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Does *M. tuberculosis* genomic diversity explain disease diversity?

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Abstract

The outcome of tuberculosis infection and disease is highly variable. This variation has been attributed primarily to host and environmental factors, but better understanding of the global genomic diversity in the *M. tuberculosis* complex (MTBC) suggests that bacterial factors could also be involved. Review of nearly 100 published reports shows that MTBC strains differ in their virulence and immunogenicity in experimental models, but whether this phenotypic variation plays a role in human disease remains unclear. Given the complex interactions between the host, the pathogen and the environment, linking MTBC genotypic diversity to experimental and clinical phenotypes requires an integrated systems epidemiology approach embedded in a robust evolutionary framework.

Keywords

Genotyping; Evolution; Genetic diversity; Virulence; Clinical outcome

Introduction

The impact of strain variation for human disease has been well established for a number of bacterial pathogens. In *Escherichia coli*, *Neisseria menigitidis*, *Haemophylus influenzae*, *Bordetella* and *Streptococcus* species, some strains are more likely to cause invasive disease and others are emerging as a consequence of vaccine escape [1-8]. In the *Mycobacterium tuberculosis* complex (MTBC), a possible role for strain diversity in human tuberculosis (TB) is increasingly being suggested from work in various infection models (reviewed in [9-13]). But if and how MTBC genomic diversity influences human disease in clinical settings remain open questions. Starting in the first half of the 20th century, studies in guinea-pigs and mice reported differences in virulence among strains of tubercle bacilli [14-24]. However, until the development of the first molecular strain-typing techniques in the early 1990s, there was a general belief that genetic diversity within MTBC was too limited to account for these differences in virulence. The highly variable outcomes in TB, which ranges from lifelong asymptomatic infection to severe extrapulmonary disease affecting multiple organs were primarily attributed to host and environmental factors [25]. Moreover, most of the pathogenesis research on TB has concentrated on the laboratory strains H37Rv and Erdman, with little attention to clinical strains. More recently however, there has been mounting interest on

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studying clinical isolates, partially because of the realization that results from laboratory strains can suffer from artefacts because of bacterial adaptation to the laboratory [26,27].

One additional difficulty in trying to link genomic diversity to phenotypic diversity in MTBC has been the lack of appropriate tools to index genomic diversity and classify strains. MTBC is a genetically monomorphic organism and some of the genotyping tools applied to other bacterial pathogens are uninformative in MTBC [28,29]. Here we start by reviewing the genotyping methods currently used in MTBC with a particular focus on their limitations. We then review the current evidence for strain phenotypic variation in MTBC from experimental and clinical studies.

Measuring genetic diversity in MTBC - Review of past and current tools

In the early 1990s, IS*6110* RFLP was established as the first gold standard for fine typing of MTBC. During the following years, molecular epidemiological studies generated important new insights into the dynamics of transmission, relapse, and re-infection in TB [30,31]. At the same time, as large international collections of MTBC strains began to accumulate, IS*6110* RFLP analysis of these strain collections identified the first genotype "families" among MTBC. These studies also highlighted that some of these strains were more successful than others, both in terms of the number of associated TB cases and in their geographic distribution [32-34]. IS*6110* RFLP typing remains used today, but it has recently been replaced as the official gold standard for epidemiological genotyping of MTBC by the PCR-based methods known as spoligotyping and MIRU-VNTR [35,36]. These techniques have the advantage of requiring less DNA than RFLP, and produce data which can easily be digitalized and compared across laboratories. They also can be performed using crude lysates or directly from patient sputum, eliminating the need for culture and formal DNA extraction.

Even though IS*6110* RFLP, spoligotyping and MIRU-VNTR genotyping of MTBC have been invaluable for molecular epidemiological studies, these techniques suffer from several drawbacks, including the propensity for convergent evolution, which limits their use for phylogenetics and strain classification [28]. Genomic deletion analysis is another PCR-based method which has been developed to partially address these limitations. This method relies on the fact that ongoing horizontal gene transfer is essentially absent in MTBC, and genetic regions deleted from any given strain cannot be reacquired. As a result, genomic deletions, or large sequence polymorphisms (LSPs) behave as unique event polymorphisms and are therefore robust phylogenetic markers for MTBC. LSP-based analyses of global strain collections have shown that MTBC adapted to humans can be separated into six main lineages associated with different geographic regions and human populations [25,37,38].

Although LSPs have proven to be ideal markers for strain classification, phylogenetic trees based on LSPs do not represent the complete picture. This is because most LSPs were originally identified through a one-way comparison to the laboratory strain H37Rv. By contrast, de novo DNA sequencing generates unbiased data, which can be used to infer phylogenies which are more likely to represent the true evolutionary history of MTBC. In this respect, it is important to highlight the difference between de novo DNA sequencing (i.e. SNP discovery) and SNP-typing which is based on previously known SNPs. Various studies have published SNP-based phylogenies of MTBC in the past, but all of these studies suffer from one or several limitations, including the representativeness of the strain sample, the number of genes analysed, or the selection of SNPs used for typing (for further review see [10,29,39]. By contrast, a more recent study by Hershberg and colleagues performed de novo DNA sequencing of 89 genes in 108 globally representative MTBC strains [40]. This study reported the most complete MTBC phylogeny to date [39].This study also highlighted the fact that human-adapted MTBC was

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more genetically diverse than previously thought, and that this diversity could be linked to human migrations.

Most recently, a study by Comas et al. [41] used next-generation DNA sequencing to compare the genomes of 23 MTBC strains. In addition to containing robust phylogenetic information (Figure 1), DNA sequence data can also be exploited to study the nature and strength of the selective forces shaping the genetic diversity within populations. Using such population genetics approaches, Comas et al. found that, as expected, essential genes in MTBC were more evolutionary conserved than non-essential genes. Surprisingly however, and in contrast to most other pathogens where antigenic genes are under diversifying selection to escape host immunity, human T cell epitopes in MTBC were under strong purifying selection and more conserved than essential genes. These findings suggest that the recognition of MTBC by the human immune system might contribute to the transmission of the pathogen.

Several other comparative genome sequencing studies of MTBC are currently under way. As whole genome sequencing is the most comprehensive and most discriminative technique to measure genetic diversity in MTBC, this technique should be used for phylogenetic analysis and classification of MTBC. Indeed, with the continuing reduction in DNA sequencing costs, we believe genome sequencing will soon be adopted as the next gold standard for routine epidemiological “genotyping” of MTBC [42].

A phylogenetic framework for strain classification

Although DNA sequence data allows inferring robust phylogenetic structures, delineating biologically meaningful groupings within a continuous spectrum of genotypic diversity is not easy, and to some extent arbitrary. Nevertheless, defining such boundaries within species is important for the purpose of strain classification. The difficulty of determining biologically meaningful groupings within related bacteria arises at multiple taxonomic levels. For example, there is still no widely accepted species concept for bacteria, and species demarcation has been based on measures of genome similarity, phenotypic or ecological clustering. MTBC is an example for which the concept of “ecotype” has been proposed to define the various (sub)-species within MTBC [43,44]. Related concepts at lower taxonomic levels include terms such as “lineage”, “sub-lineage”, “family”, “clade”, and “cluster”. Unfortunately, similar to the species concept in bacteria, little agreement exists on how to best define these groupings, and this is particularly true for MTBC. However, if we are to understand the relationships between genotype and phenotype in this organism, a proper definition of these groupings is necessary. We believe that any attempt to define definitive groupings within MTBC should be based on a genome-based and phylogenetically robust understanding of the global diversity of MTBC. Figure 2 illustrates a theoretical approach to defining a set of hierarchical groupings within a given bacterial species. For MTBC, various strain groupings have been proposed in the past, but there is still no consensus and formal agreement on how best to define these groupings. The lack of a clear definition on how to group strains in MTBC has partially been responsible for the difficulty to detect consistent associations between strain-specific genotypes and phenotypes.

A further complicating factor in the classification of MTBC has been the lack of a clear and widely accepted nomenclature for strain groupings [10]. Because whole genome sequencing is the best tool to define MTBC phylogenies, the nomenclature of MTBC should also be based on whole genome data. Figure 1 shows the global phylogeny originally published by Comas et al. which is based on 21 MTBC genome sequences [41]. The six main lineages of human-adapted MTBC defined by these data were fully congruent with the ones previously published based on LSP- and multilocus sequence analysis. Thus, we propose a simple numerical nomenclature for these six main lineages (Figure 1). Figure 1 includes a comparison to the

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various corresponding nomenclatures published previously. For the remaining of this review, we will use this numerical nomenclature combined with some group names commonly used in the past. Larger numbers of MTBC genomes will be necessary to properly define the various sub-lineages and strain families comprised within these six main lineages.

Experimental evidence for phenotypic strain diversity

To date at least 67 studies have explored strain-specific phenotypic differences in vitro or in animal models of infection (Table 1). The most commonly studied strains are laboratory or reference strains, but 38 of these studies have also included clinical isolates.

Laboratory and reference strains

The canonical strains most widely used in pathogenesis research of TB are the laboratory strains H37Rv, H37Ra and Erdman, the vaccine strain *M. bovis* BCG, and the reference clinical strains CDC1551 and HN878. The latter two were isolated during TB outbreaks in Tennessee and Texas, respectively [45,46]. H37Rv/Ra, Erdman and CDC1551 all belong to Lineage 4 (Euro-American) while HN878 belongs to Lineage 2 (East-Asia/Beijing). Here we summarize the most important phenotypic differences between these strains (Table 1). Perhaps to most striking strain-specific phenotype described so far in MTBC has been the one observed in infections with strain HN878. Compared to the laboratory strains and CDC1551, NH878 has consistently been associated with a low inflammatory immune response and increased virulence in macrophages and animal models [47-49]. Studies have also shown that BCG vaccination offered poor protection against subsequent challenge with HN878. These experimental phenotypes have been associated with the production of a particular phenolic glycolipid (PGL) [50,51]. However, not all strains belonging to Lineage 2 produce this type of PGL [52]. Interestingly, the capacity to produce PGL appears to have been lost multiple times across the MTBC phylogeny, suggesting that PGL might be selected against in human infections. In contrast to HN878, strain CDC1551 induces a particularly strong proinflammatory immune response, compared to H37Rv and Erdman [47]. However, Erdman was shown to be more virulent than CDC1551 and H37Rv, while the evidence for differences between CDC1551 and H37Rv has been conflicting. One important limitation when studying laboratory strains is that adaptation to laboratory conditions can lead to artefacts due to genetic changes acquired during growth in culture. For example, the loss of PDIM, an important cell wall component associated with mycobacterial virulence has often been documented [26]. Moreover, independent lineages of these laboratory strains have been evolving in different laboratories around the world, so that not all H37Rv strain variants will behave the same in infection models [27]. Even strain CDC1551 and HN878 have been evolving in various laboratories since their initial isolation more than ten years ago. These factors highlight the limitations of studies focusing solely on laboratory or reference strains.

Clinical strains

Clinical strains offer the advantage of representing the wildtype behaviour of virulent MTBC, particularly if the number of culture passages is kept to a minimum. A disadvantage is that the experiments need to be carefully standardized to account for the inherent differences in growth rates among clinical strains. To date, a total of 38 studies have compared the virulence and immunological characteristics of clinical strains of MTBC. Fifteen of these studies were performed before the establishment of strain genotyping, so that the reported differences in phenotype cannot be linked to differences in genotype. Among these early studies, the one consistent finding was that tubercle bacilli from South India were less virulent and more susceptible to oxidative stress when compared to isolates from Great Britain. South Indian strains were also found to be devoid of sulpholipids. Even though strains from these early studies were not genotyped, based on our current knowledge of the global phylogeography of

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MTBC (Figure 1), it is safe to assume that most of the South Indian strains belonged to Lineage 1 (Indo-Oceanic) and most of the British strains to Lineage 4 (Euro-American).

Later studies included a variety of clinical strains, in which IS*6110* RFPL and spoligotyping were used for strain typing. Among these studies, the most consistent results were from strains belonging to Lineage 2 (East Asia/Beijing). These strains induced lower levels of proinflammatory cytokines than H37Rv and other strains [53,54], and have also been associated with increased growth in human monocytes [55], which is consistent with the higher virulence reported for strain HN878. Lineage 2 strains were also associated with higher levels of necrosis, and lower levels of apoptosis, in infected macrophages, which might be partially responsible for their higher virulence [53]. In support of this possibility, in the highly attenuated strains H37Ra and BCG, the opposite was observed [56,57]. Importantly however, Lineage 2 strains exhibit a range of inflammatory and virulence phenotypes [58,59]. This could be due to the fact that Lineage 2 consists of a variety of sub-lineages, including various variants of “Beijing” strains, which might differ phenotypically [60].

Results from a series of studies reporting on strain-specific differences in virulence- and/or immune phenotypes cannot be compared because the genetic background of the strains were unknown and genetic relationships cannot be established [21,61-66]. Other studies, despite of applying strain typing, only differentiated isolates, but did not take into account the degree of relatedness between them. For example, Garcia de Viedma et al. [67] found that pulmonary strains were more virulent than extrapulmonary strains recovered from the same patients. Although these strains differed in their spoligotypes, the authors did not consider the possibility that the lung isolates from the different patients were more closely related to each other compared to the corresponding extrapulmonary isolates, or alternatively, if strains isolated from the same patient were more similar. In a study by Dormans et al. [68] strain-specific differences in virulence were found, but when comparing the genetic background of these strains, the authors found that these differences were not consistent among strains belonging to the same genotype family. Hence, in addition to mere strain classification, assessing the degree of genetic relatedness between bacterial strains by detailed phylogenetic methods, as well as quantifying genetic diversity within and between strain groupings will be crucial to understand of the effect of strain-diversity on TB.

Clinical evidence of phenotypic strain diversity

To understand the impact of MTBC genotype on TB in humans it is necessary to link findings from experimental studies to human infections. We identified a total of 33 studies that have investigated the effect of MTBC genetic diversity on clinical outcome (Table 2). Significant differences among MTBC lineages have been reported in terms of their propensity to cause secondary cases in different human population [37], their progression to active disease in recently exposed household contacts [69], and their recognition by the human immune system [70]. Most other related studies have reported associations between MTBC genotypes and disease phenotypes. For example, Lari et al. [71] found an association between Lineage 3 (CAS) strains and extrapulmonary disease. Another recent study reported an association between Lineage 4 (Euro-American) and pulmonary, as opposed to extrapulmonary TB [72]. By far the most studied strain genotype has been Lineage 2 (East-Asia, including “Beijing”). Twenty-two studies have reported on clinical and epidemiological characteristics of Lineage 2. However, the results of these studies were not always consistent across studies. While Lineage 2 has been associated with extrapulmonary [73,74] or meningeal TB [72], other studies found no such association [75]. Two studies reported an association between Lineage 2 and HIV–coinfection [76,77], while two others found no association between Lineage 2 and HIV status [78,79]. One study described an association between Lineage 2 and previous BCG

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vaccination [80], supporting the hypothesis that Lineage 2 might be emerging as a vaccine escape variant [81]. However, three studies found no such association [82-84].

Some authors have reported results which could indicate a higher virulence of Lineage 2, as suggested by some of the experimental data reviewed earlier. Two recent studies have reported an increase over the last decades of the proportion of TB cases caused by Lineage 2 in Cape Town, South Africa [85,86]. Similarly, a multi-country study has reported an increase on the number of TB cases due to Lineage 2 over time in European countries when combining data from all the countries, although not for individual countries [79]. Anh et al. [82] reported an association between Lineage 2 and younger age in TB patients from Vietnam, suggesting that Lineage 2 is also emerging in this population. By contrast, no such age-relationship was found in Singapore [87]. One possible explanation for the emergence of Lineage 2 in at least some regions of the world could be the association with antibiotic resistance (recently reviewed in [88]). In support of this possibility, some studies from Asia have reported links between Lineage 2 and treatment failure [89,90], or disease relapse [87]. By contrast in South Africa, the recent increase in Lineage 2 was due to drug-susceptible strains with the proportion of TB cases caused by drug-resistant Lineage 2 remaining constant over the years [86]. This difference between the long-term success of drug-susceptible and drug-resistant Lineage 2 strains in Cape Town highlights the possible impact of drug resistance on strain fitness [88, 91]. Even if Lineage 2 were generally more successful than the other lineages of MTBC, the available evidence is not sufficient to support a generally higher transmissibility of these strains; other mechanisms need to be considered. For example, De Jong et al. reported that transmission to secondary hosts was similar among MTBC lineages in the Gambia, but progression to active disease was higher in individuals infected with Lineage 2 [69].

Molecular mechanisms

To determine if and how strain genetic diversity in MTBC affects TB infection and disease, we also will need to understand the molecular mechanisms leading from strain genotype to the various experimental and clinical phenotypes. To date, only a few reports have explored the underlying mechanisms responsible for phenotypic differences observed in experimental studies. Reed et al. [51] reported that the hypoinflammatory and hypervirulence phenotype of strain HN878 was linked to the production of PGL by a subset of Lineage 2 strains. The reason that H37Rv and other members of Lineage 4 (Euro-American) do not produce PGL is because of an inactivating seven base pair deletion in the *pks1/15* gene which encodes a polyketide synthase involved in the production of PGL [92]. Indeed, disruption of *pks1/15* in HN878 leads to the loss of the hypoinflammatory and hypervirulent phenotype [51]. Interestingly however, insertion of an intact *pks1/15* gene into H37Rv did not result in increased virulence, highlighting a possible effect of genetic background in MTBC [58]. Other studies in Lineage 2 strains have shown that these strains accumulate triacylglycerides as a consequence of the constitutively overexpression of *dosR* [52]. This overexpression has recently been partially attributed to a 350kb duplication in some members of Lineage 2 [93].

Newton et al. [94] studied a clinical strain which caused a large TB outbreak in the UK. The authors discovered a genomic deletion affecting the gene Rv1519, which is generally absent from strains belonging to Lineage 3 (CAS). This deletion was associated with the production of the anti-inflammatory cytokine IL-10. Complementation with Rv1519 abrogated the capacity of the outbreak strain to elicit IL-10 production by infected macrophages. However, it is unknown whether the phenotype observed by Newton et al. is shared by all members of Lineage 3, or restricted to the particular outbreak strain used in the study.

Only two studies have reported phenotypic differences among MTBC strains in TB patients which could be associated with a particular genetic mutation. In a large population-based

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collection of MTBC isolates from Arkansas, Yang et al. [95] and later Kong et al. [74] found that strains isolated from patients with extrapulmonary TB were more likely than strains from pulmonary cases to carry a deletion in the phospholipase C -encoding gene *plcD*. Unfortunately these studies did not provide any information on the genetic background of these strains. This impedes exploring if these deletion events are convergent adaptations appearing several times during MTBC evolution, or characteristics of one or several evolutionary branches within the MTBC phylogeny.

More data are needed

After reviewing nearly 100 papers, the only clear message coming out of these studies is that MTBC strains differ in their virulence, immunogenicity and suceptibilty to oxidative stress in infection models. However, human studies in clinical settings have largely failed to detect common patterns in the effect of strain variation on outcome of TB. The inconsistencies in clinical studies could be due to many factors, including host and environmental factors like the nature and quality of the TB control program, nutritional aspects, as well as the extent of co-morbidities such as diabetes, which in contrast to HIV, are less frequently screened for. Different genetic variants of MTBC have been associated with different human populations, potentially reflecting some degree of host-pathogen co-evolution [37,96]. Alternatively, social aspects influencing disease transmission and treatment could be the reason for these long-term host-pathogen associations. Regardless, of the underlying mechanisms, if the various MTBC variants behave differently in different human populations, detecting consistent strain genetic effects on TB will be even more difficult.

Another limitation of most studies reviewed here were the genotyping methods used for strain characterisation. IS*6110* RFLP and spoligotyping can be used for epidemiological purposes and to classify strains into known families, but they do not provide a robust phylogenetic framework in which to position strains of interest [28]. Therefore any evolutionary discussion about putative adaptations according to the evolutionary history of these strains will be impossible. Even if a clear phylogenetic framework is available (Figure 1), it would be an oversimplification to assume that all members of a given lineage will behave the same. For example, Kremer at al. demonstrated that only the so-called “typical” Beijing strains (which are part of Lineage 2), versus the “atypical” strains, were associated with BCG vaccination [80]. Thus, intra-lineage diversity will also have to be explored in order to capture the true picture of phenotypic variation in MTBC. In this respect, whole-genome sequencing of large representative collections of strains will be crucial to define phylogenetic relationships within the main lineages of MTBC and for comparative genomic studies aiming at defining the key genetic features responsible for differential experimental and clinical phenotypes.

From genome sequencing to systems epidemiology

TB infection and disease result from a complex interaction between the pathogen, the host and the environmental [25]. TB pathogenesis is not driven by individual virulence factors but through a complex process involving many bacterial factors which interact with many components of the host immune system. Hence to understand TB, an integrative approach is needed, taking into account the many interactions between the host and pathogen factors.

Although many reports have studied human genetic susceptibility to TB [97-99], only four studies have incorporated MTBC genotypes in their investigation [72,100-102]. Interestingly, all four studies have found an interaction between a human genetic polymorphism and the particular MTBC lineage known to be associated with the corresponding geographic region and human population [10,25,37,38,40,103-105]. Furthermore, one of these studies found that the particular human polymorphism associated with disease caused by the West-African

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Lineages 5 and 6 (also known as *Mycobacterium africanum*) in Ghana has a higher frequency in African human populations than in European and Euro-American populations. Taken together, these results or consistent with the hypothesis that particular lineages of MTBC might be adapted to specific human populations [37].

In addition to interactions between host and pathogen, the outcome of TB infection and disease is shaped by environmental and socioeconomic factors. Poor living and working conditions combined with known risk factors like HIV coinfection, malnutrition, smoking, diabetes, alcohol abuse, and indoor air pollution are all contributing to the current TB epidemic [106]. Studies addressing only one of these factors at a time, and without integrating the full set of host, pathogen and environmental data, are unlikely to contribute new and relevant knowledge. To move forward, we believe only systems epidemiology, a multidisciplinary approach combining systems biology with epidemiology, will provide novel information. Systems epidemiological approaches, in which iterative rounds of mathematical predictions and empirical testing are integrated with epidemiological modelling and disease surveillance will be able to shed new light on the interactions between the biological, ecological, and sociological processes in TB [107].

Concluding remarks

There is now clear evidence that different strains of MTBC differ in virulence and immunogenicity in experimental infection models. However, if and how these differences impact on human disease remains unclear. Studies of the effect of MTBC strain diversity in clinical settings have failed to find any consistent patterns. Hence at this stage, we would answer the question as to whether MTBC diversity matters for TB in humans with a cautious “maybe”. Clearly, more data are needed. It is also important to remember that even if consistent effects of MTBC strain diversity on human disease were discovered, we still would need to determine whether these traits are selected by natural selection or are the product of other evolutionary phenomena such as random genetic drift [40]. Thus, in addition to linking MTBC genotypic diversity to phenotypic variation in the laboratory and in clinical settings, studies need to address the evolutionary forces that shape global MTBC diversity. Ultimately, only a highly integrated systems epidemiological approach building on a robust evolutionary framework will be able to determine the definitive role of MTBC genomic diversity and its relevance for global TB control.

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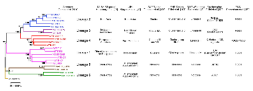

Figure 1. Neighbour-joining phylogeny based on 9,037 variable common nucleotide positions across 21 human MTBC genome sequences

Six main lineages are defined within human-adapted MTBC. The six lineages correspond to groups previously detected with a variety of genotyping techniques. Asterisks indicate spoligotype patterns within the main lineages which are considered phylogenetically unrelated based on spoligotyping, but are in fact related based on LSP analysis and genome sequencing [108] (adapted from Ref. 38). Abbreviations: MLSA=Multi Locus Sequence Analysis; LSP=Long Sequence Polymorphisms; SNP= Single nucleotide polymorphism; PGG=Principal Genetic Group.

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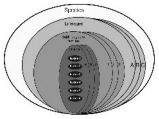

Figure 2. Theoretical approach defining a set of hierarchical strain groupings within a given bacterial species

Our capability to define different genetic entities as cluster, family, sub-lineage or lineage depends on the breadth of our sampling and molecular markers used. In this case, the species could be divided in different lineages, and within each lineage in different sub-lineages, clusters or families. The lower grouping is the strain or clone, which has been defined as a set of stocks that share many identical or similar properties due to a recent common clonal ancestry [109]. Here a clone represents genetically identical or almost identical bacteria, and can include different isolates from one patient, a transmission chain or any related units which share a very recent common ancestor.

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Table 1

Experimental studies exploring differences in phenotypes among different strains of MTBC.

Author	Year	Strain Type	Genotyping technique	Infection Model	Main Result
Dhayagude et al. [14]	1948	Clinical strains	No typing	Guinea-pigs	Strains varied in virulence but this did not correlate with the form of clinical TB.
Subbaiah et al.[17]	1960	Clinical strains	No typing	In vitro cultures	British and Indian strains showed differences in their susceptibility to hydrogen peroxide.
Sultan et al. [22]	1960	Clinical strains	No typing	Guinea-pigs	Strain-specific differences in rates of transmission in guinea-pigs.
Mitchison et al.[15]	1960	Clinical strains	No typing	Guinea-pigs	South Indian strains showed a wider range of virulence than British strains, and were, on the average, less virulent.
Subbaiah et al.[16]	1961	Clinical strains	No typing	In vitro cultures	Consistent differences in the peroxide susceptibilities.
Mitchison et al.[19]	1963	Clinical strains	No typing	Guinea-pigs and in vitro cultures	Strains with lower virulence in guinea-pigs displayed enhanced susceptibility to hydrogen peroxide in vitro.
Narayanan et al.[20]	1964	Clinical strains	No typing	Guinea-pigs and in vitro cultures	Two groups of strains could be distinguished, one with low virulence and high susceptibility to peroxide, and the other with high virulence and low peroxide susceptibility.
Collins et al.[23]	1969	Reference strains	NA	Mice	H37Rv grew more rapidly in liver, lung and spleen than H37Ra and BCG.
Alsaadi et al.[24]	1973	Reference strains	NA	Guinea-pigs	Numbers of bacteria recovered from lung and spleens were 100 fold less in H37Ra than in H37Rv.
Goren et al.[110]	1974	Clinical strains	NA	In vitro cultures	Strains showed differences in acidic lipid composition.
Grange et al.[111]	1977	Clinical strains	Bacteriophage typing	In vitro cultures	Strains of bacteriophage type 1, mainly obtained from Asian patients, had greater susceptibility to hydrogen-peroxide and/or thiophen-2-carbonic acid hydrazide than strains of other phage types.
Jacket et al.[112]	1978	Clinical and Reference strains	No typing	Guinea-pigs and in vitro cultures	Strains showing a high virulence phenotype in guinea-pigs were associated with lower susceptibility to hydrogen-peroxide compared to low virulence strains
Grange et al.[18]	1978	Clinical strains	Bacteriophage typing	Guinea-pigs and in vitro cultures	Phage type A strains, but not others, were more virulent in guinea pigs, resistant to hydrogen peroxide, resistant to thiophen-2-carbonic acid hydrazide, sensitive to thiacetazone.
Naganathan et al.[21]	1986	Clinical strains	No typing	Guinea-pigs	The distribution of the root index of virulence of isolates from patients living in the city of

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Author	Year	Strain Type	Genotyping technique	Infection Model	Main Result
					Bangalore was different, indicating a lower virulence than isolates from patients living in rural Bangalore.
O'Brien et al.[113]	1991	Reference strains	NA	Guinea-pigs macrophages	Heterogeneity in survival among strains in activated macrophages isolated from the lungs of vaccinated guinea-pigs.
Balasubramanian et al.[114]	1992	Clinical strains	No typing	Guinea-pigs	Strain differences with respect to pathology and number of bacilli recovered from spleen after intramuscular infection, and differences in infection efficiency.
McDonough et al.[115]	1993	Reference strains	NA	Murine and human macrophages	Attenuated strains differed from the virulent strain H37Rv in their abilities to replicate within macrophages,
North et al.[116]	1993	Reference strains	NA	Mice	Differential growth in virulent and attenuated strains in inmunocompromised mice.
O'Brien et al.[117]	1994	Reference strains	NA	Guineas-pigs	The in vitro resistance of mycobacteria to reactive nitrogen intermediates was positively correlated with strain virulence.
Dunn et al.[62]	1995	H37Rv, Erdman, NYH-27and *M. bovis* (Ravenel and Branch)	NA	Mice	According to host survival time, *M. bovis* Ravenel was the most virulent strain, followed, in decreasing order of virulence, by Branch, H37Rv, Erdman, and NYH-27.
Ordway et al.[61]	1995	Drug-resistant and -susceptible clinical strains	No typing	Mice	Growth patterns of several drug-resistant and drug-susceptible strains fell into three categories, avirulent (4 resistant:2 susceptibles), Virulent (2res:1sus), and fast-grower (5resis:2sus).
Schlesinger et al.[118]	1996	Reference strains	NA	Macrophages	Uptake of LAM (lipoarabinomannan)-coated microspheres by monocyte-derived macrophages was greater for Erdman LAM and intermediate for H37Rv and H37Ra LAM compared with that of buffer microspheres or microspheres coated with LAM from a non-tuberculosis strain of mycobacterium.
Lathigra et al.[119]	1996	Clinical strains	No typing	Mice and in vitro cultures	Small deletions, point mutations and nucleotide insertions were observed in two strains which did not produce the 19kD antigen.
Laochumroonvorapong et al.[120]	1997	Clinical and Reference strains *M. tuberculosis* and *M. bovis*	No typing	Human monocytes	H37Rv and H37Ra were similar both in intracellular growth rates and susceptibilities to intracellular killing following addition of exogenous hydrogen-peroxide.
Rhoades et al.[63]	1997	Clinical strains, Erdman and H37Rv.	No typing	Macrophage and in vitro cultures.	Clinical isolates exhibited a wide range of susceptibility to macrophage-generated reactive nitrogen intermediates.

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Silver et al.[56]	1998	Reference strains	NA	Human monocytes	TNFα-production by monocytes was significantly higher following infection with virulent H37Rv than with either BCG or H37Ra.
Balcewicz-Sablinska et al.[57]	1998	Reference strains	NA	Macrophages	TNFα-bioactivity was lower in macrophages infected with H37Rv than in those infected with H37Ra.
Valway et al.[45]	1998	Clinical vs Erdman	IS*6110* and pTBN12 probes	Mice	Mice infected with Erdman had 10 times fewer bacilli per lung than mice infected with clinical strains.
Zhang et al.[121]	1999	Clinical	IS*6110* RFLP	Macrophages	Isolates with the 210 genotype grew significantly more rapidly than small-cluster and unique strains in macrophages. All strains elicited production of similar amounts of cytokines and were equally susceptible to reactive nitrogen intermediates.
Manca et al.[122]	1999	Clinical isolate, CDC1551 and H37Rv	No typing	Human monocytes and in vitro culture	The clinical isolate showed a lower replication rate within monocytes than H37Rv and CDC1551.
Manca et al.[47]	1999	CDC1551, H37Rv, NH878 and HN60	NA	Mice and human monocytes	CDC1551-infected mice survived significantly longer and induced a more rapid and robust host immune response.
North et al.[123]	1999	Reference strains	NA	Mice	Mice infected with *M. bovis* Ravenel died much earlier from lung disease than H37Rv and CDC1515
Bishai et al.[124]	1999	Reference strains	NA	Rabbits	H37Rv and CDC1551 produced equal numbers of primary tubercles in rabbits, but CDC1551 tubercles were smaller and contained fewer bacilli than H37Rv tubercles.
Manca et al.[48]	2001	NH878, NHN5, HN60 and CDC1551	NA	Mice	NH878 failed to elicit the Th1-type response crucial for the control of mycobacterial infections. Survival times in immunodeficient mice infected with NH878 and H37Rv were similar.
Hoal-van Helden et al.[125]	2001	Clinical trains vs H37Rv	IS*6110* RFLP	Human macrophages	Different clinical strains were associated with high cytokine production compared with H37Rv.
Li 2002 et al.[55]	2002	Clinical strains, Beijing	IS*6110* RFPL and spoligotyping	Human blood monocytes	The four most rapidly growing isolates were members of the Beijing strain family.
Firmani et al.[126]	2002	Clinical strains vs CDC1551	NA	In vitro cultures	CDC1551 and CB3.3 were significantly more resistant to hydrogen peroxide and acidified sodium nitrite than other strains.
Danelishvili et al.[127]	2003	Reference strains	NA	Macrophages and type II alveolar epithelial cells	H37Ra elicited in more apoptosis than H37Rv strain after 5 days of infection in macrophages.
Manabe et al.[128]	2003	H37Rv and Erdman	NA	Rabbits	Fewer inhaled organisms of Erdman were required than of H37Rv to produce a visible

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					lesion. Erdman produced a larger number of tubercles than H37Rv.
Lopez et al.[129]	2003	Beijing vs H37Rv	IS*6110* RFLP	Mice	Beijing genotype strains induced significantly higher mortality than H37Rv. Previous BCG vaccination protected less against infection with Beijing strains than against the H37Rv strain.
Reed et al.[51]	2004	Reference strains	NA	Macrophages	A glycolipid (PLG) produced by HN878, W4 and W10 strains inhibited the innate immune response. In H37Rv and CDC1551, the absence of PGL production was confirmed to be associated with the previously reported 7-base-pair deletion resulting in a frameshift in what would otherwise be predicted to be a single gene encompassing *pks1* and *pks15*.
Manca et al.[50]	2004	Reference strains	NA	Mononuclear cells	Differential cytokine and chemokine responses of mononuclear cells to CDC1551 and HN878 strains, which leads to less protective immunity induced by HN878.
Petrelli et al.[64]	2004	Clinical strains and H37Rv	IS*6110* RFLP	Mice	Mice infected with Type-1 strains died earlier than those infected with other strains.
Dormans et al.[68]	2004	2 strains of each of 11 major genotype families	IS6110 RFLP	Mice	Different strains showed different virulence phenotypes, but strains of the same genotype families did not show more similar phenotypes than compared with strains of other genotype families.
Pheiffer et al.[130]	2005	Clinical Beijing strain, clinical no Beijing strain and H37Rv	IS*6110* RFLP and Spoligotyping	Plasma samples from TB patients	Beijing strain showed increased expression of α-crystallin and decreased expression of Hsp65, PstS1, and the 47 kDa protein compared to the other clinical strain and H37Rv. Different strains showed different antigen expression patterns.
Garcia de Viedma et al.[67]	2005	Clinical strains/Beijing	IS*6110* RFLP and Spoligotyping	Macrophages and mice	Extrapulmonary strains infected macrophages more efficiently than did the pulmonary strains, and showed higher infectivity in vivo.
Chacón-salinas et al. [131]	2005	H37Rv, *M. canettii* and Beijing		Macrophages	Macrophages infected with the Beijing isolate expressed the highest levels of mRNA for iNOS, IL-1b, TNFα, IL-12 cytokines and lower levels of IL-10 compared with cells infected with other genotypes.
Williams et al.[132]	2005	H37Rv, Erdman, South Indian (SI)	NA	Guinea-pigs	H37Rv and Erdman had a significantly higher pathogenic potential than SI, due to a higher bacterial counts, lower dose required to cause a disseminated infection, while SI showed a greater control of the pulmonary infection. But lesions induced by strain Erdman indicated less controlled pathogenic process than H37Rv.

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Barczak et al.[133]	2005	Reference strains	NA	Mice	Strain mixtures that contained primarily HN878 grew more quickly during the first 5 weeks of infection and were more lethal for mice.
Tsenova et al.[134]	2005	Beijing strains vs CDC1551	multiplex PCR	Rabbits	Beijing strains showed higher bacillary loads in the cerebrospinal fluid and brain, increased dissemination of bacilli to other organs, persistent levels of TNFα, higher leukocytosis, and more-severe clinical manifestations.
Manca et al.[49]	2005	HN878, W4, and CDC1551.	NA	Mice	Higher levels of type I IFNs were induced by the HN878 and W4 strains, which were associated with a decreased survival of the mice infected with HN878 or W4 relative to infection with CDC1551.
Park et al.[66]	2006	H37Rv and clinical strains	No Typing	Macrophages	Macrophages infected with the low virulence strain showed intact cell walls and organelles, and bacilli were clearly encapsulated within individual phagosomal membranes. In contrast, macrophages infected with CSU22 were necrotic, with cell swelling, lysis of cytoplasmic membranes, and release of intracellular constituents including the bacilli.
Chen et al.[135]	2006	Reference strains	NA	Macrophages	H37Rv, but not H37Ra, caused significant mitochondrial inner membrane disruption in macrophages leading to necrosis
Newton et al.[94]	2006	East African-Indian Strains (CH) vs. reference strains	LSP and MIRU	Macrophages	CH grew less rapidly and was less tolerant to hydrogen-peroxide and to acids and induced less protective IL-12p40 and more anti-inflammatory IL-10 than CDC1551 and H37Rv.
Ryoo et al.[136]	2007	Beijing vs reference.	IS*6110* RFLP	Macrophages	Beijing strains showed an increased expression of Mb1363 (probable glycogen phosphorylase GlgP) and MT2656 (halohalkane dehalogenase LinB)
Reed et al.[52]	2007	W-Beijing, Indo-oceanic and Euro-American	LSPs	In vitro culture	Beijing strains accumulated large quantities of triacylglycerides due to upregulation of the *dosR* regulon.
Tsenova et al.[137]	2007	Reference strains	NA	Mice	BCG was less efficient in protecting against HN878 dissemination to the liver and spleen than against H37Rv. HN878-induced inflammation, loss of body weight, lung and brain pathology, and signs of disease
Ordway et al.[138]	2007	HN878, H37Rv and CSU93	NA	Mice	HN878 grew faster and produced more sever pathology infections than the other strains.
Rocha-ramirez et al.[139]	2008	H37Rv, Canetii and beijing	IS*6110* RFPL and spoligotyping	Macrophages	Lipid fractions from the Beijing genotype preferentially induced macrophages to secrete high amounts of TNF-α and IL-10, but down

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Author	Year	Strain Type	Genotyping technique	Infection Model	Main Result
					regulated TLR2, TLR4 and MHC class II expression.
Palanisamy et al.[140]	2008	Reference strains	NA	Guinea-pigs	Guinea-pigs infected with Erdman, CDC1551 and HN878 had shorter survival times and more necrosis compared to H37Rv.
Honaker et al.[141]	2008	*M.bovis*, *M. tuberculosis* and BCG strains.	IS*6110* RFPL and spoligotyping	Wayne anaerobic dormancy model	BCG lacked *narK2* and *narX* induction and exhibited altered phenotypes during dormancy: *narK2/X* promoter region revealed a base substitution mutation in all tested BCG strains and *M. bovis* in comparison to the *M. tuberculosis* sequence.
Sinsimer et al.[58]	2008	Clinical Beijing strains	IS*6110* RFPL	Human monocytes	Different Beijing strains differed in their abilities to induce the pro-inflammatory cytokines depending to the genetic background of the strain.
Sohn et al.[53]	2009	Clinical Beijing strain (K strains) and H37Rv	IS*6110* RFPL	Macrophages	No significant difference in growth rate was observed between cells exposed to K-strain and those exposed to H37Rv. Levels of protective cytokines were lower in K-strain-infected cells and this strain induced significantly higher levels of necrotic cell death as opposed to than apoptosis than in H37Rv-infected cells.
Tanveer et al.[54]	2009	Clinical CAS and Beijing strains vs H37Rv	Spoligotyping	THP-1 cell line model and an ex vivo whole blood assay	Clinical isolates of CAS1 and Beijing strains displayed a lower growth index and induced reduced pro-inflammatory cytokine responses compared to H37Rv.
Marquina Castillo et al.[65]	2009	Clinical strains vs H37Rv	IS*6110* RFPL PGRS	Mice	Eight strains showed four strain phenotypes based on time to death, bacterial load, immunology kinetics and transmission to naïve mice.
Palanisamy et al.[59]	2009	Clinical strains and H37Rv	Spoligotyping	Guinea-pigs	Clinical strains showed a variety of virulence phenotypes based on pulmonary and extra-pulmonary necrosis and cavitary lesions. Drug-sensitive clinical isolates grew to higher numbers in the lungs compared to H37Rv and MDR strains.
Domenech et al.[93]	2010	Clinical and Reference strains	Spoligotyping and LSP	In vitro cultures	A 350 kb duplication is restricted to the most recently evolved sub-lineages of the Beijing strain family and is partially responsible for increased *dosR* expression.

Note: Data for this Table were identified through searches of PubMed and references from relevant reviews and articles. Search terms were "*Mycobacterium tuberculosis*", "lineage", "strain", "infection", "phenotype", "virulence", "animal model", "macrophage". Only English language papers were reviewed

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Table 2

Studies reporting different clinical outcomes in humans infected with different strains of MTBC

Reference	Year	Studied Mtb group	Genotyping technique	Main finding
Rhee et al.[142]	1999	PGGs	RFPL and *katG*-463 and *gyrA*-95	The results showed that no genotype of the *katG*-463 and *gyrA*-95-based classification system was associated with increased infectivity and pathogenicity or with increased IS*6110*-based clustering.
Anh et al.[82]	2000	Beijing	Spoligotyping	Beijing genotype strains were associated with younger age and with isoniazid and streptomycin resistance, but not with BCG vaccination.
Van crevel et al.[83]	2001	Beijing	IS*6110* RFLP and spoligotyping	Comparing Beijing with non-Beijing strains, the age distribution in the two groups was similar. No relation was found between genotype and BCG vaccination status. An equal percentage in both groups had pulmonary cavities. Significant difference between Beijing and other strains infected patients in febrile response to treatment.
Kato-Maeda A et al.[143]	2001	Different clones	IS*6110* RFLP, PGRSRFLP and genome deletions microarray	There was a statistically significant correlation between the percentage of the genome that was deleted from each clone and the percentage of the patients infected by that clone who had pulmonary cavitations revealed by chest radiography.
Lan et al.[90]	2003	Beijing	IS*6110* RFLP	The Beijing genotype was associated with treatment failure and relapse independently of drug resistance.
Hirsh et al.[96]	2004	Inter-lineage	LSP	Association between mycobacterial lineages and human populations, even if transmission took place outside of the regions where these human-bacterial associations are usually observed.
Borgdorff et al.[84]	2004	Beijing	IS*6110* RFLP Spoligotyping	Although Beijing cases were somewhat more likely to have extrapulmonary tuberculosis, the difference was not statistically significant. Beijing genotype strains were not associated with radiological presentation of pulmonary tuberculosis.
Yang et al.[95]	2005	Beijing	IS*6110* and pTBN12	Infection with a *plcD*-deleted clinical mutants was significantly associated with extrathoracic TB
Nicol et al.[75]	2005	Families/Beijing	Spoligotyping and 12-locus MIRU	Two strain families, LAM3/F11 and Beijing, predominated; but there was no overall association with extrapulmonary disease.
Kong et al.[74]	2005	Beijing	RFLP and pTBN12	A statistically significant association between *plcD* gene interruption and extrathoracic TB involvement.
European Concerted action et al.[79]	2006	Families/Beijing	IS*6110* RFLP Spoligotyping and Region A RFLP	Combining data from Western Europe showed an increase of Beijing strains over time, although not for individual countries except the Netherlands. Most countries except Asian countries showed an association between Beijing and younger age. No association between HIV status or site of disease and Beijing genotype.

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Reference	Year	Studied Mtb group	Genotyping technique	Main finding
Sun et al.[87]	2006	Beijing	IS*6110* RFLP and spoligotyping	No association between Beijing genotype and patient age or with relapse.
Caws et al.[76]	2006	Beijing	Spoligotyping	The Beijing genotype was significantly associated with HIV status, resistance to any drug, and multidrug resistance in tuberculous meningitis. The association of the Beijing genotype with drug resistance was independent of HIV status.
Kong et al.[144]	2006	Lineages 1 and 2	LSP	Extrathoracic tuberculosis was statistically significantly associated with Lineage 2 (East-Asian) but not with Lineage 1 (Indo-Oceanic).
Gagneux et al.[37]	2006	Inter-lineage	LSP	Secondary case- ratios of sympatric lineages were significantly greater in comparison with that of allopatric lineages in the same human population. Different lineages varied with regards to their ability to cause secondary cases.
Kong et al.[73]	2007	Families/Beijing/W	Spoligotyping	Patients infected by the Beijing/W lineage isolates were nearly three times as likely as patients infected with the non-Beijing isolates to have extrathoracic involvement.
Hanekom et al.[85]	2007	Beijing	IS*6110* RFLP and spoligotyping	Overabundance of one Beijing sub-lineage vs other sub-lineages, which could indicate increased transmissibility.
Maree et al.[145]	2007	PGG and Beijing	IS*6110* RFLP	There was no association between the principal genetic group of the strain or the presence of the Beijing genotype, and clinical presentation or outcome in children with tuberculous meningitis
Cowley et al. [146]	2008	Inter-lineage/W-Beijing and Lineage 1	Spoligotyping and SNP	Tuberculosis caused by Beijing strains showed an increasing in prevalence while Lineage 1 (Euro-American) strains a trend to decrease.
Twaites et al. [147]	2008	Lineage 2 and 4	LSP	Meningitis caused by the Lineage 2 (East Asian) presented with a shorter duration of symptoms. Isolates of the Lineage 4 (Euro-American) were significantly more likely than Lineage 1 (Indo-Oceanic) and Lineage 2 to be associated with the radiographic appearances of lung consolidation.
Caws et al.[72]	2008	Lineage 2 and 4	LSP	In a Vietnamese population, Lineage 4 (Euro-American) was more likely to cause pulmonary than meningeal TB, and lower mortality from meningeal TB than other lineages. A polymorphism in the TLR2 gene was associated with disease caused by the Lineage 2 (East Asian lineage including “Beijing”).
Herb et al.[100]	2008	Lineage 6 (West-African 2)	LSP	Association of the human variant G760A, which is linked to an increased risk of developing TB, was stronger in infections caused by Lineage 6 (West-African 2).
DeJong et al.[69]	2008	Inter-lineage/Beijing vs *M. africanum*	Spoligotyping and LSP	Transmission was similar, but progression to disease was significantly lower in contacts exposed to *M. africanum* than to *M. tuberculosis*. Contacts exposed to a Beijing strains were most likely to progress to disease relative to *M. africanum*.
Kremmer et al.[80]	2009	Intra-lineage/Beijing	Multiplex PCR- targets a direct repeat of IS*6110*	“Typical” Beijing strains are more frequently associated with BCG vaccination compared to “atypical Beijing strains.

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Reference	Year	Studied Mtb group	Genotyping technique	Main finding
DeJong et al.[78]	2009	Inter-lineage	Spoligotyping and LSP	Not statistically significant association among lineage and HIV.
Lary et al.[71]	2009	Inter-lineage *M.bovis* and CAS	Spoligotyping	Association of *M. bovis* and Lineage 3 (CAS) with extrapulmonary disease.
Middelkoop et al.[77]	2009	Inter-lineage/Beijing	IS*6110* RFPL	No association between IS*6110* RFPL genotpye and sex, age, type of TB disease or treatment outcome, but patients infected with Beijing strains were significantly more likely to be HIV positive.
Intemann et al.[102]	2009	Inter-lineage	Spoligotyping, IS*6110*, and pks1/15 deletion.	Human polymorphism associated to diseases caused by Lineages 2 (West-African 2) and 5 (West-African 1) but not Lineage 4 (Euro-American) in Ghana.
Van crevel et al.[101]	2009	Beijing	Spoligotyping	Beijing genotype isolates were strongly associated with tow polymorphisms in SLC11A1, a gene involved in involved in susceptibility to TB.
Van der Spuy et al.[86]	2009	Spoligo families/Beijing	IS*6110* RFLP and spoligotyping	The incidence of cases infected with strains of the Haarlem, LAM, Quebec and the Low-Copy Clades remained relatively stable, that of cases of the Beijing family increased exponentially over time.
Parwati et al.[89]	2010	Beijing	Spoligotyping	Positive sputum culture results after 6 months of treatment were more common among patients infected with Beijing strains than among those infected with non-Beijing strains.
Rakotosaminaba et al.[70]	2010	Inter-lineage	Spoligotyping	“Modern” *M. tuberculosis* strains like Lineage 2 (East-Asian) and Lineage 3 (CAS) tended to give lower IFN-γ responses than “ancient” strains like the Lineage 1 (Indo-Oceanic) in index cases and their household contacts.
Nahid et al.[148]	2010	Inter-lineage	LSP and SNP	Lineage 4 (Euro-American) sub-lineage RD724 was associated with more severe disease at baseline, and together with the East Asian lineage was associated with lower bacteriologic conversion after 8 weeks of treatment.

Note: Data for this table were identified through searches of PubMed and references from relevant reviews and articles. Search terms were “*Mycobacterium tuberculosis*”, “lineage”, “strain”, “phenotype”, “virulence”, “disease”, “clinical outcome”. Only English language papers were reviewed.