Brief Communication: Unusual Pathological Condition in the Lower Extremities of a Skeleton From Ancient Israel

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Hershkovitz *et al.* (1992) reported on a skeleton from Bet Guvrin, Israel, dating from the Byzantine period (300-600 AD). There were pathological changes in the lower extremities, which presented significant diagnostic problems. Their original diagnosis was Madura foot, based on the absence of typical medullary changes associated with osteomyelitis, and to the presence of severely ankylosed (left calcaneal talar joint) and deformed bilateral foot bones. However, Manchester (1993), in reply, noted that Madura foot is usually a predominantly unilateral condition, and that many of the surface changes were consistent with secondary pyogenic infections following plantar ulceration associated with leprous peripheral neuropathy. There was evidence that at least one joint was a typical Charcot's joint suggestive of the presence of some form of neuropathy. Manchester (1993) noted that this skeleton was from the same period as one reported by Zias (1985), which was the first ancient skeleton from which *Mycobacterium leprae* DNA was extracted and amplified (Rafi *et al*., 1994). In further reply, Hershkovitz *et al.* (1993) admitted that the pathological features could fit a diagnosis both Madura foot and leprosy, but still considered Madura foot the more likely. Here the controversy rested.

As the controversy has remained unresolved, one of the bones from the foot was sent to us by Professor Israel Hershkovitz of the Department of Anatomy and Anthropology, Tel Aviv University, Israel, for further examination. The aim was to determine whether *M.leprae* DNA was present in this specimen, as mycobacterial DNA has been shown to persist in samples of a similar age (Rafi *et al.* 1994; Donoghue *et al.* 1998). Even in the case of lepromatous leprosy, the numbers of bacilli in peripheral sites are not believed to be high. Therefore, the specimen was examined using a specific nested polymerase chain reaction (PCR), to determine whether *M.leprae* -specific DNA could be detected.

Two sets of *M.leprae* -specific nested primers were designed. The first were based on the *M. leprae* repetitive element RLEP, which is reported to be present at 28 copies/cell. These gave an outer product of 129 bp and a 99 bp-nested product. Those based on the 18kD antigen (Ag) gene gave a product of 136 bp (outer) and 110 bp (inner). The sequences and locations of these primers are listed in Table 1.

Stringent precautions were taken to avoid cross-contamination. Briefly, clean protective clothing was worn with frequent glove changes. Pipettors and surfaces were cleaned with neat household liquid detergent, rinsed with ultrapure water, and dried with ethanol before use. Sterile tubes and plugged tips were used. A separate roon was used for handling PCR products, with a different set of pipettors and protective clothing.

The DNA extraction method was essentially the second protocol described by Donoghue et al. (1998), and negative controls were processed alongside the sample. Pre-aliquoted double-strength PCR mix was used (Advanced Biotechnologies, Ltd., Epsom, UK). The final composition of the PCR mixture (50 µl) was 75 $mmol^{-1}$ Tris-HCl (pH 8.8); 20 $mmol^{-1}$ $(NH_4)_2SO_4$; 1.5 $mmol^{-1}$ $MgCl_2$; 0.01% Tween 20; 200 μmol^{-1} (each) dATP, dCTP, dGTP, and dTTP; and 1.25 units *Taq* DNA polymerase. Bovine Serum Albumin (BSA) was added to give a final concentration of 10 mM. The primer pair, BSA, and DNA preparations (5.0 µl) were added to each pre-aliquoted tube plus sufficient water to bring the volume to 50 µl. A tube with water in place of template was always included as a negative control. Primers were used at a final concentration of 60 nM (RLEP primers), 100 nM (18-kD Ag inner primers), and 200 nM (18-kD Ag outer primers).

Nested PCR was carried out using a two-tube procedure. In the first stage the outer primers were added to the PCR mix. Amplification was carried out with one cycle of strand separation at 94oC for 4 min; 25 cycles of 94^0C for 40 s; . annealing at 55 ^{o}C (18 kD Ag gene primers) or 58^{o}C (RLEP primers) for 1 min; and strand extension at 72^{o}C for 20 s + 1 s increment per cycle. There was a final 1 min at 72^{o}C for further strand extension. In the second stage of nested PCR, new tubes of PCR mix were used, with the inner primers, with 5.0µl of stage one product used as the DNA template. This second stage PCR cycle was identical to the first. PCR products were detected by agarose gel electrophoresis and recorded by Polaroid camera.

Positive results were obtained using both primer pairs, although there was an extremely weak positive with the PCR for the single-copy 18-kD Ag gene. Because of the low quantity of amplicon, only a partial sequence from the RLEP primers was obtained. However, those obtained from other archaeological samples showed a sequence identical with that of *M.leprae*. A preliminary sequence of 123 bp was obtained from the reverse inner primer, which includes part of the outer forward primer (underlined), the forward and reverse inner primers (bold) and all the intervening DNA:

GCATGTCATG[C]NNT**TGAGGTGTCGGCGTGGTC**AATGT-GCCGCACC[N]
TGAACAGGCAC[G]GTCCCCG-GCACGGTATA[G]CTATTCGCACCTGATG
TTA**TCCCTTGCACCATTTCTG**

There are 3 missing or indeterminate values and 2 doubtful results in the sequence between the inner primers, plus 2 indeterminate and one doubtful result in the forward primer region. Otherwise the sequence was identical to 30 sequences of *M. leprae* held in the gene data bank. With the exception of an internal 18 bp sequence, a BLAST search of the gene database held by the National Center for Biotechnology Information showed that this product is unique to *M.leprae*.

The primers used have been described elsewhere (Donoghue *et al.*,1999) and have been shown to be both specific and extremely sensitive, capable of detecting less than one leprosy bacillus. The pathology, plus the demonstration of *M. leprae* DNA in the specimen, indicates that the individual was suffering from leprosy (Hansen's disease).

Mycetoma, also known as Madura foot, is a local, chronic, slowly progressive disease with the classic presentation involving tumefaction, multiple draining sinuses, and grain-filled pus. Either a bacterium (actinomycetoma) or a fungal (eumycetoma) organism is the primary cause. Many different organisms have been reported as causal agents in the literature. The majority are soil environmental saprophytes (McGinnis 1996).
Hershkovitz *et al.*. (1992) thought it likely that the skeleton was of an immigrant rather than a local resident, although this was based on very limited numbers of cases of Madura foot in Israel of which they were aware. However, the current literature includes reports of cases from Great Britain, the USA, Bulgaria, and the Middle East. Therefore, it is possible that the individual was a

native of the area and had a coincidental or secondary case of Madura foot caused by local environmental organisms. Mixed or secondary infections are ubiquitous in peripheral *M.leprae* ~~infection, and possibly exacerbated by the decrease in cell-mediated immunity. A further suggestion is that a malignant condition may have arisen at the site of a trophic ulcer.~~ ~~However,~~infections, as is shown even in the present day (Dr J. Mehta, The Bandorawalla Leprosy Hospital, Pune, India, and Dr T. Oomen, Department of Pharmacology, Manipal, India, personal communications).

Although it is not possible to prove the sequence of events that led to the palaeopathological features in this individual, we conclude that the underlying disease in this case

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TABLE 1 Sequences of oligonucleotide primers for M.leprae[1]

Primer	Residue	Sequence
LP1	490-509	TGCATGTCATGGCCTTGAGG
LP2	618-599	CACCGATACCAGCGGCAGAA
LP3	505-522	TGAGGTGTCGGCGTGGTC
LP4	603-586	CAGAAATGGTGCAAGGGA
LP5	1050-1070	ATCGACTGTTGTTTGCGCAAC
LP6	1159-1139	CCAGCAACCGAAATGTTCGGA
LP7	1037-1057	TCATAGATGCCTAATCGACTG
LP8	1172-1154	GGCACATCTGCGGCCAGCA

[1] For primers LP1-LP4 the primer sequence is found in the *M.leprae* RELP3 sequence X17153. For primers LP5-LP8 the primer sequence is found in the *M.leprae* 18 kD Ag gene sequence MSGANT18K.