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Co-infection of *Mycobacterium tuberculosis* and *Mycobacterium leprae* in human archaeological samples: a possible explanation for the historical decline of leprosy

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Both leprosy and tuberculosis were prevalent in Europe during the first millennium but thereafter leprosy declined. It is not known why this occurred, but one suggestion is that cross-immunity protected tuberculosis patients from leprosy. To investigate any relationship between the two diseases, selected archaeological samples, dating from the Roman period to the thirteenth century, were examined for both *Mycobacterium leprae* and *Mycobacterium tuberculosis* DNA, using PCR. The work was carried out and verified in geographically separate and independent laboratories. Several specimens with palaeopathological signs of leprosy were found to contain DNA from both pathogens, indicating that these diseases coexisted in the past. We suggest that the immunological changes found in multi-bacillary leprosy, in association with the socio-economic impact on those suffering from the disease, led to increased mortality from tuberculosis and therefore to the historical decline in leprosy.

Keywords: ancient DNA; leprosy; tuberculosis; PCR; history of infectious diseases

1. INTRODUCTION

Tuberculosis and leprosy (Hansen's disease) have long been known to occur in antiquity (Lechat 1999; Rothschild *et al.* 2001) as they leave readily identifiable bony changes on skeletal remains (Møller-Christensen 1961, pp. 42–43; Buikstra 1981). Tuberculosis has remained at epidemic levels since the onset of industrialization in Europe and is of increasing importance in modern medicine, with an estimated one-third of the world's population infected (Kochi 1991) and more than 8.2 million new cases in the year 2000 (Corbett *et al.* 2003). Approximately 90% of those infected show no immediate symptoms owing to the activity of the cell-mediated immune response. The current high mortality levels are primarily due to co-infection with HIV and the associated immunosuppression (Rose *et al.* 2002).

Leprosy is rarely found today in Western Europe but it is still a significant disease worldwide, with more than half a million cases detected in 2002, most in Southeast Asia (World Health Organization 2003: http://www.who.int/lep/stat2002/global02.htm). Leprosy is primarily a disease of peripheral nerves and skin but also affects bones. Its clinical effects vary from the slowly developing paucibacillary or tuberculoid leprosy, to the multi-bacillary lepromatous leprosy. In this lepromatous state there is direct invasion of soft tissues around the face and mouth by *Mycobacterium leprae* (Andersen & Manchester 1992; Roberts & Manchester 1995). The type of infection is dependent on the immune response of the host. Leprosy apparently ravaged the Middle East and Europe from biblical times until the late medieval period, although diagnoses based only on written reports remain questionable. One of the effects of leprosy, particularly owing to the fear it engendered in antiquity, is that these individuals would become isolated and therefore be more likely to suffer from poor nutrition and a weakening of the immune system, thereby paving the way for opportunistic co-infection or the resurgence of a latent infection.

There have been a number of proponents of the theory that the decline of leprosy in Western Europe, from its fearsome prevalence in the twelfth and thirteenth centuries to its virtual absence by the sixteenth century (Roberts & Manchester 1995), was owing to the increasing prevalence of tuberculosis. A mathematical model of the epidemiology of leprosy and tuberculosis suggests that tuberculosis could have contributed to the historical decline of leprosy if the basic reproductive rate of leprosy was low (Lietman *et al.* 1993). There is significant evidence that a level of cross immunity exists between the two bacteria (Murhekar *et al.*

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Table 1. Primers used in this study.

name	target region	target size (bp)	primer
Mycobacterium tuberculosis complex-specific primers			
laboratory 1			
P1	IS*6110*	123	5′CTCGTCCAGCGCCGCTTCGG 3′
P2	IS*6110*		5′CCTGCGAGCGTAGGCGTCGG 3′
IS-3	IS*6110*	92	5′ TTCGGACCACCAGCACCTAA 3′
IS-4	IS*6110*		5′ TCGGTGACAAAGGCCACGTA 3′
laboratory 2			
INS-1	IS*6110*	246	5′ CGTGAGGGCATCGAGGTGGC 3′
INS-2	IS*6110′*		5′ GACGTAGGCGTCGGTGACAAA 3′
Mycobacterium leprae-specific primers			
laboratories 1 and 2			
LP1	RLEP	129	5′TGCATGTCATGGCCTTGAGG3′
LP2	RLEP		5′CACCGATACCAGCGGCAGAA3′
LP3	RLEP	99	5′TGAGGTGTCGGCGTGGTC3′
LP4	RLEP		5′CAGAAATGGTGCAAGGGA3′

1995; Ohara *et al.* 2000; Roche *et al.* 2001) and this led to the suggestion that this protected individuals with a latent tuberculosis infection from acquiring leprosy (Chaussinand 1948; Fine 1984). Another theory is that leprosy patients became more susceptible to underlying latent tuberculosis owing to the decline in immune status associated with the progression of the disease.

DNA from the causative organisms, *Mycobacterium tuberculosis* and *M. leprae*, can be detected by species-specific PCR, and the initial reports (Spigelman & Lemma 1993; Rafi *et al.* 1994) have been confirmed by many investigators, reviewed by Spigelman & Donoghue (2003). Both pathogens have no known environmental reservoir, so their presence indicates infection, if not active disease.

The aim of this study was to investigate any relationship between *M. leprae* and *M. tuberculosis* infections in human archaeological remains by using species-specific PCR to detect the DNA of these organisms. Specimens containing *M. leprae* DNA, and showing clear signs of the pathology associated with multi-bacillary leprosy, were examined for the presence of *M. tuberculosis* DNA. Specimens known to contain *M. tuberculosis* DNA were examined for *M. leprae* DNA. Subsequently a small number of previously unstudied specimens were examined for evidence of both organisms.

2. MATERIAL AND METHODS

(a) *Samples*

The skeletal material for this study came from a variety of sources. Laboratory 1 (UCL) examined samples from all sites. Laboratory 2 (Thunder Bay/Jerusalem) examined samples from Israel and Egypt.

(i) Cavum nasale samples from three skeletons from graves 11, 222 and 503 in a tenth century site at Püspökladány, Hungary. The remains from graves 222 and 503 showed pathological changes consistent with multi-bacillary leprosy (Donoghue *et al.* 2002). The skeleton from grave 11 showed only periostitis inside the maxilla.

(ii) Material was obtained from five skeletons from a leprosy hospital (according to contemporary documentary evidence), dating from the fifteenth century and located at Szombathely, Hungary. There were no morphological changes on the bones and the cause of death was stated as the plague. Samples were taken from the cavum nasale and maxilla.

(iii) Also from Hungary, samples were obtained from three skeletons buried by the eleventh–sixteenth century Basilica at Székesfehérvár. Two eleventh century skeletons (79a and 89) showed typical leprotic morphological alterations around the nasal cavity. Skeleton 89 also showed marked evidence of an abscess on the tibia. The third skeleton, from the fourteenth–sixteenth century (I/II), showed severe periostitis on both tibias but no specific pathology.

(iv) Archaeological remains from a first century AD family tomb in Akeldama, the Hinnom Valley, Israel (Gibson *et al.* 2002) were analysed. One individual was wrapped in a shroud and placed in a loculus sealed with plaster. A fragmented phalanx with an advanced pathological lesion was discovered from the chest region. The differential diagnosis included tuberculosis. Fragmented skull and other skeletal elements from the shroud loculus individual were examined, together with the pulp of a molar tooth. In addition, skeletal material, with no pathological evidence of lesions, from two infants from a wall niche was studied.

(v) Specimens from the Dakhleh Oasis, Egypt (Molto 2002), dating from the Roman period, were examined. All the skeletal samples examined from this site had evidence of pathological lesions. Two young adult male burials, B6 and B116, had pathology strongly suggestive of multi-bacillary leprosy. Samples from two other young adult males, B9 and B222, had possible leprosy pathology. Remaining samples showed early skeletal changes consistent with a number of diseases, including leprosy and tuberculosis.

(vi) Material was obtained from a small medieval cemetery at Björned, in northern Sweden, dating from the Viking period, the late tenth century to the late thirteenth century (Nuorala *et al.* 2004). Five adult individuals were selected at random. Metacarpals (2), coxae (1) and patella (2) were examined. There were no specific indications of infectious disease.

(b) *DNA extraction*

The recommended protocols of ancient DNA (aDNA) work (O'Rourke *et al.* 2000) were followed, with separate rooms for different stages of the process. The procedures have been described previously (Rothschild *et al.* 2001; Spigelman *et al.* 2002). In addition, in laboratory 1 *ca*. 25 mg of powdered sample was demineralized in Proteinase K/EDTA at 56 °C for 24–72 h. Samples were split and one aliquot incubated at 56 °C for 1 h with

Table 2. Distribution of *Mycobacterium leprae* and *Mycobacterium tuberculosis* complex-specific DNA according to specimen source and age (+, positive; −, negative; n.a., not applicable).

sample site	sample	*Mycobacterium leprae*	*Mycobacterium tuberculosis*	co-infection
tenth century Hungary Püspökladény[a]	11	+	−	−
	222	+	+	+
	503	+	+	+
	429	+	−	−
	621	−	−	n.a.
Szombathely[a] fifteenth century Hungary	6	−	+	−
	10	+	−	−
	3, 19, 20	−	−	n.a.
Székesfehérvár[a] eleventh century and fourteenth–sixteenth century Hungary	79a	−	−	n.a.
	89	+	−	−
	I/II	−	+	−
first century Israel Akeldama[b], Himmon valley	BSC	−	+	−
	BCN	−	+	−
	C1	+	+	+
	SCP	−	+	−
Fourth century Egypt, the Dakhleh Oasis[b]	B6	+	+[c]	+
	B9	−	+[d]	−
	B116	+	−	−
	B222	+[e]	+[c]	+
	B251	+	+[f]	+
	B265	+[c]	+	+
	B280[g]	+	+	+
	B377	−	+	−
	B392	+	+[c]	+
	B437	+[e]	−	−
Björned[a] tenth–thirteenth century Sweden	A4	+	+	+
	A25	−	+	−
	A1a, 2, 8	−	−	n.a.
total	32	16	18	10/24

[a] Examined by laboratory 1 only.
[b] Examined by laboratories 1 and 2.
[c] Positive in laboratory 1 only.
[d] Positive in laboratory 1 when an alternative *Taq* polymerase was used. Positive in laboratory 2 using standard technique.
[e] Positive in laboratory 2 only.
[f] Positive in laboratory 1 when PTB used in DNA extraction. Positive in laboratory 2 using standard technique.
[g] Examined by laboratory 2 only.

0.1 M *N*-phenacylthiazolium bromide (PTB), a reagent that cleaves glucose-derived protein cross-links (Poinar *et al.* 1998). Thereafter both aliquots were lysed in guanidium thiocyanate solution and DNA captured onto silica in suspension or by using a spin filter. After washing and drying, DNA was eluted from the silica, aliquoted and used immediately or stored at −20 °C. Negative extraction controls were always included. All extractions and analyses were repeated.

(c) *DNA amplification*

Details have been described previously (table 1). The *M. tuberculosis* complex was detected by targeting a specific region of the repetitive element IS*6110*. In laboratory 1 a two-tube nested PCR was used which yielded an outer product of 123 bp and a nested PCR product of 92 bp (Rothschild *et al.* 2001; Spigelman *et al.* 2002). In some cases 1 M betaine was included in the PCR mix as a facilitator. Laboratory 2 used a larger target sequence of 246 bp. *Mycobacterium leprae* was detected by amplification of the repetitive RLEP fragment, using nested PCR which resulted in an outer product of 129 bp and a 99 bp nested product (Donoghue *et al.* 2001, 2002). Negative controls were always included.

(d) *Detection and sequencing*

PCR product was detected by staining with ethidium bromide and visualizing under UV light. In London, sequencing was carried out by MWG-BIOTECH AG (Ebersberg, Germany). Elsewhere, sequencing was carried out in-house according to published protocols (Spigelman *et al.* 2002).

3. RESULTS

Negative DNA extraction and PCR controls were satisfactory. Twenty-four out of the 32 samples examined contained amplifiable DNA for one or other of these pathogens (table 2), both of which were found at all six sites. *Mycobacterium tuberculosis* complex-specific DNA was detected in 18 samples and *M. leprae*-specific DNA was found in 16 samples. Ten samples contained DNA from both organisms (figure 1). The four sites with co-infected samples were from Roman Egypt, first century Palestine, tenth century Hungary and medieval

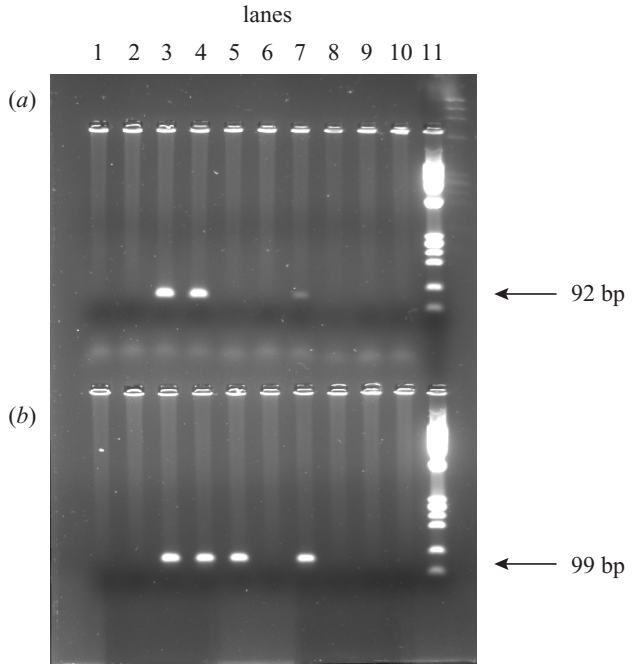

Figure 1. Gel electrophoresis analysis of (*a*) MTB IS*6110* PCR products (92 bp) and (*b*) *M. leprae* RLEP PCR products (99 bp). Lane 1: Shroud Cave sample BSC (in other extractions this sample was positive for IS*6110*), DNA extract a; lane 2: Shroud Cave sample C1, DNA extract a; lane 3: Püspökladány sample 222, DNA extract b; lane 4: Dakhleh Oasis sample B6, DNA extract b; lane 5: Dakleh Oasis sample B116, DNA extract b; lane 6: Shroud Cave sample BSC, DNA extract b; lane 7: Shroud Cave sample C1, DNA extract b; lane 8; negative control, extraction a; lane 9: negative control, extraction b; lane 10: PCR negative control; lane 11: ØX174*Hae*III molecular markers.

Sweden. Results from different laboratories showed some slight discrepancies but each obtained evidence of co-infection. When both forward and reverse strands were sequenced the consensus sequences were in complete agreement with those in the NCBI database for the target regions. Where only a single DNA strand was sequenced there were minor discrepancies.

4. DISCUSSION

DNA from both *M. leprae* and *M. tuberculosis* was detected, sometimes in the same samples, in spite of their age and the different local environmental conditions in which they were found. Approximately 30% of specimens examined, with differing degrees of bone palaeopathology, and 40% of those with DNA from one of these organisms, were harbouring both pathogens. It is believed that only 3–5% of tuberculosis infections result in bony lesions (Resnick & Niwayama 1995), so it is not surprising that some specimens with little or no pathology were positive for *M. tuberculosis* DNA. There is likely to be uneven distribution of the microbial pathogens within the sample, which could explain some of the minor discrepancies found between the different laboratories that examined this material. In addition, there were slight differences in techniques, including quantity of sample examined and PCR primers used, that may also have contributed (Spigelman *et al.* 2002).

The presence of DNA from *M. leprae* and the *M. tuberculosis* complex indicates infection, as these pathogens have no known environmental reservoir. The samples were obtained from bone, which indicates disseminated, therefore active disease. The demonstration of the presence of both pathogens in a specimen demonstrates that these diseases coexisted in the past, which is supported by palaeopathology (Manchester 1991). An examination of the literature reveals previous reports of leprosy and tuberculosis co-infections. In 1895, in the early days of medical bacteriology, Hansen noted that tuberculosis was a major cause of death in his leprosy patients in Oslo. He wrote that 'The most frequent complication which we have seen in our institutions is tuberculosis, particularly some years ago, for then the institutions were over-crowded, and subsequently the sanitary conditions were in many respects unsatisfactory' (Hansen & Looft 1895). This monograph continues with lengthy tables outlining the symptoms and pathology of patients suffering from both diseases.

In modern times it was noted that up to 26% of patients with *M. leprae* infections had concomitant tuberculosis infections diagnosed during the life of the patient (Nigam *et al.* 1979; Kumar *et al.* 1982; Rafi & Feval 1995) and at *post-mortem* (Jayalakshmi *et al.* 1987; Glaziou *et al.* 1993). In French Polynesia, Glaziou and co-workers examined the records of more than 1000 leprosy patients who had been in institutions between 1902 and 1991. They found that between 1902 and 1930, before the onset of effective antimicrobial therapy, mortality from tuberculosis in these leprosy patients was 21%. It appeared that more multibacillary patients died of tuberculosis (13%) than paucibacillary patients (4%), although in many instances there were insufficient details to distinguish between the clinical forms of the leprosy infection.

Co-infection by two or more organisms is not uncommon. Generally, one organism weakens the patient and reduces the ability of the immune system to respond adequately or rapidly enough and this allows a more virulent organism to infect the patient. In multi-bacillary (lepromatous) leprosy, patients show an impaired cell-mediated response to *M. leprae*, which is believed to be associated with HLA type (Abel *et al.* 1998). It may also be linked to a genetic defect (Kang & Chae 2001) found in a significant proportion of the population (22%). The defect is in toll-like receptor 2 (TLR2), a protein that apparently plays a vital role in triggering host defence mechanisms against microbial invasion (Krutzik & Modlin 2004). This area is still under active research, but it appears likely that an impaired cell-mediated response would allow the great predator, *M. tuberculosis*, to advance. Indeed, an examination of the incidence of leprosy and tuberculosis in Texas from 1938 to 1980 indicated that the number of cases of both diseases increased or decreased in tandem (Wilbur *et al.* 2002), although the clinical forms of leprosy were not distinguished.

It should not be forgotten that individuals suffering from leprosy often suffer social isolation and stigma (Bainson & Van den Borne 1998), especially in the past (Cule 2002). This can lead to physical and mental stress that has an adverse effect on resistance to infectious diseases. One of the populations sampled in the present study, the Dakhleh

Oasis, is known to have suffered from cribra orbitalia, an indicator of anaemic stress (Fairgrieve & Molto 2000). The authors include in their speculation on the possible causes of this condition, synergism between disease and poor nutrition. Iron is an essential nutrient for pathogenic mycobacteria and the host response of mild anaemia is beneficial to the patient (Ratledge 2004). However, with disseminated infections the severe anaemic stress found in the Dakhleh population is likely to have exacerbated both diseases. It has been suggested that the Dakhleh Oasis was used as a place for the isolation of those suffering from leprosy (Dzierzykray-Rogalski 1980), which may explain the readily-detectable presence there of both leprosy and tuberculosis.

In conclusion, the cross-immunity hypothesis appears unlikely because of the evidence of disseminated infection in the present study. We believe that the reduction in an effective cell-mediated immune response associated with multi-bacillary leprosy, coupled with the social impact of the disease, would lead to re-activation of an underlying latent tuberculosis infection, or to superinfection with *M. tuberculosis*, and to a speedier death. In time this would decrease the number of individuals suffering from leprosy, leading to the observed phenomenon of its decline.

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