Journal of Archaeological Science 36 (2009) 2408–2414

Contents lists available at ScienceDirect

Journal of Archaeological Science

journal homepage: http://www.elsevier.com/locate/jas

Mycobacterium leprae genotype amplified from an archaeological case of lepromatous leprosy in Central Asia

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ARTICLE INFO

Article history:
Received 16 December 2008
Received in revised form
23 June 2009
Accepted 25 June 2009

Keywords:
Leprosy
PCR
Genotyping
Human remains

ABSTRACT

We have amplified *Mycobacterium leprae* DNA from the skeleton of an adult human female exhibiting signs of lepromatous leprosy (LL). The remains were excavated from the site of Devkesken 6 on the Ustyurt plateau of Uzbekistan and date to between the 1st and 4th centuries AD. Recovered DNA was fragmented but of sufficient quality and quantity to allow a series of biomolecular genotyping methods to be applied. These methods included variable nucleotide tandem repeat (VNTR) typing of two microsatellite and one minisatellite regions and also single-nucleotide polymorphism (SNP) typing for nine informative loci.

Genotyping showed that the causative strain of *M. leprae* exhibited a SNP-type 3 profile, characteristic of cases associated geographically with Europe and North Africa. Further SNP sub-typing was performed and the data obtained from the Uzbek leper was compared with the same loci amplified from a case of LL recovered from Blackfriars, Ipswich, UK dating to between the 13th and 16th centuries AD. Unique group 3 subtypes were found in both the Uzbek case and Ipswich 1914. These appear to be ancestral to recent type 3 strains. Mycolic acid analysis confirmed the presence of *M. leprae* in the Uzbek samples. Phylogenetically informative SNPs and other polymorphic loci will contribute to the study of human migrations, as well as the origin and spread of leprosy.

1. Introduction

Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*, first identified as the cause of leprosy by Hansen (Hansen, 1874). It is an extremely slow-growing *Mycobacterium* and prolonged contact with infected individuals is required for transmission of the disease. Due to its low pathogenicity, the majority of people exposed to *M. leprae* do not develop the disease.

When infection does occur, this may produce disease within a spectrum of severity ranging from tuberculoid leprosy to lepromatous leprosy (LL). The tuberculoid form, affecting mainly the skin and nerves, is associated with low numbers of bacilli, a relatively efficient cell-mediated immune response and few lesions. In contrast, the lepromatous form is typified by a poor or absent cell-mediated immunity and consequently lepromatous disease is associated with a multibacillary state. There may be high circulating levels of antibodies to the *Mycobacterium* (Stanford and Stanford, 2002), but these are ineffective at controlling progression. In lepromatous disease, many tissues and organs may be affected by haematogenous dissemination, including the skeleton. Bone may also become involved through direct extension from skin and other soft tissues. The skeletal lesions associated with LL include characteristic changes to the rhinomaxillary area, the long bones of the lower legs and small bones of the hands and feet. Rhinomaxillary changes include resorption of the anterior nasal spine and rounding and widening of the lateral aspects of the pyriform aperture. There may also be pitting and/or perforation of the hard palate and destruction of the premaxillary alveolar process,

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doi:10.1016/j.jas.2009.06.026

sometimes leading to loss of the upper incisor teeth. The skeletal lesions identified with LL were first described in depth by Møller-Christensen (Møller-Christensen, 1961; Bennike, 2002).

The remains which are the subject of the present investigation were excavated from the Ustyurt plateau in Uzbekistan in the 1980s. The Ustyurt plateau covers an area of 160,600 sq km and rises to 150–300 m above sea level (Anon, 2000). The boundaries of the plateau are clearly marked by step edges, which are only possible to climb in specific locations (Olkhovskiy, 2000, p. 33). The plateau consists of a monotonous desert with large drainage basins and extensive sandy upland massifs. With the exception of sweet and salty water that can be obtained from deep wells, there are few water resources and a lack of mature vegetation present. The plateau has a continental climate (that is, hot summers, cold winters and low rainfall). Summer temperatures reach up to 47 °C and the average annual rainfall is very low, ranging from 90 mm in the south to 120 mm in the north (Yagodin et al., 2007). Most of the plateau has almost no snow cover in the winter, making it favourable place for wild animals and cattle herders to migrate in the winter. The climatic conditions on the Ustyurt are reported to have changed little over the last 3000 years (Olkhovskiy, 2000, p. 34).

The skeletal remains of the Uzbek case are those of an adult female. The skeleton is incomplete but the skull, long bones of the left arm and left leg, the left pelvis, the sacrum and the right femur are preserved. The skull shows evidence of *facies leprosa* as described by Møller-Christensen (1961) and an erosive lesion, accompanied by raised woven bone, is present on the left tibia. Diagnoses other than leprosy, considered at the time of the original report, included fungal infections, actinomycosis, the craniofacial tuberculosis of *lupus vulgaris* and treponemal disease. The small bones of the hands and feet were not preserved but osteological evidence strongly supported a diagnosis of LL (Blau and Yagodin, 2005a). Radiocarbon dating places the burial between the 1st and 4th centuries AD (Blau and Yagodin, 2005b).

Leprosy is an ancient disease which has been spread among human populations by migrations, military expansion, colonisation and along trade routes. In recent years much has been learnt about the microorganism from the *M. leprae* sequencing project (http://genolist.pasteur.fr/Leproma) and from examination of strains recovered from regions where the disease is still endemic. As a result of these initiatives, it has become clear that all present-day cases of leprosy are attributable to a single clone whose dispersal geographically can be retraced through analysis of rare single-nucleotide polymorphisms (SNPs) (Monot et al., 2005). The study of SNPs shows that leprosy originated either in the Near East or East Africa and spread with successive human movements around the world. Four main genotype profiles have been recognised (Monot et al., 2005) and recently, identification of additional phylogenetically informative SNPs allows sub-typing of the 4 main groups into a total of 16 subtypes (Monot et al., submitted for publication). This will not only facilitate detailed study of the epidemiology of leprosy and disease transmission of contemporary cases but will also assist in our understanding of the spread of the disease in prehistory through use of ancient DNA (aDNA) analysis.

The retrieval of mycobacterial DNA from cases of leprosy (and tuberculosis) is one of a few areas where aDNA analysis, undertaken in a number of laboratories, has proven to be feasible for pathogen detection and characterization (Taylor et al., 2006; Donoghue et al., 2005; Haas et al., 2000; Montiel et al., 2003). The Uzbek case is a rare example of leprosy from the archaeological record of Central Asia. It is also an important location from the perspective of human migrations across the Eurasian steppe and trade routes along the Silk Road between China to the east and the Mediterranean basin to the west. We have therefore applied aDNA methods to the Uzbek leper to confirm the osteological diagnosis as well as to type the causative strain. The SNP profile of leprosy amplified from the Uzbek case has been compared with one from a later case in Medieval England. Mycolic acid analysis was also undertaken on the Uzbek case as a further validation of the osteological diagnosis and the biomolecular study.

2. Methods

2.1. Bioarchaeological samples

2.1.1. Uzbek leper

Samples taken from the left tibia (20 mg) and skull (15 mg) were initially studied at the Windeyer Institute, UCL. These were screened for remnant *M. leprae* DNA using a sensitive PCR for the multi-copy element RLEP (Taylor et al., 2006). Samples from both skeletal sites were found to be strongly positive and were subjected to SNP typing according to the 3 loci identified by Monot et al. (2005). Four VNTR loci were also studied. These were for the TTC triplet repeat region (ML2344-ML2345, Shin et al., 2000), the AGT triplet region (ML2172-2173, Taylor et al., 2006), the *SigA* (*rpoT*) gene (Matsuoka et al., 2000) and the ML0058 locus (Taylor et al., 2006). Further samples were obtained to extend the number of SNPs studied to nine and also to allow validation of key data at a second centre (see Table 1).

2.1.2. Burial 1914, Ipswich

M. leprae DNA amplified from this case has previously been studied using VNTR analysis (Taylor et al., 2006). It was also found to be typical of European strains, having a SNP-type 3 profile in the scheme proposed by Monot et al. (2005) for the global dissemination of leprosy strains. The earlier report of this case included confirmation of the SNP 3 profile at a second centre, the University of Manchester. In the present study, additional samples (Table 1) were obtained from the left tibia of this individual and subjected to SNP sub-typing for loci 4–9 inclusive and the *rpoT* locus to compare with phylogenetic data obtained from the Uzbek case.

2.2. Contamination control precautions

Measures to prevent cross-over contamination were followed from the time of sampling. Gloves were worn and changed between handling different skeletal components. Samples of bone were removed from the skeleton using sterile disposable scalpel blades and transferred into plastic sterile containers for transport to the laboratories. The work surface was cleaned between samples, using proprietary multi-surface cleaner containing bleach. At UCL this comprises a four-laboratory approach with DNA extraction, PCR set-up, amplification and post-PCR analysis being performed in physically separated laboratories, thus minimizing the chances for air-borne contamination of ancient samples. Separate sets of pipettes were used for PCR set-up and product analysis. The former were stripped and cleaned in detergent and ethanol before each experiment. Filter tips were used routinely. Surfaces and equipment in contact with sample tubes (centrifuges, rotors, mixers, etc.) were cleaned before each assay. A more detailed account of measures taken to avoid cross-contamination have been published (Taylor et al., 2006).

2.3. DNA extraction at centre 1, UCL

Bone fragments from the Uzbek leper and burial 1914 from Ipswich were finely ground in autoclaved pestles and mortars. The NucliSens™ kit from bioMérieux was used to extract DNA from the resulting fine bone powder as previously described by Taylor et al. (2006). DNA extracts were stored at −20 °C until assayed. Extraction blanks ($n = 2$) were included to ensure that the reagents used to prepare the DNA templates were contamination free.