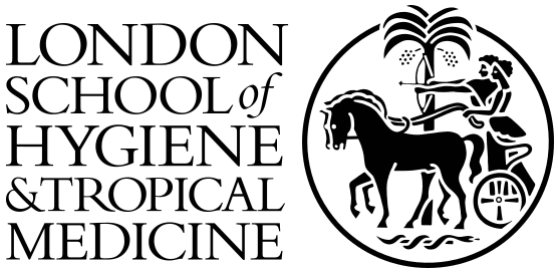

Short-form paper

Revised interpretation of the Hain Lifescience GenoType MTBC to differentiate *Mycobacterium canettii* and members of the *M. tuberculosis* complex

Running title: Hain GenoType MTBC

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Abstract

Using 894 phylogenetically diverse genomes of the *Mycobacterium tuberculosis* complex (MTBC), we simulated *in silico* the ability of the Hain Lifescience GenoType MTBC to differentiate the causative agents of tuberculosis. We propose a revised interpretation of this assay to reflect its strengths (e.g. it can distinguish some strains of *M. canettii* and variants of *M. bovis* that are not intrinsically resistant to pyrazinamide) and limitations (e.g. *M. orygis* cannot be differentiated from *M. africanum*).

Manuscript

The IVD-CE marked Hain Lifescience GenoType MTBC is the oldest and likely most widely used commercial assay to differentiate the causative agents of tuberculosis (TB) (1). Strictly speaking, these comprise *Mycobacterium canettii*, which is almost exclusively limited to the Horn of Africa, on the one hand and several species/ecotypes of the *M. tuberculosis* complex (MTBC) on the other, although most researchers and guidelines consider *M. canettii* to be part of the MTBC (2, 3). Clinically, the early identification of the precise causative agent of TB is important because it can serve as a marker for intrinsic resistance or may inform the attribution of the source of infection (e.g. in case of *M. bovis*, intrinsic resistance to pyrazinamide can usually be ruled in and a human source for the infection is unlikely (4)).

Throughout the past decade, the interpretation of the GenoType MTBC, but not its design, has been revised to reflect changes in our understanding of the causative agents of TB (1, 3, 5). More recently, several new animal species/ecotypes have been discovered, which prompted us to investigate to what extent these could be differentiated with the Hain assay using a collection of 894 diverse genomes representing *M. canettii* and major phylogenetic groups of MTBC (Figure S1 and Table S1) (6). This was possible because Hain Lifescience has filed a European patent (EP1490518B1) for its assay, which relies on a 23 rRNA probe to identify *M. canettii*/MTBC as a whole, whereas mutations in *gyrB* and the RD1^{BCG} deletion differentiate individual species/ecotypes (Figures S1 and

S2 and Table S2 (7)). Specifically, we typed all 894 genomes *in silico* for the SNP and deletion markers from the patent (Supplemental methods).

The current package insert of the GenoType MTBC lists seven binding patterns for *M. canettii* or MTBC isolates (patterns 2-8 in Figure 1 and Table S1). In 2010, however, Fabre et al. demonstrated experimentally that a minority of *M. canettii* strains yield a novel pattern, which does not feature in the package insert (8). Our simulation confirmed these results. Specifically, two of the *M. canettii* strains with the unusual experimental pattern (i.e. Percy157 and Percy525) from Fabre et al., for which genomes were available and, therefore, could be included in our study, also yielded the novel pattern *in silico* (pattern 1 in Figure 1 and Table S1) (8). The remaining five *M. canettii* genomes from Fabre et al. (i.e. Percy22, Percy32, Percy50, Percy79, and Percy301) could not be differentiated from *M. tuberculosis in silico*, which was in agreement with the experimental findings (pattern 2 in Figure 1 and Table S1) (8). Given the highly recombinogenic nature of *M. canettii*, it is not surprising that this species yields two different patterns (9, 10). All representatives of this species, including the two strains that gave the new binding pattern experimentally and *in silico*, have been found to be resistant to pyrazinamide when tested with the BACTEC MGIT 960 at 100 µg/ml, the only critical concentration recognized by the Clinical and Laboratory Standards Institute and the World Health Organization (8, 11-16). Although it is unclear whether this phenotype is due to a single mechanism shared by all strains (e.g. *rpsA* T5A) or whether different mutations are responsible in different strains (e.g. *panD* M117T or a series of *pncA* mutations (Table S3)), we recommend that the package insert is updated to include this novel pattern as "*M. canettii* (intrinsically resistant to pyrazinamide)" (13, 17-19).

Moreover, our findings suggest the following changes for the remaining seven binding patterns (Figure 1 and S1 and Table S1). First, pattern 3, currently used to differentiate *M. africanum* from the rest of the MTBC and *M. canettii*, has to be revised since our analysis showed this pattern cannot distinguish *M. africanum* from *M. orygis*, *M. pinnipedii*, nor the clade A1 ecotypes (i.e. *M. mungi*, *M. suricattae*, the chimpanzee bacillus, and the dassie bacillus) (6, 20, 21). Second, for the

sake of clarity we would separate *M. bovis* and *M. caprae* as they belong to two independent phylogenetic groups and are usually recognised as separate species/ecotypes (3). By contrast, BCG was derived from a *M. bovis* strain and is best described as *M. bovis* BCG to emphasize its intrinsic resistance to pyrazinamide (4). Finally, the current package insert features two binding patterns for "*M. bovis* subsp. *caprae*", of which one is described to occur in only 5% of cases of *M. caprae* (5). Our collection featured seven genomes consistent with this rarer pattern. However, the seven genomes did not group together phylogenetically (Figure S1). Three of the strains were isolated in 2009 from primates that were placed in quarantine upon entering the United States (22, 23). Their genomes grouped together with the *M. caprae* genomes on the phylogeny and shared the *lepA* V424V marker for this species (24). By contrast, the other four genomes were more closely related to *M. bovis*, but lacked the *pncA* H57D mutation that is responsible for intrinsic pyrazinamide resistance in this species (7, 13). Three of these isolates were isolated from humans in Malawi and the fourth from an antelope in Germany. For the latter sample, we knew the spoligotyping pattern, which we used to query the *M. bovis* spoligotype database (25). The spoligotype for the antelope isolate from 1996 (SB1898) appears to be very rare as only one identical representative was found, which was submitted from Spain in 2009. Thus, it is unclear whether these four strains represent a novel ecotype or species, but, because they are phylogenetically closer to *M. bovis* than *M. caprae*, we recommend that pattern 6 should be reported as "*M. caprae*/*M. bovis* (not intrinsically resistant to pyrazinamide)".

M. orygis has been isolated from many different animals and there is a growing recognition that it is a zoonotic source of human TB (26). Our *in silico* typing approach confirmed that *M. orygis* could be specifically identified by a mutation at codon 329 of *gyrB* (7). Since this marker is contained within the *gyrB* amplicon, we suggest it could be added to the Hain assay, as this would avoid misclassifications, such as in Rahim *et al*. in which cattle from Bangladesh were erroneously reported to have been infected with *M. africanum* instead of *M. orygis* (27).

The findings in this study are important for two reasons. First, most of our proposed changes can be implemented easily by updating the package insert of the Hain Lifescience GenoType MTBC (5). More broadly, given that whole-genome sequencing is now increasingly being used as a routine diagnostic tool, it would be possible to implement our *in silico* surveillance approach in real time to automatically flag unusual isolates for experimental follow-up. In fact, if clinical sequencing providers, such as Public Health England in the United Kingdom, were to offer this as a professional service, it could generate much-needed revenue to reduce the cost of sequencing to public health systems and, therefore, the tax payer, whilst enabling commercial companies to conduct post-marketing surveillance for genotypic assays comprehensively and cost-effectively – a win-win situation for all parties.

Figure 1. Proposed interpretation of binding patterns of Hain Lifescience GenoType MTBC.

Eight binding patterns are possible for samples that contain a single strain of MTBC or *M. canettii*. The first binding pattern is not currently included in the package insert of the GenoType MTBC (5, 8). With the exception of pattern 4 for *M. microti*, the interpretations of the remaining patterns were updated to include information about intrinsic resistance to antibiotics and/or to reflect the improved understanding of the phylogenetic diversity amongst the causative agents of TB. More information about clade A1 can be found elsewhere (6). Additional binding patterns are possible for samples that are negative, contain other bacteria, or when the assay was not carried out correctly (in these cases one or more of the conjugate control (CC), universal control (UC), or MTBC bands would be negative (5)).

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Conflicts of interest

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Clade A1: *M. mungi*, *M. suricattae*, Dassie bacillus, Chimpanzee bacillus