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Detection of Mycobacterial DNA in Andean Mummies

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The identification of genetic material from pathogenic organisms in ancient tissues provides a powerful tool for the study of certain infectious diseases in historic populations. We have obtained tissue samples from the genital areas of 12 mummies in the American Museum of Natural History collection in New York, N.Y. The mummies were excavated in the Andes Mountain region of South America, and radiocarbon dating estimates that the mummies date from A.D. 140 to 1200. DNAs were successfully extracted from all tissues and were suitable for PCR analysis. PCRs were carried out to detect *Mycobacterium tuberculosis* complex and mycobacteria other than *M. tuberculosis* (MOTB). *M. tuberculosis* complex was detected in 2 out of 12 samples, and MOTB were detected in 7 samples. This study confirmed the adequate preservation of genetic material in mummified tissues and the existence of mycobacteria, including *M. tuberculosis*, in historic populations in South America.

The identification of genetic material from pathogenic organisms in ancient tissues provides a powerful tool for the study of certain infectious diseases in historic populations. Specifically, the analysis of ancient bacterial DNA may help clarify the uncertainty of macromorphologic analyses. Moreover, the identification of bacterial DNA provides direct evidence of the occurrence and frequency of infectious diseases in historic populations and may provide information about the evolution of microorganisms and their associated diseases (5). Environmental conditions have ensured the preservation of a wealth of evidence from antiquity that provides information about diseases of the time.

Molecular studies performed to date on bacteria and other microorganisms in ancient human remains have mainly addressed typing and characterization of pathogen DNA. Several recent reports describe the isolation of *Mycobacterium tuberculosis* in ancient human skeletal remains and soft tissue remains (1, 2, 6, 8, 10–12). We used PCR analysis of genital tissue samples from 12 ancient mummies from South America in order to detect the presence of mycobacteria in this population.

Twelve dried tissue samples were obtained from mummies in the collection of the American Museum of Natural History in New York, N.Y. Archaeological findings and radiocarbon dating estimate that the mummies date to before A.D. 1220. The tissue samples, taken from histologically confirmed skin samples in the pelvic region, were in dried form. Specifically, skin samples were taken from preserved genitalia when identifiable or from adjacent skin. Positive controls of *M. tuberculosis* specimens were obtained from a clinical laboratory.

Mummy tissue samples were cut into small fragments (5 mm^3), placed in 1.5-ml microcentrifuge tubes, homogenized in 50 to 100 μl of phosphate-buffered saline (PBS) (Sigma, St. Louis, Mo.) with a homogenizer, and further diluted with 1,000 μl of PBS. After centrifugation, the supernatants were aspirated and the pellets were washed with PBS three times. The pellets were lysed in a 5 M guanidinium thiocyanate (GTC) buffer containing 5 M GTC (Sigma), 0.5% bovine serum albumin (Sigma), 80 mM EDTA, 400 mM Tris HCl (pH 7.5), and 0.5% sodium-*N*-lauroylsarcosine (Sigma) at 60°C for 1 h and then at 37°C overnight (7). DNA was extracted twice with phenol-chloroform (Sigma) at a 1:1 ratio, followed by chloroform once, and then precipitated by the addition of a 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol. The pellets were washed with 70% ethanol and air dried. They were dissolved in Tris-EDTA (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) buffer (Sigma) and stored at −20°C for later use.

PCR was carried out in 50 μl of a reaction mixture composed of 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, various concentrations of each primer, 2 U of AmpliTaq Gold DNA polymerase (Roche, Indianapolis, Ind.), 50 mM KCl, and 10 mM Tris-HCl (pH 8.3). The PCR was initiated by preheating the mixture at 95°C for 10 min, followed by temperature cycles (for GAPDH [glyceraldehyde-3-phosphate dehydrogenase], 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 40 cycles; for *M. tuberculosis*, 94°C for 1 min, 64°C for 30 s, and 72°C for 1 min for 40 cycles; for MOTB, 94°C for 1 min, 60°C for 30 s, and 72°C for 1 min for 45 cycles), in a thermal cycler (Perkin-Elmer model 9600). These temperature cycles were followed by a final extension step at 72°C for 5 min. PCR primers were used to detect the GAPDH gene (TCACT GCCACCCAGAAGACT and TTCTAGACGGCAGGTCAG GT) (15), *M. tuberculosis* (Tb-A, CTCGTCCAGCGCCGCTT CGG; Tb-B, CCTGCGAGCGTAGGCGTCGG) (4, 10), and MOTB (Tb11, ACCAACGATGGTGTGTCCAT; Tb12, CTT GTCGAACCGCATACCCT) (9, 13). To increase the detection sensitivity, a second PCR was carried out for *M. tuberculosis*. Two microliters of the first PCR product was transferred to a tube containing the second set of PCR primers (Tb-C, GCTTCGGACCACCAGCACCT; Tb-D, GCGTCGGTGAC

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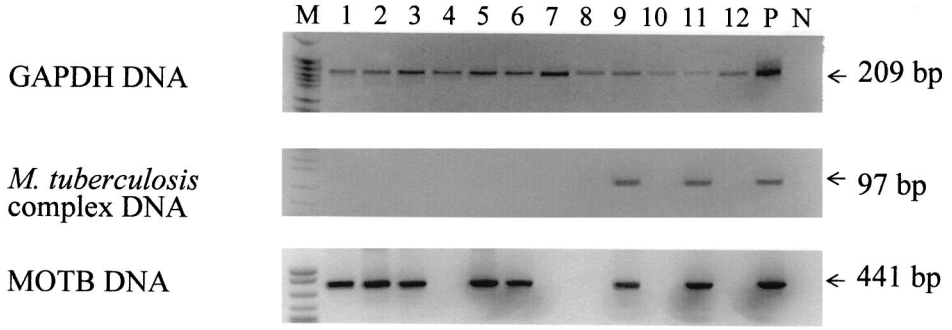

FIG. 1. Detection of GAPDH and mycobacterial DNA in mummified tissues. DNAs were extracted from the genital tissues of 12 mummies. The PCR products were examined on a 10% polyacrylamide gel. GAPDH DNA was detected in all mummified tissues, indicating that the DNA was adequate for PCR analysis. DNAs of *M. tuberculosis* complex were detected in 2 of the 12 samples, and MOTB DNAs were detected in 7 of the 12 samples. Lane M, DNA size markers (PBR322 DNA digested with *Msp*I); lanes 1 to 12, mummy samples; lane P, positive control; lane N, negative control.

AAAGGCCAC) and amplified with the appropriate temperature cycles (94°C for 1 min, 50°C for 30 s, and 72°C for 1 min for 40 cycles). The PCR products were examined by electrophoresis and visualized under UV light after being stained with ethidium bromide.

Restriction fragment length polymorphism analyses were used to ensure that the PCR products were specific for the *M. tuberculosis* complex (4, 10) and to specify MOTB. For *M. tuberculosis,* the second group of PCR products was digested with 10 U of *Sal*I (GIBCO BRL, Grand Island, N.Y.) in a 20-μl reaction mixture in the presence of the appropriate buffer at 37°C for 3 h. For MOTB (9, 13), the PCR products were analyzed by digestion with 7 U of *Bst*EII (GIBCO BRL) for 1 h at 60°C or with 10 U of *Hae*III (GIBCO BRL) for 1 h at 37°C in a 20-μl reaction mixture with the appropriate buffer.

To confirm that the DNA in mummy tissues was still intact after hundreds of years and that the DNA could be detected in and extracted from the dried samples, the GAPDH gene was used as a marker DNA for PCR. Our results showed that DNAs were efficiently isolated from mummy tissues by the 5 M GTC method and that they were adequate for PCR amplification. Human genomic DNA was detected in all samples by PCR with the GAPDH gene (Fig. 1).

In two samples (no. 9 and 11), a 97-bp DNA fragment was detected by PCR (Fig. 1) with primers specific for insertion sequence IS*6110*, which is unique to the *M. tuberculosis* complex (14). This fragment was further digested with *Sal*I and gave rise to two expected fragments of 42 and 55 bp (Fig. 2), confirming the presence of *M. tuberculosis* complex in these two samples. The IS*6110* element, which was initially identified from a clinical isolate of *M. tuberculosis,* is specific for the *M. tuberculosis* complex (*M. tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium simiae*). Generally, the IS*6110* element is present in high copy numbers in most strains of *M. tuberculosis* and low copy numbers in *M. bovis* strains (3). The specificity of detecting *M. tuberculosis* complex by PCR with the primers for IS*6110* was confirmed by Eisenach et al. (4). The primers detected strains of *M. tuberculosis*, *M. bovis*, and *M. simiae*. The IS*6110* sequence was also detected in mummified tissues (10), and it was found to be identical to that of contemporary *M. tuberculosis* as reported by Eisenach et al. (4). In this study, we applied nested PCR to detect the IS*6110* sequence in ancient DNA in order to increase sensitivity and specificity. Our results confirmed the presence of *M. tuberculosis* complex in 2 of 12 mummy samples, although we could not determine the species in the *M. tuberculosis* complex.

A 441-bp DNA fragment was amplified in seven samples (no. 1, 2, 3, 5, 6, 9, and 11) (Fig. 1) by using primers specific for the 65-kDa heat shock protein present in all MOTB (9, 13). The DNA fragments were further digested with *Bst*EII and *Hae*III to determine the species of MOTB according to the algorithm reported by Telenti et al. (13) and Rastogi et al. (9). Six samples could not be digested by *Bst*EII but were able to be digested with *Hae*III and gave rise to a 140-bp band (Fig. 3). Based on band size and patterns, they were considered to be *Mycobacterium flavescens* I. In one sample (no. 1), two DNA fragments were observed after digestion with *Bst*EII and three

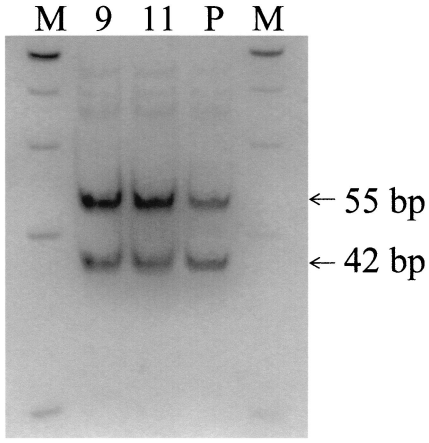

FIG. 2. Confirmation of *M. tuberculosis* complex by *Sal*I digestion. PCR products from two positive samples and one positive control were further digested with *Sal*I and separated on a 10% polyacrylamide gel. We observed two bands (55 and 42 bp), which are identical to those of the positive control (*M. tuberculosis*). These results confirmed the presence of *M. tuberculosis* complex in these two samples. Lane M, DNA size markers (PBR322 DNA digested with *Msp*I); lanes 9 and 11, mummy samples 9 and 11, respectively; lane P, positive control (*M. tuberculosis*).

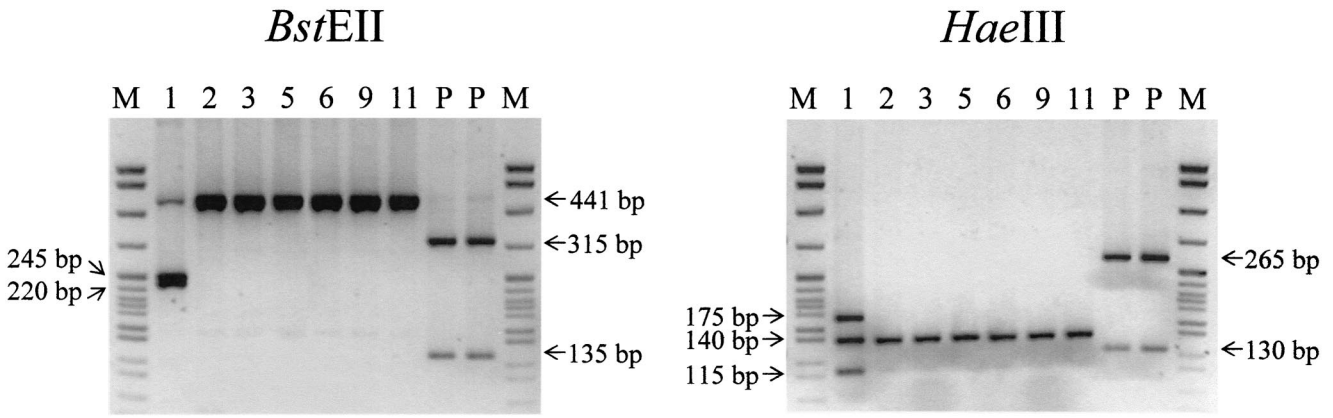

FIG. 3. Identification of MOTB by *Bst*EII and *Hae*III. PCR products from seven positive samples were further digested with *Bst*EII and *Hae*III and separated on a 2.5% agarose gel. A 441-bp band was seen in all seven samples after digestion with *Bst*EII. However, sample 1 gave rise to two bands of 245 and 220 bp. After digestion with *Hae*III, a 140-bp band was seen in all samples; however, two bands of 175 and 115 bp were observed in sample 1. The results suggest that *M. flavescens* I was present in all samples and that there was an additional species present in sample 1. Lane P, positive control (*M. leprae*); lanes M, DNA size markers; lanes 1, 2, 3, 5, 6, 9, and 11, mummy samples 1, 2, 3, 5, 6, 9, and 11, respectively.

fragments were observed after digestion with *Hae*III (Fig. 3). With these patterns, the fragments could not be assigned to a specific species. However, we believe that there may be two species present in this sample, i.e., *M. flavescens* I and another MOTB not included in the algorithm. Since these mycobacteria are present in soil and water, we do not believe that their identification indicates the presence of clinical disease; this is in contrast to *M. tuberculosis*, which must be considered pathogenic.

We also tested samples for other infectious agents, including *Mycobacterium leprae*, *Treponema pallidum*, *Leishmania* spp., herpes simplex virus, human papillomavirus, and human T-cell lymphotropic virus type 1. No PCR products were observed.

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