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Original article

Multiple loci variable number tandem repeat (VNTR) analysis (MLVA) of *Mycobacterium leprae* isolates amplified from European archaeological human remains with lepromatous leprosy

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Abstract

Molecular typing methods based on polymorphisms in single nucleotides and short tandem repeat motifs have been developed as epidemiological typing tools for *Mycobacterium leprae*. We have used a variable number tandem repeat method based on three variable loci to identify strain variation in archaeological cases of lepromatous leprosy. The panel of polymorphic loci used revealed unique profiles in five cases of leprosy, including those with identical SNP type and subtype. These were also different from profiles of three previously studied lepromatous skeletons. Whilst examination with SNP typing provides evidence for disease origins, dissemination and phylogeny, tandem repeat typing may be useful for studying cases from within a defined area or community where SNP types may be identical due to geographical constraints. We envisage the technique may be useful in studying contemporaneous burials such as those associated with leprosaria and will prove invaluable in authentication of ancient DNA analyses.
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1. Introduction

Leprosy is a chronic granulomatous disease affecting predominantly the skin and peripheral nerves. The causative organism is *Mycobacterium leprae*, an obligate intracellular pathogen first isolated by Hansen [1]. There is a spectrum of disease severity which ranges from tuberculoid leprosy (TT) through to lepromatous leprosy (LL). Tuberculoid leprosy is characterised by low numbers of bacilli, a good cell-mediated immune (CMI) response and few lesions. At the other end of the spectrum, LL is typified by susceptibility to disseminated infection and bacilli are numerous. A number of intermediate conditions are recognized; borderline tuberculoid leprosy (BT) and borderline lepromatous (BL) leprosy. Key to an effective host CMI response is the ability to recognize mycobacterial lipoproteins, activate appropriate T cell subsets and antimicrobial peptides. There appears to be an effective type 1 cytokine pattern in TT, typified by a predominance of CD4+ T cells, whereas the CD8+ T cells that mediate the type 2 cytokine pattern, including interleukin 4 (IL-4), predominate in LL [2].

Bone can be involved in all types of leprosy but more commonly in LL [3]. In lepromatous disease, many tissues may become infected via the bloodstream, including the skeleton. Bone may also become implicated through direct spread 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 may include resorption of the anterior nasal spine and rounding and widening of the lateral aspects of the pyriform aperture. There

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may also be pitting or perforation of the hard palate and destruction of the premaxillary alveolar process, sometimes leading to loss of the upper incisor teeth. The skeletal lesions identified with LL were first described in depth by Møller-Christensen [4,5].

At some point in prehistory the genome of *M. leprae* has undergone a loss of genetic material compared to other related mycobacteria such as *Mycobacterium tuberculosis.* This reductive evolution has resulted in an organism with a genome of approximately 3.3 Mb in size compared to 4.4 Mb seen in organisms of the *M. tuberculosis* complex. There has been an associated radical reduction in genes encoding protein products (1600 cf. 4000) and accumulation of over 1100 pseudogenes compared to the handful in *M. tuberculosis* [6]. The loss and inactivation of genes is thought to represent adaptation from free-living organism to specialized intracellular parasite in a specific host. Loss of metabolic functions through gene decay explains the inability to culture the organism *in vitro* [7].

An important milestone in the understanding of the disease and its origins was the sequencing of the Tamil Nadu (TN) strain of *M. leprae* (http://mycobrowser.epfl.ch/leprosy.html). Comparisons with closely related genomes and bioinformatics have led to the development of strain typing tools, both for discerning global dissemination of leprosy in prehistory and for short-term transmission *via* family members. From examination of single nucleotide polymorphisms (SNPs) in strains recovered from regions where the disease is still endemic it appears that all present-day cases of leprosy are derived from a clone which probably originated in East Africa and spread with successive human movements around the world [8]. Four main genotype profiles have been recognized and recently, identification of additional phylogenetically informative SNPs has allowed sub-typing of the four main groups into a total of 16 subtypes [9]. SNP type 4 does not show nucleotide base changes but is defined on the basis of insertion and deletions (indels) and homopolymeric tracts (HPT).

Ancient DNA (aDNA) analysis of archaeological material can contribute to our understanding of the phylogeny of the leprosy bacillus by providing data from regions where the disease is no longer extant. For example, we were able to genotype LL cases from Europe and North Africa [9]. The SNP profiles (type 3) showed a strong geographical association and were consistent with the proposed model. The approach is supported by a recent analysis of a case of LL from Japan, dating between the middle 18th–early 19th century, in which the authors showed a SNP type 1 strain in the remains of a mature adult, probably male, skeleton [10]. This is worthy of note as SNP type 3K is predominant in Japan, although SNP types 1A and ID are also found.

M. leprae strains were initially distinguished by using variable number tandem repeat (VNTR) typing based on short tandem repeat (STR) motifs [11,12] and the value of using VNTR typing in the study of short transmission chains in modern cases is now recognized [13]. Families in which several members are affected with leprosy are likely to share a common transmission source. However, in larger epidemiological studies, greater diversity is to be expected due to more complex transmission routes. The tendency for greater genetic variability in simple repeat sequences makes them ideal for studying transmission over relatively short time periods ($\sim$10 years) but limits their use in understanding the evolutionary phylogeny of mycobacteria due to their instability. Generally, there is a need for more information on the stability of specific VNTR loci in *M. leprae.* Conversely, the stability of SNPs in *M. leprae* makes them unhelpful for short-term studies of leprosy transmission.

We have used a panel of three variable and well-studied VNTR loci to type five archaeological cases with known SNP types, including those with identical subtype. We compare the data with previously studied cases and discuss the place for multiple loci VNTR analysis (MLVA) in biomolecular studies of ancient leprosy.

2. Methods

2.1. DNA extraction

Cases 503, 222 and KD271 (Hungary), burial 188 (Czech Republic) and KK ‘02 20/1 (Turkey) were processed as previously reported [14]. In brief, the procedure was as follows. Approximately 25 mg of powdered sample was demineralised 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 0.1 M N-phenacylthiazolium bromide (PTB), a reagent that cleaves glucose-derived protein cross-links [15]. Thereafter, both aliquots were lysed in guanidinium 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.

Cases G708 (Wharram Percy), Ipswich 1914 and the Uzbek leper were extracted using the NucliSens™ DNA isolation kit from bioMérieux (bioMérieux, UK Limited, Hampshire, UK). The sampling and extraction procedure have been reported previously in some detail [16].

In both protocols, extraction negative controls, consisting of reagents less bone powder, were processed alongside samples. Whenever practical, the extracts were assayed before freezing. When this was not convenient they were stored at -20 °C until assay.

2.2. PCR

The loci we have studied for screening and genotyping ancient strains of *M. leprae* are shown in Table 1. We have previously reported sequences of oligonucleotide primers used for MLVA typing [16] and SNP analysis [9].

The Excite core kit (BioGene, Cambridge, UK) was used for all PCR amplifications. SYBR green (BioGene) was included in the PCR master mixes at a final dilution of 1/55,000, and reactions were performed and monitored on a RotorGene 3000 real-time PCR platform (Corbett Research, Sydney, Australia) in a final volume of 25 μl. This included between 1 and 3 μl of DNA template. Forty-five cycles of amplification were performed for all methods. Melt analysis

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