III, randomized open-label controlled trial of a 4-month gatifloxacin-containing regimen versus standard regimen for the treatment of adult patients with pulmonary TB | IRD |
| TMC207 in patients with MDR-TB | Phase II, placebo-controlled, double-blind, randomized trial to evaluate the anti-bacterial activity, safety, and tolerability of TMC207 in subjects with newly diagnosed sputum smear+ pulmonary infection with MDR-TB | Tibotec-Virco Virology BVBA |
| OPC 67683 in patients with MDR-TB | Phase II, double-blind, placebo-controlled clinical trial, to evaluate OPC 67683 in patients with sputum culture-positive, MDR-TB | Otsuka Pharmaceutical |
| RE-MoxTB study | Phase III, double-blind controlled trial, comparing two moxifloxacin-containing treatment shortening regimens in pulmonary TB | University College, London |
| Levofloxacin and moxifloxacin for MDR-TB | Phase III, randomized, multicentre, open-label, with parallel groups comparing the effect between levofloxacin and moxifloxacin among MDR-TB patients | Seoul National University Hospital |
| Rifapentine plus moxifloxacin for treatment of pulmonary TB | Phase II randomized, open-label trial of a rifapentine plus moxifloxacin-based regimen for intensive phase treatment of smear+ pulmonary TB | Johns Hopkins University |
| Evaluation of early bactericidal activity in pulmonary TB | Phase II-dose ranging trial to evaluate the extended early bactericidal activity, safety, tolerability, and pharmacokinetics of PA-824 in adults with newly diagnosed, uncomplicated, smear+, Pulmonary TB | TB Alliance |
| Study of daily rifapentine for pulmonary TB | Phase II-randomized, open-label trial of daily rifapentine 450mg or 600mg in place of rifampicin 600mg for intensive phase treatment of smear+ pulmonary TB | Johns Hopkins University |

CDC: Centers for Diseases Control and Prevention; HIV: human immunodeficiency virus; IRD: Institut de Recherche pour le Développement; MIC: minimum inhibitory concentration; MDR-TB: multi-drug resistant tuberculosis; NIAID: National Institute of Allergy and Infectious Diseases; OBT: optimized background therapy; PK: pharmacokinetics; TB: tuberculosis; TBTC: Tuberculosis Trials Consortium; XDR-TB: extensively drug-resistant tuberculosis.

*Fluoroquinolones*

The fluoroquinolones are registered as second-line anti-TB drugs.[34] Moxifloxacin and gatifloxacin are candidates for shortening TB treatment, since they have the lowest MICs [35]and greatest bactericidal activity, as expressed in the rate of fall in CFU count.[36]

Moxifloxacin is a broad-spectrum 8-methoxy fluoroquinolone with activity against both gram-positive and gram-negative bacteria, including anaerobes.[37] It inhibits bacterial DNA gyrase, an enzyme that is essential for the maintenance of DNA supercoils, which are necessary for chromosomal replication.[38] The development of mycobacterial resistance to fluoroquinolones has been described in MDR strains[39] and in strains from HIV-infected TB patients with a low CD4 count.[40] Fluoroquinolone resistance is due to stepwise mutations in the quinolone resistance-determining region of the mycobacterial *gyrA* and *gyrB* genes.[41] No cross-resistance with the first-line anti-TB drugs has been shown, but cross-resistance within the group of fluoroquinolones was proved.[42] A study in newly diagnosed TB patients showed higher rates of *M. tuberculosis* resistance to fluoroquinolones in patients with prior exposure to fluoroquinolones than in patients who were fluoroquinolone naïve.[43] Other studies did not find such an association.[44] Moxifloxacin is metabolized by glucuronidation and sulfation (phase II metabolism) rather than by CYP450-mediated (phase I) metabolism.[45] Up to 20% of moxifloxacin is excreted unaltered in urine and 25% in feces. The AUC from 0 to 24 h (AUC 0-24) of moxifloxacin decreased by 27 to 31% when coadministered with rifampin.[46] This could be due to induction of phase II metabolic enzymes (uridine diphosphatase, glucuronosyltransferase, and sulfotransferase) by rifampin. The clinical relevance of this interaction is unknown. *In vitro* studies with moxifloxacin show MICs of 0.25 to 0.50 mg/L. The bactericidal activity of fluoroquinolones is generally considered to be concentration dependent,[47] although a recent report showed time-dependent killing as well. The ratio of AUC to MIC is thought to be the best predictor of fluoroquinolone efficacy in gram-negative, fast-multiplying bacteria. It was shown *in vitro* and *in vivo* that the greatest bactericidal activity occurs at AUC/MIC ratios of 100 to 125 or more. While it is unclear whether this also applies to the slowly multiplying *M. tuberculosis*, this observation would suggest that moxifloxacin is the fluoroquinolone with greatest efficacy, followed by gatifloxacin (AUC/MIC ratios of 96 and 68, respectively, derived from *in vitro* and *in vivo* work). Aside from the AUC/MIC ratio, the other important indicator of efficacy of

concentration-dependent killers is the Cmax/MIC ratio, which should be more than 8 to 12 for effective killing of gram-negative bacteria. Data adapted from a single-oral-dose study in healthy volunteers showed that the Cmax/MIC90 ratio of moxifloxacin 400 mg (the approved dose in humans) is 8.6.[23] *In vitro* studies and studies in mice showed enhanced bactericidal activity of moxifloxacin and isoniazid when coadministered.[48] Ethambutol adversely affected the activity of moxifloxacin *in vitro*: it reduced moxifloxacin efficacy by 80%.[49] Moxifloxacin (100 mg/Kg) was able to reduce the time to culture conversion in mice by 2 months when replacing isoniazid in the standard 6-month regimen. This reduction was not found when moxifloxacin was either added to the standard regimen or when it replaced any of the other drugs. It is hypothesized that the superior activity of 2 months of rifampin plus moxifloxacin plus pyrazinamide followed by 4 months of rifampin plus moxifloxacin (2RMZ/4RM) to 2RHZ/4RH is caused by a synergistic activity of rifampin, moxifloxacin, and pyrazinamide or antagonistic activity of rifampin, isoniazid, and pyrazinamide.[50] Moxifloxacin efficacy has also been shown in humans. EBA studies in newly diagnosed pulmonary TB patients showed comparable activity of moxifloxacin (400 mg) and isoniazid (300 mg or 6 mg/Kg).[51] The VT50 (the time needed to kill 50% of viable bacteria) of isoniazid was lower than that of both rifampin and moxifloxacin. The EBA and VT50 of combined moxifloxacin and isoniazid did not differ significantly from the two drugs in monotherapy. Based on these results, no antagonistic effect of adding moxifloxacin to the standard, isoniazid-containing regimen is expected, nor will it enhance the bactericidal activity of the regimen.[52] The effect of replacing ethambutol with moxifloxacin in the standard regimen on the 2-month sputum culture conversion rate was analyzed in 277 patients with pulmonary TB from African and North American sites.[53] No difference in percentage of negative cultures after 2 months of treatment was found. However, more patients treated with moxifloxacin had negative cultures after 4 weeks of treatment than patients treated with ethambutol (37% versus 26%; $P = 0.05$). A comparable study is ongoing in Brazil. The Gatifloxacin for TB Study Team (OFLOTUB) performed a phase II clinical trial in which ethambutol in the standard regimen was replaced by gatifloxacin, moxifloxacin, or ofloxacin.[54] The regimen with moxifloxacin caused the fastest decrease in CFU during the early phase of a biexponential fall (in a nonlinear model that differentiates between quickly and slowly eliminated bacilli). Both moxifloxacin and gatifloxacin accelerated bacillary elimination significantly in the late phase. The percentage of negative sputum cultures after 2 months of treatment did not differ significantly between the treatment groups (82% versus 77% on solid medium and 40% versus 44% on liquid medium [in MGIT] for moxifloxacin versus gatifloxacin, respectively). Two-month sputum culture conversion rates have also been evaluated in a double-blind randomized controlled trial in which isoniazid in the standard regimen was replaced with moxifloxacin (TBTC study 28). Culture conversion after 8 weeks of treatment was achieved in 60% of patients treated with the moxifloxacin-containing regimen and in 55% of patients using isoniazid (J. Grosset, presented at the 1st International Workshop on Clinical Pharmacology of Tuberculosis Drugs, Toronto, Canada, 2008). A multicenter three-armed REMoxTB trial in which the standard regimen is compared to a) a regimen of 2RHZM/2RHM and b) a regimen of 2RMZE/2RM has recently started. The possibility of combining moxifloxacin with rifapentine, two agents with a long half life, is explored in the RIFAQUIN trial (see section about rifapentine).[55] Finally, moxifloxacin could be of use in the treatment of latent TB. The combination of 3 months of once-weekly moxifloxacin and rifapentine was as effective as 6 months of isoniazid monotherapy in a mouse model for latent TB.[53] A single dose moxifloxacin of up to 800 mg was tolerated well. Little is known about the long-term tolerability in TB patients. Moxifloxacin (400 mg, administered for an average of 6.3 months) was withdrawn in 4 of 38 TB patients because of a major adverse event (including nausea, vomiting, muscle pain, tremors, insomnia, and dizziness), but no irreversible or fatal events occurred.[56] In another study, no toxicity was experienced by patients who were treated with moxifloxacin, rifampin, and isoniazid for 6 months. Prolongation of QT time has been seen in patients using moxifloxacin for a variety of other bacterial infections. In February 2008, Bayer distributed a "Dear Doctor" letter warning physicians about rare but severe hepatological and dermatological adverse events associated with moxifloxacin. In July 2008 the European Medicines Agency (EMEA) sent out a review on the association of moxifloxacin and hepatological problems. It was concluded that the benefits of moxifloxacin in treatment of respiratory tract infections outweigh the risks, but its use should be restricted.[57]

Moxifloxacin could shorten TB treatment. However, its optimal dose in TB treatment must be evaluated with respect to the recently observed decrease in AUC0-24 when coadministered with rifampin, and ambiguous results revealed from efficacy studies in mice and humans. Finally, the adverse events of moxifloxacin require extended evaluation.

Gatifloxacin has many of the favorable features of moxifloxacin.[58] However, the risk of mycobacterial resistance development and the recently found association between gatifloxacin and dysglycemic events[59] are concerns. *In vitro* studies and studies in mice showed improved activity of rifampin and isoniazid when gatifloxacin was added and even more when the regimen also included pyrazinamide,[60] but some results were contradictory: while ethambutol reduced gatifloxacin activity *in vitro*, the combination of ethambutol, ethionamide, and gatifloxacin was highly effective in mice.[61] A multicenter trial of the OFLOTUB consortium (see section on moxifloxacin) is enrolling patients at five African sites. It compares the efficacy and tolerability of a 4-month regimen of 2 months of rifampin plus isoniazid plus pyrazinamide plus gatifloxacin followed by 2 months of rifampin plus isoniazid plus gatifloxacin (2RHZG/2RHG) to the standard 2RHZE/4RH regimen. As per rifapentin, gatifloxacin has not been approved by the EMEA.

*Oxazolidinones*

Linezolid has been the first oxazolidinone to be developed and approved for clinical use. It is active against a range of bacteria, but its primary clinical role is the treatment of infections caused by aerobic Gram-positive organisms, including resistant strains such as vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* and penicillin-resistant pneumococci.[62]

*In vitro* studies have shown good activity of linezolid against different species of mycobacteria, including resistant strains. The linezolid MIC90 for *M. tuberculosis* was in the range 1–2 mg/L.[63] MICs of linezolid against other non-tuberculous mycobacteria are higher than the MIC for *M. tuberculosis*.[64] In experimental studies with the murine model of tuberculosis, oxazolidinones have shown an activity similar to isoniazid.[65] Clinical experience with the use of linezolid in the management of mycobacterial infections is still sparse. Some authors have reported successful results in the treatment of both multidrug-resistant *M. tuberculosis* infections[66] and non-tuberculous mycobacteria infections.[67] In addition to the lack of information on the efficacy of linezolid in the treatment of tuberculosis, toxicity is a matter of concern when the drug has to be used for long periods.[68] Clinical trials have shown that linezolid (600 mg twice daily in adults) is safe and generally well tolerated for courses of therapy of <28 days[69] but long-term linezolid use has been associated with reversible haematopoietic suppression, primarily thrombocytopenia[70] and neuropathy.[71] However, this drug is being used as salvage therapy when no better options are available, with some successful results.[72] Efficacy and safety of lower doses of linezolid[73] for treating MDR-TB and extensively-resistant tuberculosis (XDR-TB) are being assessed in phase II clinical trials by the TBTC and the National Institute of Allergy and Infectious Diseases (NIAID).

*Diarylquinolines*

Diarylquinolines have been identified in a process of screening various compounds for potential anti-TB activity.[74] The most active diarylquinoline (TMC207, also called R 207910, or compound J) is currently being evaluated in phase II clinical trials at a dose of 400 mg/day.

TMC207 inhibits the mycobacterial ATP synthase enzyme.[75] TMC207 has shown equal activity in susceptible and MDR strains.[76] No cross-resistance with available drugs is expected since the target of the diarylquinolines differs from that of the currently available anti-TB drugs. Mycobacteria that are resistant to TMC207 *in vitro* show mutations in the *atpE* gene, which encodes subunit c of ATP synthase.[77] Oral administration with a meal results in a twofold increase of serum TMC207 concentrations. The Cmax is reached after 5 h; the half-life is long: about 24 h in humans. PK of TMC207 show linearity with dose. TMC207 is metabolized by the CYP450 3A4 enzyme to an active *N*-monodesmethyl metabolite (M2).[78] Rifampin reduces plasma TMC207 concentrations by 50%; however, a recent study in mice showed significant activity of TMC207 even with a 50% reduction in exposure, indicating that the relevance of this interaction is questionable.[79] No drug-drug interactions were observed between TMC207 and isoniazid plus pyrazinamide (De Beule and Van Heeswijk presented at the 1st International Workshop on Clinical Pharmacology of Tuberculosis Drugs, Toronto, Canada). Steady-state concentrations in humans take more than 7 days to establish due to the extensive tissue distribution of TMC207. *In vitro* studies show MICs ranging from 0.030 to 0.120 mg/L in both fully susceptible and MDR strains.[80] The *in vitro* activity of TMC207 did not increase with increasing drug concentration, suggesting time-dependent rather than concentration-dependent killing. The activity of TMC207 is limited to mycobacterial species only. Treatment with TMC207 (25 mg/Kg), isoniazid (25 mg/Kg), and pyrazinamide (150 mg/Kg), and with TMC207, rifampin (10 mg/Kg), and pyrazinamide yielded 100% negative lung cultures in mice after only 2 months of treatment. When any of the drugs in the standard regimen was replaced with TMC207 (25 mg/Kg), the bactericidal activity improved.[74] Regimens based on the standard anti-TB drugs and/or moxifloxacin that contained both pyrazinamide and TMC207 were more active than regimens without these two drugs. A 2-month regimen of once-weekly TMC207 (125 mg/Kg), pyrazinamide (300 mg/Kg), and rifapentine (15 mg/Kg) was more active than rifampin (10 mg/Kg), isoniazid (25 mg/Kg), and pyrazinamide (150 mg/Kg) five times per week.[81] These results suggest synergistic activity of TMC207 and pyrazinamide in mice.[82] The guinea pig model was used to demonstrate sterilizing activity of TMC207. Almost complete eradication of primary and secondary lung lesions was achieved after 6 weeks of TMC207 monotherapy (15 mg/Kg), whereas the standard regimen had limited effect.[83] An EBA study was done in which patients with pulmonary TB received various doses of TMC207, rifampin, or isoniazid in monotherapy for 7 days. The EBA of both rifampin and isoniazid was better than that of TMC207. Only a dose of 400 mg TMC207 showed an EBA between days 4 and 7 of the same magnitude as that of rifampin and isoniazid in the same period.[74] Because of the pharmacokinetic interaction between rifampin and TMC207, the primary focus in the development of TMC207 is now on regimens without rifampin. Recently, a multicenter phase II study in 200 patients with MDR-TB was started in which a standard second-line, rifampin-free regimen is compared with the same regimen plus TMC207. No serious adverse events were reported in single-dose (up to 700 mg) and multiple-dose (up to 400 mg) studies in healthy, male volunteers (see supporting data in reference 77). In the EBA study, no adverse events related to the study drugs were encountered.

TMC207 has greater bactericidal activity than the standard first-line regimen in mice. It is active in susceptible and MDR strains. However, the EBA of TMC207 in humans is not as good as that of rifampin and isoniazid. The variability of serum TMC207 concentrations with food intake is a disadvantage. TMC207 could be of use in rifampin-free regimens against MDR and XDR-TB.

*Nitroimidazopyrans*

The nitroimidazopyrans have been derived from the bicyclic nitroimidazofurans that were originally developed for cancer chemotherapy but also exhibited activity against actively growing and dormant *M. tuberculosis*.[84] The compounds are structurally related to metronidazole.[85] PA-824 (a nitroimidazo-oxazine) and OPC-67683 (a dihydroimidazo-oxazole) are currently being investigated in clinical trials.

PA-824 is a prodrug that needs the mycobacterial glucose-6-phosphate dehydrogenase (FDG1) or its cofactor, coenzyme F420, to be transformed into an active form.[86] Activated PA-824 inhibits the synthesis of proteins and cell wall lipids. PA-824 activity is limited to *M. tuberculosis* complex. PA-824 is active in susceptible and resistant *M. tuberculosis* strains. No cross-resistance with standard anti-TB drugs has been observed. Mutations in the mycobacterial genes *fbiA*, *fbiB*, and *fbiC* lead to impaired coenzyme F420 synthesis and therefore resistance to PA-824.[87] Mutations in the Rv3547 gene, encoding a protein with unknown function, have been described in PA-824 resistant strains. Complementing these mutants with intact Rv3547 fully restored the ability of the mutants to metabolize PA-824. This suggests mediation of a highly specific protein, next to FGD1 and coenzyme F420, in PA-824 activity.[88] Serum PA-824 concentrations in mice are not influenced by coadministration of rifampin, isoniazid, and pyrazinamide in various combinations, and PA-824 does not influence concentrations of the latter drugs in serum.[89] PA-824 is currently being investigated in phase I clinical trials under the auspices of the Global Alliance for TB Drugs Development (GATB, TB Alliance). Studies in healthy volunteers showed a half-life of about 18 h and a time to reach Cmax of 4 to 5 h. About 65% of PA-824 is excreted in urine and 26% in feces. *In vitro* studies showed MICs of PA-824 against fully susceptible and MDR strains ranging from 0.015 to 0.25 mg/L. PA-824 activity is concentration dependent.[90] The bactericidal activity of PA-824 (25 to 50 mg/Kg) was comparable to that of isoniazid (25 mg/Kg) in mice and guinea pigs and to those of rifampin (20 mg/Kg) and moxifloxacin (100 mg/Kg) in mice.[91] PA-824 showed greater activity than isoniazid and moxifloxacin *in vitro* and in mice and comparable activity to combination therapy with rifampin and isoniazid. PA-824 (100 mg/Kg) has been incorporated in the standard regimen in mice to evaluate its potential to shorten treatment duration. Only the regimen in which isoniazid was replaced with PA-824 achieved faster lung culture conversion and a lower CFU count after 2 months of treatment than the standard regimen. However, relapse rates were the same in these regimens. The sterilizing activity of a regimen containing PA-824 (100 mg/Kg), moxifloxacin (100 mg/Kg), and pyrazinamide (150 mg/Kg) was recently found to be better than that of rifampin (10 mg/Kg), isoniazid (25 mg/Kg), and pyrazinamide (150 mg/Kg) in mice, indicating that PA-824 could be incorporated in a rifampin-free regimen to treat MDR-TB.[92] PA-824 (100 mg/Kg) was highly active in a mouse model for latent TB when combined with moxifloxacin (100 mg/Kg).[32] An extended EBA study in humans with daily PA-824 doses of 200 to 1,200 mg over 14 days is ongoing in South Africa. Results are expected soon (Spigelman, presented at the 1st International Workshop on Clinical Pharmacology of Tuberculosis Drugs, Toronto, Canada, 2008). Single PA-824 doses ranging from 50 to 1,500 mg were tolerated well by healthy volunteers, but multiple doses of 1,000 mg were associated with a moderate, reversible increase in creatinine. This renal effect of PA-824 was found to be of insignificant clinical relevance in consecutive studies (Spigelman, presented at the 1st International Workshop on Clinical Pharmacology of Tuberculosis Drugs, Toronto, Canada, 2008).

The potential of PA-824 to shorten TB treatment duration when combined with the standard, first-line anti-TB drugs was reassessed in the mouse model.[93] Mice treated with rifampin (10 mg/Kg), PA-824 (100 mg/Kg), and pyrazinamide (150 mg/Kg) remained free of relapse after 4 months of treatment, while 15% of mice treated with a 4-month regimen of rifampin, isoniazid (25 mg/Kg), and pyrazinamide relapsed. The combinations of PA-824 and pyrazinamide and of PA-824 and rifampin displayed synergistic activity in the same study. Results from this study hold promise for PA-824 to shorten TB treatment duration in combination with first-line drugs.

OPC-67683 is a mycolic acid biosynthesis inhibitor.[94] While isoniazid inhibits the synthesis of all mycolic acid subclasses, OPC-67683 inhibits methoxy and ketomycolic acid synthesis only.[95] OPC-67683 has to be activated by *M. tuberculosis* to exert its activity. Mutations in the mycobacterial Rv3547 gene found in OPC-67683 resistant *M. tuberculosis* strains suggest that this gene codes for the key enzyme in activating OPC-67683 (as well as PA-824). Studies in healthy volunteers showed a more than dose-proportional increase in systemic exposure to OPC-67683 with a stepwise increase of the dose from 5 to 400 mg. Absorption rates were higher when OPC-67683 was administered with a high-fat meal. OPC-67683 was consecutively analyzed in a different administration formula. This improved systemic absorption. The newer formula is used in ongoing evaluations of OPC-67683. OPC-67683 does not affect the activity of liver microsome enzymes, nor is it affected by activated liver enzymes.[95] Interactions with drugs that are metabolized by these enzymes are therefore not expected. The MICs of OPC-67683 are equal in drug-susceptible and -resistant *M. tuberculosis* strains and range from 0.006 to 0.024 mg/L.[94] OPC-67683 exhibits concentration-dependent activity also against intracellular *M. tuberculosis*. The *in vitro* intracellular activity of OPC-67683 was better than that of isoniazid and PA-824 and as good as that of rifampin. OPC-67683 showed sterilizing activity that was superior to that of isoniazid and equal to that of rifampin in an *in vitro* model of drug-tolerant *M. tuberculosis*, representing semidormant bacilli. No antagonism of OPC-67683 with rifampin, isoniazid, ethambutol, and streptomycin was shown *in vitro*. In mice, a regimen of OPC-67683 (2.5 mg/Kg), rifampin (5 mg/Kg), and pyrazinamide (100 mg/Kg) achieved faster eradication of bacilli than the standard RHZE regimen (5, 10, 100, and 100 mg/Kg, respectively). Whereas no mycobacterial colonies were detected after 4 months of treatment with the OPC-67683-containing regimen, colonies were still detected after 6 months of treatment with the standard regimen.[95] The EBA of 400 mg OPC-67683 in patients with pulmonary TB was low during the first 4 days. From day 4 onwards, a significant decrease in CFU was seen. This activity is currently being explored in an extended (14 days) EBA study. OPC-67683 in multiple doses up to 400 mg was tolerated well by healthy volunteers. No serious adverse events were reported.

OPC-67683 could be useful in treatment of MDR and XDR-TB. Its optimal formulation and its role in TB treatment in humans still need to be established. The low EBA is not favorable.

*Diamines*

A library of more than 60,000 compounds was generated by synthesizing ethambutol analogues with 1,2-diamine pharmacophore.[96] So far, the most promising diamine candidate from this library for TB treatment is SQ109.

SQ109 inhibits mycobacterial cell wall synthesis; the exact target is not yet known.[97] Since resistance rates to SQ109 are low, it is thought that two mycobacterial gene changes are needed to result in resistance. Therefore, SQ109 may have more than one target in *M. tuberculosis*. SQ109 undergoes a first-pass step in the liver before it enters the systemic circulation. Liver microsomes convert SQ109 in four predominant metabolites. CYP2D6 and CYP2C19 enzymes are involved in SQ109 metabolism; CYP3A4 has little effect on SQ109.[98] It has been suggested that SQ109 is a prodrug that needs activation by mycobacterial CYP enzymes. Results from a recent drug-drug interaction study in rats suggest that SQ109 induces its own metabolism.[99] SQ109 binding to plasma proteins ranges from 6 to 23% in humans, mice, rats, and dogs. Binding to tissue proteins is higher than that to plasma proteins. SQ109 has a long half-life (61 h) in humans. The MIC of SQ109 ranged from 0.16 to 0.64 mg/L in susceptible and drug-resistant MTB isolates, including ethambutol-resistant strains.[100] SQ109 also exhibits bactericidal activity within macrophages. Its activity is concentration dependent. Synergistic activity was shown *in vitro* between SQ109 and isoniazid and especially rifampin. Synergy was even present in rifampin-resistant strains. Streptomycin had an additive effect on SQ109 activity; ethambutol and pyrazinamide had no effect on the activity of SQ109. Four weeks of monotherapy with SQ109 (0.1 to 25 mg/Kg) in mice resulted in a reduction of mycobacterial load in spleen and lungs that was comparable to the effect of treatment with ethambutol (100 mg/Kg) but less than that of treatment with isoniazid (25 mg/Kg). When ethambutol (100 mg/Kg) was substituted for by SQ109 (10 mg/Kg) in an 8-week regimen of rifampin (20 mg/Kg) and isoniazid (25 mg/Kg), with or without pyrazinamide (150 mg/Kg), in mice with chronic TB, the mycobacterial load was 1.5 log10 lower than with the standard RHZE regimen.[101] No adverse events were reported in a phase I single-dose study. Multiple doses of SQ109 (up to 300 mg) were tolerated well by healthy volunteers.

SQ109 is a potential anti-TB drug that has entered phase I/II clinical trials. It has low MICs against both susceptible and resistant MTB strains. SQ109 has different and more favorable properties than ethambutol, suggesting that it should be regarded as a truly new diamine, and not just as an ethambutol analogue. SQ109 could be included in regimens containing rifampin and isoniazid, since synergism with both drugs has been shown. Clinical trials are ongoing to establish its future role in TB treatment.

*Pyrroles*

In the search for compounds with activity against mycobacteria and fungi, several pyrrole derivatives have been developed. LL3858 is being investigated in phase I clinical trials.[102] A fixed-dose combination called LL3848, containing LL3858 and the standard, first-line anti-TB drugs, is also being developed.[103]

The mycobacterial target of LL3858 is not yet known. Since LL3858 is active against *M. tuberculosis* strains that are resistant to available anti-TB drugs, the target probably differs from the targets of the currently used drugs. No data about the pharmacokinetics of LL3858 in humans are available yet. The MIC90 of LL3858 for MTB is 0.25 mg/L. LL3858 exhibits concentration-dependent activity. LL3858 (12.5 mg/Kg) reduced the mycobacterial load in mice to a greater extent than isoniazid. Regimens of 8 weeks of LL3858, isoniazid, and rifampin with or without pyrazinamide sterilized lungs and spleens of 3 of 6 and 4 of 6 mice, respectively. When the treatment period was extended to 12 weeks, complete sterilization of the target organs was achieved in 6 of 6 mice. The tolerability of LL3858 is currently being investigated in phase I clinical trials.[104]

## From mice to men III. Animal models of tuberculosis. Limits and lessons

When humans are infected with *M. tuberculosis*, they may develop primary active TB, latent TB, chronic active TB, or reactivation disease. Not all manifestations are mutually exclusive, as 10% of non-immunosuppressed individuals progress from latent to reactivation TB over their lifetimes, while HIV-infected individuals have a 10% annual risk of reactivating latent disease. Immunosuppression, HIV infection, nutritional status, intensity of exposure, BCG vaccination, and age determine, in part, individual outcomes. Less commonly

**Table 5**
Comparing features of common animal models of tuberculosis

| Model | Histopathology | | | Relative susceptibility to *M. tuberculosis* | Immunologic reagents available | Laboratory space requeriments and costs | Approximates human latent TB infection | Most common experimental uses |
|---|---|---|---|---|---|---|---|---|
| | Necrosis | Caseation | Cavitation | | | | | |
| Mouse | Variable | Usually not | No | Low | Extensive | Relative small | No; Cornell model may do so | Immunology; drug efficacy |
| Rabbit | Yes | Yes | Yes | Very low (*M. bovis* typically used) | Moderate | Relative large | No | Pathogenesis |
| Guinea Pig | Yes | Yes | Infrequent | Very high | Relatively few | Moderate | No | Vaccine efficacy; airborne transmission |
| Nonhuman primate | Yes | Yes | Yes | High | Extensive | Large | Yes | Pathogenesis; Immunodeficiency |

reported, but of increasingly recognized importance, is the role that re-exposure to TB and re-infection play in the risk of developing disease.[105] Although there are no true animal reservoirs for *M. tuberculosis*, each of these stages of infection in humans can be approached by the use of one or more of the animal models showed in Table 5.

## Conclusion

To our knowledge, control of the TB epidemic implies more than developing new drugs, however experimental models as well as the aforementioned clinical assay-based initiatives to try them in humans are crucial to be successful in the global contest to eradicate TB.

## Conflict of interest

The authors declare they have not any conflict of interest.

## References


1. Barr T, Tubach G, editors. Cliff Notes: On Steinbeck's Of Mice and men. New York City: 909 Third Avenue; 2001.
2. Burman WJ, Gallicano K, Peloquin C. Comparative pharmacokinetics and pharmacodynamics of the rifamycin antibacterials. Clin Pharmacokinet. 2001;40:327-41.
3. Diacon AH, Patientia RF, Venter A, Van Helden PD, Smith PJ, McIlleron H, et al. Early bactericidal activity of high-dose rifampin in patients with pulmonary tuberculosis evidenced by positive sputum smears. Antimicrob Agents Chemother. 2007;51:2994-6.
4. Gillespie SH. Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob Agents Chemother. 2002;46:267-74.
5. World Health Organization. Anti-tuberculosis drug resistance in the world. Report no. 4. The WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance. 2002-2007. Geneva: World Health Organization; 2008.
6. Acocella G. Clinical pharmacokinetics of rifampicin. Clin Pharmacokinet. 1978;3:108-27.
7. Ruslami R, Nijland HMJ, Alisjahbana B, Parwati I, Van Crevel R, Aarnoutse RE. Pharmacokinetics and tolerability of a higher rifampin dose versus the standard dose in pulmonary tuberculosis patients. Antimicrob Agents Chemother. 2007;51:2546-51.
8. Niemi M, Backman JT, Fromm MF, Neuvonen PJ, Kivisto KT. Pharmacokinetic interactions with rifampicin: clinical relevance. Clin. Pharmacokinet. 2003;42:819-50.
9. Heifets LB, Lindholm-Levy PJ, Flory MA. Bactericidal activity in vitro of various rifamycins against Mycobacterium avium and *Mycobacterium tuberculosis.* Am Rev Respir Dis. 1990;141:626-30.
10. Jayashree V, Nandi S, Bharat R, Shandil K, Kantharaj E, Balasubramanian V. Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis. Antimicrob Agents Chemother. 2003;47:2118-24.
11. Kreis B, Pretet S, Birenbaum J, Guibout P, Hazeman JJ, Orin E, et al. Two three-month treatment regimens for pulmonary tuberculosis. Bull Int Union Tuberc. 1976;51:71-5.
12. Long MW, Snider DE Jr, Farer LS. US Public Health Service Cooperative trial of three rifampin-isoniazid regimens in treatment of pulmonary tuberculosis. Am Rev Respir Dis. 1978;119:879-94.
13. Kochar DK, Aseri S, Sharma BV, Bumb RA, Mehta RD, Purohit SK. The role of rifampicin in the management of cutaneous leishmaniasis. QJM. 2000;93:733-7.
14. Solera JM, Rodríguez-Zapata P, Geijo J, Largo J, Paulino L, Sáez E, et al. The GECMEI Group. Doxycycline-rifampin versus doxycycline-streptomycin in treatment of human brucellosis due to *Brucella melitensis*. Antimicrob Agents Chemother. 1995;39:2061-7.
15. Kissling M, Bergamini N. Rifampicin in free combination with other antimicrobial drugs in non-Tb infections. Clinical data on 650 patients (a review). Chemotherapy. 1981;27:368-402.
16. Williams DL, Spring L, Collins L, Miller LP, Heifets LB, Gangadharam PRJ, et al. Contribution of *rpoB* mutations to development of rifamycin cross-resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1998;42:1853-7.
17. Mor N, Simon B, Mezo N, Heifets L. Comparison of activities of rifapentine and rifampin against *Mycobacterium tuberculosis* residing in human macrophages. Antimicrob Agents Chemother. 1995;39:2073-7.
18. Weiner M, Bock N, Peloquin CA, Burman WJ, Khan A, Vernon A, et al. Pharmacokinetics of rifapentine at 600, 900, and 1,200 mg during once-weekly tuberculosis therapy. Am J Respir Crit Care Med. 2004;169:1191-7.
19. Dooley K, Flexner C, Hackman J, Peloquin CA, Nuermberger E, Chaisson RE, et al. Repeated administration of high-dose intermittent rifapentine reduces rifapentine and moxifloxacin plasma concentrations. Antimicrob Agents Chemother. 2008;52:4037-42.
20. Bemer-Melchior P, Bryskier A, Drugeon HB. Comparison of the in vitro activities of rifapentine and rifampicin against Mycobacterium tuberculosis complex. J Antimicrob Chemother. 2000;46:571-6.
21. Nuermberger E, Grosset J. Pharmacokinetic and pharmacodynamic issues in the treatment of mycobacterial infections. Eur J Clin Microbiol Infect Dis. 2004;23:243-55.
22. Vernon A, Burman W, Benator D, Khan A, Bozeman L. Acquired rifamycin monoresistance in patients with HIV-related tuberculosis treated with once-weekly rifapentine and isoniazid. Lancet. 1999;353:1843-7.
23. Benator D, Bhattacharya M, Bozeman L, Burman W, Cantazaro A, Chaisson R, et al. Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. Lancet. 2002;360:528-34.
24. Jarvis B, Lamb HM. Rifapentine. Drugs. 1998;56:607-16.
25. Rosenthal IM, Williams K, Tyagi S, Peloquin C, Vernon A, Bishai WR, et al. Potent twice-weekly rifapentine-containing regimens in murine tuberculosis. Am J Respir Crit Care Med. 2006;174:94-101.
26. Rosenthal IM, Williams, Tyagi S, Vernon A, Peloquin CA, Bishai WR, et al. Weekly moxifloxacin and rifapentine is more active than the Denver regimen in murine tuberculosis. Am J Respir Crit Care Med. 2005;172:1457-62.
27. Rosenthal IM, Zhang M, Williams KN, Peloquin CA, Tyagi S, Vernon A. Daily dosing of rifapentine cures tuberculosis in three months or less in the murine model. PLoS Med. 2007;4:e344.
28. Nuermberger E, Rosenthal IM, Zhang M, Grosset J. Relative contribution of moxifloxacin versus isoniazid to rifapentine-based regimens in the murine model of tuberculosis. 1st Int Workshop Clin Pharmacol Tuberculosis Drugs, Toronto; 2008. Abstract 18.
29. Rosenthal IM, Zhang M, Grosset J, Nuermberger E. Is it possible to cure TB in weeks instead of months? 1st Int Workshop Clin Pharmacol Tuberculosis Drugs, Toronto; 2008. Abstract 19.
30. Bock N, Sterling TR, Hamilton CD, Pachucki C, Wang YC, Conwell DS, et al. A prospective, randomized, double-blind study of the tolerability of rifapentine 600, 900, and 1,200 mg plus isoniazid in the continuation phase of tuberculosis treatment. Am J Respir Crit Care Med. 2002;165:1526-30.
31. Weiner M, Bock N, Peloquin CA, Burman WA, Khan A, Vernon A, et al. Pharmacokinetics rifapentine at 600, 900, and 1,200 mg during once-weekly tuberculosis therapy. Am J Respir Crit Care Med. 2004;169:1191-7.
32. Nuermberger E, Tyagi S, Williams KN, Rosenthal I, Bishai WR, Grosset JH. Rifapentine, moxifloxacin, or DNA vaccine improves treatment of latent tuberculosis in a mouse model. Am J Respir Crit Care Med. 2005;172:1452-6.
33. Schechter M, Zajdenverg R, Falco G, Barnes GL, Faulhaber JC, Coberly JS, et al. Weekly rifapentine/isoniazid or daily rifampin/pyrazinamide for latent tuberculosis in household contacts. Am J Respir Crit Care Med. 2006;173:922-6.
34. World Health Organization. Treatment of tuberculosis. Guidelines for national programmes. 3rd ed. Geneva: World Health Organization; 2003.

35. Bonner DP. In vitro antibacterial spectrum of a new broad-spectrum 8-methoxy fluoroquinolone, gatifloxacin. J Antimicrob Chemother. 2000;45:437-46.
36. Shandil RK, Jayaram R, Kaur P, Gaonkar S, Suresh BL, Mahesh BN, et al. Moxifloxacin, ofloxacin, sparfloxacin, and ciprofloxacin against *Mycobacterium tuberculosis*: evaluation of in vitro and pharmacodynamic indices that best predict in vivo efficacy. Antimicrob Agents Chemother. 2007;51:576-82.
37. Stass H, Dalhoff A, Kubitza D, Schuhly U. Pharmacokinetics, safety, and tolerability of ascending single doses of moxifloxacin, a new 8-methoxy quinolone, administered to healthy subjects. Antimicrob Agents Chemother. 1998;42:2060-5.
38. Shindikar AV, Viswanathan CL. Novel fluoroquinolones: design, synthesis, and in vivo activity in mice against *Mycobacterium tuberculosis* H37Rv. Bioorg Med Chem Lett. 1998;15:1803-6.
39. Bozeman L, Burman W, Metchock B, Welch L, Weiner M. Fluoroquinolone susceptibility among Mycobacterium tuberculosis isolates from the United States and Canada. Clin Infect Dis. 2005;40:386-91.
40. Ginsburg AS, Woolwine SC, Hooper N, Benjamin WH Jr, Bishai WR, Dorman SE, et al. The rapid development of fluoroquinolone resistance in *M. tuberculosis.* N. Engl J Med. 2003;349:1977-8.
41. Ginsburg AS, Grosset JH, Bishai WR. Fluoroquinolones, tuberculosis, and resistance. Lancet Infect Dis. 2003;3:432-42.
42. Kam KM, Yip CW, Cheung TL, Tang HS, Leung CC, Chan MY. Stepwise decrease in moxifloxacin susceptibility amongst clinical isolates of multidrug-resistant Mycobacterium tuberculosis: correlation with ofloxacin susceptibility. Microb Drug Resist. 2006;12:7-11.
43. Ginsberg AM, Spigelman M. Challenges in tuberculosis drug research and development. Nat Med. 2007;13:290-4.
44. Huang TS, Kunin C, Shin-Jung LS, Chen YS, Tu HZ, Liu YC. Trends in fluoroquinolone resistance of Mycobacterium tuberculosis complex in a Taiwanese medical centre: 1995-2003. J Antimicrob Chemother. 2005;56:1058-62.
45. Nijland HM, Ruslami R, Suroto AJ, Burger DM, Alisjahbana B, Van Crevel R, et al. Rifampicin reduces plasma concentrations of moxifloxacin in patients with tuberculosis. Clin Infect Dis. 2007;45:1001-7.
46. Weiner M, Burman W, Leung CC, Peloquin CA, Engle M, Goldberg S, et al. Effects of rifampin and multidrug resistance gene polymorphism on concentrations of moxifloxacin. Antimicrob Agents Chemother. 2007;51:2861-6.
47. Wright DH, Brown GH, Peterson ML, Rotschafer JC. Application of fluoroquinolone pharmacodynamics. J Antimicrob Chemother. 2000;46:669-83.
48. Yoshimatsu T, Nuermberger E, Tyagi S, Chaisson R, Bishai W, Grosset J. Bactericidal activity of increasing daily and weekly doses of moxifloxacin in murine tuberculosis. Antimicrob Agents Chemother. 2002;46:1875-9.
49. Lounis N, Bentoucha A, Truffot-Pernot C, Ji B, O'Brien RJ, Vernon A, et al. Effectiveness of once-weekly rifapentine and moxifloxacin regimens against *Mycobacterium tuberculosis* in mice. Antimicrob Agents Chemother. 2001;45:3482-6.
50. Nuermberger EL, Yoshimatsu T, Tyagi S, O'Brien RJ, Vernon A, Chaisson RE, et al. Moxifloxacin containing regimen greatly reduces time to culture conversion in murine tuberculosis. Am J Respir Crit Care Med. 2004;169:421-6.
51. Pletz MWR, De Roux A, Roth A, Neumann KH, Mauch H, Lode H. Early bactericidal activty of moxifloxacin in treatment of pulmonary tuberculosis: a prospective, randomized study. Antimicrob Agents Chemother. 2004;48:780-2.
52. Gillespie SH, Gosling RD, Uiso L, Sam NE, Kanduma EG, McHugh TD. Early bactericidal activity of a moxifloxacin and isoniazid combination in smear-positive pulmonary tuberculosis. J Antimicrob Chemother. 2005;56:1169-71.
53. Burman WJ, Goldberg S, Johnson JL, Muzanye G, Engle M, Mosher AW, et al. Moxifloxacin versus ethambutol in the first 2 months of treatment for pulmonary tuberculosis. Am J Respir Crit Care Med. 2006;174:331-8.
54. Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, et al. A phase II study of the sterilising activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int J Tuberc Lung Dis. 2008;12:128-38.
55. Veziris N, Lounis N, Chauffour A, Truffot-Pernot C, Jarlier V. Efficient intermittent rifapentine-moxifloxacin-containing short-course regimen for treatment of tuberculosis in mice. Antimicrob Agents Chemother. 2005;49:4015-9.
56. Codecasa LR, Ferrara G, Ferrarese M, Morandi MA, Penati V, Lacchini C, et al. Long-term moxifloxacin in complicated tuberculosis patients with adverse reactions or resistance to first line drugs. Respir Med. 2006;100:1566-72.
57. European Medicines Agency. Questions and answers on the recommendation to restrict the use of oral formulations of moxifloxacin-containing medicines. London: European Medicines Agency; 2008.
58. Lubasch A, Keller I, Borner K, Koeppe P, Lode H. Comparative pharmacokinetics of ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin, trovafloxacin, and moxifloxacin after single oral administration in healthy volunteers. Antimicrob Agents Chemother. 2000;44:2600-3.
59. Park-Wyllie LY, Juurlink DN, Kopp A, Shah BR, Stukel TA, Stumpo C, et al. Outpatient gatifloxacin therapy and dysglycemia in older adults. N Engl J Med. 2006;354:1352-61.
60. Lu T, Drlica K. In vitro activity of C-8-methoxy fluoroquinolones against mycobacteria when combined with anti-tuberculosis agents. J Antimicrob Chemother. 2003:52:1025-8.
61. Cynamon MH, Sklaney M. Gatifloxacin and ethionamide as the foundation for therapy of tuberculosis. Antimicrob Agents Chemother. 2002;47:2442-4.
62. Yanagihara K, Kaneko Y, Sawai T, Miyazaki Y, Tsukamoto K, Hirakata Y, et al. Efficacy of linezolid against methicillin-resistant or vancomycin-insensitive Staphylococcus aureus in a model of hematogenous pulmonary infection. Antimicrob Agents Chemother. 2002;46:3288-91.
63. Alcalá L, Ruiz-Serrano MJ, Pérez-Fernández Turégano C. In vitro activities of linezolid against clinical isolates of *Mycobacterium tuberculosis* that are susceptible or resistant to first-line antituberculous drugs. Antimicrob Agents Chemother. 2003;47:416-7.
64. Wallace RJ Jr, Brown-Elliott BA, Ward SC. Activities of linezolid against rapidly growing mycobacteria. Antimicrob Agents Chemother. 2001;45:764-7.
65. Cynamon MH, Klemens SP, Sharpe CA. Activities of several novel oxazolidinones against Mycobacterium tuberculosis in a murine model. Antimicrob Agents Chemother. 1999;43:1189-91.
66. Valencia ME, Moreno V, Laguna F. Multiresistant tuberculosis caused by *Mycobacterium bovis* and human immunodeficiency virus infection. Are there new therapeutic possibilities? Enferm Infecc Microbiol Clin. 2001;19:37-9.
67. Brown-Elliott BA, Wallace RJ Jr, Blinkhorn R, Crist CJ, Mann LB. Successful treatment of disseminated Mycobacterium chelonae infection with linezolid. Clin Infect Dis. 2001;33:1433-4.
68. Migliori GB, Eker B, Richardson MD, Sotgiu G, Zellweger JP, Skrahina A, et al. TBNET Study Group. A retrospective TBNET assessment of linezolid, safety, tolerability and efficacy in multidrug resistant tuberculosis. Eur Respir J. 2009;34:387-93.
69. Rubinstein E, Isturiz R, Standiford HC. Worldwide assessment of linezolid's clinical safety and tolerability:comparator controlled phase III studies. Antimicrob Agents Chemother. 2003;47:1824-31.
70. Gerson SL, Kaplan SL, Bruss JB. Hematologic effects of linezolid:summary of clinical experience. Antimicrob Agents Chemother. 2002;46:2723-6.
71. Bressler AM, Zimmer SM, Gilmore JL. Peripheral neuropathy associated with prolonged use of linezolid. Lancet Infect Dis. 2004;4:528-31.
72. Fortún J, Martín-Dávila P, Navas E, Pérez-Elías MJ, Cobo J, Tato M, et al. Linezolid for the treatment of multidrug resistant tuberculosis. J Antimicrobl Chemother. 2005;56:180-5.
73. Park IN, Hong SB, Oh YM, Kim MN, Lim CM, Lee SD, et al. Efficacy and tolerability of daily half-dose of linezolid in patients with intractable multidrug resistant tuberculosis. J Antimicrob Chemother. 2006;58:701-4.
74. Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, et al. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis.* Science. 2005;307:223-7.
75. De Jonge MR, Koymans LH, Guillemont JE, Koul A, Andries K. A computational model of the inhibition of *Mycobacterium tuberculosis* ATPase by a new drug candidate R207910. Proteins. 2007;67:971-80.
76. Diacon AH, Pym A, Grobusch M, Patientia R, Rustomjee R, Page-Shipp L, et al. The diarylquinoline TMC207 for multidrug resitant tuberculosis. N Eng J Med. 2009;360:2397-405.
77. Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W. Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. Antimicrob Agents Chemother. 2006;50:2853-6.
78. Rustomjee R, Diacon AH, Allen J, Venter A, Reddy C, Patientia RF, et al. Early bactericidal activity and pharmacokinetics of the diarylquinoline TMC207 in treatment of pulmonary tuberculosis. Antimicrob Agents Chemother. 2008; 52:2831-5.
79. Lounis N, Gevers T, Van Den Berg J, Andries K. Impact of the interaction of R207910 with rifampin on the treatment of tuberculosis studied in the mouse model. Antimicrob. Agents Chemother. 2008;52:3568-72.
80. Huitric E, Verhasselt P, Andries K, Hoffner SE. In vitro antimycobacterial spectrum of a diarylquinoline ATP synthase inhibitor. Antimicrob Agents Chemother. 2007;51:4202-4.
81. Veziris N, Ibrahim M, Lounis N, Chauffour A, Truffot-Pernot C, Andries K, et al. A once-weekly R207910-containing regimen exceeds activity of the standard daily regimen in murine tuberculosis. Am J Respir Crit Care Med. 2009; 179:75-9.
82. Ibrahim M, Andries K, Lounis N, Chauffour A, Truffot-Pernot C, Jarlier V, et al. Synergistic activity of R207910 combined with pyrazinamide against murine tuberculosis. Antimicrob Agents Chemother. 2007;51:1011-5.
83. Lenaerts AJ, Hoff D, Aly S, Ehlers S, Andries K, Cantarero L, et al. Location of persisting mycobacteria in a guinea pig model of tuberculosis revealed by R207910. Antimicrob Agents Chemother. 2007;51:3338-45.
84. Papadopoulou MV, Bloomer WD, McNeil MR. NLCQ-1 and NLCQ-2, two new agents with activity against dormant Mycobacterium tuberculosis. Int J Antimicrob Agents. 2007;29:724-7.
85. Spigelman MK. New tuberculosis therapeutics: a growing pipeline. J Infect Dis. 2006;196 Suppl 1:S28-34.
86. Stover CK, Warrener P, Van Devanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature. 2000;405:962-6.
87. Choi KP, Bair TB, Bae YM, Daniels L. Use of transposon Tn*5367* mutagenesis and a nitroimidazopyran-based selection system to demonstrate a requirement for *fbiA* and *fbiB* in coenzyme F420 biosynthesis by *Mycobacterium bovis* BCG. J Bacteriol. 2001;183:7058-66.
88. Manjunatha UH, Boshoff H, Dowd CS, Zhang L, Albert TJ, Norton JE, et al. Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis.* Proc Natl Acad Sci USA. 2006;103:431-6.
89. Nuermberger E, Rosenthal I, Tyagi S, Williams KN, Almeida D, Peloquin CA, et al. Combination chemotherapy with the nitroimidazopyran PA-824 and first-line drugs in a murine model of tuberculosis. Antimicrob Agents Chemother. 2006; 50:2621-5.
90. Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis S, et al. Bactericidal activity of the nitroimidazopyran PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother. 2005;49:2289-93.
91. Lenaerts AJ, Gruppo V, Marietta KS, Johnson CM, Driscoll DK, Tompkins NM, et al. *Mycobacterium tuberculosis* in a series of in vitro and in vivo models. Antimicrob Agents Chemother. 2005;49:2294-301.
92. Rosenthal I, Grosset JH. Powerful bactericidal and sterilizing activity of a regimen containing PA-824, moxifloxacin, and pyrazinamide in a murine model of tuberculosis. Antimicrob Agents Chemother. 2008 52:1522-4.

93. Tasneen R, Tyagi S, Williams K, Grosset J, Nuermberger E. Enhanced bactericidal activity of rifampin and/or pyrazinamide when combined with PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother. 2008;52:3664-8.
94. Sasaki H, Haraguchi Y, Itotani M, Kuroda H, Hashizume H, Tomishige T, et al. Synthesis and antituberculosis activity of a novel series of optically active 6-nitro-2-,3-dihydroimidazo[2,1-b]oxazoles. J Med Chem. 2006;49:7854-60.
95. Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, et al. OPC-67683, a nitrodihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. PLoS Med. 2006;3:e466.
96. Jia L, Tomaszewski JE, Noker PE, Gorman GS, Glaze E, Protopopova M. Simultaneous estimation of pharmacokinetic properties in mice of three anti-tubercular ethambutol analogs obtained from combinatorial lead optimization. J Pharm Biomed Anal. 2005;37:793-9.
97. Chen P, Gearhart J, Protopopova M, Einck L, Nacy CA. Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. J Antimicrob Chemother. 2006;58:332-7.
98. Jia L, Noker PE, Coward L, Gorman GS, Protopopova M, Tomaszewski JE. Interspecies pharmacokinetics and in vitro metabolism of SQ109. Br J Pharmacol. 2006; 147:476-85.
99. Horwith G, Protopopova M, Lyer L, Mirsalis J, Li Y, Swezey R. Drug-drug interaction studies of SQ109 with first-line anti-TB drugs, abstr. 16. Abstr. 1st Int. Workshop Clin. Pharmacol. Tuberculosis Drugs. 2008; Toronto, Canada.
100. Chen P, Gearhart J, Protopopova M, Einck L, Nacy CA. Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. J Antimicrob Chemother. 2006;58:332-7.
101. Nikonenko BV, Protopopova M, Samala R, Einck L, Nacy CA. Drug therapy of experimental tuberculosis (TB): improved outcome by combining SQ109, a new diamine antibiotic, with existing TB drugs. Antimicrob Agents Chemother. 2007;51:1563-5.
102. Arrora SK, Sinha N, Sinha R, Bateja R, Sharma S, Upadhayaya RS. Design, synthesis, modelling and activity of novel antitubercular compounds, abstr. 63. Abstr. Am Chem Soc Meet. 2004.
103. Protopopova M, Bogatcheva E, Nikonenko B, Hundert S, Einck L, Nacy CA. In search of new cures for tuberculosis. Med Chem. 2007;3:301-16.
104. Tuberculosis. Ll-3858. Tuberculosis (Edinburgh). 2008;88:126.
105. Cohen T, Murray M. Incident tuberculosis among recent US immigrants and exogenous reinfection. Emerg Infect Dis. 2005;11:725-8.

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# New tuberculosis vaccines

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A B S T R A C T

The current tuberculosis (TB) vaccine, bacille Calmette-Guerin (BCG), is a live vaccine used worldwide, as it protects against severe forms of the disease, saving thousands of lives every year, but its efficacy against pulmonary forms of TB, responsible for transmission of the diseases, is variable.

For more than 80 years now no new TB vaccines have been successfully developed. Over the last decade the effort of the scientific community has resulted in the design and construction of promising vaccine candidates. The goal is to develop a new generation of vaccines effective against respiratory forms of the disease. We will focus this review on new prophylactic vaccine candidates that aim to prevent TB diseases. Two are the main strategies used to improve the immunity conferred by the current BCG vaccine, by boosting it with new subunit vaccines, and a second strategy is focused on the construction of new more effective live vaccines, capable to replace the current BCG and to be used as prime vaccines.

After rigorous preclinical studies in different animal models new TB vaccine candidates enter in clinical trials in humans. First, a small Phase I for safety followed by immunological evaluation in Phase II trials and finally evaluated in large population Phase III efficacy trials in endemic countries. At present BCG prime and boost with different subunit vaccine candidates are the more advanced assessed in Phase II. Two prime vaccines (based on recombinant BCG) have been successfully evaluated for safety in Phase I trials. A short number of live attenuated vaccines are in advance preclinical studies and the candidates ready to enter Phase I safety trials are produced under current good manufacturing practices.



## Nuevas vacunas contra la tuberculosis

*Palabras clave:*
Bacilo de Calmette-Guérin (BCG)
Tuberculosis
Vacunas subunidades
Vacunas vivas atenuadas

R E S U M E N

La actual vacuna contra la tuberculosis, bacilo de Calmette-Guerin (BCG), en uso desde 1921, protege contra las formas más graves de la enfermedad, pero su eficacia es muy variable contra las formas de tuberculosis pulmonar, por lo que la investigación y desarrollo de nuevas vacunas es un reto importante para la comunidad científica.

En la última década, importantes esfuerzos en investigación han dado como resultado el diseño y construcción de nuevos candidatos a vacunas contra la tuberculosis. El objetivo es desarrollar una nueva generación de vacunas eficaces contra las formas respiratorias de la enfermedad, responsables de la transmisión de la tuberculosis.

Esta revisión se centra en el progreso preclínico y clínico de nuevas vacunas profilácticas, dirigidas a prevenir la enfermedad. Dos son las principales estrategias: la primera busca mejorar la inmunidad conferida por BCG, revacunando con vacunas subunidades, y la segunda estrategia se centra en la construcción de nuevas vacunas vivas más eficaces, capaces de sustituir la actual BCG.

Tras rigurosos estudios preclínicos en modelos animales, algunos candidatos a la vacuna contra la tuberculosis han entrado en ensayos clínicos en humanos. Actualmente, las vacunas subunidades son las más avanzadas en estos ensayos y están siendo evaluadas en fase II en individuos previamente vacunados con BCG. Vacunas vivas basadas en BCG recombinante se están evaluado en fase I, y nuevas vacunas vivas atenuadas han mostrado buenos resultados de atenuación y protección en estudios preclínicos, y están listas para entrar en ensayos de fase I en humanos.



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## Introduction

Prophylactic vaccines are one of the most useful and cost-effective tools for reducing the morbidity and mortality associated with infectious diseases.[1] There is no other vaccine so widely used and as controversial in terms of efficacy as the current bacille Calmette-Guerin (BCG). Its protective effects in randomized controlled trials and case-control studies have provided mixed results ranging from excellent protection to no protection against tuberculosis.[2] The majority of studies have shown that BCG vaccine produces a higher degree of protection against severe forms of TB such as TB meningitis and TB disseminated, than against moderate forms of the disease.[3,4]

The effectiveness of BCG vaccine also appears to vary with latitude - the greater the distance from Ecuador, the greater the effectiveness of the vaccine. Probably exposure to nonpathogenic mycobacteria, which is more intense in warm climates, induces a degree of protective immunity in exposed populations, masking the potential BCG protection against TB.[5] Most evidence suggests that BCG efficacy is maintained after 10 years of vaccination have been described as 60 years.[6] Since today is possible to differentiate between vaccinated individual and infected with *Mycobacterium tuberculosis* (MTB) by using Interferon gamma based assays,[7] a recent study showed that BCG could protect not only TB disease but also MTB infection.[8]

Since one third of the world population is infected by MTB, new strategies that could avoid the progression of latent infection to active disease, such as immunotherapeutic vaccines designed for use post-infection in adults, could have tremendous impact on TB control. Today some therapeutic vaccines are in Phase I clinical trials for safety.[9]

Considering the natural history of tuberculosis and its wide distribution by age, there is a need for vaccines that can prevent disease having a greater efficacy in preventing respiratory forms of TB in adults and help avoiding transmission of TB. That is why research on new TB vaccine strategies, which improve the efficacy of the present BCG to prevent respiratory forms of the disease or the development of new TB vaccines more effective than the current BCG is a priority. We will focus this review on prophylactic vaccines that can prevent TB disease, with special emphasis on pulmonary forms of TB responsible for transmission of the disease.

## Bacille Calmette-Guerin the present vaccine in use against tuberculosis

BCG is the current vaccine for tuberculosis. It is a live vaccine obtained from a strain of *Mycobacterium bovis* isolated from an infected cow. Calmette and Guérin needed thirteen years and over 200 subcultures to obtain the attenuated version of the original virulent strain of *M. bovis* isolated.

BCG was used in 1921 for the first time in children, orally. Later other methods were introduced for administration, including the currently in use, intradermal injection. It is estimated that the systematic use of BCG is saving thousands of lives each year but its benefit seems to be linked to the prevention of severe childhood forms of disease, including extra pulmonary TB and the often fatal TB meningitis.[3,4] For this reason, BCG vaccination is recommended and include in the calendar of vaccination by WHO in countries with high incidence of TB. The 100 million BCG vaccinations given to infants in 2002 will have prevented 30,000 TB meningitis cases in children during their first 5 years of life, and about 11,000 cases of disseminated or miliary TB.[10] In Spain, BCG is not applied systematically in the pediatric population and is not included in the immunization schedules of the various autonomous communities, although it is present in the immunization schedule of the Basque Country.

Despite BCG being the most widely used vaccine in human history, the mechanisms of attenuation are just starting to be understood. Recent genetic studies have revealed that that the current vaccine has lost more than 100 genes in Regions of Difference (RD) from its original genome, with some regions involved in virulence such as RD1, which codes for ESAT-6 secreted protein implicated in MTB virulence.[11] BCG is a highly immunogenic complex, immunogen and which induces a cell type immunity. Originally each BCG was known by the name of the place of production, e.g., BCG (Russia), BCG (Brazil) and BCG (Japan), among others, or after the laboratories where they were produced, such as BCG Pasteur, BCG Meriéux or BCG Glaxo. The name could be followed by the number of the last passage from which it was obtained, as BCG Pasteur 1173, for example. Interestingly from 1921 BCG was generosity distributed worldwide between microbiologists, resulting in the evolution of a number of daughter substrains, as laboratories have used various procedures in the preparation of BCG changing its residual virulence and immunogenic characteristics. This lasted until in the early 1960's when batch 1331 of BCG Danish was freeze-dried and was adopted as the primary seed lot in 1966.[11] During the subcultivation process that led to attenuation, BCG has lost so many genes with respect to MTB and has been considered that BCG could be attenuated to the impotence.[12] Subculture of the original BCG strain in different laboratories and this makes that BCG is not a single organism but comprise a number of substrains that differ in genotype and phenotype with different immunological properties.[13]

The publication in 2007 of the complete sequence of the first BCG strain (Pasteur) and in 2009 BCG Tokyo, has revealed differences in genetic and molecular characteristics.[14,15] These would explain the differences found in the substrains currently used as a BCG vaccine. BCG Tokyo and BCG Pasteur are "more immunogenic" and probably more capable than other strains "less immunogenic" (BCG Glaxo, BCG Danish). Between the molecular mechanisms that contribute to attenuation, BCG substrains comprise natural mutants of major virulence factors of MTB including ESAT6, PDIM/PGL and PhoP, and differ markedly in virulence level that could be responsible for the differences in the protective efficacy and tuberculin reactivity or adverse reactions between different substrains.[11,16] The study and understanding of these new insights have extremely important implications for the development of future vaccines.

## The need for new tools for tuberculosis control, from preclinical to clinical trials

The scientific community effort has resulted in the construction of numerous vaccine candidates over the last decade. In search of candidate vaccines to enhance the effectiveness of the current BCG, there are various teams that have used different approaches. Two main strategies used to improve the immunity are subunit vaccines to "boost" the current BCG vaccine and new more efficient "prime" strategies, able to replace the current BCG, such as novel attenuated live vaccines.

Subunit vaccines mainly use antigens of the bacillus of tuberculosis to improve the immunogenicity of BCG. These MTB antigens consist generally in protein selected for their ability to be recognized by the immune system after infection in humans. These proteins can be administered directly with potent adjuvans or by inserting their genes in different viruses genetically modified such as non replicative vaccinia virus.

Live vaccines mainly consist in nonvirulent mycobacterial strains such as *Mycobacterium vaccae*[17] or the most promising use of recombinant BCG including MTB antigens or other genes from a different microorganism than MTB. Finally another approach is based in the construction from the scratch of rationally attenuated MTB strains which are based on the removal of virulence genes and immunomodulatory lipids which rending safe and more effective TB vaccines.

All vaccine candidates are evaluated to know their safety and effectiveness as degree of protection against MTB disease. In the

evaluation of new vaccines, BCG remains the "gold standard." Studies have been conducted in various animal models and the most commonly used animal model is the mouse, followed by the guinea pig. Primate models have been developed and are being used as a final testing prior to entry in clinical trials.

The advantage of mouse model is based on the amount of reagents and genetic information available, and because of its logistical and economical advantages in comparison with other models like guinea pig. Mice have certain tolerance to infection with MTB, whereas it triggers a moderate inflammatory reaction that allows the control of the bacillary concentration to a low level but without ending up eradicating it. The commonest route of infection is the intravenous route, because switches on acquired immunity very rapidly. The experimental model induced by aerosol is the most physiologic infection route and at the same time is more aggressive for the host than the intravenous one. This is because the induction of immunity is quicker after the intravenous inoculation than in the aerosol one. In this model has been demonstrated that the immunity against this infection is based essentially on the stimulus of a Th1 type response, that is to say, in the stimulation of T cells CD4+ able to produce gamma interferon and to activate the infected macrophages.[18]

Protection in new vaccines using the guinea pig model has become a compulsory experiment since the extreme sensibility that has demonstrated this animal against MTB, and the toxic response generated allowing the comparison between different TB vaccine candidates.[19] On the other hand, the necessity to evaluate the protection of any new vaccine before to carry on human clinical trials in an experimental model closer physiologically to humans has lead to the development of the primate model. In this model the protection mechanisms against the MTB infection are established.[20]

Once the candidate is proven safe and effective, is proposed for study in clinical trials in humans. Clinical trials are divided into 3 consecutive phases, phases I-III, which correspond to the actual development stage, and a phase IV or post-marketing pharmaco vigilance and be released. The first phase of the trial, Phase I, aims to determine the safety and biological effects, including immunogenicity and takes place in a small group of healthy volunteers. Phase II focuses on the immunogenicity and to determine the effectiveness of the vaccine in a limited number of volunteers. Since there is not a clear correlation of protection in humans, the only way to demonstrate the efficacy of a new vaccine candidate is to test in a population with a high incidence of tuberculosis and compare unvaccinated, with gold standard BCG vaccinated. Phase III aims to assess the safety and effectiveness in vaccinated and unvaccinated volunteers by large double-blind trials. Phase III of the vaccine against tuberculosis is predicted long (3-4 years) in the absence of biological markers of protection and require the study of their effectiveness in reducing disease in immunized individuals, the participation of thousands of individuals. According to the Global Plan to Stop TB to have a new licensed vaccine available for 2015, it is estimated that at least 20 vaccine candidates should enter phase I safety trials, with about half going forward for immunological evaluation in phase II trials and three / four being evaluated in phase III efficacy trials.[10]

## Improving bacille Calmette-Guerin: subunit vaccine candidates

Numerous subunit vaccines have been developed using different experimental approaches. The justification for these vaccines is that a few antigens can achieve the same protection that comes with the complete bacteria and their use would provide the vaccine safe, reproducible and without posing problems for application to immunocompromised individuals. To date subunit vaccines alone have not demonstrated a better protection than BCG in different animal model tested and is for that that the new strategy fort subunit vaccines is to be used as boost Improving the effectiveness of BCG.[21] These approaches are aimed at enhancing the protection of BCG. The experiments consist of priming revaccinated animals with BCG and boosting with subunit vaccine candidates.

Subunit antigens have been chosen by different approaches. The study of the immune response in healthy individuals, who have been in contact with MTB, has identified several antigens that would be key to containing tuberculosis infection. The immunization consists of a recombinant fusion protein Mtb72 and adjuvant that provokes a good cellular immune response. This candidate vaccine of GlaxoSmithKline Biologicals (Rixensart, Belgium), Mtb72 has passed Phase I in humans and in collaboration with Aeras Global TB Vaccine Foundation is allowing its progress in clinical trials for safety and immunity, involving adults previously infected with TB or vaccinated with BCG.[22,23]

Another promising approach is the use of ESAT 6 that has been developed from antigens of the bacillus of tuberculosis. It is a major secreted protein. In experimental animals infected, treated and reinfected with MTB, it was noted that at the time of reinfection animals developed a strong T cell response to ESAT-6 making this protein relevant as a source of protective immunity. By merging with another major antigen Ag85B, derivatives have been built of this protein. This subunit vaccine administered with different adjuvants causes an immune response in mouse and primate models inducing protection against infection with MTB. Currently this candidate is in Phase I within the European TB Vaccine Initiative TBVI.[24,25]

Adrian Hill's group at the University of Oxford has used the non replicative modified vaccinia virus Ankara (MVA) to introduce an antigen of the bacillus of tuberculosis (Ag85A). An experiment involving prime vaccination of guinea pigs with BCG, boost with the virus carrying Ag85A and boost with Ag85A protein with an adjuvant, shows greater protection than BCG alone in this animal models.[19] Phase I studies with this candidate have been published[26] and currently is in Phase II clinical evaluation for efficacy and safety in previously BCG vaccinated individuals.[27]

## Development of prime live vaccines based on recombinant bacille Calmette-Guerin

BCG-based vaccines consist of recombinant BCG derived from genetically modified strains of BCG aiming to increase their immunity. Different groups have chosen this strategy to build their candidates. The group of Marcus Horwitz of UCLA (University of California, Los Angeles, USA) developed through genetic engineering techniques rBCG30 strain. It is based on the Tice BCG strain, enhanced with MTB antigen Ag85B, and its overexpression is achieved by increasing the stimulation of the immune system.[28,29] rBCG30 has been noted that the first live vaccine candidate has passed Phase I clinical evaluation. However, as a consequence of less than expected immunogenicity, rBCG30 is no longer in clinical development.

Different strategy is used in the construction of rBCG: RD1 based on the introduction of MTB genes that are absent in BCG coding for ESAT6.[30,31] Such as approach has shown higher protection in animal models but rigorous residual virulence studies are ongoing for regulatory approval to enter in clinical trials.

A third strategy is to increase cellular immunity conferred by BCG by inserting the gene that encodes for a protein of *Listeria monocytogenes*. This gene inside the infected macrophage increased presentation of BCG antigens to other immune system cells. This line of research is led by the group of Stefan Kaufmann, director of Max Planck Institute in Berlin and today this candidate is successfully exiting Phase I safety trials, with plans to enter Phase II in TB endemic countries for safety and immunogenicity.[32,33]

## Prime vaccines based in new live attenuated vaccines

Classical vaccine candidates have to mimic natural infection as closely as possible without causing disease.[10] Provided that immune

response evolved to provide protection against infectious diseases, the optimal development of a protective immune response by a vaccine should reproduce the steps and processes elicited during the establishment of natural immunity.[34] Epidemiological and animal studies indicate that previous infection with tuberculosis confers relative protection against subsequent disease due to post exposure.[35,36]

The improvement of live attenuated vaccines has often been limited by a lack of tools for genetic manipulation of mycobacteria. Pioneering studies performed in Europe by the group of Brigitte Gicquel[37,38] and USA by the group of William Jacobs[39] were able to develop the mycobacterial genetic tools today in use. This strategy is more laborious, meaning starting from scratch and demonstrating in a first stage the attenuation of the vaccine candidate constructed and, secondly, a higher immunity to TB.

Pantothenic acid (vitamin B5) is an essential molecule for the synthesis of coenzyme A and acyl-carrier proteins, two important molecules in fatty acid metabolism and biosynthesis of polyketides among other metabolic reactions. A double-deletion mutant of MTB in the *panC* and *panD* genes involved in the pantothenate synthesis has been constructed.[40] This auxotrophic mutant is attenuated when tested in Balb/c and SCID mice, and conferred protection when used as subcutaneous vaccine in mice challenged with low aerosol doses of virulent H37Rv. In order to increase attenuation and stability of attenuated phenotype a deletion of virulence region RD1 was performed.[41] A representative of this strategy is the mc²6020 strain constructed by inactivation of the *panCD* and *lysA* genes involved in pantothenate and lysine metabolism respectively.[40]). Protection levels equivalent to BCG were generated in the lungs and spleen of vaccinated guinea pigs, with reduced dissemination of infection to the spleen at five weeks after aerosol challenge with MTB.[42]

Another strategy to rationally attenuate MTB consists on inactivation of *secA*. This gene encodes a component of a mycobacterial protein secretion system involved in inhibiting the host immune system and consequently promoting MTB survival within the host. Conversely, inactivation of *secA* results in increased host cell apoptosis and increased priming of antigen-specific $CD8^+$ T cells *in vivo*. These results pave the way for a new approach to improving live vaccine candidates; the *secA* mutant is currently in preclinical development.[43]

In order to build a new vaccine from a MTB a rational inactivation of major its virulence genes, was performed by the by the Group of the University of Zaragoza, in collaboration with the Pasteur Institute. The hypothesis to build a new live vaccine arose from studies on molecular epidemiology of multidrug-resistant MTB strains, assuming that if only a few MTB complex strains are transmitted in a different way than the rest, they have some characteristic that makes them increase their virulence. Therefore, if we decipher the role of the gene in MTB know how to increase their virulence, is possible to inactivate these genes to decrease its virulence. In the late 1990´s the group focus their studies on the strain which caused the largest outbreak of multidrug-resistant TB described in Europe,[44] later described as an *M. bovis* XDR strain.[45] It was found that *phoP* gene, annotated as a possible regulator of virulence genes in the genome of MTB, was highly expressed in this strain, so it was hypothesised that its inactivation may reduce the virulence of MTB. Using genetic engineering techniques *phoP* gene was inactivated. The inactivation of this single gene led to an important attenuation both the cellular model as in the mouse model. Subsequent studies have shown attenuation in the mouse model with reduced immune system (SCID mice, severe combined immunodeficiency) and higher attenuation than BCG Pasteur.[46]

Today we know that the *phoP* gene regulates 2% of the genes of MTB most involved with virulence, including complex lipids, which could be the basis of its high attenuation. Trials of protection against infection, conducted in collaboration with national and international teams from Spain, England, France and Mexico, have shown promising results in the mouse model and superior protection and greater immunity to BCG in the guinea pig model.[20,46] Immunity conferred by the *phoP* mutant is under study. Experiments with primates performed in the PBRC in the Netherlands, were very encouraging.[20]

Another important question that arises is whether after MTB attenuation the sensitivity profile to antituberculous drugs could be changed. The *phoP* mutant strain was found to be fully sensitive to ethambutol, isoniazid, rifampicin and streptomycin. And the mutant was more sensitive to isoniazid than MTB wild type and this could be due to changes in the cell envelope of the *phoP* mutant.[47,48] All these results indicate that MTB *phoP* mutant is sensitive to major antituberculous drugs and, in case of hypothetical infection with the vaccine strain, it would be possible to be treated.

Inoculation of a high dose of vaccine candidates in guinea pigs is a study inspired by the BCG validation standards for toxicity. In one such study, animals were inoculated with $2.5 \times 10^6$ CFU (colony-forming units) of attenuated vaccine (50 times the standard vaccination dose).[49] Data indicate the lack of toxicity of *phoP* mutant strain since the health status of the animals was satisfactory, as evidenced by the constant increase of their weight and lack of pathology after the end of a 6 month follow up. Determination of DTH in guinea pigs at the end of this study reflected similar values to the ones obtained 4 or 6 weeks after immunization with BCG, thus reflecting the conservation of the immune response.

The use of post-exposure infection models for checking toxicity when vaccines are administered in a therapeutic way is based on previous data showing that this administration can be dangerous because of potential induction of the "Koch phenomenon". A number of vaccine candidates have recently been tested to assess the effectiveness and lack of toxicity after post exposure vaccination. The results suggest that although most vaccine candidates are unlikely to evoke the "Koch phenomenon", extreme caution should be taken to avoid serious reactions in previously infected individuals in clinical trials. By using previous validated models of post-exposure infection in guinea pigs and mice to address this question, and we can conclude that no toxic effects have been developed in any of the cases, as it has been demonstrated after examining the bacillary concentration and histology of the tissues.[49] In fact, in the guinea pig post-exposure model, the administration of *phoP* mutant vaccine decreased the pathology, which could be related to a kind of protective effect, although this was not confirmed by a reduction in the bacillary counts. In any case, the lack of toxicity in these models gives an idea about how safe this vaccine is, including the potential secure profile to be used in subjects with latent TB infection. All together extended safety studies encourage the use of *phoP* mutant strain as a starting point for the construction of a next generation attenuated live vaccines.[49]

For live vaccines based on attenuated MTB, a consensus document was developed at the Geneva conference and the presence of at least two non-reverting independent mutations in the mycobacterial genome was recommended in order to avoid reversion and elimination of antibiotic resistance markers.[50] Regulatory issues are fundamental for the development of new tuberculosis vaccines[51] and a second Geneva Consensus include recommendations for novel live TB vaccines prior to entry in phase I safety trials, criteria through to Phase III, review of manufacturing considerations and considering requirements and associated issues related to the use of these new vaccines within an existing BCG vaccination programme.[52]

## Future challenges

Vaccination against tuberculosis poses great challenges. One third of the world population is infected with TB bacilli, for this whom investigation in so-called "therapeutic vaccines" aimed at reducing the time of treatment with conventional drugs is ongoing. Another challenge in vaccination against TB is to protect the population

**Table 1**
Tuberculosis vaccine candidates using a heterologous "prime-boost strategy" to complement the immune response induced by current bacille Calmette-Guerin (BCG)

| Type of vaccine | Product | Sponsor | Status 2010 | Product description |
|---|---|---|---|---|
| Viral vector | MVA-85A Aeras-485 | Oxford/Aeras | Phase I, Phase II | Mofdified vaccinia Ankara vector expressing MTB antigen 85A |
| Viral vector | Aeras-402 Crucell Ad35 | Crucell/Aeras | Phase I, Phase II | Replication-deficient adenovirus 35 vector expressing MTB antigens 85A, 85B, TB10.4 |
| Recombinant protein | Mtb72f | GSK/Aeras | Phase I, Phase II | Recombinant protein composed of a fusion of MTB antigens Rv1196 and Rv0125 and adjuvant |
| Recombinant protein | Hyrbid +IC-311 | SSI/Intercell/TBVI | Phase I, Phase II | Adjuvanted recombinant protein composed of MTB antigens 85B and ESAT-6 |

**Table 2**
New recombinant bacille Calmette-Guerin (BCG) replacement BCG vaccine as "prime" in clinical trials

| Type of vaccine | Product | Sponsor | Status 2010 | Product description |
|---|---|---|---|---|
| Recombinant BCG Live | rBCG 30 | UCLA/INH/Aeras | Phase I | rBCG Tice strain expressing 30Kda MTB Antigen 85B |
| Recombinant BCG Live | VPM 1002 | Max Planck TBVI | Phase I | rBCG Prague strain expressing listeriolysin and carries a urease deletion mutation |

**Table 3**
New live vaccines to replacement bacille Calmette-Guerin (BCG) vaccine as "prime" in pre clinical and good manufacturing practices (GMP)

| Type of vaccine | Product | Sponsor | Status 2010 | Product description |
|---|---|---|---|---|
| Recombinant Live | *MTBVAC01*<br>Δ *phoP*<br>Δ *fad26 (DIM)* | Universidad de Zaragoza<br>Institut Pasteur<br>BIOFABRI TBVI | GMP | Live vaccine based on attenuation of MTB by inactivation of *phoP* and *fadD26* genes |
| Recombinant Live | *MTB*<br>(Δ *lysA*, Δ *panCD*, Δ *secA2*) | Albert Einstein<br>College of Medicine | Preclinical studies | Non-replicating, MTB satin auxotrophic for lysine and pantothenate, attenuated for *secA2* |

currently vaccinated with BCG. Recent trials using protein subunit vaccines in animals previously vaccinated with BCG are giving good results. Live rationally constructed vaccines derived from a *phoP* based mutant strain of tuberculosis, are in advanced preclinical stage. Live vaccines rationally attenuated by the inactivation of genes that regulate virulence, are promising vaccine candidate because of low cost of production, and by the tremendous experience, both in the use, production and distribution of BCG. In addition live vaccines are easily produced and affordable.

Today, after almost 100 years since the development of BCG, there is more than a dozen of developing preventive vaccine candidates in human trials or in advanced preclinical stages with excellent results. Due to the lake of correlation of protection the real value of their efficacy could be assed in a large scale phase trials in countries with a high incidence of TB. The development of a new vaccine that confers higher level of protection is a challenge for the scientific community that would, with a low cost, consider the eradication of tuberculosis in the medium term and replace the current BCG in the long term.[53]

For the future panorama on TB vaccines against TB there are 3 scenarios that could help to the Millennium goal to eradicate TB as a public health problem in 2050 (http://www.stoptb.org/global/plan/): *a)* a subunit vaccine to increase the immunity of individuals vaccinated with BCG administered after vaccination with BCG (boost); *b)* a new vaccine to replace BCG with a birth dose (prime); and *c)* a new vaccine to replace BCG with a birth dose plus a subunit vaccine (Tables 1-3).

Developing new vaccines against TB. Currently there are 12 vaccines in a research designed to improve the immunity of BCG and others to replace BCG.

Aeras: Foundation in the United States for the development of new vaccines against tuberculosis sponsored by the Bill Gates Foundations. http://www.aeras.org/

Tuberculosis Vaccine Initiative (TBVI). European foundation for the development of new vaccines against tuberculosis (http://www.tbvi.eu/).

In 2006, a new initiative called Stop TB was launched according to the Millennium Development Goals, it aims to reduce the global burden of tuberculosis in 2015 and posing as a goal to eliminate TB as a public health problem in 2050 (http://www.stoptb.org/globalplan/).

For a complete list of vaccine candidates including post infection and immunotherapy consult the Stop TB partnership working group on new TB vaccines (www.stoptb.org/wg/new_vaccines/).The document "vaccine pipeline" include a complete list of TB vaccine candidates in 3 sections: *a)* in clinical trials; *b)* in advanced preclinical studies and good manufacturing practices, and *c)* next generation.

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## Conflict of interest

C. Martín and B. Gicquel are named inventors on a composition of matter patent for *Mycobacterium tuberculosis phoP* attenuated mutant filed by the University of Zaragoza. There are no other conflicts of interest.

## References

1. Almond JW. Vaccine renaissance. Nat Rev Microbiol. 2007;5:478-81.
2. Fine PE. Variation in protection by BCG: implications of and for heterologous immunity. Lancet. 1995;346:1339-45.
3. Trunz BB, Fine P, Dye C. Effect of BCG vaccination on childhood tuberculous meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-effectiveness. Lancet. 2006;367:1173-80.
4. Bonifachich E, Chort M, Astigarraga A, Díaz N, Brunet B, Pezzotto SM, et al. Protective effect of Bacillus Calmette-Guerin (BCG) vaccination in children with extra-pulmonary tuberculosis, but not the pulmonary disease. A case-control study in Rosario, Argentina. Vaccine. 2006;24:2894-9.

5. Demangel C, Garnier T, Rosenkrands I, Cole ST. Differential effects of prior exposure to environmental mycobacteria on vaccination with *Mycobacterium bovis* BCG or a recombinant BCG strain expressing RD1 antigens. Infect Immun. 2005;73:2190-6.
6. Aronson NE, Santosham M, Comstock GW, Howard RS, Moulton LH, Rhoades ER, et al. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. Jama. 2004;291:2086-91.
7. Ewer K, Deeks J, Álvarez L, Bryant G, Waller S, Andersen P, et al. Comparison of T-cell-based assay with tuberculin skin test for diagnosis of *Mycobacterium tuberculosis* infection in a school tuberculosis outbreak. Lancet. 2003;361:1168-73.
8. Eisenhut M, Paranjothy S, Abubakar I, Bracebridge S, Lilley M, Mulla R, et al. BCG vaccination reduces risk of infection with *Mycobacterium tuberculosis* as detected by gamma interferon release assay. Vaccine. 2009;27:6116-20.
9. Vilaplana C, Montane E, Pinto S, Barriocanal AM, Domenech G, Torres F, et al. Double-blind, randomized, placebo-controlled Phase I Clinical Trial of the therapeutical antituberculous vaccine RUTI. Vaccine. 2010;28:1106-16.
10. Young D, Dye C. The development and impact of tuberculosis vaccines. Cell. 2006;124:683-7.
11. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science. 1999; 284:1520-3.
12. Behr MA, Small PM. Has BCG attenuated to impotence? Nature. 1997;389:133-4.
13. Behr MA. BCG--different strains, different vaccines? Lancet Infect Dis. 2002;2:86-92.
14. Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci USA. 2007;104:5596-601.
15. Seki M, Honda I, Fujita I, Yano I, Yamamoto S, Koyama A. Whole genome sequence analysis of Mycobacterium bovis bacillus Calmette-Guerin (BCG) Tokyo 172: a comparative study of BCG vaccine substrains. Vaccine. 2009;27:1710-6.
16. Leung AS, Tran V, Wu Z, Yu X, Alexander DC, Gao GF, et al. Novel genome polymorphisms in BCG vaccine strains and impact on efficacy. BMC Genomics. 2008;9:413.
17. Von Reyn CF, Mtei L, Arbeit RD, Waddell R, Cole B, Mackenzie T, et al. Prevention of tuberculosis in Bacille Calmette-Guerin-primed, HIV-infected adults boosted with an inactivated whole-cell mycobacterial vaccine. Aids. 2010;24:675-85.
18. Orme IM, McMurray DN, Belisle JT. Tuberculosis vaccine development: recent progress. Trends Microbiol. 2001;9:115-8.
19. Williams A, Hatch GJ, Clark SO, Gooch KE, Hatch KA, Hall GA, et al. Evaluation of vaccines in the EU TB Vaccine Cluster using a guinea pig aerosol infection model of tuberculosis. Tuberculosis (Edinb). 2005;85:29-38.
20. Verreck FA, Vervenne RA, Kondova I, Van Kralingen KW, Remarque EJ, Braskamp G, et al. MVA.85A boosting of BCG and an attenuated, *phoP* deficient *M. tuberculosis* vaccine both show protective efficacy against tuberculosis in rhesus macaques. PLoS ONE. 2009;4:e5264.
21. Brooks JV, Frank AA, Keen MA, Bellisle JT, Orme IM. Boosting vaccine for tuberculosis. Infect Immun. 2001;69:2714-7.
22. Reed SG, Coler RN, Dalemans W, Tan EV, DeLa Cruz EC, Basaraba RJ, et al. Defined tuberculosis vaccine, Mtb72F/AS02A, evidence of protection in cynomolgus monkeys. Proc Natl Acad Sci USA. 2009;106:2301-6.
23. Von Eschen K, Morrison R, Braun M, Ofori-Anyinam O, De Kock E, Pavithran P, et al. The candidate tuberculosis vaccine Mtb72F/AS02A: tolerability and immunogenicity in humans. Hum Vaccin. 2009;5:475-82.
24. Dietrich J, Andersen C, Rappuoli R, Doherty TM, Jensen CG, Andersen P. Mucosal administration of Ag85B-ESAT-6 protects against infection with *Mycobacterium tuberculosis* and boosts prior bacillus Calmette-Guerin immunity. J Immunol. 2006;177:6353-60.
25. Van Dissel JT, Arend SM, Prins C, Bang P, Tingskov PN, Lingnau K, et al. Ag85B-ESAT-6 adjuvanted with IC31® promotes strong and long-lived *Mycobacterium tuberculosis* specific T cell responses in naïve human volunteers. Vaccine. 2010;28:3571-81.
26. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med. 2004;10:1240-4.
27. Whelan KT, Pathan AA, Sander CR, Fletcher HA, Poulton I, Alder NC, et al. Safety and immunogenicity of boosting BCG vaccinated subjects with BCG: comparison with boosting with a new TB vaccine, MVA85A. PLoS One. 2009;4:e5934.
28. Hondalus MK, Bardarov S, Russell R, Chan J, Jacobs WR Jr, Bloom BR. Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. Infect Immun. 2000;68:2888-98.
29. Hoft DF, Blazevic A, Abate G, Hanekom WA, Kaplan G, Soler JH, et al. A new recombinant bacille Calmette-Guerin vaccine safely induces significantly enhanced tuberculosis-specific immunity in human volunteers. J Infect Dis. 2008;198:1491-501.
30. Pym AS, Brodin P, Majlessi L, Brosch R, Demangel C, Williams A, et al. Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. Nat Med. 2003;9:533-9.
31. Brodin P, Majlessi L, Brosch R, Smith D, Bancroft G, Clark S, et al. Enhanced protection against tuberculosis by vaccination with recombinant *Mycobacterium microti* vaccine that induces T cell immunity against region of difference 1 antigens. J Infect Dis. 2004;190:115-22.
32. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Eddine AN, et al. Increased vaccine efficacy against tuberculosis of recombinant *Mycobacterium bovis* bacille Calmette-Guerin mutants that secrete listeriolysin. J Clin Invest. 2005;115: 2472-9.
33. Kaufmann SH. Envisioning future strategies for vaccination against tuberculosis. Nat Rev Immunol. 2006;6:699-704.
34. Rosenthal KS, Zimmerman DH. Vaccines: all things considered. Clin Vaccine Immunol. 2006;13:821-9.
35. Von Reyn CF, Vuola JM. New vaccines for the prevention of tuberculosis. Clin Infect Dis. 2002;35:465-74.
36. Ziegler JE, Edwards ML, Smith DW. Exogenous reinfection in experimental airborne tuberculosis. Tubercle. 1985;66:121-8.
37. Martin C, Timm J, Rauzier J, Gómez-Lus R, Davies J, Gicquel B. Transposition of an antibiotic resistance element in mycobacteria. Nature. 1990;345:739-43.
38. Ranes MG, Rauzier J, Lagranderie M, Gheorghiu M, Gicquel B. Functional analysis of pAL5000, a plasmid from *Mycobacterium fortuitum:* construction of a "mini" *Mycobacterium-Escherichia coli* shuttle vector. J Bacteriol. 1990;172:2793-7.
39. Jacobs WR Jr, Tuckman M, Bloom BR. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature. 1987;327:532-5.
40. Sambandamurthy VK, Wang X, Chen B, Russell RG, Derrick S, Collins FM, et al. A pantothenate auxotroph of *Mycobacterium tuberculosis* is highly attenuated and protects mice against tuberculosis. Nat Med. 2002;8:1171-4.
41. Sambandamurthy VK, Derrick SC, Hsu T, Chen B, Larsen MH, Jalapathy KV, et al. *Mycobacterium tuberculosis* DeltaRD1 Deltapan CD: a safe and limited replicating mutant strain that protects immunocompetent and immunocompromised mice against experimental tuberculosis. Vaccine. 2006;24:6309-20.
42. Sambandamurthy VK, Derrick SC, Jalapathy KV, Chen B, Russell RG, Morris SL, et al. Long-term protection against tuberculosis following vaccination with a severely attenuated double lysine and pantothenate auxotroph of *Mycobacterium tuberculosis*. Infect Immun. 2005;73:1196-203.
43. Hinchey J, Lee S, Jeon BY, Basaraba RJ, Venkataswamy MM, Chen B, et al. Enhanced priming of adaptive immunity by a proapoptotic mutant of *Mycobacterium tuberculosis*. J Clin Invest. 2007;117:2279-88.
44. Samper S, Martín C, Pinedo A, Rivero A, Blázquez J, Baquero F, et al. Transmission between HIV-infected patients of multidrug-resistant tuberculosis caused by *Mycobacterium bovis*. Aids. 1997;11:1237-42.
45. Samper S, Martín C. Spread of extensively drug-resistant tuberculosis. Emerg Infect Dis. 2007;13:647-8.
46. Martín C, Williams A, Hernández-Pando R, Cardona PJ, Gormley E, Bordat Y, et al. The live *Mycobacterium tuberculosis phoP* mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine. 2006;24:3408-19.
47. Pérez E, Samper S, Bordás Y, Guilhot C, Gicquel B, Martín C. An essential role for phoP in *Mycobacterium tuberculosis* virulence. Mol Microbiol. 2001;41:179-87.
48. Gonzalo Asensio J, Maia C, Ferrer NL, Barilone N, Laval F, Soto CY, et al. The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in *Mycobacterium tuberculosis.* J Biol Chem. 2006; 281:1313-6.
49. Cardona PJ, Asensio JG, Arbues A, Otal I, Lafoz C, Gil O, et al. Extended safety studies of the attenuated live tuberculosis vaccine SO2 based on phoP mutant. Vaccine. 2009;27:2499-505.
50. Kamath AT, Fruth U, Brennan MJ, Dobbelaer R, Hubrechts P, Ho MM, et al. New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. Vaccine. 2005;23:3753-61.
51. Brennan MJ, Fruth U, Milstien J, Tiernan R, De Andrade Nishioka S, Chocarro L. Development of new tuberculosis vaccines: a global perspective on regulatory issues. PLoS Med. 2007;4:e252.
52. Walker KB, Brennan MJ, Ho MM, Eskola J, Thiry G, Sadoff J, et al. The second Geneva Consensus: recommendations for novel live TB vaccines. Vaccine. 2010;28:2259-70.
53. Martín C. Tuberculosis vaccines: past, present and future. Curr Opin Pulm Med. 2006;12:186-91.