# The genomics of mycobacteria

M.N. Viale [(1)], M.J. Zumárraga [(1)], F.R. Araújo [(2)], A.M. Zarraga [(3)], A.A. Cataldi [(1)], M.I. Romano [(1)] & F. Bigi [(1)*]

(1) Instituto de Biotecnología, Instituto Nacional de Tecnología Agropecuaria (INTA), Los Reseros y Nicolás Repetto, CP 1686 Hurlingham, Argentina
(2) Brazilian Agricultural Research Corporation (EMBRAPA), Campo Grande, Parque Estação Biológica - PqEB. Brasília, Mato Grosso do Sul, DF-Brazil
(3) Instituto de Bioquímica y Microbiología, Facultad de Ciencias, Universidad Austral de Chile, Independencia 641, Valdivia, Región de los Ríos, Chile
*Corresponding author: bigi.fabiana@inta.gob.ar

**Summary**
The species *Mycobacterium bovis* and *Mycobacterium avium* subspecies *paratuberculosis* are the causal agents, respectively, of tuberculosis and paratuberculosis in animals. Both mycobacteria, especially *M. bovis*, are also important to public health because they can infect humans. In recent years, this and the impact of tuberculosis and paratuberculosis on animal production have led to significant advances in knowledge about both pathogens and their host interactions. This article describes the contribution of genomics and functional genomics to studies of the evolution, virulence, epidemiology and diagnosis of both these pathogenic mycobacteria.

**Keywords**
Diagnosis – Epidemiology – Evolution – Genomics – Mycobacteria – Paratuberculosis – Tuberculosis – Virulence.

## Introduction

The genus *Mycobacterium* includes known pathogens that cause serious diseases, such as tuberculosis or leprosy in mammals. Many of the pathogenic species of this genus are found in the *Mycobacterium tuberculosis* complex (MTBC) and cause tuberculosis in a number of mammals, including humans. MTBC species and their main hosts (shown in parentheses) are: *M. tuberculosis* and *M. africanum* (humans); *M. bovis* (cattle); *M. microti* (vole); *M. canettii* (humans and animals); *M. caprae* (ruminants and humans); *M. pinnipedii* (marine mammals); *M. orygis* (animals and humans in Africa and Asia); *M. suricattae* (meerkat) and *M. mungi* (mongoose).

Another major group of mycobacteria is the *M. avium* complex (MAC). The members of this complex range from environmental mycobacteria, which cause opportunistic infections in immunocompromised humans, to pathogens of poultry and other livestock species. The MAC comprises the subspecies *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *silvaticum* and *M. avium* subsp. *paratuberculosis* (*MAP*). *MAP* is the species of greatest veterinary importance, principally because it causes paratuberculosis, a granulomatous enteritis that not only has a huge economic impact on ruminant production but also has zoonotic potential, as it may cause Crohn's disease in humans (1, 2).

*Mycobacterium bovis* is the causal agent of bovine tuberculosis and is one of the two species of the genus *Mycobacterium*, along with *MAP*, of greatest impact on livestock production. This article therefore describes the contribution of genomic tools to the study of both these species.

## Evolution

Extensive genome sequencing has identified at least 150 species of the genus *Mycobacterium*, divided into two major groups: rapidly growing mycobacteria, which include most of the free-living saprophytic species widely distributed in the environment, and slow-growing mycobacteria, which include MTBC and MAC species, as well as *M. intracellulare*, all of which are pathogens of mammals and birds (3).

A comparison of the genomes of mycobacteria species with other phylogenetically related species, such as *Corynebacterium* or *Streptomyces*, revealed that one distinction of the genus *Mycobacterium* is that it expands

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lipid metabolism genes. Among species of pathogenic and free-living mycobacteria there was also an expansion of genes encoding the metabolism of saturated fatty acids and genes encoding molybdenum; in the case of pathogenic mycobacteria, there was also an expansion of genes encoding DNA replication, repair and recombination. At the outset, the genome of mycobacteria was likely to have been shaped by gene transfer and gene duplication (4). However, unlike the hypothesis postulated for some species of nontuberculous mycobacteria (5), genomic studies (6) have shown there to be virtually no genetic exchange between MTBC members.

Owing to its wide host range, the species *M. bovis* was initially thought to be the common ancestor from which pathogens with a restricted ecological niche, including *M. tuberculosis*, were derived. However, after the first genome of *M. tuberculosis* was sequenced – that of strain H37Rv – a new evolutionary scenario was put forward for the MTBC. Genomic analysis of regions of difference (RD) between species established *M. tuberculosis* as the MTBC's common ancestor (7). Subsequently, genome sequencing confirmed this evolutionary scenario, with *M. tuberculosis* as the most ancestral species, followed by *M. bovis* and, lastly, bacillus Calmette-Guérin (BCG).

The complete genome sequence of *M. bovis* was published in June 2003 (8), five years after publication of the *M. tuberculosis* genome sequence (9). While the genome of *M. bovis* is more than 99.95% identical to that of *M. tuberculosis*, it has seven deleted RD ranging from 1 kilobase (kb) to 12.7 kb in length. So the main evolutionary force to have shaped the *M. bovis* genome could be said to be gene deletion. One interesting finding was that many of the missing or altered genes in *M. bovis* are also missing or altered in *M. leprae*, an obligate intracellular pathogen that underwent a significant reduction in its genome (8).

Despite major advances in understanding the evolutionary mechanisms leading to the emergence of MTBC species, particularly *M. bovis*, it is still difficult to explain how this bacterium – a microorganism whose genome has suffered a substantive loss of genomic regions and has no unique genes – has come to inhabit a broader ecological niche than its ancestor, *M. tuberculosis*. Certainly, part of the answer lies in the more than 1,200 mutations of a single nucleotide (single nucleotide polymorphism [SNP]) in coding regions or genes that distinguish *M. bovis* from *M. tuberculosis*. The genes showing the greatest variation between the two species are those encoding cell wall and secreted proteins. In particular, extensive variation is found in genes encoding the PE-PGRS and PPE protein families (8). These proteins are expressed on the surface of mycobacteria and are believed to be primarily responsible for the antigenic variation causing differing immune responses to *M. tuberculosis*, more than 10% of whose genome consists of these genes (10). In *M. bovis*, some of the genes in the ESAT-6 family of antigens are also affected. The ESAT-6 protein is a powerful human T-cell-stimulating antigen that belongs to a family of over 20 proteins, including other T-cell antigens, such as CFP10 and CFP7 (11). Owing to their amplifying effect, a key finding was gene variants or gene mutations encoding transcriptional regulators that determine differences between *M. bovis* and *M. tuberculosis*. Of a total of 206 transcriptional regulators and two-component systems, 19 genes were identified with one or more non-synonymous SNPs (unpublished data) and one gene encoding a truncated regulator (*Rv0931c*) in *M. bovis* (12). Recently it has also been suggested that a mutation in the *phoR* gene of most strains of *M. bovis* could explain the low transmission of this species to humans. PhoR and PhoP form a two-component system that activates the expression of many proteins that are important for the interaction of *M. tuberculosis* with the host (13). Another noteworthy feature is high-level expression of the humoral antigens MPB70 and MPB83 in the bovine bacillus compared with the human bacillus, owing to a mutation in a gene encoding a protein that regulates gene expression (14), as well as polymorphism between *M. bovis* and *M. tuberculosis* with regard to several genes involved in the synthesis or transport of complex lipids (8).

In recent years, high-throughput RNA sequencing (which has uncovered the transcriptome) has also helped to improve our understanding of the evolutionary processes leading to the speciation of mycobacteria and their adaptation to different ecological niches. Although transcriptome studies of two species of closely related lineage – *M. bovis* and *M. marinum* (the latter is a species with a wide host range and the genomic characteristics of environmental mycobacteria) – have shown a high degree of conservation in their transcriptomes, they suggest that an important evolutionary force to have differentiated the two transcriptomes was the larger number of promoter regions (-10 boxes) in *M. bovis* than in *M. marinum* (15). SNP-driven promoter differences likely underpin many of the transcriptional differences between species of the same complex that are evolutionarily closely related, such as *M. bovis* and *M. tuberculosis* (15). Furthermore, the combination of genome and transcriptome analysis has led to the discovery that the presence of SNPs in antisense transcripts leads to the production of non-coding RNAs that regulate gene expression through mechanisms that degrade or inhibit transcription of their complementary mRNA (16).

With regard to the MAC, the first genome to be completely deciphered was that of the bovine strain *M. paratuberculosis* K-10 (17). The genome of this strain is a circular chromosome of 4,829,781 base pairs (bp) encoding 4,350 open reading frames, 45 tRNAs and one rRNA operon. The main difference between *MAP* and the other MAC members is that the former has between 14 and 18 copies of the insertion

sequence (IS) IS*900* throughout its entire genome (18). This sequence of 1,451 bp became a very useful tool for the differential diagnosis of this mycobacterium, so much so that analysis of the patterns of polymorphism of this sequence enabled *MAP* to be further subdivided into strain types: type I (or *MAP*-S), which is common in sheep, and type II (or *MAP*-C), which is common in cattle and goats. A third *MAP* strain type was originally isolated from a bison in the United States (19); referred to as 'Bison type', this type has also been isolated from bison and humans in India (20).

By sequencing several MAC strains, the likely evolutionary scenario of members of this complex was established. One model proposes a first phase in which an ancestral clone of *M. hominissuis* evolved through inversions in the genome, coupled with the acquisition and loss of genomic sequences, creating two independent pathogenic clones. One contained *M. avium* and *M. hominissuis*, while the other gave rise to the original pathogenic clone of *MAP* (proto-*MAP*). In a second phase, the two main *MAP* lineages, *MAP*-S and *MAP*-C, arose from the original clone of proto-*MAP* following independent genomic events (21). A slightly different evolutionary scenario proposes that the common ancestral strain *M. hominissuis* gave rise to the *MAP*-S strain, followed by a second genomic event that gave rise to *MAP*-C (22). A later analysis based on a comparison of the insertion sequence IS*1311*, which was undertaken to determine the evolution of the Bison type strain, was to confirm the latter hypothesis, i.e. that *M. hominissuis* is the parent strain that gave rise to *MAP*-S, which then evolved into the lineage *MAP*-C, subsequently giving rise to the United States bison-type strain and later to the Indian Bison-type strain (23).

# Virulence

Despite their close genetic relationship, MTBC members have a highly diverse profile of virulence. While the effect of virulence on pathogenesis, diagnosis and vaccine efficacy has been studied exhaustively in relation to *M. tuberculosis* (24, 25, 26), very little information is yet available on *M. bovis*.

There have been many studies aimed at linking the virulence of a species or strain to its genetic profile. One example is a comparative study carried out in Argentina of two strains of *M. bovis* (04-303 and 534): one isolated from a wild boar in La Pampa province and the other from a bovine in Santa Fe province. Even though the two strains were phylogenetically related, they presented a very different phenotype of virulence when tested in various hosts, with the strain isolated from the wild boar showing heightened virulence (27, 28). Other comparative studies of the two strains determined the differences in their *in vitro* transcriptomes (29). The DNA microarray and quantitative real-time polymerase chain reaction (RT-qPCR) techniques demonstrated that the genes *mce4D*, *Mb2607/Mb2608* and *Mb3706c* were over-expressed in the virulent strain 04-303, whereas *alkB*, *Mb3277c* and *Mb1077* were over-expressed in the attenuated strain 534. In addition, 49 genes were identified that were expressed differently in the two strains: a total of 35 genes were expressed more in the virulent strain and 14 genes were expressed more in the attenuated strain. Most of the genes whose expression varies according to strain showed the same differential expression in bovine macrophage during *in vitro* culture. These results would indicate that differences in gene expression patterns might explain the phenotypic differences in the virulence of the two strains. Subsequently, the genomes of these two Argentine strains were sequenced (30; data not yet published) and, based on these data, the sequences of 345 virulence-related genes were analysed using the AF2122/97 genome as the reference strain. Compared to this strain, there were mutations in 11 of these genes in strain 04-303 and in 15 genes in strain 534. The non-synonymous mutations that predict amino acid changes were located in the *hrcA, hpx* and *esat6* genes of the virulent strain 04-303, and in the *irtA, pks10, clgR* and *tetR* genes of attenuated strain 534. The mutation of strain 04-303 in *hrcA* seems particularly important because it encodes the heat-inducible transcriptional repressor HrcA. This transcriptional regulator is involved in transcriptional control of the heat-inducible family of proteins Hsp60/GroES (31), which are considered important for the virulence of several pathogens (32).

Despite intensive research efforts, there is not yet much information on the molecular basis of *MAP* pathogenicity. One of the most notable differences between *MAP* and the other MAC and MTBC members is that no mycobactin (a siderophore) is produced in *MAP*. In *M. tuberculosis*, iron transport was found to be associated with a group of ten genes (*mbt*A-J) (33). *MAP* genome analysis revealed that the first of the ten genes – *mbtA* – is shorter than in *M. bovis*, *M. tuberculosis* and *M. avium*. Truncation of this gene, which plays a crucial role in the cascade of reactions resulting in the production of mycobactin, is thought to be the reason why its production is attenuated in *MAP* (17), making it necessary to add it to the culture medium used for *in vitro* growth.

Another difference between *MAP* and *M. tuberculosis* is that *MAP* has fewer proteins of the PE/PPE family. In *MAP*, there are only six genes encoding PE proteins and 36 genes encoding PPE homologues (which account for 1% of the genome), compared with the 38 and 68, respectively, in *M. tuberculosis* (17). Nor are genes encoding the PE-PGRS protein subfamily found in *MAP*, which may suggest a more limited and less variable immune response than in *M. tuberculosis* and *M. bovis*.

The virulence factors of the genus *Mycobacterium* include *mce* genes, whose gene expression products are essential

for cell entry and survival within macrophages (34, 35, 36, 37, 38, 39). In *MAP*, the *mce* genes form eight operons distributed throughout the genome, including two copies of *mce5* and *mce7*, just as in *M. smegmatis*. A comparison between the *MAP* K10 genome and the genome of other MAC members revealed significant differences in several genomic regions. One such region includes four open reading frames of the *MAP* genome (*MAP2189*, *MAP2190*, *MAP2192* and *MAP2193*) – homologous with the *mce* family – which were classed as divergent in the genome of non-paratuberculosis MAC strains (40). This could give *MAP* a specific advantage when it comes to macrophage infection and/or virulence mechanisms. Indeed, a recent study describes how a natural mutant of *MAP* with a 16 kb deleted region including an entire *mce* operon and PE/PPE genes shows reduced ability to survive in different cell models, supporting the theory that these genes are crucial to *MAP* virulence (41).

Another very important virulence factor in the genus *Mycobacterium* is the mycolid acids of its cell wall, which are responsible for entry into the host cells and for suppressing or evading the defence mechanisms of the immune system. An analysis of the *MAP* genome revealed that there were around 80 more of the genes putatively involved in lipid metabolism in *MAP* than there were in *M. tuberculosis*. This difference between *MAP* and *M. tuberculosis* might point to variations in metabolism and lipid biosynthesis that may determine the presence/absence of antigens on the cell surface, which would affect the host's immune defence mechanism (17).

# Molecular epidemiology

The reference method for typing *M. tuberculosis* is analysis of restriction fragment length polymorphism (RFLP) using the insertion sequence IS*6110* as the probe. However, as most *M. bovis* strains have only one copy of this element, the RFLP method is of little use. While other types of probe – such as direct repeats (DR) or polymorphic guanine-cytosine-rich repetitive sequence (PGRS) – increase differentiation, they also make the interpretation of results more complex.

The molecular epidemiology of bovine tuberculosis has been facilitated by the implementation of PCR-based techniques because this has reduced the turnaround time for results, does not require large amounts of DNA to implement and, in some cases, can be carried out directly on clinical material with no need for prior isolation. One of the most widespread PCR-based techniques is reverse line blot hybridisation spoligotyping (42). The presence or absence of polymorphic sequences, called 'spacers', defines hybridisation patterns (spoligotypes) and leads to interspecies and intraspecies differentiation within the MTBC (42). Spoligotyping is the best choice for large-scale studies because it allows long-standing relationships to be determined. A spoligotype (SB0140) was identified that predominates in countries that have had trade relations with the United Kingdom (43) and which, together with its ancestor, SB0130, forms the European 1 clonal complex, both missing spacer 11 (44). Other clonal complexes have also been described, including: European 2, which predominates on the Iberian peninsula and is characterised by the lack of spacer 21 (45); Africa 1, identified by the lack of spacer 30 and prevalent in several West African countries (46); and Africa 2, which lacks spacers 3 and 7 and is found in East Africa (47).

More discriminatory markers are required to establish recent relationships, such as variable numbers of tandem repeats (VNTR), or more specifically, major polymorphic tandem repeats (MPTR) (48), exact tandem repeats (ETR) (49, 50) and mycobacterial interspersed repetitive units (MIRU) (51). Although the maximum discriminatory power is obtained by combining 24 loci – MIRU2, MIRU4 (ETR D); MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU26, MIRU27 (QUB5), MIRU31 (ETR E), MIRU39, MIRU40, ETR A, ETR B, ETR C, Mtub04, Mtub21, Mtub29, Mtub30, Mtub34, Mtub39, QUB11B, QUB26 and QUB4156 (52) – 96% of total resolution is achieved by combining 15 loci. Recently, this scheme has been optimised for bronchoalveolar lavage fluid sampling (53). Smaller VNTR panels have also been described for *M. bovis*, depending on the population structure and epidemiological status of individual regions (54, 55). Genotyping can resolve individual cases. It was used to demonstrate, for the very first time, the transmission of the same *M. bovis* clone between two humans in Argentina (56).

Whole-genome sequencing (WGS) is a new tool that could be incorporated into tuberculosis control and surveillance programmes because the mutation rate of the *M. tuberculosis* genome is low – only 0.39 SNPs per genome per year (57). By comparison, traditional molecular methods have limited discriminatory power, particularly in regions where low prevalence has been achieved. WGS is fast and reliable but still very costly; sometimes the information generated is stored in public databases, such as TB Database (www.tbdb.org); Wellcome Trust Sanger Institute (www.sanger.ac.uk/resources/downloads/bacteria/mycobacterium.html); TubercuList (genolist.pasteur.fr/TubercuList), BoviList (genolist.pasteur.fr/BoviList); and NCBI (www.ncbi.nlm.nih.gov/genome). As WGS also provides information on the physiology, profile of virulence and drug resistance of the prevalent pathogen, it is fast being included as a tool for diagnosis and epidemiological studies.

The simultaneous SNP analysis of different genes, which is known as multilocus sequence typing (MLST), makes it possible to identify phylogenetic and epidemiological

relationships between different strains. With respect to mycobacteria, MLST has been used mainly in *M. tuberculosis*; as yet, little information is available concerning *M. bovis*. Up till now, the information generated has been more useful from a phylogenetic than from an epidemiological standpoint. The technology consists of sequencing what are considered essential genes and using the corresponding strains to detect the different alleles and establish an allelic profile. An MLST scheme was used for *M. tuberculosis* strains (58), but it was not as good as IS*6110*-RFLP for discrimination at the strain level, although it was better than spoligotyping (58). A study using an SNP scheme proposed previously (59) was able to classify *M. bovis* strains into three main lineages, and to distinguish virulent *M. bovis* strains from the *M. bovis* bacillus Calmette-Guérin (*M. bovis* BCG) strain (60). It must also be borne in mind that homoplasy can occur with both spoligotyping and VNTR as a result of convergent evolution, making SNPs particularly useful for defining strain lineages (61).

A combination of PCR for the *hsp65* gene, followed by PCR restriction-enzyme analysis (PRA), is used to differentiate mycobacteria to the species level (62). However, it does not differentiate between MAC bacteria or bacteria of the *M. tuberculosis* complex. A more recent study using a larger gene fragment successfully differentiated *MAP* from other MAC bacteria, and even differentiated between *MAP*-C and *MAP*-S (63).

RFLP analysis using the insertion sequence IS*900* has been used extensively to type *MAP* (64, 65, 66). However, higher polymorphism is achieved by determining MIRU-VNTR than by using RFLP. A number of MIRU-VNTR loci have been identified in MAC genomes, with varying discriminatory power, which is extremely important for the epidemiology of paratuberculosis (67, 68, 69, 70, 71, 72).

Single nucleotide polymorphisms are the commonest polymorphisms in the MAC. Studying them using MLST of conserved genes encoding enzymes or structural proteins has made it possible to determine variability between MAC subspecies and strains (73).

Another tool for *MAP* differentiation and subtyping is the analysis of multilocus short sequence repeats (MLSSR), which achieves high discriminatory power (74, 75). In a study based on the allelic variability of 11 MLSSR, it was possible to differentiate 33 *MAP* strains from different geographic areas and different hosts, belonging to 20 subtypes (74). Another MLSSR analysis conducted in the United States detected 61 *MAP* genotypes out of a total of 211 *MAP* strains and 56 *M. avium* strains, although the degree of differentiation in *M. avium* was low (76).

A combination of different techniques can provide more epidemiological information about *MAP*, as described in a study analysing strains from different hosts in South America by determining MIRU-VNTR and MLSSR. Seven MIRU-VNTR genotypes and seven MLSSR were detected, giving a combined total of nine genotypes. The results revealed a predominance of one MIRU-VNTR genotype (INMV1) and one MLSSR genotype (genotype A), which are also common in the United States and Europe (77). In another study in Germany and Luxembourg, 91 isolates from symptomatic dairy cattle were also genotyped by MIRU-VNTR and MLSSR, which led to the detection of 11 MIRU-VNTR genotypes and 6 MLSSR, giving a combined total of 25 genotypes (78). These studies demonstrate the usefulness of such techniques for *MAP* genotyping and their potential application in control programmes.

A major challenge in developing countries is to secure government policies for implementing such technologies in programmes for the surveillance, control and eradication of bovine tuberculosis and paratuberculosis.

# Diagnosis

Most of the studies on the detection of *M. bovis* by PCR are based on identifying two insertion sequences that were first identified in the pre-genomic era: IS*6110* and IS*1081*. The IS*6110* sequence was adopted for the diagnosis of human tuberculosis because the *M. tuberculosis* genome often contains multiple copies of it. Paradoxically, amplification based on IS*6110* proved to be more sensitive for detecting *M. bovis*, which has only one copy of this sequence, than amplification based on IS*1081*, of which *M. bovis* has five copies (79).

There have been several reports of *M. bovis* detection by PCR that also allows for simultaneous differentiation of other members of the *M. tuberculosis* complex. For example, a multiplex PCR based on the simultaneous amplification of the rRNA operon (conserved in all mycobacteria), the *mur*A gene (conserved throughout the *M. tuberculosis* complex) and the RD4 region (missing only in *M. bovis*) made it possible to correctly distinguish membership of the genus *Mycobacterium*, the MBTC and, specifically, *M. bovis* (80).

A complete bioinformatics scan of genomic sequences led to the development of *Seek*TB, a two-step quantitative PCR (qPCR) using 16 pairs of oligonucleotides, which is able to distinguish all eight members of the *M. tuberculosis* complex (81). Furthermore, the authors demonstrated that RD7, which is present in *M. tuberculosis*, is missing from *M. bovis* (82). This genomic difference was used to establish a PCR to distinguish *M. bovis* from *M. tuberculosis* in suspected cases of zoonotic tuberculosis or bovine tuberculosis caused by *M. tuberculosis* (83). However, this method is unable to distinguish *M. bovis* from *M. caprae*, *M. pinnipedii*, *M. microti*

or *M. africanum*, as RD7 is missing from all these species too.

Genomic studies have shown that region TbD1 is present in all members of the *M. tuberculosis* complex except the so-called 'modern' strains of *M. tuberculosis*, which are those primarily responsible for human tuberculosis in the Americas, Europe and North Asia. Thus, PCR based on TbD1 targets all the mycobacteria causing tuberculosis in animals (84). Recently, the simultaneous identification of bacteria belonging to the *M. tuberculosis* complex and to the MAC by single-tube tetraplex qPCR assay was described (85). This method uses IS*1311* to identify the MAC, *devR* to identify the *M. tuberculosis* complex, and the internal transcribed spacer (ITS) located between the 16S and 23S rRNA genes to identify the genus *Mycobacterium*.

As regards paratuberculosis, although the gold standard for diagnosing *MAP* is culture and isolation from faeces, PCR tends to be used for final confirmation of its presence. PCR-based detection techniques are used for locating it in both faeces and milk (86, 87, 88, 89). The specific sequence most commonly used to make a diagnosis is IS*900*. Different techniques have been devised based on this sequence, including *in situ* hybridisation, analysis of genomic polymorphisms using this probe as a marker, nested PCR and loop-mediated isothermal amplification (LAMP). Methods based on real-time PCR have also been developed that not only detect the presence of bacteria but are also able to quantify the bacterial load in a sample. A study using this technique, based on IS*900* combined with IS*Map02* (the latter being an insertion sequence of which there are six copies in the *MAP* genome), found high sensitivity and specificity for detection of the bacterium and put forward the method as a useful tool for paratuberculosis control and eradication programmes (90).

Genomic analysis for antigen prediction is a well-developed discipline that uses algorithms to scan the genome in search of domains with affinity for major histocompatibility complex (MHC) class I or class II. Based on experimental data obtained using a model antigen – ESAT6 – the ProPred programme, which predicts epitopes recognised by human MHC (HLA-DR), was found to correctly predict epitopes of *M. bovis*-specific antigens (91). Comparative studies of the mycobacterial genomes available in 2006 identified 42 *M. bovis*-specific genes (92). Based on pools of peptides from each of the 42 proteins, Mb2890, Mb3895 and Mb2555 were found to be the most antigenic and specific proteins. However, cross-reactions with *MAP* occurred even though none of the three proteins is encoded in this species. In another study, based on bioinformatics scanning of the *M. bovis* genome and a literature review, secreted proteins were selected because, according to previous findings, the most antigenic proteins tended to be secreted (93). A total of 119 putative secreted proteins were identified, including several from the ESX family – which are antigenic and closely linked with *M. tuberculosis* complex virulence – that showed stimulation of Th1 cells (94).

Whole-genome sequencing makes it easy to identify unique *MAP* sequences to implement or improve diagnostic tests. An analysis of the whole genome of strain K10 has revealed some 161 unique genomic regions of *MAP* with potential application in diagnosis, the longest of which is 15.9 kb (17). Indeed, the immunogenicity of some of the proteins encoded by these unique regions has been confirmed (95, 96). The combination of genomic information, molecular tools and immunological tests is extremely useful in the differential diagnosis of *MAP*.

# Conclusions

Whole-genome sequencing of pathogenic mycobacteria, in particular that of *M. tuberculosis* in 1998, could be defined as the turning point after which advances in knowledge concerning these pathogens and their host interactions gathered pace dramatically. However, it was prior to this – in the pre-genomic era – that the evolutionary basis of the genus *Mycobacterium* was established and the main virulence mechanisms of pathogenic species were deciphered. Pre-genomic studies also facilitated the development of molecular typing methods, which have been used until now for epidemiological studies and diagnosis. Many questions remain that are expected to be answered using genomics, transcriptomics and systems biology, particularly questions concerning the processes by which mycobacteria interact with their hosts and adapt to ecological niches. These new, high-performance disciplines are also expected to contribute to enhancing disease control tools, as well as to developing next-generation vaccines and new and better diagnostic methods.

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