J. Biol. Res. 2005 n. 1 - Vol. LXXX - Rubbettino - Soveria Mannelli

Preservation and Identification of ancient M. tuberculosis complex DNA in Egyptian mummies

Albert R. Zink[1], Stephanie Köhler[1], Nasim Motamedi[1], Udo Reischl[2], Hans Wolf[2], Andreas G. Nerlich[1]

[1] Division of Paleopathology, Institute of Pathology, Academic-Teaching Hospital München-Bogenhausen, 81925 Munich, Germany,

[2] Institute of Medical Microbiology and Hygiene, University of Regensburg, 93053 Regensburg, Germany

KEY WORDS: Egyptian mummies, ancient DNA, tuberculosis

Abstract

For years we have investigated the presence and molecular evolution of tuberculosis in Pre Dynastic and Early Dynastic Egyptian mummy material from Abydos (c. 3500-2800 BC), Middle and New Kingdom until the Late Period in Thebes-West (c. 2050 – 500 BC). We have analysed 160 bone and soft tissue samples from different time periods and populations for the occurrence of M. tuberculosis complex DNA. All positive specimens were genetically characterised by spoligotyping and mutation analysis. Molecular analyses revealed excellent state of preservation of the specimens. Research showed a high incidence of M. tuberculosis during all time periods. We further detected specific MTB strain differences with M. africanum in some of the Middle Kingdom samples and "modern" M. tuberculosis strains in the New Kingdom to Late Period material. These results demonstrate that aDNA is excellently preserved in ancient Egyptian mummies allowing the reconstruction of occurrence, frequency, molecular evolution and spread of tuberculosis in Pharaonic populations.

Introduction

Tuberculosis is presently still one of the major causes of death world-wide. Approximately one third of the world population is infected with bacteria of the *Mycobacterium tuberculosis* complex (MTB), with about 8 million new cases annually, leading to 2 – 3 million deaths each year (WHO, 2000). There is increasing evidence that tuberculosis was present in a variety of historic populations of the New and Old World dating back several thousand years ago as shown either by morphology or molecular biology. Thereby, it has been demonstrated that positive molecular evidence for MTB has been obtained up to 3500 BC from human remains (Spigelman & Lemma, 1993; Salo *et al*., 1994; Nerlich et al., 1997; Crubezy *et al.*; 1998, Taylor et al., 1999, Fletcher et al., 2003, Zink *et al*., 2001, 2002a, 2003a, 2004, Donoghue et al., 2004). The as yet oldest molecular evidence for tuberculosis comes from a 17500 years old bison bone from the Natural Trap Cave in Wyoming (Rothschild et al., 2001). Taken together, there exists unequivocal evidence that tuberculosis has been present in the Old and New World long before regular contacts started at the end of the 15th century. Therefore, it seems to be surprising that the American-European contact has lead to a terrible burst of tuberculosis in the American Indians (Verano and Ubelaker, 1992). However, it has still not been possible to develop an evolutionary time-scale of TB evolution. Some authors estimate the origin of *the M. tuberculosis* complex dating back some 10 - 20,000 years (Sreevatsan et al., 1997). Most of the work on ancient mycobacterial DNA was done for the identification of TB in historic times. A few further studies have dealt with the frequency of this infectious disease in populations from different times and geographic regions (Faerman et al., Zink et al., 2001). The application of spoligo- and genotyping techniques in mycobacterial aDNA studies have paved the way to identify the mycobacterial evolution (Fletcher et al. 2003, Zink et al., 2003b). Most interestingly, none of these studies provided evidence for the presence of *M. bovis* in historic times, in contrast to the theory that *M. tuberculosis* evolved from *M. bovis* by cattle to human transmission during domestication (Cockburn 1963). These results were supported by Brosch et al. (2002), who proposed a new evolutionary scenario that starts with an ancient form *of M. tuberculosis* and developed into different *M. bovis* strains at his end.

In this work we present the results of our molecular analyses of 160 ancient Egyptian mummy samples. Thereby, we were able to identify different *M. tuberculosis* and *M. africanum* strains and investigate genetic variable regions and partial gene sequences in the TB positive tested mummy material. This allows a unique insight into the evolution of tuberculosis in ancient Egypt and underlines the excellent state of preservation of ancient DNA in Egyptian mummies.

Material

In this study we analyzed bone and soft tissue samples from 160 ancient Egyptian mummies and skeletons for the presence of *Mycobacterium tuberculosis* complex DNA. The material derived form the predynastic to early dynastic period, Abydos, Upper Egypt, (3500 – 2650 BC), a tomb shaft adjacent to the main tomb complex of TT196 (26th dynasty), exclusively used during the Middle Kingdom to Second Intermediate Period (c. 2050 – 1650 BC) and several tomb complexes, which were built in the Middle or New Kingdom and used until the Late Period (c. 1500 - 500 BC). The bone specimens were tested for the presence of the IS6110 and further characterised by spoligotyping and gene mutation analysis.

Methods

The pretreatment and DNA extraction of the ancient bone samples were performed as described previously (Zink et al., 2001). For the PCR amplification of mycobacterial DNA a primer pair for a 123bp fragment of the insertion sequence IS 6110 was used. The corresponding PCR-products were identified by gel electrophoresis due to the resulting size of the amplified fragment and by the banding pattern following restriction endonuclease digestion (Hae III). The nucleotide sequences were determined by automated sequencing. In parallel, a 202 bp fragment of the human b-actin gene was amplified, to test for the presence of amplifyable DNA and to assure that the PCR reaction was not inhibited. Spoligotyping was applied to the samples with a positive signal for the IS6110 region for further analysis. The resulting spoligotyping patterns were compared to the international database SpolDB3 (Filliol et al., 2002).
A detailed description of the PCR conditions and all further analysis steps has been published previously (Zink et al., 2001, Zink et al., 2003b).
In a further step, we performed a genotypic of the TB positive specimens. Therefore, we tested the specimens for the absence or presence of a *M. tuberculosis* specific deletion (TbD1) and the loss of variable region RD9 which is characteristic for *M. africanum, M. microti* and *M. bovis* strains. Additionally, partial gene sequences of *oxyR, pncA, mtp40, katG* and *gyrA* were analysed to detect different strains and genetic groups of *M. tuberculosis*.
During the whole working procedure several precautions were taken to avoid any contaminations following the widely accepted standards for working with ancient DNA (see Kolman & Tuross, 2000).

Results

In 73 (45,6%) of the 160 investigated samples a fragment of the human b-actin gene could be amplified. Moreover, 38 samples (23,8%) from all three different time periods were tested positive for the presence of mycobacterial DNA (Fig. 1). Most of the samples with morphological bone or soft tissue alterations typical for tuberculosis (Figs. 2 and 3) were

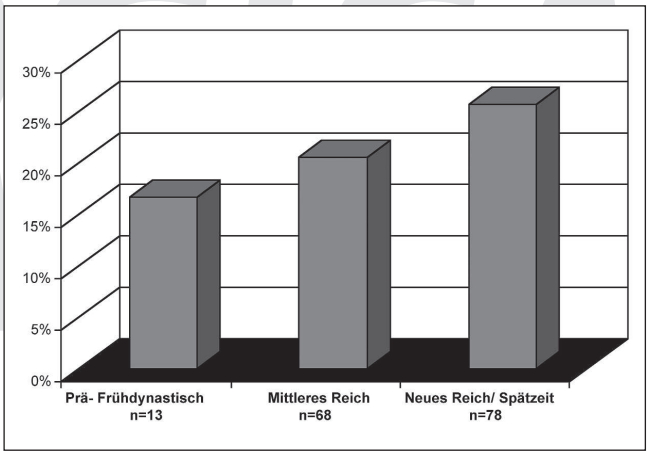

Fig. 1 - Tuberculosis frequency in three different time periods of ancient Egypt.

confirmed by the molecular analysis. Additionally, in samples with non-specific alterations and also specimens without any morphological lesions *M. tuberculosis* complex DNA could be amplified in 20% respectively 18% of the cases.
In this study 16 of the 38 positive samples provided a complete spoligotyping signature, which could be compared to the international spoigotyping database SpolDB3 (Fig. 4). 20 cases showed an incomplete, patchy hybridisation pattern and 2 cases showed no spoligotyping signature. 'Thereby, ubiquitous *M. tuberculosis* signatures could be detected in the New Kingdom to Late Period samples, which are clearly related to the modern *M. tuberculosis* type. One Early Dynastic sample showed hybridisation signals between position 33 to 36 and could therefore probably represent an ancestral *M. tuberculosis* strain. In concordance with our former studies two samples of the Middle Kingdom tomb were clearly characterised as *M. africanum* strains.

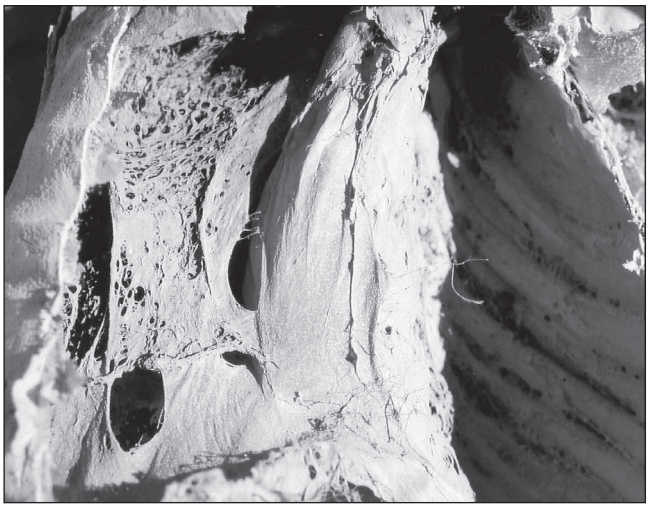

Fig. 2 - Torso of a male mummy (age<35years) presenting with extensive pleural adhesions of the right lung to the chest wall.

We found no evidence for *M. bovis* specific patterns, whereby samples with incomplete or without spoligotyping signatures cannot be further identified and attributed to a certain *M. tuberculosis* complex strain.
The *M. tuberculosis* specific deletion TbD1 was present in 8 samples from all different time periods and deleted in six

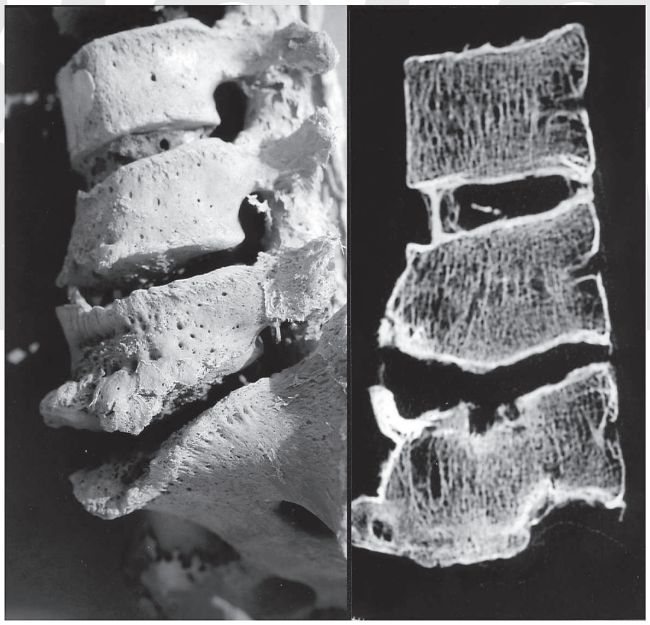

Fig. 3 - Lumbar spine of the mummy showing severe anterior destruction of two lumbar vertebral bodies (left) and on CT scans osteolytic destruction inside the vertebral body with osteosclerotic margins are seen (right).

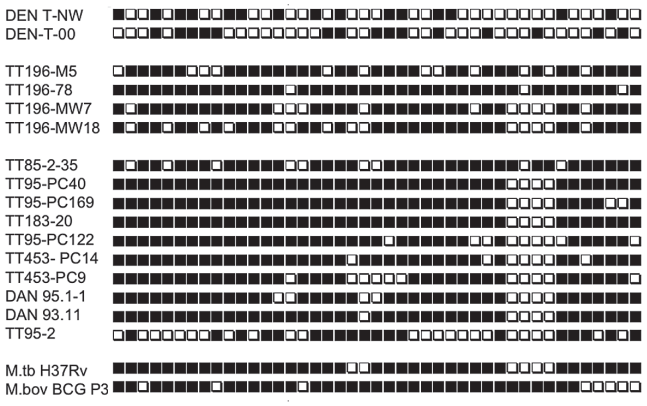

Fig. 4 - Spoligotyping results of ancient Egyptian mummy samples.

cases from the New Kingdom to Late Period specimens. This means that we have found evidence for ancestral *M. tuberculosis* strains in all different time periods, whereas «modern» *M. tuberculosis* strains were only detected in the New Kingdom to Late Period material. In six samples from all time periods sequences inside RD9 could be successfully amplified. In three cases of the Middle Kingdom RD9 was deleted, which is characteristic for *M. africanum* strains. This confirms the spoligotyping results and thereby substantiate the finding of *M. africanum* strains in the Middle Kingdom. The amplification and sequencing of *oxyR* and pncA in seven, respectively four, ancient Egyptian samples substantiated the presence of *M. tuberculosis* or *M. africanum* strains. Moreover, 4 samples of the New Kingdom to Late Period samples could be classified as genetic group 2 or 3 by katG mutations. The occurrence of TbD1 deletions and katG mutations in the New Kingdom to Late Period samples clearly show that «modern» *M. tuberculosis* strains existing at least 2500-3500 years in Egypt.

Discussion

The results of molecular study on ancient Egyptian mummies and skeletons provide evidence for an excellent preservation of ancient DNA in these samples. There was some controversy on the survival of DNA in specimens coming from a hot and dry climate, leading to the suggestion that no ancient DNA can be retrieved from Egyptian mummies (Marota et al., 2002). However, this assumption based on a study on papyrus DNA and overlooked some important facts enhancing the preservation of biomloecules in Egyptian mummies (Zink et al., 2003c). In the ongoing scientific debate, the high annual temperature in Egypt, among other reasons, was considered to be responsible for an almost complete DNA decay in Egyptian mummies (Gilbert et al., 2005). This argument was clearly disproved by the findings of one of Gilbert's co-authors. Poinar et al. (2003) found ancient DNA in material from the arid zone of the desert of Nevada, where the mean annual temperature is about the same as in Egypt. Again, other factors such as the mummification procedure and the lower temperature inside the rock tombs were disregarded and have led to an incorrect appraisal of the DNA survival condition in ancient Egypt (Zink and Nerlich, 2005). Moreover, in another study we could demonstrate a good biomoelcular preservation of the ancient Egyptian bone samples by a high collagen content and the amino acid profiles of the extracted protein, which agreed with the DNA preservation found in the studied cases (Zink et al, 2005).

There exists a lot of evidence that tuberculosis infections affected both human and animals several thousand years ago (for review see Donoghie et al., 2004). Up to now, no evidence has been found for the presence of *M. bovis* in ancient material ranging from bison bone material, ca. 17000 BP (Rothschild et al., 2001) to 18th century Hungarian mummies. Most interestingly, also the mycobacterial DNA isolated from the 17000 years old bison sample revealed a spoligotype more closely related to *M. africanum* then showing a typical *M. bovis* signature. In our study we also found no evidence for the occurrence of *M. bovis* in ancient Egypt. Taken together, these findings clearly support the new evolutionary scenario published by Brosch et al. (2002), that puts *M. bovis* at the end of the evolutionary lineage derived from a supposed *M. tuberculosis* complex precursor. Therefore, it becomes more and more unlikely that human tuberculosis evolved form bovine tuberculosis during domestication in Neolithic times as long believed (Cockburn, 1963). Further studies of ancient human and animal material offers the only possibility to find ancient *M. bovis* strains and thereby, clarify the evolutionary pathway of bovine tuberculosis.

A first attempt to genotype ancient mycobacterial findings was done by Fletcher et al. (2003) who could show that *M. tuberculosis* strains isolated from 18th century Hungarians belonged to genetic groups 2 and 3, representing modern *M. tuberculosis* strains. Therefore, it was suggested that the TbD1 deletion occurred before the 18th century in Europe and the dramatic increase of tuberculosis cases in Europe during the subsequent periods was mainly due to the spread of modern

M. tuberculosis strains (Brosch et al., 2002). However, little is known on the evolution and spread of the M. tuberculosis complex before recent times and how far back dates the origin of mycobacteria's diversity. In this study we found evidence that the RD9 deletion occurred at least 4000 years ago in ancient Egypt. This confirmed our previous spoligotyping results, where we found probable *M. africanum* specific signatures in some of our Middle Kingdom samples (Zink et al., 2003b). The *M. tuberculosis* specific deletion TbD1 was still present in samples from all different time period of our ancient Egyptian samples. In contrast, the loss of TbD1 could only be detected in 6 samples from the New Kingdom to Late Period tombs in Thebes-West. Most interestingly, three of the TbD1 deleted samples also showed a katG mutation and could be classified as genetic group 2 or 3 according to Sreevatsan et al. (1997). Our findings clearly extend the initial results provided by the investigation of the Hungarian mummies of the 18th century (Fletcher et al., 2003) and draw back the occurrence of the Tbd1 deletion to the New Kingdom/ Late Period, 3500 years ago. We cannot exclude an earlier onset of modern *M. tuberculosis*, but we have found no evidence for the loss of TbD1 in the Middle Kingdom or Pre to Early Dynastic material.

Literature Cited

Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM,. Pym AS, Samper S, van Soolingen D, Cole ST. 2002. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Nat Acad Sci USA 99:3684-3689.

Cockburn A. 1963. The evolution and eradicaton of infectious disease. Johns Hopkins Press, Baltimore.

Crubézy É, Ludes B, Poveda J-D, Clayton J, Crouau-Roy B, Montagnon D. 1998. Identification of *Mycobacterium* DNA in an Egyptian Pott's disease of 5400 years old. CR Acad Sci Paris 321:941-951.

Donoghue H, Spigelman M, Greenblatt CL, Bar-Gal GK, Lev-Maor G, Matheson C, Vernon K, Nerlich AG, Zink AR. 2004. Ancient DNA from the *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* in archaeological material - verification and characterization. Lancet Infect Dis 4:584-592.

Faerman M, Jankauskas R, Gorski A, Bercovier H, Greenblatt CL. 1997. Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA analysis. Ancient Biomolecules 1: 205-214.

Filliol I, *et al.* 2002. Global distribution of Mycobacterium tuberculosis spoligotypes. *Emerg Infect Dis* 8:1347-1349.

Fletcher HA, Donoghue HD, Taylor GM, van der Zanden AGM, Spigelman M. 2003. Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. Microbiology 149:143-151

Gilbert MT, Barnes I, Collins MJ, Smith C, Eklund J, Goudsmit J, Poinar H, Cooper A. 2003. Long-term survival of ancient DNA in Egypt: Response to Zink and Nerlich.
Am J Phys Anthropol. 2005 Feb 15; [Epub ahead of print]

Kolman CJ, Tuross N. 2000. Ancient DNA analysis of human populations. Am J Phys Anthropol 111: 5-23

Marota I, Basile C, Ubaldi M, Rollo F. 2002. DNA decay rate in papyri and human remains from Egyptian archaeological sites. Am J Phys Anthropol 117:310–318.

Nerlich AG, Haas CJ, Zink A, Szeimies U, Hagedorn HG. 1997. Molecular evidence for tuberculosis in an ancient Egyptian mummy. Lancet 350:1404

Poinar H, Kuch M, McDonald G, Martin P, Pa¨a¨bo S. 2003. Nuclear DNA from sloth coprolithes. Curr Biol 13:1150–1152.

Rothschild BM, Martin LD, Lev G, Bercovier H, Bar-Gal GK, Greenblatt C, Donoghue H, Spigelman M, Brittain D. 2001. *Mycobacterium tuberculosis* complex DNA from an extinct bison dated 17,000 years before the present. Clin Infect Dis 33:305-311

Salo WL, Aufderheide AC, Buikstra J, Holcomb TA. 1994. Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proc Natl Acad Sci USA 91:2091-2094.

Spigelman M, Lemma E. 1993. The use of polymerase chain reaction to detect *Mycobacterium tuberculosis* in ancient skeletons. Int J Osteoarchaeol 3:137-143.

Sreevatsan S, Pan X, Stockbauer K, Connell N, Kreiswirth B, Whittam T, Musser J. 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci USA 97:9869-9874.

Taylor GM, Goyal M, Legge AJ, Shaw RJ, Young D. 1999. Genotypic analysis of *Mycobacterium tuberculosis* from medieval human remains. Microbiology 145:899-904.

Verano JW, Ubelaker DH, ed. 1992. Disease and Demography in the Americas. Washington, DC: Smithsonian Institution Press

World Health Organization. 2000. The WHO/IUATLD global project on anti-tuberculosis drug resistance surveillance. Anti-drug resistance in the world. Report 2. Prevalence and trends. WHO/CDS/TB/2000.278. Communicable Diseases. Geneva, Switzerland: World Health Organization.

Zink A, Haas CJ, Reischl U, Szeimies U, Nerlich A. 2001. Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. J Med Microbiol 50:355-366.

Zink AR, Grabner W, Nerlich AG. 2002a. Molecular study on human tuberculosis in contemporary tissue samples and time delineated populations from ancient Egypt. J Paleopathol 14:59-68.

Zink AR, Reischl U, Wolf H, Nerlich AG. 2002b. MiniReview – Molecular analysis of ancient microbial infections. FEMS Microbiol Lett 213: 41-147.

Zink A, Grabner W, Reischl U, Wolf H, Nerlich AG. 2003a. Molecular study on human tuberculosis in three geographically distinct and time delineated populations from ancient Egypt. Epidemiol Infect 130:239-249.

Zink A, Sola C, Reischl U, Grabner W, Rastogi N, Wolf H, Nerlich AG. 2003b. Characterisation of Mycobacterium tuberculosis Complex Findings from Egyptian Mummies by Spoligotyping. J Clin Microbiol 41:359-367.

Zink A, Nerlich A. 2003c. Molecular analyses of the "Pharaohs" – Feasibility of molecular studies in ancient Egyptian material. Am J Phys Anthropol 121: 109-111

Zink AR, Sola C, Reischl U, Grabner W, Rastogi N, Wolf H, Nerlich AG. 2004. Molecular identification and characterisation of Mycobacterium tuberculosis complex in ancient Egyptian mummies. Int. J. Osteoarchaeol14:404-413.

Zink A, Grabner W, Nerlich AG. 2005. Molecular identification of human tuberculosis in recent and historic bone tissue samples. A study on the role of molecular techniques for the study of historic tuberculosis. Am J Phys Anthropol 126:32-47.

Zink AR, Nerlich AG. 2005. The long-term survival of ancient DNA in Egypt. Am. J. Phys. Anthropol Feb 15 [Epub ahead of print].