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**Title :** The paradoxes of *Mycobacterium tuberculosis* molecular evolution and consequences for the inference of tuberculosis emergence date

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## **Author contributions**

Rima Zein-Eddine, Fiona Hak, Adrien Le Meur , Ccharlotte Genestet, Gaëtan Senelle performed data curation and formal analyses of data at the basis of this work. Guislaine Refrégier created the model. Christophe Guyeux, Oana Dumitrescu, Christophe Sola, Guislaine Refrégier participated in the funding acquisition. Guislaine Refrégier wrote the initial draft and performed the final editing. Christophe Guyeux, Adrien Le Meur, Christophe Sola participated in editing the manuscript. Adrien Le Meur created the figure.

# 1 **The paradoxes of *Mycobacterium tuberculosis* molecular evolution and consequences for** 2 **the inference of tuberculosis emergence date**

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20 REVIEW

## 21 **Abstract**

22 The date of tuberculosis emergence has been the subject of such a long debate that it almost  
23 resembles a Graal quest. New studies joining archaeological efforts with sequencing methods raise  
24 high hopes for solving the question. Yet, inferring the emergence of the disease based on samples  
25 from epidemics that occurred relatively long after this emergence requires a molecular clock for  
26 the *Mycobacterium tuberculosis* complex. Several clocks inferred from different types of loci  
27 and/or different samples, using both sound reasoning and reliable data, are actually very different,  
28 which we call the paradoxes of *M. tuberculosis* molecular evolution.

29 After having presented these paradoxes, we will remind the limits of the molecular clocks used in  
30 the different studies such as assumption of homogeneous substitution rate. We will then review  
31 recent results that shed new light on the characteristics of *M. tuberculosis* molecular evolution:  
32 traces of diverse selection pressures, the impact of host dynamics, etc. We provide some ideas on  
33 what to do next to get nearer to the Graal. Among them, the collection of additional remains from  
34 ancient tuberculosis seems still essential.

35 **Keywords:** mutation rate, substitution rate, positive selection, purifying selection, pathogen.

36 **Highlights:** Tuberculosis is still the first deadly infectious disease caused by a bacterium. It has  
37 imposed a high death toll on humankind for millennia, but what key events underlie this success  
38 is still not clear. More specifically, the date of its wide success, which we will refer to as tuberculosis  
39 emergence, is still highly debated, between 70,000 to 6,000 years Before Present. We discuss the  
40 relative reliability and the different assumptions at the basis of these datings and provide a model  
41 of all parameters affecting pathogens molecular clocks. We argue that remains with ancient *M.*  
42 *tuberculosis* are still needed to shed light on tuberculosis main diversification events.

43

44

## 45 **1. Introduction**

46 Identifying the role of microorganisms in infectious diseases was key to the increase in life expectancies  
47 that has characterized the XX<sup>th</sup> century [1, 2]). Reconstructing the history of a specific disease helps  
48 understanding future threats and thus avoid them or limit their impact. Phylogenetics and  
49 phylogenomics infer the common ancestry and evolutionary history of organisms based on DNA  
50 sequences, either from a selection of genes or from complete genomes [3]. It has proven to be a very  
51 powerful tool to disentangle the origin of recent epidemics [4, 5]. On a wider timescale, the picture is  
52 however less clear as exemplified by the debates on the precise origin of 1918 flu [6-8].

53 Tuberculosis is both an ancient disease and the presently most deadly bacteria-related infectious  
54 disease. Works of art and bones from Predynastic Egypt and the Middle Kingdom testify to the  
55 presence of the disease [9-11]. Tedious work from archaeologists continue to fuel the evidence of past  
56 traces of tuberculosis [12, 13], suggesting a high prevalence of the disease already 10,000 y Before  
57 Present (BP): Baker et al. identified osteological evidence highly suggestive of tuberculosis in Syria  
58 around 10,000 y Before Present in 10% of the remains [12]. Yet the road seems still long to make a  
59 clear history of tuberculosis disease using only osteological evidences of the disease. Interdisciplinary  
60 studies mixing archaeology and phylogenomics emerge to try to provide definite answers on the global  
61 history of tuberculosis [14], potentially reducing the interest in further archaeological explorations  
62 with or without ancient DNA. We however want here to draw attention to the hypotheses underlying  
63 these inferences. Here, we first present the different dates of tuberculosis emergence according to  
64 various phylogenetical inferences, and we clarify the hypotheses of the underlying models. Second, we  
65 will present compelling data that show how much these hypotheses are violated in the bacterium  
66 evolution. We will focus on *M. tuberculosis* complex *sensu stricto* (hereafter referred to as MTBC) that  
67 includes all human-adapted tuberculosis lineages L1-L9 and the animal strains. We exclude *M. canettii*

68 despite its high sequence identity with the rest of the lineages, due to its poor contribution to the  
69 present human epidemics and widely different timescales [15].

70

## 71 **2. Various Inferences regarding the age of tuberculosis and the relying evidences**

72 Most phylogenomics studies infer divergence dates using a molecular clock in a Bayesian framework.  
73 They assume that DNA evolves at the same pace along the whole phylogeny. This molecular clock is  
74 usually assessed either from recent heterochronous sampling of recent epidemics, or from dated  
75 fossils [16]. Pathogens codiverging with hosts (and vice-versa) benefit from a third possibility: relying  
76 on the partner's phylogeny [17]. We detail below the different types of studies that tried to infer the  
77 date of tuberculosis emergence.

78 The emergence date of tuberculosis was first estimated using synonymous mutations from four *M.*  
79 *tuberculosis* genes and the application of a mutation rate derived from *E. coli* and *S. typhimurium* [18].  
80 The use of other bacteria's rate seems reasonable as they evolve drug resistance in similar proportions  
81 in lab experiments (David 1971). This study suggested that *M. tuberculosis* clonal expansion dates back  
82 to 15,300-20,400 years Before Present (BP). However, a closer look at the data shows that this  
83 inference derived from only four synonymous mutations from a sample of 31 isolates. In addition,  
84 these rare mutations occurred in a heterogeneous subselection of 4 genes with very diverse functions  
85 (housekeeping genes targeted by drugs, antigens) [18]. In addition, it is difficult to reconstruct how  
86 well the selected isolates represented the global diversity. They might have corresponded only to L4  
87 as it was the most widely sampled lineage at this time, or to modern MTBC (including L2 and potentially  
88 L3 lineages), or to the whole diversity including L1 and the L5-L6-animal strains group. If the proposed  
89 dating stands for L4 emergence, this would mean that global MTBC expansion would be twice older.  
90 Kapur et al. dating might therefore only roughly reflect the actual date of tuberculosis emergence.

91 The second type of estimation derived from the mutation rate of minisatellites data [19]. This approach  
92 has the advantage of circumventing potential biases due to low single nucleotide mutation rate. The  
93 diversity of MIRU profiles leads to date the MTBC Most Recent Common Ancestor (MRCA) to 40,000  
94 years BP. This dating corresponds to a coalescence time of main L4 sublineages such as LAM (L4.3) of  
95 7,000 years BP [19].

96 Another type of estimations is based on a molecular clock derived from *M. tuberculosis* ancient DNA.  
97 The corresponding studies took both synonymous and non-synonymous mutations without  
98 distinction. This choice is motivated by the quest of precision in the mutation rate estimation. It is  
99 supported by the clonal evolution of MTBC and an average ratio between synonymous and non-

100 synonymous mutations (dN/dS) close to 1 ( $\sim 0.7$ ) [20]. Such a ratio suggests that no selection occurred.  
101 Yet, another possibility is the combination of positive/diversifying selection (dN/dS higher than 1) to  
102 negative/purifying selection (dN/dS below 1) (see further discussion below). The first ancient genome  
103 was sequenced from a Peruvian mummy infected by *M. pinnipedii* [21]. The inferred mutation rate was  
104 estimated at  $\sim 5 \cdot 10^{-8}$  subst/site/year. Applying this rate to the global phylogeny induces to date MTBC  
105 MRCA at  $\sim 6,000$  y before present [21]. Interestingly, new estimations based on L4 MTBC isolated from  
106 Hungarian mummies come out with similar estimations (Brynildsrud et al, 2017; Sabin et al, 2020).

107 At last, another study inferring a molecular clock used the codivergence of *M. tuberculosis* with its  
108 human hosts. The peculiar history and very restricted expansion of L4.2.2 sublineage allowed Refrégier  
109 al (2016) to support the same molecular clock as Bos et al., dating MTBC MRCA to  $\sim 6,000$  BP. Of note,  
110 the authors do not argue in favour of this hypothesis as discussed below (Refrégier et al, 2016).  
111 Interestingly, this article pinpoints the fact that the molecular clock inferred from heterochronous  
112 sampling of recent epidemics is around twice faster as found by several independent studies at around  
113  $10^{-7}$  subst/site/year [22-24].

114 Apart from the studies using molecular clocks, Comas et al. dated MTBC clonal expansion using the  
115 human phylogeny. Based on some similarities between the phylogenetic trees and geographic patterns  
116 of *M. tuberculosis* and modern *H. sapiens* involved in the Out-of-Africa migration event, emergence  
117 was reevaluated to  $\sim 70,000$  y before Present, questioning the Neolithic nature of tuberculosis  
118 emergence (Comas et al, 2013). The corresponding global mutation rate deriving from this inference  
119 is  $\sim 3 \cdot 10^{-9}$  subst/site/year which is around 1 order of magnitude less than that of *Y. pestis* and 3 orders  
120 less than *S. pneumoniae*, and also 1 order of magnitude less than that of studies based on ancient DNA  
121 [25].

122 Altogether, the estimated dates of tuberculosis emergence span a very wide time range, going from  
123 6,000 to 70,000 years BP. Younger inferences were obtained using both synonymous and non-  
124 synonymous mutations of ancient MTBC isolates, whereas inferences based solely on synonymous  
125 mutations or minisatellites data or codivergence with the host come up with elder age of emergence.  
126 We will next examine deeper why inferences based on both synonymous and non-synonymous  
127 mutations might be erroneous.

128

129

130 **3. Assumptions at the basis of molecular dating**

131 Errors of several orders of magnitude can happen when inferring dates with sequences that do not  
132 evolve with constant substitution rates *i.e.* with constant molecular clock [26]. Let us remind here that  
133 substitution rate differs from the mutation rate by two characteristics: substitution looks at  
134 accumulated modifications in the population after selection action, and substitution rate is usually  
135 measured per time (per year) instead of per generation. Altogether, both the bacterial multiplication  
136 rate, whether intra or interpatient, and selection are evolutionary forces controlling the impact of  
137 mutation rate on the molecular clock (Grey ovals, Fig. 1). We will now detail how substitution rate (and  
138 thus molecular clock) can vary among genes and how selection and bacterial multiplication may  
139 influence it.

140 Many nucleotides in a genome do not evolve with the same substitution rate. This is obviously true  
141 between genes when one has undergone horizontal gene transfer and thus changed cellular  
142 environment that controls mutation rate, nucleotide concentrations, for all types of mutations [27].  
143 The divergence between single nucleotide substitution rate is largely inflated between synonymous  
144 and non-synonymous mutations as a consequence of selection: positive/diversifying selection is  
145 characterized by the fixation of non-synonymous mutations conferring a selective advantage to the  
146 organism, and negative/purifying selection corresponds to the elimination of non-synonymous  
147 mutations that reduce organism fitness. This is actually the reason why the non-  
148 synonymous/synonymous substitution ratio, the dN/dS, is indicative of different selection pressures  
149 between genes [20]. Several studies have in fact documented the existence of positive/diversifying  
150 selection pressures on MTBC genes. This includes genes targeted by drugs but also other genes  
151 involved in host-pathogen interaction [28-31]. Recent exploration of trajectories of dN/dS showed  
152 strikingly different dN/dS trajectories supporting a major past selection pressure on epitopes, and a  
153 more recent pressure on toxin-antitoxin systems [32]. The selection on MTBC epitopes may relate to  
154 the reciprocal selection of MTBC onto human genome [33]. Experimental evolution selecting for  
155 biofilm formation also showed a high propensity for adaptation of MTBC, especially active upon  
156 regulatory proteins, as well as an important role for epistatic interactions [34] (gene/sequences types  
157 in red in Fig. 1). Moreover, mutation rate also depends on DNA repair systems and on oxidative damage  
158 as recently shown by Moreno-Molina *et al.* [35] and Genestet *et al.*[31]. Last, duplicated genes such as  
159 PE-PPE undergo occasional intragenomic recombination, which should also be taken into account if  
160 their mutations are taken into account [36]. Obviously thus, the pace at which non-synonymous  
161 mutations accumulate in different types of genes varies due to selection, potentially heterogeneous  
162 mutation rate. This advocates against the common practice of merging synonymous and non-  
163 synonymous mutations when building a molecular clock [37]. The current studies using ancient DNA  
164 all suffer from this limitation.

165 We showed that intra-host selection acting upon non-synonymous mutations impacts MTBC molecular  
166 clock. We want to underline in addition that these selections are likely to have varied through time  
167 and space. Indeed, intra-host selection depends on patient's drug treatment, but also on the  
168 characteristics of the immune system (efficiency, adaptation). These characteristic first depend on the  
169 host genetics [33]. They obviously also highly depend on other diseases. For instance, HIV-status is a  
170 strong predictor of tuberculosis disease and tuberculosis regained strength with HIV dissemination  
171 around the 80ies [38], but other diseases such as diabetes are also drivers of tuberculosis [39]. And of  
172 course recently, COVID has shown to have indirectly impacted tuberculosis transmission by reducing  
173 the diagnostic measures efficiency [40]. Other diseases may have greatly impacted the ability of the  
174 immune system to fight tuberculosis over time and space, either because they exhaust the body,  
175 reduce immune efficiency or because they may provide cross-immunity such as leprosy [41](Fig. 1).  
176 Other key parameters for MTBC opportunities to disperse are: demography, which is impacted by  
177 social parameters such as wars, economic wealth, etc and impacts migration; ecological changes linked  
178 to present global warming or past cooling (the Little Ice Age) [42]

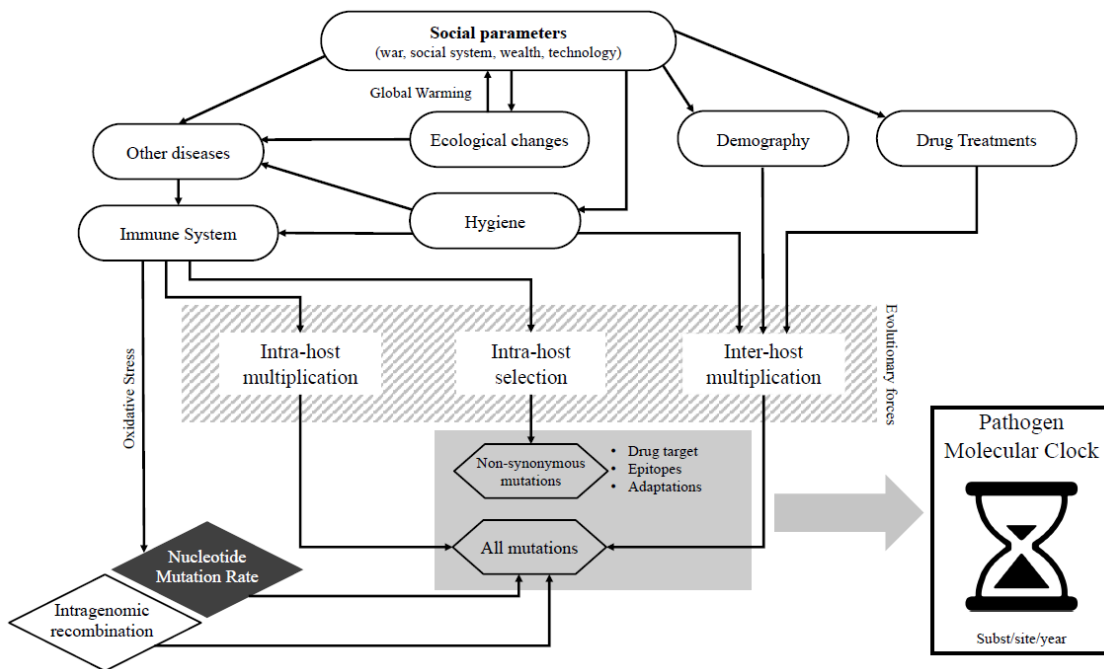
179 A last important point to bear in mind when considering molecular clock *versus* mutation rate is that  
180 mutation rate is usually measured as a number of nucleotide changes per generation. This is because  
181 most mutations are linked to replication infidelity and DNA repair [43-45]. But generations can have  
182 different length time. Molecular clock may thus largely depend on the pace at which MTBC multiplies  
183 inside the host (intrahost multiplication). Ford et al questioned this point by comparing the  
184 accumulation of MTBC mutations in infected macaques carrying either latent or active tuberculosis  
185 [46]. They could not detect a difference between the two categories but the power they had to do so  
186 was limited due to the low number of replicates and the relatively short time of investigation, which  
187 allowed to accumulate 0 to 3 substitutions in 91 to 507 days depending on the animal survival. In  
188 addition, the study did not discriminate between synonymous and non-synonymous mutations.

189

190 We thus showed that non-synonymous mutations may have diverse substitution rates *i.e.* molecular  
191 clocks, with one key evolutionary force at play being selection. This should prompt us to focus on  
192 synonymous mutations. The synonymous mutation rate was explored shortly after the sequencing of  
193 the second human MTBC strain, CDC1551 [47]. Interestingly, this rate appeared to vary highly between  
194 genes. Recent deep exploration of diversity also suggests that various mutation rates prevail along the  
195 genome of bacteria [48]. Synonymous mutations could thus be an incomplete solution to the problem  
196 of identifying a reliable molecular clock.

197





199

200

**Figure 1 – Parameters affecting molecular pathogens' clock, a model built for MTBC**

201

We distinguish two types of targets: all mutations or only non-synonymous mutations (grey hexagons).

202

The main drivers of the difference between mutation and the molecular clock are intra and inter-host

203

multiplication and intra-host selection (evolutionary forces rectangle). Parameters affecting intra and

204

inter-host processes are indicated above. They include community parameters (social characteristics,

205

demography), global disease status (hygiene, other diseases prevalence) which impacts directly or

206

indirectly the immune status of the population and the opportunities for transmission *i.e.* inter-host

207

multiplication.

208

209

#### 4. Conclusions

210

211

Since the model of epidemiologic transition, pathogens are thought to have shaped the history of

212

humankind (Caldwell 2001). *Mycobacterium tuberculosis*, with its incredibly low mutation rate, pushes

213

researchers working on phylogenetic inferences to merge data that clearly have a very diverse

214

dynamics due to selection, but also due to demography, the impact of other diseases and mutation

215

rate. Altogether, we have shown that no safe dating can be presently provided for tuberculosis

216

emergence. This holds for major diversification events of the agent of tuberculosis such as Lineage 4

217

expansion. As a reminder, we may speak for major diversification event for this lineage as 9 sublineages

218

have been consistently considered at an equivalent position in the taxonomy (L4.1 to L4.9). This major

219 diversification event could be due to Roman Empire expansion or Christian Crusades. In addition to the  
220 emergence date of tuberculosis, this 1,000 y uncertainty on this major diversification event in MTBC is  
221 also far from being solved.

222 Now that the reliability of sequencing is improving, that powerful methods exist and that the amount  
223 of data is exploding, turning towards synonymous mutations and implementing in rich phylodynamics  
224 model may help revisiting the possible history of tuberculosis. Yet, all assumptions underlying  
225 phylodynamics, including host demography, MTBC mutation rate, selection, etc. should be kept in  
226 mind to avoid exaggerated inferences. Independent confirmations are needed. They may come from  
227 other diseases, human history, etc. Altogether, archaeological research, with more bones to gnaw on,  
228 will definitively be of great help to strengthen or lower support of phylogenetic conclusions.

229

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240 Rima Zein-Eddine, Fiona Hak, Adrien Le Meur , Ccharlotte Genestet, Gaëtan Senelle performed data  
241 curation and formal analyses of data at the basis of this work. Guislaine Refrégier created the model.  
242 Christophe Guyeux, Oana Dumitrescu, Christophe Sola, Guislaine Refrégier participated in the funding  
243 acquisition. Guislaine Refrégier wrote the initial draft and performed the final editing. Christophe  
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245 Adrien Le Meur created the figure.

246

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