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Evolutionary changes in the genome of *Mycobacterium tuberculosis* and the human genome from 9000 years BP until modern times

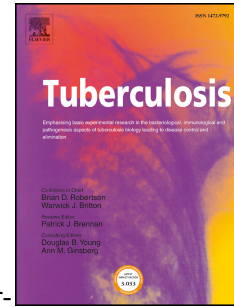
Dr Mark Spigelman, Helen D. Donoghue, Ziad Abdeeb, Suheir Ereقات, Issa Sarie, Charles, L. Greenblatt, Ildikó Pap, Ildikó Szikossy, Israel Hershkovitz, Gila Kahila Bar-Gal, Carney Matheson

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1 **Evolutionary changes in the genome of *Mycobacterium tuberculosis* and the human**
2 **genome from 9000 years BP until modern times**

3
4 Mark Spigelman^{1,2,3++}, Helen D Donoghue^{1,4+}, Ziad Abdeeb⁵, Suheir Ereqat⁵, Issa Sarie⁵, Charles,
5 L. Greenblatt³, Ildikó Pap⁶, Ildikó Szikossy⁶, Israel HersHKovitz², Gila Kahila Bar-Gal⁷, Carney
6 Matheson⁸

7
8 ¹Centre for Clinical Microbiology, Division of Infection & Immunity, University College London,
9 London, UK

10 ²Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel Aviv University, Tel
11 Aviv, Israel

12 ³Kuvin Center for the Study of Infectious & Tropical Diseases and Ancient DNA, Hadassah Medical
13 School, The Hebrew University, Jerusalem, Israel

14 ⁴Centre for the History of Medicine, Division of Biosciences, University College London, London,
15 UK

16 ⁵Al-Quds Nutrition and Health Research Institute, Faculty of Medicine, Al-Quds University, Abu-
17 Deis, P.O. Box 201760, West Bank, Palestine

18 ⁶Department of Anthropology, Hungarian Natural Science Museum, Budapest, Hungary

19 ⁷Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel

20 ⁸Paleo-DNA Laboratory, Departments of Anthropology and Biology, Lakehead University, Thunder
21 Bay, Ontario, Canada

22 + Equal First authors

23 Email addresses:

24 spigelman@btinternet.com; h.donoghue@ucl.ac.uk; zabdeen13@gmail.com;

25 sereqat@med.alquds.edu; isarie63@gmail.com; charlesg@ekmd.huji.ac.il; papildi@hotmail.com;

26 anatom2@post.tau.ac.il; gila.kahila@mail.huji.ac.il; cmatheso@lakehead.ca;

27

28 *Corresponding author: Dr Mark Spigelman, 2 Clarence Terrace, Regents Park, London NW1 4RD
29 Telephone: 44(0)2072249095 e-mail: spigelman@btinternet.com

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32 **Summary**

33 The demonstration of *Mycobacterium tuberculosis* DNA in ancient skeletons gives researchers
34 an insight into its evolution. Findings of the last two decades sketched the biological relationships
35 between the various species of tubercle bacilli, the time scale involved, their possible origin and
36 dispersal. This paper includes the available evidence and on-going research. In the submerged
37 Eastern Mediterranean Neolithic village of Atlit Yam (9000 BP), a human lineage of *M.*
38 *tuberculosis*, defined by the TbD1 deletion in its genome, was demonstrated. An infected infant at
39 the site provides an example of active tuberculosis in a human with a naïve immune system. Over
40 4000 years later tuberculosis was found in Jericho. Urbanization increases population density
41 encouraging *M. tuberculosis*/human co-evolution. As susceptible humans die of tuberculosis,
42 survivors develop genetic resistance to disease. Thus in 18th century Hungarian mummies from
43 Vác, 65% were positive for tuberculosis yet a 95-year-old woman had clearly survived a childhood
44 Ghon lesion.

45 Whole genome studies are in progress, to detect changes over the millennia both in bacterial
46 virulence and also host susceptibility/resistance genes that determine the NRAMP protein and
47 Killer Cell Immunoglobulin-like Receptors (KIRs). This paper surveys present evidence and
48 includes initial findings.

49

50 **Key words:** Ancient DNA; evolution; KIR historical specimens; *Mycobacterium tuberculosis*;
51 *SLC11A1* gene; Solute Carrier family genes

52

53 1. Introduction

54 Microbial infections played a key role in shaping life on earth and have been a major selector for
55 the evolution of all present species. Evidence exists that demonstrate infectious diseases were
56 already present in our remote ancestors.^{1,2} Considering the impact of *Mycobacterium tuberculosis*
57 (MTB), in all probability it has had a greater influence on the genetic selection of the *Homo sapiens*
58 population than any other infectious agent.

59 The molecular identification of human pathogens in ancient human remains has recently opened
60 new scientific fields that provide considerable insight into the history and evolution of host,
61 pathogen and their interaction. This allows us to track changes in the ancestral tubercle bacillus as
62 it became more and more exposed to the internal environment and immune system of its human
63 host. Conversely, it is possible to track changes in the genes of the human population that confer
64 resistance or susceptibility to disease over time.

65 TB is related to population density,³ transmitted from human to human living in close contact.
66 However, the origin of the disease, the earliest hosts of MTB and its evolution remain unclear. The
67 evolution of the bacteria cannot be considered in isolation. It is important to realise how TB has
68 influenced the human development over the millennia, particularly our resistance/susceptibility
69 genes. MTB experienced an evolutionary bottleneck when it became an obligate pathogen and has
70 a clonal relationship with different human lineages.⁴ Subsequent co-evolution has resulted in the
71 majority of TB infections being latent. In past eras of low human population density, MTB adapted
72 over time in response to host-adaptive changes and *vice versa*. This process, which can be
73 defined as mutualism, is a biological interaction between individuals of two different species where
74 both individuals derive a fitness benefit. As the host becomes more resistant, strains better able to
75 colonise the resistant host will predominate, thus starting off another cycle. More virulent MTB
76 strains will attack their human host, killing the most susceptible and leaving the more resistant as
77 survivors. However, when human populations were sparse, this could break the chain of
78 transmission of the pathogen. The development of antibiotics has shortened the mutualistic cycle
79 significantly, but the combination of HIV co-infection, antimicrobial therapy and increased global
80 human population density is leading to the emergence of some MTB strains that are both more
81 transmissible but also more virulent.⁵

82

83 2. The impact of palaeomicrobiological investigations of archaeological human material

84 2.1 Questions to be addressed

85 Archaeologists should seek to correlate research questions with historical events. For example,
86 did past invasions introduce new pathogens, or more virulent strains of pathogens into susceptible
87 populations? Thousands of indigenous peoples in the Americas died from exposure to European
88 strains of MTB, measles and smallpox.⁶ Another possible scenario is that invaders may have

89 brought new pathogens with them on return to their place of origin. A good example of this is the
90 introduction by European colonialists of venereal syphilis from South America.

91 A further question one has to ask is what was the genetic status of *Homo erectus* or
92 predecessor species regarding the underlying genetic basis of host resistance and susceptibility to
93 tuberculosis. Did ancestral hominids have the precursors of modern host susceptibility/resistance
94 genes or were these acquired late? Is the 'Out of Africa' theory of the origin of human TB proposed
95 by Gutierrez et al⁷ capable of being verified by a study of human remains, or will these show that
96 TB developed in several areas and that this is the explanation for the variability of the organism in
97 different geographical areas?

98 The majority of TB patients in the world today never progress to active disease. The World
99 Health Organisation estimates that approximately one-third of the global population is infected but
100 only 10% of immunocompetent progress to active disease during their lifetime.⁸ Our current
101 immunity may be the result of Darwinian selection only, or may depend upon whether particular
102 genes are switched on or off – a mechanism that can result in rapid adaptation. It must be
103 remembered that other non-genetic factors influence human susceptibility to infection such as
104 dietary deficiencies, stress and trauma.⁹ Long-term climatic changes have an impact on vegetation
105 and agriculture¹⁰ whereas local variations in climate may influence transmission of MTB by
106 infectious aerosols. Temperature changes will determine whether humans spend more time in the
107 open air or enclosed spaces, for example.

108 2.2 Significant findings

109 With the first reported finding of MTB DNA in ancient skeletons based on amplification of a small
110 (123 bp) DNA target that was specific for the MTB-complex¹¹ a new era of research into microbial
111 pathogen evolution became possible. In addition to skeletal remains, calcified and mummified
112 tissues also proved to be good sources of MTB ancient DNA (aDNA)^{Mic 12}. Our knowledge was
113 enhanced with the finding of MTB in a 17000-year-old Pleistocene bison from Natural Trap Cave,
114 Wyoming.¹³ Spoligotyping revealed that the Pleistocene bison lesions contained aDNA from the
115 *M. tuberculosis* complex, possibly MTB or *Mycobacterium africanum*, but distinct from
116 *Mycobacterium bovis*. The consensus bison spoligotyping pattern was compared with the
117 combined database collated by the National Institute of Public Health and Environment (RIVM),
118 Utrecht, The Netherlands and the Veterinary Science Division, Department of Agriculture and
119 Rural Development, Belfast, N. Ireland. No exact matches were found on the database. However,
120 in a computer analysis comparing a library of defined species, the highest similarity was from *M.*
121 *africanum* (82.3%), then *M. tuberculosis* - MTB (76.6%), with *M. bovis* having only 72.7% similarity.

122 The original aDNA findings in the Pleistocene bison were confirmed ten years later by finding
123 species-specific MTB cell wall lipid biomarkers.¹⁴ We have used this method of independent
124 confirmation of our MTB aDNA findings since 1998¹⁵ because lipid analysis uses methods based
125 on the direct detection of femtogram quantities of target molecules, with no need for any

126 amplification. This is a more rigorous method of independent confirmation than sending part of the
127 specimen to another laboratory for analysis.

128

129 The Pleistocene bison contained MTB-complex aDNA but the particular lineage has not yet been
130 identified. The earliest known human MTB was detected and characterised in samples from the
131 submerged Neolithic site of Atlit Yam, a 9000-year-old settlement submerged in the sea off the
132 coast of Haifa in Israel.¹⁶ The findings were confirmed by lipid analysis and the preservation was
133 sufficiently good that it was possible to confirm that the MTB had experienced the TbD1 deletion,
134 found only in human lineages. This is of particular significance as this was a Pre-Pottery site with
135 the earliest evidence of animal domestication in the Levant.

136 We were fortunate as a group to secure samples from two large collections of natural mummies
137 – one from the 18th to early 19th century from Vác, Hungary and the second from early Christian
138 Nubia dated to 500-1400 CE at Kulubnarti in Northern Sudan. The importance of these collections
139 was that the DNA preservation is well above average as in both locations the bodies were naturally
140 mummified with no chemicals used. Indeed, the Kulubnarti material demonstrated co-infections of
141 MTB with *Leishmania* spp, and using the Hungarian material, it was possible to determine the main
142 MTB genetic lineages and perform molecular typing.¹⁷ Our work on the Pleistocene bison together
143 with the Hungarian Vác mummies was cited and assisted in developing the hypothesis proposed in
144 an excellent early paper on MTB evolution by Brosch *et al.*¹⁸

145 To fill the time gap between the Nubian Kulubnarti mummies and the Atlit Yam skeletal remains,
146 specimens from the Bronze Age township of Jericho have been examined. Initially bones from
147 early excavations from the 1950's were studied, in a collaboration involving colleagues from
148 Munich, Al Quds University and Jerusalem. Unfortunately, although these specimens yielded
149 possible MTB aDNA, this could not be confirmed independently. Material from the excavation of
150 Ain es-Sultan refugee camp area, where ancient Jericho (Tel es-sultan) ~4000 BC has yielded
151 MTB aDNA, which has been confirmed by lipid analysis. The infecting pathogen was from a TbD1-
152 deleted MTB lineage. At present a metagenomic study on this specimen is in progress at
153 McMasters University.

154 The Hungarian mummy project based on 265 bodies, most wholly or partially mummified, from a
155 sealed crypt, is unique as there is contemporaneous archival information about many of the
156 individuals. This enabled the identification of some family groups and also made it possible to
157 study TB in a large population from a fixed period and single location.¹² It was possible to type the
158 MTB aDNA within a family and to show that each member was infected with a slightly different
159 strain.¹⁷ Recently, lung tissue from the older daughter in this family group has been shown by non-
160 enriched whole genome sequencing, to contain two different strains of MTB, with apparent
161 sequential deletions, that appear to be ancestral to a modern outbreak strain in Germany.¹⁹ In
162 contrast, MTB aDNA was found in a calcified lymph node from the mediastinum of a 95-year-old
163 mummy, where initially all tissues were negative but an X-ray showed the calcified node. This

164 demonstrates that in this well-preserved group of mummies it is possible to identify cases of active
165 and of latent infection.²⁰ It was these finding that led to our interest in host susceptibility and
166 resistance genes.

167 3. Host susceptibility and resistance

168 In addition to the retrieval of the pathogen DNA, a pilot study is investigating the genes believed
169 to be responsible for susceptibility or resistance to the disease to determine if these genes differ in
170 any way between those who were infected and those who appear immune. The study of the host
171 susceptibility/resistance factors in the mummies and their descendants will give information on the
172 role of host genetics in the pathogenesis of infectious disease, and contribute to the design of new
173 therapeutic strategies. The study involves two host targets, the *SLC11A1* gene (previously named
174 *NRAMP*) and Killer Cell Immunoglobulin-like Receptor genes (KIRs). The plan is to seek any
175 correlation between presence and absence of tuberculosis, with the presence of certain alleles in
176 these resistance genes. Already, our initial research on material from Hungarian and Sudanese
177 mummies has revealed some interesting genetic patterns.

178 KIRs are members of a group of regulatory molecules found on subsets of lymphoid cells, first
179 identified by their ability to impart some specificity on natural killer (NK) cytotoxicity. The *KIR* locus,
180 which maps to chromosome 19q(13.4) within the 1 Mb *Leukocyte Receptor Complex (LRC)*,
181 contains a family of polymorphic and highly homologous genes. KIR genes are tandemly arrayed
182 over a physical distance of about 150 Kb, displaying the remarkable feature of gene content
183 variation among haplotypes. The KIR molecules recognize the Human Leukocyte Antigen (HLA)
184 class I molecules, which are encoded by genes within the Major Histocompatibility Complex (MHC)
185 chromosome 6.²¹ Interactions between KIR isotypes that inhibit natural killer (NK) cell activity and
186 specific HLA class I allotypes protect healthy cells from spontaneous destruction by NK cell
187 mediated cytotoxicity. Other KIR isotypes stimulate the activity of NK cells demonstrating that KIR
188 play a significant role in the control of the innate immune response. Recent studies report a greater
189 repertoire of inhibitory KIR genes among TB patients than controls²² and a direct association of
190 certain KIR and HLA-C genes²³ with resistance to pulmonary TB. Different KIR genes have a role
191 in inhibiting or increasing susceptibility towards TB and the complimentary MHC ligands need to be
192 tested for the functional relevance of the associated genes.²⁴

193 A contemporaneous study of the *SLC11A1* gene is in progress at Lake Head University. The
194 promoter region has been studied in modern populations and been linked to a number of infections
195 and autoimmune diseases, caused by *M. tuberculosis*, *M. bovis*, *Mycobacterium leprae*,
196 *Mycobacterium lepraemurium*, *Salmonella typhimurium* and *Leishmania donovani*. The
197 identification of sequence variants has prompted research into the evolution of nuclear genes,
198 inheritance patterns, selective pressures, and changes in both allele frequencies and disease
199 linkages over time. Linkage studies can help ascertain the resistance and susceptibility factors of
200 diseases and can assist modern medicine by providing a better understanding of the infectious

201 processes themselves.^{25,26} The Allele 2 variation of the promoter region was found to be present in
202 every patient infected with tuberculosis, indicating that this level of allelic expression may well be
203 related to the resistance or susceptibility of an individual to infectious diseases. Allele 3 seems to
204 produce the highest level of *SLC11A1* expression, which confers a resistance to microbial infection
205 to the individual, but increases susceptibility to autoimmune diseases. Conversely, Allele 2
206 produces the lowest level of *SLC11A1* expression, conferring individual resistance to autoimmune
207 diseases, but also a greater susceptibility to microbial infections. It is possible that this
208 contradiction in allelic expressions may have resulted from inverse selective pressures, serving to
209 maintain both alleles within the human population. Allelic variants of *SLC11A1* have been identified
210 as risk factors for paediatric TB.²⁷ Other studies of host susceptibility and resistance genes have
211 indicated that different human lineages may exhibit differing susceptibilities to TB infection.²⁸ There
212 is also limited evidence that genetic expression may vary according to sex and age.²⁹ An intriguing
213 finding is that human genetic susceptibility varies according to the differing clinical forms of TB.³⁰

214 Limited data are now available on amplified aDNA (Tables 1 and 2) from 18 individuals from 18th
215 century Vác, Hungary and early Christian Nubia (Table 2).²⁵ The promoter microsatellite
216 polymorphisms of the *SLC11A1* gene look encouraging as patterns are emerging (Table 2). Both
217 the KIR and *SLC11A1* studies are on-going and results will be disclosed on completion.

218

219 **4. Conclusions**

220 This study seeks to show the progress that has been achieved in paleomicrobiological research
221 over the last two decades and indicates its contribution to the study of human pathogen co-
222 evolution. Understanding the adaptations that the host and the pathogen have undergone through
223 history, together with the resistance/susceptibility adaptations, may shed light on future interactions
224 of humans with MTB. It is highly important to understand the process of mutualism – the biological
225 interaction between individuals of two different species, where each derives a fitness benefit – in
226 the present era of personalized medicine.

227

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231 pioneer the early mycolic acid work on the bison bone.

232

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234 Not required

235

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240

241 **Author contributions**

242 MS conceived the original aDNA studies and HD, GKB-G, SE and CM performed experiments.

243 HD, GKBG, CG and CM analysed ancient DNA data. MS, IH, ZA, IP and IS provided data and

244 supplied specimens. Is S is head archaeologist of the Jericho excavations MS wrote the first and

245 final drafts, HD prepared revised drafts and all authors approved the final version.

246

247 **Competing interests**

248 None declared

249

250 **Table legends**

251 **Table 1.** The SLC11A1 gene promoter microsatellite primer set

252 ~~**Table 2.** The repeats identifying each SLC11A1 allele~~

253 **Table 2.** Genotypes of the *SLC11A1* gene found in Hungarian and Nubian Mummies.

254

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Table 1. The SLC11A1 gene promoter microsatellite primer set

Primer	Sequence
C1	ACT CGC ATT AGG CCA ACG AG
C2(FAM)* (6FAM)	TTC TGT GCC TCC CAA GTT AGC

346
347
348

- The antisense primer marked with florescence dye
- The primer was published by Bellamy *et al.*, 1998²⁵

349

Table 2. Genotypes of the *SLC11A1* gene found in Hungarian and Nubian Mammies

Sample	Allele	Genotype	<i>M. tuberculosis</i> infection
1	2/3	Heterozygote	Positive chest
2	2/3	Heterozygote	Positive chest
3	2	Homozygote	Positive chest and abdomen
4	2	Homozygote	Positive chest and abdomen
5	2	Homozygote	Positive chest and abdomen
6	2/3	Heterozygote	Positive right lung and abdomen
7	2	Homozygote	Positive chest
8	2/3	Heterozygote	Positive chest, abdomen and plura
9	3	Homozygote	Positive left chest, left lung, left pelvis and abdominal wall
10	#		Positive soft tissue, pleura, rib
11	3	Homozygote	Not Infected
12	3	Homozygote	Not Infected
13	3	Homozygote	Not Infected
14	2/4	Heterozygote	Unknown
15	3/4	Heterozygote	Unknown

350

Mutation present – to be confirmed

351

Allele 1(201bp) = A(CA)₅TG(CA)₅TG(CA)₁₁C; Allele 2(199bp) = A(CA)₅TG(CA)₅TG(CA)₁₀C;

352

Allele 3 (197bp) = A(CA)₅TG(CA)₅TG(CA)₉C; Allele 4(199bp) = A(CA)₅TG(CA)₉C

353

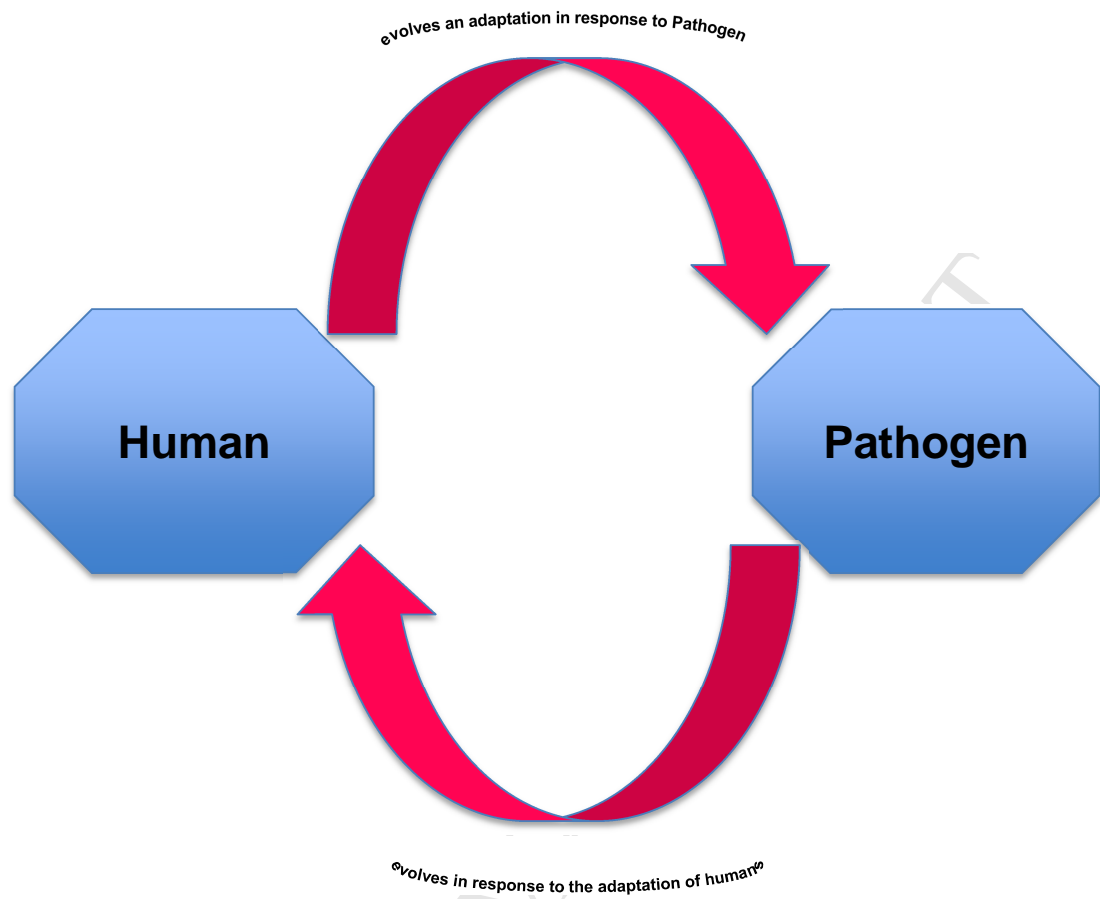


Figure 1: Co-evolution between human and pathogens
Evolution of one species in response to characteristics of another