CONCISE COMMUNICATIONS

Identification of *Treponema pallidum* Subspecies *pallidum* in a 200-Year-Old Skeletal Specimen

Connie J. Kolman,[1,a] Arturo Centurion-Lara,[2] Sheila A. Lukehart,[2] Douglas W. Owsley,[3] and Noreen Tuross[1]

[1]*Smithsonian Center for Materials Research and Education, Suitland, Maryland;* [2]*Department of Medicine, University of Washington, Seattle;* [3]*Department of Anthropology, Smithsonian Institution, Washington, DC*

Treponema pallidum subsp. *pallidum*, the causative agent of venereal syphilis, was detected in a 200-year-old skeletal specimen from Easter Island. An initial diagnosis of treponemal infection was confirmed by extensive purification of immunoglobulin that reacted strongly with *T. pallidum* antigen. Extracted DNA exhibited a single-base polymorphism that distinguished *T.p.* subsp. *pallidum* from 4 other human and nonhuman treponemes. Extensive precautions against contamination of the subject matter with modern treponemal DNA were employed, including analysis of archaeological and modern specimens in 2 geographically separate laboratories. Molecular determination of historical disease states by using skeletal material can significantly enhance our understanding of the pathology and spread of infectious diseases.

Reconstructing the evolutionary history of infectious diseases such as venereal syphilis, influenza, and AIDS holds enormous potential for advancing our understanding of their current pathology and for guiding health policies designed to control them. However, the diagnosis of disease states in ancient populations is generally limited to a classification of osseous and tissue lesions that typically are pathognomonic only in the extreme stages of a few diseases. Immunologic assays can provide an initial identification of pathogen class, but in some cases, more detailed nucleotide sequence information is needed to distinguish among pathogens. Subspecies- and strain-specific DNA polymorphisms are currently being detected for many pathogens with increasing speed because of the advent of polymerase chain reaction (PCR)–based methodologies and whole genome sequencing [1, 2]. Knowledge of the pervasive nature of contamination in ancient DNA (aDNA) technology has enhanced our ability to detect contamination, permitting a high degree of confidence when analyzing ancient, nonhuman organisms [3].

An ideal opportunity to demonstrate the efficacy of molecular methods in elucidating specific disease states exists with *Treponema pallidum* subsp. *pallidum*, the causative agent of ve-nereal syphilis. Four treponematoses have been described in humans with causative agents that are distinguished at the species and subspecies level: venereal syphilis/*T.p.* subsp. *pallidum*, endemic syphilis/*T.p.* subsp. *endemicum*, yaws/*T.p.* subsp. *pertenue*, and pinta/*T. carateum*. Definitive evidence for the existence of distinct treponemal subspecies has been difficult because, until recently, it was not possible to discriminate between different treponemes. All human treponemes appear morphologically identical and exhibit antigenic cross-reactivity. Furthermore, the genomes of all noncultivable treponemes are highly homologous, and efforts to distinguish between subspecies based on individual DNA polymorphisms have been plagued with difficulty. However, Centurion-Lara et al. [1] have recently identified a single-base mutation in the 5′ untranslated region of the 15-kDa lipoprotein (tpp15) [4] that is unique to *T.p.* subsp. *pallidum* and creates an *Eco*47III site.

The diagnosis of a specific treponematosis is equally difficult in skeletal specimens. With the exception of pinta, which is essentially a dermatologic disease, skeletal markings left by the other 3 treponematoses are virtually indistinguishable, with differences only in the distribution and frequency of lesions. Variability in osteological lesions reported for different treponemal syndromes [5] would require multiple and virtually complete skeletal remains from any given site or period to determine an accurate diagnosis. The difficulty of diagnosis in skeletal specimens is further compounded by the similarity of treponemal lesions to other conditions, such as chronic osteomyelitis, symmetric osteoporosis, rickets, hereditary anemia, and metastatic carcinoma [6–8].

The growing number of pathogen aDNA studies [9, 10] attests to the merit of nucleic acid–based identification of disease states in archaeological specimens. However, these studies suffer

from the common practice of inclusion of a positive PCR control of DNA extracted from modern specimens. Modern DNA can inadvertently contaminate the archaeological specimen and provide template for the PCR even when control DNA is stored separately from aDNA samples [3]. The finding of identical DNA sequences from an archaeological specimen and control DNA, as determined in previously published studies [9, 11], precludes convincing proof that ancient DNA was extracted and analyzed.

Herein, we report the use of 2 molecular methods, immunological assay and determination of DNA sequence variation, to identify the presence of *T.p.* subsp. *pallidum* in a skeletal specimen from Easter Island, RHO235, with markings indicative of treponemal infection. The general diagnosis of treponemal infection was confirmed through extensive purification of IgG that reacted strongly with *T. pallidum* antigen. This approach has the distinct advantage of being far less prone to contamination and false-positive results than aDNA PCR amplification. However, IgG reactivity cannot distinguish among treponemal subspecies. Subspecies identification was provided by direct DNA sequence determination of PCR amplicons and DNA sequence determination of cloned amplicons that confirmed the presence of the *T.p.* subsp. *pallidum*-specific mutation in the 5′ flanking region of *TPP15*. All experiments on the Easter Island specimen were conducted at the Smithsonian Center for Materials Research and Education (SCMRE), while all experiments utilizing modern treponemal DNA, such as primer optimization, were conducted at the University of Washington (UW). These results confirm the first definitive diagnosis of venereal syphilis based on DNA variation in a skeletal specimen. Confirmation and classification of treponemal subspecies present in clinical, forensic, and paleopathological specimens will greatly enhance epidemiologic, evolutionary, and anthropological studies of *T. pallidum*.

Materials and Methods

Sample. Specimen RHO235 was excavated on Easter Island. Abnormal skeletal lesions included anterior curvature and subperiosteal thickening of the diaphyses of the tibiae, that is, "saber shins" [12]. The bone collagen was dated by accelerator mass spectrometry radiocarbon measurement that produced an uncalibrated age of 240 ± 50 years before the present (TO-7540).

IgG purification. Femoral bone was powdered by using a Spex mill (Spex Industries, Edison, NJ). Fifteen grams of powdered bone was dialyzed against 0.5 M EDTA, pH 7.3, at 4°C for 3 days, after which the solution was concentrated by YM30 (Amicon, Beverly, MA) filtration to a volume of ~2 mL. Immunoglobulins of the IgG class were isolated by high-performance liquid chromatography (HPLC) purification over a protein A column (Pierce, Rockford, IL) that was equilibrated with phosphate-buffered saline, pH 7.4. Samples were loaded in a 0.1 M phosphate buffer, pH 8.0, and eluted with 0.1 M sodium citrate, pH 3.0-3.5. The HPLC eluant was immediately brought to neutrality upon collection in tubes preloaded with 0.4 mL Tris-HCl, pH 8.5.

ELISA. Purified IgG fractions and whole bone extracts were tested in an ELISA assay against *T. pallidum* antigen (Clark Laboratories, Jamestown, NY). Reactivity was assayed by optical density measurements at 490 nm. Each sample was tested in 2 separate experiments in duplicate or triplicate as sample volume allowed. A second Easter Island specimen with no evidence of treponemal infection, RHO267, was processed as a negative control. High- and low-positive controls were analyzed to establish the positive range for immunoreactivity.

DNA extraction and purification. Decalcification of bone fragments was performed as described earlier for ~3 weeks. EDTA solution was removed from the dialysis tubing after 1.5 weeks and replaced with fresh solution, which was removed after an additional 1.5 weeks. The 2 EDTA extracts were processed separately. Extracts were dialyzed against 6 changes of double distilled water and were concentrated by partial freeze-drying. Further purification was not necessary.

DNA amplification. Primers L243 (5′-GAGCAGGATGTC-TCTATGAGTTATAAAGAG-3′) and H123 (5′-GAAGCCACT-ACCGATGTGCG-3′) were used to amplify a portion of the 5′ flanking region of *TPP15*. Amplification was performed in a 25-μL reaction containing GeneAmp buffer (PE Applied Biosystems, Foster City, CA), 1.75 mM MgCl$_2$, 200 μM of each dNTP, 0.5 μM of each primer, 400 μM spermidine, and 3.0 U AmplitaqGold (PE Applied Biosystems). Thermal cycler conditions consisted of an initial 12-min incubation at 95°C followed by 50 cycles of 1 min at 94°C, 2 min at 60°C, and 1 min at 72°C and a final 5-min extension step at 72°C. Primers were tested and PCR conditions were optimized at the UW by using *T. pallidum* Nichols strain.

Restriction fragment length polymorphism analysis. Restriction digests were performed directly on amplification products by use of 10 U of *Eco*47III (New England Biolabs, Beverly, MA) and overnight incubation at 37°C. Digestion products were analyzed by electrophoresis through 4% Metaphor gels (FMC BioProducts, Rockland, ME).

DNA sequence analysis. PCR products were separated by gel electrophoresis, excised as agarose bands, purified by use of a GeneClean II kit (Bio101, La Jolla, CA), and resuspended in 10 μL of ddH$_2$0. PCR products were then either sequenced directly or ligated into pCR-Script SK(+) by using the PCR-Script Cloning kit and cloned in XL1-Blue MRF′ cells (Stratagene, La Jolla). Sequencing was performed by using M13 or PCR primers, DNA Sequencing Kit, FS version (PE Applied Biosystems), and a Perkin-Elmer 373 DNA Sequencer. DNA sequence data were collected for both light and heavy strands between bp −223 and −145 of the 5′ flanking region of *TPP15*.

Contamination precautions. All analyses of archaeological material were conducted at the SCMRE. No studies of modern treponemes were ever conducted at the SCMRE. Positive PCR controls were never used. All pre-PCR protocols were performed in a laboratory designed with positive hepa-filtered air pressure and dedicated to aDNA work. PCR and post-PCR analyses were conducted in separate wings of the building. All reagents were purchased as ready-made solutions (Amresco, Solon, OH). Disposable lab coats, gloves, filter tips, dedicated pipetmen, and disposable laboratory ware were used throughout the analyses. All PCRs involving modern treponemal DNA—for example, optimization of primers—were performed at the UW.

Table 1. Bone sample reactivity with *Treponema pallidum* antigen.

Sample	OD$_{490\ nm}$ (SD)[a]	No. of tests
RHO235, purified IgG	1.12 (.03)	4
RHO235, whole bone extract	.11 (.04)	6
RHO267, purified IgG	.06 (.03)	4
RHO267, whole bone extract	.09 (.03)	6
High-positive control	.94 (.05)	6
Low-positive control	.45 (.03)	6

[a] Optical density reading at 490 nm (± SD).

Results

Immunological assay. Purified IgG fraction from specimen RHO235 demonstrated significant antigen binding that was indicative of treponemal reactivity (table 1). The amount of binding observed with a similar fraction from negative control RHO267 was not in the positive range established by the treponemal IgG controls. The extensive purification of IgG that was performed herein was necessary for antigen binding, as evidenced by the negligible binding observed with whole bone extracts from either sample.

DNA analysis. PCR amplifications were recovered only from the second EDTA DNA extract collected during days 12–24 of the bone decalcification procedure. Spermidine was essential for successful amplification. Amplification was extremely dependent on the amount of DNA extract added to the reaction, and 0.04 μL extract/10 μL PCR was empirically determined to be the optimal concentration.

Successful amplification by use of the second EDTA extract produced a fragment of the expected size of 120 bp. *Eco*47III digestion produced a fragment consistent with the expected size of 92 bp (figure 1). It was not possible to resolve the smallest digestion fragment of 28 bp from primer dimer products. DNA sequence was determined directly from amplification fragments and from cloned amplicons. All DNA sequences identified the 5′ flanking region of *TPP15*. The cytosine at position −150, diagnostic for *T.p.* subsp. *pallidum* [1], was present in all DNA sequences (figure 1).

Discussion

Reconstruction of the origin and evolution of disease states is significantly enhanced by a comparison of data from modern and ancient specimens. A decade ago, diagnosis of infectious diseases in ancient populations was generally limited to a visual inspection of skeletal and soft-tissue remains. In the current study, immunological analysis of a skeletal specimen from Easter Island confirmed a diagnosis of treponemal infection, and DNA sequence analysis enabled specific identification of *T.p.* subsp. *pallidum*, the causative agent for venereal syphilis. Skeletal material is a common tissue source at archaeological sites, and previous research suggests that extraction of amplifiable DNA is typically more successful from bone than from

soft tissue [13]. The archaeological record furnishes excellent time depth and geographical breadth, and often a synchronous historical record is available for comparison. Thus, skeletal material represents a valuable source of information on infectious
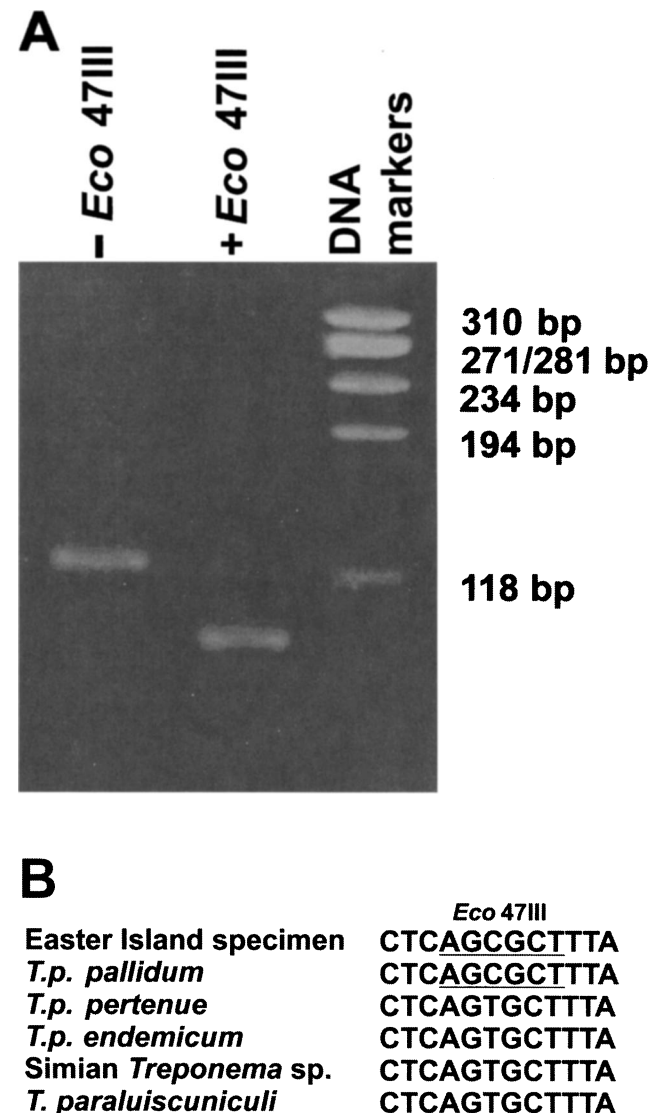

A

310 bp
271/281 bp
234 bp
194 bp

118 bp

B

	Eco 47III
Easter Island specimen	CTC**AGCGCT**TTA
T.p. pallidum	CTC**AGCGCT**TTA
T.p. pertenue	CTCAGTGCTTTA
T.p. endemicum	CTCAGTGCTTTA
Simian *Treponema* sp.	CTCAGTGCTTTA
T. paraluiscuniculi	CTCAGTGCTTTA

Figure 1. Analysis of DNA extracted from Easter Island specimen, RHO235. *A*, DNA was amplified with primers specific for the 5′ untranslated region of a 15-kDa lipoprotein, tpp15, and then digested with *Eco*47III. Undigested and digested polymerase chain reaction (PCR) products were electrophoresed in the first and second lanes, while ΦX174/*Hae*III DNA size markers were run in the third lane. *B*, comparison of DNA sequence from the Easter Island specimen and published sequences from 3 human pathogenic treponemes (*Treponema pallidum* subspecies *pallidum*, *T.p.* subsp. *pertenue*, *T.p.* subsp. *endemicum*), 1 simian treponeme, and 1 rabbit treponeme [1]. Sequence was determined for the Easter Island specimen directly from amplification products and from cloned PCR amplicons. The recognition site for *Eco*47III is underlined.

diseases, particularly for those diseases with significant osseous involvement, such as treponematosis; venereal syphilis is estimated to have caused skeletal lesions in 0.5%–1.0% of urban populations prior to the advent of penicillin treatment [14].

Identification of *T.p.* subsp. *pallidum* DNA in a skeletal specimen represents the first nucleic acid–based confirmation of a morphologically identified treponemal infection. The age of the specimen is consistent with exposure to venereal syphilis during one of the European voyages of the 1700s, although variations in the ^{14}C calibration curves make it impossible to rule out a date earlier by ~100 years. Analysis of additional specimens with treponemal-type lesions is now warranted to aid in the reconstruction of the evolution of treponematosis. Debate over the origin of venereal syphilis has focused on whether or not the disease was endemic to the New World prior to European contact. Molecular determination of *T. pallidum* subspecies in archaeological specimens from critical geographic and temporal periods could provide the answer to this long-standing question and also furnish essential information on the evolutionary relationship of the other treponemal subspecies. Reconstruction of the evolutionary history of venereal syphilis holds enormous potential for advancing our understanding of its pathology and for guiding future research in areas such as spread of the disease and vaccine development.

Acknowledgments

References

1. Centurion-Lara A, Castro C, Castillo R, Shaffer JM, Van Voorhis WC, Lukehart SA. The flanking region sequences of the 15-kDa lipoprotein gene differentiate pathogenic treponemes. J Infect Dis 1998;177:1036-40.
2. Fraser CM, Norris SJ, Weinstock GM, et al. Complete genome sequence of *Treponema pallidum,* the syphilis spirochete. Science 1998;281:375-88.
3. Kolman CJ, Tuross N. Ancient DNA analysis of humans. Am J Phys Anthropol (in press).
4. Purcell BK, Swancutt MA, Radolf JD. Lipid modification of the 15 kDa major membrane immunogen of *Treponema pallidum.* Mol Microbiol 1990;4:1371-79.
5. Rothschild BM, Rothschild C. Treponemal disease revisited: skeletal discriminators for yaws, bejel, and venereal syphilis. Clin Infect Dis 1995;20: 1402-08.
6. Williams HU. Human paleopathology. Arch Pathol 1929;7:839-902.
7. Steinbock RT. Paleopathological diagnosis and interpretation. Springfield, IL: Thomas Books, 1976:137.
8. Ortner DJ, Putschar WGJ. Identification of pathological conditions in human skeletal remains. Washington, DC: Smithsonian Institution Press, 1985: 104-234.
9. Salo WL, Aufderheide AC, Buikstra J, Holcomb TA. Identification of *Mycobacterium tuberculosis* DNA in a pre-Columbian Peruvian mummy. Proc Natl Acad Sci USA 1994;91:2091-4.
10. Drancourt M, Aboudharam G, Signoli M, Dutour O, Raoult D. Detection of 400-year-old *Yersinia pestis* DNA in human dental pulp: an approach to the diagnosis of ancient septicemia. Proc Natl Acad Sci USA 1998;95: 12637-40.
11. Jackson PJ, Hugh-Jones ME, Adair DM, et al. PCR analysis of tissue samples from the 1979 Sverdlovsk anthrax victims: the presence of multiple *Bacillus anthracis* strains in different victims. Proc Natl Acad Sci USA 1998;95: 1224-29.
12. Owsley DW, Gill GW, Ousley SD. Biological effects of European contact on Easter Island. In: Larsen CS, Milner GR. In the wake of contact: biological responses to conquest. New York: Wiley-Liss, 1994:161–77.
13. Höss M, Dilling A, Currant A, Pääbo S. Molecular phylogeny of the extinct ground sloth *Mylodon darwinii.* Proc Natl Acad Sci USA 1996;93:181-5.
14. Baker BJ, Armelagos GJ. The origin and antiquity of syphilis. Curr Anthropol 1988;29:703-37.