Tuberculosis origin

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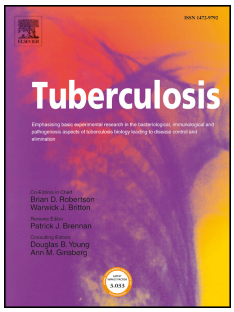

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Tuberculosis origin: the Neolithic scenario

Israel Hershkovitz[a†], Helen D. Donoghue[b†], David E. Minnikin[c], Hila May[a], Oona Y-C. Lee[c], Michal Feldman[a], Ehud Galili[d], Mark Spigelman[a,e], Bruce M. Rothschild[f], Gila Kahila Bar-Gal[g]

[a]Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel-Aviv University, Tel-Aviv, Israel

[b]Centres for Clinical Microbiology and the History of Medicine, University College London, London, UK

[c]Institute of Microbiology and Infection, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, UK

[d]Israel Antiquities Authority, Jerusalem, and Zinman Institute of Archaeology, Haifa University, Israel

[e]Kuvin Center for the Study of Infectious and Tropical Diseases, Hebrew University-Hadassah Medical School, Jerusalem, Israel

[f]Biodiversity Institute and Departments of Anthropology and Geology, University of Kansas, Lawrence KS 66045, USA,

[g] The Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel

Email addresses:

anatom2@post.tau.ac.il; h.donoghue@ucl.ac.uk; d.e.minnikin@bham.ac.uk; hilamay@gmail.com; leeoy@bham.ac.uk; michalfe@gmail.com; udi@israntique.org.ilspigelman@btinternet.com; bmr@ku.edu; gila.kahila@mail.huji.ac.il;

***Corresponding author**: Professor Israel Hershkovitz, Department of Anatomy and Anthropology, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv 69978, Israel
Tel: 972-3-6409495, Fax: 972-3-6408287, e-mail: anatom2@post.tau.ac.il

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30 † These authors share senior authorship

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Summary

This paper follows the dramatic changes in scientific research during the last 20 years regarding the relationship between the *Mycobacterium tuberculosis* complex and its hosts – bovids and/or humans. Once the *M. tuberculosis* and *M. bovis* genomes were sequenced, it became obvious that the old story of *M bovis* evolving into the human pathogen should be reversed, as *M. tuberculosis* is more ancestral than *M. bovis*. Nevertheless, the timescale and geographical origin remained an enigma.

In the current study human and cattle bone samples were examined for evidence of tuberculosis from the site of Atlit-Yam in the Eastern Mediterranean, dating from 9250-8160 (calibrated) years ago. Strict precautions were used to prevent contamination in the DNA analysis, and independent centers used to confirm authenticity of findings. DNA from five *M tuberculosis* genetic loci was detected and had characteristics consistent with extant genetic lineages. High performance liquid chromatography was used as an independent method of verification and it directly detected mycolic acid lipid biomarkers, specific for the *M. tuberculosis* complex. These, together with pathological changes detected in some of the bones, confirm the presence of the disease in the Levantine populations during the Pre-pottery Neolithic C period, more than 8,000 years ago.

Key words:

Ancient DNA; Neolithic; origin of tuberculosis; paleopathology;

1. Introduction

Human tuberculosis (TB) persists as a global epidemic with disproportionate effects on low-income populations. Modern genetic data supported by the archaeological evidence indicate that the *Mycobacterium tuberculosis* complex (MTBC) may have co-existed with humans for at least 15,000 years since the Neolithic.[1,2] The disease reached near-epidemic proportions in the rapidly urbanizing and industrializing societies of Europe and North America in the 18th and 19th centuries.[3]

Despite extensive research over period of more than 100 years, the timing, cause and geographical origin of TB in humans is still under debate. Until the end of the previous century, it was commonly believed that animals, especially bovine, transmitted the ancestral *Mycobacterium* to humans – divergent evolution. As infection with tuberculosis spreads in two major ways, by the respiratory route directly from another infected person (e.g., *M. tuberculosis*) or by the gastrointestinal route mainly by drinking milk infected or milk products with the bovine tubercle bacillus (*Mycobacterium bovis*),[4] the notion that newly domesticated cattle, sheep or goats in the Eastern Mediterranean region during the agricultural revolution (ca. 8,300-5,500 BC), is the source of the disease in humans, became common.[5] Even when this idea of zoonotic transmission of *M. bovis* to Early Neolithic farmers was widespread, we pointed out the following criticisms:[6] 1) It was unclear when and how the *M. bovis* spread among domesticated cattle; 2) The oldest known human skeletal evidence of TB from the Mediterranean region, other then those of the Pre-Pottery Neolithic (PPN) C site of Ain Gaazal,[7] were all roughly dated to a much later period – the fourth millennium BC or later.[6,8] Also, this later date was reflected by pathological and molecular findings reported for Egyptian mummies (some dating back to the XXIst Dynasty) and skeletons (the oldest dated to 3300 BC) that were reported to have tuberculosis pathology;[9,10] 3) The spread of TB from cattle to human occurs largely by drinking infected milk, yet milk consumption did not start until the "Secondary Products Revolution" in the fifth-fourth millennium BC.[11] Furthermore, according to Keusch et al.,[12] by two years of age virtually all Neolithic children

were lactase-deficient, i.e., they lacked the ability to metabolize milk. Biological tolerance of adult populations to bovine milk and milk products only began in the Neolithic period.[13] In this case only infants would have consumed milk and thus contracted bovine TB; 4) When considering TB infection, herd size is of greater relevance than human population size.[14] With few exceptions, the harsh unpredictable Mediterranean environmental conditions, including large arid zones and hilly topography, are suitable for goats but not for raising large herds of cattle. Based on the above arguments, at that time we rejected the 'domesticated-bovine-hypothesis' for TB and concluded that the appearance of human TB was probably associated with the beginning of urbanization in the Fertile Crescent region during the fifth-fourth millennium BC, during the Chalcolithic-Early Bronze Age c. 3.500 BC.

In the last decade of the 20th century it was shown that the identification of *M. tuberculosis* DNA in ancient bones is possible.[15] Less than 10 years later, the plethora of molecular studies of the MTBC – both ancient and modern – showed that there is no direct evolutionary relationship between *M. bovis* and *M. tuberculosis* but these were divergent evolutionary lineages, with *M. tuberculosis* being more ancestral.[16] Genetic analysis of the pathogen from a Pleistocene bison bone (17,000 years) showing tubercular-like infection indicated greater similarity to *Mycobacterium tuberculosis* and *M. africanum* rather than to *M. bovis*.[17] Furthermore, the overwhelming majority of studies that have examined MTB complex aDNA by spoligotyping[17,18] demonstrate that the organisms are not *M. bovis*. The sole exception to date is the detection of *M. bovis* in a group of Iron Age semi-nomadic pastoralists from Siberia dating from the 4th century BC to 4th century AD.[19] Further genetic studies, based on coalescence analysis have even suggested the possibility of human to bovine transmission of TB, whereby the most ancestral human MTB may have infected livestock and through a parallel evolutionary process established tuberculosis in cattle (*M. bovis*) and goats (*Mycobacterium caprae*).[20] Nonetheless, this and other DNA studies adhered to two basic notions: the first that the origin of the disease in humans is within the Fertile Crescent; the second that the transition from human to domesticated animal hosts is linked to the development of agriculture some 13,000 years ago.[1,16,20]

As TB is still one of the leading infectious diseases worldwide, with an estimated 1.4 million deaths in 2011[21] the questions of the time and conditions surrounding the emergence of *M. tuberculosis* are important. The primary aim of the current research was to present both the published and later findings from the Pre-pottery Neolithic C site of Atlit-Yam in an attempt to answer those questions.

1.1 Background on the site and its inhabitants

Atlit-Yam is one of the major submerged sites discovered and studied during the 1980s and 1990s. Hershkovitz et al.(2008)[22] gives the full bibliography that describes the site, its structures and occupation. The site is located 300 to 500 m offshore and 8-12 m below sea level in the North Bay of Atlit, 10 km south of Haifa (34º56' E, 32º42.5' N). Stone foundations of several rectangular structures, paved floors, long straight walls, hearths, round megalithic structures, storage and production installations, and water wells have been discovered, all embedded in dark clay. The structures and installations are sparsely scattered over the site with wide-open spaces between them. The site was dated to the end of the Pre-Pottery Neolithic period (PPNC). Radiocarbon dates on charcoal and waterlogged plant remains range from 8180 to 7250 years BP (9250-8160 BP calibrated). The rich, well-preserved finds of Atlit-Yam include botanical and faunal remains, stone, flint and bone tool assemblages, and human bones. The site is one of the earliest prehistoric Mediterranean fishing villages ever excavated. Human bones were revealed in ninety-one different locations at the site, of which forty-six were recognized as graves dug into the clay. Most burials (70%) were located in specific areas, adjacent to walls or installations. No grave showed evidence of stone construction, or surface marking. Burials were mainly primary, containing mostly (75%) single interments, situated around the rectangular structures and rarely in within them. In some cases, grave goods were added to the graves. Secondary burials were rare. Grave goods were found in fifteen burials.

The health status of the Atlit-Yam population was relatively good, as attested by the life span of the population. The pathologies identified are mainly associated with infectious diseases, such as ear infections due to diving (auditory exostosis), spondylolysis due to

intensive rowing activities, anemia due to the marshy environment and probably tuberculosis following cattle domestication.[22] Dental wear associated with weaving fishing nets and dental diseases was also identified.

2. Materials and Methods

The remains of 64 individuals from Atlit-Yam were examined for TB lesions. All human bones are housed at Tel Aviv University. Identification of TB was based on both morphological (macro and micro) and molecular analyses. All cases with bony lesions indicative of TB were sampled for MTBC aDNA, either directly from the lesion itself or from a bony area with a rich blood supply.

2.1. Morphological analysis

Osseous criteria for TB: As many infectious diseases tend to produce similar bone changes, osseous criteria alone are not sufficient to reach a definite diagnosis of TB.

2.1.1. Osseous criteria for the presence of TB in infants, children and adolescents

All skeletons were inspected for the following gross osseous changes, all of which are indicative for potential presence of tuberculosis in sub adult and children: (a) convoluted engraving on the inner aspect of the cranial bones, a phenomenon termed '*Serpens Endocrania Symmetrica*' (SES);[23] (b) periosteal reactive bone of tubular bones characterized by destruction of the cortex and formation of an expanded shell of periosteal reactive bone;[24] (c) growth deficit and/or intrauterine growth retardation; (d) deformity of long bones (due to foci destroying a growth plate);[25] (e) presence of multiple lesions throughout the skeleton.

2.1.2. Osseous criteria for the presence of TB in adults

Osseous changes, indicative for potential presence of tuberculosis in adults are: (a) presence of SES;[23] (b) presence of hypertrophic osteoarthropathy;[23] (c) local destruction and cavitation in cancellous bone; (d) local changes in the epiphyses of long bones, mainly undermining and resorptive grooving along the line of the synovial attachments; (e) bony ankylosis;[25] (f)

cavitation and or collapse (wedge-shape vertebra) of vertebral body; (g) destruction of hip and/or knee joints; (h) proliferative bone reaction on the ribs.[24]

2.2. Histological sections

Fragments of affected bones were used for histological sections. The bones were cleaned with water (ultrasonic bath) and immersed in alcohol (90%). The bones were then embedded in methylmethacrylate. The tissue block was cut into 150 μm thick sections using a slow-speed diamond saw (Isomet: Buehler). The sections were ground and polished (Phoenix Beta: Buehler) to a final thickness of 15-30 μm and surface stained with H&E.

2.3. Molecular analysis-Human bones

All molecular work was conducted in dedicated aDNA laboratories, taking strict precautions against contamination. DNA was extracted from two Atlit-Yam samples, an adult female and an infant, using guanidine thiocyanate lysis buffer and silica-based purification. The extracted DNA was amplified via PCR and characterized using deletion analysis, spoligotyping and sequencing.[22] The presence or absence of the *M. tuberculosis*-specific deletion (TbD1) was determined by targeted PCR[22] and by spoligotyping pattern.[18] Negative PCR findings are not proof of absence, due to the damage and breakdown of aDNA over time and the localization of pathogen molecular markers within the host. However, a positive result does confirm TB, especially in combination with typical TB-associated morphology, histology and biochemistry.

2.4. Molecular analysis-cattle bones

Samples were taken from five cattle bones with no visible pathological changes and were processed as described above.

2.5. Lipid biomarkers

Extraction, derivatisation and high performance liquid chromatography (HPLC) analysis of mycobacterial cell wall mycolic acids was carried out on samples from both the infant and adult. For examination of lipid biomarkers an established protocol was carried out.[22]

3. Results

3.1. Paleopathology

The skeletal remains of well-preserved individuals from the site of Atlit-Yam were examined for lesions consistent with a possible diagnosis of tuberculosis. Among the 64 specimens studied, three specimens showed bone pathology suggestive of tuberculosis: a – an adult woman buried together with an infant (Fig. 1); these skeletons were later sampled for molecular examination (see below); b – an adult male. The infant, though small in size, was estimated (on a very fragmented skeleton), to be less then 1 year old based on crown development and long bone dimensions. The infant shows SES on the inner aspect of the cranial bones (Fig. 2c) and hypertrophic osteoarthropathy (HOA) lesions – a periosteal reaction of tubular bones characterised by the formation of an expanded shell of periosteal reactive bone on the long bones (Fig. 2a,b). Both lesions are indicative of tuberculosis. The woman, estimated to be around 25 years old based on teeth attrition, epiphyseal ring ankylosis and separated symphysis pubis, had a periosteal reaction affecting the distal diaphysis of one tibia, a bony change associated with HOA. The adult male exhibited a destruction of the anterior vertebral body of a thoracic vertebra (Fig. 3), known as Pott's disease and characteristic of TB.[26] No proliferative bone reaction was observed on the ribs. The histological analysis (Fig. 2b) clearly shows that the new bone formation rests on the original bone surface without infiltrating or destroying it. This indicates that the inflammatory process originates in the periosteum and/or the surrounding soft tissue, and not in the medullary cavity, as the consistency of the compact bone is undisturbed.

3.2. Molecular analysis

Ancient DNA analysis was conducted on the ribs and several limb bones of the woman and from the long bones of the infant. *Mycobacterium tuberculosis* (MTB) complex DNA was detected in the bones of both the woman and infant.[22] Multi-copy IS*6110* and IS*1081* amplicons were obtained and sequenced from the rib of the woman and the infant long bone. The results were replicated in two laboratories: at UCL an IS*6110* 123bp product from the woman (right rib) and a 92 bp nested IS*6110* product from the infant were obtained,

sequenced and found to be identical to contemporary *M. tuberculosis* sequences.[22] Additionally, a 104 bp sequence of the IS*1081* gene fragment obtained from the infant long bones was found to be identical to contemporary *M. tuberculosis* sequences.[22] The amplification and direct sequences of the IS*6110* gene region were successfully replicated at the Hebrew University of Jerusalem.

A TbD1 flanking PCR, based on a single site on the DNA strand, was successfully amplified for the infant sample and a complete DNA sequence for the 128bp amplicon with the outer primers was obtained[22] identical to that in the *M. tuberculosis* reference sequence. Nested PCR was also successful. Spoligotyping was successfully performed on both adult and infant specimens. There were several faint or dubious positives, and it was noted that spacers 33, 35, 37-43 were present and that spacers 2, 8, 21, 34 and 36 were either absent or only faintly positive on three or more occasions. However, a consensus spoligotype, based on any positive result, contained no missing spacer regions.

None of the 5 bones of cow analyzed for MTB aDNA yielded positive results.

3.3. Lipid biomarkers

Long-chain fatty acids were extracted as pentafluorobenzyl (PFB) esters, and fractions corresponding to PFB mycolates were obtained.[22] After treatment with pyrenebutyric acid (PBA) these fractions produced PBA-PFB mycolates, which, after reverse phase HPLC, gave profiles closely similar to standard *M. tuberculosis*.[22] Further normal and reverse phase HPLC gave detailed profiles for each sample, reinforcing the identity with *M. tuberculosis*.

4. Discussion

The current study sought answers to three basic questions regarding TB, namely when, where and how did *M. tuberculosis* first infect humans and cause disease? The morphological (macro and micro) examination, molecular investigations and lipid analysis have shown clearly that people at the Atlit-Yam site dated to the Pre-pottery Neolithic C period (6,200-5,500 BC) were infected by *M. tuberculosis* and that it was of a TbD1-deleted lineage. Further support for this finding is from a contemporaneous PPNC site of Ain Gaazal, in Jordan,

where vertebrae with osseous lesions typical of those caused by the TB bacillus were found.[7] Not surprisingly, TB appeared several hundred years later in the early Neolithic populations of central Europe, ca. 5400-4800 BC.[2] There are archaeological and genetic studies[27] indicating that early farmers from the Near East started migrating into Europe during the 6th millennium BC. Did they (or their cattle) carry the TB bacillus with them? The genetic evidence for Near-Eastern origins of European cattle[28] appears to be significant. Interestingly, sub-typing the aDNA of the bacillus found in the Neolithic European site of Derenburg revealed that, in contrast to modern European *M. tuberculosis* lineages, four MTBC strains still harbored the TbD1 region.[2] In the world today, such TbD1-intact strains are found mainly in the Far East and Pacific Rim. Also at Derenburg, one strain was found to belong to the RD9-deleted MTBC lineage that includes *M. africanum* and *M. bovis*.

Current data suggest that the MTBC is as old as 40,000 years.[20] However, it is notable that there are no documented cases of TB among human populations prior to the PPNC period. Of more than a thousand Natufian and Pre-Pottery Neolithic A and B skeletons excavated in the eastern Mediterranean region, none demonstrated osseous lesions associated, directly or indirectly, with TB. This contrasts with the evidence for the rise of infectious diseases among early farmers compared to their preceding hunter/gatherers.[29] Furthermore, there are global data to suggest that the transition to farming and animal husbandry not only subjected humans to new pathogens but also increased the risk of infectious diseases due to living conditions and diet.[30] It therefore seems the presence of cattle was pertinent for TB after all. Atlit-Yam is the only Neolithic site where cattle bones dominate the zooarcheologic record and where cattle were a major component of the diet. In the absence of detectable *M. bovis*, the cattle may be important by supporting a larger and denser human population, thus indirectly encouraging the conditions for the long-term maintenance and transmission of *M. tuberculosis*.

Finally we conclude that the infant had disseminated primary tuberculosis: the only DNA sequences for single copy sites were obtained from the infant material, which suggests a higher bacterial load during life. In infants less than a year old the present risk of developing

active disease on infection with *M. tuberculosis* is high due to the inadequacy of their immune system. The size of the infant's bones, and the extent of the bony changes, suggest a case of acquired neonatal tuberculosis, where an adult suffering from contagious pulmonary tuberculosis infects an infant shortly after birth. Childhood tuberculosis is closely linked with adult disease, and is usually a sentinel event in the community, demonstrating recent transmission. In the absence of any effective treatment, advanced tuberculosis carried significant mortality for both mother and child, so it is unsurprising for a presumed mother and child to succumb and be buried together. We believe that these are the earliest confirmed cases of the disease. Based on the spoligotype and TbD1 deletion, the genetic lineage resembles the Principal Genetic Group PPG1b. The relationship between genetic variants of *M. tuberculosis*, geographical location and the presentation of disease is poorly understood at present. Our study, we believe, provides a marker in real-time to indicate how this major pathogen has changed its relationship with its human host.

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Ethical approval

Not required

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Author contributions

I.H. and E.G. conducted the archaeological excavation; I.H., H.M. and M.F. assessed the palaeopathology; H.D.D. did the DNA molecular analysis in London and shares senior authorship with I.H.; D.E.M. and O.Y-C.L. analyzed lipid biomarkers; M.S. coordinated the project; B. M. R. was a leading researcher in the TB project; G.K.B. performed PCR and spoligotyping in Israel and initialized the second stage of the TB aDNA study at Atlit-Yam.

All authors discussed the results and commented on the manuscript.

Competing interests

The authors declare no conflict of interest

Figure legends

Figure 1: The mother and the child from Atlit Yam. Both were confirmed positive for TB by both morphological and aDNA analysis

Figure 2: Evidence for TB on the infant long bones: new bone formation on the shaft of a long bone - HOA (a), radiating appearance of the appositional bone on the infant long bone (b), grooves (SES) on the inner table of the calvaria (c).

Figure 3: Beveled thoracic vertebra of an adult person suggestive of TB

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