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Journal of Archaeological Science 34 (2007) 2091–2100

Journal of Archaeological SCIENCE

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

Digging deeper into the limits of ancient DNA research on syphilis

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Received 6 September 2006; received in revised form 18 January 2007; accepted 25 February 2007

Abstract

The search for the origins of syphilis has a long history in the medical and anthropological literatures. If we know more about the emergence of the pathogen that causes the disease in humans we will understand its evolution through time and space as well as shed light on its current state in living populations. Ancient DNA techniques used to isolate *Treponema pallidum* subsp. *pallidum* DNA from archaeological human specimens provide direct evidence of its existence in the past. However to date, only Kolman et al. (1999) have been successful in this endeavour, while other attempts have failed (e.g., Barnes and Thomas, 2006; Bouwman and Brown, 2005). Why has there been little success? This paper serves to compliment and add relevant information to Bouwman and Brown's and Barnes and Thomas' discussion concerning our inability to apply ancient DNA techniques to study venereal syphilis in past human populations.

Our approach utilized 15 different human specimens from different geographies and different temporal periods: eight samples come from medically diagnosed individuals archived during the American Civil War period; six originate from the United Kingdom and predate 1492 with four of these samples having been previously analyzed by Bouwman and Brown and one sample comes from historic Canada. Human mitochondrial and amelogenin DNA, as well as several genes from the *Treponema* organism were analyzed revealing the relatively good preservation of human multi-copy and single copy DNA but not treponemal DNA. This study also incorporates a unique molecular experiment using rabbits infected with venereal syphilis to help illustrate that treponemal DNA disseminates to bone early during the first stages of infection but is not present in later stages of the disease using the techniques presented in this study.

Keywords: Ancient DNA; Syphilis; Treponemal DNA; Human bone; Rabbit bone; Paleopathology

1. Introduction

For many physical anthropologists, epidemiologists, molecular biologists and other researchers, the search for ancient pathogen DNA (aDNA) is considered the ultimate methodology for studying disease in the past. In fact, the molecular approach appears to have provided a great deal of information regarding pathogens such as *Mycobacterium tuberculosis* (e.g., Bathurst and Barta, 2004; Fletcher et al., 2003; Rothschild et al., 2001; Taylor et al., 1996; Taylor et al., 2005), *Mycobacterium leprae* (e.g., Donoghue et al., 2005; Haas et al., 2000; Taylor et al., 2000), Influenza (e.g., Reid et al., 1999), *Plasmodium falciparum* (e.g., Sallares and Gomzi, 2001), *Trypanosoma cruzi* (e.g., Aufderheide et al., 2004), and *Yersinia pestis* (e.g., Drancourt et al., 1998), etc. However, recent research (e.g., Barnes and Thomas, 2006; Bouwman and Brown, 2005; Gilbert et al., 2004) is questioning earlier optimistic results and suggesting that ancient pathogen DNA may not always be available for study. Understanding the reasons why is an important step in limiting the destruction of precious human material that may be studied using other methods (e.g., macroscopy and histology).

Venereal syphilis is a complex disease with symptoms that wax and wane through three distinct stages when left untreated.

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0305-4403/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jas.2007.02.007

The primary stage of syphilis infection is generally isolated to a cutaneous lesion, known as a chancre, at the site of inoculation. This lesion disappears within several weeks with other symptoms appearing in the secondary phase of the disease in the possible forms of a skin rash, condyloma lata, lymphadenopathy as well as other systemic symptoms due to hematogenous dissemination of the bacteria. Once again, signs and symptoms disappear within several weeks and years may pass (typically 2–10 years) before the tertiary stage of syphilis is encountered. Typically, it is during this final stage where skeletal alterations (e.g., gummatous and nongummatous lesions primarily affecting the tibial, cranial vault and nasal areas) are most predominant. In addition, patients can also suffer from cardiovascular and neurological implications as well. The chronic nature of this disease and its ability to affect bone have created the opportunity to study its impact on human populations in antiquity.

The first issue concerning our understanding of the pathophysiology of syphilis and its feasibility in a molecular study in ancient or archived specimens pertains to whether the *Treponema* bacteria and/or its DNA will disseminate to the skeletal matrix. Although the presence of preserved treponemal organisms in bone is contentious, spirochetes have been found in osseous materials by several researchers. For example, in 1923 Schneider (cited by Jaffe, 1972:928) reported observing upon autopsy resting spirochetes in bone lacunae in congenitally affected infants. The author suggests that with bone turnover, the spirochetes can be released causing a new bout of infection, but with immune response and lesion formation, spirochetes are killed and degenerated. This leads to the question as to why no organisms are found in areas of bone lesions. Several years later, Bauer (1944) also reported seeing large numbers of spirochetes, but this time in the upper and lower jaws (e.g., tooth germ, pulp, uncalcified and calcified dentine, dental follicle and enamel epithelium) of a macerated fetus with congenital syphilis. In retrospect, it is quite possible that he was observing *Treponema denticola* spirochetes, which are regularly found in mouths of individuals with periodontal disease (Riviere et al., 1991), and not *T.p.* subsp. *pallidum* spirochetes. To the best of our knowledge, no new studies have been able to duplicate this observation due to the differences in clinical research approaches (i.e., concentration on sera, lesion exudates, spinal fluid or organs as targeted areas for analysis and not bone) and the fact that few people retain bone lesions (if they manifest them) at their time of death due to chemotherapy used either for syphilis treatment or other penicillin-treatable illnesses. To address this question more specifically, this paper reviews the history of diagnostic studies of syphilis and outlines a unique rabbit experiment using molecular techniques that test the hypothesis that *T.p.* subsp. *pallidum* disseminates throughout the skeleton.

Today, clinical molecular research provides proof that treponemal DNA can be targeted via PCR from different biological samples from people in various stages of the disease (e.g., Kouznetsov et al., 2005; Marfin et al., 2001; Pietravalle et al., 1999). For example, Hollier et al. (2001) were able to detect DNA from amniotic fluid taken from mothers in all three stages of syphilis, including the early latent stage (4/5 were successful). This study demonstrates that ''spirochetemia can occur throughout the course of *T. pallidum* infection'' (Marfin et al., 2001:163). Since *T. pallidum* DNA can be isolated during later stages of the disease, it stands to reason that it should be possible to isolate it from skeletons displaying bone lesions.

The hypothesis that the detection of treponemal DNA should be possible in archaeological remains also stems from earlier studies using paraffin-embedded tissue (e.g., Burstain et al., 1991). Due to their promising results, Burstain et al. (1991:66) predicted that archival specimens could be used to help ''resolve a number of questions concerning syphilis in the pre-antibiotic and perhaps even the prehistoric eras''. Zoechling et al. (1997) also presented an impressive study using formalin-fixed, paraffin-embedded biopsy specimens from skin lesions of patients with secondary and tertiary syphilis.[1] They were able to isolate treponemal DNA from both late secondary and tertiary syphilis (gumma) specimens, but at a relatively low success rate (4/6 and 1/7, respectively). What is so interesting about this particular research is that the specimens obtained from the patients are dated between 1966 and 1981. Therefore, these 'archival' samples represent the closest simulation of 'ancient' samples found in the literature. Also, Zoechling et al. (1997) amplified a relatively small fragment (196 bp) using nested PCR (original target was of a 379-bp fragment from the 47-kDa membrane protein), a technique which resembles aDNA methodologies for isolating smaller amplicons.

Attempts to isolate treponemal (syphilitic) DNA from ancient human material was first tried on muscle tissue from mummified individuals displaying 'tertiary treponematosis' excavated from El Morro, Arica and San Miguel de Azapa, Chile (Rogan and Lentz paper presented in the Paleogenetics Workshop at the World Congress on Mummy Studies, 1995). Rogan and Lentz were successful in amplifying DNA most likely of spirochete origins, but not specific to *T. pallidum*. A molecular approach was also attempted to confirm venereal syphilis in Maria of Aragon, a mummy from the abbey of San Domenico Maggiore in Naples from the Renaissance period (Rollo et al., 1996). Desiccated cellular material was scraped from a linen dressing and tested using a nonspecific 16S ribosomal rRNA gene with the results showing an 85% base similarity to *T. pallidum* when directly sequenced. Unfortunately, when the amplicons were cloned, no *T. pallidum* DNA could be found, but instead many other genera were isolated (e.g., *Propionibacterium*, *Mycobacterium*, *Peptostreptococcus*, *Clostridium* and *Capnocytophaga*) (unspecific results were also observed by Barnes and Thomas, 2006 and Bouwman and Brown, 2005). Despite this, the researchers believe that indirect confirmation of venereal syphilis was obtained through the historical medical documents which prescribed 'salivation cures' to those suffering from '*Morbus gallicus*' (venereal syphilis)

[1] Unfortunately the work performed for this study had a limited adherence to aDNA standards: disposable labware was used for all processes; a separate room was used for extraction procedures; and negative controls remained negative; but positive amplification products were checked via restriction enzyme digestion only without any direct sequencing or cloning involved.

and the presence of human oral cavity microorganisms (i.e., *Propionibacterium*, *Peptostreptococcus* and *Capnocytophaga*) in the bandage (Rollo et al., 1996).

The only successful amplification *specific* to *T. pallidum* subsp. *pallidum* DNA was performed on a 200-year old femur from Easter Island of an individual possessing saber shins (Kolman et al., 1999). Although they were able to isolate DNA from the 5′-flanking region of the 15-kDa lipoprotein that is specific to *T. pallidum* subsp. *pallidum*, these authors used 15 g of femoral bone. This extremely large amount of bone material is unsettling considering the invasive nature of aDNA research; normally most methods use less than 1 g of material to extract DNA.

These successful PCR amplifications from patients and archival samples in the early to latent stages of syphilis, and an archaeological sample showing pathognomic lesions indicative of the disease, provided the aDNA community with the expectation of future success in isolating syphilitic DNA from human skeletal remains. However, it should be pointed out that the lack of 100% success rates even from modern clinical samples, the lack of 100% base similarity when correct band sizes were obtained and the use of such large sample quantities, suggests that isolating *T. pallidum* DNA from archaeological samples would be extremely difficult if not impossible to carry out.

The present study's approach to studying syphilis in the past is similar to Barnes and Thomas (2006), and Bouwman and Brown (2005); we all used Kolman et al.'s (1999) PCR assay to first test the samples, but went on to use other areas of the 15-kDa lipoprotein gene and other genes within the *Treponema* genome in hopes of isolating other areas of immunological interest for confirmation (e.g., *Tpr* gene family (Bouwman and Brown, 2005), *polA* gene, 47-KDa membrane protein and the Glycerophosphodiester Phosphodiesterase [GPD] gene [the latter three were employed in this work]). We also amplified and isolated endogenous mtDNA from the control region to check for molecular preservation within the samples and attempted sex determination of the individuals via amplification of the amelogenin gene. This study and that of Bouwman and Brown (2005) tested some of the same specimens from the Kingston-upon-Hull site (skeletons 805, 932, 1121 and 1216). Results presented here are in agreement with the previous authors in that there was a lack of success in amplifying treponemal DNA even though human endogenous DNA as well as other pathogen DNA could be isolated. This paper summarizes our results and provides a more in depth discussion on the limits of biomolecular syphilitic analysis including the results of a molecular experiment that was designed to simulate acute versus chronic treponemal infection in rabbits.

2. Materials and methods

2.1. Human samples

Bone samples are listed in Table 1. Individuals sampled for this study were selected from different geographic areas and from different time periods and were chosen based either on the presence of skeletal alterations indicative of syphilis and/or from historical documentation or medical reports indicating the individual suffered from syphilis. Eight of the 15 samples were acquired from individuals who lived during the period of the American Civil War (1861–65) and are from a collection housed at the Armed Forces Institute of Pathology, Washington D.C. Six samples are from individuals who lived in the United Kingdom all dating to pre-Columbian times (four from Hull, England [1450–75], one from Gloucester, England [mid-15th century] and one from Whithorn, Scotland [1300–1450]) and one from Belleville, Ontario dating to the mid-1800s.

The samples from the Civil War period are important because they are accompanied with written historical data stating that these individuals suffered and/or died from the complications of syphilis. Consequently, syphilis diagnosis is provided by the attending physicians from the time period. The remaining skeletal samples provide a putative presence of syphilis via pathognomonic osseous alterations (e.g., caries sicca, osteitis, periostitis, etc.).

2.2. Rabbit samples

In addition to the human archaeological and archival specimens, several different tissue samples were taken from two New Zealand white male adult rabbits donated by Dr. Hsi Liu from the Centers for Disease Control and Prevention (CDC) in Atlanta: 'Rabbit #12' (samples were taken from three of five injection sites: left inguinal [groin] lymph node, right testicle, liver, kidney, spleen, heart, right and left femora) was injected subcutaneously five times on the back seven months prior to sampling and 'Rabbit C' (inguinal [groin] lymph node, axillary [arm pit] lymph node and left femur were used for sampling) was injected intra-testicularly only 10 days (therefore in primary stage) prior to euthanasia (both were injected with similar concentrations of the organism *T.p.* subsp. *pallidum*). These two rabbits were in different stages of the disease. The former was in the later stage as all lesions where injections took place had healed by the time of sampling. The second rabbit still had lesions at the site of injection with live spirochetes having been harvested that same day.

2.3. Human DNA extraction

Each bone (approximately 1 g sample) and whole tooth sample were subjected to a 1.5-min soak in 1 N HCl, then transferred to 1 N NaOH solution for 1.5 min, followed by several rinses (approximately 10–20 min each) in (''ultrapure'') DNA/RNA free water. The samples were then placed in a UV cross-linker and rotated every 20 min until all sides were irradiated. When the samples were dry, they were ground to a powder in a SPEX™ liquid nitrogen grinding mill (ATS Scientific Inc., Oakville, ON, Canada).

Extractions were performed on different days for those individuals where multiple samples were taken, as well as for nonpathological and pathological bones to ensure against cross-contamination. DNA was extracted using the Yang

Table 1
Summary list of samples and results of mtDNA and Amelogenin PCRs

Lab Reference	Origin	Site Reference #	Bone Element	Non/Pathological	mtDNA Reproducibility	Comparison to Cambridge	Haplogroup	Morphological Sex	DNA Sex
1a	*Civil War Period, USA*	1001949 MM2880-2	articular surface of tibia	np	++	16294;16296	T		nr
1b			bone dust (no decontamination)	?	+				nr
2a		1001949 MM2880-4	articular surface of tibia	np	++		H		nr
2b			bone dust (no decontamination)	?	++				nr
3		1002700 MM1015[mr]	fibula end	np	++	16298;16313	preV	M	M?
4		1001245	rib	np	++	16187;16189;16223;16264; 16270; 16278;16311	L1b		nr
5a		1002814 MM1154[mr]	skull?	p?	+			M	nr
5b			skull	p (healed?)	++	16223	?		M?
5c			skull	p?	+				nr
6a		1000304 MM1622[hd]	long bone	np?	nr				nr
6b			long bone with lots of trabeculae	np?	nr				nr
6c			bone dust	?	nr				M
7		295209	skull	np	++		H	F	nr
8		1000873 MM2510	skull	np?	++	16212;16213;16223; 16278;16390	L2		3 M
9a	*Hull, England*[a]	HMC94-SK805	fibula	P	++	16294;16296;16304	T	M	nr
9b			long bone	np	++				3 M
10a		HMC94-SK932	humerus	p	+			M	nr
10b			tooth	np	+				3 M
10c			long bone	p	++	16263	U4		M
11a		HMC94-SK1121	fibula	p	+	16183;16189	H	F	nr
11b			tibia	p	+				F
12a		HMC94-SK1216	scapula	np	+			M	nr
12b			long bone	p	++		H		nr
12c			tibia	p	++				nr
13	*Whithorn, Scotland*		healed? gumma of skull	p	nr				nr
14	*Blackfriars, England*	SK77	rib	p	++		H	F	F
15a	*Belleville, Canada*	B302	proximal radius	np	+			M	M
15b			mid femur	p	++		H		3 M

NP = nonpathological sample; *P* = pathological sample; ? = uncertainty in bone element and/or state due to lack of observable criteria; mr = medical report associated with remains; hd = historical observation of bones by a doctor. ++ Indicates consistent replicate results were obtained on at least three independent PCRs with sequencing in both the forward and reverse directions; + indicates forward or reverse sequencing could only be obtained from multiple PCRs; M and F denote male and female sexes, respectively; 3M means that 'male' was successfully identified on three separate amplification reactions; ''?''suggests the sexing is tentative; and nr refers to 'no results' obtained from the PCR.

[a] All individuals from Hull were also analyzed by Bouwman and Brown (2005).

et al.'s (1998) method including multiple blank extractions to assess the presence of exogenous DNA contamination.

2.4. Rabbit DNA extraction

Tissue extractions of the rabbit material were carried out using the QIAamp DNA Mini Kit™ in a laboratory where no human, faunal or pathogen work had been performed previously. This laboratory is also completely different from the modern DNA lab used to test PCR primer efficiency and specificity, and separated from the laboratories of the McMaster aDNA Centre. Tissue was homogenized by cutting it into minute pieces with a sterile scalpel that was only used once per tissue and bone was ground with a 100% commercial bleach-sterilized mortar and pestle.

2.5. PCR conditions and DNA analysis

Amplifications were carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin–Elmer Applied Biosystems, Foster City, CA) in a standard 50 μl reaction volume containing 5 μl of DNA extract, 10× buffer (50 mM KCl and 10 mM Tris–HCl), various magnesium concentrations, 200 μM dNTPs, 1.0 mg/ml BSA, 30 pmol (0.3 μM) of each primer and 2.5 and 5 U AmpliTaq Gold polymerase (Perkin–Elmer Applied Biosystems, Foster City, CA). Multiple assays were used in order to isolate DNA from different sources: human mitochondrial DNA (mtDNA), amelogenin and treponemal DNA (Table 2). This top-down approach from endogenous multi-copy to single-copy DNA and finally to single-copy pathogen DNA tests the molecular preservation within the samples with each proceeding step ultimately becoming more difficult to perform as DNA quantity decreases.

All PCRs were optimized for magnesium ion concentration and annealing temperature. For all PCRs at least one negative control was used and for mtDNA (K562 − Invitrogen) and treponemal DNA synthetic positive controls were used to assess contamination and PCR success, respectively. This assessment was accomplished by sequencing the K562 (Invitrogen) mtDNA amplicon and comparing it to all ancient, positive results and also by synthesizing a shorter treponemal DNA amplicon than the target to control any laboratory contamination. Each of the PCR amplifications was carried out with an initial denaturing of 94 °C for 12 min and a final extension of 72 °C for 7 min. All PCR assays were tested on all of the samples in at least three independent amplifications.

PCR amplification reactions were run out on 8% non-denaturing polyacrylamide gels at 200 V for 30 min. Positive samples were purified using Qiagen QIAquick™ PCR Purification Kits and sequenced in forward and reverse directions using the primers mentioned in Table 2 via an automatic sequencer, ABI PRISM® 3100 Genetic Analyzer, at a central facility on the McMaster University campus (MOBIX). Sequences were then aligned via BioEdit (Hall, 1999) against the Cambridge Reference Sequence (Anderson et al., 1981) for polymorphisms in mtDNA and against *T.p.* subsp. *pallidum* DNA (15-kDa lipoprotein (Centurion-Lara et al., 1998); GPD (Cameron et al., 1999); 47-kDa membrane protein (Zoechling et al., 1997) and *polA* (Liu et al., 2001)). Mitochondrial DNA polymorphisms were also transcribed into the computer program mtRadius (Fluxus Technology Ltd.) in order to calculate haplogroup affiliation. The program mtRadius is a geographic information system that searches for the closest matches to a given mtDNA control region sequence and illustrates their geographic placement on a map (Röhl et al., 2001).

All ancient extracts were prepared in an isolated room dedicated to aDNA and all PCR mixes were set up in another laboratory in a laminar flow hood at the McMaster aDNA Centre. All modern DNA work (e.g., establishing PCR assay efficiency)

Table 2
Details of PCRs

Target locus	Primers	Product length (bp)	Magnesium concentration (mM)	Annealing temperature	Cycling numbers	Reference
Human DNA						
HVRI	5′-ATACTTGACCACCTGTAGTAC 5′-TGATTTCACGGAGGATGGTG	281	2.5	55	40	This study
Amelogenin	5′-TCATGAACCACTNCTCAGG 5′-CTGATTCTAAGATAGTCACA 5′-GGTAAAATACTAACTTTGGGCA	154 − X 119 − Y	2.5	50	50	This study
Treponemal DNA						
15-kDa lipoprotein gene	5′-GAGCAGGATGTCTCTATGAGTTATAAAGAG 5′-GAAGCCACTACCGATGTGCG	120	1.75	60	50	Kolman et al. (1999)
15-kDa − ARMS	5′-GCGTGTTCTTGAGCGTCTGAGC 5′-CAACTGGGTATACACCTACACAAG	147	1.75	69	50	This study
GPD − ARMS	5′-ACATACACTAGATCCGATCCTCTT 5′-AGTTGCAGTTTATCCGTGGGTTG	169	1.75	67	50	This study
Polymerase I gene[a]	5′-TGCGCGTGTGCGAATGGTGTGGTC 5′-CACAGTGCTCAAAAACGCCTGCACG	377	3	65	50	Liu et al. (2001)
47-kDa membrane protein	5′-GACCCAAGCGTTACTAAGATGG 5′-ACCGCAACTGGGACAAACTTCAT	196	1.6	67	50	Zoechling et al. (1997)

Each primer set begins with the forward primer. Sensitivity of the PCRs was taken as face value from the original publications, but were tested in house for mtDNA (1 pg) and amelogenin (1 pg), and the ARMS treponemal DNA (1-25 pg).

[a] Although this amplicon represents a rather large target by aDNA standards, it was used on the advice of Dr. Liu et al. (2001) due to its sensitivity.

was performed in a geographically separated laboratory on different days from any aDNA work to decrease possibilities of cross-contamination. All other precautions standard to aDNA analysis (e.g., disposable clothing, filtered pipette tips, sterilization of stainless steal-ware, plastic ware and solutions via autoclaving and UV irradiation, bleaching all surfaces, etc.) were also followed. All ancient DNA amplifications were accompanied by at least one extraction blank and two water blanks.

3. Results and discussion

3.1. mtDNA and amelogenin DNA preservation

We followed a similar rationale as previous workers by attempting to amplify multi-copy human DNA first and then moving towards single copy DNA. This allowed for a better assessment of DNA preservation for each specimen. Although the human-handling history prior to laboratory manipulation of the bone samples used in this work is unknown, no laboratory personnel contaminated them while at our centre as each laboratory individual's mtDNA was compared to the samples and no one possessed the disease under study (data not shown).

Molecular results are summarized in Table 1. It should be noted that all extraction and PCR blanks were negative for all amplification reactions. For the region of the human mitochondrial genome 13 of the 15 individuals (~87%) produced replicate PCR sequences in forward and reverse directions. Subsequent PCR tests where known concentrations of modern K562 mtDNA were spiked/added to the remaining two negative samples, remained unchanged (i.e., negative) showing that inhibition was present (von Hunnius unpublished PhD thesis, 2004). The negative results for the Civil War sample MM1622 (Lab Reference 6) and the difficulty in consistently obtaining other Civil War sample DNA can be explained by the conservation methods used by the Armed Forces Institute of Pathology circa 1893: pathological samples were macerated by boiling, dried at 100 °F, degreased by benzene (often placed in sunlight to speed up the process) and if the bones needed to be whitened due to discoloration, they were exposed to chlorine, both in a gas and liquid form (Army Medical Museum, unpublished document). Each one of these steps and the chemical reactions involved are known to heavily degrade DNA and inhibit PCR. Negative results for the Whithorn sample (Lab Reference 13) are also likely attributed to the type of sample taken: a skull fragment. The Whithorn gumma sample was relatively small in size consisting of only flat, thin cortical bone that has been noted by others as containing DNA of lesser quality/quantity than long bones (Alonso et al., 2001). This is most likely related to the increased number of cells (and DNA) found in the thick, dense cortex of long tubular bones.

Since a mitochondrial haplogroup should normally only be assigned when 340 bp of the HVR1 region is obtained, the results presented in Table 1 should be treated as tentative as only 281 bp were amplified. With this in mind, the majority of these individuals' haplogroups are of European descent which is in accordance to our original assumptions of the sites where these samples originated. The only exceptions to this are individuals Civil War 1001245, MM1154 and MM2510. Unfortunately records available for these three individuals do not provide any hints to their geographical origins. What is considerably interesting is the replication of the mtDNA polymorphism at position 16263 for individual HMC-SK932 that was also found by Bouwman and Brown (2005).

In contrast to Barnes and Thomas (2006) and Bouwman and Brown (2005), this study also attempted to amplify endogenous nuclear DNA from the amelogenin gene to see if lower copy DNA was preserved. Results at this level show that sex could be identified at the molecular level for four of the nine individuals (~44%) where sex was observed morphologically or by medical records. Two other individuals have tentative sex typing as 'males' as the X chromosome amplicon was not visualized on the gel which is most likely explained by allelic drop out (the assay preferentially targets the Y chromosome to be the shorter amplicon in order to limit falsely identifying males as females). This translates into relatively well preserved DNA in a good portion of the individuals used in this analysis by aDNA standards.

3.2. Treponemal DNA preservation

Four different areas of the *Treponema* genome using five different PCR assays (15-kDa lipoprotein gene [(Kolman et al., 1999) and the 15-kDa ARMS test], the DNA Polymerase I gene [*polA*] (Liu et al., 2001), the 47-kDa membrane protein gene (Zoechling et al., 1997) and the GPD gene ARMS test) all produced negative results for these 15 samples.

As discussed briefly by Barnes and Thomas (2006) and Bouwman and Brown (2005), unsuccessful treponemal DNA amplification is most likely plagued by its inability to survive outside the hosts' environment. Studies have shown that *T. pallidum* is easily destroyed by heat, antiseptics, and detergents (Plorde, 1994; Pusey, 1933; Schouls, 1992) and is rapidly killed by drying (Cates, 1998; Plorde, 1994) because of its rather fragile outer membrane (Radolf et al., 1989; Schouls, 1992). Unlike most Gram-negative organisms, *T. pallidum* outer membranes do not contain lipopolysaccharides (LPS) (Schouls, 1992:91] that are known to form ''a confluent and impermeable physical barrier, restricting access of lytic components'' (Wilks and Sissons, 1997:171). Without a sturdy cellular membrane, *T. pallidum* DNA is exposed to many physical and chemical elements that can alter and destroy DNA bonds. This crucial structure is most likely why *Mycobacterium tuberculosis* with its thick, robust cell wall (Brennan and Nikaido, 1995; Schouls, 1992; Wilks and Sissons, 1997) has the ability to survive throughout the lifetime of a mammalian host and the degradation process after death, which has allowed researchers to isolate it from many different specimens of antiquity (e.g., Fletcher et al., 2003; Spiegelman and Donoghue, 1999; Taylor et al., 1996; Taylor et al., 2005).

Organism viability is not the only issue when it comes to aDNA research; the resistance of the organisms' DNA to degradation is also vital. It is important to note that unlike human genomic DNA, bacterial genomes are not associated with histone molecules (Wilks and Sissons, 1997:164]. Histones allow DNA to pack into dense configurations that act to protect it

from degradation (i.e., depurination) *in vivo* (Clark and Felsenfeld, 1971), but in the postmortem environment, histones break down and DNA preservation is then dependent on binding to hydroxyapatite (HAP) crystals in the mineral portion of bone (Martinson, 1973; Okasaki et al., 2001). Therefore, treponemal DNA preservation is dependent on HAP binding which must occur very soon after the death of the individual, as the acidic environment that arises during decay will lyse the bacterial membrane. This process would likely be time and quantity dependent as there would be competition with larger amounts of endogenous DNA available for the binding process.

Research on *T. pallidum* DNA preservation in different environments has looked at DNA survivability and PCR testing to a limited degree but current results are nonetheless impressive (e.g., Villanueva et al., 1998). For example, Wicher et al. (1992) were able to detect DNA in swabs (from early skin syphilitic lesions) immersed in TE buffer and kept at room temperature for 90 days (7/8), swabs stored dry at room temperature for 90 days (3/3), as well as punch biopsies taken from healing skin lesions kept in TE buffer for 90 days (10/12). In another experiment, Wicher et al. (1998) obtained positive amplification results from biopsies taken from healed lesions at one month after infection (5/8) and then after three months storage (10% success). Such a poor outcome after the passage of time suggests that for samples retrieved from more adverse burial environments, positive amplification would be negligible to impossible.

Another possibility to discuss is the theory that by the time bone lesions manifest, no *T. pallidum* bacteria (and hence, no DNA) exist at the site of infection. Different studies have noted that by the tertiary stage (where the majority of bone lesions develop; however, skeletal alterations have been observed in the secondary stage as well (e.g., Bauer and Caravati, 1967; Ehrlich and Kricun, 1976; Gomez Martinez et al., 2003; Gurland et al., 2001; Jaffe, 1972; Newman and Saunders, 1938; Ollé-Goig et al., 1988; Reynolds and Wasserman, 1942; Shore et al., 1977; Squires and Weiner, 1939; Thompson and Preston, 1952; Waugh, 1976; Wile and Sinear, 1916; Wile and Welton, 1940), individuals are no longer considered infectious because the number of spirochetes has decreased dramatically (Knox et al., 1976). Many researchers believe that the observed bone lesions are actually a hyper-allergenic response (delayed hypersensitivity) (Metzger, 1976; Schell and Musher, 1983; Smith, 1976; Musher and Baughn, 1998) possibly due to the degraded remnants of the bacteria at that particular site (Jaffe, 1972; Resnick and Niwayama, 1995) or to treponemal antigens (Salazar et al., 2002). Schneider first suggested this (cited by Jaffe, 1972:928) in 1923 when he observed spirochetes in 'normal' bone lacunae, but not in lacunae of bone lesions suggesting that the spirochetes were released upon bone turnover and degraded by the immune response. Metzger (1976:323) suggests instead that the late lesions are the result of an immunologic response against treponemal antigens. Although the process is still poorly understood, it appears that it may be the combination of the two (Cotran et al., 1999).

It is also possible that the individuals sampled did not have syphilis and in fact suffered from another infectious agent as syphilis manifests with a relatively similar pathological repertoire as several other diseases (e.g., tuberculosis, Paget's). With further investigation, historical documents for Civil War individuals MM2880-2 and 4 (Army Medical Museum unpublished document, 1868) and 1001245 (Otis unpublished document, 1868) suggest that they may not have been suffering from syphilis at the time of their death. In addition several individuals in Bouwman and Brown's (2005: Table 6) research were identified with ''treponemal pathology'', but were positive for *M. tuberculosis* DNA. However, this does not preclude the possibility that these individuals may have been co-infected. We note that the IS6110 insertion sequence for *M. tuberculosis* (following the nested approach by Taylor et al., 1996) was also targeted in all of our samples (unpublished data), but even though the correct band size was obtained for four of the individuals, sequencing displayed substantial deviation from the expected.

The majority of what is known concerning the organism that causes syphilis and its immunopathogenesis is the result of studies performed on rabbits infected with the bacteria. In addition, all new PCR assays specific to *Treponema* infections are tested using harvested spirochetes generally from rabbits. In conjunction, these PCR studies have looked into bacterial dissemination through time by trying to isolate treponemal DNA from multiple tissues within the rabbit: blood, skin, testes, inguinal lymph nodes, spleen, heart and brain (e.g., Burstain et al., 1991; Wicher et al., 1992, 1998). However, none of these tests have looked at spirochete dissemination into bone, particularly at different stages of the disease. This is an important avenue of research as syphilis generally produces bone lesions during later stages of the disease and if treponemal DNA cannot be found within this tissue medium in clinical samples, its chances of being amplified in archaeological specimens remain slim.

To test whether treponemal organisms penetrate bone in both acute and chronic states, several tissue types were sampled from two adult New Zealand white male rabbits considered to be in these two different stages of syphilis infection. All samples were subjected to the five different PCR protocols and our results (Table 3) show that indeed, treponemal DNA does disseminate to the bone. However, the most important finding from this experiment is that although treponemal DNA could be isolated from the site of injection from the ''chronic rabbit'' (also observed by Wicher et al., 1998), it could only be isolated from the bone during the acute stage. Sequencing of a subset of these amplicons revealed that the correct sequence was amplified (data not shown). This is the first time syphilitic DNA has been identified from bone of a positive clinical sample using the PCR technique. It shows that the bacteria actually disseminate to the bone fairly early on in the course of the disease (and that it should theoretically be found in human bone as well), but is not present in later or dormant forms (i.e., it could not be isolated and amplified via multiple PCR techniques).

The rabbit model approach could be further expanded to test a range of burial environments so that a simulated archaeological context could be achieved. With experiments like this and others that may follow, our understanding of treponemal DNA preservation in bone will be greatly enhanced.

Table 3
Results of rabbit tissue testing

Rabbit	Sample Taken	Kolman et al. (1999)	15-kDa ARMS	GPD ARMS	47-kDa	*polA*
Rabbit 12	Control tissue: skin from area not injected					
	Injection 1: tissue from area of healed injection site with hair					
	Injection 2: tissue from area of healed injection site with hair	×				
	Injection 3: tissue from area of healed injection site with hair					
	Left pelvic lymph node					
	Right testicle					
	Liver					
	Kidney		×	×		
	Spleen		×	×		
	Heart					
	Right femur: sampled the midshaft with marrow for experiment					
	Left femur: sampled the midshaft with tissue for experiment					
Rabbit C	Pelvic lymph node	×	×	×	×	×
	Armpit lymph node	×	×		×	×
	Left femur: sampled the midshaft bone only for experiment		×			×

Samples taken from adult New Zealand white male rabbits injected with *Treponema pallidum* subsp. *pallidum* and the results of the molecular tests that identify this bacteria. × = Positive result.

4. Conclusions

At the outset of this work in 1999 when human endogenous DNA amplification was successful, it was naively believed that this particular study could expect success retrieving *T.p.* subsp. *pallidum* DNA because of the achievement of Kolman et al. (1999). It is possible that the sheer size of the sample Kolman et al. (1999) used may have been responsible for their success. Therefore, it may be suggested that treponemal DNA existed in the subsequent tested samples, but not for the genes analyzed. This seems a weak hypothesis since Bouwman and Brown (2005) also examined the four individuals from the Hull site that we studied. They also targeted the multicopy *Tpr* gene (only single copy genes were analyzed in this work and that of Barnes and Thomas, 2006). Excluding human endogenous and TB DNA, a total of five different genes with 13 different assays were used to try and isolate treponemal DNA from these UK samples with no results. These data, as well as the above reasoning, provide impressive evidence as to the limits of amplifying ancient treponemal DNA.

In addition, this study tested the idea that later stages of syphilis infection have little or no organism DNA by using rabbits in acute and latent/chronic phases of the disease. This approach demonstrated that the organism does reach many different areas of the body, including bone, at an impressively rapid rate. However, at later stages, organisms were not found within the bone medium with any of the PCR assays used here. These interesting results reveal a distinction of organism invasion of the bone during different stages of the disease. Although this experiment was performed using the rabbit model, it is our closest approximation to human pathogenesis that can be ethically tested. Considering the results obtained, it can be postulated that within humans similar conclusions will ensue. In other words, in later stages of the disease syphilitic organisms will most likely *not be* located in bone and the possibility of isolating them by PCR will be negligible. These results provide an additional piece of evidence as to why no treponemal amplicons can be isolated within the bone samples exemplifying tertiary osseous manifestations.

In the end, the results and the discussion presented here and elsewhere (Barnes and Thomas, 2006; Bouwman and Brown, 2005) should serve as a warning for paleopathologists who hope that the new and exciting world of ancient DNA can help them with their observations of diseases in the past. We should not be so quick to test precious human material when not much is known about the organism's ability to infiltrate bone and/or survive in dynamic and harsh burial environments.

Acknowledgements

This work was supported in part by the Social Sciences and Research Council of Canada Scholarship #752-2001-1058,

Dr. Ronald V. Joyce Ontario Graduate Scholarships in Social Sciences (OGS), Raymond Moore Graduate Scholarship in Science and Technology (OGSST), the Department of Anthropology and the School of Graduate Studies, McMaster University. The authors wish to thank Dr. Liu, Dr. Cox and the Centers for Disease Control and Prevention for their advice and their generous contribution of *Treponema* DNA and the rabbits used in this project; Anthea Boylston (University of Bradford) and Charlotte Roberts (University of Durham) for granting access to and sampling the UK specimens; and Dr. Paul Sledzik from the National Museum of Health and Medicine (a division of the Armed Forces Institute of Pathology, Washington, D.C.) for donating samples from the American Civil War time period. Dr. Ann Herring, Dennis Ng and Dr. Jodi Lynn Barta are gratefully acknowledged for their support and technological expertise. The authors also would like to thank the two anonymous reviewers for their helpful comments.

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