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MINIREVIEW

# Importance of the Genetic Diversity within the *Mycobacterium tuberculosis* Complex for the Development of Novel Antibiotics and Diagnostic Tests of Drug Resistance

**Claudio U. Köser,[a,b] Silke Feuerriegel,[c] David K. Summers,[d] John A. C. Archer,[e] and Stefan Niemann[c]**

Department of Medicine, University of Cambridge, Cambridge, United Kingdom[a]; Health Protection Agency, Cambridge, United Kingdom[b]; Molecular Mycobacteriology, Research Center Borstel, Borstel, Germany[c]; Department of Genetics, University of Cambridge, Cambridge, United Kingdom[d]; and Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia[e]

**Despite being genetically monomorphic, the limited genetic diversity within the *Mycobacterium tuberculosis* complex (MTBC) has practical consequences for molecular methods for drug susceptibility testing and for the use of current antibiotics and those in clinical trials. It renders some representatives of MTBC intrinsically resistant against one or multiple antibiotics and affects the spectrum and consequences of resistance mutations selected for during treatment. Moreover, neutral or silent changes within genes responsible for drug resistance can cause false-positive results with hybridization-based assays, which have been recently introduced to replace slower phenotypic methods. We discuss the consequences of these findings and propose concrete steps to rigorously assess the genetic diversity of MTBC to support ongoing clinical trials.**

Our understanding of the genetic diversity of the *Mycobacterium tuberculosis* complex (MTBC), which encompasses a number of species that cause tuberculosis (TB), has witnessed two key transitions. Initially, the differentiation of clinical isolates of MTBC relied on a few characteristics, such as colony morphologies, or the ability to grow in the presence of certain chemicals or phages (68, 170). These methods had limited discriminatory powers and were replaced by molecular genotyping methods (IS*6110*, spoligotyping, and mycobacterial interspersed repetitive unit-variable number tandem repeats [MIRU-VNTRs] [102]). Although these offered a higher resolution, they were still merely surrogates for the underlying genome diversity and ongoing evolution of MTBC. Over the past few years, however, researchers have been able to interrogate genomic diversity directly using either traditional Sanger sequencing or, more recently, whole-genome sequencing (143).

The results of these efforts have been discussed elsewhere and are beyond the scope of this minireview (2, 3, 5, 21, 22, 39, 43, 49, 58, 60, 62, 114, 145, 146). Instead, we will focus on aspects that are of immediate relevance to the development of novel antibiotics and diagnostic tests to detect drug resistance. Specifically, we will review the mounting evidence that the genetic diversity within MTBC, albeit limited (2), lies at the heart of both intrinsic and acquired drug resistance. This diversity has not always been considered in the past. By taking this into account, we propose improved standards to avoid these shortcomings in the future.

## KNOWN IMPACT OF GENETIC DIVERSITY

**Intrinsic differences in drug susceptibility.** The most significant impact of the genetic diversity of clinical MTBC isolates occurs when genetic changes result in intrinsic drug resistance. The clinical impact of intrinsic resistance will depend on the importance of the antibiotic and the frequency of the strains in question. The most prominent manifestation of this phenomenon concerns pyrazinamide (PZA), which is one of four first-line drugs used in combination with isoniazid (INH), rifampin (RIF), and ethambutol (EMB). The inclusion of PZA in the regimen allows for treatment in 6 months, rather than 9 months (132).

*Mycobacterium bovis* is PZA resistant due to the H-to-D change at position 57 (H57D) change in *pncA* (*Rv2043c*) (76) and accounts for about 3.1% of human TB cases worldwide (107), whereas *Mycobacterium canettii* is resistant by an unknown mechanism and is generally limited to the Horn of Africa (49, 85, 149). It is believed that most patients will be cured even if infected with an intrinsically PZA-resistant strain, but more-detailed investigations are required to determine whether these patients are at a higher risk of TB relapse or accumulating further drug resistance (107). This is primarily due to the fact that patients in developing countries, where *M. bovis* and *M. canettii* are mostly found, do not have access to diagnostics that can identify the precise member of MTBC with which they are infected (49, 66, 107, 132, 148). Yet even in well-resourced countries, *M. bovis* is not always identified, which contributes to fatal outcomes in some cases (7). Moreover, clonal spread of resistant variants of *M. tuberculosis* can also have significant consequences in developed countries. For example, a recent study of PZA monoresistant *M. tuberculosis* strains, which account for 6.2% of TB cases among Canadian-born patients in Quebec, found that these patients had significantly worse clinical outcomes compared to patients infected with fully susceptible isolates (171).

Having been derived from *M. bovis*, the bacillus Calmette-Guérin (BCG) vaccine strain, which is one of the oldest and most widely used vaccines worldwide and is also used for the treatment for bladder carcinoma (100), is intrinsically resistant to PZA and is also resistant to cycloserine (47, 48). The *cycA* (*Rv1704c*) G122S mutation is partially responsible for the latter intrinsic resistance

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Address correspondence to Claudio U. Köser, cuk21@cam.ac.uk.



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**TABLE 1** Known mutations that cause false-positive results with commercial genotypic susceptibility assays

| Antibiotic(s) | Gene | Mutation(s)[a] | Assays affected |
|---|---|---|---|
| Rifampin | *rpoB* (*Rv0667*) | Q510Q caG/caA, F514F ttC/ttT, D516D gaC/gaT | Cepheid Xpert MTB/RIF, Hain GenoType MTBDR*plus*, INNO-LiPA RIF.TB, and Nipro LiPA |
| Fluoroquinolones | *gyrA* (*Rv0006*) | T80A A90G | Hain GenoType MTBDR*sl* and Nipro LiPA |

[a] The Q510Q caG/caA mutation was a change from caG to caA in the glutamine codon at position 510.

(32). Moreover, the more recently derived BCG strains display elevated MICs for isoniazid, presumably due to a mutation in *mmaA3* (*Rv0643c*), and some of these strains are cross-resistant to ethionamide (ETH), which in some countries is the preferred drug for treating BCG disease with presumed central nervous system involvement (15, 87, 136, 166). Fortunately, disseminated BCG is very rare and can be treated with either a high dose of INH or combination therapy (15).

The most recently described manifestation of intrinsic resistance involves *M. canettii*, which, in addition to being intrinsically PZA resistant as discussed above, is also potentially resistant to the novel drug PA-824 due to an unknown mechanism (to be discussed in more detail below) (49, 55, 85, 149). Should the latter resistance be confirmed *in vivo*, the consequences for use of the proposed three-drug regimen of PA-824, moxifloxacin, and pyrazinamide, which is currently in phase 2 clinical trials (41), will depend on the local distribution of the MTBC strains. *M. canettii* is believed to be rare across the world, with the exception of the Republic of Djibouti where it accounts for approximately 11% of active TB cases, although more accurate data concerning its prevalence and spread are required (49, 69, 71, 85). Importantly, TB caused by *M. canettii* is clinically and radiologically indistinguishable from disease caused by the remaining members of the MTBC (85). As a result, empirical treatment with PA-824–moxifloxacin–pyrazinamide might not be effective in this part of the world. Whether this potential limitation also extends to delamanid (OPC-67683) and metronidazole is unclear (55).

In contrast to the above examples, the genetic diversity in MTBC can also render some strains more susceptible to antibiotics. The BCG strains that share the RD2 deletion are susceptible to macrolides *in vitro*, whereas all other members of MTBC are resistant against this class of antibiotics due to an intact copy of *ermMT* (*erm37*, *Rv1988*) (10, 26). Whether this difference can be exploited clinically is still open to debate (136). Similarly, the *gyrA* (*Rv0006*) T80A A90G double mutations result in hypersusceptibility to fluoroquinolones (FQs) (16, 23, 101, 126, 138). Last, it appears that *M. canettii* is more susceptible to the drug combination of trimethoprim-sulfamethoxazole (also known as co-trimoxazole) than MTBC, which, until recently, had been falsely assumed to be intrinsically resistant against this pair of antibiotics (59, 85, 91, 92, 117, 160, 172).

**Differences in selected drug resistance.** In addition to the underpinning drug resistance that exists prior to the start of treatment, the genetic diversity within MTBC also affects the nature of resistance mutations selected during treatment (MTBC acquires resistance by chromosomal mutations rather than from a vast lateral gene pool). When analyzing INH resistance in different lineages, Gagneux et al. found that the Euro-American lineage was more likely to harbor the *katG* (*Rv1908c*) S315T mutation, whereas resistance in the Beijing lineage was associated with mutations elsewhere in the gene. In the Indo-Oceanic lineage, *inhA* (*Rv1484*) promoter mutations were more common (61). In addition, the level of resistance conveyed by particular mutations can vary between different lineages (51). These and related findings (11, 75, 98) not only inform which mutations should be targeted by genotypic assays (see next section) but also highlight the clinical value of knowing the cause of drug resistance. Thus, low-level INH resistance caused by promoter mutations in *inhA* might be overcome with a high dose of the antibiotic, whereas this is not possible for the accompanying ETH cross-resistance. Conversely, when *katG* mutations are responsible for INH resistance, treatment with ETH remains an option (27). To allow for the practical application of these findings, Warren et al. have proposed a diagnostic algorithm based on the Hain GenoType MTBDR*plus* assay (112, 165).

**Confounding of genotypic susceptibility testing.** To overcome the limitations imposed by the low growth rate of MTBC, genotypic antibiotic susceptibility tests have been introduced and either have been endorsed by the World Health Organization or are currently under evaluation (11, 72, 73, 98, 119, 144). Given that these hybridization-based assays interrogate the DNA rather than the amino acid sequence, they detect both synonymous and nonsynonymous mutations, unless the genetic diversity in the target regions was taken into consideration for the design of the respective probes (4, 12, 24, 83, 155). For example, additional probes had to be included for the assay manufactured by Nipro Corp. to cover several neutral or silent mutations in *pncA* (13, 108, 144). This included the S65S mutation, which is shared by most but not all Delhi/CAS strains (151). In contrast, the A46A synonymous mutation, a marker for *M. canettii* (42, 49, 76, 85, 149), was not compensated for, thereby allowing the assay to rely on this mutation as a surrogate for the aforementioned intrinsic PZA resistance. Additional probes were not required for the *katG* assay developed by the same group, since their chosen probes do not cover the known polymorphisms within this gene (11).

In marked contrast, a synonymous mutation in *rpoB* (*Rv0667*) has been recently shown to cause false-positive resistance results for the key first-line antibiotic RIF in three commercial assays (Hain GenoType MTBDR*plus*, INNO-LiPA RIF.TB, and Cepheid Xpert MTB/RIF) (9). Even though this mutation (F514F) (Table 1) was found to be rare in Barcelona, Spain, with a frequency of only 1.4%, it still had a large effect on the positive predictive value of the Cepheid Xpert MTB/RIF assay, given that true RIF resistance is also rare in this region (two false-positive results compared to only six true-positive results) (111). The results of this assay therefore have to be analyzed with caution, particularly since RIF resistance has been used as a surrogate for INH (and hence multidrug) resistance, despite not being always appropriate (147, 157). Another synonymous mutation in *rpoB* (D516D) has the same effect with the aforementioned assay by Nipro Corp. (108), and Q510Q causes false-positive results with the Cepheid Xpert MTB/RIF (168) and, presumably, the other three assays discussed

above. Similarly, the A90G mutation in the hypersensitive *gyrA* double mutant (T80A A90G), alluded to in the previous section, does give a false-positive resistance result for the second-line FQs if analyzed using the Hain GenoType MTBDR*sl* test (Table 1) (16, 23, 126). The probe design in the assay by Nipro Corp. should result in the same limitation, although this remains to be confirmed experimentally (12, 108). Notably, T80A is a marker for the *M. tuberculosis* Uganda genotype, which was formerly known as *M. africanum* subtype II (36, 39, 71, 138). Consequently, the limitation of these tests appears to be confined to some strains of this genotype, but the precise frequency of this double mutation both among Uganda strains and the wider MTBC diversity has to be determined further.

The genetic diversity has also confounded several recent studies seeking to investigate the molecular basis of drug resistance (90, 91, 128). For example, Wang et al. had initially proposed that the *Rv2629* D64A mutation might be the missing resistance mechanism in about 5% of RIF-resistant strains which lack resistance mutations in *rpoB* (96, 164). However, Chakravorty et al. found that the overexpression of *Rv2629* does not result in RIF resistance (29). In addition, phylogenetic analyses showed that the D64A mutation is not associated with RIF resistance but instead constitutes a marker for the Beijing lineage (29, 74, 97, 115). As a result, an assay using high-resolution melting analysis that relies on this change has been developed to allow for the rapid identification of Beijing strains (8). The usefulness of this assay has recently been called into question by a report that identifies this mutation in non-Beijing strains as well (174).

In a similar way, we showed that the T202A mutation in *thyA* (*Rv2764c*) is a marker for the Latin American Mediterranean (LAM) lineage of *M. tuberculosis* which includes the KwaZulu-Natal (KZN) strain family which is endemic in South Africa (54, 78). Yet, contrary to two prior studies (103, 134), rather than rendering this lineage intrinsically resistant against *para*-aminosalicylic acid (PAS), the mutation is just a phylogenetic polymorphism (54, 57).

## NEW STANDARDS FOR FUTURE STUDIES

**Data presentation.** Past studies have not always fully exploited the data they gathered on the genetic diversity within MTBC, given that their primary focus lay elsewhere. For instance, most clinical studies of TB focus on elucidating the basis of drug resistance. Although synonymous mutations could affect mRNA stability, no such mutation has ever been implicated in drug resistance in MTBC. Consequently, some studies have not reported or specified synonymous mutations at all or have listed them separately from the nonsynonymous mutations (25, 130, 131, 153). This precludes readers from determining whether the mutation in question is a secondary mutation, which frequently accompanies complete loss-of-function mutations in nonessential genes. The same dilemma arises with studies that focus exclusively on synonymous mutations for phylogenetic purposes (18). Crucially, the way in which data are presented means that, where multiple genes are involved in resistance to the same antibiotic, it is not always clear which mutations occur together in the same strain. As a result, it cannot be determined whether an individual mutation is necessary and sufficient for resistance.

In order to use all of the above-mentioned information in the most effective way, we propose that future sequence-based studies should include all mutations detected in their supplementary data or in GenBank, even if their focus is on only a subset of sequence changes. For studies of drug resistance mutations, the format of these supplements should be similar to that used by the Tuberculosis Drug Resistance Mutation Database (TBDReaMDB) (see below) (139).

**Data analysis.** The association between the LAM lineage, including the KZN strain family, and the *thyA* T202A mutation discussed earlier went unreported for methodological reasons. In late 2007, the Broad Institute sequenced the genomes of three KZN strains (KZN 4207, KZN 1435, and KZN 605), all of which contained the *thyA* T202A mutation (79, 86). However, mutations were called relative to F11, another LAM strain with the T202A mutation (complete *M. tuberculosis* F11 genome [GenBank accession no. CP000717.1]), and consequently, this mutation was filtered out. For the same reason, Ioerger et al. also failed to recognize the mutation in strains KZN 4207, KZN 2475, and KZN 506 (78). Only Das et al. (37), who analyzed the former trio of KZN strains independently, used H37Rv as a reference strain. Yet, they did not establish the link with PAS resistance in their discussion (37).

To be clear, the method used by the Broad Institute and Ioerger et al. was the most appropriate for their particular interest, namely, to quantify the differences between closely related strains. Indeed, the normalization of sequence data against a close relative allows for the discovery of changes in areas that are deleted in more distantly related strains. However, mutations should also be called relative to the genome of the *M. tuberculosis* H37Rv laboratory strain (34), thereby anchoring the sequence data in the larger body of MTBC experimental data (89). This issue of normalization also partially explains why two errors in *rpsL* (*Rv0682*) and *gidB* (*Rv3919c*), two streptomycin resistance-mediating genes, were not addressed until recently (28, 89).

**Data dissemination.** To date, several hundred papers have reported sequencing MTBC strains to determine the genetic basis of drug resistance. However, until recently, no centralized database existed to collect this information. The TBDReaMDB has been an important step toward addressing this shortcoming, although the stringent criteria for inclusion of papers have meant that many mutations remain to be added (139). Nonetheless, the strength of the database is that it does not merely house a list of mutations. Instead, it includes additional information such as which other mutations coincide with a particular mutation, the frequency of the mutation in question, and the geographic location of the study.

A second notable development that is of particular relevance to identify changes of phylogenetic relevance for intrinsic resistance is the continued development of the Broad TB database (TBDB) (63). In addition to offering a user-friendly browser to analyze several near-complete genomes, it houses the data of the *M. tuberculosis* Phylogeographic Diversity Sequencing Project. This project resequenced 31 strains selected in a systematic manner to cover the various lineages and species of MTBC (35, 36, 63, 71). Importantly, these data should provide a sound basis for future studies to avoid the branch collapse that affected some of the prior studies of MTBC diversity (6, 62, 115, 146). Indeed, this resource has already acted as a catalyst by identifying some phylogenetic markers within resistance genes (36). In addition, it highlighted the link between antibiotic resistance and antigenic variation. Specifically, the plethora of nonsense and frameshift mutations reported for *tlyA* (*Rv1694*), which encodes a dual-function 2′-O-

methyltransferase and hemolysin (129), not only results in resistance to capreomycin and viomycin but also abolishes the translation of known epitopes in this protein (35, 80, 95, 104, 105, 123).

Yet, the effect on resistance of many more changes remains to be explored. Perhaps the most remarkable genetic difference to be investigated is the 350-kb duplication identified recently in some Beijing/W strains (44). This duplication encompasses almost 8% of all genes (*Rv3128c-Rv3427c*) and is potentially relevant for eight antibiotics. First, it was recently shown that promoter-up mutations of *whiB7* (*whmC*, *Rv3197A*), which encodes a transcriptional activator, result in cross-resistance to kanamycin and streptomycin (173). It seems plausible that a gene duplication might have a similar effect and lead to an increase in transcription. However, preliminary results indicated that the MICs for both drugs were not significantly different (44). Second, the overexpression of *mfpA* (*Rv3361c*) results in FQ resistance (53, 70). Again, preliminary results have fortunately ruled out this possibility for the Beijing strains with the duplication (44). The same applies to the duplication of the racemase Alr (Rv3423c), the target of cycloserine (17, 44). Third, MICs for isoxyl, an antibiotic which was used in the 1960s for the treatment of TB and has since then emerged as a potential candidate for the treatment of drug-resistant TB, might be affected due to the duplication of DesA3 (Rv3229c), a stearoyl coenzyme A $\Delta^9$-desaturase required for oleic acid biosynthesis that is inhibited by this drug (46, 88, 116, 125, 163). Fourth and last, the effect of the duplication of *fbiA* (*Rv3261*) and *fbiB* (*Rv3262*) on susceptibility to the three nitroimidazoles (metronidazole, delamanid, and PA-824), which are currently in phase 2 and 3 clinical trials, has to be clarified (33, 65). Given that both genes are required in activation, rather than encoding the target, of both drugs, intrinsic resistance is not expected. Moreover, the duplication includes a number of additional genes which in different lineages are essential *in vitro* or are required for optimal growth during infection or survival within macrophages (93, 133, 140, 141). Consequently, these genes might not be good candidates for future antibiotics.

**Experimental design.** For practical reasons, new drugs are usually tested against a small number of strains during the pretrial phase (77). Similarly, clinical trials sample only a limited portion of the global MTBC diversity, thereby potentially missing intrinsic drug resistance (55). To address this possibility for BTZ043, a benzothiazinone (99, 154), a promising drug candidate, Pasca et al. (120) found that 240 clinical isolates from three hospitals in Europe and one in Russia were equally susceptible to the antibiotic. Although this study was a step in the right direction, it sampled only a part of the MTBC diversity. Similarly, the phase 2a trials of PA-824 and delamanid included only *M. tuberculosis* as the sole MTBC representative, and therefore, it remains unclear whether *M. canettii* can be treated with these antibiotics (40, 41).

To control for this factor in a systematic manner, well-characterized reference collections encompassing representatives of all major MTBC genotypes and species must be tested *in vitro* and *in vivo*, given that even the differences among the various stocks of the H37Rv laboratory strains can be significant (38, 89). The identity of the clinical isolates in these collections should be confirmed using multiple deletions or single-nucleotide polymorphisms rather than traditional techniques to avoid the pitfalls of homoplasy (1, 36, 52, 62, 84, 109, 135, 142). It was such an approach that allowed us to raise the possibility of intrinsic drug resistance of *M. canettii* against PA-824 (55).

The same concerns apply to studies seeking to elucidate the resistance mechanism of current drugs. In this context, phenotypic susceptibility results must be analyzed with care, as these results can be flawed (14, 81, 121, 122, 156, 158). Particularly when genotypic and phenotypic results are inconsistent for PZA, the experiment should be repeated, with different techniques if possible, to avoid false associations (31, 106). The most prominent example is probably the T47A mutation in *pncA*, which was first reported for the aforementioned Beijing/W family in a large outbreak in New York City, New York, in the 1990s (19, 150) and leads to increased MICs close to the resistance breakpoint, resulting in poor reproducibility of susceptibility testing (45, 56, 82, 110, 149, 152, 167).

Furthermore, associations between mutations and drug resistance should also be confirmed by alternative techniques. These include the generation of isogenic mutants via allelic exchange (67, 137, 161), *in vitro* selection experiments (127), crystal structures (124) and, where possible, the direct measurement of the activity of the enzyme in question (16, 94, 118, 126). Moreover, association studies of mutations with the treatment outcome of patients are required (169).

In practice, the strains available from TDR Tuberculosis Strain Bank and the genomes from TBDB should provide a valuable resource to control for intrinsic drug resistance (50, 63, 64, 162). However, the discovery of a major duplication in some Beijing strains highlights the importance of establishing additional collections, especially with strains from countries such as India that are highly diverse but have been given less attention than other lineages (30, 44). The same limitations apply to our understanding of the population structure of *M. africanum* WA1 (WA1 stands for West African 1) strains. RD711 has been regarded traditionally as a marker for this lineage (62), as reaffirmed recently in a review by de Jong et al. (39). However, five *M. africanum* WA1 strains that lack this deletion have been described in a different collection (76, 159). Consequently, RD711 appears merely to define a sublineage of *M. africanum* WA1, rather than the lineage as a whole, possibly leading to an underestimate of the prevalence of *M. africanum* WA1 (76, 159). Moreover, a further subclassification of this phylogenetic lineage into WA1a and WA1b is warranted but is not reflected by those strains sequenced so far for TBDB (55, 63).

## CONCLUDING REMARKS

The shortcomings of some past studies should act as cautionary tales to ensure that in the future, sequence data are used to its fullest extent. This is especially important given the increasing impact of whole-genome sequencing on the study of TB (115). The MTBC population structure should be assessed rigorously when evaluating new antibiotics and diagnostic methods to control for the possibility of intrinsic drug resistance and false-positive resistance results. Ideally, this should occur before as well as throughout clinical trials (113). Last, it is imperative that researchers are committed to the dissemination of their results via the available databases and structure their data accordingly. Similarly, drug companies should make their antibiotics available for testing by researchers.

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